

Transposon Tn21, Flagship of the Floating Genome

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INTRODUCTION

With the rich insights provided by the burgeoning information on entire prokaryotic genome sequences, it is important to recall that a significant percentage of the DNA in natural isolates of many eubacteria is in the form of extrachromosomal elements called plasmids. Many of these elements are transferable even very widely across species and genus boundaries. In addition to genes for their own maintenance and transfer, these ubiquitous genetic elements frequently carry embedded mobile genetic elements called transposons, which are capable of moving from one carrier replicon to another.

Much of what we understand about DNA replication and recombination in general has been learned through the study of such plasmids and transposons in the eubacteria. Although they are major agents of the horizontal gene exchange which complicates simple phylogenetic analyses of whole bacterial genome sequences, there are no compendia of the complete

sequences of the genomes of these peripatetic members of the “floating genome.” We have compiled and annotated sequence information for one of the first recognized, largest, and most widely distributed examples of this group, the multiply composite transposon Tn21.

This analysis has been stimulated by the demonstrated involvement of the Tn21-family in the global dissemination of antibiotic resistance determinants (16–18) and consequent interest in the processes involved in the accumulation and exchange of resistance loci by plasmids and transposons. Horizontal and vertical transfer of antibiotic resistance genes in members of the family *Enterobacteriaceae* and *Pseudomonas* is mostly due to large broad-host-range plasmids and to the transposons they carry and share with other replicons (17). Many transposons encoding multiple antibiotic resistances in gram-negative members of the *Enterobacteriaceae* belong to the Tn21 subgroup of the Tn3 family of transposable elements (29, 63). Transposon Tn21 and many of its closest relatives carry within them a potentially independently mobile DNA element called an integron. The integron encodes a RecA-independent, site-specific integration system that is responsible for the acquisition of multiple small mobile elements called

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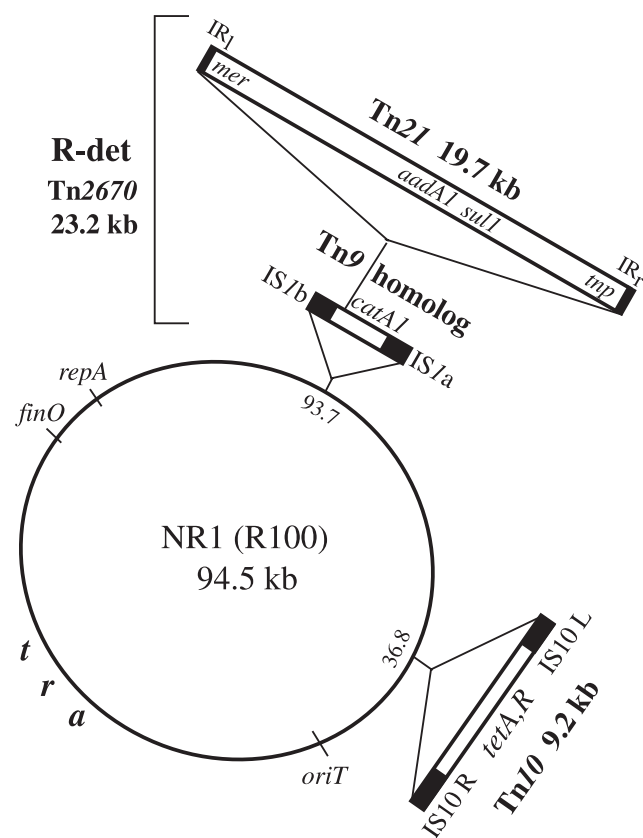


FIG. 1. NR1, an IncFII self-transmissible, multiple-antibiotic resistance R plasmid. Inserts indicate mobile genetic elements.

gene cassettes that encode antibiotic resistance genes (35, 60, 74, 104, 109). Tn21 also carries the genes involved in its own transposition (*tnp*) and a mercury resistance (*mer*) operon (29, 98).

Transposon Tn21 itself is carried by plasmid NR1 (R100), which was originally isolated from *Shigella flexneri* in Japan in the late 1950s (70). NR1 is a 94.5-kb, self-transmissible, multiple-antibiotic resistance plasmid (Fig. 1) and is the archetype of a large collection of similar R-plasmids that have been discovered worldwide (18, 116). NR1, which belongs to the FII incompatibility group of R-plasmids, was initially considered to have two components: a resistance transfer factor (RTF), which carries the genes for self-transmissibility (*tra*) and autonomous replication (*rep*), and a resistance determinant (R-det), which contains a composite Tn9-like transposon, carrying the *catA1* (*catI*) gene, and transposon Tn21 (120). The R-det component is bound by direct repeats (*IS1a* and *IS1b*) and is itself transposable as Tn2670 (37, 43). Tn21 is an independent transposon within the R-det and is bounded by inverted repeats *IR*₁ and *IR*₂ (19, 28, 127). Located in the RTF region of NR1 is transposon Tn10, which carries *tetR* (*tet* repressor) and *tetA* (confers tetracycline resistance) and is bounded by *IS10R* and *IS10L* (46). The other major functional loci within the RTF are the origin of conjugative transfer (*oriT*) gene and the transfer (*tra*) genes.

Recently, the last section of the Tn21 transposon was sequenced (5), revealing that Tn21 itself includes four discrete mobile elements: a class 1 integron, the *aadA1* gene cassette contained in the integron, and two insertion sequences, *IS1326* and *IS1353* (Table 1 and Fig. 2). We have assembled five

TABLE 1. Transposon Tn21 and the mobile elements it contains

Element	Coordinates	Size (bp)
Tn21	1–19671	19,671
In2	4040 ^a –15038	10,999
<i>aadA1</i> cassette ^b	5395–6250	856
<i>IS1326</i>	8276–10719, 12333–12360 ^c	2,470
<i>IS1353</i>	10720–12332	1,612

^a Duplication of 5 bp due to insertion of In2; coordinates 4035 to 4039 and 15039 to 15543.

^b The cassette is located within In2 but is independently mobile.

^c The sequence of *IS1326* is interrupted by the insertion of *IS1353*, which creates a 2-bp target site duplication.

overlapping GenBank sequences (accession no. X04891, M10791, X12870, U42226, and J01730 [Table 2]) and updated the annotation of the assembled sequence of 19,671 bp (GenBank accession no. AF071413 [Tables 3 to 5]). Due to the growing interest in integrons, we present the Tn21 sequence with the transposition genes on the left and the *mer* genes on the right, since this allows the components to be viewed in the orientation familiar to those studying the integron itself.

TRANSPOSITION COMPONENTS

Tn3-Like Transposable Elements—Subtype Tn21

Tn3-like transposable elements are characterized by flanking inverted repeats of about 38 bp and have two genes, *tnpA* and *tnpR*, which encode a transposase and resolvase, respectively (29, 95). The *tnpR* gene and the *res* site (the site at which the resolvase acts) are located just upstream of *tnpA*. In the Tn21 transposon subgroup, the *tnpA* and *tnpR* genes are transcribed in the same direction. The TnpA proteins of this transposon family are at least 70% homologous to each other, as are their TnpR proteins (29). Transposases from this family share, as do many other transposases, a DD(35)E motif, which may form part of the catalytic center (125).

Tn21, a Class II Replicative Transposon

Transposition of transposon Tn21 is carried out by the transposase, TnpA (29). The sites of insertion, defined by a 5-bp duplication of target DNA, are usually AT rich but have no other obvious consensus sequence (77). Replicative transposition of Tn21 involves specific recognition of and binding to the terminal inverted-repeat (*IR*) elements of Tn21 by TnpA, which then mediates joining of the donor and recipient replicons (77). This cointegrate intermediate contains a copy of Tn21 and is resolved by the action of the *tnpR* gene product, a resolvase which acts at a specific site (*res*) adjacent to the *tnpR* gene (85).

The resolvase catalyzes site-specific recombination between supercoiled DNA and two directly repeated copies of the transposon DNA (102). Resolvase subunits bind to the three subsites (I, II, and III) of *res* within both recombination loci (100). These protein-DNA assemblies at each *res* site then interact with each other to form a synaptic complex (101) in which the resolvase separates the cointegrate to give a new recombinant replicon with one copy of the transposon and the original donor replicon, which retains its copy. In the Tn21 subgroup of transposons, the transposase-*IR* interactions and the resolvases are interchangeable; e.g., Tn21 *tnpA* can act on the Tn501 *IR*s and Tn21 *tnpR* can act on the Tn501 *res* site (29).

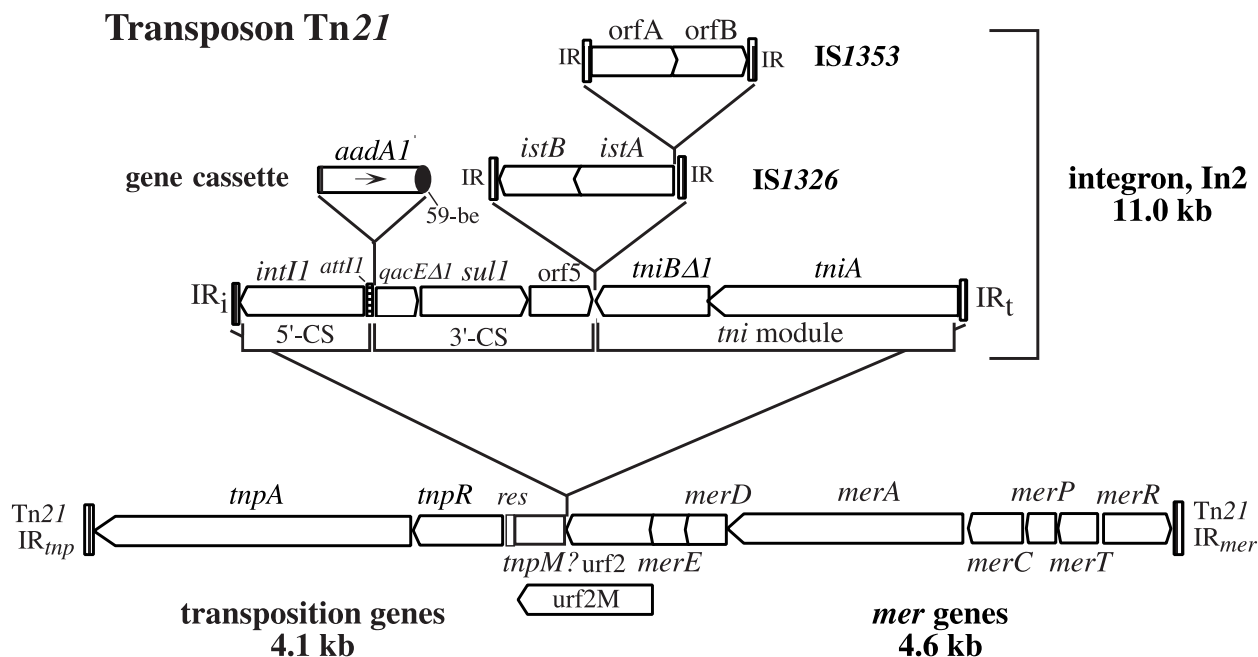


FIG. 2. Symbols for the transposition (*tnp*) region, the integrator, and the *mer* operon of transposon Tn21 (GenBank accession no. AF071413). Vertical bars indicate flanking inverted repeats (IR) of transposons and insertion sequences (IS). The *tnp* region consists of genes for the transposase (*tnpA*), the resolvase (*tnpR*), the putative transposition regulator (*tnpM*), and the resolution site (*res*) for Tn21. The 5'-CS of the integrator includes the integrase gene (*intI1*) and the *attI1* insertion site. The *aadA1* gene cassette contains the aminoglycoside adenylyltransferase gene and the 59-be. The arrow indicates the direction of transcription. The 3'-CS includes genes encoding resistance to quaternary ammonium compound disinfectants (*qacEΔ1*) and sulfonamide resistance (*sul1*), an ORF (*orf5*) of unknown function, and two insertion sequences (IS1353 is inserted into IS1326). The *tni* (transposition of the integrator) gene region has suffered deletion in Tn21, and only *tniA* and a portion of *tniB* remain. The mercury resistance (*mer*) operon consists of the regulatory genes *merR* and *merD* and the structural genes *merT*, *merP*, *merC*, and *merA*. There are two unknown reading frames, *urf1* (also called *merE*) and *urf2*, downstream of *merD*. *urf2M* is a hypothetical gene defined in this work, which may have existed before integrator insertion.

***tnp* transposition module.** The positions of the important features of the *tnp* region of Tn21 are presented in Table 3, and those of the IR_{*mer*} region are presented in Table 4. The *tnp* region begins with the left IR (designated IR_{*tnp*}) and extends to include the *res* site. The right end of Tn21 (designated IR_{*mer*}) lies beyond the end of the *merR* gene. These left and right Tn21 IRs are 38-bp imperfect repeats (three base differences). *tnpA* is the largest structural gene in the transposon, encoding a 988-amino-acid (aa) transposase which terminates 5 bp into IR_{*tnp*} (115). An independent promoter for *tnpA* has been suggested (29, 42) to occur within the last 80 bp of the *tnpR* gene. *tnpR* is 561 bp and terminates 2 bp before the putative initia-

tion codon of *tnpA*. The predicted promoter region for the *tnpR* gene (and possibly the *tnpA* gene) is located within the *res* site (22, 31). The 129-bp *res* site has three resolvase binding sites (I, II, and III) with the recombination crossover point located in *resI* (85).

Putative *tnpM*. An adjacent putative gene of undefined function, *tnpM* (351 bp), was originally defined as starting at the

TABLE 2. Sequenced regions comprising Tn21 (GenBank accession no. AF071413)

Region	Coordinates	Reference(s)	Original GenBank accession no.
<i>tnpA</i>	1–3056	115	X04891
<i>tnpR</i> , <i>tnpM</i>	3057–4105	42	M10791
<i>tnpR</i> , < <i>tnpM</i> ^b		22	K01726 ^a
<i>intI1</i> , <i>aadA1</i> , (<i>qacEΔ1</i> , <i>sul1</i>)	4106–7539	109	X12870
<i>intI1</i> , < <i>aadA1</i>		63	M33633 ^a
<i>qacEΔ1</i> , <i>sul1</i> , <i>orf5</i> , <i>istB</i> , <i>istA</i> , <i>orfA</i> , <i>orfB</i> , <i>tniBΔ1</i> , <i>tniA</i>	7540–14933	5, 33, 104	U42226
<i>orf2</i> , <i>merE</i> , <i>D</i> , <i>A</i> , <i>C</i> , <i>P</i> , <i>T</i> , <i>R</i>	14934–19671	7, 65, 66	J01730
< <i>merD</i> , <i>A</i> , <i>C</i> , <i>P</i> , <i>T</i> , <i>R</i>		1	K03089 ^a

^a Not used in Tn21 assembly AF071413 but partial duplications of other regions used.

^b < indicates a partial gene sequence.

TABLE 3. Features of the *tnp* transposition region of Tn21

Feature	Coordinates ^a
Tn21 IR _{<i>tnp</i>}	1–38
<i>tnpA</i> gene.....	(34–3000)
<i>tnpA</i> – 10.....	(3063–3068)
<i>tnpA</i> – 35.....	(3086–3091)
<i>tnpR</i> gene.....	(3003–3563)
<i>tnpR</i> – 10.....	(3591–3596)
<i>tnpR</i> – 35.....	(3614–3618)
<i>resIII</i>	(3576–3605)
<i>resII</i>	(3610–3652)
<i>resI</i>	(3668–3704)
Recombination crossover point.....	(3684–3685)
<i>tnpM</i> gene.....	(3689–4039)
<i>tnpM</i> – 10 ^b	(4086–4091)
<i>tnpM</i> – 35 ^b	(4109–4114)
Alternate <i>tnpM</i> – 10 ^b	(4094–4099)
Alternate <i>tnpM</i> – 35 ^b	(4115–4120)

^a Parentheses indicate that the ORF or control site is on the complementary strand in the sequence compiled in GenBank accession no. AF071413.

^b There are two possible –10 and –35 promoter regions for *tnpM*; however, neither conform well to the consensus sequence for σ^{–70} RNA polymerase. Both lie within the left end of In2.

TABLE 4. Features of the *mer* region of Tn21

Feature	Coordinates ^a
Tn21 IR _{mer}	19634–19671
<i>merR</i> gene	(19204–19638)
<i>merTPCAD</i> –35	(19193–19198)
<i>merTPCAD</i> –10	(19168–19173)
<i>merR</i> –10	(19166–19171)
<i>merR</i> –35	(19145–19150)
<i>merT</i> gene	(18782–19132)
<i>merP</i> gene	(18493–18768)
<i>merC</i> gene	(18035–18457)
<i>merA</i> gene	(16289–17983)
<i>merD</i> gene	(15909–16271)
<i>merE</i> gene	(15676–15912)
urf2 putative gene	(14972–15679)
urf2M putative gene ^b	(3689–4039) + (15044–15679)

^a Parentheses indicate that the ORF or control site is on the complementary strand in the sequence compiled in GenBank accession no. AF071413.

^b Newly defined here (see the text).

junction of the *tnp* region and the integron IR_i (leftward inverted repeat) and terminating within *resI* (42). The ribosomal binding site and the promoter –10 and –35 regions for this putative *tnpM* gene are located within the integron, in IR_i and in the noncoding region downstream of the *intI1* gene, respectively.

Although the roles of *tnpA* and *tnpR* have been defined, regulation of the *tnp* genes in Tn21 is less well understood. Studies by de la Cruz and Grinstead with Tn21 (19) and by Tanaka et al. with Tn2603 (112) showed that transposition and resolution functions were intact in deletion derivatives which left the *tnpA*, *tnpR*, and *tnpM* reading frames intact but disrupted the putative –35 promoter region of *tnpM*. These authors concluded that *tnpM* was not necessary for transposition. However, Hyde and Tu (42) proposed that the *tnpM* gene

produces a protein which enhances Tn21 transposition and suppresses cointegrate resolution. The basis for their proposal was the finding that a strain carrying a nonsense mutation in the *tnpM* reading frame was less effective than its parent in effecting Tn21 transposition and resolution. Furthermore, they observed that when a multicopy derivative carrying only Tn21 *tnpM* was present, transposition of Tn21 increased by 2.5-fold and resolution decreased 22-fold. They also noted an open reading frame (ORF) in Tn501 (*orf2*) at the same relative location and orientation, whose product was 84.6% similar to the putative Tn21 TnpM protein (Fig. 3). These similarities led Hyde and Tu to examine whether transposition and resolution of Tn501 was susceptible to modulation by Tn21 *tnpM*. They found that with the Tn21 *tnpM* gene in *trans*, transposition of Tn501 also increased by 2.5-fold and resolution decreased by 1.5-fold. However, no TnpM protein has been demonstrated. Thus, at present, neither the exact nature of the control for TnpA and TnpR nor the role of the putative TnpM is understood. We offer here the possible alternative that the *tnpM* reading frame is actually derived from a longer ORF which was interrupted by the insertion of In2 (see below). These alternatives are not necessarily mutually exclusive.

MER OPERON

Mercury Resistance

Divalent mercuric ion, Hg(II), has a high affinity for thiol groups of proteins and for this reason is extremely toxic in biological systems. The current model of the mercury resistance (Hg^r) mechanism of gram-negative bacteria posits that Hg(II) enters the periplasm and is bound to a pair of cysteine residues in MerP which transfer Hg(II) to cysteine residues in MerT or MerC. The latter move the ion across the cytoplasmic membrane in a series of ligand exchange reactions, resulting in

TABLE 5. Features of the In2 region of Tn21

Feature	Coordinates ^a	Feature	Coordinates
In2 boundaries	4040–15038	<i>sulI</i> gene	6700–7539
5' conserved segment	4040–5394	Potential 59-be	7534–7567
IR _i	4040–4064	<i>orf5</i> gene	7667–8167
<i>intI1</i> gene	(4242–5255)	IS1326 boundaries	8276–12360
P _{int} –10	(5285–5290)	IS1326 IR	8276–8301
P _{int} –35	(5308–5313)	<i>istB</i> gene	(8343–9128)
P _c (P1) –35	5140–5145	<i>istA</i> gene	(9115–10638)
P _c (P1) –10	5163–5168	<i>istAB</i> –10	(10711–10716)
P2 –35	5259–5264	IS1353 boundaries	10720–12332
3G insertion that creates P2	5278–5280	IS1353 IR	10720–10731
P2 –10	5282–5287	<i>orfAB</i> –35	10738–10743
<i>attI1</i> site	5362–5400	<i>orfAB</i> –10	10762–10767
Core site, GTTRRRY gene cassette	5394–5400	<i>orfA</i> gene	10791–11429
<i>aadA1</i> gene cassette	5395–6250	Frame shift	11396–11402
5' portion of 59-be	5395–5400	Stem-loop	11407–11462
<i>aadA1</i> gene	5416–6195	<i>orfB</i> gene	11390–12304
alternate start, <i>aadA1</i> gene	5404	IS1353 IR	12355–13215
78% identical to <i>merO</i>	5491–5508	IS1326 IR	12335–12360
3' portion of 59-be	6197–6250	<i>istAB</i> –35	(12348–12353)
Inverse core site, RYYAAC	6197–6203	<i>tni</i> region	(12355–14990)
Central IR in 59-be	6209–6225	<i>tniBΔI</i> gene	(12355–13215)
Central IR in 59-be	6229–6243	<i>tniA</i> gene	(13218–14933)
Core site, GTTRRRY	6250–6256	<i>tniAB</i> –10	(14963–14968)
3' conserved segment	6251–8275	<i>tniAB</i> –35	(14985–14990)
<i>qacEΔI</i> gene	6359–6706	IR _t	15014–15038
Sequence diverges from <i>qacE</i>	6650–6651	5-bp direct repeat of target	15039–15043

^a Parentheses indicate that the ORF or control site is on the complementary strand in the sequence compiled in GenBank accession no. AF071413.

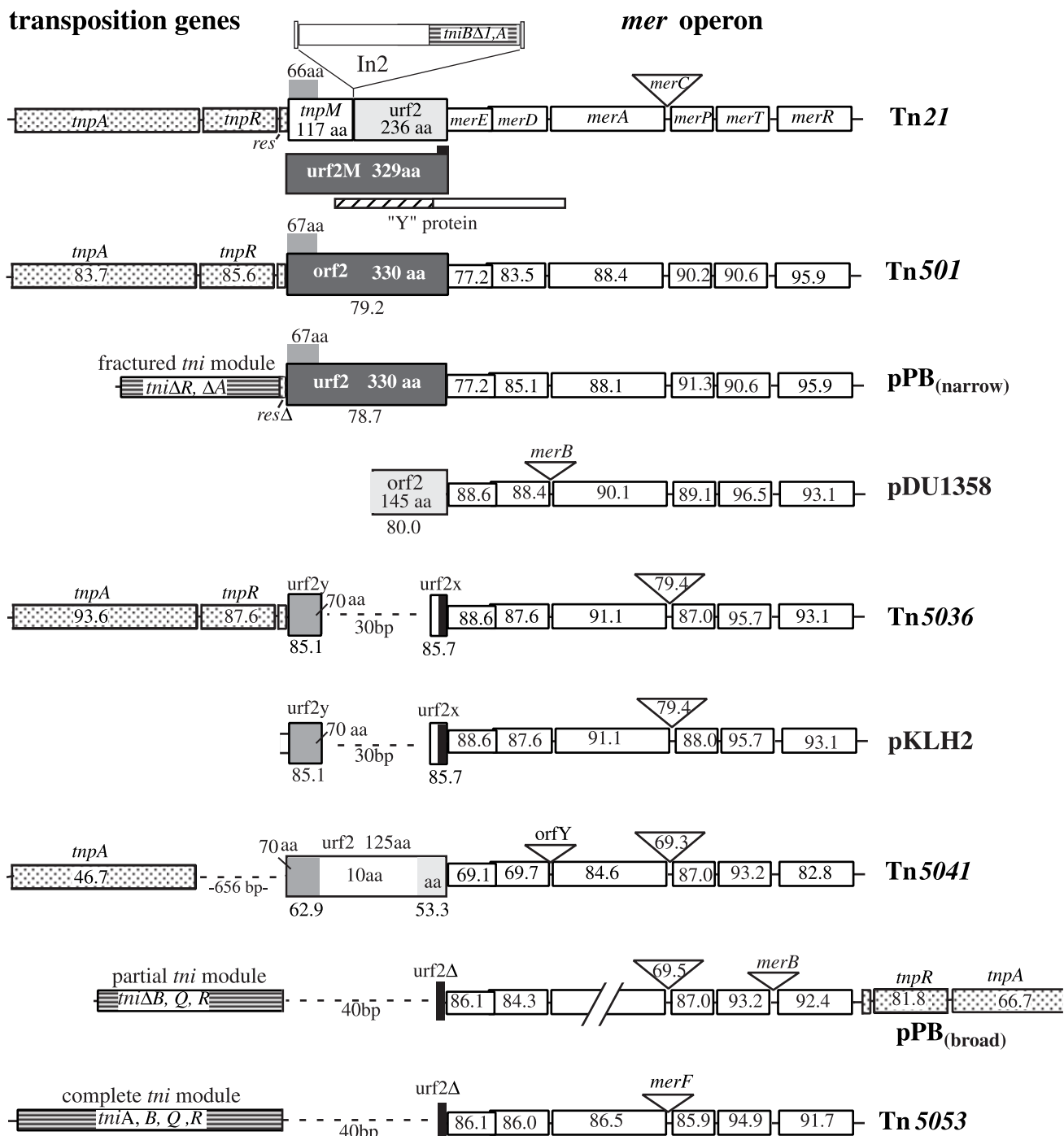


FIG. 3. Defined or hypothetical proteins of Tn21, the closely related Tn501 and Tn5036, the distantly related transposons Tn5041 and Tn5053, and other mer operons in plasmids pDU1358, pKLH2, and pPB (narrow- and broad-spectrum mercury resistance operons). References are given in Table 6. Numbers in boxes and triangles representing mer or transposition genes are percent amino acid similarities to corresponding proteins of Tn21. Tall shaded boxes represent regions similar to putative Tn21 Urf2 (light gray), to the last 66 aa of putative TnpM (medium gray), or to hypothetical Tn21 Urf2M (dark gray). Black boxes represent small regions (5 to 7 aa) of similarity to the first 7 aa in Urf2M. Numbers below the tall shaded boxes are percent similarities to corresponding proteins in Tn21. Horizontal striped boxes represent tni genes, and spotted boxes represent tnp genes. The slashed box represents a region similar to Tn21 Urf2M and Y proteins. merA of the pPB broad-spectrum mer operon has not been sequenced.

the delivery of Hg(II) to the active site of the cytoplasmic flavin disulfide oxidoreductase, mercuric reductase (MerA) (98). Mercuric reductase catalyzes the reduction of Hg(II) to the highly volatile and less reactive monoatomic gas, Hg⁰, which diffuses from the cell and from the medium.

mer Genes

The mer region (Table 4) (1, 65, 66), flanked on its right by Tn21 IR_{mer} and on its left by the rightward IR (designated IR_r) of the integron, is sufficient to confer resistance to Hg(II). The

TABLE 6. Sequences of *mer* loci from gram-negative bacteria

Common name	Source and genes sequenced	Accession no.	References
Tn21	Plasmid NR1 from <i>Shigella flexneri</i> <i>merRTPCAD</i> , <i>urf1</i> , <i>urf2</i>	AF071413	7, 65, 66; this work
Tn5036	Tn5036; plasmid pKLH256 from <i>Enterobacter cloacae</i> <i>merRTPCAD</i> , <i>urf1</i> (<i>E</i>), <i>urf2X</i> , <i>urf2Y</i>	Y09025	124
pMER610	Plasmid pMER610 from <i>Alcaligenes</i> sp. <i>merRTPCAD</i> <i>urf1</i> (<i>E</i>), <i>urf2X</i> , <i>urf2Y</i>	Y08993	124
pKLH272	Plasmid pKLH272 from <i>Enterobacter agglomerans</i> <i>merRTPCAD</i> , <i>urf1</i> (<i>E</i>), <i>urf2X</i> , <i>urf2Y</i>	Y08992	124
pKLH2	Plasmid pKLH2 from <i>Acinetobacter calcoaceticus</i> <i>merRTPCADE</i> , <i>urf2X</i> , <i>urf2Y</i>	L04303	47
pDU1358	Plasmid pDU1358 from <i>Serratia marcescens</i> <i>merRTPABD</i> , <i>orf1</i> , <i>orf2</i>	M24940, Z49200, M15049	27
Tn5053	Tn5053; <i>Xanthomonas</i> sp. strain W17 chromosome <i>merRTPFADE</i> , <i>urf2</i>	L40585	49
pPB	Plasmid pPB from <i>Pseudomonas stutzeri</i> <i>merRTPAD</i> , <i>urf1</i> , <i>urf2</i> and <i>merRBTPCAD</i> , <i>urf1</i> , <i>urf2Δ</i>	U80214, U81032, U90263	84
Tn501	Plasmid pVS1, Tn501 in <i>Pseudomonas aeruginosa</i> <i>merRTPAD</i> , <i>orf1</i> , <i>orf2</i>	Z00027	6, 65
Tn5041	Tn5041, <i>Pseudomonas</i> strain KHP41 <i>merRTPCAYD</i> , <i>urf1</i> , <i>urf2</i>	X98999	124
<i>Thiobacillus</i>	<i>Thiobacillus ferrooxidans</i> E-15 chromosome <i>merR2</i> , <i>merC2</i> , <i>orf1</i> , <i>orf2</i> , <i>orf3</i> , <i>merC1</i> , <i>merR1</i> and <i>urf</i> , <i>merC</i> , <i>merA</i>	X57326 D90110	44 45

mercury resistance operon of Tn21 contains two regulatory genes, *merR* and *merD*, and four structural genes, *merT*, *merP*, *merC*, and *merA*, encoding the NADH-dependent flavin oxidoreductase, mercuric reductase (MerA); two inner membrane proteins (MerT and MerC); and a periplasmic protein (MerP). Further genes may also play a role in mercury resistance.

Metalloregulator (*merR*). The *merR* gene is at the opposite end of the transposon to the *tnpA* gene, and, like *tnpA*, *merR* terminates 5 bp into the IR bounding Tn21. The *merR* gene and the *merTPCAD* genes are transcribed divergently from a compact 71-bp promoter/operator (*merOP*) region in which their respective -10 RNA polymerase recognition hexamers overlap by 4 bp (39, 73). MerR binds a dyadic operator (*merO*) in the 19-bp spacer between the *merTPCAD* -35 and -10 promoter hexamers, and in the absence of Hg(II) it represses their expression. Since the dyadic binding site is also superimposed on the *merR* transcriptional start position, MerR represses its own synthesis (96). The metal binding domain of MerR, containing residues from Cys80 to Cys128, is sufficient to form a single, dimeric, trigonally coordinate, high-affinity Hg(II) binding site (126). When MerR binds Hg(II), an allosteric change in MerR allows transcription of the *mer* structural genes without changing the level of slightly leaky repression of MerR (86, 107). MerR also sequesters RNA polymerase at the *merT* promoter, forming a stable preinitiation complex (39). MerR contacts the α , β , and σ^{70} subunits of RNA polymerase both in the preinitiation and Hg(II)-activated promoter complexes (51).

Mercury transport genes (*merT*, *merP*, and *merC*). The products of the first three structural genes of the *mer* operon are involved in uptake of Hg(II) into the cell. These small genes are tightly spaced, with the *merT* gene (351 bp) separated by only 13 bp from the *merP* gene (276 bp) and *merP* separated from the *merC* gene (423 bp) by 35 bp. Both MerT and MerC are inner membrane proteins (106) and are predicted to have three and four transmembrane-spanning α -helices, respectively. MerP is located in the periplasm (106) and is composed of four β -strands and two α -helices (103). Both cysteine residues (Cys33 and Cys36) in MerP are required for Hg(II) binding in vitro (90), and this is one of only two proteins of the operon whose structure is presently known (103). MerP is believed to transfer Hg(II) to the amino-terminal cysteine

pair (Cys24 and Cys25) of MerT (36, 67). How Hg(II) is then transferred inside the cell to MerA is unknown. The MerP sequence constitutes the index example of the HMA motif found in several prokaryotic metal transporters (62, 72, 97) and in the human Cu transporters, MNK and WND (8).

The *merC* gene is apparently not required for Hg(II) resistance, if MerP and MerT are present, since there are functional *mer* operons without *merC*, e.g., those found in transposons Tn501 and Tn5053 (Fig. 3) (98). Moreover, deleting *merC* from the Tn21 *mer* locus had no effect on Hg(II) resistance (36). However, the *Thiobacillus merC* loci do not have the *merT* or *merP* genes but have at least three distinct *merC* genes (Table 6) (40), and *Thiobacillus MerC* can function alone as a mercury transport system in *Escherichia coli* (52). Tn21 MerC binds Hg(II) (91), possibly via a heavy-metal consensus binding region similar to those found in MerP and in the amino-terminal region of MerA. The actual interactions of MerP, MerT, MerC, and MerA remain to be defined.

Mercuric reductase gene (*merA*). *merA*, which begins 51 bp on the 3' side of *merC*, encodes a cytoplasmic, redox enzyme (564 aa) and is the largest gene in the *mer* operon. MerA belongs to the class I pyridine nucleotide-disulfide oxidoreductases and has sequence and structural homology to glutathione reductase and other disulfide oxidoreductases (93). The amino terminus of *merA* and of the *merP* gene (signal sequence excluded) are extensively homologous, indicating a fusion between ancestral genes for MerP and for glutathione reductase. MerA functions as an α_2 homodimer, and two pairs of cysteine residues, a redox-active pair (Cys135 and Cys140) from one subunit of MerA and a carboxyl-terminal pair (Cys561 and Cys562) from the other subunit of MerA, form the catalytic site (21). MerA uses two hydrides from two NADPH molecules for the reduction of flavin adenine dinucleotide, which in turn reduces Hg(II) bound to the redox-active cysteines of the enzyme (89, 93). Studies by Miller et al. (64) have shown that pyridine nucleotide complexed dimers of the enzyme are asymmetric. Since the reduced enzyme EH2-NADPH complex is the reductant of Hg(II), these observations suggest that the enzyme may function asymmetrically during catalysis. Miller et al. (64) have proposed an alternating-sites model for the catalytic reduction of Hg(II), where both subunits of the dimer function in catalysis but the steps are staggered, allowing sub-

units to reverse roles after part of the reduction so that the enzyme can both bind Hg(II) very tightly and reduce it (64).

Secondary regulatory gene (*merD*). The most promoter-distal experimentally defined gene of the *mer* operon, *merD* (363 bp), begins 17 bp downstream of *merA* (54). The MerD protein is made in very small amounts (54). The suggested role of MerD is that of a down-regulator of the *mer* operon which binds weakly to the *merOP* region; MerD null mutants show increased operon expression (69).

Putative *merE* (*urf1*). Two other reading frames at the end of the *mer* operon in Tn21 are also frequently found in other *mer* operons (Fig. 3), but experimental definition for them is lacking. The first of these, which overlaps the 3' end of *merD* by 4 bp, is a 237-bp ORF variously designated *orf1* (6, 27), *urf1* (7, 84, 124), *urf* (45), or *merE* (47, 49) (Table 6). Putative *merE* has a GTG start codon and an appropriately placed Shine-Dalgarno consensus sequence (AGGAGG) and encodes a predicted polypeptide of 78 aa. There is no evidence to indicate whether this ORF is transcribed or translated, and no null or nonsense mutations in it have been examined for their effect on Hg resistance. However, 10 highly similar ($80.4\% \pm 8.6\%$ as determined by GAP; Genetics Computer Group, Inc. [GCG], Madison, Wis.) putative MerE protein sequences (listed in Table 6) from other *mer* loci of gram-negative bacteria have been reported. Of the 10 putative *merE* genes, 9 lie in the same position and orientation as the *urf1* in the Tn21 *mer* locus (Fig. 3) and the other is located 5' to the *merC* gene of a *Thiobacillus mer* locus (Table 6) (45). The putative Tn21 *merE* is also 58.0% similar to *merT* (previously called ORF2) in the *mer* locus of a gram-positive bacterium, *Bacillus* sp. strain RC607 (114). These 12 sequences are the only homologs of Tn21 *merE* in the database.

Cysteine pairs are characteristically found in Mer proteins, and putative MerE has a cysteine pair (Cys28 and Cys30) which, with adjacent residues (LTCPCHL), is conserved in all of these putative MerE polypeptides. The predicted secondary structure of MerE would have two transmembrane helices with the cysteine pair positioned approximately in the middle of the first helix (87, 99). That the first cysteine pair is also found in the same predicted position in MerT, MerC, and MerF suggests that MerE may play a role in Hg(II) transport.

urf2 and the Hypothetical urf2M

Overlapping the 3' end of *merE* by 4 bp is the second reading frame of unknown function, *urf2* (708 bp), whose stop codon lies 67 bp inside the right end of the integrin rightward boundary at IR_r. However, if the integrin and one of its flanking 5-bp direct repeats were removed, *urf2* would fuse in frame with the putative *tnpM* gene to produce a 987-bp hypothetical gene which we designate Tn21 *urf2M* (Fig. 3). Its 328-aa predicted product, Urf2M, is 76.9% identical and 79.3% similar to the predicted 329-aa product of *orf2* found in transposon Tn501. Transposon Tn501 is a Tn21 subgroup transposon with a gene organization similar to that of Tn21 but lacking both *merC* and the integrin (29). Also, the predicted product of *urf2* in the *mer* operon of plasmid pPB is 78.7% similar to Tn21 Urf2M. The predicted product of *orf2* (an incomplete sequence) in the *mer* operon of plasmid pDU1358 is 80.0% similar to Tn21 Urf2M.

Further, part of Tn21 Urf2M (226 of 328 aa) (Fig. 3) is also about 62% similar to a group of hypothetical proteins which belong to the YEGE/YHDA/YHJK/YJCC family of proteins (4, 121). These putative proteins (referred to here as Y proteins) are characterized by transmembrane regions and tyrosine kinase domains as found in two-component signal reg-

TABLE 7. Comparison of codon usage in *tnpA*, *merA*, and the *tnp-mer* junction of Tn21 family transposons and a distantly related transposon, Tn5041

Gene A	Genes compared ^a		Gene B	Length (bp)	<i>D</i> ^{2b}
	Length (bp)	Length (bp)			
Tn21 <i>tnpA</i>	2,967	Tn501 <i>tnpA</i>	2,967	0.62	
		Tn5036 <i>tnpA</i>	2,973	0.97	
		Tn5041 <i>tnpA</i>	2,988	2.55	
Tn21 <i>merA</i>	1,695	Tn501 <i>merA</i>	1,686	0.41	
		Tn5036 <i>merA</i>	1,686	0.50	
		Tn5041 <i>merA</i>	1,680	2.72	
Tn21 <i>urf2M</i>	987	Tn21 <i>merA</i>	1,695	1.00	
		Tn21 <i>tnpA</i>	2,967	3.21	
		Tn501 <i>orf2</i>	990	1.31	
		Tn501 <i>merA</i>	1,686	0.98	
		Tn501 <i>tnpA</i>	2,967	3.61	

^a Tn21, Tn501, and Tn5036 belong to the Tn21 family of transposons; Tn5041 is distantly related to them (124).

^b The *D*² statistic, calculated with the GCG program Correspond, compares the pattern of codon usage generated by the GCG program CodonFrequency (20). *D*² is the sum over all codons of the residual equation $D^2 = (\text{frequency}_{\text{codon table 1}} - \text{frequency}_{\text{codon table 2}})^2$. *D*² increases as patterns of codon usage become more dissimilar. *D*² values of <3.0 are consistent with a common origin for the genes (24). (The significance of *D*² decreases with sequence sizes of 1,000 bp).

ulators (41). The carboxyl termini of the Y proteins are $62.3\% \pm 1.0\%$ similar ($40.3\% \pm 3.5\%$ identical) to Tn21 Urf2M beginning at aa 22 and ending 35 aa into the beginning of putative TnpM (a total of 226 contiguous residues). This similarity to the carboxyl termini of the Y proteins also occurs in Tn501 Orf2 and pPB Urf2.

These similarities suggest that before In2 insertion, a distinct insertion or deletion occurred, forming an ORF (encoding a 328-aa product) between the *mer* and *tnp* genes or within a gene at the 3' end of an ancestral *mer* operon. Possible remnants of this event are the last 66 aa of Tn21 TnpM, the last 67 aa of Tn501 Orf2 and pPB Urf2, and the last 70 aa of Tn5041 Urf2, pKLH2 Urf2y, and Tn5036 Urf2y, which are conserved (including three conserved cysteine pairs) (Fig. 3). Ribosomal binding sites in front of possible translation start sites occur in the genes encoding each of the aforementioned 66-, 67-, and 70-aa predicted products, suggesting that a smaller gene may be present in the larger ORFs.

To assess the possible origin of Tn21 *urf2M* and Tn501 *orf2*, we compared codon usage patterns of Tn21 *urf2M* with those of the Tn21 and Tn501 *merA* and *tnpA* genes (Tables 7 and 8). The GCG program Correspond (24) compares two codon frequency tables (20) and yields the residuals statistic *D*² (Table 7, footnote b), which, when greater than 3, indicates different codon usage patterns. *D*² values of <3.0 are consistent with a common origin for the genes (24). We largely limited our analysis to sequences of >1,000 bp since we observed that *D*² increased when the sequence was small.

The *D*² values, 0.98 to 1.31, indicated that Tn21 *merA*, Tn501 *merA*, and Tn501 *orf2* have similar codon usage patterns to Tn21 *urf2M* (Table 7). Higher *D*² values (3.21 and 3.61) indicated that Tn21 *tnpA* and Tn501 *tnpA* have dissimilar codon usage patterns compared to Tn21 *urf2M*. Codon usage in each of the two ORFs which comprise the putative Tn21 *urf2M* (*tnpM* and *urf2*) shows that each is as distant from *tnpA* and as close to *merA* as is the putative composite gene; thus, by the *D*² statistic, neither component gene is related to *tnpA* or to *merA* any differently than the putative fused gene. We also noted a

TABLE 8. Comparative codon usage in Tn21

Tn21 gene	Length (bp)	D^{2a} with respect to:										
		<i>tnpA</i>	<i>intI1</i>	<i>aadA1</i>	<i>qacEΔ1</i>	<i>sul1</i>	<i>istAB</i>	<i>orfAB</i>	<i>tniA</i>	<i>urf2M</i>	<i>merA</i>	
<i>tnpA</i>	2,967	0										
<i>intI1</i>	1,014	1.28	0									
<i>aadA1</i>	792	5.09	4.31	0								
<i>qacEΔ1</i>	348	10.50	7.98	5.83	0							
<i>sul1</i>	840	1.69	1.85	3.97	8.20	0						
<i>istAB</i>	2,310	4.30	4.50	1.89	5.41	3.53	0					
<i>orfAB</i>	1,554	3.25	2.82	1.64	5.08	2.52	1.75	0				
<i>tniA</i>	1,716	0.62	0.88	4.08	9.36	1.10	4.05	2.67	0			
<i>urf2M</i>	987	3.20	3.47	4.54	6.80	3.33	3.27	1.53	3.26	0		
<i>merA</i>	1,695	2.73	3.40	4.93	8.84	3.90	3.74	2.25	2.78	1.00	0	

^a See Table 7, footnote b.

surprisingly low D^2 value of 1.13 between one of the genes which encodes the Y protein CY02B10.18C (*Mycobacterium tuberculosis*) and Tn21 *urf2M*. On the basis of these comparisons, Tn21 *urf2M* is more likely to have been associated longer with the *mer* genes than with the *tnp* genes, although its function in mercury resistance and its relationship to the Y proteins is unknown. An alternative hypothesis is that the origins of these ORFs cannot be discerned by codon usage comparisons because they are nonfunctional and therefore are freer to drift than is a functional gene.

In2 INTEGRON

Integrations are a major vehicle for the spread of multiple-antibiotic resistance, and for class 1 integrations, this has been aided by the piggybacking of integrations on other transposons as in Tn21 and its relatives. Integrations provide the cassette-associated resistance genes with two advantages: a "built-in" expression system and ready mobility. Since the promoters located in the 5'-conserved sequence (5'-CS) of the integration (upstream of the inserted cassette) are functional in a wide range of bacteria, the integration can effect the coexpression of several cassette-associated antibiotic resistance genes (11). Cassette-associated resistance genes also harness several mechanisms for their dispersal. Not only are cassettes themselves mobile (10), but also the integrations into which they incorporate are transposable (5). Finally, integrations often occur in plasmids, many of which have broad host range, assisting interspecies spread (16, 81, 104). Integrations and gene cassettes have been reviewed elsewhere (34, 35, 80, 81).

The integration found in Tn21 is a class 1 integration, designated In2 (104), that includes one integrated gene cassette, the *aadA1* cassette. At 11 kb, In2 accounts for over half of the total length of Tn21. In2 is flanked by imperfect inverted repeats of 25 bp (IR_i and IR_j) and bounded by a 5-bp direct duplication of the target sequence (5, 7), indicating that it has transposed into an ancestral *tnp-mer* transposon to create Tn21. In2 does not include a complete set of transposition (*tni*) genes and cannot transpose itself (5). However, movement of In2 has occurred, presumably by using transposition proteins supplied in *trans*, because close relatives of In2 are found in other independent locations (5).

In2 is a composite element that contains a single cassette and two insertion sequences (ISs). The backbone of In2 consists of three distinct regions: 5'-CS, the 3'-conserved segment (3'-CS), and vestiges of the *tni* transposition gene module (Fig. 2). The 5'-CS and 3'-CS lie on either side of the cassette insertion point, which in In2 is occupied only by the *aadA1* cassette. A remnant of the *tni* module is located to the right of

two IS elements, IS1326 and IS1353. One or more deletions originating at the right boundary of IS1326 may have eliminated most of the *tni* genes. Similarly, a deletion originating at the left end of IS1326 has removed part of the 3'-CS (5). IS1353, which is located within IS1326, is presumed to have been acquired after the integration moved into Tn21, because close relatives of Tn21, e.g., Tn2411, that do not contain IS1353 are known (5, 28). The *aadA1* cassette is also found in Tn2411 and may have been present in the In2 progenitor that inserted into the *tnp-mer* transposon. These six regions are described in detail below.

5'-Conserved Segment

The 5'-CS or *intI* module of class 1 integrations extends from the left IR, designated IR_i , to the boundary of the first integrated cassette (Fig. 2). The 5'-CS includes three repeats at its left end that are predicted to bind the *tni* transposase (79) (described in more detail below): the *intI1* gene, encoding the class 1 integrase; the promoter regions for the *intI1* and *aadA1* genes; and the bulk of the *attI1* site, the IntI1-specific recombination site into which cassettes are inserted. IntI1 is a member of the integrase family of site-specific recombinases (14, 26, 74) and catalyzes recombination between various pairs of recombination sites (59-be [see below] \times 59-be, 59-be \times *attI1*, and *attI1* \times *attI1*) in experimental conduction assays (32, 38, 60, 61, 82, 105). IntI1 also catalyzes the integration of additional gene cassettes at *attI1* (10) and both the excision and rearrangement of gene cassettes (12, 13). Experimentally, the *attI1* site consists of at least 34 bp but no more than 64 bp of the 5'-CS and includes 6 to 10 bp at the first adjacent cassette (Fig. 4) (82), although a shorter length has recently been claimed (38). There are four IntI1 binding sites in *attI1*: two sites appear to consist of a simple integrase recombination site made up of a pair of inversely oriented IntI1-binding sites and two additional IntI1-binding sites (DR1 and DR2) that enhance the efficiency of the simple recombination site (Fig. 4) (14, 25).

Integrations also include a promoter, P_c (formerly known as P_{ant} or $P1$), that directs transcription of the cassette-borne gene(s) (Fig. 4). The sequences of the 5'-CS of class 1 integrations found in several different locations are >99% identical. The most frequent differences between the sequences of the 5'-CS from different integrations occur within the -10 or -35 regions of the P_c promoter (104), giving rise to variants with different strengths (9, 11, 55); the variant of P_c in In2 is the weakest. However, the 5'-CS of In2 contains a unique insertion of three G residues (bases 5278 to 5280 [Table 5]; bold G's in

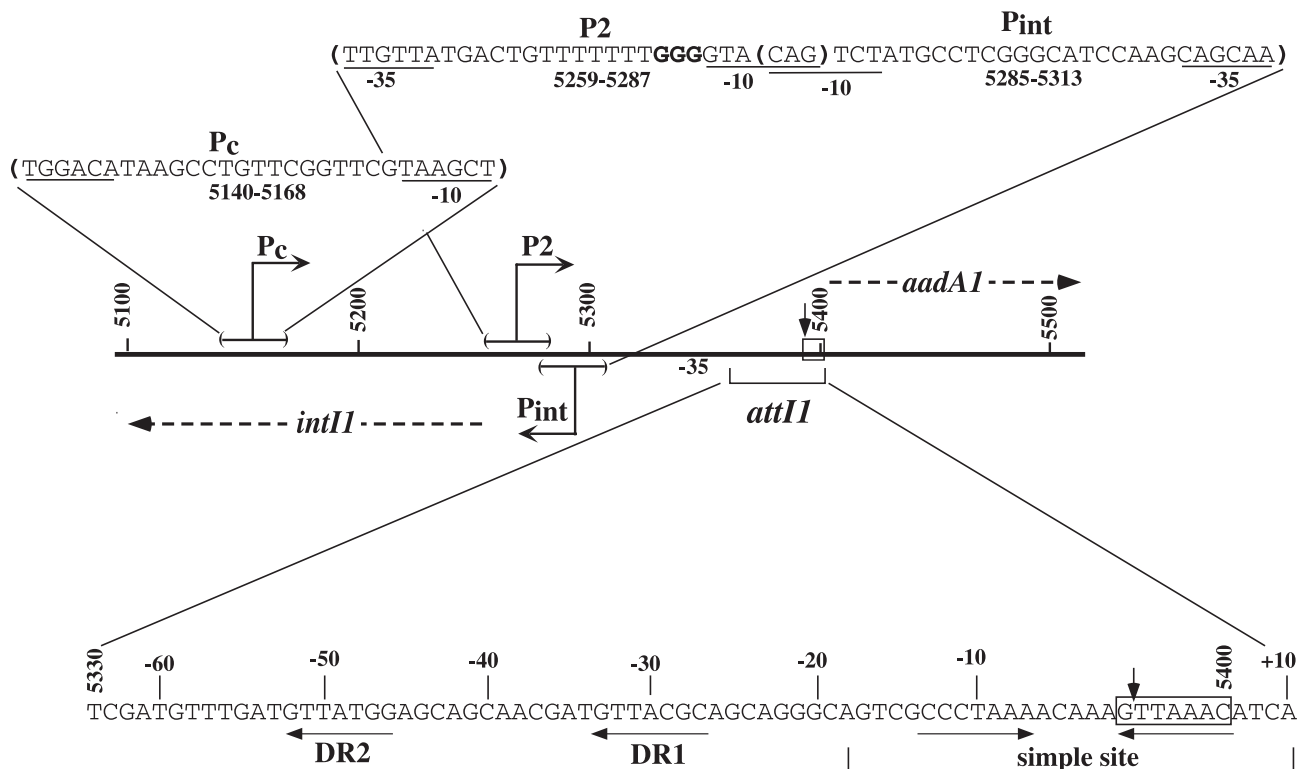


FIG. 4. In2 promoter and *attI1* regions. The direction and position of transcription from the promoters P_c and P_2 for the *aadA1* gene and transcription from the promoter P_{int} for the *intI1* gene are indicated by arrows with parentheses. Translation start sites of *aadA1* and *intI1* are indicated by the dashed arrows. The *attI1* site includes a 64-bp region (sufficient for full recombination site activity for *attI1*). The numbering scheme relates to the number of base pairs to the left (negative) and to the right (positive) of the recombination crossover point (designated position 0), which is marked with a vertical arrow. A box surrounds the *attI1*-*aadA1* recombination site. Integrase binding sites DR1 and DR2 (closely related to the 7-bp consensus sequence, GTTRRRY) and the "simple site" recognized by IntI1 are underscored with arrows. The numbers 5100 to 5500 indicate the location in the GenBank sequence AF071413.

Fig. 4) that increases the spacing between a distinct set of potential -10 and -35 hexamers from 14 to 17 bp, thereby creating a second promoter, P2, which is more efficient than P_c (11, 55). P2 is a secondary promoter found only in In2 and close relatives. Constitutive transcripts from both P_c and P2 can be detected (11); there is no information on whether either is subject to regulation by other factors.

***aadA1* Cassette**

In2 includes a single gene cassette containing the *aadA1* gene, which determines resistance to streptomycin and spectinomycin. The AAD(3'') protein is an aminoglycoside adenylytransferase that inactivates these antibiotics by adding an adenylyl or other nucleotidyl group. The cassette also contains an IntI1-specific recombination site, the *aadA1* 59-base element (59-be; also called *attC*) located downstream of the gene

(Fig. 5). The *aadA1* cassette thus has the features typical of gene cassettes, i.e., a gene and a 59-be. The *aadA1* cassette was presumably incorporated into In2 by IntI1-mediated recombination between the *attI1* site and the 59-be in a free circular *aadA1* cassette. Experimental evidence for the free circular intermediate and for cassette integration has been reviewed extensively elsewhere (34, 35, 80).

The *aadA1* cassette is organized compactly (Fig. 5). The first possible in-frame ATG codon for *aadA1* is only 8 bp from the cassette boundary but is not preceded by a consensus ribosome binding site within the cassette. Alternatively, translation of *aadA1* may initiate at a GTG codon, 12 bp downstream, which is preceded by a ribosome binding site. Translation of the *aadA2* gene in a related cassette is initiated in this manner (3). The 59-be of the *aadA1* cassette lies only 1 bp from the termination codon of the gene.

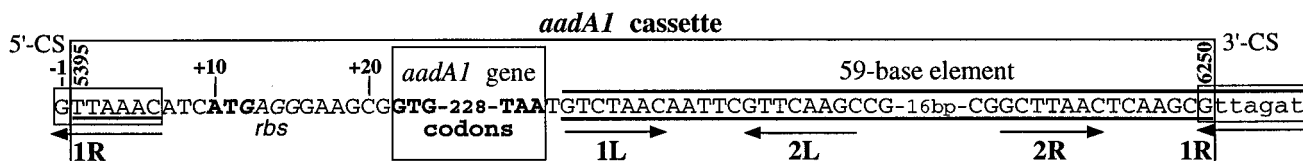


FIG. 5. The *aadA1* cassette containing the *aadA1* (aminoglycoside adenylytransferase) gene and 59-be. The base pairs comprising the 59-be in its free circular form are indicated by the heavy line below them, and those comprising its integrated form are indicated by the heavy line above them. Small boxes surround the composite *attI1/aadA1* (left) and *aadA1/qacE* 59-be (right) recombination sites. The putative initiation codon of the *aadA1* gene and its stop codon are in bold (large box). The putative ribosomal binding site (rbs) is marked in italics. Arrows indicate imperfect inverted repeat sequences. The numbering scheme is as described in the legend to Fig. 4.

In the *aadA1* cassette, the 59-be is actually 60 bp long and has features common to this family of recombination sites (13, 32, 105). Since the 59-be, of the free circular cassette form, is separated into two parts by the recombination crossover, the 59-be immediately downstream from *aadA1* in In2 actually includes 54 bp of the *aadA1* 59-be (from the circular cassette form) and at least 6 bp (lowercase in Fig. 5) of the adjacent module, which is the 3'-CS of In2. This composite 59-be is called the *aadA1/qacE* 59-be. The terminal 6 bp of the *aadA1* 59-be in the circular cassette form lie at the beginning of the integrated *aadA1* cassette and constitute part of the composite *attI1/aadA1* recombination site of In2. The outer 25 bp of all 59-be are relatively highly conserved (13, 105). Within each 25-bp consensus region is a simple site configuration (1L-2L and 2R-1R [Fig. 5]), as found with other integrase recognition sites, and each simple site includes a pair of inversely oriented 7-bp core sites. Only 8 base of the composite core sites are completely conserved: three each in 1L and 1R (ACC and GTT) and one each in 2L and 2R (A and T) in all 59-be (105).

3'-Conserved Segment

In early studies of class 1 integrons, the 3'-CS was defined as a conserved region containing three ORFs (*qacEΔ1*, *sulI*, and *orf5*) located to the right of the different cassette arrays found in various integrons (Fig. 2) (104). More recent studies (5, 33) have revealed that the length of the 3'-CS region is variable and that some integrons have additional sequence (*orf6* or portions of *orf6*) downstream of *orf5*. However, not all class 1 integrons have this region (Fig. 6); e.g., Tn402, also recently called Tn5090 (79), does not have a 3'-CS region.

The *qacE* gene encodes an inner membrane protein with four transmembrane segments and determines resistance to quaternary ammonium compounds (antiseptics and disinfectants) by an efflux mechanism (71, 88). The last 66 bp of *qacEΔ1* (encoding 115 aa) differs from the complete *qacE* gene (encoding 110 aa); the *qacEΔ1* gene confers only marginal resistance to quaternary ammonium compounds (75). The *sulI* gene (formerly *sulI*) encodes a sulfonamide-resistant dihydropteroate synthase (279 aa), which can replace the normal bacterial enzyme targeted by this group of drugs (111). The function of *orf5* is not known; however, the predicted protein is 38% identical and 47% similar to a puromycin *N*-acetyltransferase (gi 1346911) of *Streptomyces alboniger* and is 36% identical and 50% similar to a putative acetyltransferase (gi 3449263) of *Streptomyces coelicolor* (121). Although in In2, transcription originating from P_c or P_2 in the 5'-CS is likely to extend into the 3'-CS, affording expression at these three 3'-CS genes, a weak promoter that is sufficient for expression of sulfonamide resistance has also been identified upstream of the *qacEΔ1* gene (30).

In Tn402, the *qacE* gene is associated with a 59-be and hence is part of a mobile cassette (81), whereas *qacEΔ1* is not mobile. The *sulI* and adjacent sequence may also have originally been part of cassettes which integrated at *attI1* and subsequently became immobilized by loss of their adjacent 59-be recombination sites (Fig. 6) (5, 104). Thus, the 3'-CS appears to contain vestiges of the earliest insertion events. The presence of the *sulI* gene would explain the wide distribution of integrons containing this region, since sulfonamides were the first widely used antimicrobial agents.

tni Transposition Module

Of the class 1 integrons, where sufficient sequence is available, only Tn402 (Fig. 6) carries the full complement of four *tni* genes (5, 79), believed to encode transposition functions on the

basis of their similarity to the *tni* genes of Tn5053 (Fig. 3) (48). The *tniA*, *tniB*, and *tniQ* genes of Tn5053 are required for transposition, and the fourth gene, *tniR*, encodes a resolvase which resolves the cointegrates as does TnpR of Tn21 (48). In In2, only one complete gene, *tniA*, and a truncated version of the *tniB* gene are present. All class 1 integrons, including Tn402, have complex ends (79). Both ends have three copies of a 19-bp sequence, the outermost of which overlaps the terminal 25-bp IRs. By analogy to Tn7, these three 19-bp repeats are believed to function as transposase binding sites, although the identity of the Tni protein involved is not known.

IS1326 Insertion Sequence

The insertion sequence IS1326 has terminal 26-bp IRs and includes two ORFs that overlap by 14 bp (5). These ORFs have been designated *istA* (1,524 bp) and *istB* (786 bp) due to the similarity of their predicted products to IstA and IstB, required for transposition in IS21 (83). IstA has a DD(45)E transposase motif and a helix-turn-helix motif, and IstB has a distinctive set of nucleoside triphosphate binding domains (5). These two genes have share a common promoter upstream of *istA*. However, in In2, IS1353 has inserted between the -10 and -35 promoter regions of IS1326, and thus *istAB* may not be expressed. IS1326 also occurs in other integrons, e.g., integrons In0 and In5 in plasmids pVS1 and pSCH884, respectively (5).

IS1353 Insertion Sequence

The acquisition of IS1353 by In2 appears to be a recent event that occurred after the movement of the In2 to its present location in Tn21 (5). IS1353 is a member of the IS3 family (5) and has the characteristic 5'-TG and CA-3' at the outer ends of its terminal 12-bp IRs. IS1353 is flanked by a direct duplication of 2 bp of the target sequence. It contains two ORFs, *orfA* and *orfB*, transcribed in the opposite direction from *istA* and *istB* of IS1326. A potential promoter for *orfA* and *orfB* lies 24 bp upstream of the putative OrfA translational start. OrfB has a DD(35)E transposase motif similar to other transposases of the IS3 family (23). A potential frameshift motif, A₆G, and a predicted mRNA stem-loop are present where the *orfA* and *orfB* reading frames overlap, suggesting that (like other IS3 relatives, IS911 and IS150), three polypeptides, OrfA, OrfB, and a fusion protein (OrfAB) that is the actual transposase, may be produced (78). The initiation codon of OrfB has yet to be determined; it may be at the ATT codon 3 bp upstream of the A₆G motif (as seen in IS911) or at the ATG codon 6 bp downstream of the A₆G motif.

Tn21 EVOLUTION

Transposons are important for bacterial evolution and adaptation because they carry traits that can be useful for the host cell in varying environments; Tn21 provides its host cell with both antibiotic and mercury resistance determinants. The Tn21 subgroup of transposons is phylogenetically identified on the basis of *tnp* genes, *res* sites, and IR ends, but its members vary considerably in the number and types of additional genes (29, 58) they carry. Moreover, some transposons in this subgroup do not carry either a *mer* region or any resistance genes. The ubiquity and variety of elements in the Tn21 subgroup is the result of several recombination processes (29).

Two characteristics of the transposition machinery of the Tn21 subgroup of transposons foster their ready association with many different genetic loci. The first of these characteristics is that both the resolvase and the transposase have rather broad substrate specificities for the DNA sites which are their

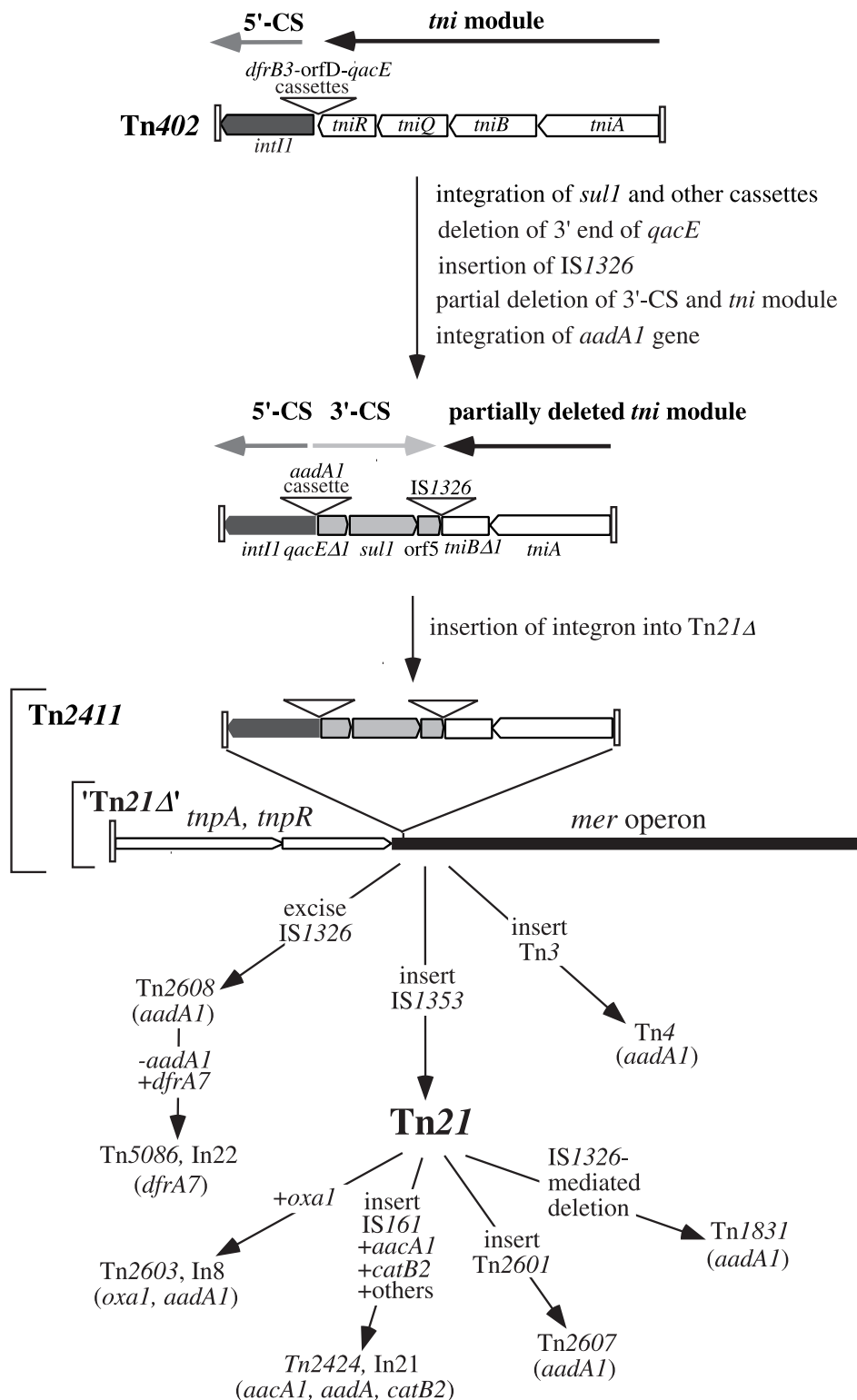


FIG. 6. Evolution of Tn21. Dark gray represents the 5'-CS, and light gray represents the 3'-CS. Cassette insertion is indicated by plus (+), and cassette excision is indicated by minus (-).

substrates. For example, the Tn21 TnpA transposase recognizes several different IR sequences and can also mediate one-ended transposition to generate novel joint molecules (29). In addition, the various TnpR resolvases act on each

other's *res* sites, yielding distinct rearrangements of these transposons (29). The second characteristic of the Tn21 subgroup which enhances their variety is the placement of the *res* site to one side of *tnpA* and *tnpR* (Fig. 2) rather than between

them (as found in the Tn3 transposon family). This arrangement minimizes the segregation of *tnpR* and *tnpA* which might otherwise occur in aberrant resolution events and results in a relatively stable resolvase-transposase "module." In addition to these properties of the Tn21 subgroup, two or more Tn21-like elements are often found in the same bacterial strain, typically on large conjugative plasmids, where host-mediated homologous recombination can also contribute to their reassortment (59).

Moreover, when an integron is present, two IntI1-mediated, RecA-independent, site-specific recombination processes occur: (i) insertion, rearrangement, or excision of antibiotic resistance gene cassettes (10, 12, 13) and/or (ii) formation and resolution of cointegrates. Both processes involve recombination between various pairs of specific recombination sites (59-be \times 59-be, 59-be \times *attI1*, and *attI1* \times *attI1*) (32, 38, 60, 61, 82, 105), thus enabling the rapid development of new arrays of antibiotic resistance genes (Fig. 6). Further variation results from the acquisition of insertion sequences, transposons, and integrons and subsequent deletions arising at their ends and mediated by them (5).

Model for Tn21 Evolution

Several transposons, considered to be very close relatives of Tn21, have nearly identical DNA sequences or restriction maps in the *tnp* and *mer* regions but differ in that they carry no or different antibiotic resistance genes. Tanaka et al. (112) first proposed that those Tn21-like transposons which confer multiple antibiotic resistance had descended from an ancestral mercury resistance transposon like Tn501 by successive insertions of antibiotic resistances and/or insertion sequences. Their restriction endonuclease analysis indicated similarity between the *mer* and the *tnp* regions of Tn2613, which lacks antibiotic resistance genes, and the corresponding regions of Tn2608, which has a 6.3-kb insertion between the *mer* and *tnp* regions and has also gained streptomycin and sulfonamide resistances. These observations suggested that Tn2613 might reasonably be considered the predecessor of Tn2608. However, Tn21 itself has a slightly different restriction map in the Tn21 IR_{mer} and in the *tnpM* regions from those of both Tn2613 and Tn2608, suggesting that neither of these transposons was the immediate predecessor of Tn21.

A more recent model for the evolution of Tn21 and other family members (5) is based on restriction maps, heteroduplex analyses, and sequence data for a larger group of these transposons (19, 53, 63, 94, 110, 113, 117, 119, 122, 123). In this analysis, those considered members of the family are similar to Tn21 in carrying the same *tnpRA* transposition genes and an integron in the same position as In2. They may or may not carry the complete *mer* locus, and they also differ from Tn21 in either the identity or number of gene cassettes in the integron or by the acquisition or loss of other IS elements or transposons. For example, the original Tn21, isolated in Japan, includes a 1.6-kb region corresponding to IS1353 that is not present in related isolates from Germany (29), which suggests that Tn21 acquired IS1353 after the global spread of an ancestor such as Tn2411 that does not contain this IS (5).

Brown et al. (5) proposed that Tn2411 arose from a hypothetical ancestor called Tn21 Δ , which lacks an integron but contains the *mer* operon (Fig. 6). In Tn21 Δ , *urf2M* would be present (as seen in *orf2* and *urf2* adjacent to *mer* operons in Tn501 [6] and pPB [84], respectively [Fig. 3]). The ancestor of In2 (without IS1353) inserted into Tn21 Δ , perhaps via a transposition event catalyzed in *trans* by another transposon with a complete set of *tni* transposition genes. The alternative, i.e.,

that the In2 ancestor might have been complete and later lost its *tni* genes by a deletion event occurring on or after insertion of IS1353, seems unlikely since integrons with very similar organization to In2 are found in other locations (5).

Subsequently, transposon Tn2411 became widely distributed, experiencing integration, excision, or exchange of gene cassettes, insertion of transposons or insertion sequences, and IS-mediated deletions or excisions (Fig. 6), which have given rise to the examples of this group described so far (19, 53, 63, 94, 110, 119, 122, 123) and probably many others.

Model for In2 Evolution

The In2 integron is believed to have been a highly evolved element (Fig. 6) before it integrated into Tn21 Δ . Brown et al. (5) have proposed that the central components of In2 evolved from a progenitor, similar to Tn402, which had experienced several genetic events prior to insertion into Tn21 Δ . These genetic events would include the creation of the 3'-CS via the integration of *qacE*, as well as *sull1*, and other putative cassettes and subsequent events to immobilize them. After the insertion of IS1326, this IS caused the partial deletion of the adjacent 3'-CS and *tni* module. The insertion of IS1353 occurred after the integron moved into Tn21 Δ and is likely to be the latest event in the creation of both In2 and Tn21.

Codon Usage Comparisons

Tn21 can be viewed as an assemblage of genes captured by transposition genes: the *tnp* genes acquired the *mer* operon, and the *tni* genes acquired the integrase system. After these two independent events, the integron progenitor, itself an assemblage of genes captured by either the *tni* module or the integrase module, inserted into Tn21 Δ , followed by insertion of IS1353 (Fig. 6). Some hints about the variety of sources of these many components is given by their codon usage patterns.

Codon usage comparisons of Tn21 genes point to significant diversity in their origins (Table 8). Codon usage comparisons of *merA* and *urf2M* reveal low D^2 values with respect to each other but not with respect to other integron or *tnp* genes, with the exception of *orfAB* of IS1353. Codon usage in *aadA1*, as in the majority of genes found in integrated cassettes (2), is quite dissimilar to that in other genes within the integron, presumably reflecting diverse origins of the cassette-associated genes. In Tn21, the exception to this appears to be the low D^2 values between *aadA1* and *istAB* (IS1326) and *orfAB* (IS1353). High D^2 values are also observed between *qacE* Δ 1 (a former cassette) and all other Tn21 genes compared with it. Codon usage comparisons of genes within the integron revealed low D^2 values between *intI1*, *sull1*, and *tniA*, suggesting that these genes originated from closely related microorganisms which are different from *E. coli* or *Pseudomonas aeruginosa* (2, 79). This observation supports the view that *sull1* was integrated early in the evolution of class 1 integrons (104). Comparison of codon usage in Tn402 genes revealed similar low D^2 values (0.81) between *int* and *tniA* as for those genes in Tn21 and high D^2 (9.27 to 6.98) values between *qacE* and other genes within this integron (79). The low D^2 values between most of the Tn21 genes and the *orfAB* genes in IS1353 suggest that perhaps IS1353 was acquired while Tn21 was in a host with codon utilization similar to that of the ancestral host of Tn21.

Tn21 SAILS THE WORLD

The Tn21 family is widely distributed in both clinical and environmental isolates of gram-negative bacteria. All characterized examples of the Tn21 family have been found on large

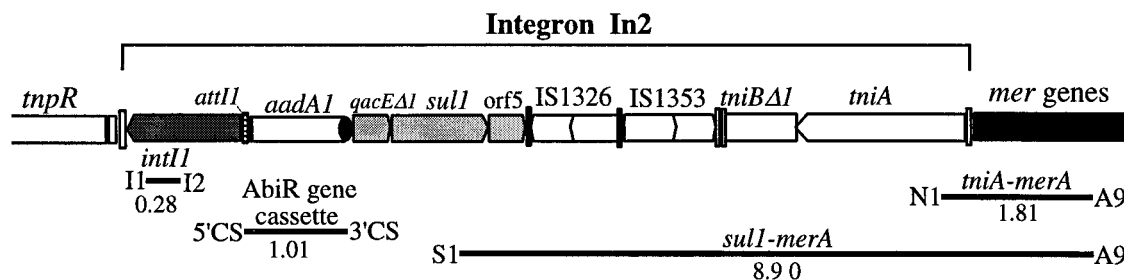


FIG. 7. PCR primers used to detect integron components and physical linkage between *mer* operons and class 1 integrons in gram-negative bacteria. Primers I1 and I2 are located in the *intI1* gene, and intergenic primers, 5'-CS and 3'-CS, lie between the *intI1* gene and the antibiotic resistance (AbiR) gene cassette and between the AbiR gene cassette and the *qacEΔ1* gene, respectively (56). S1 and N1 primers are located in the *sul1* and *tniA* genes of the integron, and primer A9 is located in the *merA* gene of the *mer* operon (57). Numbers under bars indicate the expected size (in kilobases) of the PCR products for Tn21.

conjugative plasmids. Although bacterial conjugation was once believed to be restricted in host range, it now appears that this mechanism of transfer permits genetic exchange between many different bacterial genera in nature (16, 50).

Environmental Occurrences

Pearson et al. observed the widespread distribution of Tn21- and Tn501-related *tnpA* and *tnpR* sequences in soil bacteria from three sites (two mercury-polluted sites and one pristine site); 20 of 30 isolates hybridized to transposase and resolvase gene probes (76). They observed extensive recombination between different transposition genes and reassortment of the *mer* and the transposition gene regions. Further evidence for widespread distribution includes a recent report of the occurrence of Tn21-, Tn501-, and Tn3-related *tnpA* sequences in total bacterial DNA from the marine environment (15). The Tn21 *tnpA* sequence occurred once per 10^3 or 10^4 bacteria.

Clinical Occurrences

Apart from the above environmental isolates, Tn21 carriage of integrons and integrated cassette-associated resistance genes in gram-negative pathogens is also well documented (32, 53, 80). The Tn21 family encodes resistances against both older and newer antibiotics. Strains of bacteria resistant to streptomycin, an antibiotic rarely used medically in the past 30 years but still extensively used in animal husbandry, are frequently found (68). In a 1995 study, over half (53%) of 49 clinical isolates with transferable resistance to aminoglycosides carried integrons with *aadA1* (25%) or *aacA4* (75%) gene cassettes (92). In an earlier (1992) study, 807 unselected gram-negative clinical isolates were screened to determine the distribution of the integron and Tn21 transposition genes (128). A total of 19% of the isolates carried some part of the *tnpA* *tnpR*, or *intI1* gene and 8% of the isolates had all of the genes, although linkage of *tnp* genes and integrons was not demonstrated. Besides complete Tn21-like transposons, a great variety of defective transposons were observed in many genera of bacteria (128).

Tn21 and Tn21-like transposons with integrons have also been observed in primate intestinal bacteria that were challenged with Hg(II) released from dental amalgams (108). There was a strong association of multiple-antibiotic resistance with two of the most abundant phylogenetic types of Hg resistance locus (locus 1 and locus 4) in these bacteria (58, 118). Using PCR, we have established (Fig. 7) that *mer* locus 1 carries a In2-like integron and that some examples of *mer* locus 4 carry a class 1 integron similar to In4 in Tn1696 (119). In both *mer* locus types, the adjacent integrons carry from one to

four antibiotic resistance genes. Hg exposure experienced by the fecal flora of these animals led to enrichment for strains carrying Hg resistance loci closely linked to integron multiple-antibiotic resistant transposons.

The causes of the dissemination of the Tn21 family of transposons are presently unknown but may be due to the chance association of the integron and *mer* at a time when the rich ecosystem of the human intestinal microflora was experiencing increasing antibiotic usage coinciding with exposure to Hg due to its use in the treatment of syphilis and in amalgam dental restorations.

CONCLUDING REMARKS

Tn21 and its relatives are major agents in the dissemination of mercury resistance and antibiotic resistance genes in gram-negative bacteria. Understanding the molecular processes involved in the accumulation and exchange of resistance genes by these integron-carrying transposons will inform efforts to control the spread of these genes as well as others which will arise in response to newer antibiotics. The regulation of antibiotic resistance gene cassette movement and stability, the factors that influence the recombination processes in the transposon, and the question whether there are environmental selection pressures other than antibiotics that contribute to their spread are subjects of intense study in many laboratories worldwide. Like mariners of old, Tn21 has left its kin in many ports and picked up barnacles along the way. As others of its family come to light, we expect that there will be answers to many of the questions posed above. We encourage others working on similar complex transposable elements and plasmids to compile and deposit their sequences. Such data will be essential to understanding the still-evolving relationships between the ubiquitous, peripatetic "floating" genome and the more constant cellular chromosomes.

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