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Characterization of In0 of *Pseudomonas aeruginosa* Plasmid pVS1, an Ancestor of Integrons of Multiresistance Plasmids and Transposons of Gram-Negative Bacteria

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Many multiresistance plasmids and transposons of gram-negative bacteria carry related DNA elements that appear to have evolved from a common ancestor by site-specific integration of discrete cassettes containing antibiotic resistance genes or sequences of unknown function. The site of integration is flanked by conserved segments coding for an integraselike protein and for sulfonamide resistance, respectively. These segments, together with the antibiotic resistance genes between them, have been termed integrons (H. W. Stokes and R. M. Hall, Mol. Microbiol. 3:1669–1683, 1989). We report here the characterization of an integron, In0, from *Pseudomonas aeruginosa* plasmid pVS1, which has an unoccupied integration site and hence may be an ancestor of more complex integrons. Codon usage of the integrase (*int*) and sulfonamide resistance (*sul1*) genes carried by this integron suggests a common origin. This contrasts with the codon usage of other antibiotic resistance genes that were presumably integrated later as cassettes during the evolution and spread of these DNA elements. We propose evolutionary schemes for (i) the genesis of the integrons by the site-specific integration of antibiotic resistance genes and (ii) the evolution of the integrons of multiresistance plasmids and transposons, in relation to the evolution of transposons related to Tn*21*.

Horizontal and vertical transfer of antibiotic resistance genes in bacteria has been shown to be mostly due to R plasmids and to the transposons they carry (16, 22, 26). The evolution of some of these elements, specifically the Tn*21*-like transposons and related plasmids, by site-specific integration of antibiotic resistance genes has been described in recent years (3, 11, 32, 42, 60, 61). These elements have been shown to possess a common structure, the integron (Fig. 1A), which has been described as a novel family of potentially mobile DNA elements made up of two conserved segments between which discrete units are integrated as cassettes (48). The 5′-conserved segment encodes a putative site-specific recombinase, Int (also called TnpI [28, 29]), that shows similarity to the phage integrases (33, 37, 48). This segment also contains a common promoter region for expression of the integrated cassettes, P1-P2 (3), also designated P(P2) (48). The 3′-conserved segment carries a gene for sulfonamide resistance (*sul1* [48, 49]) and two open reading frames (ORFs), ORF4 and ORF5 (48). The integrated cassettes found between these segments most often encode antibiotic resistance determinants, such as genes for aminoglycoside-modifying enzymes, β-lactamases, trimethoprim-resistant dihydrofolate reductases, and chloramphenicol resistance. The occasional presence of cassettes of unknown function and phenotype suggests a general mechanism for the evolution of integrons by site-specific integration of gene cassettes. It has been suggested that an ancestral structure of the integrons should carry the Int recombinase and a sulfonamide resistance gene that may or not be linked to mercury resistance genes (49, 61). Such a structure, consisting of the 5′- and the 3′-conserved segments without integrated cassettes, could serve as substrate for in vivo or in vitro recombination experiments, leading to the elucidation of the role of Int and of the molecular mechanisms involved in the integration of gene cassettes and consequently in the evolution of the integrons.

pVS1 is a 29-kb nonconjugative plasmid of *Pseudomonas aeruginosa* which carries the mercury-resistance transposon Tn*501* and a sulfonamide resistance gene (*sul1*) which is not in the transposon (21, 47). The map of the sulfonamide resistance region of pVS1 (21) revealed some colinearity with the hypothetical ancestral integron's structure. We present in this report the characterization of the integrase and sulfonamide resistance gene region of pVS1 and show that it contains the 5′- and 3′-conserved segments of the integron (48) but has no integrated cassettes. The codon usage of the genes (*int* and *sul1*) and of ORF5 of In0 of pVS1 suggests that they originate from closely related organisms; this is in contrast to the codon usage of other antibiotic resistance genes which are part of integrated cassettes of other integrons. On the basis of these results and the analysis of published data, we present schemes for (i) the evolution of multiresistance integrons by integration of antibiotic resistance gene cassettes and (ii) the evolution of the general structure of the integrons with regard to the genesis of Tn*21*-like transposons from the ancestral mercury resistance transposon Tn*2613* (55, 60).

(These results were presented in part at the 89th Annual Meeting of the American Society for Microbiology, New Orleans, La., 1989 [5].)

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. aeruginosa* plasmid pVS1 (Sul^r Hg^r::Tn*501*) (21, 47) was isolated from *P. aerug-*

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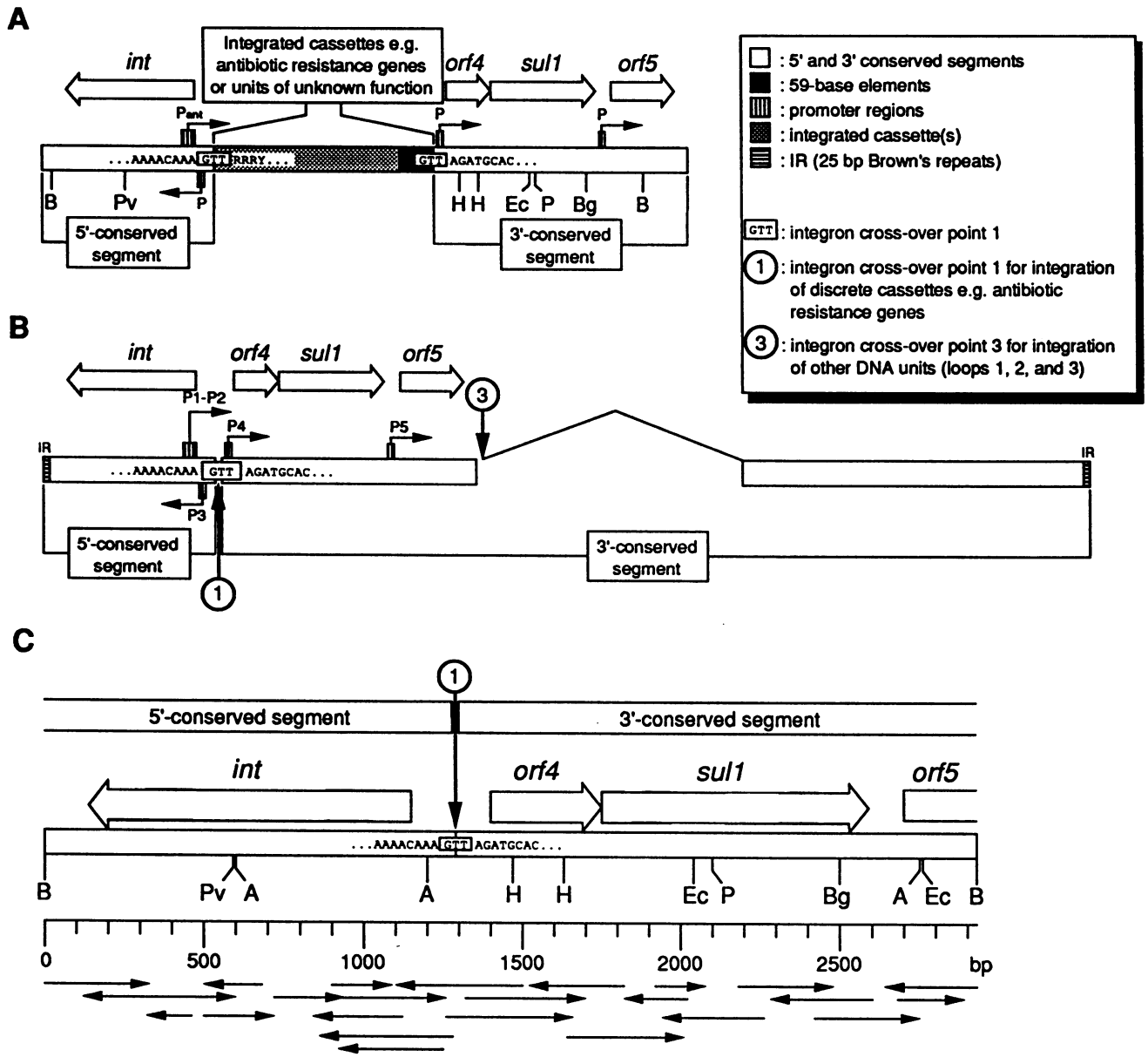

FIG. 1. Integrons. (A) General structure of the integrons (adapted from references 3 and 48). The structure is shown with a single integrated gene cassette; there may be none (as in pVS1) or more than one, each with a 59-base element at its downstream end. (B) Redefinition of the basic integron structure, based on that of Brown et al. (10), Rådström et al. (39), Rådström (38), and this work. The 25-bp inverted Brown's repeats (IR) and the 2.7-kb DNA segment found downstream of crossover point 3 are added to the integron structure shown in panel A. This structure is equivalent to InB (Fig. 4). In In0 of pVS1, the 2.8-kb loop 2, not conserved in all integrons, is inserted at crossover point 3. (C) Molecular cloning of the *int* and *sul1* genes of plasmid pVS1. Partial restriction map of the 2.9-kb sulfamethoxazole-resistant *Bam*HI insert of pLQ860, sequence of the junction between the 5'- and 3'-conserved segments, and location of the putative crossover point 1 (GTT [28]). The length and direction of arrows indicate the extent of sequencing reactions. A, *Ava*I; B, *Bam*HI; Bg, *Bgl*II; Ec, *Eco*RV; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II.

inosa strain PAO25 by the method of Beaulieu et al. (1) and purified by isopycnic ultracentrifugation on a cesium chloride-ethidium bromide gradient. *Escherichia coli* NM522 (*hsd*Δ5 Δ(*lac-pro*) [F′ *pro*$^+$ *lacI*^{q}ZΔM15]) served as the host strain for cloning. Cloning vectors pTZ18R and pTZ19R and helper phage M13KO7 were purchased from Pharmacia Canada Ltd.

Microbiological media and antibiotics. All microbiological media (Difco) were obtained from BDH. Ampicillin sulfate and kanamycin sulfate were obtained from ICN Biochemicals. Sulfamethoxazole was purchased from Sigma. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was purchased from Boehringer Mannheim Canada Ltd.

Enzymes. Restriction endonucleases were purchased from Boehringer Mannheim Canada Ltd., Bethesda Research Laboratories, or New England BioLabs and used according to the manufacturers' conditions. T4 DNA ligase was purchased from Bethesda Research Laboratories.

Cloning of the *sul1* gene, transformation, and selection of recombinant clones. Shotgun cloning of the *sul1* gene was performed by ligation of *Bam*HI-, *Eco*RI-, and *Hin*dIII-cleaved pVS1 into phagemid vector pTZ19R. Transformation of $CaCl_2$-treated *E. coli* NM522 was performed by the method of Maniatis et al. (27), and selection of recombinant clones was done on solid media containing ampicillin (50 μg/ml) and sulfamethoxazole (500 μg/ml). Screening of recombinant plasmids was performed by the miniscale method of Birnboim and Doly (2). Large-scale isolation of plasmid DNA from recombinant clones was performed by the method of Bissonnette et al. (3).

DNA sequencing. pTZ single-stranded templates, derived from pLQ860, pLQ861, and subclones made in pTZ18R and pTZ19R, were isolated and sequenced with the Sequenase 2.0 DNA sequencing kit (United States Biochemical Corp.) and [α-^{35}S]dATP (NEN-Dupont), as described by Bissonnette et al. (3). Polyacrylamide gel electrophoresis under denaturing conditions was performed with the LKB Macrophor Sequencing System, and autoradiography was done on X-Omat XAR-5 films (Eastman Kodak). Nucleotide sequence data were analyzed by the Genetics Computer Group (GCG) software (13).

Nucleotide sequence accession number. The sequence described in this paper has been assigned GenBank accession number M73819.

RESULTS

Molecular cloning of the *sul1* gene of plasmid pVS1. The ligation of *Bam*HI, *Eco*RI, and *Hin*dIII fragments of pVS1 into pTZ19R yielded three libraries from which sulfamethoxazole-resistant clones were selected. Two clones bearing a 2.9-kb *Bam*HI fragment in opposite orientations, pLQ860 and pLQ861, were further characterized. A restriction map (Fig. 1) revealed colinearity of restriction sites of part of this insert, the 5′-conserved segment, with the regions upstream of resistance genes of several plasmids, including the *oxa1* gene of Tn*2603* (32) and of the following part, the 3′-conserved segment, with the *sul1* gene of Tn*21*, R388, and R46 (48, 49). In addition, the restriction map of the region downstream of *sul1* in pVS1 (21) shows colinearity with the corresponding region of Tn*2411* (23), which differs from Tn*21* only by the absence in the former of a 1.45-kb DNA region (loop 1 [30]). Therefore, the integron carried by pVS1, In0, can be considered as a less-evolved version, lacking the *aadA1* gene, of the integron of Tn*2411*.

Nucleotide sequencing of the *sul1*-bearing insert of pLQ860 and pLQ861. In order to establish the relationship of the In0 integron of plasmid pVS1 with the integrons of multiresistance plasmids and transposons, we sequenced the 2.9-kb *Bam*HI fragment encoding sulfamethoxazole resistance of pLQ860 and pLQ861. The nucleotide sequence of this fragment (Fig. 2) is nearly identical to the corresponding regions of the 5′- and 3′-conserved segments of the integrons. The 5′-conserved segment of pVS1 (nucleotides 1 to 1285) is more than 99% conserved among all the other segments characterized to date, while the 3′-conserved segment (nucleotides 1289 to 2935) is identical to that of Tn*21*, R388, and R46 (48, 49). The GTT (nucleotides 1286 to 1288) cannot be unambiguously assigned to either segment, since it is duplicated in integrons containing inserted gene cassettes.

ORFs of pVS1. In0 contains two long ORFs, coding for polypeptides of 337 amino acids (Int) and 279 amino acids (Sul1), in addition to two other ORFs, ORF4 and ORF5. It is not known whether ORF4 is expressed even though it shows similarity with a *Staphylococcus aureus* ethidium bromide resistance (*ebr*) gene (39). On the In1 integron of plasmid R46, Guerineau et al. (17) have demonstrated the presence of a promoter region on the 3′-conserved segment that yields transcripts, starting at position 1376 of our sequence, i.e., 20 bases upstream from the potential initiation codon of ORF4, and encompassing ORF4 and *sul1*. Although ORF4 has a potential ribosome binding site (GGAG), its presence was not detected in *E. coli* minicells containing derivatives of plasmid R46 (17). The sequence of the corresponding ORF in Tn*402* (which, unlike other integrons, lacks *sul1* and ORF5) diverges from ORF4 of all other integrons at a point near its carboxyl end. ORF4 of Tn*402* shows a greater extent of homology with *ebr* than do the others (39), suggesting that it is a more ancient ORF4, which may have been interrupted by the addition of a *sul1*-ORF5 unit to an integron more ancient than In0, as suggested by Stokes and Hall (48). If ORF4 is translated in some integrons, it may be involved in translational coupling with *sul1*. There is as yet no evidence for the expression of ORF5, although its putative product shows an interesting similarity (Z value of 10; $Z = (x - m)/\sigma$, where x is the local alignment score, m is the mean of random scores, and σ is the standard deviation of random scores [25]) with the puromycin acetyltransferase of *Streptomyces alboniger* (24).

Codon usage. Using the GCG program CORRESPOND (13), we analyzed the codon usage of *sul1*, *int*, ORF5, and ORF4 and compared it with that of antibiotic resistance genes found on other integrons (Table 1). The CORRESPOND program looks for similar patterns of codon usage by comparing codon frequency tables. D-square values lower than 3, as calculated by this program, are significant and might reflect a common or close origin for the analyzed genes.

When the codon usage of the genes and ORFs found on In0 are compared, significant D-square values (1.85 to 2.47) are obtained among *int*, *sul1*, and ORF5; this suggests that these genes originate from closely related microorganisms which are different from *E. coli* (3.61 to 6.23) or *P. aeruginosa* (3.44 to 4.02). Stokes and Hall (48) proposed that *sul1* was integrated early in the evolution of the integrons, and the codon usage analysis shown in Table 1 correlates with this hypothesis. In contrast, the D-square values of correspondence between the codon usage of ORF4 and that of *sul1*, *int*, and ORF5 are largely above (7.98 to 10.38) the values obtained among the last three; this indicates a different origin for ORF4, even if its incorporation into the integron preceded that of *sul1* and ORF5. The high values obtained for the majority of genes found as integrated cassettes in integrons, including the streptomycin-spectinomycin resistance genes *aadA1* and *aadA2*, point to a significant diversity in their origins.

DISCUSSION

Sequence at the junction of the conserved segments. One of the interesting features of the integrons is their ability to integrate antibiotic resistance gene cassettes, a phenomenon that is probably associated with their response to antibiotic selection. One characteristic of these integrated cassettes is the presence of potential stem-loop structures, the 59-base elements (11, 14, 19, 48), or recombinational boxes (44) found at the 3′ end of the integrated cassettes. At the junction between the 5′- and 3′-conserved segments (crossover point 1) of pVS1, there is no sequence resembling these elements. In pVS1, the absence of a 59-base element or

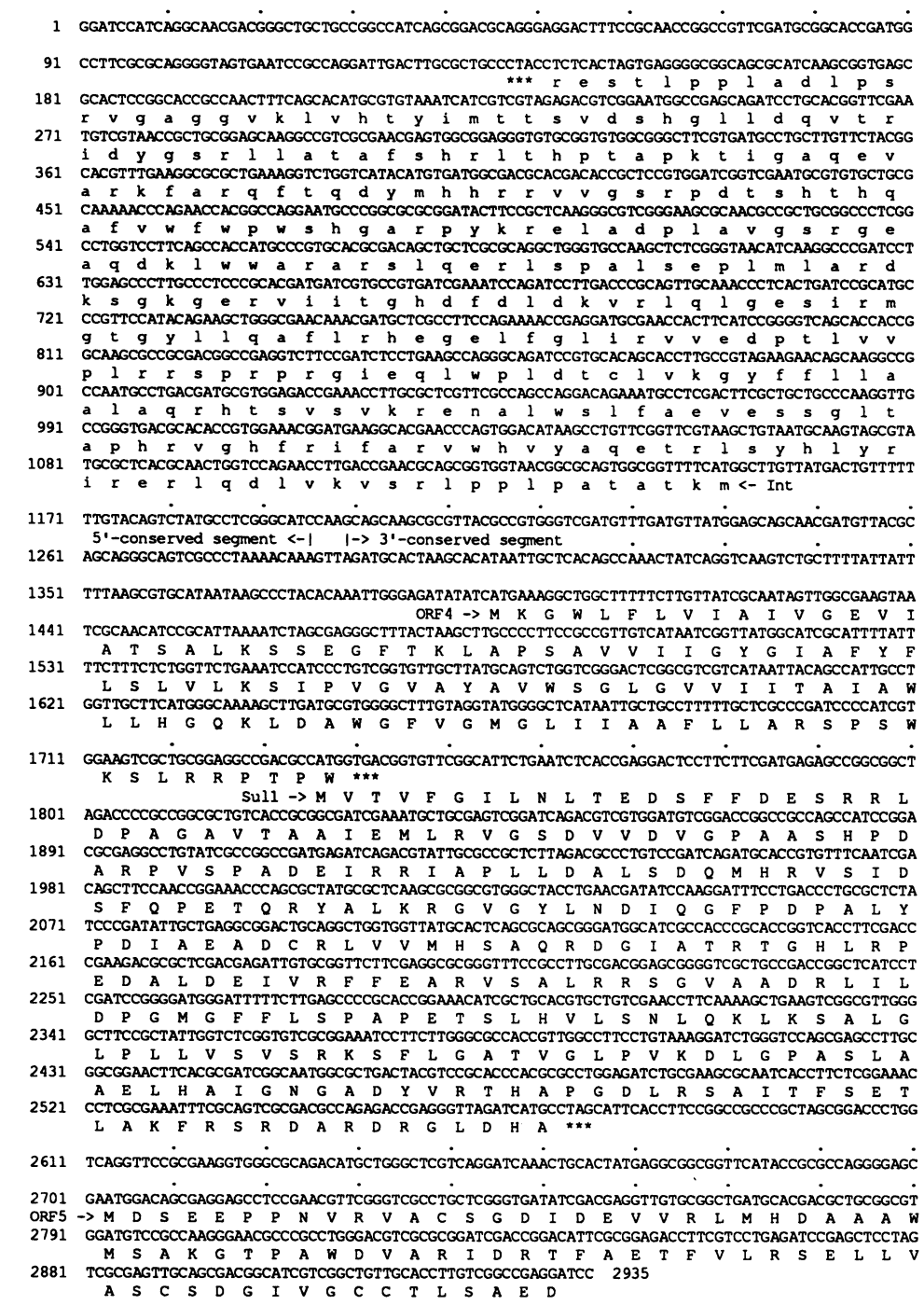

FIG. 2. Nucleotide sequence of the 2.9-kb *Bam*HI fragment of plasmid pVS1 encoding *sul1* and deduced primary structures of ORF4, Sul1, the N-terminal portion of ORF5, and Int (in lowercase letters). The junction between the 5'- and 3'-conserved segments (GTT; crossover point 1) is indicated by parallel lines. The putative sulfonamide resistance promoter is at bases 1035 to 1040 (−35) and 1058 to 1063 (−10), and the putative integrase promoter is on the complementary strand at bases 1205 to 1200 (−35) and 1182 to 1177 (−10).

similar structure is consistent with its status as a less-evolved integron. The presence of such an element has been reported to be required on at least one of the sequences participating in the Int-mediated crossing over at the GTT sequence at crossover point 1 (28). The presence of 59-base elements in integrated gene cassettes and their absence in pVS1 suggest that these elements are associated with the incoming genes. The occasional presence, in association with antibiotic resistance gene cassettes, of DNA cassettes of unknown function containing these elements at their 3' ends, such as X in Tn*1696* (61) and Y in R46 (19), pBP11 (31), Tn*2410* (4), and Tn*402* (39), suggests that any chromosomal

TABLE 1. Comparison of codon usage of genes carried by In0 with that of other genes

Gene	Source	D-square[a]					
		sul1	*int*	ORF5	ORF4	*aadA1*	*aadA2*
5′- and 3′-conserved segments							
sul1	R388 (In3), pVS1 (In0)	0.00	**1.85**	**2.47**	8.20	4.00	3.58
int	pVS1 (In0)	**1.85**	0.00	**2.26**	7.98	4.31	3.84
ORF5	R46 (In1)	**2.47**	**2.26**	0.00	10.38	7.69	6.56
ORF4	R46 (In1), pVS1 (In0)	8.20	7.98	10.38	0.00	5.77	4.03
Microorganism genes							
E. coli low[b]		**2.36**	**2.53**	5.40	3.38	**0.71**	**0.71**
E. coli high[b]		4.23	3.61	6.23	9.79	4.33	4.36
P. aeruginosa[c]		3.82	3.44	4.02	11.64	6.23	4.14
Integrated genes							
aacA1a	pBWH100 (In14)	9.83	10.49	13.77	5.80	4.43	6.48
aacA1b	pMG7 (In23)	4.52	4.57	6.80	6.39	**1.79**	3.38
aacA2	pSCH884 (In5)	3.81	3.33	4.26	8.90	3.35	4.54
aacC1	R135 (In15), Tn*1696* (In4)	5.47	5.55	6.12	6.80	**2.14**	3.76
aadA1	Tn*21* (In2)	4.00	4.31	7.69	5.77	0.00	**2.53**
aadA2	pSa (In6)	3.58	3.84	6.56	4.03	**2.53**	0.00
aadB	pDGO100 (In7)	4.52	4.81	5.83	8.39	**2.39**	4.88
cmlA	Tn*1696* (In4)	5.85	5.57	7.58	5.82	4.29	**1.73**
dhfrI	pLMO150 (In17), pLMO229 (In18)	6.42	7.00	9.39	4.50	**2.54**	4.34
dhfrII	R388 (In3)	7.94	8.41	12.26	7.35	4.38	7.26
dhfrV	pLMO20 (In13)	4.87	5.37	8.45	7.27	**1.90**	3.87
oxa1	Tn*2603* (In8)	10.41	11.50	14.16	6.31	4.87	6.85
oxa2	R46 (In1), pBP11 (In9)	4.82	4.59	7.13	4.33	**1.64**	3.27
pse2	pMON234 (In12)	7.46	7.85	9.49	6.52	3.04	4.59
pse4	pMON709 (In19)	7.40	8.08	11.13	**2.38**	4.71	**2.64**
Mercury resistance gene, *merA*	Tn*501*	3.43	3.36	4.25	9.34	5.28	**2.45**

[a] The D-square values generated by the GCG program CORRESPOND (13) for each pair of genes are shown. Values likely to be significant, i.e., <3.0, are in boldface type. Data for *sul1* (49; this study), *int* (this study), ORF5 and ORF4 (48), *aacA2* (46), *aacA1b* (8), *aadA1* (49), *aadA2* (36, 38, 53), *aadB* (43), *dhfrII* (52, 62), *dhfrV* (49), *oxa2* (19), *aacA1a* (56), *aacC1* (61), *cmlA* (3), *dhfrI* (51), *oxa1* (32), *pse2* (20), *pse4* (7), and *merA* (9) were from the respective references.
[b] Codon usage tables for *E. coli* are available in the GCG data base (13). Low and high indicate the levels of expression.
[c] A codon usage table for *P. aeruginosa* was constructed from the compilation of West and Iglewski (59).

gene first becomes associated with a recombination-promoting 59-base element and then becomes associated with an integron. Alternatively, the 59-base element may be supplied by the first integrated cassette, eliminating the requirement for the element on the incoming gene and thus favoring tandem integrations. The discovery and sequencing of chromosomal relatives of integron-associated resistance genes should provide clues to the origin and function(s) of the 59-base elements.

Evolution of the integrons of multiresistance plasmids and transposons. The integron was originally defined by Stokes and Hall (48) as the region between the points of sequence divergence between Tn*21* and R46 and included the 1.4-kb 5′-conserved segment encoding the integrase gene; the 2.0 kb 3′-conserved segment encoding ORF4, *sul1*, and ORF5; and the resistance gene cassettes integrated at the GTT at the junction of these segments. The divergence between Tn*21* and R46 at the end of the 5′-conserved segment corresponds to one of the 25-bp inverted repeats originally found by Brown et al. (10) as the extremities of an extra 11.2-kb region found in Tn*21* when its sequence is aligned with the related transposon Tn*501*. However, the other end of the integron, the divergence at the end of the 3′ segment, now appears to be due merely to the presence of the 2.8-kb loop 2 in Tn*21* and its absence in R46. We therefore propose to redefine the integron (Fig. 1B) as the region between the 25-bp Brown's repeats. Not all of the 11.2 kb between the Brown's repeats in Tn*21* is characteristic of the integron; much of it represents recently acquired insertions. Figure 3 presents a scheme for the evolution of the integrons, principally but not exclusively by site-specific integration of antibiotic resistance genes at crossover point 1. To elaborate this scheme, we used data for integrons for which the sequence has been determined or for which restriction maps are sufficiently precise. We also considered the frequency of occurrence of the integrated genes, e.g., the nonspecific insertion (at crossover point 2) of the almost totally conserved *sul1*-ORF5 region and the site-specific integration (at crossover point 1) of the frequently encountered *aadA1* gene probably preceded other insertions. The numbering of the integrons is not related to the evolutionary status of the integrons or to the other rearrangements observed in the region downstream of crossover point 1.

The proposed evolution of the integron In0 of pVS1 by insertion of a *sul1*-ORF5 unit into InA is based on the recent characterization by Rådström et al. (39) of Tn*402*, which contains an integron (In16) made up of an intact 5′-conserved segment, a *dhfrIIc* gene and the Y unit, both integrated at crossover point 1, and a shortened 3′-conserved segment that contains ORF4, lacks *sul1* and ORF5, but has a 2.25-kb DNA unit in their place in a region referred to as crossover point 2. Further downstream, they demonstrated the presence of a 2.7-kb fragment which is also conserved in the integrons of Tn*21*, Tn*2411*, and Tn*5086* (50). The ancestral integron InA would therefore contain only the 1.4-kb 5′-conserved segment (beginning at the 25-bp Brown's re-

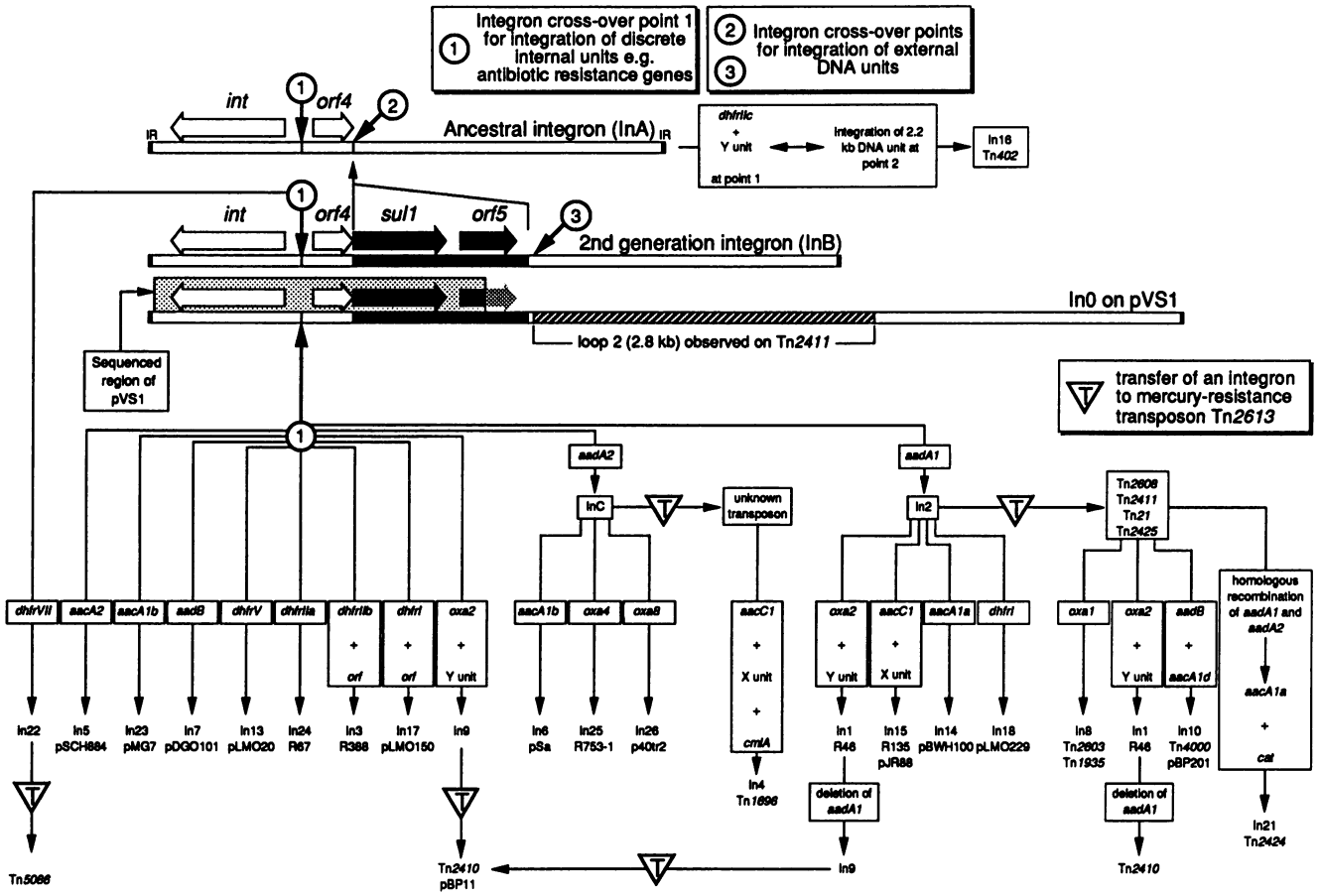

FIG. 3. The evolution of the integrons of multiresistance plasmids and transposons by site-specific integration of gene cassettes at crossover point 1. The correlation between the structure and the evolution of the ancestral integron InA, its evolution to In0 by integration of a *sul1*-ORF5 unit at crossover point 2, and the site-specific recombination events that occurred at crossover point 1 which led to the genesis of the multiresistance integrons for which sufficient sequence data or restriction maps are available is shown. The order of integration for elements that arose from multiple integrations (large boxes showing two or more integrated cassettes) is not determined. Sources of data for Tn*402* (39); pSCH884 (46); pMG7 (8); pDGO101 (11); Tn*5086* (50); pLMO20 (49); R67, R388, pLMO150, and pLMO229 (51); pBP11 (31); Tn*2410* (4); pSa (53); R753-1 (6, 54); p40tr2 (6); Tn*1696* (3, 61); R46 (19, 48); R135 (61); pJR88 (57); pBWH100 (56); Tn*2608*, Tn*21*, Tn*2411*, and Tn*2425* (30, 55); Tn*2603* (32); Tn*1935* (12); Tn*4000* and pBP201 (43); and Tn*2424* (34, 35) are the respective references. The numbering of the integrons used here (18, 48) is not related to the evolutionary status of the integrons.

peat and containing the integrase gene), crossover point 1, and the shortened 3′-conserved segment (3.1 kb) containing ORF4 and the 2.7-kb fragment and ending in the other Brown's repeat. Presently, Tn*402* is the only example of an integron lacking the *sul1*-ORF5 region. Since *sul1* and ORF5 are both absent in Tn*402* and present in all other integrons, they were probably integrated together or consecutively very early in the evolution of the integrons. To simplify the evolutionary pathways, the integration of *sul1* and ORF5 into InA at crossover point 2 could be considered as a single event. Subsequent integration of antibiotic resistance genes other than *sul1* would take place at crossover point 1, i.e., between the 5′-conserved segment and the now complete 3′-conserved segment. This 3′-conserved segment would contain another crossover point (3) where the integration of DNA elements of various sizes (loops 1, 2, and 3 [see below]) can occur. According to restriction maps and heteroduplex analysis, some plasmids such as R388 seem to lack the distal 2.7-kb region of the 3′-conserved segment adjacent to *sul1* and ORF5 (29, 39, 42, 58). This segment could have been deleted or additional segments could have been integrated at crossover point 3 by recombinational events of unknown nature. The second possibility would result in the 2.7-kb DNA segment being located further away on the plasmids.

This 2.7-kb fragment has been sequenced (38), and one of the ORFs shows significant similarity (41) with the *tnsB* gene of Tn*7* (*Z* value of 13) (15) and with the transposase of Tn*552* (*Z* value of 16) (40). It is thus highly likely that the integron is a relatively inefficient, possibly site-specific, transposon which is more efficiently disseminated as part of Tn*21*-like transposons. The existence of integrons on plasmids (R388, R46, and pSa) unrelated to Tn*21* indicates their potential mobility. One integron, Tn*402*, has been reported to transpose to two sites (presumably specific) in bacteriophage lambda (45). At present, no integron other than Tn*402* has been observed to transpose.

Although the evolution of the integrons and of the Tn*21*-like transposons has been closely related in the past (29, 32, 60), distinctions in the following three events should be noted: (i) the evolution of the integrons by the site-specific integration of discrete cassettes at crossover point 1, (ii)

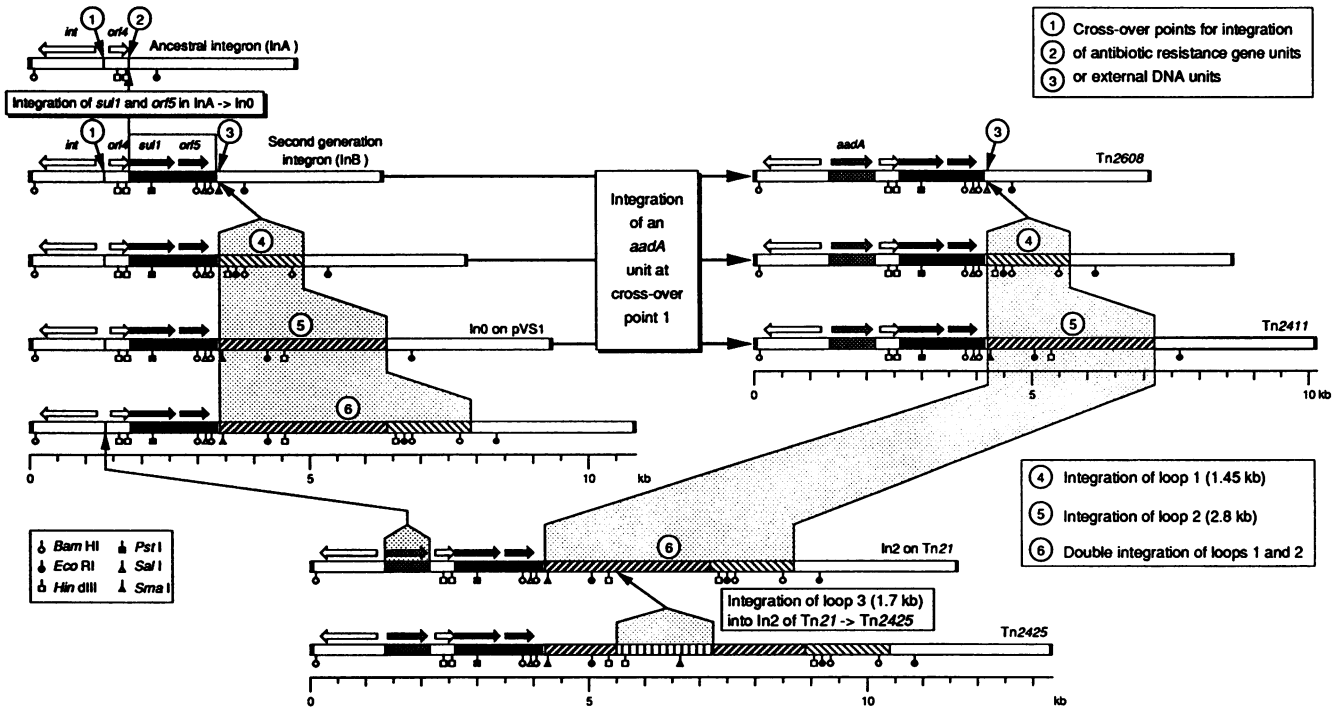

FIG. 4. Evolution of the integrons and relation with the evolution of Tn*21*-like transposons. Potential recombination events that generated these elements, prior to their integration into a mercury resistance transposon related to Tn*2613*, include integration of antibiotic resistance genes at crossover point 1 and DNA elements (insertion sequences or other elements) at crossover points 2 and 3. At crossover point 1, we show, for simplicity, only the site-specific integration of *aadA* (*aadA1* or *aadA2*) gene cassettes into InA or In0 in the generation of In2-like integrons. Restriction sites for the potential and characterized elements are shown. Maps are adapted from Tanaka et al. (55) (Tn*2608*), Itoh et al. (21) (pVS1), and Meyer et al. (30) (Tn*2411*, Tn*21*, and Tn*2425*).

other internal rearrangements of the integrons at crossover points 2 and 3, and (iii) the transposition of integrons to mercury resistance transposon Tn*2613* (10, 55, 60).

We propose two distinct evolutionary trees (Fig. 3 and 4); one summarizes the evolution of the integrons by site-specific integration of antibiotic resistance genes at crossover point 1, and the other links the evolution of the integrons to the evolution of the Tn*21*-like transposons. Since integron evolutionary intermediates have been discovered both on plasmids and on Tn*21*-like transposons, it is probable that the evolution by integration of DNA units at the crossover points can occur either on plasmids or mercury-resistance based transposons and is not dependent on Tn*21* transposition genes (*tnpMRA*).

Evolution of the integrons by site-specific integration of antibiotic resistance gene units at crossover point 1. The evolution of integrons and transposons has certainly been accelerated by selective pressure resulting from the intensive use of antibiotics in the last 40 years and is facilitated by the involvement of site-specific recombination in gene insertions. This Int-mediated mechanism has integrated DNA cassettes, most of which contain antibiotic resistance genes, from still unknown origins to a specific site, downstream of an efficient promoter, similar to that found on pVS1 and Tn*21*, the whole acting as an expression cassette (61) in which efficient expression levels can be attained (48). In this respect, the integron is a natural expression vector.

The most frequent gene cassettes encountered in the multiresistance integrons are the streptomycin-spectinomycin resistance *aadA1* and *aadA2* genes, with all of the others being rarer. Tandem integrations at crossover point 1 frequently include one or the other of these genes; this phenomenon could be related to the early emergence of In2-like integrons (*aadA-sul1*), since streptomycin clinical availability and thus selective pressure preceded that of other aminoglycosides, trimethoprim, and oxacillin. Also, the 59-base element supplied by the first cassette may favor subsequent integrations on either side of the *aadA* cassette, as shown for the *aadA* stems in Fig. 3. The *aadA2* branch presumably evolved in parallel to the *aadA1* branch, and the existence of an In2-like integron that would carry *aadA2* instead of *aadA1* is proposed. Since integrons are borne on both plasmids and transposons, their potential transposition to mercury-resistance transposon Tn*2613* is included in the proposed tree in order to explain the generation of multiresistance transposons, but this transfer could presumably occur at any time during the evolution of an integron by insertions at crossover point 1.

TEM-like β-lactamase, tetracycline resistance, classical chloramphenicol acetyltransferase (*cat*), and aminoglycoside phosphotransferase (*aph*) genes are usually found in genetic environments other than integrons. The apparent absence of the above-mentioned genes on the integrons may be related to their presence on other plasmids or transposons (e.g., Tn*3*, *-5*, *-9*, and *-10*) which are distinct from the integrons and which have their own efficient mechanisms of dissemination.

Structure of the integrons and evolution of the Tn*21*-like transposons. For years, investigators have proposed that the Tn*21*-like transposons have evolved from a mercury-resis-

tance transposon related to Tn*2613* (42, 55, 60). Most of these models do not adequately consider the involvement of a sulfonamide resistance element lacking streptomycin resistance in the evolutionary events that led to the genesis of this large family of transposons, because such elements are infrequent. The Tn*21*-like transposons Tn*1831*, Tn*2411*, Tn*21*, and Tn*2425* carry an In2-like (*aadA1-sul1*) integron and differ from one another by the presence or absence of three DNA elements (loops), visualized by electron microscopy of heteroduplexes and confirmed by restriction mapping (30). These loops of 1.45 kb (loop 1), 2.8 kb (loop 2), and 1.7 kb (loop 3; IS*161*) are found alone or in combination on these transposons. Since different combinations of these loops occur on Tn*21*-like transposons and since pVS1 carries an integron resembling that of Tn*2411* (i.e., containing loop 2) but lacking *aadA1*, we propose an evolutionary pathway originating from the ancestral integron InA with integration events at crossover points 1, 2, and 3 to explain the genesis of Tn*21* and Tn*2425* (Fig. 4). The alternate pathways described in this scheme include In2-like (*aadA-sul1*) and In0-like (*sul1*) integrons, some of which have been mapped on various plasmids and transposons. In this scheme, we show the evolution of plasmid-borne integrons by insertions of the loops at crossover point 3 and the transposition of integrons from plasmids to the mercury resistance transposon Tn*2613*. In0 of pVS1, which has not yet been found on a transposon, is a potential direct intermediate in the evolution of Tn*2411*, which lacks *aadA1*. At crossover point 1, we consider, for simplicity, only the site-specific integration of *aadA* (*aadA1* or *aadA2*) gene cassettes into InA or In0, in the generation of In2-like integrons. Other genes can insert into less-evolved structures; Tn*5086* (50) may have evolved by integration of a *dhfrVII* unit into an InB integron lacking loops 1, 2, and 3. The existence of integrons whose restriction maps (and thus presence or absence of loops) are related to those of evolutionary intermediates such as InB, In0, and In2 argues in favor of the model shown in Fig. 4. The evolutionary tree of Wiedemann et al. (60) relies on deletion and integration events of a Tn*21*-based In2 structure. Our model in Fig. 4 is more additive, like that of Tanaka et al. (55), although it requires more than two steps between Tn*2613* and Tn*21*. It arrives at each element by the most direct pathway and considers that Tn*21* is a relatively evolved element, resulting from multiple insertions at crossover points 1, 2, and 3. Although deletions can and do occur (60), models involving multiple deletion events are more complicated and less probable. Only one, in the generation of Tn*2410*, is involved in our scheme.

In summary, we show that *P. aeruginosa* plasmid pVS1 carries one of the less-evolved integrons characterized to date, In0. Correspondence between codon usage of *int*, *sul1*, and ORF5 suggests that these genes originate from closely related organisms. In contrast, the differences in codon usage that exist among the antibiotic resistance genes that are part of integrated cassettes of integrons that evolved from In0, point to a diversity of origins of these genes. Evolutionary schemes elaborated from the data presented here and from others published in recent years point to In0 as an intermediate in the evolution of the integrons by (i) site-specific integration of antibiotic resistance gene units at crossover point 1 and (ii) insertion of other DNA units (loops 1, 2, and 3) at crossover point 3. In0 itself seems to be derived from an ancestral integron (InA) from which trimethoprim resistance transposon Tn*402* is directly descended. It will be of interest to determine the origins of the integrated antibiotic resistance gene cassettes, a discovery that would potentially permit the reconstitution of the proposed evolutionary pathways, by in vivo or in vitro experiments using integrase for site-specific integration at integron target sites.

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