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# IntI2 Integron Integrase in Tn*7*

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**Integrons can insert and excise antibiotic resistance genes on plasmids in bacteria by site-specific recombination. Class 1 integrons code for an integrase, IntI1 (337 amino acids in length), and are generally borne on elements derived from Tn*5090*, such as that found in the central part of Tn*21*. A second class of integron is found on transposon Tn*7* and its relatives. We have completed the sequence of the Tn*7* integrase gene, *intI2*, which contains an internal stop codon. This codon was found to be conserved among *intI2* genes on three other Tn*7*-like transposons harboring different cassettes. The predicted peptide sequence (IntI2*) is 325 amino acids long and is 46% identical to IntI1. In order to detect recombination activity, the internal stop codon at position 179 in the parental allele was changed to a triplet coding for glutamic acid. The sequences flanking the cassette arrays in the class 1 and 2 integrons are not closely related, but a common pool of mobile cassettes is used by the different integron classes; two of the three antibiotic resistance cassettes on Tn*7* and its close relatives are also found in various class 1 integrons. We also observed a fourth excisable cassette downstream of those described previously in Tn*7*. The fourth cassette encodes a 165-amino-acid protein of unknown function with 6.5 contiguous repeats of a sequence coding for 7 amino acids. IntI2*179E promoted site-specific excision of each of the cassettes in Tn*7* at different frequencies. The integrases from Tn*21* and Tn*7* showed limited cross-specificity in that IntI1 could excise all cassettes from both Tn*21* and Tn*7*. However, we did not observe a corresponding excision of the *aadA1* cassette from Tn*21* by IntI2*179E.**

Integrons are genetic elements that permit tandem integration and expression of mobile cassettes coding for antibiotic resistance genes (18, 31, 37). The gene cassettes move by a conservative site-specific recombination mechanism, catalyzed by an integrase encoded by the integron 5′-conserved segment (5′-CS) (29, 37). The integrase belongs to the phage integrase family of recombinases (3), now called tyrosine recombinases (13). Integration of cassettes generally occurs into a target site *attI*, at the junction between the 5′-CS and the first cassette (Fig. 1) (11, 32). A second type of cassette recombination sites are the *attC* sites (59-base elements) which occur either at the 3′ end of each inserted cassette (17, 18, 31, 38) or in a free cassette circle (10). The initially described type of integron (referred to as class 1) is generally borne on elements similar to Tn*5090* (30) or on related defective transposons (8), which sometimes "piggyback" on competent transposons to form Tn*21* and its relatives. In addition, a second class of integron occurs on the nonreplicative transposon Tn*7*. Tn*7* preferentially inserts into a unique site in bacterial chromosomes (16, 21, 23) from which it is transferred to other bacterial cells by less-specific transposition onto a conjugative plasmid (46). The question of whether or not Tn*7* carries a functional integron is interesting because it would add to the multiplicity of systems for gene exchange among microorganisms.

The integron of Tn*7* (Fig. 1) has an organization similar to that of the class I integrons and carries three resistance gene cassettes—*dfrA1*, *sat*, and *aadA1* (38, 39, 40)—close to an open reading frame, IntI2*, that is related to the class 1 integrase, IntI1, of Tn*21* (Fig. 1) (19, 29). A variation of the cassette content in Tn*7* was previously observed among related elements such as Tn*1825* and Tn*1826* (42) and Tn*4132* (49). Since two cassettes in Tn*7*, *dfrA1* and *aadA1*, have also been observed in class 1 integrons (14, 40), some cassettes appear to have been transferred among integron classes. Part of the integrase gene on Tn*7* was included in a sequence by Simonsen et al. (36). The nucleotide sequence included an internal stop codon (TAA) in the *intI2* gene. We reverified the presence of the internal stop codon which we also found to be present in the *intI2* genes of Tn*1825* and Tn*1826* (Fig. 1). The sequence for *intI2* in Tn*7* was completed here by sequencing the previously unknown 3′ end of the gene, confirming that *intI2* is closely related to *intI1* of Tn*21* and also resembles an integrase gene from a third integron class (*intI3*) (1).

We show here experimentally that the second class of integrase in Tn*7* can promote recombination between cassette recombination sites present in the same plasmid (excision) or in different plasmids (cointegration), but only after changing the termination codon to a sense codon. Recombination activity was assayed in vivo by expressing the Tn*7* integrase from a repaired form of *intI2*, *intI2*179E*, in which the internal stop codon has been altered into a triplet (GAG) encoding glutamic acid. Analysis of cassette recombination involving the class-specific *attI* sites revealed that the Tn*7* integrase seems to be more restricted to recognition of its own *attI* site than the Tn*21* integrase.

The three previously defined cassettes borne on Tn*7* mediate resistance to trimethoprim (*dfrA1* [15, 40]), streptothricin (*sat* [39, 42]) and spectinomycin (*aadA1* [14]). We checked for further excisable units and found a fourth cassette downstream of those described previously. This new cassette lacked the

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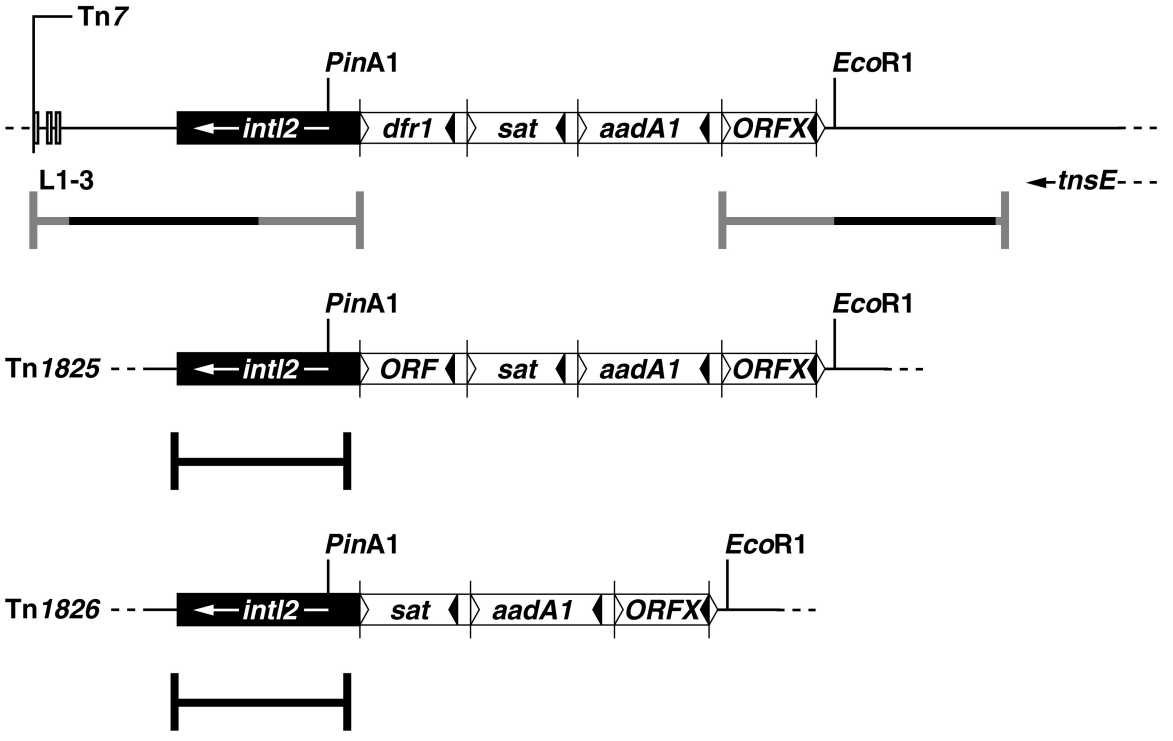


FIG. 1. Maps of the integron regions in Tn*7* and in the related transposons Tn*1825* and Tn*1826*. Integron sequences are represented by boxes. Black boxes indicate the conserved sequence (5′-CS-2) with the integrase gene, *intI2*. Cassettes are shown by white boxes. Leftward-pointing filled arrowheads denote inverse core sequences inside the cassettes, and rightward-pointing open arrowheads denote core sequences where DNA strand transfer occurs. Vertical lines at cores indicate cassette borders. The *Pin*AI and *Eco*RI restriction enzyme cut sites used for cloning the cassette regions are indicated. Nucleotide sequences determined in this study are underlined. Newly determined sequences are indicated with heavy black lines, while heavy gray lines indicate previously determined sequences that were checked in the present study. Vertical boxes denote the 22-bp repeats, L1-3, for transposase binding, which are clustered close to the left Tn*7* end (2).

palindromic *attC* site that is generally found at the 3′ end of cassettes.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids are listed in Table 1. *Escherichia coli* strains were cultured in twofold-concentrated YT broth (27), Luria broth (6), or IsoSensitest medium (Oxoid) supplemented with the appropriate antibiotic(s) at 37°C. The antibiotic concentrations used were as follows: ampicillin (50 μg $ml^{-1}$), spectinomycin (50 μg $ml^{-1}$), trimethoprim (50 μg $ml^{-1}$), streptothricin (2 μg $ml^{-1}$), chloramphenicol (40 μg $ml^{-1}$), streptomycin (50 μg $ml^{-1}$), sulfathiazole (0.5 mM), and nalidixic acid (25 μg $ml^{-1}$).

**Standard DNA techniques.** Construction of recombinant DNAs, transformations, and restriction enzyme digestions were done according to standard procedures (34). Plasmid DNA preparations were performed with the Wizard Minipreps DNA Purification System (Promega) according to the manufacturer's instructions. Restriction enzymes, T4 DNA polymerase, polynucleotide kinase, and exonuclease III were from Boehringer Mannheim. T4 DNA ligase was from New England Biolabs. 5′-Methyl-dCTP and oligonucleotides (see Table 2) were purchased from Pharmacia LKB Biotechnology. For DNA sequencing, the dideoxyribonucleotide chain-terminating method developed by Sanger et al. (35) was used with synthetic primers listed in Table 2. The radioactively labeled compound [α-$^{35}$S]dATP was from Amersham, and T7 DNA polymerase Sequenase version 2.0 was from U.S. Biochemical Corp., Cleveland, Ohio. Electrophoresis gels for sequencing contained 6% acrylamide-*N*,*N*′-methylenebisacrylamide (30:1) and 40% urea.

**DNA sequencing strategies.** The integrase genes from Tn*7*, Tn*1825*, and Tn*1826* were amplified with PCR primers Tn7-int32 and Tn7-int55 (Table 2) by using *E. coli* DH5α R483 (Tn*7*), IE972 (Tn*1825*), and IE970 (Tn*1826*) as templates. At least three independent amplification products of each integrase gene were sequenced on both strands by cloning in both orientations in M13mp18 and M13mp19. The DNA sequence was determined by using seven different oligonucleotides: universal 17-nucleotide primer specific for M13mp18 and M13mp19, Tn7-int32, Tn7-int36, Tn7-int37, Tn7-int38, Tn7-int53, and Tn7-int55 (Table 2). Three separately amplified products of the 860-bp DNA fragment obtained with primers Tn7left1 and Tn7left2 were cloned in both orientations in M13mp18 and M13mp19 and sequenced on both strands by using Tn7left1, Tn7left2, Tn7left3, Tn7left4, Tn7left5, Tn7left6, Tn7left7, Tn7left8, and Tn7left9 as primers (Table 2). The 797-bp *Eco*RI-*Hin*dIII fragment located between the *aadA1* and *tnsE* genes in Tn*7* was cloned in M13mp18 and M13mp19. Subclones were obtained by using restriction enzymes *Sau*3A and *Xba*I and the nucleotide sequence was determined on both strands by using Tn7right1 and the universal 17-nucleotide primer specific for the M13 vectors.

**Mutagenesis of the internal stop codon in the *intI2* gene.** Directed point mutations in the internal stop codon replacing amino acid 179 in *intI2* of Tn*7* were made according to the procedure described by Vandeyar et al. (44). A 1.6-kb *Sph*I-*Hpa*I fragment containing the Tn*7* integrase gene from plasmid R483 was cloned in M13mp18. M13 clones were grown on *E. coli* strain JM105. Extracted single-stranded phage DNA was used as a template for the mutagenesis reaction, together with phosphorylated mutagenesis primer Tn*7*int*179E (Table 2). Mutant phage DNA was propagated in *E. coli* CU9276. Mutants were detected by restriction enzyme cleavage of a *Sac*I site that was created by changing the stop codon (TAA) into a codon for glutamic acid (GAG). The mutants were further confirmed by DNA sequencing. Subcloning of the mutated gene into pUC19 resulted in plasmid 636*179E (T535G, A537G). A clone carrying the corresponding parental gene fragment was called plasmid 636.

**PCR assay for detection of site-specific recombination.** Divergent primers specific for the cassettes of Tn*7* or Tn*21* (Table 2) were used for inverse PCR (28) to detect site-specific recombination between cassette ends. The cassette region from Tn*7* was cloned on a 2.9-kb *Pin*A1-*Eco*RI fragment into the *Xma*I and *Eco*RI sites in the polylinker of vector pSU18 and pSU19 to form plasmids 670 and 671. Plasmids 2123 and 2280 contained the *Sph*I-*Hin*dIII cassette region from the integron of Tn*21* cloned in pSU18 and pSU19, respectively (Table 1). The cassette clones, together with an integrase overproducing plasmid (636*179E or 2101, Table 1), were introduced into *E. coli* DH5α. Template DNA

TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Description[a] | Source or reference |
|---|---|---|
| *E. coli* | | |
| DH5α | *supE44* Δ*lacU169* (Φ80 *lacZ′*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA* | 47 |
| HB101 | $F^-$ *hsdS20*($r_B^-$ $m_B^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20* ($Sm^r$) *xyl-5 mtl-1 supE44* $\lambda^-$ | 7, 34 |
| JM105 | *thi rpsL endA sbcB15 hspR4* Δ(*lac-proAB*) F′ *traD36 proAB lacI*$^q$ ZΔM15 | 48 |
| CU9276 | *hsdR17 mcrAB recA1* Δ(*lac-proAB*) F′ *traD36 proAB lacI*$^q$ZΔM15 | 44 |
| IE972 | V601::*thr leu thi* $Rif^r$ (Tn*1825*) | 43 |
| IE970 | J53::*met pro* (Tn*1826*) | 43 |
| Plasmids | | |
| R388 | $Tp^r$ $Su^r$ $Tra^+$ | 38, 41 |
| R388Δ1 | R388 deleted for the *dfr2b* and *ORFA* cassettes; $Su^r$ $Tra^+$ | 20 |
| R483 | $Tp^r$ $Stri^r$ $Sp^r$ | 4 |
| pUC18/19 | $Ap^r$ | 48 |
| pSU18/19 | $Cm^r$ | 5 |
| 636 | 1,620-bp *Sph*I-*Hpa*I fragment from R483 in pUC19; $Ap^r$ | This study |
| 636*179E | Plasmid 636 with a point mutation, changing the stop codon in the integrase gene at position 179 to a glutamic acid; $Ap^r$ | This study |
| 670 | 2,919-bp *Pin*A1-*Eco*RI fragment from R483 in pSU18; $Cm^r$ $Tp^r$ $Stri^r$ $Sp^r$ | This study |
| 671 | 2,919-bp *Pin*A1-*Eco*RI fragment from R483 in pSU19; $Cm^r$ $Tp^r$ $Stri^r$ $Sp^r$ | This study |
| 674 | 352-bp *Sph*I-*Pst*I PCR fragment (Tn*7-107*, cup2) from plasmid 670 (deleted for the *dfr1* and *sat* cassettes) in pSU19; $Cm^r$ | This study |
| 675 | 439-bp *Xba*I-*Pst*I PCR fragment (Tn*7-107*, cup2) from plasmid 670 (deleted for the *dfr1* and *sat* cassettes) in pSU18; $Cm^r$ | This study |
| 678 | Plasmid 670 deleted for the *dfr1* and *sat* cassettes; $Cm^r$ $Sp^r$ | This study |
| 699 | Plasmid 670 deleted for the *dfr1* cassette; $Cm^r$ $Stri^r$ $Sp^r$ | This study |
| 2101 | 1,176-bp *Rsa*I-*Bam*HI of Tn*21* in pUC18; $Ap^r$ | 20 |
| 2123 | 1,618-bp *Sph*I-*Hin*dIII PCR fragment (pSph, pHin) from Tn*21* in pSU18; $Cm^r$ $Sp^r$ | 20 |
| 2213 | 900-bp *Sph*I-*Pst*I PCR fragment (pSph, cup2) from Tn*21* in pSU19; $Cm^r$ | 20 |
| 2280 | 1,618-bp *Sph*I-*Hin*dIII PCR fragment (pSph, pHin) from Tn*21* in pSU19; $Cm^r$ $Sp^r$ | This study |

[a] $Sm^r$, streptomycin resistance; $Stri^r$, streptothricin resistance; $Ap^r$, ampicillin resistance; $Sp^r$, spectinomycin resistance.

was prepared by boiling one loop of bacteria in 200 μl of water. Four sets of divergent PCR primers specific for the cassettes in Tn*7* were used (cdo11+cup11, sat1up2+sat1do2, cdo2+cup2, and Tn*7*Fling+Tn*7*CRS+185, Table 2). PCR products arise when recombination correctly juxtaposes the two primers, either by circularization of an excised cassette or by tandem duplication of a cassette. The PCRs were run for 30 cycles at 94, 60, and 72°C by using a DNA thermal cycler 480 (Perkin-Elmer). Oligonucleotides specific for sequences flanking the mobile cassettes in the integrons of Tn*7* and Tn*21* (Table 2) were used for convergent PCR to detect cassette deletions. The PCR products obtained were cloned into the polylinker of pUC19, and the crossover points were confirmed by nucleotide sequencing.

**Phenotypic test to detect cassette deletions.** Overnight cultures of DH5α harboring the clone overproducing integrase (plasmid 636*179E or plasmid 2101) and the plasmid to be tested were grown in IsoSensitest broth supplemented with ampicillin and under selection for the plasmid-borne cassettes and then diluted 1,000-fold in IsoSensitest broth without selection. DNA was prepared after cultivation at 37°C overnight, and 100 ng was used for the transformation of plasmid-free DH5α. Loss of resistance to antibiotics due to cassette deletions was checked among 600 colonies grown on chloramphenicol plates. Analogous experiments performed in the presence of plasmid 636wt (with the internal stop codon in the *intI2* gene) did not result in any loss of antibiotic resistance cassettes.

**Mating-out assay for site-specific recombination.** Donor *E. coli* cells were constructed by introducing R388 (trimethoprim resistant [$Tp^r$], sulfonamide resistant [$Su^r$]) or R388Δ1 ($Su^r$) into HB101 by conjugation, followed by transformation of the nonconjugative pSU18-clone (chloramphenicol resistant [$Cm^r$], Table 1) to be analyzed and the clone producing integrase (plasmid 636*179E or plasmid 2101). Exponentially growing cultures of donor cells (0.5 ml) were mixed with 0.5 ml of the recipient strain (DH5α, nalidixic acid resistant [$Nal^r$]) in stationary phase. The mix was pelleted, and the cells were resuspended in 100 μl of Luria broth, spread on a Millipore 25-mm (pore-size) filter on a fresh IsoSensitest agar plate, and incubated at 37°C for 2 h. Filters were washed in 1 ml of 0.9% NaCl solution, and dilutions were plated on agar plates supplemented with nalidixic acid and either sulfathiazole or chloramphenicol to determine the recombination frequency. The recombination frequencies (incidence of cointegrate formation between either R388 or R388Δ1 and pSU18 derivatives) are given as the number of $Cm^r$ transconjugants per $Su^r$ transconjugant. Plasmid fusions in $Nal^r$ and $Cm^r$ transconjugants were analyzed by PCR by using either a cassette-specific primer (cup2 for the *aadA1* cassette, sat1-down2 for the *sat* cassette, and cup11 for the *dfrA1* cassette) and a primer hybridizing to the conserved sequence in R388 or R388Δ1 (pSph). To analyze the reciprocal crossover product in the cointegrates, a primer specific for *qacE*Δ*1* (pHin), together with a vector-specific primer, was used. The PCR products were analyzed by nucleotide sequencing to confirm crossover points.

**Nucleotide sequence accession number.** The nucleotide sequences reported in this study have been assigned accession numbers AJ001816 and AJ002782 in the EMBL, GenBank, and DDBJ databases. The former sequence is a revision of the previously released sequence L10818.

## RESULTS

**Sequence characterization of the left end of Tn*7*.** In this work we have sequenced the complete *intI2* gene, and all areas of Tn*7* beyond those that have previously been analyzed, i.e., *dfrA1* (15, 36), *sat* (42), *aadA1* (14), and *tnsA-tnsE* (15a). The new data cover regions on either side of the cassettes, as indicated by horizontal lines in Fig. 1. The proteins coded by the new gene *intI2* (325 codon triplets) and by *intI1* (337 codon triplets) of Tn*21* showed 46% amino acid identity, which indicates a closer relation than to other tyrosine recombinases and a possible role of IntI2* in incorporation of cassettes into Tn*7*. However, translation of *intI2* is interrupted by an internal ochre codon (TAA) at amino acid position 179 (Fig. 2A and B), which must be suppressed or replaced in order to produce the full-length product. Unless read-through of TAA is allowed, a shorter and probably inactive polypeptide of 178 amino acids would be produced. The *intI2* genes of both transposons Tn*1825* and Tn*1826* (Fig. 1) (42), which carry cassette arrangements different from those of Tn*7*, were sequenced

(underlined areas in Fig. 1; positions 940 to 1970 in Fig. 2A). The sequences of the complete *intI2* genes were identical, and all have a stop codon interrupting translation at position 179.

The amino acid identity between IntI1 and IntI3 from *Serratia marcescens* isolate AK9373 (1) is higher (59%) than between IntI2* and either IntI1 or IntI3 (46% in both instances) or IntI4 (48%) (Fig. 2B). The IntI1 (9, 26), IntI3 (F. Gagnon and P. H. Roy, unpublished results; M. Gullberg, K. Hansson, and L. Sundström, unpublished results), and IntI4 (33) proteins have been shown to be functional. We provide evidence below that the IntI2* protein is functional after mutagenesis of the internal stop codon to a glutamic acid codon (IntI2*179E). The sequence between the *intI2* gene in Tn*7* and the divergently transcribed cassettes showed no obvious resemblance to the corresponding regions in Tn*21* or in the class 3 integron in *S. marcescens*.

The 936-bp sequence (position 1 to 936 in Fig. 2A) downstream of the integrase gene *intI2* revealed no open reading frame of significant length. The beginning of this sequence contained the cluster of three transposase binding sites—L1, L2, and L3 (23)—with a defined role in transposase binding (2). It is an interesting coincidence that the 3′ end of the integrase gene *intI1* in Tn*5090* (30) is located even closer to similar binding sites that appear to be involved in transposon mobility.

**Sequence of the region between *aadA1* and *tnsE*.** The third cassette in Tn*7* contains the *aadA1* gene encoding an adenylyltransferase, which inactivates streptomycin and spectinomycin (14) (Fig. 1). The partially sequenced region downstream of *aadA1* was checked and extended to fill in a previously unsequenced region. The beginning of the palindromic *attC* site (59-base element), occurring at the 3′ end of each cassette, generally is an inverse complement of the first nucleotides in the 5′ end of the same cassette (20). This complementarity between bases 4240 to 4248 and bases 4761 to 4769 (Fig. 2A) indicated the possibility of a fourth cassette downstream of *aadA1* in Tn*7*. The cassette contains an open reading frame of 165 amino acids (named ORFX; Fig. 1 and 2A). ORFX was not followed by an *attC* site of the usual palindromic structure, such as those at the downstream ends of the *dfrA1*, *sat*, and *aadA1* genes. Functional studies (see below) defined the terminal crossover points of the cassette. The function of the 165-amino-acid polypeptide remains unknown, but a series of 6.5 peculiar repetitions of a sequence coding for seven amino acids was observed (Fig. 2C). The amino acid sequence is almost completely conserved among the repeats, whereas the nucleotide sequence varied somewhat, hinting that ORFX might have a biological function.

The nucleotide sequence between ORFX and the *Hin*dIII site at position 5716 contained no open reading frame of known function. An 85-nucleotide region located just 3′ of the newly identified cassette showed full identity to a sequence downstream of a gene described in *Arabidopsis thaliana* (GenBank/EMBL file no. M55553 [24]), but this may be due to a cloning artifact in the latter.

**Excision of a fourth cassette, ORFX, in Tn*7*.** The inverse PCR assay (see Materials and Methods and Fig. 3A) allowed detection of recombination products corresponding to excision of a unit containing the 165-amino-acid ORFX. The bands indicative of recombination were observed in the presence of overproduced IntI1 or IntI2*179E (see below). The crossover points were determined to be at positions 4239 to 4243 and positions 4776 to 4780 (Fig. 2A) by nucleotide sequencing of PCR products (data not shown). Although sequence identities at the ends of the cassette did not permit exact determination of the crossover point, it most likely is between G and TT in the recombination sites as shown previously (20, 32). The 3′ end of the cassette lacks a complete *attC* sequence but shows identity to 13 bases, CTAATAAAATGTT (nucleotides 2215 to 2227 in Fig. 1), of *attI2* in Tn*7*.

TABLE 2. Oligonucleotides

| Oligonucleotide | Sequence | Positions[d] |
|---|---|---|
| cdo2 | TATCTGCAGTTTGGAGAATGGCAGCGC | 465–488[a] |
| cup2 | TATCTGCAGTTOGCGCTTAGCTGG | 469–450[a] |
| cdo11 | TATCAAGCTTTACATCTGACAATGAG | 787–808[b] |
| cup11 | TATAAGCTTGAACGTGTTACGACCGC | 792–770[b] |
| Tn7int*179E | CCCAGCAATAAAAGAGCTCATTGAGCAAGC | 1393–1364 |
| Tn7SRS-107 | TATCTAGAAACAGAGTGTCTTG | 2118–2131 |
| Tn7CRS+185 | TTAAAGCTTATGGAGTTGTCGTAGTTGC | 4424–4402 |
| Tn7 Fling | ATAAAGCTTACGACAATAGTCCATC | 4426–4444 |
| Tn7-int32 | ATTGGATCCTGATTGATAAGTAG | 940–954 |
| Tn7-int36 | AGTGACACGCTTGCTAAC | 1123–1140 |
| Tn7-int37 | AGTTGTCGTCTTGCTGAATAAG | 1338–1359 |
| Tn7-int38 | TAACTTGGTTGCGAGTATCC | 1542–1561 |
| Tn7-int53 | ATCATCTGCATGACTCCGTTG | 1202–1182 |
| Tn7-int55 | TATGCATGCTAGAATAGGCTGTATAG | 1962–1944 |
| Tn7left1 | TAGAATTCGTAGCGTCGTAAGCTAATAC | 95–115 |
| Tn7left2 | TTGGATCCTGCGGATGGACTGATG | 973–955 |
| Tn7left3 | TCCTATATCCAACTGATCAT | 204–223 |
| Tn7left4 | ATAAACAATCATAGAGGG | 875–858 |
| Tn7left5 | ACAGTAACAAGCATTACGAGG | 415–435 |
| Tn7left6 | TTGTTACTCCAAGAGCTTG | 585–603 |
| Tn7left7 | AATGGTCACTGTGATTGACAG | 787–807 |
| Tn7left8 | TCTGAAGCACATTTCTTATAC | 629–651 |
| Tn7left9 | ATGTTAATGGGCTATTACAATG | 391–369 |
| Tn7right1 | AAAGAAGATCAGACTCGTAG | 5278–5259 |
| Universal 17-nt[e] | GTAAAACGACGGCCAGT | |
| Reverse 16-nt | AACAGCTATGACCATG | |
| sat1up2 | ATGGAACGATCTAGCCTCTAT | 249–269[c] |
| sat1do2 | TGTGCGCGACTCCTTTGCCTC | 319–299[c] |
| pHin | GGCAAGCTTAGTAAAGCCCTCGCTAG | 2055–2030[a] |
| pSph | TGATCCGCATGCCCGTTCCATACAG | 709–733[a] |

[a] Published sequence (37).
[b] Published sequence (39).
[c] Published sequence (38).
[d] Primer positions are as in Fig. 2A except as noted.
[e] nt, nucleotide.

**Substitution of the internal stop codon in the Tn*7* integrase gene with a sense triplet.** To test whether the IntI2* protein of Tn*7* could be activated, we changed the internal ochre codon (TAA) into a triplet (GAG) coding for glutamic acid, the corresponding amino acid found in IntI1. After site-directed mutagenesis, we cloned the 1.6-kb *Sph*I-*Hpa*I fragment carrying the *intI2*179E* gene into pUC19 to form plasmid 636*179E. The ability of this clone to promote recombination between cassette sites was tested by using the inverse PCR assay (primers designed to give a product only if recombination has occurred) and the convergent PCR assay (primers arranged to give a smaller product if recombination has occurred) as described in Materials and Methods. As a recombination substrate, we used plasmid 670 containing the cassette region from the Tn*7* integron (Fig. 3A). The inverse primers specific for the *dfrA1*, *sat*, *aadA1*, and ORFX cassettes revealed recombination products in the presence of plasmid 636*179E,

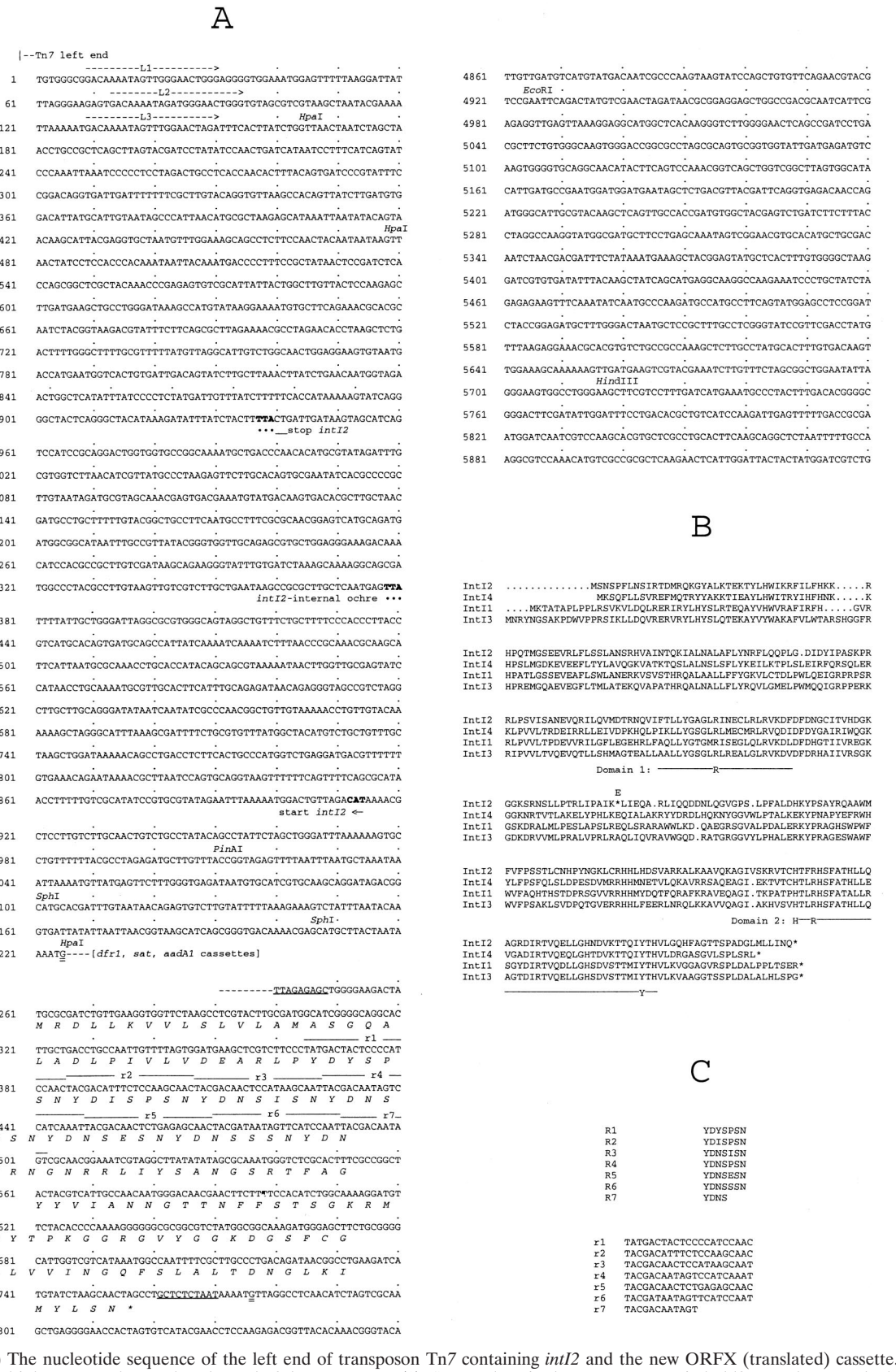


FIG. 2. (A) The nucleotide sequence of the left end of transposon Tn*7* containing *intI2* and the new ORFX (translated) cassette. L1 to L3 indicate the multiple repeats clustered at the end of the transposon (2). Stop codons in the *intI2* gene are in boldface and are marked with three dots below the sequence. The start codon of the gene is shown with an arrow. The G in the GTT sequences at the ORFX cassette borders are underlined by double dashed lines. Underlined sequences mark the core and inverse core in the ORFX cassette. The peculiar repetitions in ORFX

from which the repaired Tn*7* integrase (IntI2*179E) was overexpressed (Fig. 3A). These results were confirmed by PCR with convergent primers binding on either side of a given cassette (Fig. 3A). Sequencing of the PCR products revealed that DNA strand crossover promoted by IntI2*179E occurred between the GTT sequences at the ends of the cassettes (data not shown). Plasmid 636 encoding IntI2* failed to promote cassette recombination in any of the assays used here.

**IntI1 and IntI2*179E show different substrate specificities.** We tested the ability of IntI1 and IntI2*179E to mediate crossovers at *attC* or *attI* sites in Tn*7* and Tn*21*. All cassettes except the first cassette in an integron have *attC* sites at both ends, which should be recognized by all integron integrases. Both IntI1 and IntI2*179E mediated recombination between the *attC* sites at the ends of the *aadA1* cassette in Tn*7* (plasmid 670; Fig. 3A) (45). Qualitative detection of recombination was done by using the convergent and inverse PCR assays (Fig. 3A).

In contrast to the *attC* sites, the *attI* site includes the end of the class-specific 5′-CS and the beginning of the first cassette in an integron. Apart from an abundance of A, these conserved sequences are unique for each class (20) and recombination involving these *attI* sites could be class specific. Plasmid 2280 contains the *aadA1* cassette adjacent to the 5′-CS-1 (*attI1* of Tn*21* [38]), whereas in plasmid 678 *aadA1* has been inserted adjacent to the 5′-CS-2 (*attI2* of Tn*7*) (Fig. 3B). IntI2*179E mediated recombination, as assayed by PCR with both divergent and convergent primers, between the *attI2*×*attC* sites of *aadA1*, excising this cassette when it was adjacent to 5′-CS-2 in plasmid 678 (Fig. 3B) but not when it was adjacent to 5′-CS-1 (*attI1*×*attC*; plasmid 2280). IntI1, however, mediated both *attI1*×*attC* and *attI2*×*attC* recombination, excising the *aadA1* cassette when adjacent to either *attI1* or *attI2*, and therefore seems to have a wider site specificity than IntI2*179E.

Which cassette is incorporated adjacent to the 5′-CS also affects the *attI* site (20). The *attI2* sites including the first six bases of the *dfrA1* or *sat* cassettes (plasmids 670 [Fig. 3A] and 699) were also shown by PCR detection to recombine in the presence of either IntI1 or IntI2*179E.

We conclude that IntI2*179E has a narrower specificity for *attI* sites compared to IntI1, which can mediate recombination involving either *attI1* or *attI2*. The result was the same regardless of which cassette was inserted at *attI*, and the specificity therefore depends mainly on the conserved sequence 5′ of the crossover point.

**Quantitation of excision frequency as measured by phenotypic tests.** The loss of antibiotic resistance markers borne on cassettes gives quantitative information of how efficient recombination is between cassette end sites. Cassette excision studied by the phenotypic tests for trimethoprim, streptothricin (39), and spectinomycin with plasmid 670 as substrate showed that the *dfrA1*, *sat*, and *aadA1* cassettes were lost at frequencies of 1.1, 13, and 10%, respectively, in the presence of IntI2*179E (Table 3). Deletion rates were also measured for the *aadA1* cassette located in the first position in class 1 and class 2 integrons (plasmids 2280 and 678 in Table 3). In the presence of IntI2*179E, the *aadA1* cassette was lost at 0.33% when adjacent to 5′-CS-2, whereas no loss was detected when the same cassette was adjacent to 5′-CS-1. IntI1-mediated excision of the *aadA1* cassette from both plasmids 2280 and 678 took place, but excision was much more efficient when the cassette was adjacent to 5′-CS-1 (59% from 2280) than when the cassette was adjacent to 5′-CS-2 (0.7% from 678). This demonstrates that, while IntI1 possesses some degree of specificity for its own *attI* site, IntI2*179E discriminates against a class 1 *attI* site. IntI1 also seems to be a more efficient enzyme than IntI2*179E since it can delete the *aadA1* cassette when the latter is adjacent to 5′-CS-2 more efficiently than does IntI2*179E (Table 3). However, IntI2* may have undergone genetic drift, or glutamate may not be the optimal amino acid at position 179.

**Cassette deletion is dependent on position and direction of transcription through the Tn*7* integron.** Phenotypic tests were used to determine the frequencies of integrase-mediated excision of cassettes from the Tn*7* integron cloned in plasmids 670 and 671 to yield both orientations of the cassette arrays in the polylinker sites of pSU18 (Table 3). In plasmid 671, the cassettes were transcribed from the integron promoter in 5′-CS-2 (positions 1930 to 1959, Fig. 2A), as well as from the *lac* promoter in the vector. In plasmid 670, the *lac* promoter was reversed compared to the cassettes such that transcription from the integron promoter alone ensured expression of the phenotype (resistance). In the presence of IntI1 integrase the *dfrA1*, *sat*, and *aadA1* cassettes were lost at frequencies of 1.5, 5.2, and 73%, respectively. Placing the *lac* promoter in tandem with the cassette promoter (plasmid 671) significantly reduced the rate of cassette excision for all three cassettes tested (Table 3). Among 600 colonies checked the *aadA1* cassette was lost from plasmid 671 at 18%, whereas no excision of either *sat* or *dfrA1* cassettes was observed (Table 3).

The effect of the placing the *lac* promoter in tandem with the cassette promoter was similar with IntI2*179E. The excision rates for all cassettes were much lower with plasmid 671 than with plasmid 670 (Table 3). Our results thus suggest that the orientation of a cassette relative to the vector promoter influences the rate of excision. The effect of the vector promoter is noteworthy; antisense transcription or reduced forward transcription enhances loss of cassettes.

**Site-specific recombination between integrons of classes 1 and 2.** Plasmid clones of the Tn*7* integron containing all cassettes (plasmid 670) or two of the cassettes (*aadA1* and ORFX; plasmid 678) efficiently formed cointegrates with the conjugative plasmid, R388 (harboring a class 1 integron with two complete cassettes, *dfrB2* and ORFA) in the presence of either IntI1 or IntI2*179E (Fig. 4). This mating-out assay for cointegration was first described by Martinez and de la Cruz (25; see

are marked with horizontal lines above the sequence and are indexed by r1 to r7. The sequence at positions 4782 to 4856 is identical to a sequence in *Arabidopsis* (M55553). (B) Comparison of the amino acid sequences of the translated *intI1*, *intI2*, *intI3*, and *intI4* (also called Vch*intIA*). The well-conserved domains 1 and 2, with the four amino acids marked that are highly conserved among all members of the tyrosine integrase family, are underlined. (C) Nucleotide sequences and translated amino acid sequences of the 6.5 repetitions in ORFX. The sequence from positions 940 to 1970 was found to be identical in Tn*1825* and Tn*1826*.

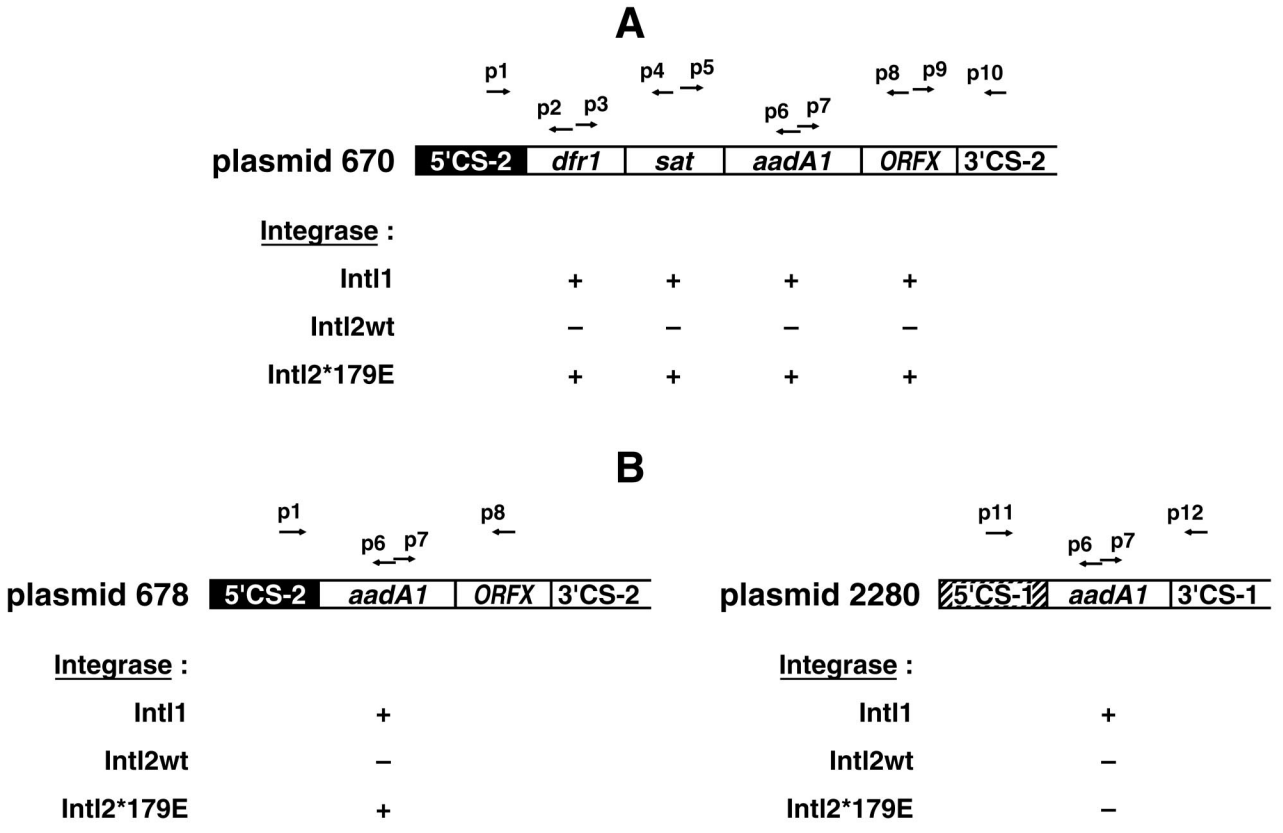


FIG. 3. Site-specific recombination between cassette ends promoted by IntI1 or IntI2*179E from cloned integron fragments of class 1 and class 2 integrons. Recombination was detected by divergent and convergent PCR as described in Materials and Methods. The ability of the different integrases to promote excision of the tested cassettes is denoted by plus and minus signs. Identical results were obtained with divergent and convergent PCR assays. (A) Plasmid 670 carrying the cassette region from Tn*7* (class 2 *attI* site). (B) Plasmid 678 with the cassette region from Tn*7* deleted for the *dfrA1* and *sat* cassettes and plasmid 2280 carrying the cassette region from Tn*21* (Class 1 *attI* site). White boxes indicate cassettes, and arrows indicate the positions of the PCR primers used in this study. Black boxes indicate the conserved sequence (5′-CS-2) from the class 2 integron of Tn*7*, while the conserved sequence from the class 1 integron in Tn*21* is hatched (5′-CS-1). The primers used are denoted as follows: p1, Tn7SRS-107; p2, cup11; p3, cdo11; p4, sat1do2; p5, sat1up2; p6, cup2; p7, cdo2; p8, Tn7CRS+185; p9, Tn7Fling; p10, reverse; p11, pSph; and p12, pHin.

also Materials and Methods). The most efficient recombination site of R388 is an *attC* downstream of ORFA (26), and most cointegrates result from crossovers at that site. Plasmid clones harboring an *attI2* site (derivatives 674 and 675 in Fig. 4) were transferred with R388 at frequencies in the range of 100 per million matings. The rates were similar in the presence of IntI1 and IntI2*179E. For crossing over between *attI1* (plasmid 2213) and R388, however, the IntI1 recombinase gave high rates (72,000 per million matings) of recombination, while IntI2*179E gave only background rates, thus confirming a specificity of the IntI2*179E protein for its own *attI2* site.

Site-specific recombination has been demonstrated between two *attI1* sites in the absence of the palindromic *attC* sites (20). To exclude the efficient *attC* sites from the recombination system a modified R388 (R388Δ1 [20]) harboring only an *attI1* site was used. The frequencies of crossover between the *attI1* in R388Δ1 and an *attI2* (plasmids 674 and 675 in Fig. 4) were measured. In the presence of IntI1, the recombination rates for both plasmids were 13 to 19 $\times$ $10^{-6}$, whereas IntI2*179E gave insignificant recombination. In comparison, the recombination between two *attI1* sites was previously shown to occur at 430 $\times$ $10^{-6}$ in the presence of IntI1 (20). These results again indicate a specificity of the IntI2*179E protein for its own *attI2* site.

The crossover sequences in recombination between an *attI1* and an *attI2* were localized to within the core triplets GTT by nucleotide sequencing across crossover points in cointegrates formed by site-specific recombination. These results agree with previous data for the products of recombination between two *attI* sites of class 1 (20).

## DISCUSSION

Tn*7* is a remarkably widespread transposon which commonly accounts for trimethoprim resistance ($Tp^r$) in gram-

TABLE 3. Deletion frequencies of cassettes borne on class 1 and 2 integrons promoted by class 1 and 2 integrases

| Plasmid[a] | *attI* class | Integrase | Cassette deletion frequencies (%)[b] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | *dfr1* | *sat* | *aadA1* | *dfr1 aadA1* | *sat dfr1* | *aadA1 sat* | *aadA1 sat dfr1* |
| 670 | 2 | IntI2* | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 | IntI2*179E | 1.1 | 13 | 10 | 0.17 | 1.5 | 0.8 | 0 |
| | 2 | IntI1 | 1.5 | 5.2 | 73 | 0.33 | 0.50 | 2.0 | 0.17 |
| 671 | 2 | IntI2* | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2 | IntI2*179E | 0 | 0.67 | 0.67 | 0 | 0 | 0 | 0 |
| | 2 | IntI1 | 0 | 0 | 18 | 0 | 0 | 2.5 | 0 |
| 2280 | 1 | IntI2* | –[c] | – | 0 | – | – | – | – |
| | 1 | IntI2*179E | – | – | 0 | – | – | – | – |
| | 1 | IntI1 | – | – | 59 | – | – | – | – |
| 678 | 2 | IntI2* | – | – | 0 | – | – | – | – |
| | 2 | IntI2*179E | – | – | 0.33 | – | – | – | – |
| | 2 | IntI1 | – | – | 0.7 | – | – | – | – |

[a] See Fig. 3 for maps of cloned integron fragments in plasmids 670, 671, 2280, and 678.
[b] Phenotypic tests were used as described in Materials and Methods to detect cassette excisions. DNA fragments were cloned in pSU18 or pSU19 to obtain substrates in either direction in respect to the *lac* promoter (in plasmid 671 the *lac* promoter is directed from the 5′ end of the cassettes, and in plasmids 670, 2280, and 678 the promoter is directed from the 3′ end of the cassettes). Excision rates are given as the percentage of sensitive colonies among 600 to relevant antibiotics.
[c] –, Not done.

negative bacteria. Tn*7* uses a nonreplicative transfer due to the production of a 5′ cleavage mechanism involving TnsA and shows a remarkable hierarchy of target specificities controlled by two proteins, TnsD and TnsE (12). In addition to its complex transposition mechanism, Tn*7* has been postulated to carry an integron. Tn*7* is known to carry two cassettes, *dfrA1* and *aadA1*, identical to those in integrons as well as a third, *sat* (39). We have now completed the nucleotide sequence of Tn*7*. This analysis disclosed the presence of a fourth cassette downstream of those previously described, which all encode resistance to antibiotics used in human or veterinary medicine. The new cassette described here could not be assigned a resistance phenotype, but a biological function seems possible due to the conservation of internal 7-amino-acid repeats in the gene product. A particularly notable feature of the integrase gene in Tn*7*, *intI2*, is an internal stop codon at amino acid position 179.

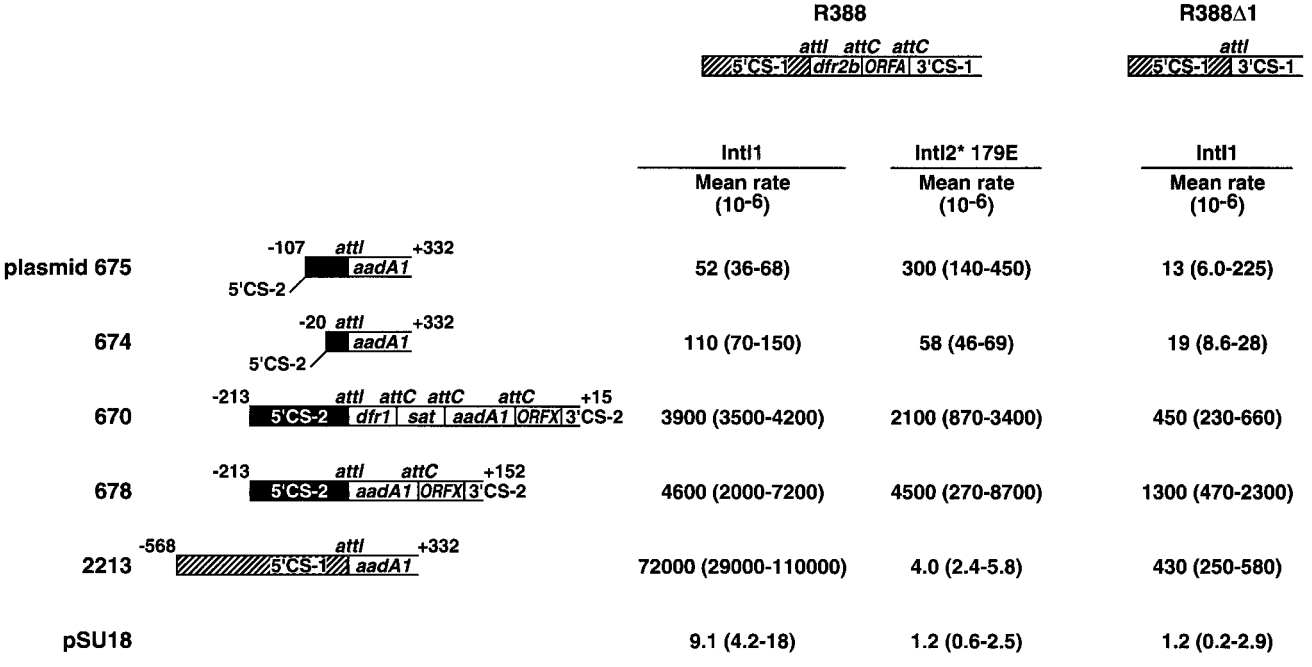


FIG. 4. Structures of cloned integron fragments and recombination rates detected with the mating-out assay as described in Materials and Methods. Integron fragments from either Tn*21* or Tn*7* were tested for recombination with wild-type R388 and R388Δ1 in the presence of IntI1 or IntI2*179E. Conserved sequences from class 1 integrons are hatched (5′-CS-1) and from class 2 are in black boxes (5′-CS-2). Open white boxes indicate cassettes. The lengths of 5′ and 3′ flanks from the closest cassette border are given above the cloned integron fragments. Recombination frequencies are given as mean transfer frequency of million transferred R388 or R388Δ1, with ranges given within brackets. All experiments were repeated at least four times. Only background recombination rates were detected between the tested pSU18 clones and R388Δ1 with IntI2*179E.

There are at least two possible explanations for the existence of an internal stop codon in the gene. Either the integrase gene is a pseudogene which is no longer active or the stop codon may permit a certain level of activity in ochre suppressor strains. Also, it is known that integrases often bind DNA through a helix-turn-helix DNA recognition motif in the N-terminal moiety of the protein. This makes it possible that a short, truncated form of the protein could bind to the same DNA sites as the complete integrase and have a regulatory function. Either the short form of the gene product could control the access to DNA-binding sites or it could autoregulate a promoter. The conservation of the stop codon throughout members of the Tn*7* family carrying different cassettes in their integrons, found by the sequencing of the integrase genes of Tn*1825* and Tn*1826* described here, makes it possible that the short form of IntI2* could be an inhibitor polypeptide controlling the action of integrase.

The ability of Tn*7* to use both site-specific and nonspecific modes of transposition (22, 46) could explain why it has become so widespread and persistent in bacterial populations. The integron of Tn*7* has acquired a $Tp^r$ cassette which has probably contributed to its success. In contrast, there is a limited variety of cassette arrangements in the class 2 integrons, and this may be directly attributable to the internal stop codon of *intI2*. Again, the conservation of the internal stop codon makes it possible that the trimethoprim cassette in Tn*7* and the open reading frame cassette in Tn*1825* may have been inserted by *trans* action of IntI1 in cells where both classes of integron were present.

Mutagenesis of the stop codon to a glutamate codon restores activity to IntI2*. Both IntI1 and IntI2*179E efficiently excise cassettes (other than cassettes in first position) by *attC*×*attC* recombination. Excision of cassettes in the first position, however, occurs by *attI*×*attC* recombination, and the *attI* sites differ between class 1 and class 2 integrons. IntI1 and IntI2*179E differ in their ability to recognize these sites; while IntI1 recognizes both *attI1* and *attI2* (and thus can excise a cassette in first position in either class of integron), IntI2*179E recognizes only its own *attI* site and is unable to excise cassettes in first position in class 1 integrons. Cointegrate formation by *attI*×*attC* recombination confirms this specificity. Also, the low-level *attI*×*attI* recombination between class 1 and 2 forms of *attI* can be carried out by IntI1 but not by IntI2*179E. We cannot explain the observed effects of transcription on the frequencies of excision. Reduced excision frequency was observed in the presence of strong unidirectional transcription; it may be that RNA polymerase interferes with binding of the integrase.

In spite of the efficiency of Tn*7* as a transposon, class 1 integrons have shown a far greater degree of cassette diversity than the Tn*7*-based class 2 integrons. Although the internal stop codon is an obvious explanation, the greater efficiency and looser specificity of IntI1 may also be important. Most integrons make up part of defective transposons lacking two of the four transposition genes borne on functional Tn*5090* (30), which is a competent transposon. This disadvantage can be circumvented by the "piggybacking" of these defective transposons on an efficient transposon, as in the Tn*21* family of transposons. Finally, the evolutionary success of an integron is no doubt determined by two other important factors: the resistance cassettes it carries and the host range of the plasmid on which it occurs.

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