Site-Specific Recombination System Encoded by Toluene Catabolic Transposon Tn4651

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The 56-kb class II toluene catabolic transposon Tn4651 from *Pseudomonas putida* plasmid pWW0 is unique in that (i) its efficient resolution requires, in addition to the 0.2-kb resolution (*res*) site, the two gene products TnpS and TnpT and (ii) the 2.4-kb *tnpT-res-tnpS* region is 48 kb apart from the *tnpA* gene (M. Tsuda, K.-I. Minegishi, and T. Iino, J. Bacteriol. 171:1386-1393, 1989). Detailed analysis of the 2.4-kb region revealed that the *tnpS* and *tnpT* genes encoding the putative 323- and 332-amino-acid proteins, respectively, were transcribed divergently with an overlapping 59-bp sequence in the 203-bp *res* site. The motifs (the R-H-R-Y tetrad in domains I and II with proper spacing) commonly conserved in the integrase family of site-specific recombinases were found in TnpS. In contrast, TnpT did not show any significant amino acid sequence homology to the other proteins that are directly or indirectly involved in recombination. Analysis of site-specific recombination under the *Escherichia coli recA* cells indicated that (i) the site-specific resolution between the two copies of the *res* site on a single molecule was catalyzed by TnpS, (ii) the functional *res* site was located within a 95-bp segment, and (iii) TnpT appeared to have the role of enhancing the site-specific resolution. It was also found that TnpS catalyzed the site-specific recombination between the *res* sites located at two different molecules to form a cointegrate molecule. Site-specific mutagenesis of the conserved tyrosine residue in TnpS led to the loss of both the resolution and the integration activities, indicating that such a residue took part in both types of recombination.

Many prokaryotic mobile elements have been identified that carry various xenobiotic-degrading genes (33, 38, 41). The most extensively characterized xenobiotic-degrading genes are the toluene-degrading (*xyl*) genes on plasmid pWW0 from *Pseudomonas putida* mt-2 (3, 24). We have previously demonstrated that all of the *xyl* genes on this 117-kb plasmid were located on Tn4651, a 56-kb transposon belonging to the class II family (Fig. 1) (36). In general, one end of the typical class II transposon is occupied by the transposase (*tnpA*) and resolvase (*tnpR*) genes and the resolution (*res*) site, and transposition of such a mobile element occurs by a two-step process (29). In the former step, TnpA recognizes the short (<50 bp) terminal inverted repeats (IRs) and mediates the formation of the cointegrate of the donor and target replicons with two copies of transposon, one at each junction. In the latter step, TnpR catalyzes resolution of the cointegrate by site-specific recombination between the two copies of the *res* site, giving rise to the donor replicon and the target replicon with an insert of the transposon. Tn4651, however, showed some unique features in the organization of the transposition-related genes (Fig. 1). The Tn4651 *tnpA* gene located at one end was, contrary to those of other typical transposons, transcribed in an inward direction (13). Furthermore, the efficient site-specific resolution of the Tn4651-mediated cointegrate required the two gene products, TnpS and TnpT, and the 2.4-kb region carrying *tnpS-res-tnpT* was 48 kb apart from the *tnpA* gene (34). Although the

cointegration step of Tn4651 has been extensively analyzed by Kivisaar's group (13, 14, 15, 32), no detailed analysis of the resolution system has been carried out in more than a decade.

Prokaryotic site-specific recombinases can be divided into the two major families, the resolvase and integrase families, on the basis of similarity of the amino acid sequences, conserved motifs, and molecular mechanisms of recombination (23, 29). Most members of the resolvase family represented by the TnpR proteins of the class II transposons catalyze only the intramolecular recombination between the two copies of the recombination site (12). In contrast, almost all of the members of the integrase family represented by the phage-encoded integrases catalyze both the intramolecular and intermolecular recombinations, and this family also includes the recombinases that take part in integration of the gene cassettes into the integrons and their excision (11) and the transposition of conjugative transposons (27, 30). The TnpI proteins encoded by the two unusual class II transposons, Tn4430 and Tn5401, also belong to the integrase family, and they catalyze the site-specific resolution of their respective cointegrates (5, 21). However, it has not been investigated whether these two proteins are also able to catalyze the intermolecular site-specific recombination. Some members of the integrase family require additional accessory proteins for the intramolecular recombination. For instance, excision of lambda phage DNA from its lysogenic chromosome of *Escherichia coli* requires, in addition to integrase (Int), the phage-encoded excisionase (Xis), as well as the integration host factor (IHF) encoded by the host chromosome (18).

In the present study, we analyzed the Tn4651-encoded site-specific resolution system under *E. coli recA* backgrounds. We

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FIG. 1. Structure of Tn4651. Restriction site abbreviations: A, *Aat*II; Ap, *Apa*I; B, *Bam*HI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; N, *Nco*I; Sa, *Sac*I; Sb, *Sac*II; Sm, *Sma*I; St, *Stu*I; and X, *Xba*I. Restriction sites derived from the vectors are in parentheses. (A) Schematic structures of Tn4651 (13, 35) and the related transposon Tn5041 (17). The following abbreviations and symbols are used: *xyl*, toluene-degrading genes; U, upper pathway operon; M, *meta* pathway operon; *mer*, genes for resistance to mercuric ion; TnpC, a regulator that controls negatively the TnpA expression at the posttranscriptional level (13); filled arrowhead, IR; and open arrowhead, IS1246 (26). The arrow indicates the transcriptional direction of the genes and operons. (B) Detailed structure of the *tnpS-res-tnpT* region. The numbers above the restriction sites are the base positions obtained by defining the upstream *Xba*I site as position 1. The short arrow below the map is the sequence in the primer used for amplification of DNA fragments or for primer extension analysis. Note that the *tnpS* and *tnpT* genes had been indicated to be located within the *Xba*I-*Sac*II and *Aat*II-*Sma*I fragments, respectively (34). (C) Structures of the pUC- or pBR322-based plasmids carrying *tnpS*, *tnpT*, and both genes. The detailed procedures for construction of these plasmids are described in Materials and Methods. The size of the Km^r gene on pGEN7K is arbitrary. While pGEN59 and pGEN77 carry the wild-type *tnpS* gene, pGEN95 and pGEN96 carry the Y293F mutation (triangle) in the *tnpS* gene.

show that (i) TnpS is a site-specific recombinase belonging to the integrase family and catalyzes both intra- and intermolecular site-specific recombinations and (ii) TnpT appears to have a role to enhance the former type of recombination.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Routine cultivation of *E. coli* and *P. putida* cells was performed at 37 and 30°C, respectively. Luria-Bertani (LB) broth was used as a liquid medium and was solidified with 1.5% agar to prepare LB agar plate (7). Final concentrations of the supplements added to media were

as follows: ampicillin (AMP), 50 µg/ml; kanamycin, 50 µg/ml; chloramphenicol, 30 µg/ml; streptomycin, 100 µg/ml; tetracycline (TET), 10 µg/ml; nalidixic acid, 20 µg/ml; IPTG (isopropyl-β-D-thiogalactopyranoside), 0.1 mM.

DNA manipulations and construction of plasmids. Established procedures were employed for preparation and manipulation of plasmid DNA, agarose gel electrophoresis, and transformation of *E. coli* cells (28). PCR was, unless otherwise stated, carried out by using *ExTaq* DNA polymerase (Takara), and the primer sequences are available upon request. To clone the PCR fragments into appropriate plasmids, the following recognition sites were added in the 5' parts of the primers: *Hind*III in S1; *Eco*RI in S2; *Sca*I in RES1, RES2, and RES32; and *Kpn*I in RES6 (Fig. 2 and 3A).

Insertion of the 2.4-kb *Xba*I-*Sma*I fragment containing the Tn4651 *tnpS-res-*

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Reference or source
Strains		
<i>E. coli</i>		
DH5 α	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 Δ(lacZYA-argF) ϕ80lacZΔM15</i>	28
HB101	<i>supE44 supF58 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1</i>	28
JM109	F' (<i>traD36 proAB lacI^q lacZΔM15</i>) <i>recA1 relA1 endA1 gyrA96 thi Δ(lac-proAB) hsdR17 supE44</i>	42
<i>P. putida</i>		
PT205	KT2440 carrying pMT1405	35
PT280	PpN1 Sm ^r	37
Plasmids		
pUC118	Cloning vector; Ap ^r	39
pUC119	Cloning vector; Ap ^r	39
pGEM5Zf(+)	Cloning vector; Ap ^r	Promega
pBluescript II SK(+)	Cloning vector; Ap ^r	28
pTrc99A	<i>trc</i> -driven expression vector; Ap ^r	2
pKK223-3	<i>tac</i> -driven expression vector; Ap ^r	1
pUC4K	Km ^r Ap ^r	31
R388	Tra ⁺ Tp ^r Su ^r	4
pMT299	pACYC184 derivative; Cm ^r Tc ^r	37
pMT1405	pWW0::kan; Tol ⁺ Km ^r ; kan outside of Tn4651	35
pGEN1	pUC119 derivative carrying the <i>tnpS-res-tnpT</i> region; Ap ^r	This study
pGEN7	pUC118 derivative carrying the <i>res-tnpT</i> region; Ap ^r	This study
pGEN7K	pGEN7 derivative carrying kan; Ap ^r Km ^r	This study
pGEN21	pTrc99A derivative carrying the <i>tnpT</i> gene expressed from <i>trc</i> promoter; Ap ^r	This study
pGEN59	pKK223-3 derivative carrying the <i>tnpS</i> gene expressed from <i>tac</i> promoter; Ap ^r	This study
pGEN77	pGEN21 derivative carrying the <i>tac-tnpS</i> region from pGEN59; Ap ^r	This study
pGEN95	pKK223-3 derivative carrying the mutant <i>tnpS</i> gene expressed from <i>tac</i> promoter; Ap ^r	This study
pGEN96	pGEN21 derivative carrying the mutant <i>tac-tnpS</i> region from pGEN95; Ap ^r	This study
pGEN101	pMT299 derivative carrying the <i>res1-kan-res1</i> fragment; Tc ^r Km ^r	This study
pGEN101R	pGEN101 derivative lacking kan and one copy of <i>res1</i> ; Tc ^r	This study
pGEN102	pMT299 derivative carrying the <i>res2-kan-res2</i> fragment; Tc ^r Km ^r	This study
pGEN102R	pGEN102 derivative lacking kan and one copy of <i>res2</i> ; Tc ^r	This study
pGEN103	pMT299 derivative carrying the <i>res3-kan-res3</i> fragment; Tc ^r Km ^r	This study
pGEN106	pMT299 derivative carrying the <i>res6-kan-res6</i> fragment; Tc ^r Km ^r	This study
pGEN388	R388 derivative carrying the <i>res1-kan</i> fragment; Tra ⁺ Km ^r	This study
pGEN389	R388 derivative carrying the <i>res2-kan</i> fragment; Tra ⁺ Km ^r	This study

^a kan, pUC4K-derived Km^r gene; Tol⁺, toluene degradation; Tp^r, trimethoprim resistance;

tnpT region into the corresponding sites of pUC119 gave rise to pGEN1 (Fig. 1C). The 1.32-kb *AatII-SacI* fragment containing the *tnpT* gene was excised from pGEN1 and inserted into the corresponding sites of pGEM5Zf(+) so that the vector-derived *ApaI* site was located just outside of the *AatII* site. Excision by *ApaI* and *SacI* digestion of the *tnpT* gene from the resulting plasmid and insertion into the corresponding sites of pBluescript II SK(+) gave rise to the plasmid in which the *tnpT* gene was flanked by the two *KpnI* sites. Such a *KpnI* fragment was inserted into the *KpnI* site of pUC118 to generate pGEN7, and insertion of the pUC4K-derived, *BamHI*-flanked kanamycin resistance (Km^r) gene into the *BamHI* site in pGEN7 led to construction of pGEN7K (Fig. 1C). Excision of the 1.13-kb *NcoI-EcoRI* fragment containing the *tnpT* gene from pGEN1 and its subsequent insertion into corresponding sites just downstream of the *trc* promoter of pTrc99A generated pGEN21. The DNA fragment containing the *tnpS* gene (base positions 17 to 1056 in AB077820) (Fig. 1B) was amplified by PCR by using pGEN1 as a template and primers S1 and S2. The PCR product was digested by *EcoRI* and *HindIII* and inserted into the corresponding sites of pKK223-3 that are located just downstream of the *tac* promoter. The resulting plasmid was designated pGEN59 (Fig. 1C). pGEN77 was constructed by excision of the *BamHI-HindIII* fragment containing the *tac-tnpS* fragment from pGEN59 and its insertion into corresponding sites of pGEN21.

In order to change the tyrosine residue of TnpS to phenylalanine, the megaprimer PCR method (6) was employed for site-directed mutagenesis of *tnpS* by using KOD DNA Polymerase (Toyobo). The first PCR was carried out with the primers S1 and SA179T and pGEN59 as a template (Fig. 1C). The amplified fragment was used as the megaprimer for the second PCR with S2 as another primer. The amplicon containing the mutant *tnpS* gene was digested by *EcoRI* and *HindIII* and inserted into the corresponding sites of pKK223-3 to obtain pGEN95 (Fig. 1C). Excision of the *BamHI-HindIII* fragment containing the *tac*

promoter and the mutant *tnpS* gene from pGEN95 and its subsequent insertion into the corresponding sites of pGEN21 gave rise to pGEN96.

Determination of transcription start site by primer extension. Plasmid pMT1405 (pWW0::kan) (35) was introduced into *P. putida* PT280 (Table 1) that was shown not to carry the Tn4651 *tnpS-res-tnpT* region (unpublished data). An RNeasy Mini Kit (Qiagen) was employed to prepare the total RNA from 1-ml samples of LB cultures of PT280 and PT280(pMT1405). A primer extension experiment in a 20- μ l reaction volume was performed according to the protocol of Aloka, Ltd. (Tokyo, Japan), with the IRD-41-labeled oligonucleotide primer PETnpS or PETnpT (Fig. 1B). (The primer sequences are available upon request.) Gel electrophoresis was carried out by using a Li-Cor 4000 automated DNA sequencer.

Site-specific recombination assays. To analyze the site-specific resolution of Tn4651 in this study, a pACYC184-based plasmid, pMT299 (Table 1), was used to construct its derivative carrying the pUC4K-derived Km^r gene that was flanked by two copies of the *res* site or its part (Fig. 2). JM109 cells carrying the resulting plasmid were subjected to transformation of the pMB1-based plasmid that carried *tnpS*, *tnpT*, or both genes whose expression was under the control of the *tac* or *trc* promoter. The resulting transformant cells were briefly cultivated in LB medium with or without the addition of IPTG and then plated on LB agar plates containing appropriate antibiotics. The colonies thus obtained were examined for sensitivity to kanamycin and for the profiles of the residing plasmids. Excision of the circular DNA form consisting of the *res* site and the Km^r gene was also examined by PCR with primers 4Km1 and 4Km2 (Fig. 6).

To investigate the site-specific integration activity of the Tn4651-specified resolution system, a DNA fragment containing one copy of the *res* site, together with the Km^r gene, was inserted into conjugative plasmid R388 (Fig. 2). The resulting plasmid was introduced into the DH5 α cells that had contained the

FIG. 2. Construction of pMT299 and R388 derivatives carrying various parts of the *res* site. Only the relevant restriction sites are depicted, and their abbreviations are as described in the legend of Fig. 1 except for the following: Hc, *HincII*; P, *PstI*; and Sc, *ScaI*. The filled arrow indicates the *res* site. The white arrowheads above some plasmids indicate the primers used for amplification of parts of the *res* sites. The PCR product with primers M4 and RES1 was used to obtain *res1* present on pGENKres1, pMT299res1, pGEN101, and pGEN388; that with M4 and RES2 was used to obtain *res2* on pGENKres2, pMT299res2, pGEN102, and pGEN389; and that with M4 and RES3 was used to obtain *res3* on pGENKres3, pMT299res3, and pGEN103. For construction of pGENres6, PCR was carried out by using pMT299res2 as the template with primers RES6 and 184E. See Fig. 3 for details. The bold line indicates the pMT299-derived region. The *Km*^r gene in all of the plasmids is transcribed in a leftward direction.

pMT299 derivative carrying one copy of the *res* site and the pMB1-based plasmid carrying *tnpS*, *tnpT*, or both genes. The strain harboring these three plasmids was employed as the donor to mate with HB101 on a membrane filter (34), and the TET resistance-streptomycin resistance (Tc^r Sm^r) transconjugants were selected

to detect the mobilization of the pMT299-based replicon. Such transconjugants were examined for the physical analysis of the plasmids.

Determination and analysis of nucleotide sequence. The DNA fragments cloned on pUC18 and pUC19 were sequenced with an ABI 310 automated DNA

FIG. 3. Structure of the Tn4651 *res* site. (A) Sequence of the *res* site. The numbers at the left are the base positions obtained by taking the upstream *Xba*I site (Fig. 1B) as position 1. The transcription start sites of *tnpS* and *tnpT* are indicated by closed circles. The putative -35 and -10 sequences for *tnpS* and *tnpT* and the putative SD (S.D.) sequence for *tnpT* are boxed, and the predicted translation start sites of the *tnpS* and *tnpT* genes are shown by bold arrows. The 9-bp IRs are indicated by a pair of thin arrows, and the putative IHF-binding site (9) is doubly overlined. The oligonucleotides for PCR amplification of various parts of the *res* site are indicated as white boxes above or below the sequence. (B) Summary of the Tn4651 site-specific recombination analysis. The structure of one of the two copies of the *res* region on each plasmid is schematically drawn. The number above the structure indicates the nucleotide position (Fig. 1B). Open and filled arrows indicate the 9-bp IRs and the putative IHF binding site, respectively. Ssr, detection of site-specific resolution of the plasmid on the agar plates; PCR, detection of the excised and nonreplicating DNA by PCR with primers 4Km1 and 4Km2 (see Fig. 6); Ssi, detection of site-specific integration between the *res* sites located at two different DNA molecules (Table 2); +, detected; -, not detected.

sequencer (Applied Biosystems) according to the protocols recommended by the manufacturer. The nucleotide sequence reported here has been deposited in the DDBJ-EMBL-GenBank database under accession number AB077820. The computer analysis of the sequence was performed with the software programs GENETYX 10 (SDC, Inc., Tokyo, Japan) and BLAST 2 (National Institute of Genetics, Mishima, Japan).

RESULTS

Sequence and expression analysis of *tnpS-res-tnpT* region.

The 2,368-bp sequence of the Tn4651 *Xba*I-*Sma*I fragment encoding its resolution system (34) was determined in this study (accession number AB077820) (Fig. 1B). The 203-bp sequence (nucleotides from position 1045 [*Aat*II site] to position 1247 [*Sac*II site]) containing the *res* site is shown in Fig. 3A. The 1,247-bp *Xba*I-*Sac*II fragment had the two candidate open reading frames (ORFs) for *tnpS*: the one located at positions 358 to 1032 and the other located at positions 1056 to 88 on the complementary strand. Since deletion of the *Stu*I fragment from positions 225 to 363 had led to loss of the TnpS activity (34), the latter ORF encoding a 323-amino-acid protein was concluded to be *tnpS*. The 1,324-bp *Aat*II-*Sma*I frag-

ment had the three candidate ORFs for *tnpT*: the ORF from positions 1237 to 2232; the ORF from positions 1481 to 1888; and the ORF from positions 1931 to 2182. The first ORF encoding a 332-amino-acid protein was postulated to be *tnpT* because the DNA fragment covering at least positions 1247 to 2018 (*Sac*II and *Stu*I sites, respectively) had been indicated to be required for TnpT activity (34).

BLAST searches revealed that TnpS had homology with the integrase family of site-specific recombinases (23), showing 36 to 38% identity and 53 to 55% similarity with the putative integrase from the atrazine catabolic plasmid pADP-1 of a *Pseudomonas* strain (U66917) (22), the integrase-like protein from *Coxiella burnetii* (Y15898), and the OrfI protein of Tn5041 (X98999) (17). The proteins in this family invariably have the conserved R-H-R-Y tetrad in domains I and II with proper spacing (23), and these structural characteristics were also found in TnpS (Fig. 4). In contrast, TnpT showed 29% identity and 47% similarity only with the OrfQ protein of Tn5041 (X98999) (17).

The transcription start sites of *tnpS* and *tnpT* were determined in the *P. putida* background by the primer extension

FIG. 4. Comparison of TnpS with other members in the integrase family of site-specific recombinases. The consensus sequences in this family are depicted at the top of the figure; the invariant R-H-R-Y tetrad residues in the family are indicated by the capital and boldface letters, and the highly conserved amino acid residue is indicated by the small letter (23). The number beside the amino acid sequence is the amino acid position. The mutagenized tyrosine residue in TnpS is indicated by an arrowhead. The accession numbers of the nucleotide sequences for the proteins are as follows: Tn4651 TnpS (AB077820); pADP-1 Int, putative integrase from plasmid pADP-1 (U66917); *Coxiella* Int, integrase-like protein from *C. burnetii* (Y15898); and Tn5041 OrfI, integrase-like protein from Tn5041 (X98999).

method (data not shown), and these two genes were found to be transcribed divergently with an overlapping 59-bp sequence in the 203-bp *res* site (Fig. 3A). On the basis of this information, the putative promoter sequences of *tnpS* and *tnpT* and the putative ribosome-binding (Shine-Dalgarno [SD]) sequence of *tnpT* are shown in Fig. 3A. However, we could find no putative SD sequences for *tnpS*. The *tnpS* gene was inserted just downstream of the *tac* promoter and its associated SD sequence of pKK223-3 to construct pGEN59, and the *tnpT* gene was inserted just downstream of the *trc* promoter and its associated SD sequence of pTrc99A to construct pGEN21 (Fig. 1C). The addition of 0.1 mM IPTG to the *E. coli* JM109(pGEN21) cells led to very severe growth inhibition, while such treatment of the JM109(pGEN59) cells did not. The mutant derivatives of JM109(pGEN21) were isolated that could grow normally in the presence of IPTG, and they were categorized into the three groups. The first group had the insertion of an unknown sequence(s) in *tnpT*, and the second one had the defects in the *trc* promoter, resulting in no transcription of *tnpT*. The last group has been suggested to have an unknown chromosomal mutation(s) since transformation of the new JM109 cells by the plasmids prepared from the mutants still produced growth inhibition in the host cells.

Site-specific resolution activity of *tnpS-res-tnpT* region. To analyze the Tn4651-encoded resolution system under an *E. coli*

recA background, a pACYC184-based Tc^r Km^r plasmid, pGEN101, was constructed in which a Km^r gene was flanked by the two copies of the 203-bp *res* site (*resI*) (Fig. 2 and 3B). We also constructed pGEN77 by insertion of the *tac-tnpS* region of pGEN59 into pGEN21 that had the *trc-tnpT* gene (Fig. 1C). We thereafter introduced pGEN21, pGEN59, or pGEN77 into JM109(pGEN101). Since the prolonged cultivation of JM109(pGEN101)(pGEN21) that overexpressed TnpT alone, but not the two other strains, on LB plates containing 0.1 mM IPTG led to the very severe growth defect, the three JM109 derivatives were cultivated only for 1 h at 37°C in AMP- and TET-containing LB medium with or without IPTG. After such treatment, the cells were plated on LB agar plates containing AMP and TET, and the 150 colonies obtained from each experiment were examined for resistance to kanamycin. Cultivation of JM109(pGEN101)(pGEN21) and JM109(pGEN101)(pGEN59), even in the presence of IPTG, generated no kanamycin-sensitive (Km^s) colonies, and physical analysis of the residing plasmids (Fig. 5A, lanes 1 and 2) revealed that the structure of pGEN101 remained unaffected. In contrast, JM109(pGEN101)(pGEN77) led to formation of the Km^s colonies at frequencies of 6 or 9% when the cells were cultivated in the absence or presence, respectively, of IPTG. The addition of IPTG did not lead to a drastic increase in the frequency of formation of the Km^s colonies for unknown rea-

FIG. 5. Resolution and cointegration of plasmids carrying Tn4651 *res* site. (A) Resolution of plasmid that carried the Km^r gene flanked by two directly repeated copies of the *res* site. The JM109 derivative carrying the substrate and helper plasmids was cultivated for 48 h in LB medium containing AMP and TET, and the cleared lysate prepared from each culture was analyzed by agarose gel electrophoresis. The substrate plasmids used were as follows: pGEN101, lanes 1 to 5; pGEN102, lane 6; pGEN103, lane 7; pGEN106, lane 8. The helper plasmids were as follows: pGEN21, lane 1; pGEN59, lane 2; pGEN77, lanes 3, 4, 6, 7, and 8; pGEN96, lane 5. The cleared lysates prepared from the LB cultures of the Km^r and Km^s derivatives of JM109(pGEN101)(pGEN77) were loaded in lanes 3 and 4, respectively. In lanes 1 and 2, pGEN21 and pGEN59 overlap pGEN101. oc, open circular DNA; chr, chromosome. (B) Cointegration of two plasmids, each carrying the Tn4651 *res* site. The donor strain DH5α (pGEN101R)(pGEN388)(pGEN77) was mated with HB101, and the Tc^r transconjugants were selected. Agarose gel electrophoresis of cleared lysates prepared from the donor strain (lane 1) and the transconjugant (lane 2) was done.

sons. The leaky transcription from the *trc* and *tac* promoters in the absence of IPTG (20) might have allowed expression of TnpS and TnpT sufficient for the resolution. The Km^s derivatives contained, in addition to pGEN77, a novel plasmid (pGEN101R) that was smaller than pGEN101 (Fig. 5A, lane 4). Analysis of pGEN101R by restriction digestion and sequencing confirmed that this plasmid was formed by site-specific resolution, indicating that both *tnpS* and *tnpT* were required for efficient resolution. pGEN101R was also detected in the cleared lysate prepared from the Km^r colonies of JM109(pGEN101)(pGEN77) after their prolonged cultivation in LB containing AMP and TET (Fig. 5A lane 3), indicating that pGEN101R was gradually generated from pGEN101 by site-specific resolution.

Similar resolution experiments were also carried out by using pGEN101-related plasmids, pGEN102 and pGEN103, that carried the left 160-bp (*res2*) and 130-bp (*res3*) sequences, respectively, of *res1* (Fig. 2 and 3B). In the presence of pGEN77, pGEN102 but not pGEN103 resolved site specifically to generate the Km^s colonies at a frequency of 5 or 9% in the absence or presence, respectively, of IPTG. The Km^s derivatives carried pGEN102R (Fig. 5A, lane 6) that was the resolved product of pGEN102. Prolonged cultivation of the Km^r cells of JM109(pGEN77)(pGEN103) in LB medium containing AMP and TET did not generate the resolved derivative of pGEN103 (Fig. 5A, lane 7). These results indicated that (i) the rightmost 73-bp sequence of *res1* was not required for efficient resolution and (ii) the rightmost 30-bp sequence of *res2* was important for resolution.

To investigate whether the excision of the Km^r gene was correlated with formation of the circular DNA form consisting of the Km^r gene and one copy of the *res* site, PCR detection of such excision events was performed with the primers 4Km1 and 4Km2 (Fig. 6A). Use of the cleared lysates from the Km^r cells of JM109(pGEN101)(pGEN77) and JM109(pGEN102)(pGEN77), but not from those of JM109(pGEN103)(pGEN77), led to successful amplification of the DNA molecules with the expected sizes (Fig. 6B, lanes 3, 7, and 8). These results also supported the conclusion that loss of the Km^r gene occurred by its site-specific excision. The excision events were also detected by PCR from the cleared lysates prepared from JM109(pGEN101)(pGEN59) (Fig. 6B, lane 2) and JM109(pGEN102)(pGEN59) (data not shown), although these two strains did not generate Km^s derivatives (<0.6%). Therefore, TnpT was not certainly required for site-specific resolution but might play a role in enhancing the frequency of site-specific resolution.

The 95-bp sequence covering the rightmost portion of *res2* (positions 1110 to 1204 in Fig. 3A) was designated *res6*, and we constructed a pGEN101-related plasmid, pGEN106, in which the Km^r gene was flanked by two copies of *res6* (Fig. 2 and 3B). The JM109(pGEN106)(pGEN77) strain did not generate Km^s derivatives (<0.6%), and the resolved plasmid derived from pGEN106 was not detected when the cleared lysate prepared from the Km^r cells was analyzed by agarose gel electrophoresis (Fig. 5A, lane 8). However, PCR analysis of the cleared lysate (Fig. 6, lane 9) supported the formation of the excised circular DNA consisting of the Km^r gene and a copy of *res6*. PCR analysis of the cleared lysate prepared from JM109(pGEN106)(pGEN59) led to the same observation (data not shown). The right end of *res6* (Fig. 3B) had the IR of a 9-bp sequence with

FIG. 6. PCR detection of site-specific resolution. (A) Schematic drawing for PCR detection of the excised and nonreplicating DNA consisting of the *res* site and Km^r gene. White arrowheads indicate the primers. (B) Agarose gel electrophoresis of the PCR-amplified excised DNA fragments. PCR with primers 4Km1 and 4Km2 was performed by using the cleared lysate prepared from the JM109 derivative carrying the following combinations of plasmids: pGEN101 and pGEN21, lane 1; pGEN101 and pGEN59, lane 2; pGEN101 and pGEN77, lane 3; pGEN101 and pTrc99A, lane 4; pGEN101 and pGEN95, lane 5; pGEN101 and pGEN96, lane 6; pGEN102 and pGEN77, lane 7; pGEN103 and pGEN77, lane 8; pGEN106 and pGEN77, lane 9. In lane M, size markers are indicated in kilobases.

a 2-bp spacer and a putative consensus sequence for the binding site of the *E. coli* IHF protein (9).

Site-specific integration activity of Tn4651-specified recombination system. Since TnpS had the motif commonly conserved in the integrase family of site-specific recombinases (Fig. 4), we anticipated that TnpS alone or in concert with TnpT might be able to mediate site-specific integration of a *res*-carrying plasmid into another plasmid that also carried the *res* site. We expected that such a recombination event could be detected effectively by conjugal mobilization of the nontransmissible plasmid by the coresiding transmissible plasmid only when both plasmids carried the functional *res* site. pGEN101R that carried *res1* and the Tc^r gene was employed as the former type of plasmid, and pGEN388 (Fig. 2) was constructed as the latter type of plasmid by insertion of *res1* and the Km^r gene into transmissible plasmid R388 (4). The DH5 α derivative carrying pGEN101R, pGEN388, and one of the pMB1-based plasmids (pGEN21, pGEN59, or pGEN77) was used as the donor to mate with HB101, and mobilization of pGEN101R was examined by selecting Tc^r Sm^r transconjugants (Table 2). When we used pTrc99A that did not carry *tnpS* or *tnpT*, pGEN101R was mobilized at a frequency of ca. 10^{-6} per pGEN388 transfer, and each Tc^r transconjugant carried a sin-

TABLE 2. Site-specific integration mediated by Tn4651 *res* site^a

Expt	Tra ⁻ plasmid ^b	Tra ⁺ plasmid ^b	Helper plasmid ^c		Mobilization of Tra ⁻ plasmid ^d
			<i>tnpS</i>	<i>tnpT</i>	
1	pMT299	pGEN388	—	—	$<1.0 \times 10^{-7}$
2			—	+	$<1.0 \times 10^{-7}$
3			+	—	$<1.0 \times 10^{-7}$
4			+	+	$<1.0 \times 10^{-7}$
5	pGEN101R	pGEN388	—	—	9.0×10^{-7}
6			—	+	1.0×10^{-6}
7			+	—	3.7×10^{-3}
8			+	+	4.7×10^{-3}
9			Mutant	+	9.0×10^{-7}
10	pGEN102R	pGEN389	—	—	2.9×10^{-6}
11			+	—	3.7×10^{-4}
12	pMT299res6	pGEN389	—	—	$<1.0 \times 10^{-7}$
13			+	—	3.7×10^{-4}

^a The DH5 α derivative carrying the Tra⁻ and Tra⁺ plasmids, as well as the helper plasmid, was mated with HB101 to select the transconjugants that exhibited the TET resistance specified by the Tra⁻ plasmid.

^b The *resI* sequence on pGEN101R and pGEN388, the *res2* sequence on pGEN102R and pGEN389, and the *res6* sequence on pMT299res6.

^c The following plasmids were used: pTrc99A in experiments 1, 5, 10, and 12; pGEN21 in experiments 2 and 6; pGEN59 in experiments 3, 7, 11, and 13; pGEN77 in experiments 4 and 8; and pGEN96 in experiment 9. Use of pKK223-3 instead of pTrc99A gave rise to similar results (data not shown). + and —, presence and absence of gene.

^d Mean values of at least two independent experiments taking the transfer frequency of the Tra⁺ plasmid as 1.0.

gle species of plasmid (data not shown) that could revolve into the two parental plasmids in the presence of pGEN77. Restriction analysis of the fused plasmid and sequence analysis of its *resI*-containing regions confirmed that the plasmid was formed by reciprocal recombination between the two copies of *resI*. Since neither *tnpS* nor *tnpT* was involved in the unexpected recombination, we did not further investigate the mechanism for such a low-frequency recombination event. Supply of TnpT also resulted in such a low-frequency mobilization of pGEN101R. A >1,000-fold increase in mobilization of this plasmid was observed when TnpS alone and when TnpS and TnpT simultaneously were supplied. The transconjugants thus obtained carried a single species of plasmid (Fig. 5B) whose structure was the same as that residing in the transconjugants that were obtained by use of DH5 α (pGEN101R)(pGEN388)(pTrc99A) as the donor. These results indicated that TnpS alone could efficiently promote site-specific integration without involvement of TnpT. Use of *res2* and *res6* on the two plasmids, pGEN389 and pMT299res6 (Fig. 2 and 3B), respectively, also generated the cointegrate (Table 2), indicating that *res2*, and most probably *res6*, was sufficient for site-specific integration.

Importance of tyrosine residue of TnpS in site-specific recombination. As described above, TnpS had the R-H-R-Y tetrad commonly conserved in the integrase family of recombinases, and the tyrosine residue in this tetrad in this family has been demonstrated to be a catalytic center of the recombination reaction (23). To investigate whether this residue in TnpS was essential for the site-specific recombination, its tyrosine residue at position 293 (Fig. 4) was changed to phenylalanine by site-directed mutagenesis. The *tnpS* mutant versions of pGEN59 and pGEN77 were constructed and designated pGEN95 and pGEN96, respectively (Fig. 1C). Use of these mutant plasmids in the site-specific resolution and integration assays revealed that the mutant TnpS protein lost both the

activities (Fig. 5, lane 6; Fig. 6, lanes 5 and 6; and Table 2), indicating that the tyrosine residue played an essential role in both types of site-specific recombination.

DISCUSSION

To investigate the Tn4651-encoded site-specific resolution system in this study, we constructed *E. coli recA* strains harboring the two compatible plasmids: one carrying the Km^r gene flanked by two copies of the *res* site or its parts and the other carrying *tnpS*, *tnpT*, or both genes (Fig. 1C, 2, and 3B). Investigation of site-specific resolution by detection of the Km^s colonies revealed that the 160-bp *res2* sequence contained the functional *cis*-acting site. Based on this assay system, *res6* that corresponded to the rightmost 95-bp portion of *res2* was considered not to be functional. However, *res6* was indicated to be functional according to more sensitive but qualitative detection of the excised DNA molecule by PCR (Fig. 6B, lane 9). The 65-bp sequence present on *res2* but absent in *res6* (Fig. 3) might be important to some extent for effective resolution of the cointegrate. The right end of *res6* was occupied by the 9-bp IRs (Fig. 3). In many site-specific recombination systems, such IRs but with different sequences have been demonstrated to be indispensable for efficient binding of the recombinases and/or their accessory proteins to catalyze recombination (40), and the 9-bp IR in *res6* of Tn4651 might have a similar role.

The *res2* sequence, and most probably the *res6* one, could act as the substrate for intramolecular resolution and intermolecular integration reactions, and analysis in this study revealed that (i) both reactions could be catalyzed by TnpS that had the structural properties characteristic of the integrase family and (ii) TnpT was most likely to enhance the former type of reactions. The site-specific recombination system of Tn4651 could therefore be considered to be apparently analogous to those encoded by integrons in which the integrases are able to catalyze both the excision and the integration reactions and, to a lesser extent, analogous to those encoded by lysogenic phages and some conjugative transposons (e.g., Tn916 and Tn5276) (18, 25, 30). With respect to the latter group of mobile elements, the small (<100 amino acid residues) accessory proteins collectively designated recombination direction factors (RDFs) (e.g., lambda Xis protein) (19) are, in addition to the integrases, required absolutely for site-specific excision, and some RDFs have been shown to bind to specific recognition sequences at the *att* sites so as to introduce sharp DNA bends (8). However, TnpT was much larger than RDFs and did not show homology to any RDFs. It is at present unknown whether TnpT also has the ability to bind to the Tn4651 *res* site since our attempt to overproduce the TnpT protein alone in *E. coli* led to a very toxic effect on the growth of the host cells. Our observation that such a toxic effect could most probably be suppressed by the chromosomal but uncharacterized mutation(s) indicated that TnpT might interact with the unknown host product(s). Identification of the mutated chromosomal gene(s) might provide us with some clues to elucidate the molecular mechanism of TnpT in the resolution. There is also a possibility that TnpT interacts directly with TnpS to enhance the resolution frequencies. Such possible titration of the free form of TnpT by TnpS might be able to account for our observation that the attempt to overexpress both TnpS and

TnpT simultaneously in *E. coli* resulted in a less toxic effect on the host cells. Site-specific recombination between the two *res* sites, one on the pACYC184 replicon and the other on the R388 replicon, was detected at a low frequency without the involvement of TnpS or TnpT (Table 2). Construction of the latter R388 derivative was associated with disruption of the site-specific integrase gene of *Int3*, an integron on R388 (10). Such a low-frequency integration event might therefore be most likely ascribed to the activity of an unknown integrase protein encoded by the host *E. coli* chromosome. However, BLAST searches of the protein databases did not clarify the candidate proteins that displayed similarity with TnpS.

A 15-kb mercury transposon, Tn5041 (Fig. 1A), from a *Pseudomonas* strain (17) is closely related to Tn4651 in that (i) these two transposons share >95% identity in the genes and sites involved in cointegration (i.e., *tnpA* and terminal IRs) and (ii) the Tn4651 *tnpA* product could catalyze the cointegration of Tn5041 (16, 17). The 2.3-kb region of Tn5041 probably responsible for the cointegrate resolution is separated by 9 kb from the *tnpAC* genes, and this 2.3-kb region carries the putative *res* site flanked by the two genes, *orfI* and *orfQ*, that are postulated to be transcribed divergently (17). Furthermore, the sizes of *orfI*, *orfQ*, and the *res* site of Tn5041 are similar to those of the *tnpS*, *tnpT*, and *res* site, respectively, of Tn4651, and *OrfI* that possesses the motifs conserved in the integrase family has been speculated to be the counterpart of TnpS. However, the resolution-related region of Tn5041 was not so homologous as to hybridize with the corresponding region of Tn4651 by Southern analysis (17). The Tn5041-encoded resolution system remains to be elucidated since the resolution activity of Tn5041 assayed by a system similar to ours was very weak in the *E. coli* cells (16). An analysis of the Tn5041-encoded resolution system in *P. putida* and further investigation of the functional exchangeability of TnpS and *OrfI* and of TnpT and *OrfQ* will uncover more clearly the degree of relatedness between the two transposons.

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