

Functional Analysis of Unique Class II Insertion Sequence *IS1071*

Masahiro Sota,^{1,2*} Hirokazu Yano,² Yuji Nagata,² Yoshiyuki Ohtsubo,² Hiroyuki Genka,²
 Hisashi Anbutsu,^{2†} Haruhiko Kawasaki,³ and Masataka Tsuda²

Department of Environmental Simulation, Institute for Environmental Sciences, Rokkasho, Aomori 039-3212,¹ Department of Environmental Life Sciences, Graduate School of Life Sciences, Tohoku University, Katahira, Sendai 980-8577,² and Department of Applied Biochemistry, Graduate School of Agriculture and Biological Sciences, Osaka Prefecture University, Sakai, Osaka 599-8531,³ Japan

Received 20 June 2005/Accepted 29 September 2005

Various xenobiotic-degrading genes on many catabolic plasmids are often flanked by two copies of an insertion sequence, *IS1071*. This 3.2-kb IS element has long (110-bp) terminal inverted repeats (IRs) and a transposase gene that are phylogenetically related to those of the class II transposons. However, the transposition mechanism of *IS1071* has remained unclear. Our study revealed that *IS1071* was only able to transpose at high frequencies in two environmental β -proteobacterial strains, *Comamonas testosteroni* and *Delftia acidovorans*, and not in any of the bacteria examined which belong to the α - and γ -proteobacteria. *IS1071* was found to have the functional features of the class II transposons in that (i) the final product of the *IS1071* transposition was a cointegrate of its donor and target DNA molecules connected by two directly repeated copies of *IS1071*, one at each junction; (ii) a 5-bp duplication of the target sequence was observed at the insertion site; and (iii) a *tnpA* mutation of *IS1071* was efficiently complemented by supplying the wild-type *tnpA* gene in *trans*. Deletion analysis of the *IS1071* IR sequences indicated that nearly the entire region of the IRs was required for its transposition, suggesting that the interaction between the transposase and IRs of *IS1071* might be different from that of the other well-characterized class II transposons.

Bacterial class II (Tn3-like) transposons generally carry the genes for their transposition (*tnpA*, *tnpR*, and *res*) and one or more phenotypic traits between their terminal inverted repeats (IRs), which have sizes of less than 50 bp (Fig. 1A and B) (23). These transposons move by a two-step and replicative mechanism (6, 23). In the first step, the *tnpA* product (transposase) acts at the IRs to generate a cointegrate of the donor and target molecules connected by two directly repeated copies of the transposon, one at each junction. In the second step, the cointegrate resolves at the resolution (*res*) sites by means of the *tnpR* product (resolvase). A 5-bp duplication of the target sequence is generated upon transposition. The transposases of the class II transposons are able to catalyze their transposition even when the *tnpA* gene and cognate IRs are located on separate molecules (6, 23). Several class II transposons have been reported to play an important role in the wide dissemination of various catabolic gene clusters, such as toluenylenes, naphthalene, and carbazole (17, 29–31). To date, three major groups (Tn3, Tn21, and Tn4651) of class II transposons have been characterized in detail with respect to their structural and functional aspects (Fig. 1A).

IS1071 is a 3.2-kb insertion sequence (IS) that was originally identified in a chlorobenzoate-catabolic transposon, Tn5271, from *Comamonas testosteroni* BR60 (20). On the basis of structural features of its 110-bp IRs and 2,913-bp *tnpA* gene, *IS1071* has been considered to belong to the class II transposons (7, 20).

However, this IS element shows the uniqueness in its long (110-bp) IRs and its lack of the resolution function. The identification of many *IS1071* sequences in close proximity to various xenobiotic-degrading genes on self-transmissible plasmids from environmental bacteria, e.g., *Pseudomonas* (18), *Comamonas* (2, 13), and *Wautersia* (3), indicates that *IS1071* must have been involved in the recruitment of catabolic genes to these plasmids and in the dissemination of these genes among various host strains. We have also identified a haloacetate-catabolic *IS1071*-composite transposon, TnHad1, on an IncP-1 β plasmid, pUO1, from *Delftia acidovorans* strain B (Fig. 1A) (24, 25). TnHad1 is located within a defective class II transposon, TnHad2, which is a Tn21-related transposon that lacks the *tnpA* and *tnpR* genes (Fig. 1A) (24). We have previously reported that the two intact copies and one truncated copy of *IS1071* in TnHad2 might have been incorporated into an ancestor of TnHad2 (24). However, no clear transposition events of the TnHad1-specified *IS1071* element were observed.

No functional analysis of *IS1071* has been carried out since its discovery more than a decade ago. Our functional analysis of *IS1071* in this study has indicated that (i) efficient transposition of *IS1071* occurred in two specific host strains, (ii) *IS1071* had the functional features of the class II transposons, and (iii) almost the entire region of the 110-bp IR was required for transposition.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains and plasmids used are listed in Table 1. Luria broth (LB) and LB agar (1) were used throughout this study. *Escherichia coli* cells were cultivated at 37°C and the others at 30°C. The agents added to the media were as follows: ampicillin, 100 μ g/ml; chloramphenicol, 50 μ g/ml; kanamycin, 50 μ g/ml; nalidixic acid, 30 μ g/ml; tetracycline, 10 μ g/ml; sulfathiazole, 350 μ g/ml.

DNA methodology. Standard methods were used for extracting plasmid DNA, DNA digestion with restriction endonucleases, ligation, gel electrophoresis, and transformation of bacterial cells (1). The PCR was carried out

* Corresponding author. Present address: Department of Biological Sciences, 222 Life Sciences North, University of Idaho, Moscow, ID 83844-3051. Phone: (208) 885-4031. Fax: (208) 885-7905. E-mail: sota@uidaho.edu.

† Present address: Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology (AIST), Higashi 1-1-1, Tsukuba, Ibaraki 305-8566, Japan.

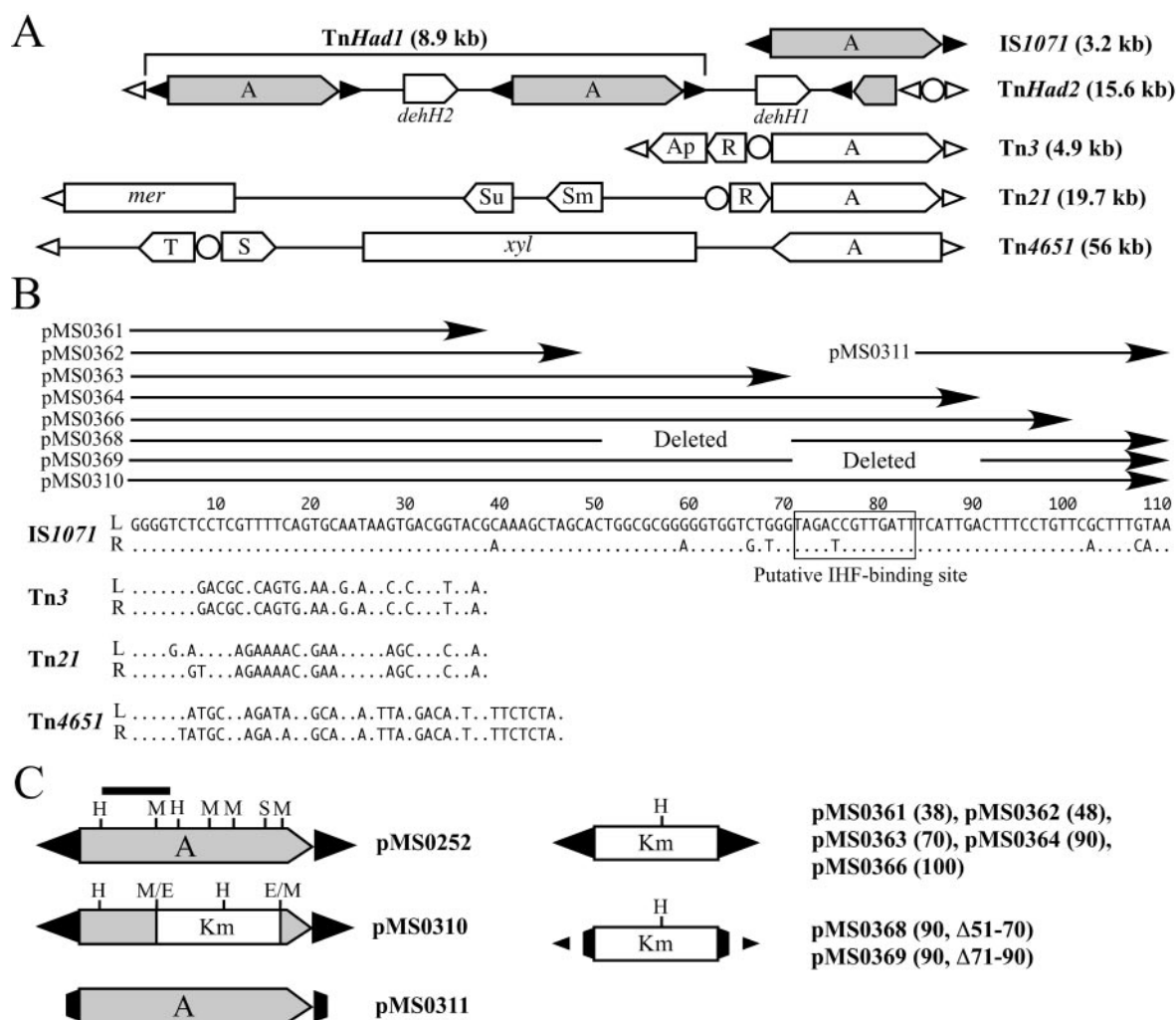


FIG. 1. Structures of class II transposons, their IR sequences, and *IS1071* derivatives. (A) Schematic structures of the class II transposons. The sizes are arbitrary. The black and white arrowheads indicate the terminal IR sequences of *IS1071* and those of the representative transposons, respectively, and the circle represents the *res* site. The pentagon shows the orientation of the gene. Abbreviations: A, *impA* gene; R, S, and T, genes for cointegrate resolution; Ap, Sm, Su, and *mer*, genes for resistance to ampicillin, streptomycin, sulfonamide, and mercury, respectively; *xyl*, genes for degradation of toluene and xylenes. (B) Comparison of IR sequences. The left (L) and right (R) ends of *IS1071* are defined as those located upstream and downstream, respectively, of the *impA* gene, and those of other transposons are defined as the distal and proximal ends, respectively, to the *impA* gene. A dot indicates a nucleotide identical to that in the left IR of *IS1071*. The arrows indicate the IR sequences that are carried in the *IS1071* derivatives on the plasmids depicted. The boxed sequence represents the putative IHF-binding site. (C) Structures of the *IS1071* derivatives on the pMS plasmids. The black bar indicates the DNA fragment used as a probe for the Southern analysis (Fig. 3). E, H, M, and S represent EcoRI, HindIII, MunI, and Sall sites, respectively. The values in parentheses are the lengths of the mutant IRs in the pMS plasmids.

with *ExTaq* DNA polymerase (TAKARA BIO). Purification of PCR-amplified DNA fragments was done with a GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences) according to the manufacturer's protocol. Nucleotide sequencing was performed with an ABI PRISM model 310 sequencer (Applied Biosystems). Southern hybridization (1) was done with an ECL Random-Prime Labeling and Detection System (Amersham Biosciences) according to the manufacturer's protocol.

PCR amplification and construction of plasmids. Primer 1071Kpn (5'-ACG TGGTACCGGGGCTCCTCGTTTTCAGT-3') contained a KpnI site (underlined) and the outermost 20-base sequence of the 110-bp IR of *IS1071* (bold letters). This primer was used for PCR amplification of the entire *IS1071* sequence with pUO1 as the template. The product was cloned into the KpnI site of pBBR1MCS-3 (15) to generate pMS0252 (Fig. 1C). The MunI fragment in the *IS1071* element on pMS0252 was replaced by the 1.3-kb pUC4K-derived EcoRI fragment carrying a *Km^r* determinant (26), and the resulting *Km^r-IS1071* derivative was inserted into the KpnI site on broad-host-range plasmid pNIT6012 (Table 1) to construct pMS0310 (Fig. 1C). Seven pNIT6012-based plasmids,

pMS0361, pMS0362, pMS0363, pMS0364, pMS0366, pMS0368, and pMS0369, carried the *Km^r-IS1071* derivatives with mutant IRs (38-, 48-, 70-, 90-, 100-, 90-, and 90-bp IRs, respectively), and the IRs in the last two plasmids lacked internal 20-bp sequences at different positions (Fig. 1B and C). These plasmids were constructed by PCR with appropriate primers. Primer 1071-84 (5'-GGCCGC TAGCTCATTGACTTTCTGTTTC-3') had an NheI site (underlined) and the 18-bp sequence (bold letters) that annealed to the internal nucleotides (positions 84 to 101, Fig. 1B) of the 110-bp IRs of *IS1071*. This primer was used to amplify an *IS1071* derivative lacking the outermost 83-bp sequences at both ends. Cloning of the amplified product into the XbaI site of pBBR1MCS (16) generated pMS0311 (Fig. 1C). Primers 1071pro1 (5'-TTTTGTGACGCGGGTCTCCTC GTTTTCAGT-3') and 1071pro3 (5'-TTTTAGATCTCGTGAACCTCAAAA GTGGGA-3') were used to amplify the 145-bp sequence upstream of the putative *impA* gene of *IS1071* (Fig. 2A). The product flanked by Sall (GTTCGAC, underlined in 1071pro1) and BglII (AGATCT, underlined in 1071pro3) sites was inserted between the corresponding sites in promoter-probe vector pCB182 (22) to construct pMS0321 (Fig. 2B). The BamHI-PstI fragment of pMS0321 con-

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
<i>Agrobacterium tumefaciens</i> C58	Free of IS1071	35
<i>Comamonas testosteroni</i> JCM5832	Free of IS1071; type strain	JCM ^a
<i>Delftia acidovorans</i> B	Harboring pUO1	14
<i>D. acidovorans</i> B123	pUO1-cured derivative of strain B, more than one copy of IS1071 in the chromosome	14
<i>Escherichia coli</i> DH5 α	<i>recA lacZ</i> Δ M15; free of IS1071	1
<i>E. coli</i> HB101	<i>recA</i> ; free of IS1071	1
<i>E. coli</i> JM109	Nal ^r <i>recA hsdR17</i> ; free of IS1071	1
<i>Pseudomonas alcaligenes</i> JCM5967	Free of IS1071; type strain	JCM ^a
<i>P. putida</i> PpN1	Free of IS1071	4
Plasmids		
pUO1	Hg ^r , haloacetate degradation, Tn <i>Had1</i> , Tn <i>Had2</i> (Fig. 1A)	14
pBBR1MCS	Cm ^r , broad-host-range cloning vector	16
pBBR1MCS-3	Tc ^r , broad-host-range cloning vector	15
pCB182	Ap ^r , promoter-probe vector, <i>lacZ</i> gene without its promoter sequence (Fig. 2B)	22
pME6012	Tc ^r , broad-host-range vector, pVSI and p15A replicons	8
pNIT6012	Tc ^r , pME6012 derivative carrying the <i>oriT</i> sequence of RP4 at BamHI site	Laboratory collection
pMS0252	Tc ^r , pBBR1MCS-3 derivative carrying IS1071 (Fig. 1C)	This study
pMS0310	Tc ^r Km ^r , pNIT6012 derivative carrying a Km ^r -IS1071 derivative with 110-bp IRs (Fig. 1B and C)	This study
pMS0311	Cm ^r , pBBR1MCS derivative carrying an IS1071 derivative that lacked the outermost 83-bp sequence in both IRs (Fig. 1B and C)	This study
pMS0321	Ap ^r , pCB182 derivative carrying the 145-bp sequence upstream of the <i>tnpA</i> gene in IS1071 between BglII and Sall sites (Fig. 2A and B)	This study
pMS0324	Cm ^r , pBBR1MCS derivative carrying the BamHI-PstI fragment of pMS0321 (Fig. 2B)	This study
pMS0326	Cm ^r , pBBR1MCS derivative carrying the BglII-PstI fragment of pCB182 (Fig. 2B)	This study
pMS0361	Tc ^r Km ^r , pNIT6012 derivative carrying a Km ^r -IS1071 derivative with the outermost 38-bp sequences of the IRs (Fig. 1B and C)	This study
pMS0362	Tc ^r Km ^r , pNIT6012 derivative carrying a Km ^r -IS1071 derivative with the outermost 48-bp sequences of the IRs (Fig. 1B and C)	This study
pMS0363	Tc ^r Km ^r , pNIT6012 derivative carrying a Km ^r -IS1071 derivative with the outermost 70-bp sequences of the IRs (Fig. 1B and C)	This study
pMS0364	Tc ^r Km ^r , pNIT6012 derivative carrying a Km ^r -IS1071 derivative with the outermost 90-bp sequences of the IRs (Fig. 1B and C)	This study
pMS0366	Tc ^r Km ^r , pNIT6012 derivative carrying a Km ^r -IS1071 derivative with the outermost 100-bp sequences of the IRs (Fig. 1B and C)	This study
pMS0368	Tc ^r Km ^r , pNIT6012 derivative carrying a Km ^r -IS1071 derivative whose IRs have a 20-bp deletion at positions 51 to 70 (Fig. 1B and C)	This study
pMS0369	Tc ^r Km ^r , pNIT6012 derivative carrying a Km ^r -IS1071 derivative whose IRs have a 20-bp deletion at positions 71 to 90 (Fig. 1B and C)	This study
pUC4K	Ap ^r Km ^r , cloning vector, Km ^r gene is flanked by the EcoRI, BamHI, Sall, and PstI sites	26
R388	Tp ^r Su ^r , conjugal plasmid free of transposons, IncW replicon	32

^a JCM, Japan Collection of Microorganisms.

taining the 145-bp fragment and a promoterless *lacZ* gene was cloned to the corresponding sites in pBBR1MCS to construct pMS0324 (Fig. 2B). The BglII-PstI fragment of pCB182 that carried the promoterless *lacZ* gene was cloned between the BamHI and PstI sites in pBBR1MCS to generate pMS0326 (Fig. 2B). Primers 1071-287 (5'-GGTCTGGCGCTCCATATTTGGTTTCTGCGC-3') and 1071-1358 (5'-GCAGCTTGGCAAGGTACTCGATGGCAGGAT-3') were used to amplify the 1.1-kb portion of IS1071 (Fig. 1C). The product was used as a probe for Southern hybridization.

Transposition assays. Transposition of the IS1071 derivatives was assayed by the "mating-out" experiments described previously (24). For this purpose, we introduced pMS0252 and R388 (32) into the bacterial strains listed in Table 2. The resulting strains were employed as donors to mate with *E. coli* JM109 on a membrane filter, and Tc^r Nal^r transconjugants were selected. The transposition frequency was expressed as the number of Tc^r Nal^r transconjugants per number of Su^r Nal^r transconjugants. The Tc^r Nal^r transconjugants were analyzed for their plasmid profiles. Complementation of the IS1071 *tnpA* mutation and transposition of the Km^r-IS1071 derivatives with mutant ends were also examined by the mating-out experiment. The donor strain was *C. testosteroni* JCM5832 harboring

the following three plasmids: (i) R388; (ii) pMS0311, a pBBR1MCS-based plasmid carrying the *tnpA* gene of IS1071; and (iii) pMS0310 or one of the other pNIT6012-based plasmids carrying a Km^r-IS1071 derivative with mutant ends (Fig. 1B and C). Such a JCM5832 derivative was mated with JM109, and Km^r Nal^r transconjugants were selected and analyzed for their plasmid profiles.

β -Gal assays. The bacterial strains harboring pMS0324 or pMS0326 (Fig. 2B) were grown to the early stationary phase in LB containing chloramphenicol and used for β -galactosidase (β -Gal) assays according to the method of Miller (19).

Nucleotide sequence accession number. Our partial sequencing and the previously deposited sequences of IncW plasmid R388 revealed its complete sequence (33,913 bp), and the sequence has been deposited in the DDBJ/EMBL/GenBank databases under accession number BR000038.

RESULTS

Transposition of IS1071. Although we previously detected a very low frequency of transposition (1.3×10^{-7}) of the pUO1-

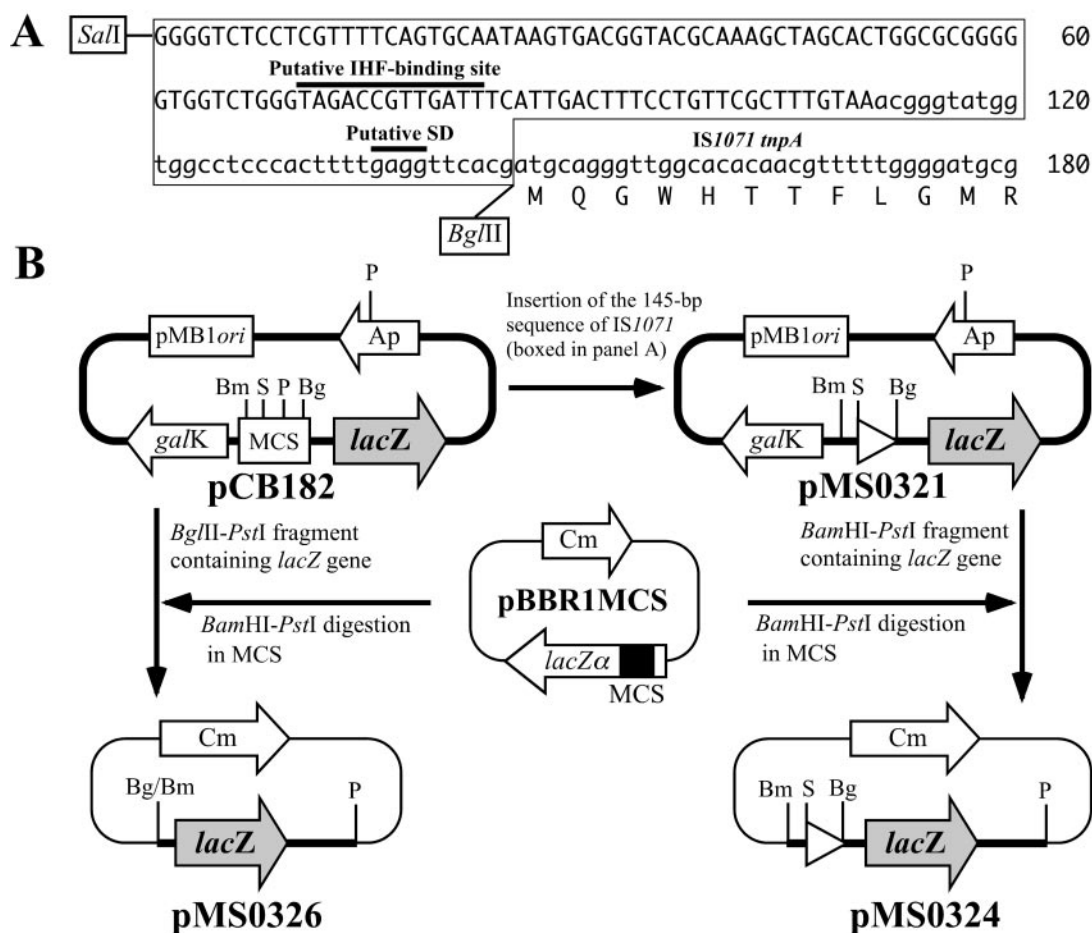


FIG. 2. Construction of plasmids used for *tnpA* promoter analysis. (A) Nucleotide sequence upstream of the *tnpA* gene of *IS1071*. Capital letters indicate the 110-bp IR sequence of *IS1071*. The boxed region (145 bp) was amplified by PCR with primers 1071pro1 and 1071pro3 and used to construct pMS0321. (B) Construction of plasmids. Abbreviations for restriction endonucleases are as follows: B, BamHI; Bg, BglII; P, PstI; S, SalI. Plasmid pCB182 is a promoter-probe vector and carries a multiple-cloning site (MCS) between the *galK* and *lacZ* genes, each of which lacks its promoter sequence (22). The BglII-PstI fragment of pCB182 that carried the promoterless *lacZ* gene was cloned between the BamHI and PstI sites in pBBR1MCS (16) to generate pMS0326. The 145-bp sequence of *IS1071* (white arrowhead) flanked by SalI and BglII sites (see panel A) was inserted between the corresponding sites in pCB182 to generate pMS0321. Subsequent cloning of the BamHI-PstI fragment of pMS0321 containing the *lacZ* gene between the corresponding sites in pBBR1MCS led to the construction of pMS0324.

derived *IS1071* element in *E. coli* (24), our subsequent and repeated attempts to detect such a transposition event in any *E. coli* strains were unsuccessful. Therefore, in this study we investigated *IS1071* transposition in several environmental bacterial strains listed in Table 2. These strains, harboring pMS0252 (=pBBR1MCS-3::*IS1071*) and R388, were employed as the donor host strains in the mating-out experiments. Tc^r Nal^r transconjugants were obtained at high frequencies when *C. testosteroni* JCM5832 and *D. acidovorans* B123 were employed as the donor host strains (Table 2) but not with any of the other donor strains. Restriction and Southern analyses of the plasmids from several Tc^r Nal^r transconjugants revealed that these plasmids were cointegrates of pMS0252 and R388 connected by two directly repeated copies of *IS1071*, one at each junction, since each plasmid had (i) two (4.2- and 3.5-kb) fragments identical to those from pMS0252 (Fig. 3A) and (ii) two copies of *IS1071* (Fig. 3B). It was theoretically possible that these cointegrates were generated by nonreplicative transposition of an *IS1071*-composite transposon from dimers of

TABLE 2. Transposition of *IS1071*

Host strain ^a	Proteobacterial division	Transposition frequency ^b	
		pBBR1MCS-3	pMS0252
<i>A. tumefaciens</i> C58	α	<1.2 × 10 ⁻⁷	<8.7 × 10 ⁻⁸
<i>C. testosteroni</i> JCM5832	β	<3.3 × 10 ⁻⁷	1.5 × 10 ⁻³
<i>D. acidovorans</i> B123 ^c	β	<1.4 × 10 ⁻⁵	4.0 × 10 ⁻⁴
<i>E. coli</i> HB101	γ	<9.8 × 10 ⁻⁹	<1.6 × 10 ⁻⁸
<i>P. alcaligenes</i> JCM5967	γ	<9.7 × 10 ⁻⁸	<2.1 × 10 ⁻⁸
<i>P. putida</i> PpN1	γ	<1.9 × 10 ⁻⁸	<1.4 × 10 ⁻⁸

^a *D. acidovorans* B123 carried more than one copy of *IS1071* in its chromosome, and the other strains were free of *IS1071* (data not shown).

^b Transposition of *IS1071* was detected by the cointegration between pMS0252 and R388 (32). Plasmid pMS0252 is a pBBR1MCS-3 derivative carrying *IS1071* (Fig. 1C). The transposition frequency is expressed as the number of Tc^r Nal^r transconjugants per number of Su^r Nal^r transconjugants. A value with the symbol < means that no Tc^r Nal^r transconjugants were obtained in the experiment. All values are averages from at least three independent experiments.

^c The transfer frequency of R388 was low (2 × 10⁻⁴ per donor cell) for an unknown reason(s) when *D. acidovorans* B123 was used as the donor host.

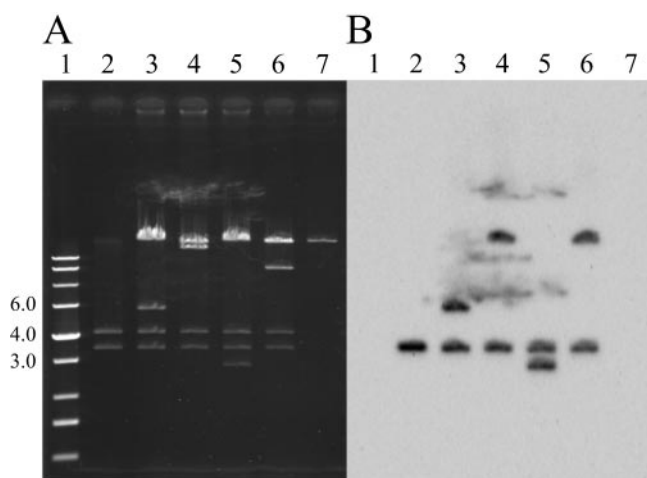


FIG. 3. Analysis of IS1071-mediated cointegrates by agarose gel electrophoresis (A) and Southern hybridization patterns with an internal 1.1-kb fragment of IS1071 (Fig. 1C) as a probe (B). All samples were digested with Sall, which cleaved IS1071 at one site (Fig. 1C). Lane 1, marker DNA; lane 2, pMS0252; lanes 3 to 6, cointegrates of pMS0252 and R388 obtained in the independent experiments; lane 7, R388. The sizes (in kilobases) of three fragments in the marker DNA are shown on the left.

pMS0252. This possibility was unlikely since agarose gel analysis did not reveal the preferential presence of the pMS0252 dimers in the two host strains (data not shown). Sequence analysis of the insertion sites indicated that IS1071 transposed to various sites in R388 with concomitant generation of a 5-bp duplication of the target sequence. These results demonstrated that (i) IS1071 is highly mobile, but only in specific bacterial strains; (ii) IS1071 is duplicated upon transposition; and (iii) IS1071 generates a 5-bp duplication of the target sequence. Subsequent transposition experiments in this study were carried out with *C. testosteroni* JCM5832 since this strain was free of IS1071.

Complementation of cointegration function. The *tnpA* mutations of the class II transposons are usually complemented efficiently by supplying their cognate wild-type *tnpA* genes in *trans* (23). To investigate whether this was the case with IS1071, we conducted a mating-out experiment with *E. coli* JM109 as a recipient strain and a *C. testosteroni* JCM5832 derivative harboring pMS0311 (the supplier of *tnpA*), pMS0310 (the Km^r-IS1071 carrier) (Fig. 1B and C), and R388 as a donor strain. Km^r Nal^r transconjugants were obtained at a frequency of 3.7×10^{-3} (Table 3, experiment 1). All of the 100 transconjugants examined showed resistance to tetracycline, which was encoded by the vector portion (pNIT6012) of pMS0310. This suggested that all the transconjugants carried the fusion plasmids that have portions of pMS0310 and R388. Detailed analysis of the plasmids from several transconjugants with restriction endonucleases further revealed that they were cointegrates of the two plasmids connected by two directly repeated copies of the Km^r-IS1071 derivative, one at each junction (data not shown). The absence of the R388::Km^r-IS1071 plasmids in the 100 transconjugants indicated that the *C. testosteroni* JCM5832-encoded DNA recombination systems (e.g., RecA system) did not function efficiently to resolve the cointegrates rapidly. These results

indicated that (i) the wild-type *tnpA* gene of IS1071 is able to complement its mutation in *trans* and (ii) the final product of the IS1071 transposition is a cointegrate of its donor and target DNA molecules. The Km^r Nal^r transconjugants were also obtained at very low frequencies when the *tnpA* gene was not supplied (Table 3, experiment 1). However, all the plasmids residing in these transconjugants had an identical structure in which pMS0310 and R388 were fused but not connected by the two copies of Km^r-IS1071 (data not shown). Such a structure also supported the idea that the IS1071 derivative was not involved in the cointegration. We did not further examine the mechanism by which such a fusion product was formed because IS1071 transposition was not involved.

Transposition of IS1071 derivatives with mutant ends. IS1071 has 110-bp IRs, the outermost 38-bp sequences of which are similar to the IRs of Tn3 and Tn21 (Fig. 1B). Considering that Tn3 and Tn21 are able to transpose by using their 38-bp IRs (23), we constructed several Km^r-IS1071 derivatives with shorter IRs and investigated their transposition. A *C. testosteroni* JCM5832 derivative harboring pMS0311, R388, and a pNIT6012-based plasmid carrying a Km^r-IS1071 derivative with mutant ends (Fig. 1B and C) was mated with JM109. Use of the seven pNIT6012 derivatives generated the Km^r Nal^r transconjugants at frequencies of 10^{-6} to 10^{-7} , and these frequencies were very similar regardless of the presence or absence of the *tnpA* gene (Table 3, experiments 2 to 8). Furthermore, the transconjugants in each experiment had a fusion product of R388 and the pNIT6012-based plasmid that was not generated by Km^r-IS1071 (data not shown). These results strongly suggest that IS1071-mediated cointegration required almost the entire region of its 110-bp IRs.

Transcriptional activity of the *tnpA* gene. To know why IS1071 was transposable in the *Comamonas* and *Delftia* cells but not in the *Agrobacterium*, *E. coli*, and *Pseudomonas* cells (Table 2), we investigated the promoter activity of the IS1071-specified 145-bp sequence that was located just upstream of its *tnpA* gene (Fig. 2A). Plasmid pMS0324, which carried this sequence in front of a promoterless *lacZ* gene (Fig. 2B), was introduced into the strains listed in Table 2, and the β -Gal activities of the resulting strains were assayed. As shown in Table 4, *C. testosteroni* JCM5832, *Agrobacterium tumefaciens*

TABLE 3. Transposition of Km^r-IS1071 derivatives with mutant IRs^a

Expt	Donor of Km ^r -IS1071 ^b (IR length, bp)	Transposition frequency ^c	
		pBBR1MCS	pMS0311
1	pMS0310 (110)	8.5×10^{-7}	3.7×10^{-3}
2	PMS0366 (100)	1.5×10^{-6}	8.7×10^{-6}
3	pMS0364 (90)	2.4×10^{-6}	6.0×10^{-6}
4	pMS0363 (70)	8.0×10^{-7}	4.2×10^{-7}
5	pMS0362 (48)	8.7×10^{-7}	3.8×10^{-6}
6	pMS0361 (38)	4.7×10^{-7}	2.9×10^{-6}
7	pMS0368 (90, Δ 51–70)	3.7×10^{-6}	8.7×10^{-6}
8	pMS0369 (90, Δ 71–90)	3.1×10^{-6}	2.8×10^{-6}

^a The donor strain was a *C. testosteroni* JCM5832 derivative harboring the three following plasmids: (i) R388, (ii) a pNIT6012 derivative carrying a Km^r-IS1071 derivative with the mutant IRs, and (iii) either pBBR1MCS or pMS0311, a pBBR1MCS derivative that carried the *tnpA* gene of IS1071 (Fig. 1B and C).

^b The IR sequences and structure, respectively, of each Km^r-IS1071 derivative are shown in Fig. 1B and C.

^c The transposition frequency is expressed as the number of Km^r Nal^r transconjugants per number of Su^r Nal^r transconjugants. All values are averages from at least three independent experiments.

TABLE 4. Promoter analysis of the *tnpA* gene^a

Host strain	IS1071-mediated cointegration	β-Gal activity (Miller units)	
		pMS0324	pMS0326
<i>A. tumefaciens</i> C58	No	142 ± 31	7.6 ± 2.1
<i>C. testosteroni</i> JCM5832	Yes	9.2 ± 0.6	ND ^b
<i>D. acidovorans</i> B123	Yes	ND	ND
<i>E. coli</i> DH5α	No	ND	ND
<i>P. alcaligenes</i> JCM5967	No	ND	ND
<i>P. putida</i> PpN1	No	33 ± 4.0	ND

^a β-Gal activity was assayed by the method of Miller (19). Plasmid pMS0324 carries a *tnpA* promoter-*lacZ* transcriptional fusion, whereas pMS0326 lacks the *tnpA* promoter region and was used as a negative control. The nucleotide sequence upstream of the *tnpA* gene is shown in Fig. 2A, and the structures of the plasmids used in this analysis are shown in Fig. 2B. The values are averages from three independent experiments and are shown with the standard deviations.

^b ND, not detected in a 60-min reaction.

C58, and *Pseudomonas putida* PpN1 cells harboring pMS0324 showed low β-Gal activities but the activities were not detected in *E. coli* DH5α, *D. acidovorans* B123, and *Pseudomonas alcaligenes* JCM5967. These results were inconsistent with the transposition experiments since transposition of IS1071 in *A. tumefaciens* C58 and *P. putida* PpN1 was not detected despite the positive transcriptional activity of the *tnpA* promoter in these hosts. Thus, differences in promoter activity cannot be the cause of the observed differences in transposition activity among the strains examined.

DISCUSSION

In this study, we showed that (i) IS1071 transposed by a replicative mode to generate a cointegrate of its donor and target molecules as the final product, (ii) a 5-bp duplication of the target sequence was generated upon transposition, and (iii) a *tnpA* mutation of IS1071 was efficiently complemented by the supply of the wild-type *tnpA* gene in *trans*. The experimental data obtained in this study confirm that IS1071 is classified as a class II transposon. A remarkable difference between IS1071 and other typical class II transposons is the absence of the resolution function. Such a function is not absolutely required for completion of the transposition reaction of IS1071 since the host-specified RecA system is also able to resolve the cointegrate by homologous recombination between two directly repeated copies of a transposon (6). It has been proposed that the class II transposons would have evolved from an IS1071 or IS1071-like element by acquisition of the resolution systems (7, 20). This is consistent with the fact that the phylogenetic tree of the enzymes for the resolution systems does not agree with that of the transposases from various class II transposons (7); for example, the transposases of Tn5403 (accession no. X75779) and Tn5393 (M95402) have 62% amino acid identity but their resolvases share only 35% identity. It would be interesting to examine by what special molecular mechanisms the resolution function was acquired.

The length (110 bp) of the IRs is another remarkable characteristic of IS1071 since the IRs of other prokaryotic class II transposons are less than 50 bp long (Fig. 1B). Our deletion analysis in this study revealed that the outermost 100-bp part of the IRs was not sufficient for the transposition of IS1071

(Table 3). It is known that transposases of several class II transposons cooperatively bind to their IRs with the integration host factor (IHF) (12, 34). A well-studied example is Tn4652, a deletion derivative of Tn4651 from *P. putida* KT2440 (12, 34). Tn4652 requires the IHF for its efficient transposition (10, 12). It has been considered that an unidentified *Pseudomonas*-specific host factor(s) activates the transcription of the *tnpA* gene promoter and that binding of the *Pseudomonas* IHF to the ends of Tn4652 is also required for its transposition (10, 12). Since the IHF generally binds to the flanking region of its target sequence (12) and IS1071 carries a putative IHF-binding site in its both IRs (Fig. 1B), it is likely that lack of the IHF-binding sites in the mutant IRs of IS1071 might be a reason why our IS1071 mutants were unable to transpose. The apparent requirement of the long IR sequences for IS1071 transposition also indicates that the specific recognition and binding of the IS1071 transposase to its IRs might be different from those of other typical class II transposons such as Tn3 and Tn21. In vivo binding experiments of the IS1071 transposase and the IHF with its cognate IRs will provide some clues to clarify the unique interaction between the transposase and IRs.

In this study, high-frequency transposition of IS1071 was detected only in two β-proteobacteria and not in any of the other bacteria tested (Table 2), which belong to the α- and γ-proteobacteria. Since *E. coli* JM109, used as the recipient cell in the transposition experiments, has an *hsdR* (restriction-defective) mutation, the host-specific detection of the IS1071 transposition was not due to the restriction of the transferred cointegrates in JM109. It should be noted that our experiment with pMS0252 and R388 cannot detect the IS1071 transposition if (i) IS1071 transposes into R388 by a simple insertion mechanism or (ii) the cointegrate of pMS0252 and R388 resolves rapidly by the host-encoded DNA recombination systems (6). However, this idea is not valid because (i) we never obtained the R388::Km^r-IS1071 plasmid and (ii) the cointegrate of pMS0252 and R388 was stably maintained in *A. tumefaciens*, *P. putida*, and *P. alcaligenes* (data not shown). The strain-specific transposition of IS1071 is consistent with our database searches, which showed that IS1071 and its remnants are, in addition to their preferential localization on the broad-host-range IncP-1β plasmids (18, 21, 27, 28), mainly distributed on the chromosomes of several β-proteobacterial strains such as *D. acidovorans* P4a (9), *Wautersia metallidurans* CH34 (accession no. X90708), and *Burkholderia xenovorans* LB400 (5). Moreover, the nucleotide sequence of IS1071 is highly conserved (>99%) in many bacterial strains, indicating that this element might have been maintained only in a limited number of closely related bacterial strains. One possible explanation for the host-specific transposition of IS1071 is that its transposition requires or is inhibited by some host-specific factor(s). The other possibility is the defect in posttranscriptional steps of the *tnpA* transcript (translation, holding, and maturation, etc.). It could also be envisioned that the host specificity is involved in the codon usages of the host cells, because (i) taxonomically closely related organisms have similar codon usages (11) and (ii) both *C. testosteroni* and *D. acidovorans* are members of the family Comamonadaceae (33). These ideas are consistent with our preliminary experiment in that IS1071 transposed only at a very low frequency (9.6×10^{-7}) in *E. coli* cells, even when its *tnpA* gene was expressed under the control of a *tac* promoter. We do not know which one of the three ideas is

the most plausible. It is of great interest to uncover the specific host factor(s) of *Comamonas* and *Delftia* cells that plays a crucial role in IS1071 transposition.

ACKNOWLEDGMENTS

We are grateful to Eva M. Top (University of Idaho) for critically reviewing the manuscript and Masaru Nagai (Institute for Environmental Sciences) for experimental assistance.

This work was carried out under contract with the Ministry of Education, Culture, Sports, Science and Technology. This work was also supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology and the Ministry of Agriculture, Forestry, and Fisheries (HC-05-2323-5), Japan.

REFERENCES

- Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1994. Current protocols in molecular biology. Wiley, New York, N.Y.
- Boon, N., J. Goris, P. De Vos, W. Verstraete, and E. M. Top. 2001. Genetic diversity among 3-chloroaniline- and aniline-degrading strains of the *Comamonadaceae*. *Appl. Environ. Microbiol.* **67**:1107–1115.
- Clement, P., D. H. Pieper, and B. Gonzalez. 2001. Molecular characterization of a deletion/duplication rearrangement in *tfd* genes from *Ralstonia eutropha* JMP134(pJP4) that improves growth on 3-chlorobenzoic acid but abolishes growth on 2,4-dichlorophenoxyacetic acid. *Microbiology* **147**:2141–2148.
- Dean, H. F., and A. F. Morgan. 1983. Integration of R91-5::Tn501 into the *Pseudomonas putida* PPN chromosome and genetic circularity of the chromosomal map. *J. Bacteriol.* **153**:485–497.
- Goris, J., P. De Vos, J. Caballero-Mellado, J. Park, E. Falsen, J. F. Quensen III, J. M. Tiedje, and P. Vandamme. 2004. Classification of the biphenyl- and polychlorinated biphenyl-degrading strain LB400¹ and relatives as *Burkholderia xenovorans* sp. nov. *Int. J. Syst. Evol. Microbiol.* **54**:1677–1681.
- Grindley, N. D., and R. R. Reed. 1985. Transpositional recombination in prokaryotes. *Annu. Rev. Biochem.* **54**:863–896.
- Grindley, N. D. F. 2002. The movement of Tn3-like elements: transposition and cointegrate resolution, p. 272–302. *In* N. L. Craig, R. Craigie, M. Gellert, and A. M. Lambowitz (ed.), *Mobile DNA II*. ASM Press, Washington, D. C.
- Heeb, S., Y. Itoh, T. Nishijyo, U. Schneider, C. Keel, J. Wade, U. Walsh, F. O'Gara, and D. Haas. 2000. Small, stable shuttle vectors based on the minimal pVS1 replicon for use in gram-negative, plant-associated bacteria. *Mol. Plant-Microbe Interact.* **13**:232–237.
- Hoffmann, D., S. Kleinstuber, R. H. Muller, and W. Babel. 2003. A transposon encoding the complete 2,4-dichlorophenoxyacetic acid degradation pathway in the alkali-tolerant strain *Delftia acidovorans* P4a. *Microbiology* **149**:2545–2556.
- Horak, R., and M. Kivisaar. 1998. Expression of the transposase gene *tnpA* of Tn4652 is positively affected by integration host factor. *J. Bacteriol.* **180**:2822–2829.
- Ikemura, T. 1985. Codon usage and tRNA content in unicellular and multicellular organisms. *Mol. Biol. Evol.* **2**:13–34.
- Ives, H., R. Horak, R. Teras, and M. Kivisaar. 2004. IHF is the limiting host factor in transposition of *Pseudomonas putida* transposon Tn4652 in stationary phase. *Mol. Microbiol.* **51**:1773–1785.
- Junker, F., and A. M. Cook. 1997. Conjugative plasmids and the degradation of arylsulfonates in *Comamonas testosteroni*. *Appl. Environ. Microbiol.* **63**:2403–2410.
- Kawasaki, H., N. Tone, and K. Tomomura. 1981. Plasmid-determined dehalogenation of haloacetates in *Moraxella* species. *Agric. Biol. Chem.* **45**:29–34.
- Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop II., and K. M. Peterson. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**:175–176.
- Kovach, M. E., R. W. Phillips, P. H. Elzer, R. M. Roop II., and K. M. Peterson. 1994. pBBR1MCS: a broad-host-range cloning vector. *BioTechniques* **16**:800–802.
- Maeda, K., H. Nojiri, M. Shintani, T. Yoshida, H. Habe, and T. Omori. 2003. Complete nucleotide sequence of carbazole/dioxin-degrading plasmid pCAR1 in *Pseudomonas resinovorans* strain CA10 indicates its mosaicity and the presence of large catabolic transposon Tn4676. *J. Mol. Biol.* **326**:21–33.
- Martinez, B., J. Tomkins, L. P. Wackett, R. Wing, and M. J. Sadovsky. 2001. Complete nucleotide sequence and organization of the atrazine catabolic plasmid pADP-1 from *Pseudomonas* sp. strain ADP. *J. Bacteriol.* **183**:5684–5697.
- Miller, J. H. 1992. Short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Nakatsu, C., J. Ng, R. Singh, N. Straus, and C. Wyndham. 1991. Chlorobenzoate catabolic transposon Tn5271 is a composite class I element with flanking class II insertion sequences. *Proc. Natl. Acad. Sci. USA* **88**:8312–8316.
- Schlüter, A., H. Heuer, R. Szczepanowski, L. J. Forney, C. M. Thomas, A. Pühler, and E. M. Top. 2003. The 64,508 bp IncP-1β antibiotic multi-resistance plasmid pB10 isolated from a waste-water treatment plant provides evidence for recombination between members of different branches of the IncP-1β group. *Microbiology* **149**:3139–3153.
- Schneider, K., and C. F. Beck. 1986. Promoter-probe vectors for the analysis of divergently arranged promoters. *Gene* **42**:37–48.
- Sherratt, D. 1989. Tn3 and related transposable elements: site-specific recombination and transposition, p. 163–184. *In* D. E. Berg and M. M. Howe (ed.), *Mobile DNA*. American Society for Microbiology, Washington, D.C.
- Sota, M., M. Endo, K. Nitta, H. Kawasaki, and M. Tsuda. 2002. Characterization of a class II defective transposon carrying two haloacetate dehalogenase genes from *Delftia acidovorans* plasmid pUO1. *Appl. Environ. Microbiol.* **68**:2307–2315.
- Sota, M., H. Kawasaki, and M. Tsuda. 2003. Structure of haloacetate-catabolic IncP-1β plasmid pUO1 and genetic mobility of its residing haloacetate-catabolic transposon. *J. Bacteriol.* **185**:6741–6745.
- Taylor, L. A., and R. E. Rose. 1988. A correction in the nucleotide sequence of the Tn903 kanamycin resistance determinant in pUC4K. *Nucleic Acids Res.* **16**:358.
- Tralau, T., A. M. Cook, and J. Ruff. 2001. Map of the IncP1β plasmid pTSA encoding the widespread genes (*tsa*) for *p*-toluenesulfonate degradation in *Comamonas testosteroni* T-2. *Appl. Environ. Microbiol.* **67**:1508–1516.
- Trefault, N., R. De la Iglesia, A. M. Molina, M. Manzano, T. Ledger, D. Perez-Pantoja, M. A. Sanchez, M. Stuardo, and B. Gonzalez. 2004. Genetic organization of the catabolic plasmid pJP4 from *Ralstonia eutropha* JMP134 (pJP4) reveals mechanisms of adaptation to chloroaromatic pollutants and evolution of specialized chloroaromatic degradation pathways. *Environ. Microbiol.* **6**:655–668.
- Tsuda, M., and H. Genka. 2001. Identification and characterization of Tn4656, a novel class II transposon carrying a set of toluene-degrading genes from TOL plasmid pWW53. *J. Bacteriol.* **183**:6215–6224.
- Tsuda, M., and T. Iino. 1990. Naphthalene degrading genes on plasmid NAH7 are on a defective transposon. *Mol. Gen. Genet.* **223**:33–39.
- Tsuda, M., K. Minegishi, and T. Iino. 1989. Toluene transposons Tn4651 and Tn4653 are class II transposons. *J. Bacteriol.* **171**:1386–1393.
- Ward, J. M., and J. Grinsted. 1982. Physical and genetic analysis of the Inc-W group plasmids R388, Sa, and R7K. *Plasmid* **7**:239–250.
- Wen, A., M. Fegan, C. Hayward, S. Chakraborty, and L. I. Sly. 1999. Phylogenetic relationships among members of the *Comamonadaceae*, and description of *Delftia acidovorans* (den Dooren de Jong 1926 and Tamaoka et al. 1987) gen. nov., comb. nov. *Int. J. Syst. Bacteriol.* **49**:567–576.
- Wiater, L. A., and N. D. Grindley. 1988. γδ transposase and integration host factor bind cooperatively at both ends of γδ. *EMBO J.* **7**:1907–1911.
- Wood, D. W., J. C. Setubal, R. Kaul, D. E. Monks, J. P. Kitajima, V. K. Okura, Y. Zhou, L. Chen, G. E. Wood, N. F. Almeida, Jr., L. Woo, Y. Chen, I. T. Paulsen, J. A. Eisen, P. D. Karp, D. Bovee, Sr., P. Chapman, J. Clendenning, G. Deatherage, W. Gillet, C. Grant, T. Kutayin, R. Levy, M. J. Li, E. McClelland, A. Palmieri, C. Raymond, G. Rouse, C. Saenphimmachak, Z. Wu, P. Romero, D. Gordon, S. Zhang, H. Yoo, Y. Tao, P. Biddle, M. Jung, W. Krespan, M. Perry, B. Gordon-Kamm, L. Liao, S. Kim, C. Hendrick, Z. Y. Zhao, M. Dolan, F. Chumley, S. V. Tingey, J. F. Tomb, M. P. Gordon, M. V. Olson, and E. W. Nester. 2001. The genome of the natural genetic engineer *Agrobacterium tumefaciens* C58. *Science* **294**:2317–2323.