

## Presence of a Group II Intron in a Multiresistant *Serratia marcescens* Strain That Harbors Three Integrons and a Novel Gene Fusion

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We analyzed the role of integrons in the dissemination of antibiotic resistance in a recent multiresistant clinical isolate, *Serratia marcescens* SCH88050909 (SCH909). This isolate harbors three integrons, all on a 60-kb conjugative plasmid. By PCR, hybridization, and sequencing analyses, we found that integron 1 has the *dfrA1* and *ant(3'')-Ia* cassettes. The first cassette in integron 2 contains the *ant(2'')-Ia* gene, separated from its *attC* site (59-base element) by a 1,971-bp insert containing a group II intron; this intron codes for a putative maturase-reverse transcriptase on the complementary strand and is the first such intron to be found associated with an integron. The *attC* site is followed by a novel aminoglycoside resistance gene, *ant(3'')-Ii-aac(6')-IId*, which has been characterized for its bifunctional ANT(3'')-I and AAC(6')-II activities. DNA sequence analysis of this fused cassette suggests that insertion and excision due to the integrase activity could have an important role in the evolution of aminoglycoside resistance genes. This gene is followed by an unknown open reading frame with a typical *attC* site and a partial cassette composed of the beginning of the *bla*<sub>OXA-10</sub> cassette interrupted by *IS1*. The sequence downstream of *IS1* revealed that the *bla*<sub>OXA-10</sub> cassette is incomplete and that the 3' conserved segment of this integron is absent. Integron 3 is in a Tn1696-like transposon with the *aac(3)-Ia* cassette followed by three unknown cassettes and *ant(3'')-Ia*. The presence of the group II intron and the relationship of group II introns in eubacteria with mobile elements suggest a possible role of this element in events such as cassette formation and/or plasmid evolution.

The spread of antibiotic resistance genes among bacterial strains is an increasing problem in nosocomial infections (7). Over 40 antimicrobial resistance genes are located within integrons as mobile DNA elements called cassettes and account for a significant proportion of the antibiotic resistance genes found in gram-negative bacteria (26). Integrons are often located in plasmids or transposons, thus enabling the rapid spread of the gene cassettes among a wide variety of bacterial species (2, 3, 23). In fact, several aminoglycoside resistance proteins, such as ANT(3'')-I and AAC(6')-I, which are common in the multiresistant gram-negative bacterial population, are coded for by gene cassettes [*ant(3'')-Ia*, *ant(3'')-Ib*, and *aac(6')-Ib*] (14). Their expression depends on their insertion into the variable region of integrons, where the genes are transcribed from a common promoter in the 5' conserved segment (13).

The origins of the cassette-associated genes are not known (9, 27). It has been proposed that cassettes may have originated from transcripts that were converted to DNA, involving the activity of an as-yet-unidentified reverse transcriptase (RT) (27). The palindromic *attC* sites (59-base elements) found at the end of each cassette either may have been present within the original transcripts, for example, as transcription terminators, or may have been added at a later stage (27). It has been also speculated that secondary sites for IntI1, defined as the degenerate pentanucleotide GWTMW, can facilitate the asso-

ciation of new genes into integrons and may have been the substrate for the creation of *attC* sites during evolution (6, 9).

The goal of our work is to elucidate the role of the antimicrobial resistance mechanisms carried on integrons in a multiresistant *Serratia marcescens* strain. We describe a novel *ant(3'')-Ii-aac(6')-IId* gene cassette, inserted into the variable region of a class I integron, that codes for a bifunctional ANT(3'')-I-AAC(6')-II mechanism. The role of the integrase activity in the formation of this fused cassette is discussed. The role of a group II intron located between the *ant(2'')-Ia* resistance gene and its *attC* site is discussed.

### MATERIALS AND METHODS

**Bacterial strains and plasmids.** *S. marcescens* SCH88050909 is a clinical isolate from Greece that was collected because of its multiple aminoglycoside resistance at Schering-Plough Corporation in 1988. Here, we use only the last three numbers of the Schering strain designation (SCH909) (14). A nalidixic acid-resistant (Nal<sup>r</sup>) derivative of *Escherichia coli* C600 was used as a recipient in conjugation experiments, and *E. coli* NM522 was used as a recipient in transformation studies. Plasmid pTZ19 (Pharmacia-LKB Biotechnology, Uppsala, Sweden) was used for cloning of DNA fragments.

**Media and culture conditions.** Bacterial strains were grown on Luria-Bertani agar and in Luria-Bertani broth supplemented with one or more of the following antibiotics, as appropriate: ampicillin (50 µg/ml), gentamicin (25 µg/ml), nalidixic acid (50 µg/ml), or streptomycin (25 µg/ml).

**Antibiotic susceptibility.** Determination of the aminoglycoside resistance profile (AGRP) (30) was done by using aminoglycoside disks (Schering-Plough Corporation, Kenilworth, N.J.) (17) on Mueller-Hinton agar. The 11 aminoglycosides included fortimycin, amikacin, tobramycin, apramycin, dibekacin, gentamicin, isepamicin, netilmicin, 5-episisomicin, 2'-N-ethylnetilmicin, and 6'-N-ethylnetilmicin. Assignment of enzymatic mechanisms to each strain was based on the unique pattern of resistance to these aminoglycosides (17). In addition, the MICs in liquid medium were determined with an inoculum of 10<sup>5</sup> to 10<sup>6</sup> CFU/ml.

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**DNA techniques.** Preparation of DNA was performed as described by Sambrook et al. (29). Plasmid DNA was prepared by using a Wizard purification kit supplied by Promega. DNA was digested with various restriction enzymes, and the fragments were separated in horizontal gels of 0.8 to 1.0% (wt/vol) agarose dissolved in 0.4 M Tris-acetate-0.01 M EDTA. Gels were stained with ethidium bromide, and the DNA was visualized by UV transillumination at 302 nm. Transformation procedures were performed as described by Sambrook et al. (29). Conjugation was carried out with brain heart infusion agar for 4 h at 37°C, and the transconjugants were selected on Mueller-Hinton agar medium containing ampicillin (50 µg/ml).

PCR amplifications were carried out with 100-µl volumes containing 10 ng of DNA, 10 µl of 10× PCR buffer, 10 µl of 10× deoxynucleoside triphosphate mix (2 mM each dATP, dCTP, dGTP, and dTTP), 10 µl of each primer stock solution (2.5 pmol of each primer per µl), and 60 µl of sterile distilled water. Each reaction mixture was covered with 75 µl of mineral oil. *Taq* DNA polymerase from Promega was added (1 µl of a 3-U/µl diluted solution) after 12 min at 94°C (hot-start method). To amplify the DNA in the thermocycler (Perkin-Elmer Cetus, Emeryville, Calif.), we used a three-step profile described previously (14). The gene probes and/or primers used for PCRs were specific for the following nucleotide sequences: β-lactam resistance genes *bla*<sub>CTX-M-2</sub> (ATGACTCAGAG CATTTCGC and TCACCTTATTCGGGACCAC), *bla*<sub>PER-2</sub> (1), and *bla*<sub>TEM</sub> (14); aminoglycoside resistance genes *aac*(6′)-*Ib*, *ant*(3′′)-*Ia*, *aac*(3)-*Ia*, *aac*(3)-*Ila*, and *ant*(2′′)-*Ia* (14); trimethoprim resistance gene *dfzA1* (14); and the 5′ conserved segment (5′ CS) and the integrase gene of class I integrons (Sulpro3) (14). DNA was transferred to nylon filters by Southern blotting and hybridized with appropriate probes as described by Sambrook et al. (29). Autoradiography was done with Kodak X-Omat AR film.

The 5.0-kb *Hind*III fragment of pSm909 containing integron 3 and *Tn1696*-related sequences, the 2.3-kb *Bam*HI fragment of pSm909 containing the integrase gene, the *ant*(2′′)-*Ia* gene, and part of the group II intron from integron 2, and the 4.2-kb *Hinc*II fragment containing the remainder of the group II intron, *ant*(3′′)-*Ii*-*aac*(6′)-*IId*, open reading frame (ORF) O, *bla*<sub>OXA-10Δ</sub>, *IS1*, and unknown sequences from pSm909 were cloned into the corresponding sites of pUC19.

**DNA sequencing and analysis.** DNA was purified by using a QIAquick kit according to the manufacturer's instructions (Qiagen Inc., Studio City, Calif.). Sequencing was done on both DNA strands by using an ABI 373 sequencer. Internal oligonucleotide primers were used when necessary to ensure that both strands were sequenced. The nucleotide sequences were analyzed by using Genetics Computer Group programs.

**Nucleotide sequence accession numbers.** The nucleotide sequences of the variable regions of integrons 2 and 3 in *S. marcescens* SCH909 have been deposited in GenBank under accession numbers AF453998 and AF453999, respectively.

## RESULTS

**Characterization of the antimicrobial resistance genes of *S. marcescens* strain SCH909.** The MICs for multiresistant *S. marcescens* SCH909 and its transconjugant in *E. coli* C600 are shown in Table 1. Strain SCH909 showed susceptibility to cefoperazone, aztreonam, imipenem, norfloxacin, ciprofloxacin, nalidixic acid, netilmicin, and amikacin; reduced susceptibility to cefoxitin, cefotaxime, and ceftazidime; and resistance to gentamicin, tobramycin, neomycin, streptomycin, ampicillin, cephalothin, piperacillin, trimethoprim, sulfamethoxazole, and chloramphenicol. We tested for the presence of *bla*<sub>SHV</sub>, *bla*<sub>TEM</sub>, *bla*<sub>CTX-M-2</sub>, *bla*<sub>PER-2</sub>, *cmlA*, *catB2*, and *Tn3* (where *bla*<sub>TEM</sub> is usually located) by PCR and the *aac*(3)-*Ila* gene by dot blot hybridization in this strain. The *bla*<sub>TEM</sub> gene and *Tn3* were harbored by this strain. Sequence analysis of the *bla*<sub>TEM</sub> PCR product revealed that the gene was *bla*<sub>TEM1</sub>, conferring resistance to ampicillin but not to third-generation cephalosporins, such as cefotaxime. The genetic determinants for the intermediate susceptibility and/or resistance to cefoxitin, cefotaxime, ceftazidime, gentamicin, tobramycin, neomycin, streptomycin, ampicillin, cephalothin, piperacillin, trimethoprim,

TABLE 1. MICs of antibiotics for *S. marcescens* SCH909, *E. coli* C600 transconjugant, and control *E. coli* ATCC 25922

Antibiotic	MIC (µg ml <sup>-1</sup> ) for:		
	<i>E. coli</i> C600	<i>S. marcescens</i> SCH909	<i>E. coli</i> C600 transconjugant
Gentamicin	1	64	128
Tobramycin	0.5	128	128
Amikacin	1	2	2
Netilmicin	0.5	8	8
Streptomycin	1	64	2
Neomycin	0.5	64	64
Ampicillin	4	>256	>256
Cefoxitin	2	4	16
Imipenem	0.25	0.5	0.5
Cephalothin	4	256	256
Cefotaxime	0.06	16	16
Cefoperazone	0.12	8	8
Ceftazidime	0.25	16	16
Piperacillin	1.0	>128	>128
Aztreonam	0.12	4	4
Norfloxacin	0.125	0.25	0.125
Ciprofloxacin	0.0625	0.0625	0.0625
Nalidixic acid	>64	4	>64
Chloramphenicol	4	32	32
Trimethoprim	1	16	16
Sulfamethoxazole	16	>512	>512

sulfamethoxazole, and chloramphenicol were transferable and carried on a 60-kb conjugative plasmid (Table 1).

**Evidence for the presence of three integrons of class I in *S. marcescens* strain SCH909.** By PCR with primers 5′-CS and 3′-CS from the 5′ and 3′ conserved segments of the integrons, we amplified the variable regions of the integrons. With this pair of primers, there was only one PCR product with a 1,500-bp length, suggesting the presence of only one integron in SCH909. Sequencing of this PCR product revealed the presence of two cassettes, *dfzA1* and *ant*(3′′)-*Ia*, in that order, within the integron. However, PCR mapping with primers from the 5′ conserved segment of the integron in conjunction with primers from various resistance gene cassettes showed that there were three different cassettes, *dfzA1*, *ant*(2′′)-*Ia* (*aadB*), and *aac*(3)-*Ia* (*aacCI*), each immediately adjacent to an integron 5′ conserved segment (Fig. 1). Therefore, PCR mapping of integrons (14) must not be limited to the use of primers from the 5′ and 3′ conserved sequences. The PCR bias toward smaller products may mask larger integrons in the same strain, or the 3′ conserved segment may be missing (see below).

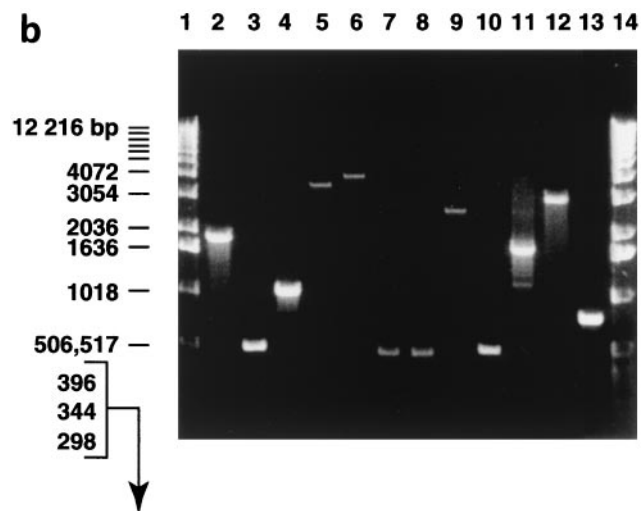
The finding of three integrons by PCR analysis was corroborated by hybridization to the whole genome of SCH909 and to the transconjugant digested with *Hind*III. Copies of the *intI1* gene were found in the 15.0-, 13.0-, and 5.0-kb *Hind*III fragments by use of the Sulpro3 and 5′-CS probes. The 3′ conserved segment of the integrons was found in the 15.0- and 5.0-kb *Hind*III DNA fragments, suggesting that it may be missing in one integron.

**Analysis of the three variable regions of the integrons.** The nucleotide sequences of the 1.5-, 5.2-, and 3.1-kb regions from the variable regions of the three integrons found in SCH909 were determined (see Materials and Methods). Integron 1 contains the *dfzA1* and *ant*(3′′)-*Ia* genes, and its variable region is identical to that of pLMO229 (32).

a

1. *IntI1* *dfrA1* *ant(3'')-Ia* *qacEΔ1* *sulI* *orf5*2. *ant(2'')-Ia* *Smtr* *ant(3'')-Ii-aac(6')-IId* *orfO*  $\Delta$ *bla*<sub>oxa-11</sub>-*IS1* *orf*3. *aac(3)-Ia* *orfP* *orfP* *orfQ* *ant(2'')-Ia*

b



Integron 2 (GenBank accession no. AF453998) contains an *ant(2'')-Ia* gentamicin resistance gene which is separated from its *attC* site by a 1,971-bp insert containing an ORF on the complementary strand (Fig. 1a). While the *ant(2'')-Ia* gene was identical, the *ant(2'')-Ia attC* site possessed only 84.5% identity with that of the *ant(2'')-Ia* gene already described (4). The complementary strand of the insert showed 48.1% identity with a group II intron from *Pseudomonas alcaligenes* (36). The product of this ORF, called *Smtr*, from the bottom strand showed 39.2% identity with the maturase of this intron, although the ORF length for the putative maturase and/or RT

FIG. 1. Mapping of integrons of *S. marcescens* SCH909. (a) General structures of integrons 1, 2, and 3 found in *S. marcescens* SCH909. The arrows show the direction of transcription. (b) PCR amplification from SCH909. The PCR products were separated by electrophoresis through 1.0% agarose. Lanes 1 and 14, 1-kb DNA ladder; lanes 2 to 13, SCH909 with the following primers: lane 2, Sulpro3 and 3'-CS; lane 3, Sulpro3 and *dfrA1*; lane 4, Sulpro3 and *ant(3'')-Ia*; lane 5, Sulpro3 and *ant(3'')-Ii*; lane 6, Sulpro3 and *aac(6')-Ib*; lane 7, Sulpro3 and *ant(2'')-Ia*; lane 8, Sulpro3 and *aac(3)-Ia*; lane 9, *aac(3)-Iacooh* and 3'-CS; lane 10, *ant(3'')-Iacooh* and 3'-CS; lane 11, *aac(3)-Iacooh* and *ant(3'')-Ia*; lane 12, *ant(2'')-Iacooh* and *aac(6')-Ib*; and lane 13, *tnpR* and BLA-TEM. Primer Sulpro3 is a rightward primer in the 5' conserved segment. Primers with gene names are leftward primers near the beginning of their respective cassettes. Primers with "cooh" are rightward primers near the end of their respective cassettes. Primer 3'-CS is a leftward primer in the 3' conserved sequence. These primers are used to determine gene order in integrons (14).

differed. The putative RT from SCH909 possessed all seven polymerase-like domains characteristic of RT activity (Fig. 2). In addition to the seven RT-like domains and like the maturase from *P. alcaligenes* (intron *xln6*), a domain, z, which is characteristic of non-long terminal repeat retroelements (19), was detected at the N-terminal end of the RT from SCH909. Also, domain x was found downstream of the seven RT domains in *Smtr*. Domain x has been suggested to be involved in binding of the intron RNA during reverse transcription and splicing. A noncoding region immediately downstream of the putative RT could fold into typical V and VI RNA secondary structures (Fig. 3). Domain V is the only component other than domain I that is absolutely essential for group II ribozyme activity (16). A 1,971-bp sequence (from bp 534 to bp 2504 of integron 2)

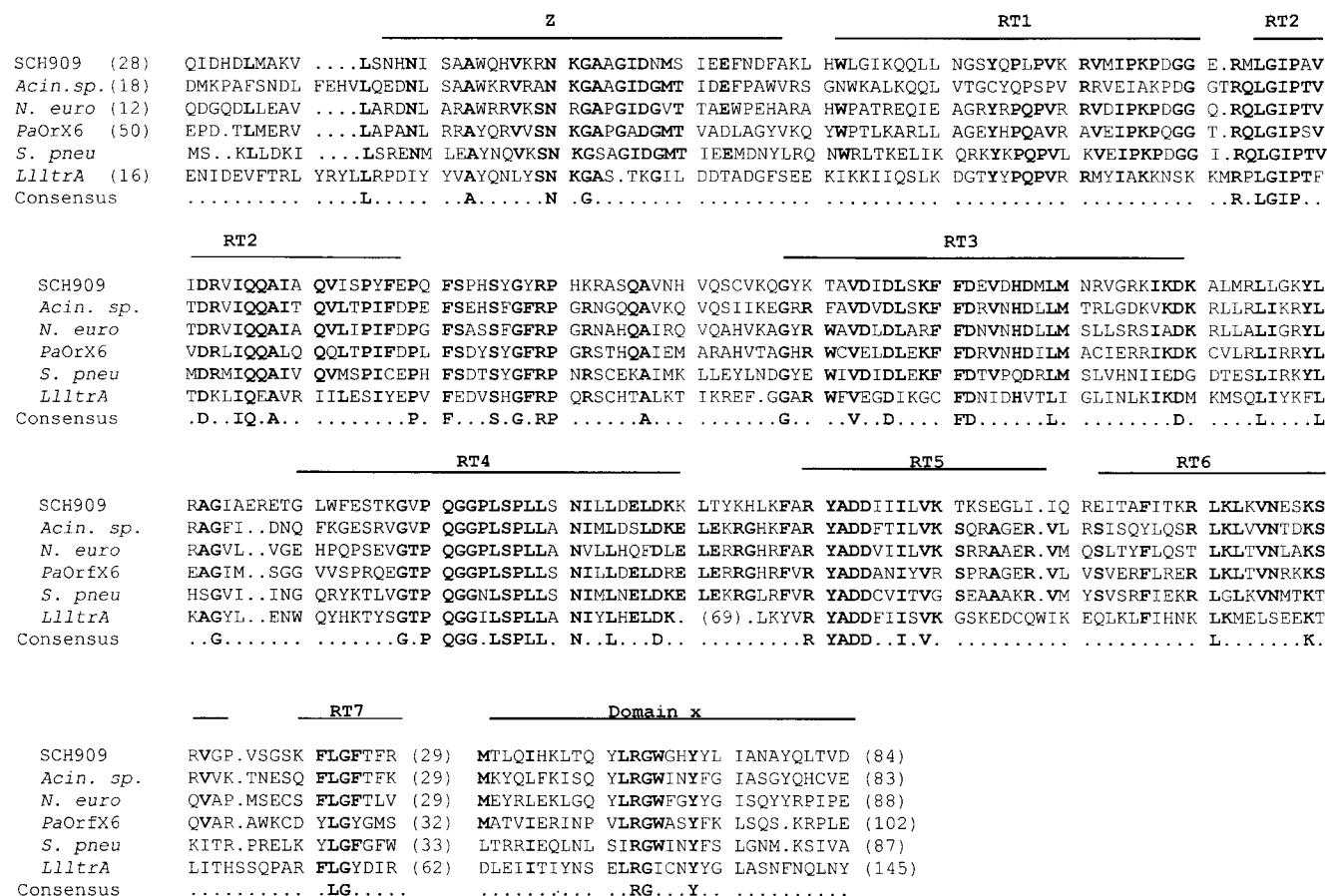


FIG. 2. Protein alignment of the putative maturase, Smtr, in SCH909 with eubacterial group II intron-encoded proteins from an *Acinetobacter* sp. (*Acin.sp.*) (J. H. Yum et al., unpublished; GenBank accession number AF369871; ORF II), *N. europaea* (*N. euro*) (JGI, unpublished), *P. alcaligenes* (*PaOrX6*) (36), *Streptococcus pneumoniae* (*S. pneu*) (5), and *Lactococcus lactis* (*LlItrA*) (18). Consensus amino acids (four or more identical in the six sequences) are in bold letters. Domains conserved among intron-encoded proteins are denoted by the lines above the alignment. RT1 through RT7, RT-like domains (19); z, domain of undetermined function in non-long terminal repeat retroelements; x, maturase-specific domain (19). Numbers in parentheses indicate numbers of amino acids not shown.

could fold into the typical RNA secondary structure of a central wheel with six spokes that define the six major ribozyme domains of group II introns (data not shown).

This region is followed by a novel aminoglycoside resistance gene [*ant(3'')-II-aac(6')-IId*] which codes for a fusion protein involved in streptomycin, spectinomycin, and gentamicin resistance [ANT(3'')-I-AAC(6')-II profile; see below]. This gene is followed by an ORF (ORF O) whose product has weak similarity to *qacE* multidrug transporters, with a typical *attC* site [95% identity to *aac(6')-IIa* 59-base element] and the beginning of a *bla<sub>OXA-10</sub>* β-lactamase cassette interrupted by *ISI*. At the other end of *ISI*, the sequence does not return to that of *bla<sub>OXA-10</sub>*, indicating possible recombination between two copies of *ISI*. Also, PCR analysis with internal and specific primers for the *bla<sub>OXA-10</sub>* gene revealed that the whole gene is not present in strain SCH909. Therefore, the 60-kb plasmid from SCH909 has evolved by several insertions and recombinations.

Integron 3 (GenBank accession no. AF453999) contains another aminoglycoside resistance gene cassette, *aac(3)-Ia*, followed by two identical ORF P cassettes, a different ORF Q cassette, and *ant(3'')-Ia* (Fig. 1). The sequence to the left of the integrase of

integron 3 shows that this integron is part of a *TnI696*-like transposon (data not shown). ORF P and ORF Q are identical to ORF X(A) and ORF X(B) of *Klebsiella oxytoca* plasmid pACM1 (24), which has only one copy of the former.

Sequencing of the 5' conserved segments of integrons 1 and 2 revealed integrase genes identical to that of *Tn21* (13), while that of integron 3 is like that of *TnI696*. Unlike the situation in *TnI696*, the 19-bp duplication which permits the expression of the *aac(3)-Ia* gene as a translational fusion which begins before the *attI* site (35) is absent. In SCH909, the *aac(3)-Ia* gene probably uses an initiation codon internal to the cassette.

**Characterization of a new aminoglycoside resistance gene, the *ant(3'')-II-aac(6')-IId* gene cassette.** Plasmid DNA from strain SCH909 was digested with *HincII*. The fragments were ligated to the *HincII* site of pTZ19r and introduced by transformation into *E. coli* NM522. The transformants selected on ampicillin-gentamicin were screened for inserts by agarose gel electrophoresis, and a plasmid containing an insert of 4.2 kb was chosen (pGM172). The fragment from a *Sau3AI* partial digest was subcloned into the *BamHI* site of pTZ19r to yield plasmid pGM172-7, which contained a 2.3-kb insert that con-

ferred resistance to gentamicin, streptomycin, and spectinomycin on *E. coli* NM522. The AGRP of pGM172 and pGM172-7 revealed the combination of ANT(3'')-I plus AAC(6')-II mechanisms (Tables 2 and 3). We found an ORF spanning 1,392 nucleotides from the start codon ATG at position 2563 to the stop codon TAA at positions 3952 to 3954 in the sequence of integron 2 (GenBank accession number AF453998). The first 789 nucleotides of this ORF possessed 99.2 and 82.7% identities, respectively, with the novel *ant(3'')-If* (*aadA6*) (22) and *ant(3'')-Ig* (*aadA7*) (15) over the entire length and 76.4 and 77.1% identities, respectively, with *ant(3'')-Ia* and *ant(3'')-Ib* over a 785-bp length. There is no stop codon at the position corresponding to the *ant(3'')-Ia* and *ant(3'')-Ib* stop codons. We called this part of this ORF *ant(3'')-Ii* (*aadA9*).

To test the AGRP of the *ant(3'')-Ii* sequence alone, two oligonucleotide primers were used for subcloning the fragment that possessed significant homology with the *ant(3'')-I* genes (the upper primer was the M13 15-base sequencing primer, 5' to 3', CCCAGTCACGACGTT; the lower primer was aadA9L, 5' to 3', CGCGGATCCTTAGGCACCAAGCAATTTAGT). Plasmid pGM172 was used as a template for the PCR, which yielded a product of 2,112 bp. A *Bam*HI site was located in the PCR product 945 bp upstream of the *ant(3'')-Ii* initiation codon. A stop codon (TAA), at the location corresponding to the *ant(3'')-Ia* and *ant(3'')-Ib* stop codons, and a second *Bam*HI site were included in the lower primer. The PCR product was digested with *Bam*HI, yielding a 1,731-bp *Bam*HI fragment; this fragment was ligated to the *Bam*HI site of pTZ19r, and the clone was introduced by transformation into *E. coli* NM522. Transformants selected on streptomycin-ampicillin were screened for inserts by agarose gel electrophoresis. A hybrid plasmid (named pST2) containing the 1,731-bp insert was chosen. The AGRP determination of pST2 corresponded, as expected, to an ANT(3'')-I mechanism. The *ant(3'')-Ii-aac(6')-IId* gene cassette is the second in the variable region of the integron and is expressed from a promoter directed toward it from within the class II intron. We are attempting to locate this promoter by primer extension.

Downstream of the *ant(3'')-Ii* sequence, there is a sequence from positions 3457 to 3954 constituting a gene that was previously called *aac(6')-Ib'* but that we renamed (because of the AGRP) *aac(6')-IId*, since the AGRP of the protein is that of an AAC(6')-II, i.e., amikacin sensitive and gentamicin resistant. The *ant(3'')-Ii-aac(6')-IId* gene confers resistance to streptomycin, spectinomycin, and gentamicin, while the cloned *ant(3'')-Ii* sequence confers resistance only to streptomycin and spectinomycin. Nucleotide sequence determination indicated total identity between the *aac(6')-IId* sequence from SCH909 and the *aac(6')-Ib'* sequence from *Pseudomonas aeruginosa* BM2687 (12), which confers resistance to gentamicin but not to amikacin. Like other *aac(6')-Ib'* genes, the *aac(6')-IId* sequence has a T-to-C transition that results in a leucine-to-serine substitution at position 90. This point mutation is responsible for the altered substrate specificity (25).

The *ant(3'')-Ii-aac(6')-IId* gene cassette extends from positions 2563 to 3954. At the 3' end of the *ant(3'')-Ii-aac(6')-IId* gene, there is a typical *attC* site that has a 74-nucleotide sequence (positions 3949 to 4022) with an approximate 20-base similarity at each end that is related to the *attC* site (59-base element) consensus sequence (31). This 74-bp sequence showed 91.9% identity

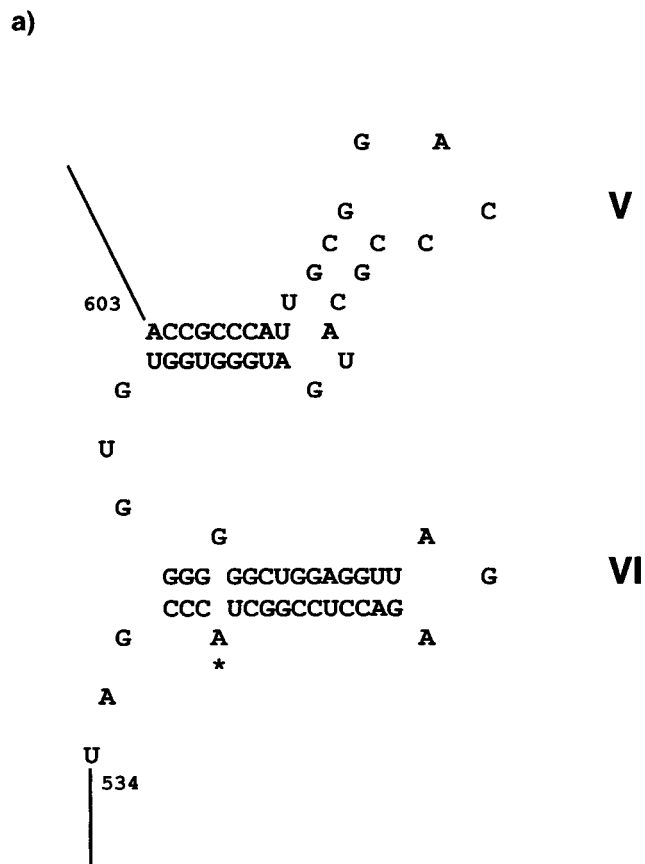


FIG. 3. General features of Smtr and its insertion site. (a) Secondary structure model of domains V and VI of the group II intron, Smtr, within integron 2 from SCH909. The boundary of the intron and the *ant(2'')-Ia attC* site is indicated by a vertical line. The bulging adenine residue involved in lariat formation is indicated by an asterisk. (b) Nucleotide sequence alignment of the *ant(2'')-Ia* stop codon and its *attC* site (59-base element) (4) with the *ant(2'')-Ia* gene and Smtr-intron boundary from SCH909). *ant(2'')-Ia* stop codons are indicated by asterisks. Exon sequences are shown in bold letters. (c) Nucleotide sequence alignment of the group II introns of SCH909 and *N. europaea*. The latter is adjacent to an *attC* site on one end; there is no recognizable ORF on the other end.

with the *aac(6')-Ib'* *attC* site from *P. aeruginosa* BM2687 (12) and 89.2% identity with the *attC* site of *aac(6')-Ib* from Tn1331 (33).

## DISCUSSION

SCH909 has three class I integrons on a single plasmid, two containing integrase genes identical to that of pVS1 (2) and a third identical to that of Tn1696 (35). There are multiple mechanisms for resistance to some antibiotics, e.g., gentamicin [*aac(3)-Ia*, *ant(2'')-Ia*, and *ant(3'')-Ii-aac(6')-IId*] and streptomycin [*ant(3'')-Ii-aac(6')-IId* and two copies of *ant(3'')-Ia*]. Integron 2 contains two unusual features: a fused aminoglycoside resistance gene, *ant(3'')-Ii-aac(6')-IId*, and a group II intron located precisely at the junction of the *ant(2'')-Ia* gene and its *attC* site.

At least two hypotheses can be proposed for the origin of the fused protein: the bifunctional protein could have been created by cassette fusion, or it could have been encoded by an ances-



(34), and fusion with the 5' conserved sequence in pCFF04, which can use a 19-bp repeat in a manner identical to that used for the expression of *aac(3)-Ia* in Tn1696 (35).

An alternate explanation for the origin of the bifunctional protein is that *ant(3'')-Ii-aac(6')-IId* was an ancestral aminoglycoside gene from which *aac(6')-Ib*, *aac(6')-Ib'*, *aac(6')-IIa*, *aac(6')-IIb*, *ant(3'')-Ia*, and *ant(3'')-Ib* evolved by deletion and cassette formation due to selective pressure. There is another case of a bifunctional enzyme described in the aminoglycoside resistance protein family, that encoded by *aac(6')-Ie-aph(2'')* and confined to gram-positive species.

In intron 2, the *ant(2'')-Ia* gene is separated from its *attC* site by a group II intron that we call Smtr. At least two possible routes that would lead to a group II intron within a cassette can be envisaged. The simplest explanation is a direct insertion of Smtr, mediated by one of the two retrotransposition pathways, retrohoming or ectopic insertion (8, 16, 18, 19), at the inverted core site at the junction of *ant(2'')-Ia* and its *attC* site. The SCH909 group II intron Smtr begins with GTACG and ends with GAT, in accord with consensus sequences for the ends of group II introns. The target DNA needed for reverse splicing to occur in Ll.LtrB, the only bacterial group II intron that has been shown to be functional for splicing and mobility in vivo, is rather specific and contains the target site TG'GTTA (20). In contrast, although the complete recognition site of group II intron xln6 from *P. alcaligenes* has not been determined, it contains TTGT'TA (36). The SCH909 group II intron Smtr forms a subgroup with xln6 and also with a group II intron found in 14 copies in genomic DNA of *Pseudomonas putida*, with a site specificity of TTTTGT'T. The complement of the consensus inverted core site of the *attC* site (59-base element) is AATTGT'TAGGC, where the prime corresponds to the point of intron insertion. Notably, in the integron context, the group II intron is "upside down," so that the *attC* site is exon 1 while the *ant(2'')-Ia* structural gene is exon 2. It is unknown what effect transcription arriving from exon 2, in contrast to the situation in Ll.LtrB, where external transcription occurs from exon 1, has on intron functions.

Taking into account the special features of mobility of group II introns, splicing (to remove the intron), retrohoming (in which RNA invades double-stranded DNA), and ectopic insertion (in which RNA invades RNA) (5a, 8, 16, 18, 19), an alternative explanation for the presence of Smtr could involve a role in one or more steps in the formation of cassettes from preexisting structural genes and *attC* recombination sites. It was proposed that cassettes could be formed by reverse transcription (10) mediated by RT activity (27), although no role for introns was mentioned by these authors. A possible mechanism for cassette formation would involve two independent transpositional events placing one intron immediately downstream of a structural gene (such as a resistance gene) and another adjacent to an *attC* site. The next event would involve recombination, possibly *recA* mediated, between the two introns. The next step would involve splicing out of the intron, followed by final reverse transcription to conserve the newly formed cassette at the DNA level. An interesting example of one of these possible intermediates was found in the partial genome sequence of *Nitrosomonas europaea* (Joint Genome Institute [JGI], unpublished data), in which one copy of a novel group II intron which we call Netr is precisely inserted at the

inverted core site of an *attC* site, in the same manner as in SCH909 (Fig. 3c). The other end of this element is not associated with the stop codon of a structural gene; thus, it may represent the intron-*attC* intermediate. A second copy of Netr is adjacent to a putative gene cassette, and there is no *attC* site at its other end; thus, it may be an example of a gene-intron intermediate. The target specificity of the intron, as mentioned above, may explain the precise juxtaposition in many cassettes in integrons of the structural gene stop codon and the inverted core site of the *attC* site. In SCH909, the *ant(2'')-Ia* gene is identical to that of pDGO100 (4), whereas the *ant(2'')-Ia attC* site shows only 84.5% identity with *ant(2'')-Ia attC* sites already described (3). The SCH909 sequence may represent an unspliced intermediate in an independent event of *ant(2'')-Ia* cassette formation.

Recently, three integron sequences submitted to GenBank and containing a gene for  $\beta$ -lactamase VIM-2 from *Acinetobacter* spp. (J. H. Yum et al., unpublished data; accession number AF369871), *P. aeruginosa* (K. Lee et al., unpublished data; accession number AY029772), and *S. marcescens* (K. Lee et al., unpublished data; accession number AY030343) indicated the presence of a group II intron (identical among these three but distinct from that of SCH909) between *ant(3'')-Ia* and its *attC* site (in the first two) or between the *qacF* gene and an *attC* site identical to that of *ant(3'')-Ia* rather than that of *qacF* (in the third). These may be further examples of independent events of cassette formation.

A few scattered *attC* sites (59-base elements), apparently not associated with structural genes and not making up parts of integrons, are found in some partially sequenced genomes, such as those of *Shewanella putrefaciens* (The Institute for Genomic Research, unpublished data) and *N. europaea* (JGI, unpublished). These genomes also contain functional integron integrases (70; G. Léon and P. H. Roy, unpublished data), and closely related integrases have been found in other strains of these species (28). These environmental organisms may serve as reservoirs of integron components.

Group II introns have been described, until now, as being inserted close to mobile elements in eubacteria (11, 21). Moreover, as mobile elements by themselves and located, in the case of SCH909, within antimicrobial resistance gene cassettes, they contribute to DNA rearrangements leading to R plasmid evolution. The elucidation of the mechanism of the formation of integron cassettes remains a key to the understanding of their relationship with the group II introns and their role in aminoglycoside resistance gene evolution and in the accumulation of resistance genes by integrons. We are attempting to determine whether the intron RNA can be spliced to yield a template in which cassette formation could be completed by reverse transcription. If splicing occurs, intron mobility will be tested to determine whether it can be targeted to an *attC* site within a cassette, e.g., *ant(2'')-Ia* (retrohoming), to an *attC* site not associated with a cassette, and to the region of the stop codon of a resistance gene. In *N. europaea*, the two copies of a group II intron are in the latter two contexts, which may be earlier intermediates in cassette formation. Recombination between them would produce a putative later intermediate like that in SCH909, and this process is also being attempted.

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