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Characterization of Insertions of IS*476* and Two Newly Identified Insertion Sequences, IS*1478* and IS*1479*, in *Xanthomonas campestris* pv. campestris

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Thirty-two plasmid insertion mutants were independently isolated from two strains of *Xanthomonas campestris* pv. campestris in Taiwan. Of the 32 mutants, 14 (44%), 8 (25%), and 4 (12%) mutants resulted from separate insertions of an IS*3* family member, IS*476*, and two new insertion sequences (IS), IS*1478* and IS*1479*. While IS*1478* does not have significant sequence homology with any IS elements in the EMBL/GenBank/DDBJ database, IS*1479* demonstrated 73% sequence homology with IS*1051* in *X. campestris* pv. dieffenbachiae, 62% homology with IS*52* in *Pseudomonas syringae* pv. glycinea, and 60% homology with IS*5* in *Escherichia coli*. Based on the predicted transposase sequences as well as the terminal nucleotide sequences, IS*1478* by itself constitutes a new subfamily of the widespread IS*5* family, whereas IS*1479*, along with IS*1051*, IS*52*, and IS*5*, belongs to the IS*5* subfamily of the IS*5* family. All but one of the IS*476* insertions had duplications of 4 bp at the target sites without sequence preference and were randomly distributed. An IS*476* insertion carried a duplication of 952 bp at the target site. A model for generating these long direct repeats is proposed. Insertions of IS*1478* and IS*1479*, on the other hand, were not random, and IS*1478* and IS*1479* each showed conservation of PyPuNTTA and PyTAPu sequences (Py is a pyrimidine, Pu is a purine, and N is any nucleotide) for duplications at the target sites. The results of Southern blot hybridization analysis indicated that multiple copies of IS*476*, IS*1478*, and IS*1479* are present in the genomes of all seven *X. campestris* pv. campestris strains tested and several *X. campestris* pathovars.

Insertion sequences (IS) are mobile DNA elements capable of mediating various types of DNA rearrangements such as transposition, deletion, inversion, and cointegration. They are usually 0.8 to 2.5 kb long and encode a transposase protein. Most IS carry 10- to 40-bp inverted repeats (IR) at their ends and generate direct repeats of short target sequences upon insertion (7). To date, more than 500 IS elements have been isolated from both eubacteria and archaea. Except for those highly similar variants from the same or related hosts, IS elements are considered heterogeneous at the nucleotide sequence level. Many can be grouped into families on the basis of conservation of motifs in their presumptive transposase amino acid sequences and their terminal nucleotide sequences. The IS*3* and IS*5* families are the two largest families (14).

Members of the IS*3* family contain two overlapping open reading frames (ORFs), and the transposase protein is generated by programmed ribosomal frameshifting between the two ORFs. The transposase C-terminal region contains the characteristic DD(35)E motif, i.e., conservation of the acidic amino acid triad with several additional residues and 35 residues between the last two conserved acidic residues, D and E. Most members carry the dinucleotide TG at the 5′ ends (14). It has been suggested that the acidic amino acid triad interacts with the terminal 2 or 3 bp of the element to correctly position the IS ends in the catalytic site during transposition (10, 14). The IS*3* family can be divided into the subgroups IS*407*, IS*2*, IS*3*, IS*51*, and IS*150* on the basis of alignment of the transposase sequences (14). The IS*5* family, on the other hand, is relatively heterogeneous. Either the members contain two ORFs and produce transposase by frameshifting, like the IS*3* family members, or they contain a long ORF which covers most of the length of one strand and encodes transposase. In any case, their transposases carry another type of DDE motif, i.e., a spacing of 71 to 76 residues between the first two conserved acidic residues and a spacing of 40 to 67 residues between the last two acidic residues, in addition to conservation of D(1)GY in the region containing the second acidic residue and conservation of R(3)E(6)K in the region containing the third acidic residue (14, 18). All members carry the nucleotide G at the 5′ end. The IS*5* family can be divided into the subgroups IS*5*, IS*427*, IS*903*, IS*1031*, IS*H1*, and IS*L2* on the basis of alignment of the transposase sequences, particularly the residues near the three conserved acidic residues (14).

Xanthomonas campestris is a plant-pathogenic bacterium, consisting of more than 125 pathovars based on host specificity (26). Kearney et al. (11) isolated a mutant strain of *X. campestris* pv. vesicatoria from a pepper field and demonstrated that the mutant had an insertion of IS*476* in the *avr* locus, leading to virulence on an otherwise resistant pepper cultivar. This implies that the bacteria may extend the host range to include plants previously resistant through transposition. In order to investigate the role that IS elements play in the diversity of this species, first we systemically analyzed IS elements from an important pathovar, *X. campestris* pv. campestris, the agent causing crucifer black rot. A plasmid system developed by Gay et al. (8) was used to isolate IS elements in bacteria. In this system, the broad-host-range plasmid pUCD800 carrying the sucrose-sensitive *Bacillus subtilis sacB* gene with its *cis*-regulatory sequence, *sacR* was transferred into bacteria, and plasmid mutants with an insertion of chromosomal IS element in the *sacRB* gene were identified from survivors on agar plates containing 5% sucrose. We report here the successful isolation of 32 plasmid insertion mutants of *X. campestris* pv. campestris by

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source[a]
Strains		
E. coli DH5α	F$^-$ *hsdR17 recA1 supE44 endA1 gyrA96 relA1* Δ(*argF-lacZYA*)*U169 thi-1* ϕ80d *lacZ*ΔM15 λ$^-$	BRL
E. coli HB101	F$^-$ *hsdR20 ara-14 leuB6 proA2 recA13 lacY1 galK2 rpsL20 xyl-5 thi-1 mtl-1 supE44* λ	2
E. coli RR1	F$^-$ *hsdR20 ara-14 leuB6 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ	1
X. campestris pv. campestris 11 (Xc11)	Ap^r, Taiwanese strain	Y.-H. Tseng
X. campestris pv. campestris 11A	Ap^r, a spontaneous avirulent mutant of Xc11	Y.-H. Tseng
X. campestris pv. campestris 17	Ap^r, Taiwanese strain	Y.-H. Tseng
X. campestris pv. campestris 2	Taiwanese strain	K.-C. Tzeng
X. campestris pv. campestris 6	Taiwanese strain	K.-C. Tzeng
X. campestris pv. campestris 85	Taiwanese strain	K.-C. Tzeng
X. campestris pv. campestris 88	Taiwanese strain	K.-C. Tzeng
X. campestris pv. begoniae	ATCC 49082	ATCC
X. campestris pv. citri 60	Taiwanese strain	K.-C. Tzeng
X. campestris pv. dieffenbachiae 65	Taiwanese strain	K.-C. Tzeng
X. campestris pv. glycinea 69	Taiwanese strain	K.-C. Tzeng
X. campestris pv. mangiferaeindicae 38	Taiwanese strain	K.-C. Tzeng
X. campestris pv. phaseoli 73	Taiwanese strain	K.-C. Tzeng
X. campestris pv. vesicatoria 64	Taiwanese strain	K.-C. Tzeng
X. campestris pv. oryzae 1	Taiwanese strain	K.-C. Tzeng
Agrobacterium tumefaciens LBA4404	Str^r	Clontech
Erwinia carotovora subsp. carotovora ZL4	Taiwanese strain	K.-C. Tzeng
Pseudomonas solanacearum RD4	Taiwanese strain	K.-C. Tzeng
Rhizobium leguminosarum 128C53	*str-29*, harboring a 200-kb, Km^r plasmid pVW3JI	CCRC
Plasmids		
pUCD800	Km^r; carrying sucrose-sensitive *sacRB*	8
pRK2013	Km^r; mobilization helper plasmid	5
pUC18	Ap^r; α-*lacZ*/MCS[b] or cloning vector	28

[a] BRL, Bethesda Research Laboratories; ATCC, American Type Culture Collection; CCRC, Culture Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan.

[b] α-*lacZ*/MCS, DNA encoding the α-fragment of β-galactosidase (the *lacZ* gene product) with a multiple cloning site.

using this system. Twenty-six (81%) were characterized and had insertions of IS*476* and two newly identified IS elements, IS*1478* and IS*1479*. Their insertion specificity and distribution in related bacteria are presented.

MATERIALS AND METHODS

Bacterial strains, plasmids, oligonucleotides, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Oligonucleotides were obtained from the Regional Instruments Center at National Chung Hsing University and Gibco BRL. Bacteria were grown in Luria broth (LB) (19) at 37°C (for *Escherichia coli*) or 28°C (for other bacteria). To select for sucrose-sensitive mutants, a modified TYG medium (4) was used, in which 5% glucose was substituted for 5% sucrose. Kanamycin or ampicillin at 50 μg per ml was added to the medium to avoid segregation of the plasmids.

Triparental mating. Plasmid pUCD800 was transferred into *X. campestris* pv. campestris 11 (Xc11) and 17 (Xc17) by conjugation with pUCD800-containing *E. coli* DH5α (donor), promoted by pRK2013-containing *E. coli* HB101 (helper). Mating was performed on solid medium as outlined by Simon (23) with slight modifications. Briefly, about 10^8 mid-log-phase cells each of the donor and helper cells were mixed with 10^9 mid-log-phase cells of Xc11 or Xc17 on a membrane disk on a nonselective LB plate. The plate was incubated at 28°C for 24 h, and bacterial cells on the disk were resuspended in LB and plated on medium containing ampicillin and kanamycin in order to select the transconjugants.

Genome and plasmid DNA extraction. The alkaline lysis method described by Sambrook et al. (19) was used for plasmid extraction. For extraction of the total cellular DNA, bacterial cells were gently lysed by sodium dodecyl sulfate (SDS)-proteinase K treatment followed by the phenol-chloroform extraction and ethanol precipitation methods described by Scordilis et al. (22). Since *Xanthomonas* cells secrete exopolysaccharides which will coprecipitate with the DNA, *Xanthomonas* cells were washed with buffer consisting of 10 mM Tris-HCl, pH 7.6, and 1 M NaCl prior to DNA extraction.

DNA manipulation and Southern hybridization. Restriction enzyme digestions were performed according to the instructions provided by the suppliers. Cloning, PCR, plasmid transformation, and random primer labeling were performed by the methods described by Sambrook et al. (19). Southern hybridization was performed with Hybond N membranes (Amersham), using the protocols provided by the manufacturer. After hybridization with the labeled probe, the nylon membrane was washed under high-stringency conditions, namely, two 30-min washes with 2× SSC–0.1% SDS (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature, followed by one 30-min wash with 0.5× SSC–0.1% SDS at room temperature and one 30-min wash with 0.1× SSC–0.1% SDS at 50°C.

DNA sequencing. Double-strand DNA sequencing was performed on both strands of the plasmid DNA template by the dideoxy-chain termination method (20) using Sequenase version 2.0 DNA sequencing kit and ^{35}S-dATP (Amersham). When a long sequence reading was preferred, the DNA fragment was cloned into plasmid pUC18 before sequencing. For resolving G-C compressions, dITP was used according to the instructions provided by Amersham.

Nucleotide sequence accession number. The nucleotide sequences of IS*476*B, IS*1478*A, IS*1479*A, and IS*1479*B have been deposited in GenBank under accession nos. U62552, U59749, U56973, and U56974, respectively.

RESULTS AND DISCUSSION

Isolation and grouping of 32 independent plasmid insertion mutants. Plasmid pUCD800 was successfully transferred into two strains of *X. campestris* pv. campestris, Xc11 and Xc17, by conjugation promoted by triparental mating with pRK2013. Four independent subcultures of pUCD800-containing Xc17 and three independent subcultures of pUCD800-containing Xc11 were collected, and dilution plating was performed on agar plates containing 5% sucrose. For each subculture, one plate with about 40 survivors was used to screen for plasmid insertion mutants. Since plasmid DNA extracted from *Xanthomonas* cells is generally refractory to enzymatic digestion, plasmid DNA of each survivor was extracted, transformed into *E. coli* DH5α, and extracted again from the transformant for

TABLE 2. Characteristics of the 32 independent plasmid insertion mutants

Mutant	Isolated from:	Size (kb) of insertion	No. of restriction enzyme sites[a] in the insertion								Group
			B	D	EI	EV	H	K	P	S	
Xc11-12	Xc11	1.2	0	0	0	0	1	0	0	1	I
Xc11-8	Xc11	1.2	0	0	0	0	1	0	0	1	I
Xc11-6	Xc11	1.2	0	0	0	0	1	0	0	1	I
Xc11-10	Xc11	1.2	0	0	0	0	1	0	0	1	I
Xc11-14	Xc11	1.2	0	0	0	0	1	0	0	1	I
Xc11-17	Xc11	1.2	0	0	0	0	1	0	0	1	I
Xc17-2	Xc17	1.2	0	0	0	0	1	0	0	1	I
Xc17-48	Xc17	1.2	0	0	0	0	1	0	0	1	I
Xc17-6	Xc17	1.2	0	0	0	0	1	0	0	1	I
Xc17-47	Xc17	1.2	0	0	0	0	1	0	0	1	I
		1.4	0	1	0	1	0	0	0	0	
Xc17-49	Xc17	1.2	0	0	0	0	1	0	0	1	I
Xc17-51	Xc17	1.2	0	0	0	0	1	0	0	1	I
Xc17-3	Xc17	1.2	0	0	0	0	1	0	0	1	I
Xc11-1	Xc11	1.5	0	0	1	1	0	0	2	1	II
Xc11-2	Xc11	1.5	0	0	1	1	0	0	2	1	II
Xc11-13	Xc11	1.5	0	0	1	1	0	0	2	1	II
Xc17-13	Xc17	1.5	0	0	1	1	0	0	2	1	II
Xc17-15	Xc17	1.5	0	0	1	1	0	0	2	1	II
Xc17-25	Xc17	1.5	0	0	1	1	0	0	2	1	II
Xc17-14	Xc17	1.5	0	0	1	1	0	0	2	1	II
Xc17-7	Xc17	1.5	0	0	1	1	0	0	2	1	II
Xc11-25	Xc11	1.2	0	0	0	0	0	0	2	0	III
Xc17-27	Xc17	1.2	0	0	0	0	0	0	2	0	III
Xc17-5	Xc17	1.2	0	0	0	0	0	0	2	0	III
Xc17-4	Xc17	1.2	0	0	0	0	0	0	2	0	III
Xc11-7	Xc11	1.3	0	0	0	0	1	0	1	0	IV
Xc17-35	Xc17	1.3	0	0	0	0	1	0	1	0	IV
Xc11-5	Xc11	0.8	0	0	0	0	1	0	1	0	
Xc17-17	Xc17	0.8	0	0	0	0	0	0	1	0	
Xc11-15	Xc11	1.2	0	0	0	1	0	0	0	0	
Xc11-11	Xc11	2.1	0	1	1	0	1	0	0	1	
Xc17-1	Xc17	7.0	1	0	1	2	0	0	5	2	

[a] Restriction enzyme sites abbreviations: B, *Bam*HI; D, *Dra*I; EI, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sma*I.

restriction mapping with *Bam*HI and *Pst*I. The restriction mapping results showed that about one-third of the survivors were plasmid insertion mutants, all of which had insertions in the 2.6-kb *Bam*HI-*Pst*I *sacRB* fragment. One mutant (Xc17-47) had an additional 1.4-kb insertion in the plasmid other than that in the 2.6-kb *Bam*HI-*Pst*I *sacRB* region. These mutant plasmid DNAs were further mapped with *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, and *Sma*I, and their restriction patterns were compared. For each subculture, one insertion mutant of the same plasmid restriction pattern was collected. In this way, we collected a total of 32 independent plasmid insertion mutants. The restriction patterns of the 32 mutant plasmids were compared with that of pUCD800 (8, 24), and information about the insertion fragments, such as their locations within *sacRB*, their sizes, as well as restriction enzyme sites carried by the insertion fragments, was obtained and is shown in Table 2. Twenty-seven of the 32 mutants could be divided into four groups, group I to IV, with 13, 8, 4, and 2 members, respectively, on the basis of their restriction enzyme sites and size of insertion.

The 13 group I mutants and mutant Xc11-11 had insertions of IS*476* in their plasmids. Two mutants from each of the group I, II, and III mutants were randomly picked for complete sequence determination of the insertion fragments. The mutants selected included mutants Xc11-12 and Xc17-3 from the group I mutants, Xc11-2 and Xc11-13 from the group II mutants, and Xc17-4 and Xc17-5 from the group III mutants (Table 2). Based on the location of the insertion in the 2.6-kb *Bam*HI-*Pst*I *sacRB* DNA fragment and the nucleotide sequence of the *sacRB* fragment (24), for each insertion fragment to be sequenced, one pair of 16-mer oligonucleotides identical to the *sacRB* sequence close to the insertion site was synthesized and used as primers to sequence from the neighboring *sacRB* region into the insertion fragment. Oligonucleotide primers identical to the end sequences of the readings were again synthesized, and sequencings were performed to read further inward into the insertion fragments, and so forth. The complete sequences of the six insertion fragments could thus be obtained.

The sequencing results indicated that both group I insertion fragments were 1,226 bp long and flanked by 4-bp direct repeats of the target sequences. The sequence of the insertion fragment in mutant Xc11-12 was identical to that of the IS*476* sequence (accession no. M28557; 12, 16), whereas the sequence in mutant Xc17-3 had differences from that of the IS*476* sequence at three positions, i.e., G466C, C467G, and C1051G substitutions. We named the former IS*476*A, and we named the latter IS*476*B. IS*476*A (IS*476*) was originally isolated in *X. campestris* pv. vesicatoria and was characterized as an IS*407* group member of the widespread IS*3* family (14, 16). IS*476*B, carrying the three base substitutions and thus changes of A118R and H312D in its presumptive transposase sequence, belongs to the IS*407* group as well.

A 1.2-kb *Bsp*MI fragment comprising 97% of IS*476*A sequence was excised from the Xc17-3 mutant plasmid and used as a probe for Southern hybridization with the 32 mutant

Group I

Xc11-12 gatcaaaaatgacgattgacggcattacgtctaacgATAT$_{1524}$⟶ATATttacatgcttggttatgtttctaattct
Xc11-8 aaaatatctcttctgcaaaaggcctggacgtttgggACAG $_{723}$⟶ACAGctggccattacaaaacgctgacggcact
Xc11-6 atttaacaaagcatactatggcaaaagcacatcattCTTC$_{1273}$⟶CTTCcgtcaagaaagtcaaaaacttctgcaaa
Xc11-10 gttaaaaatggatcttgatcctaacgatgtaaccttTACT$_{1633}$⟵TACTtactcacacttcgctgtacctcaagcga
Xc11-14 tagtatttgaagcaaacactggaactgaagatggctACCA$_{1218}$⟶ACCAaggcgaagaatctttatttaacaaagca
Xc11-17 aaaaacgtatcaaaatgtacagcagttcatcgatgaAGGC$_{1108}$⟵AGGCaactacagctcaggcgacaaccatacgc
Xc17-2 agcaacagtattaacctttactaccgcactgctggcAGGA $_{529}$⟵AGGAggcgcaactcaagcgtttgcgaaagaaa
Xc17-48 ggcaaaagcacatcattcttccgtcaagaaagtcaaAAAC$_{1292}$⟵AAACttctgcaaagcgataaaaaacgcacggc
Xc17-6 ctcccgcggatcaaaaatgacgattgacggcattacGTCT$_{1516}$⟵GTCTaacgatatttacatgcttggttatgttt
Xc17-47 ttatgtttctaattctttaactggcccatacaagccGCTC$_{1576}$⟶GCTGaacaaaactggccttgtgttaaaaatgg
Xc17-49 cgctgacggcactgtcgcaaactatcacggctaccaCATC $_{778}$⟶CATCgtctttgcattagccggagatcctaaaa
Xc17-51 ttaactggcccatacaagccgctgaacaaaactggcCTTG$_{1592}$⟵CTTGtgttaaaaatggatcttgatcctaacga
Xc17-3 cggcgctctcggtatgattgagctaaacgatgattaCACA$_{1372}$⟵CACActgaaaaaagtgatgaaaccgctgattg
Xc11-11 attacacactgaaaaaagtgatgaaaccgctgattgcatc$_{1404}$⟶$_{512}$ccgcactgctggcaggaggcgcaactcaag

Group II

Xc11-1 ca**aa**cggcgctctcggtatg**a**ttgagctaaa**c**gaTGA**TTA**$_{1368}$⟵TGA**TTA**cacactgaaaaaagtgatgaaaccgc
Xc11-2 ca**aa**aatgacgattgacggc**a**ttacgtctaa**c**gaTAT**TTA**$_{1527}$⟶TAT**TTA**catgcttggttatgtttctaattctt
Xc11-13 ca**aa**aatgacgattgacggc**a**ttacgtctaa**c**gaTAT**TTA**$_{1527}$⟵TAT**TTA**catgcttggttatgtttctaattctt
Xc17-13 gg**aa**ctgaagatggctacca**a**ggcgaagaat**c**ttTAT**TTA**$_{1238}$⟵TAT**TTA**acaaagcatactatggcaaaagcaca
Xc17-15 ta**aa**aatggatcttgatcct**a**acgatgtaac**c**ttTAC**TTA**$_{1635}$⟵TAC**TTA**ctcacacttcgctgtacctcaagcga
Xc17-25 ta**aa**aatggatcttgatcct**a**acgatgtaac**c**ttTAC**TTA**$_{1635}$⟶TAC**TTA**ctcacacttcgctgtacctcaagcga
Xc17-14 ta**aa**aatggatcttgatcct**a**acgatgtaac**c**ttTAC**TTA**$_{1635}$⟶TAC**TTA**ctcacacttcgctgtacctcaagcga
Xc17-7 ga**aa**aatatcaagttcctga**a**ttcgattcgt**c**caCAA**TTA** $_{683}$⟵CAA**TTA**aaaatatctcttctgcaaaaggcctg

Group III

Xc11-25 aaacttctgca**a**agcgataa**aa**aa**c**gcacggctgagT**TA**G$_{1328}$⟵T**TA**Gcaaacggcgctctcggta**tg**attga**g**ct
Xc17-27 acacactgaaa**a**aagtgatg**aa**ac**c**gctgattgcatC**TA**A$_{1467}$⟵C**TA**Acacagtaacagatgaaat**tg**aacgc**g**cg
Xc17-5 acacactgaaa**a**aagtgatg**aa**ac**c**gctgattgcatC**TA**A$_{1467}$⟵C**TA**Acacagtaacagatgaaat**tg**aacgc**g**cg
Xc17-4 cttctattgac**a**gctggaaa**aa**cg**c**tggcggcgtctT**TA**A $_{894}$⟵T**TA**Aagacagcgacaaattcga**tg**caaat**g**at

FIG. 1. Insertion junction sequences in the group I, II, III, and Xc11-11 mutant plasmids. Only about 30 bp of the *sacRB* sequences flanking the insertions is listed. For the group I and the Xc11-11 mutant plasmids, arrows indicate insertions of IS*476* variants and their orientations. For the group II and group III mutant plasmids, arrows indicate insertions of IS*1478* and IS*1479* variants separately and their orientations. Numbers to the left of the arrows indicate the *sacRB* coordinates of the bases 5′ to the insertions. For the Xc11-11 mutant plasmid, the number to the right of the arrow indicates the *sacRB* coordinate of the base 3′ to the insertion. Duplicated target sequences are indicated by capital letters. Bases in the flanking *sacRB* sequences that are conserved within the same group of mutant plasmids are in bold type.

plasmids. The result showed that the 13 group I and Xc11-11 mutant plasmids hybridized with the probe (data not shown). Note that the Xc11-11 insertion fragment was 0.9 kb longer than the 13 group I insertion fragments and carried one additional *Eco*RI site and one additional *Dra*I site (Table 2). Two 15-mer primers identical to the IS*476*A sequence from bp 102 to 88 and from bp 1096 to 1110 were synthesized, and the insertion junction sequences in the 13 group I and the Xc11-11 mutant plasmids were determined by sequencing outward into *sacRB* with the two primers. The results showed that all 13 group I insertion fragments had about 100 bp of the IS*476*A(B) (either IS*476*A or IS*476B*) terminal sequences and were flanked by duplications of 4-bp target sequences. The Xc11-11 insertion fragment had about 100 bp of the terminal IS*476*A(B) sequences but was not flanked by a 4-bp target site duplication (Fig. 1). We concluded that the 13 group I mutants and mutant Xc11-11 all had insertions of IS*476* variants in their plasmids. The facts that *X. campestris* pv. campestris has at least two IS*476* variants and the sequence of one is identical to that of an IS*476* copy derived from *X. campestris* pv. vesicatoria suggest that horizontal transfer occurred between the two bacteria in recent years.

The 13 group I mutants carried 4-bp target site duplications at the insertion junctions without sequence preference, whereas mutant Xc11-11 carried a duplication of 952 bp at the insertion junction. The insertion junction sequences in the 13 group I mutants were aligned. As shown in Fig. 1, there was no sequence conservation or preference in either the 4-bp target sequences or the *sacRB* sequences upstream and downstream from the insertion sites. The 13 insertions were randomly distributed in *sacRB* in both orientations at about equal frequencies. In addition to IS*476*, seven IS*407* group members of the IS*3* family were reported, i.e., IS*407* in *Pseudomonas cepacia*, IS*511* in *Caulobacter crescentus*, IS*1222* in *Enterobacter agglomerans*, IS*1400* in *Yersinia enterocolitica*, IS*D1* in *Desulfovibrio vulgaris*, IS*Rm6* in *Rhizobium meliloti*, and IS*R1* in *Rhizobium lupini* (6, 14). Their 4-bp target duplications were GGAT (for IS*407*) (27), GCGG (for IS*511*) (15), TTTC (for IS*1400*) (3), AAGG and AATT (for IS*D1*) (6), CCCA (for IS*Rm6*) (29), and TGCC (for IS*R1*) (17). (Target duplication for IS*1222* was not detected.) It seemed that these six IS*407* group members had at least two successive identical bases within the 4-bp target sequences. IS*476* is distinct from them in that it did not have this or any type of specificity in its 4-bp target sequence.

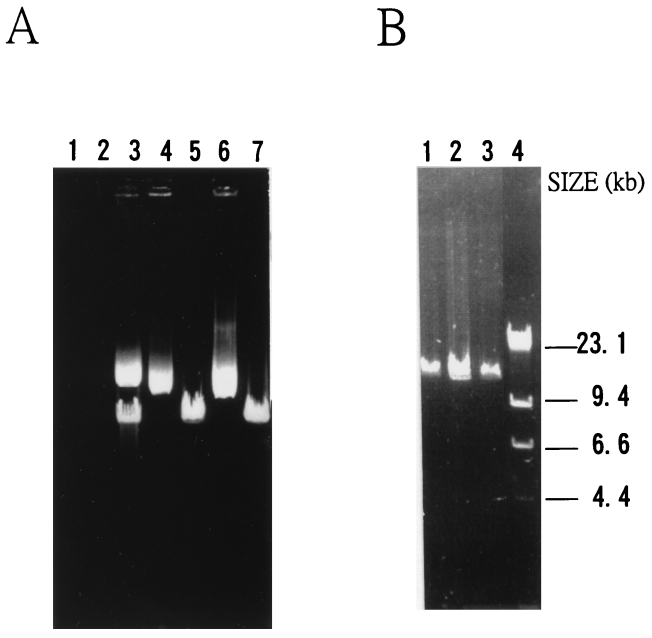

FIG. 2. (A) Agarose gel electrophoresis of pUCD800 DNAs isolated from Xc11, Xc17, *E. coli* DH5α, *E. coli* RR1, and *E. coli* HB101 (lanes 3 to 7). Endogenous plasmid preparations of Xc11 (lane 1) and Xc17 (lane 2) were electrophoresed simultaneously. (B) Agarose gel electrophoresis of *Eco*RI-digested pUCD800 DNAs isolated from *E. coli* DH5α, *E. coli* RR1, and *E. coli* HB101 (lanes 1 to 3). The *Hin*dIII digests of λ DNA (lane 4) were used as size markers, and the sizes of the resulting fragments are indicated to the right of the gel.

That the IS*476* insertion detected in *X. campestris* pv. vesicatoria had the target duplication of GATG further supports our observation (11).

The Xc11-11 mutant plasmid carrying an insertion of IS*476* without obvious target sequence duplication was further examined through detailed restriction mapping and sequencing with primers identical to several regions in *sacRB*. The result indicated that mutant Xc11-11 actually had a duplication of 952 bp (from *sacBR* coordinates 513 to 1464) at the insertion site. The 952-bp region contains one *Dra*I site and one *Eco*RI site, resulting in an extra *Dra*I site and an extra *Eco*RI site in the Xc11-11 insertion fragment compared with the mapping results of the group I mutant plasmids (Table 2 and Fig. 1). To further study this, plasmid pUCD800 DNAs were extracted from Xc11 and Xc17 and *E. coli* DH5α, HB101 (both *recA*), and RR1 (*recA*$^+$) backgrounds and analyzed by agarose gel electrophoresis. As shown in Fig. 2A, plasmid preparations from Xc17 and *E. coli* RR1 contained only the large species, while preparations from *E. coli* DH5α and HB101 contained only the small species. Interestingly, both species were present in about equal amounts in the Xc11 preparation. The DNA preparations from the three *E. coli* backgrounds were digested with *Eco*RI, which cut pUCD800 once and analyzed by agarose gel electrophoresis. As shown in Fig. 2B, all three preparations demonstrated DNA fragments of the size of pUCD800 (14.5 kb), indicating that the small species was the monomer and the large species was the dimer (or multimer). A two-step mechanism for generation of Xc11-11 mutant plasmid is thus proposed. Insertion of IS*476* occurred first in the pUCD800 dimer molecule in Xc11 at coordinate 1464 in one *sacRB* copy, and an IS-mediated adjacent deletion occurred later from one end of IS*476* to coordinate 512 in the other *sacRB* copy.

The eight group II mutants had insertions of IS*1478*, which itself constitutes a new subgroup of the IS*5* family. The two insertion fragments in the two randomly picked group II mutants, Xc11-2 and Xc11-13, displayed identical 1,506-bp nucleotide sequence. Both carry perfect inverted repeats of 20 bp at their ends and were flanked by 6-bp direct target repeats. A Blast search in the EMBL/GenBank/DDBJ database did not reveal significant nucleotide sequence similarity with any known IS element. This newly identified insertion sequence was named IS*1478*A, as we recently found a chromosomal copy carrying at least one base substitution in the sequence, which was named IS*1478*B (unpublished data).

IS*1478*A contains a long ORF, from ATG at bp 118 to TGA at bp 1485, presumably encoding a transposase protein of 455 amino acids. A σ^{70}-like promoter (bp 61 to 89) and a ribosome binding site (RBS) (bp 104 to 108) with an IR (bp 62 to 107) inbetween were found upstream of the ORF. Only in the case of transcription from upstream into the IS could the mRNA form a stable stem-loop structure ($\Delta G = -29.2$ kcal/mol [25]). Located within the stem structure, the RBS could then be obscured, perturbing translation of the transposase. This would be a means of preventing transposition of an IS from external activation, as postulated for IS*10* and IS*150* (13, 21). Figure 3A shows the first 120-bp IS*1478*A sequence with these sequence elements indicated.

The predicted IS*1478*A transposase sequence, which is shown in Fig. 4A, carries the DDE motif characteristic of the IS*5* family, such as conservation of D(1)GY in the region containing the second conserved acidic residue, conservation

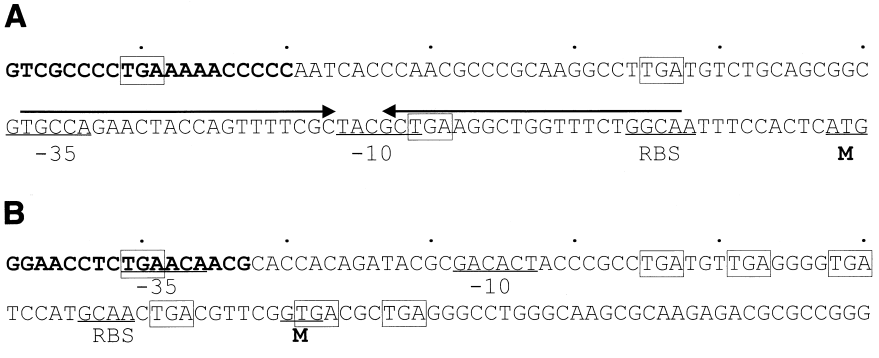

FIG. 3. The first 120-bp nucleotide sequences of IS*1478*A (A) and IS*1479*A (B). The left terminal IR sequences are in bold type. Predicted RBS, the −35 and −10 regions of σ^{70}-like promoters, and the methionine (M) start codons for translation into the cognate transposase proteins are indicated. Imperfect IRs are indicated by arrows over the sequence, and TGA stop codons are boxed.

A

```
IS1478A MHTRRPAAEHMPAEELFRSRLENQIDLRHPLAQLSQRMPWTALEQALSSR  50
IS5           MSHQLTFADSEFSSKRRQTRKEIFLSRMEQILPWQNMVEVIEPF  44
IS1479A             MTLRAWASARDAPGDLPGRDGAGRPVA-AIARAGRAA  36

IS1478A LPATQAGGGRPALPVRLIAGLLYLKHAYDLSDEAVCERWLENPYWQFFTG 100
IS5     YPK--AGNGRRPYPLETMLRIHCMQHWYNLSDGAMEDALYEIASMRLF-A  91
IS1479A LSRV-GRPGRQPYALATMLRIHLLQQWYALSDPAMEEALHEIPTLRRF-A  84

IS1478A EVVFQTRLPCDASSLTRWRQRLDEAGMEELLAHTINAAHAMQAVDARELS 150
IS5     RLSLDSALP-DRTTIMNFRHLLEQHQLARQLFKTINRWLAEAGVMMTQGT 140
IS1479A QLGGLDNVP-DETTILNFRRLLETHGLAARCWKRSSAS-GAQGPEPAVGH 132

IS1478A RVIVDTTVQEKAIAYPTDSRLLEVARKKLVLLAKRYGIGLRQSYARQGPA 200
IS5     --LVDATIIEAP---------------------------------------- 150
IS1479A DRRCDADRGA---------------------------------------- 142
         TiVDATi(consensus for the IS5 subgroup)
          l    l

IS1478A LSRKAGRYAHARQFKRMQRVLRRQRTVLGRVLRDIARKLDQVEPGVRERI 250
IS5     ---------------------------------------------------- 150
IS1479A ---------------------------------------------------- 142

IS1478A AVWLERAQRLYTQRPKDKQKLYALHAPEVECIGKGKARQAYEFGVKVGIA 300
IS5     -----------------SSTKNKEQQRDPEMHQTKKGNQWH-FGMKAHIG 182
IS1479A -----------------SSTKNADHARDPEMHQTKKGNQWY-FGMKAHIG 174

                                                  + +
IS1478A VTACKGLVVGARSFPGNPYDGDTLAEQLEQTRGLLQDLSVEPTVAIVDLG 350
IS5     VDAKSGLTHSLVTTAANEHDLNQLGNLL----------HGEEQFVSADAG 222
IS1479A VDEFSGLVHHVHCTAANVADVTVTHTLL----------HGKEDSVFGDSG 214
                                                v-aDaG

        +                                               +
IS1478A YRG---REVDG--------------VQVL--HRGKAKTLTRRQWRWIKRR 381
IS5     YQGAPQREELAEVDVDWLIAERPGKVRTLKQHPRKNKTAINIEYMKASIR 272
IS1479A YTGADKREELQDCEAAFFIAAKRSVLQAIGNKRERAREQRWEHF-KASVR 263
        Y-tG  (consensus for the IS5 subgroup)  k----iE--KA-lR
                                                r           v

          +     +
IS1478A QAVEPVIGHLKDDCRLRRCRLKGAQGDALHVLGCAAGYNLRWLLRWIAFL 431
IS5     ARVEHPFRIIKRQFGFVKARYKGLLKNDNQLAMLFTLANLFRADQMIRQW 322
IS1479A AKVEHPFRVIKRQFGYTKVRYRGLAKNTAQVLTLFALSNLWMKRKQLLPA 313

IS1478A RAWMRAMGWPSFSAVPLSPMTLGA 455
IS5     ERSH                     326
IS1479A MGSVRL                   319
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B

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IRL:
IS1478A GTCGCCCCTGAAAAACCCCC
IS5       GGAAGGTGCGAACAAG
IS1479A   GGAACCTCTGAACAACG

IRR:
IS1478A GTCGCCCCTGAAAAACCCCC
IS5       GGAAGGTGCGAATAAG
IS1479A   GGATGGTCTGATCAACG
```

FIG. 4. (A) Alignment of the transposase amino acid sequences of IS*1478*A, IS*5*, and IS*1479*A. Amino acids are indicated in the single-letter code. Shaded amino acids are identical or homologous amino acids in two of the three sequences. Shaded and boxed amino acids are conserved in the three sequences. Amino acids conserved in the D(1)GY and R(3)E(6)K signatures for the IS*5* family members (18) are indicated by plus signs above the sequence. Consensus sequence motifs for the IS*5* subgroup (14) are indicated under the three sequences, in which capital letters indicate conservation and lowercase letters indicate predominance of that particular amino acid. The three conserved acidic residues of the DDE motif are shown as large bold letters. Note that the third conserved acidic residue, E, in IS*5* (and IS*1479*A) is shown at different positions according to the two reports (14, 18). Homologous amino acids are grouped as follows: I, L, V, and M; F, Y, and W; H, K, and R; E and D; N and Q; G and A; S and T; C; and P (18). Gaps introduced in the sequences to maximize the alignment are indicated by the dashes. (B) Alignment of the left and right IR (IRL and IRR, respectively) sequences of IS*1478*A, IS*5*, and IS*1479*A. Nucleotides identical in two of the three elements are shaded. Nucleotides conserved in the three elements are shaded and boxed. Gaps introduced in the sequences to maximize the alignment are indicated by the dashes.

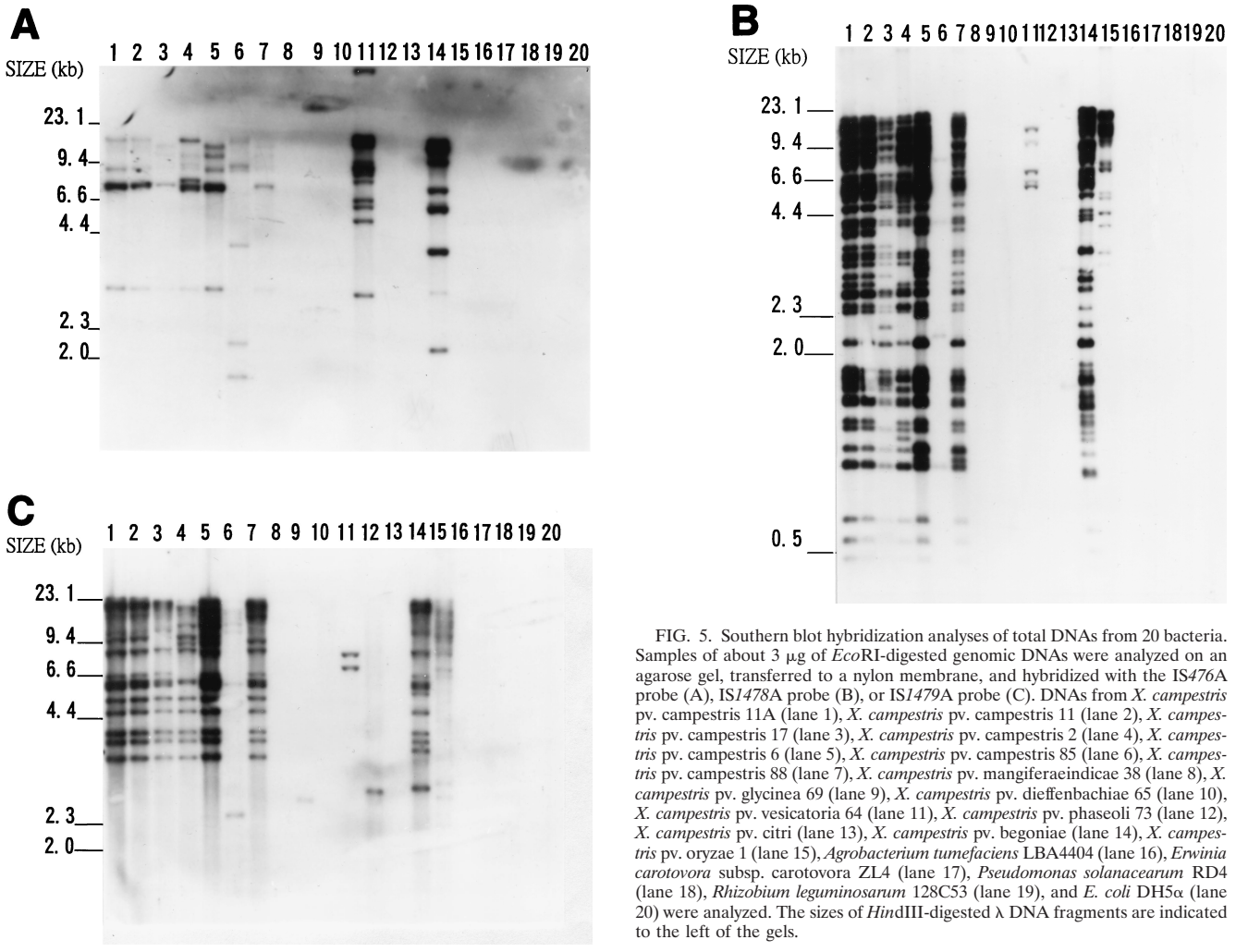

FIG. 5. Southern blot hybridization analyses of total DNAs from 20 bacteria. Samples of about 3 μg of *Eco*RI-digested genomic DNAs were analyzed on an agarose gel, transferred to a nylon membrane, and hybridized with the IS*476*A probe (A), IS*1478*A probe (B), or IS*1479*A probe (C). DNAs from *X. campestris* pv. campestris 11A (lane 1), *X. campestris* pv. campestris 11 (lane 2), *X. campestris* pv. campestris 17 (lane 3), *X. campestris* pv. campestris 2 (lane 4), *X. campestris* pv. campestris 6 (lane 5), *X. campestris* pv. campestris 85 (lane 6), *X. campestris* pv. campestris 88 (lane 7), *X. campestris* pv. mangiferaeindicae 38 (lane 8), *X. campestris* pv. glycinea 69 (lane 9), *X. campestris* pv. dieffenbachiae 65 (lane 10), *X. campestris* pv. vesicatoria 64 (lane 11), *X. campestris* pv. phaseoli 73 (lane 12), *X. campestris* pv. citri (lane 13), *X. campestris* pv. begoniae (lane 14), *X. campestris* pv. oryzae 1 (lane 15), *Agrobacterium tumefaciens* LBA4404 (lane 16), *Erwinia carotovora* subsp. carotovora ZL4 (lane 17), *Pseudomonas solanacearum* RD4 (lane 18), *Rhizobium leguminosarum* 128C53 (lane 19), and *E. coli* DH5α (lane 20) were analyzed. The sizes of *Hin*dIII-digested λ DNA fragments are indicated to the left of the gels.

of R(3)E(6)K in the region containing the third acidic residue, and 36 residues between the second and third conserved acidic residues (14, 18). The 5′ terminal G nucleotide characteristic of the IS*5* family members is conserved in IS*1478*A (14). Therefore, IS*1478*A belongs to the IS*5* family. A BLAST search with nonredundant protein database revealed only low level of resemblance to the IS*5* transposase. IS*1478*A is distinct from all other IS*5* family members in its terminal 3-bp sequence (5′-GTC-3′), size of direct target repeats (6 bp), and spacing between the first two conserved acidic residues in its transposase DDE motif (192 residues) and cannot be grouped with any of the five existing subgroups (14). A new IS*1478* subgroup of the IS*5* family is thus proposed. In fact, IS*1478*A is distinct from all reported DDE-type IS elements in that the spacing between the first two conserved acidic residues (192 residues) in the transposase DDE motif is about triple the sizes of the others (51 to 85 residues) (14).

Transposase sequences and the terminal IR sequences from IS*5* and IS*1478*A were aligned, and the results are shown in Fig. 4A and B, respectively. Those sequences from IS*1479*A, which belongs to the IS*5* subgroup of the IS*5* family (see below), were aligned for comparison. The alignments clearly showed that limited but significant homologies exits among the transposase sequences and the terminal IR sequences of the three IS*5* family members.

Southern hybridization with the 32 mutant plasmids was performed with the PCR-amplified IS*1478*A DNA fragment as a probe. The result indicated that only the eight group II mutant plasmids hybridized to the probe (data not shown). Two 15-mer primers identical to the IS*1478*A sequence from bp 82 to 68 and from bp 1420 to 1434 were synthesized, and insertion junction sequences in the eight group II mutant plasmids were determined by sequencing outward into *sacRB* with the two primers. As shown in Fig. 1, all eight group II insertion fragments had about 80 bp of the IS*1478*A terminal sequences and were flanked by duplications of 6-bp target sequences, indicating that all of the eight group II mutants had insertions of IS*1478*A or its variants in their plasmids.

The four group III mutants had insertions of IS*1479*, which belongs to the IS*5* subgroup of the IS*5* family. Nucleotide sequencing with the two randomly picked group III mutants, Xc17-4 and Xc17-5, indicated that the two insertion fragments were 1,154 bp long and flanked by 4-bp directed target repeats (Fig. 1). The two sequences differ at six positions (G610, G643, C661, A677, G679, and C943 for the Xc17-4 insertion fragment; A610, A643, T661, G677, A679, and G943 for the Xc17-5 insertion fragment), but have the same 17-bp imperfect terminal IRs. Both sequences carry an ORF starting with GTG at bp 80 and ending with TAA at bp 1039, presumably encoding a transposase protein of 319 amino acids. A putative RBS

and a σ^{70}-like promoter were found upstream of the ORF. Several TGA stop codons were found in the 5′ noncoding regions of the two sequences, which might be a means of premature terminating the readthrough transcript from the external promoter due to tight coupling of transcription and translation in prokaryotic organisms (13). Figure 3B shows the first 120-bp sequence with these sequence elements indicated. A Blast search of the nucleotide sequences in the EMBL/GenBank/DDBJ database indicated that both sequences have 73% sequence homology with IS*1051* in *X. campestris* pv. dieffenbachiae (accession no. X70380), 62% homology with IS*52* in *Pseudomonas syringae* pv. glycinea (accession no. M14366), and 60% homology with IS*5* in *E. coli* (accession no. J01735). These two 1,154-bp insertion fragments were named IS*1479*A and IS*1479*B. IS*1479*A and IS*1479*B differ in one amino acid residue (T200 for IS*1479*A and A200 for IS*1479*B) in their presumptive transposase sequences, and like IS*1051*, IS*52*, and IS*5*, carry the DDE motif of the IS*5* subgroup of the IS*5* family (14), which is shown in Fig. 4A. IS*1479*A(B), therefore, belongs to the IS*5* subgroup of the IS*5* family. The presumptive transposase sequences and the terminal IR sequences of IS*1478*A, IS*1479*A, and IS*5* were aligned, and the results are shown in Fig. 4A and B, respectively.

Southern hybridization with the 32 mutant plasmids was performed with PCR-amplified IS*1479*A DNA fragment as a probe. The result indicated that only the four group III mutant plasmids hybridized to the probe (data not shown). Two 15-mer primers identical to the IS*1479*A sequence from bp 76 to 62 and bp 1081 to 1095 were synthesized, and insertion junction sequences in the other two group II mutant plasmids were determined by sequencing outward into *sacRB* with the two primers. As shown in Fig. 1, insertion fragments in these two group III mutant plasmids had about 70 bp of the IS*1479*A(B) terminal sequences and were flanked by duplications of 4-bp target sequences. Thus, the four group III mutants all had insertions of IS*1479* variants in their plasmids.

Insertions of IS*1478* and IS*1479* had preferred orientations and target site specificities. The junction sequences of the eight IS*1478* insertions and four IS*1479* insertions, shown in Fig. 1, were examined in more detail. It was found that the eight IS*1478* insertions demonstrated conservation of PyPuNTTA (Py is a pyrimidine, Pu is a purine, and N is any nucleotide) in their 6-bp target sequences. Insertions of IS*1478* showed a strong preference in one orientation and were not random, as three insertions occurred at *sacRB* coordinate 1695 and two occurred at coordinate 1587. When ca. 30-bp *sacRB* sequences up- and downstream from the five IS*1478* insertion sites were aligned, a consensus sequence $AAN_{16}AN_{10}C$ 8 bp upstream from the insertion sites was found. Similar phenomena were observed for the four IS*1479* insertions. These included conservation of sequence PyTAPu for the 4-bp target duplications, two insertions detected at *sacBR* coordinate 1467, and consensus sequences AN_8AAN_2C 15 bp upstream and TGN_5G 22 bp downstream from the insertion sites. It is likely that an A 20 bp upstream or a sequence $AN_{(10\ or\ 12)}C$ upstream from the insertion site is important for selection of target sites by these two IS*5* family members. Recently, Hu and Derbyshire (9) examined 63 insertion sites of an IS*5* family member, IS*903*, and found preferences for insertions at four regions in a 55-kb plasmid, and several sites occurred more than once. When one preferred region was cloned in a plasmid and the insertion sites were examined, they observed strong preference of insertions of IS*903* in one orientation and conservation of 5-bp sequences on both sides of the target sequences. Although we examined only eight IS*1478* and four IS*1479* insertion sites, our results are fairly consistent with theirs.

Presence of IS*476*, IS*1478*, and IS*1479* in strains of *X. campestris* pv. campestris and in related bacteria. It was suspected that IS*476*, IS*1478*, and IS*1479* might be widespread in nature. Twenty bacteria were chosen for examination of the presence of the three elements. These bacteria included *Agrobacterium tumefaciens*, *Erwinia carotovora* subsp. *carotovora*, *E. coli*, *Pseudomonas solanacearum*, *Rhizobium leguminosarum*, *X. campestris* pv. begoniae, *X. campestris* pv. citri, *X. campestris* pv. dieffenbachiae, *X. campestris* pv. glycinea, *X. campestris* pv. mangiferaeindicae, *X. campestris* pv. oryzae, *X. campestris* pv. phaseoli, *X. campestris* pv. vesicatoria, and six strains of *X. campestris* pv. campestris, including Xc11 and Xc17. A spontaneous avirulent mutant of Xc11 (Xc11A) was also included. Southern blot hybridization of *Eco*RI-digested genomic DNAs of these bacteria was performed with either the 1.2-kb *Bsp*MI IS*476*A DNA fragment or the PCR-amplified IS*1478*A or IS*1479*A DNA fragment as a probe. Figure 5 shows the hybridization results, which indicate that all seven strains of *X. campestris* pv. campestris, *X. campestris* pv. vesicatoria, and *X. campestris* pv. begoniae had multiple copies of the three elements in their genomes. In addition, *X. campestris* pv. citri, *X. campestris* pv. glycinea, *X. campestris* pv. oryzae, and *X. campestris* pv. phaseoli had multiple copies of either IS*1478*, IS*1479*, or both in their genomes. Of the 20 bacteria tested, only *X. campestris* pv. mangiferaeindicae, *X. campestris* pv. dieffenbachiae, and the five non-*X. campestris* bacteria did not contain any of the three elements. Thus, the three insertion elements are widespread only in *X. campestris* pathovars. It is interesting that six of the seven *X. campestris* pv. campestris strains tested and *X. campestris* pv. begoniae carried more than 20 copies of IS*1478* in their genomes (Fig. 5B).

There are other six plasmid insertion mutants, including the two group IV mutants, that did not show homology with either IS*476*, IS*1478*, or IS*1479*. Insertions in these mutants ranged from 0.8 to 7.1 kb in size (Table 2). They accounted for 19% of the total insertion mutants isolated and were not analyzed in this study.

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