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Functional Characterization of IS*1999*, an IS*4* Family Element Involved in Mobilization and Expression of β-Lactam Resistance Genes

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IS*1999* and a point mutant derivative, IS*1999.2*, have been described inserted upstream of emerging antibiotic resistance genes bla_{VEB-1} and bla_{OXA-48}. 5′ Rapid amplification of cDNA ends experiments revealed that expression of these β-lactamase genes was driven by the outward-directed promoter, P_{out}, located in the IS*1999* elements. These findings led us to study IS*1999*-mediated gene mobilization. Thus, the transposition properties of IS*1999* and of IS*1999*-based composite transposons, made of two copies of IS*1999* in different orientations, were investigated. IS*1999* or IS*1999*-based composite transposons were capable of transposing onto the conjugative plasmid pOX38-Gen. Sequence analysis of the insertion sites revealed that IS*1999* inserted preferentially into DNA targets containing the consensus sequence NGCNNNGCN. Transposition was more efficient when at least one left inverted repeat end was located at an outside end of the transposon. The transposition frequency of IS*1999.2* was 10-fold lower than that of IS*1999*, and transposition frequencies of the putative natural transposon, Tn*1999*, were below detection limits of our transposition assay. This reduced transposition frequency of IS*1999.2*-based elements may result from a lower transcription of the transposase gene, as revealed by reverse transcription-PCR analyses.

Insertion sequences (ISs) are present in most bacterial genomes analyzed and may represent up to 2 to 5% of genomic DNA. They play an important role in assembling sets of "accessory" functions in bacteria and in dissemination of resistance genes (6, 15, 19, 22). Many IS elements can transmit these gene arrays in the form of composite transposons, where two flanking IS elements cooperate in mobilizing an intervening DNA segment.

ISs of the IS*4* family are delimited by short imperfect inverted repeat sequences (IR). These ISs encode a transposase that is required for transposition and are capable of inserting into a target molecule, leading to the duplication of several base pairs (directly repeated sequences) at the site of insertion (6).

IS*1999* was initially identified in clinical *Pseudomonas aeruginosa* isolates from Thailand (8, 17, 32). In these strains, IS*1999* was inserted into the integron-specific recombination site, *attI1*, upstream of the integron-borne bla_{VEB-1} gene that encodes an extended-spectrum β-lactamase (17, 23, 25). IS*1999* encodes a putative transposase of 402 amino acids (17) that shares 71% amino acid identity with IS*10*, the best-characterized IS*4* family member (6). IS*1999* is 1,328 bp long, has 21-bp imperfect terminal repeats, and generates a 9-bp target site duplication after transposition (17). An outward-directed promoter, P_{out}, located close to the left inverted repeat (IRL) of IS*1999* was characterized (1). In previous work, P_{out} together with the promoter P_{ant} of class 1 integrons was shown to increase the expression of the bla_{VEB-1} gene in *P. aeruginosa* (1).

Recently, IS*1999.2*, an isoform of IS*1999*, has been detected in *Klebsiella pneumoniae* (21). This IS was located upstream of another unrelated antibiotic resistance gene, bla_{OXA-48}, that encodes a broad-spectrum β-lactamase of a different type (21). The present work describes the analysis of the genetic environment of bla_{OXA-48} that revealed the presence of two identical copies of the IS*1999* isoform inserted on either side of the resistance gene. The frequent association of IS*1999* with resistance genes of clinical interest led us to study IS*1999*-mediated antibiotic resistance gene mobilization.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Clinical strains *P. aeruginosa* 1 (bla_{VEB-1}), *K. pneumoniae* 11978 (bla_{OXA-48}), and *Escherichia coli* DH10B (pA-1) harboring the natural plasmid of *K. pneumoniae* 11978 have been previously described (8, 21). *E. coli* DH10B (Life Technologies, Eragny, France) was used as the bacterial host in electroporation experiments. The recombination-deficient strain *E. coli* DH5α (pOX38-Gen) and the rifampin-resistant *E. coli* DH10B Rif^r were used for transposition and conjugation experiments (12). The low-copy-number cloning vector pBBR1MCS.3 was used for cloning experiments (13). Bacterial cells were grown in trypticase soy (TS) broth or on TS agar plates (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France) with antibiotic solutions when required.

Antimicrobial agents and susceptibility testing. Routine antibiograms were determined by the disk diffusion method on Mueller-Hinton agar (Sanofi Diagnostics Pasteur). The antimicrobial agents (and their sources) were the following: ticarcillin (GlaxoSmithKline, Marly-Le-Roi, France), gentamicin (Schering-Plough, Levallois-Perret, France), rifampin (Aventis, Paris, France), tetracycline (Sigma, Saint Quentin Fallavier, France), and kanamycin (Euromedex, Mundolsheim, France). The antibiotics, with abbreviations and concentrations used for selection, were the following: kanamycin (Kan; 30 μg/ml), ticarcillin (Tic; 50 μg/ml), tetracycline (Tet; 15 μg/ml), gentamicin (Gen; 7 μg/ml), and rifampin (Rif; 200 μg/ml).

Nucleic acid extraction. Recombinant plasmids and pOX38-Gen-derivative plasmids were extracted using Plasmid Mini-Midi kits and the Very Low Copy Plasmids purification protocol, respectively (QIAGEN, Courtaboeuf, France). Extractions of whole-cell DNAs were as described elsewhere (18). Total RNAs from *E. coli* DH10B (pA-1) were extracted with an RNeasy Maxi kit (QIAGEN) according to the recommendations of the manufacturer.

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TABLE 1. Sequences of primers used in this study[a]

Primer	Sequence (5′→3′)	Accession no.	Primer location (bp)	Reference or source
IS1999A	CAGCAATTCTTTCTCCGTG	AF133699	1205–1223	21
IS1999B1	CAAGCACAACATCAAGCGC	AF133699	2189–2171	21
IS1999B2INV	TCGTTTTAGGTGAAGTTCTGG	AF133699	3475–3495	This work
IS1999IRLext	TGCGCTTCCACCCTAATTTG	AF133699	3292–3611	This work
IS1999IRRext	ggatccccggaATTCGCCA	AF133699	1190–1183	This work
IS1999IRRext2	TGGGATTGGAGGTACTCAGGC	AF133699	1260–1240	This work
IS-1Kpn	TTAA**ggTACC**GCTAACTTTGTTTTAGGG	AF133699	3695–3668	This work
IS-2Eco	ctc**TgaatTC**ATAAATCAGCCATAGCATAGC	AF133699	1142–1164	This work
IS-3Eco	GTA**gaATTC**GCCAATCAGTTGCTC	AF133699	1195–1172	This work
IS-4Pst	TGT**cTGcaG**TTATGGAGCAGCAACGATG	AF133699	1094–1121	This work
IS-1Eco	TT**gAATTc**CCGCTAACTTTGTTTTAGGG	AF133699	3695–3668	This work
IS-1Sac	TTAAATTA**CCGCgg**ACTTTGTTTTAGGG	AF133699	3695–3668	This work
IS-4Eco	T**GaaTtc**TGTTATGGAGCAGCAACGATG	AF133699	1094–1121	This work
IS-stopXmn	TCAC**GAAAGGTTTC**CTCatcaTTGCATACGTTTGG	AF133699	1461–1494	This work
OXA-48A	TTGGTGGCATCGATTATCGG	AY236073	1280–1299	21
OXA-48B	GAGCACTTCTTTTGTGATGGC	AY236073	2023–2003	21
IS1999.2F	ATCCGCTTTTTTTACAGGCCGA	AY648695	4374–4395	This work
bla$_{OXA-48}$GSP1	AAAGCATGTAGCATCTTGCTCATAC	AY236073	1674–1650	This work
bla$_{OXA-48}$GSP2	ACAGGCACAACTGAATATTTCATC	AY236073	1614–1591	This work
bla$_{OXA-48}$GSP3	TCTGTCCATCCCACTTAAAGACTT	AY236073	1544–1521	This work
GapA1	ATCAACGGTTTTGGCCGTAT	X02662	502–521	Corvec
GapA2	GTTGATAACTTTAGCCAGCGG	X02662	972–952	Corvec
TnpA*1999*-GSP1	GCTTAGCCAAAACCAACCAT	AF133697	649–630	This work
TnpA*1999*-GSP2	TAGGGGATAGGACTTCTCAT	AF133697	496–477	This work
TnpA*1999*-GSP3	ATGCGCTTGATGTTGTGCTT	AF133697	289–269	This work

[a] Nucleotides that were not complementary to the sequence submitted to the GenBank database under the accession number AF133699 are shown by lowercase letters; the restriction sites introduced into the primer sequences are boldfaced; the two successive TGA stop codons that are located in the IS-stopXmn primer (inverse sequence) are underlined.

The presence of episomal plasmids in the host strains was checked using a Plasmid Mini kit (QIAGEN). DNA extracted from agarose gel slices and PCR products were purified using QIAquick columns (QIAGEN).

PCR experiments and hybridization. *Taq* DNA polymerase (Roche Diagnostics, Meylan, France) and *Pfu* DNA polymerase (Promega Corporation, Madison, Wis.) were used as recommended. Standard PCR amplification experiments (27) were performed with primers listed in Table 1.

Southern blot hybridizations (27) were performed using whole-cell DNAs of *P. aeruginosa* 1, *K. pneumoniae* 11978, *E. coli* DH10B (pA-1), and *E. coli* DH10B. Four micrograms of each DNA was digested with 10 U of the restriction enzyme EcoRV. Hybridizations were performed under high-stringency conditions using the ECL nonradioactive labeling and detection kit (Amersham Pharmacia Biotech, Orsay, France). PCR-generated probes consisted of a 985-bp fragment internal to IS*1999* using IS1999A and IS1999B1 as primers and whole-cell DNA of *P. aeruginosa* 1 as template as well as of a 744-bp fragment internal to *bla*$_{OXA-48}$ using OXA-48A and OXA-48B as primers and whole-cell DNA of *K. pneumoniae* 11978 as template (Table 1).

Cloning experiments and sequencing. T4 DNA ligase and restriction endonucleases were used according to the recommendations of the manufacturer (Amersham Biosciences). In order to study the transposition of IS*1999*, an omega fragment (ΩKm) from plasmid pHP45Ω-Km (24), made of a kanamycin resistance gene [*aph*(*3′*)-*IIa*] flanked by transcriptional and translational termination sequences, was introduced into IS*1999* (Fig. 1). A 1.3-kb fragment containing the left IR up to the end of the transposase gene was amplified with primers tailed with restriction sites for KpnI (IS-1Kpn) and EcoRI (IS-2Eco) and genomic DNA from *P. aeruginosa* 1 as template (Table 1). The primers IS-3Eco and IS-4Pst were used to amplify a 102-bp fragment containing the right IR (IRR) of IS*1999* (Table 1). The digested PCR products were mixed in a four-way ligation together with an EcoRI-restricted ΩKm fragment (2.2 kb) and the KpnI-PstI-restricted pBBR1MCS.3 vector in order to create the tagged insertion sequence IS*1999*.Kan, yielding plasmid pIS*1999*.Kan (Fig. 1).

In order to obtain a construct in which the transposase gene was in opposite orientation relative to P_{lac}, DNA fragments of 1.3 kb and of 102 bp were amplified by PCR with the pairs of primers IS-2Eco/IS-1Sac and IS-3Eco/IS-4Pst, respectively. These DNA fragments were subsequently restricted with the appropriate enzymes and mixed in a four-way ligation together with EcoRI-restricted ΩKm fragment and PstI-SacII-restricted pBBR1MCS.3 vector to create pIS*1999*as.Kan (Fig. 1).

A similar PCR-based cloning strategy was used for the following constructs (Fig. 1). Fragments of 1.3 kb were amplified with the pairs of primers IS-1Kpn/IS-4Eco and IS-4Eco/IS-1Sac, digested with the appropriate enzymes, and mixed in a four-way ligation with EcoRI-restricted ΩKm fragment and KpnI-SacII-restricted pBBR1MCS.3 vector to create pTn*LL*, carrying two copies of IS*1999* in inward and opposite orientations. Fragments of 1.3 kb were amplified with the pairs of primers IS-1Kpn/IS-4Eco and IS-1Eco/IS-4Pst, digested with the appropriate enzymes, and mixed in a four-way ligation with EcoRI-restricted ΩKm fragment and KpnI-PstI-restricted pBBR1MCS.3 vector to create pTn*LR*, carrying two copies of IS*1999* in the same orientation. Fragments of 1.3 kb were amplified with the pair of primers IS-1Eco/IS-4Pst, digested with the appropriate enzymes, and ligated with EcoRI-restricted ΩKm fragment and PstI-restricted pBBR1MCS.3 vector to create pTn*RR*, carrying two copies of IS*1999* in outward and opposite orientations.

The introduction of two stop codons in the IS*1999* transposase sequence was performed as follows. A 1.1-kb fragment was amplified with the primers IS-1Kpn and IS-stopXmn using the genomic DNA of *P. aeruginosa* 1 as template (Table 1). The purified PCR product was restricted using KpnI and XmnI prior to its introduction into the KpnI-XmnI-restricted plasmid pIS*1999*.Kan, yielding plasmid pIS*1999*stop.Kan (Fig. 1).

SacII-PstI-restricted whole-cell DNA of *E. coli* DH10B (pA-1) was cloned into a SacII-PstI-restricted pBBR1MCS.3 vector (Fig. 1), giving rise to plasmid pTn*1999*.

The IS*1999* isoform (IS*1999*.2) was cloned in the same environment as pIS*1999*.Kan as follows. A 1.3-kb fragment was amplified with the primers IS1999.2F and IS1999A, with genomic DNA of *E. coli* DH10B (pA-1) as template (Table 1), and then it was digested with BclI-XmnI (this fragment contained five nucleotide substitutions relative to the sequence of IS*1999*). The BclI-XmnI fragment was purified and introduced into the BclI-XmnI-restricted pIS*1999*.Kan plasmid, generating pIS*1999*.2.Kan (Fig. 1).

Ligation products were electroporated into *E. coli* DH10B, as previously described (18), and selection was performed on TS agar plates containing the appropriate antibiotics.

Sequencing of the insert of each construct was performed using laboratory-designed primers on an ABI PRISM 3100 automated sequencer (Applied Biosystems, Les Ullis, France).

Mating-out assay and transposition experiments. The transposition of single tagged ISs and composite transposons onto the conjugative plasmid pOX38-Gen

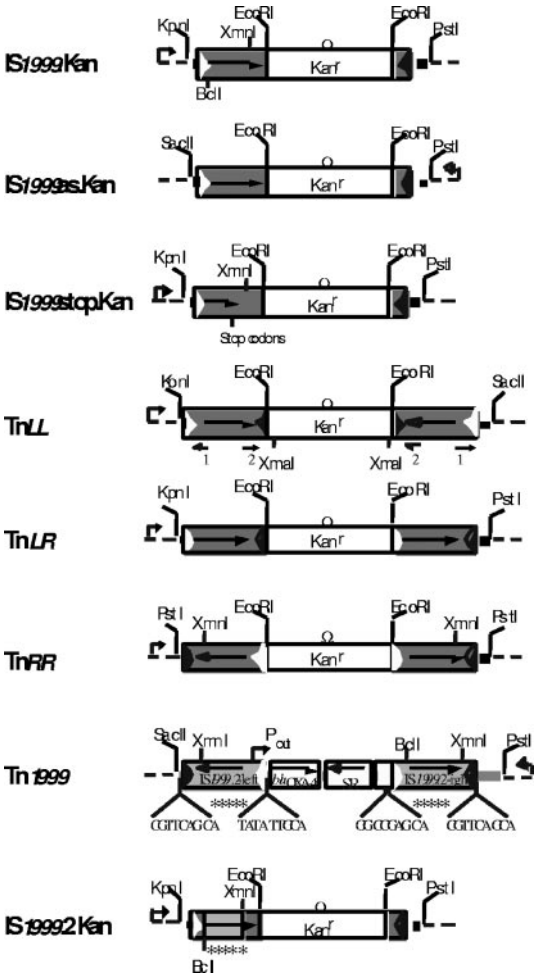

FIG. 1. Schematic map of the constructs used in this study. All constructs were cloned into the multiple cloning site of the pBBR1MCS.3 shuttle vector. Restriction sites that were used for cloning are indicated. The coding regions are shown as boxes, with an arrow indicating the orientation of their transcription. The broken arrows indicate the P_{lac} promoter. The IS*1999* left and right inverted repeats are shown by empty and filled triangles, respectively. IS*1999* and iso-IS*1999* elements are represented as dark gray boxes and light gray boxes, respectively. Ω Kan^r is an omega fragment conferring resistance to kanamycin. The environments of IS*1999* in *P. aeruginosa* 1 and of Tn*1999* on the plasmid pA-1 are shown by dark and gray lines, respectively. Dashed lines represent the cloning vector. The 9-bp sequences located on each side of IS*1999*.2-left and of IS*1999*.2-right are indicated on the Tn*1999* representation. The five nucleotide substitutions in the IS*1999*.2 sequence are symbolized with five stars. The position of the primers IS1999B2INV (1) and IS1999IRRext2 (2), used in inverse PCR experiments, are symbolized by small arrows on the Tn*LL* representation. The location of the P_{out} promoter responsible for the bla_{OXA-48} transcription is represented by a small broken arrow on the Tn*1999* representation.

was investigated by mating-out assay in liquid medium (18). The recombinant plasmids pIS*1999*.Kan, pIS*1999*as.Kan, pIS*1999*stop.Kan, pIS*1999*.2.Kan, pTn*LR*, pTn*LL*, pTn*RR*, and pTn*1999* (Fig. 1) were electroporated into *E. coli* DH5α (pOX38-Gen) for transposition experiments. The experiments were performed in triplicate in three independent experiments. For each strain tested, one colony that grew on agar plates for 24 h was cultured under weak agitation in 1 ml TS broth at 37°C for 3 h. The inoculum was then used as a donor for mating assays with *E. coli* DH10B Rif^r as recipient. Mating was done by incubating 800 μl of recipient and 200 μl of donor strains under low agitation at 37°C for an additional 3 h. Mating was stopped by vigorous vortexing and cooling on ice. One-hundred-microliter aliquots of serial 10-fold dilutions were then plated onto TS agar plates with gentamicin, with rifampin, and with or without ticarcillin for pTn*1999* and with gentamicin, with rifampin, and with or without kanamycin for the other plasmids. The transposition frequency was calculated by dividing the number of Gen^r Kan^r Rif^r or Gen^r Tic^r Rif^r transconjugants by the number of Gen^r Rif^r transconjugants. All the Gen^r Kan^r Rif^r or Gen^r Tic^r Rif^r colonies were screened for tetracycline susceptibility to exclude those that may have arisen from nontranspositional events.

Insertion site determination. Plasmid pOX38-Gen carrying various insertions of either IS1999.Kan alone or IS*1999* composite transposons was extracted from independent and randomly chosen *E. coli* DH10B Rif^r transconjugants. Direct sequencing of the ends of IS*1999* using the primers IS1999IRLext and IS1999IRRext (Table 1) was performed in order to determine the precise sites of insertion of IS*1999*.Kan elements, whereas the insertion site for composite transposons was determined by an inverse PCR technique as follows. Plasmid DNAs were digested with XbaI (Fig. 1). After inactivation of the endonuclease, an intramolecular ligation was performed. Two microliters of the ligation mixture was used as template with IS1999B2INV and IS1999IRRext2 primers for inverse PCR amplification, and then the PCR-generated products were sequenced (Table 1).

Transcription initiation. RNA was extracted using the RNA Quick kit (QIAGEN) according to the recommendation of the manufacturer. Reverse transcription (RT) and rapid amplification of cDNA ends (RACE) were performed with the 5′RACE system, version 2.0 (Invitrogen, Life Technologies, Cergy Pontoise, France). Five micrograms of total RNAs extracted from cultures of *E. coli* DH10B (pA-1) and the bla_{OXA-48}GSP1, bla_{OXA-48}GSP2, and bla_{OXA-48}GSP3 antisense bla_{OXA-48} gene-specific primers (Table 1) were used to determine the bla_{OXA-48} transcription initiation site. Similarly, five micrograms of total RNAs extracted from culture of *E. coli* DH10B (pIS*1999*.Kan) and *E. coli* DH10B (pIS*1999*.2.Kan) with TnpA*1999*-GSP1, TnpA*1999*-GSP2, and TnpA*1999*-GSP3 antisense gene-specific primers (Table 1) were used to determine the transcription initiation site of the transposase gene of IS*1999* and IS*1999*.2.

RT-PCR conditions. Five micrograms of total RNAs extracted from culture of *E. coli* DH10B (pIS*1999*.Kan) and *E. coli* DH10B (pIS*1999*.2.Kan) was DNase treated for 15 min at 25°C in a final volume of 16 μl containing 2 U of RNase-free DNase (Roche). EDTA was added to a final concentration of 2.5 mM, and the DNase was inactivated by a 10-min incubation at 65°C. Five microliters of DNase-treated RNA was reverse transcribed in a final volume of 25 μl using 200 U of the Moloney murine leukemia virus based SuperScript III reverse transcriptase as recommended by the manufacturer (Invitrogen) and 2 μM of the reverse primer (TnpA*1999*-GSP1 and GapA2; see below). The reaction mixture was incubated for 1 h at 42°C, followed by a 5-min incubation at 95°C. Ten microliters of the cDNA was used for amplification of specific IS*1999-tnpA* mRNA using primers IS1999B1 and TnpA*1999*-GSP2 (Table 1), yielding a 228-bp fragment, and 10 μl was used for amplification of gap (4, 7) (encoding D-glyceraldehyde-3-phosphate dehydrogenase, used as an external control) using primers GapA1 and GapA2 and yielding a 470-bp fragment in the following conditions: 12 min of initial denaturation at 94°C, followed by 35 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 55°C, and 1 min of extension at 72°C, with a final extension step of 10 min at 72°C. Both PCR products were detected on a 2% agarose gel. Each RT-PCR was performed in triplicate. The band intensities were estimated using the Taxotron software (Institut Pasteur).

TABLE 2. Frequency of the transposition of IS*1999* and its derivatives

Transposon	Transposition frequency (±SD[a])
IS*1999*.Kan	3.7×10^{-6} (1.7×10^{-6})
IS*1999*as.Kan	1.7×10^{-6} (2.1×10^{-7})
IS*1999*stop.Kan	$<1.0 \times 10^{-7}$
IS*1999*.2.Kan	2.6×10^{-7} (8.7×10^{-8})
Tn*LR*	4.0×10^{-6} (9.2×10^{-7})
Tn*LL*	3.6×10^{-6} (1.0×10^{-6})
Tn*RR*	3.7×10^{-7} (2.6×10^{-7})
Tn*1999*	$<1.0 \times 10^{-7}$

[a] Standard deviations calculated from three independent cultures are indicated in parentheses.

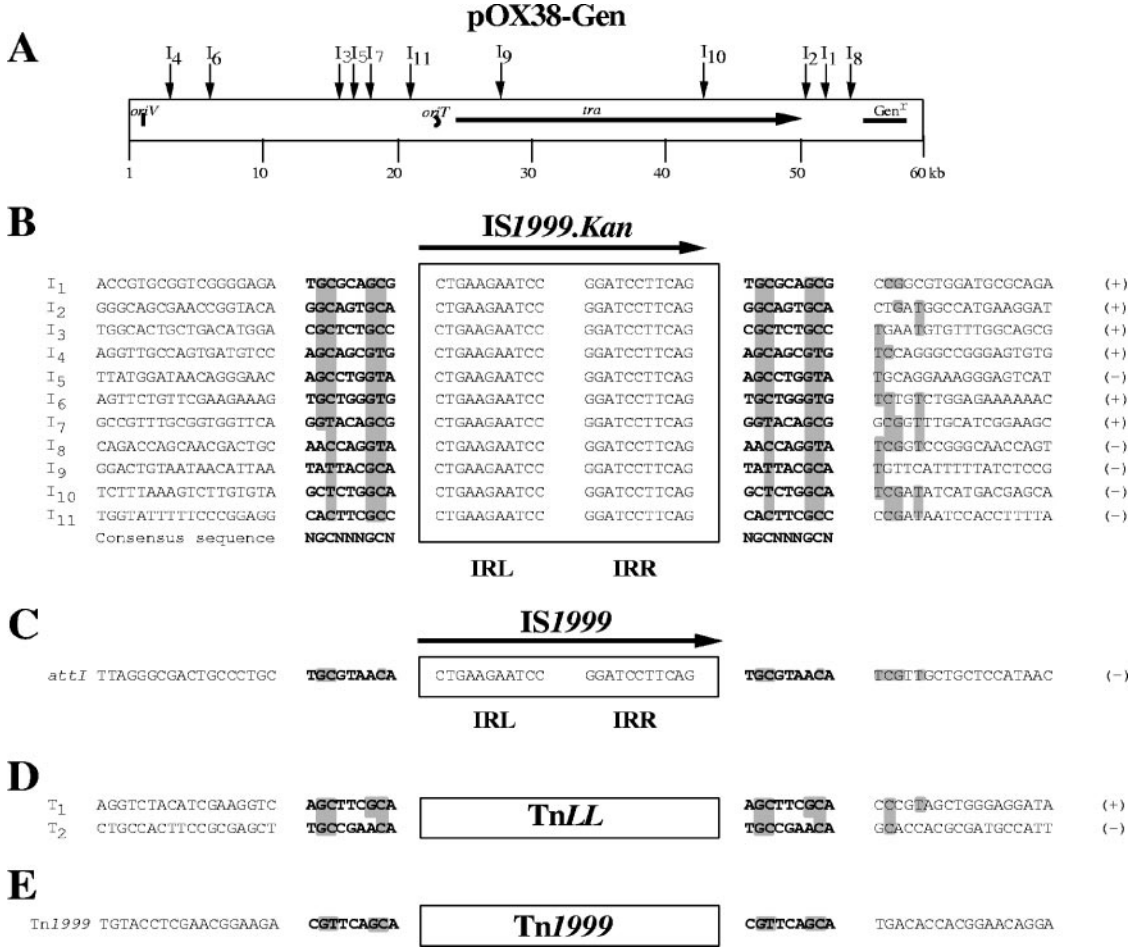

FIG. 2. Target sites of IS*1999* insertions. (A) Map positions of IS*1999*.Kan and Tn*LL* insertions in plasmid pOX38-Gen. Insertions of the tagged insertion sequence (I1 to I11) and of the composite transposons (T1 and T2) are indicated by a vertical arrow. The origin of replication (*oriV*), the origin of transfer (*oriT*), the *tra* genes required for plasmid transfer, and the gentamicin resistance gene (Gen^r) are indicated on the pOX38-Gen representation. Nucleotide sequence alignment of the 11 pOX38-Gen::IS*1999*.Kan transconjugants (B), of the *P. aeruginosa* 1 *attI1*::IS*1999* junctions (C), of two representative pOX38-Gen::Tn*LL* insertions (D), and of the Tn*1999* environment (E) are shown. Nucleotide sequences of the end regions of IS*1999*.Kan, IS*1999*, Tn*LL*, and Tn*1999* are boxed. Target site sequences duplicated after transposition are indicated by boldface letters. Gray boxes indicate conserved nucleotides in the environment of IS*1999*.Kan and IS*1999* insertions. Orientation of the insertion sequences of IS*1999*.Kan and Tn*LL* in plasmid pOX38-Gen are indicated by pluses and minuses.

Nucleotide sequence accession number. The nucleotide sequence of Tn*1999* has been submitted to the EMBL/GenBank nucleotide sequence database under the accession number AY236073.

RESULTS

Transposition of IS*1999* by mating-out assay. The transposition ability of IS*1999*.Kan was investigated by mating-out assays with the *E. coli* DH5α strain, harboring pIS*1999*.Kan (Kan^r) and the gentamicin-resistant plasmid pOX38-Gen (Gen^r) (a transfer-proficient F plasmid derivative) as the donor and *E. coli* DH10B Rif^r as the recipient.

Transposition events that resulted from conjugation of pOX38-Gen:IS*1999*.Kan were selected as Gen^r Kan^r Rif^r colonies and screened for susceptibility to tetracycline (Tet^s) at a frequency of 3.7×10^{-6} (Table 2). Susceptibility of *E. coli* (pOX38-Gen::IS*1999*.Kan) transconjugants to tetracycline indicated that resistance to kanamycin was neither due to plasmid cointegration (pIS*1999*.Kan::pOX38-Gen) nor to selection of rifampin-resistant *E. coli* DH5α (pIS*1999*.Kan). This suggests that a transpositional event had occurred. As a control, no Gen^r Kan^r Rif^r Tet^s transconjugant was obtained when IS*1999*stop.Kan was used (Table 2).

Plasmids pIS*1999*.Kan and pIS*1999*as.Kan were isogenic, except for the P_{lac} promoter orientation. The plasmid pIS*1999*.Kan carries the P_{lac} promoter in a sense orientation with the transposase gene and might lead to its overexpression, whereas pIS*1999*as.Kan carries the P_{lac} promoter in an antisense orientation. Compared to pOX38-Gen::IS*1999*.Kan, transposition events that resulted from conjugation of pOX38-Gen::IS*1999*as.Kan were screened as Gen^r Kan^r Rif^r Tet^s colonies at a lower frequency (1.7×10^{-6}). The similarity between the transposition frequencies obtained with pIS*1999*.Kan and pIS*1999*as.Kan suggested that the transposition frequency of a single IS*1999* element is independent of its orientation within the cloning vector. More-

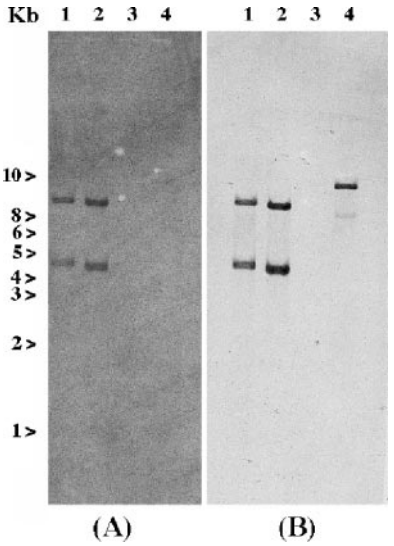

FIG. 3. Hybridizations of EcoRV-digested whole-cell DNAs of *E. coli* DH10B (pA-1) (lane 1), *K. pneumoniae* 11978 (lane 2), *E. coli* DH10B (lane 3), and *P. aeruginosa* 1 (lane 4). (A) bla_{OXA-48}-specific hybridization. (B) IS*1999*-specific hybridization.

over, it suggested that the P_{lac} promoter in pIS*1999*.Kan (located in the cloning vector) had no significant effect on the transposase expression (Table 2).

Target site preference of insertion element IS*1999*. Several randomly chosen Gen^r Kan^r Rif^r Tet^s transconjugants isolated from independent experiments were analyzed. The insertion sites of IS*1999*.Kan were determined by DNA sequencing of the external neighboring regions of the inverted repeats. A 9-bp target site duplication, consistent with a transposition event, was observed in all the studied insertion events (Fig. 2).

To determine whether IS*1999* had a target site preference, locations of 11 insertion events were mapped onto plasmid pOX38-Gen. IS*1999*.Kan insertions occurred in 11 different sites, and alignment of the insertion site sequences (I1 to I11) revealed a consensus motif (NGCNNNGCN) (Fig. 2). Indeed, most target sites had a purine at positions 2 and 7 with a preference for a guanosine (G) residue. The 11 target sites had a pyrimidine at positions 3 and 8, with C favored over T (Fig. 2).

Characterization of a putative natural transposon, Tn*1999*. Recently, IS*1999* was found upstream of the bla_{OXA-48} gene that was located on the natural plasmid (pA-1) (21). Southern hybridization experiments were performed with whole-cell DNA of *E. coli* DH10B (pA-1) that was digested with EcoRV (EcoRV cuts in bla_{OXA-48} and not in IS*1999*). Fragments internal to IS*1999* or to bla_{OXA-48} were used as probes. Two fragments hybridizing with both probes were detected, suggesting the existence of two copies of IS*1999* located on each side of bla_{OXA-48} that may be part of a composite transposon (Fig. 3).

Whole-cell DNA of *E. coli* DH10B (pA-1) was restricted with several enzymes that cut at neither IS*1999* nor the bla_{OXA-48} sequence. Hybridization with an IS*1999*-specific probe revealed that a SacII-PstI restriction generated a single fragment of 6.1 kb (data not shown). This SacII-PstI-restricted fragment was cloned and sequenced, identifying the structure of a 4.9-kb putative transposon named Tn*1999*. Tn*1999* was made of two copies of IS elements in outward and opposite orientations that flanked a region originating from a *Shewanella* sp. genome (20). Tn*1999* included two open reading frames encoding (i) a class D β-lactamase (OXA-48) that confers resistance to most β-lactams, including carbapenems, and (ii) a transcriptional regulator of the LysR family that shares 98% amino acid identity with that identified in the genome of *Shewanella oneidensis* MR-1 as well as a portion of an open reading frame encoding an acetyl-coenzyme A carboxylase multifunctional enzyme, AccADC (Fig. 1).

The IS elements of Tn*1999* had identical sequences but were isoforms of IS*1999* and were named IS*1999*.2. Indeed, IS*1999*.2-left and IS*1999*.2-right differed from the sequence of IS*1999* (GenBank accession number AF133697) by five nucleotide substitutions. However, none of these substitutions led to changes of the amino acid sequence of the IS*1999* transposase, and one of them was located next to the P_{in} +1 transcription start site (Fig. 4).

Using the 5′RACE technique, the promoter responsible for bla_{OXA-48} expression was mapped. Transcription initiation occurred at a thymidine residue (nucleotide position 115 from the inner terminus of the IS*1999*.2-left element). As expected, the deduced promoter region named P_{out} was identical to that previously identified for IS*1999* (1). P_{out} is made of a −35 promoter region (CAGTAT) separated by 17 bp from a −10 region (TAGGAT), and it is similar to the outward-directed promoter identified in IS*10* (−35 [CAGAAT] and −10 [TAAAAT]) (30).

The genetic environment of Tn*1999* was analyzed. Tn*1999* was inserted in a Tir gene encoding a transfer inhibition protein that shares 93% amino acid identity with that of plasmid pCTXM-3 in a *Citrobacter freundii* strain (GenBank accession number AF550415). The 9-bp sequences that were located on each side of IS*1999*.2-left (CGTTCAGCA/TATATTGCA) or IS*1999*.2-right (GGCCGAGCA/CGTTCAGCA) matched the consensus target sequence (Fig. 1 and 2). The 9-bp duplication (CGTTCAGCA) located on each side of the structure suggested that insertion into the Tir gene may have occurred by a transposition event.

Transposition of IS*1999*-based composite transposons. The ends of IS*1999* are not identical. (i) IS*1999* contains two IR (IRL and IRR) that share 81% nucleotide identity. (ii) Moreover, analysis of the IS*1999* nucleotide sequence revealed that IS*1999* carries a putative IHF (integration host factor) binding site (bp 30 to 42) adjacent to the IRL (Fig. 4).

In order to evaluate whether two IS*1999* elements were able to transpose in the form of various composite transposons, different constructs were made using two copies of IS*1999* (i) in inward and opposite orientations (Tn*LL*), (ii) in the same orientation (Tn*LR*), or (iii) in outward and opposite orientations (Tn*RR*) (Fig. 1). Tn*LL* transposition involves the action of transposase at two IRL, Tn*RR* transposition involves two IRR, and Tn*LR* transposition involves one IRL and one IRR, as for a single IS*1999* transposition. In these constructs, each insertion sequence could transpose either independently of each other or together under a composite transposon structure.

The composite transposons Tn*LL* and Tn*LR* transposed at similar frequencies (ca. 4×10^{-6}) (Table 2). However, the

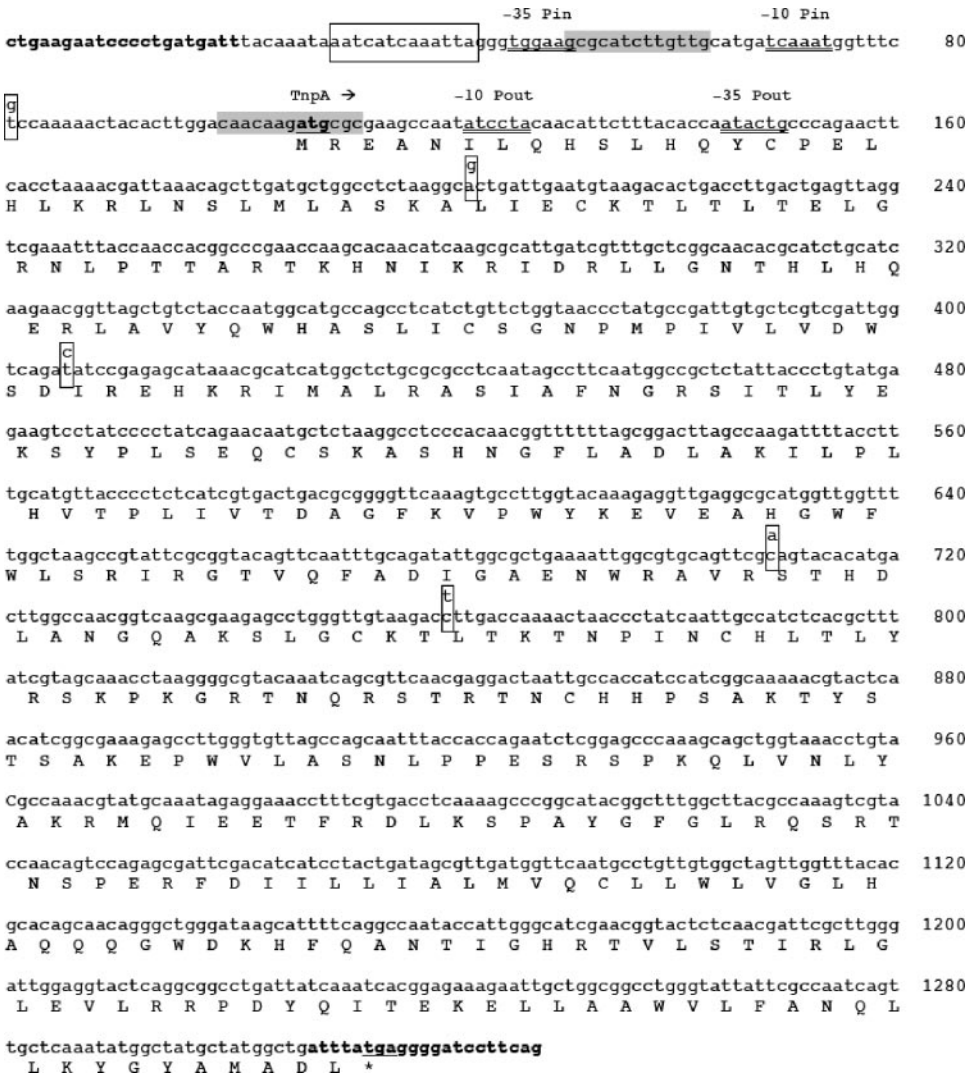

FIG. 4. Nucleotide sequence of IS*1999*. The deduced amino acid sequence is designated in a single-letter code below the nucleotide sequence. The start and stop codons of the transposase gene are underlined. The transposase gene name, followed by an arrow indicating its translational orientation, is indicated above the initiation codon. IS*1999* left and right inverted repeats (IRL and IRR) are boldfaced. The boxes represent the putative IHF binding site (bp 30 to 42). The complementary inverted repeat sequences are shown by gray boxes. The promoter sequences −10 and −35 of P_{out} and P_{in} promoters are double underlined and indicated above the sequence (GenBank accession no. AF133697). Nucleotide changes relative to IS*1999.2* are indicated above each nucleotide and are boxed.

Tn*RR* transposition frequency was at least 10-fold lower than that of Tn*LL* or of Tn*LR*. This result suggested that transposition of a composite transposon flanked by two outside IRR ends (e.g., which do not contain any IHF binding site) is less efficient than other transposition events which involve at least one IRL end (containing an IHF binding site).

Analysis of the genetic environment of several insertions of composite transposons using IS*1999* elements revealed in all cases a duplication of 9 bp, confirming that a transposition event had occurred. Sequence of the insertion sites matched to the consensus target site and two representative insertions (T1 and T2) are shown in Fig. 2.

Transposition assay using the putative transposon, Tn*1999*. The transposition ability of Tn*1999* was investigated using plasmid pTn*1999*. However, despite several attempts, no Gen^r Tic^r Rif^r Tet^s transconjugants were obtained, suggesting either that the Tn*1999* transposition frequency might be lower than 10^{-7} (limit of detection of our transposition assay) or that this structure, flanked by two IS*1999*.2 elements, may not be able to transpose.

We have shown previously that transposition of Tn*RR* but not of Tn*1999* was detectable. Tn*1999* and Tn*RR* had similar structures. Both structures were made of two IS elements in outward and opposite directions located on each side of a 2.2-kb central region. However, the structures of Tn*1999* and Tn*RR* have two major differences that may affect their transposition frequency: the nature of the central region and the nature of the IS elements. Indeed, one structure was made of two copies of IS*1999*.2 (isoform of IS*1999*), whereas the other was made of two copies of IS*1999*.

The transposition ability of IS*1999*.2.Kan was investigated (Fig. 1). Transconjugants that resulted from conjugation of

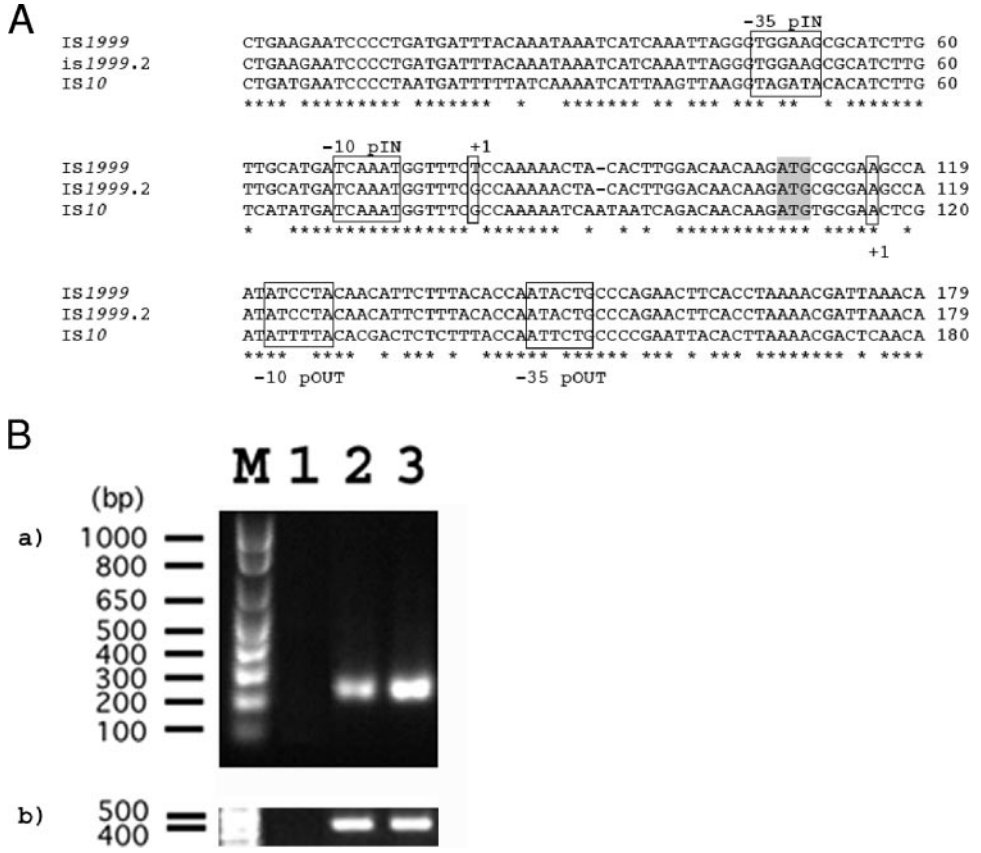

FIG. 5. A. Nucleotide sequence alignments of the promoter regions of IS*1999*, IS*1999.2*, and IS*10*. The start codon of the transposase gene is boldfaced. The boxes represent the conserved boxes of P_{out} and P_{in}. The complementary inverted repeated sequences are shown by gray boxes. The promoter sequences −10 and −35 of P_{out} and P_{in} promoters are boxed and indicated above the sequence. B. RT-PCR results. M, molecular size marker (100 bp; Invitrogen). Lane 1, no RNA (negative control); lane 2, RNA from IS*1999*.2-containing cells; lane 3, RNA from IS*1999*-containing cells. Panel a represents IS*1999*-specific primers, and panel b represents the gap-specific primers.

pOX38-Gen::IS*1999*.2.Kan were screened as Gen^r Kan^r Rif^r Tet^s colonies at the frequency of 2.6×10^{-7} (Table 2). Thus, the transposition efficiency of IS*1999*.2 was lower than that of IS*1999*.

Using the 5′RACE technique, the promoter responsible for the IS*1999-tnpA* expression was precisely mapped. Transcription initiation occurred at a thymidine residue for IS*1999-tnpA* and at a guanosine residue for IS*1999.2-tnpA* (both located at nucleotide position 81) (Fig. 5A). P_{in} is made of a −35 promoter region (TGGAAG) separated by 17 bp from a −10 region (TCAAAT) and is similar to the P_{in} promoter identified in IS*10* (−35 [CAGAAT] and −10 [TAAAAT]) (30).

RT-PCR experiments were performed, and the band intensities were normalized according to the gap gene amplification results (Fig. 5B). A factor of three to four differences in band intensities was observed for P_{in} of IS*1999* versus that of IS*1999.2*, suggesting that IS*1999-tnpA* is more efficiently transcribed.

DISCUSSION

The association of IS*1999* with emerging antibiotic resistance genes triggered our interest in the study of IS*1999*-mediated gene mobilization. We showed that IS*1999* was capable of transposition into DNA targets containing a consensus sequence. Another important feature for IS*1999* is the inclusion of strong outward-directed promoter sequences (P_{out}) involved in the expression of downstream-located genes. This promoter was similar to, and at the same location as, that previously identified for IS*10* (30).

IS*1999* and IS*10* sequences share significant nucleotide identity (67%) and conserved genetic features. (i) IS*1999* and IS*10* possess a similar P_{in} promoter driving the transposase expression and a similar outward-directed promoter, P_{out} (Fig. 4). When cloned on a multicopy plasmid, IS*10* is subjected to a phenomenon called multicopy inhibition (31). Indeed, an increase of the copy number of IS*10* led to an increase in the intracellular amount of the antisense RNA generated from the P_{out} promoter. This antisense RNA, by interacting with the sense RNA generated from P_{in}, inhibits the transposase expression (31). The IS*1999* P_{out} transcript is antisense to the transposase promoter P_{in}, and IS*1999* elements were cloned on a low-copy plasmid. Thus, the multicopy inhibition is likely to occur in our study and may lead to an underestimation of the transposition frequency of IS*1999*. Another point known to be responsible for a transposition frequency decrease is the length of dependence. Indeed, as has been shown for other transposons, the transposition frequency decreases with the length

of the DNA fragment inserted within the IR (5–6, 16, 26, 29). In our study, the kanamycin resistance gene was inserted in order to tag IS*1999* and led to an additional 2.2-kb increase of the space between the IR. However, this increase likely has little effect on IS*1999* transposition, since the length dependence is only valid for longer DNA fragments.

(ii) Both IS*1999* and IS*10* carry two complementary inverted repeats in the IRL region, one of which overlaps the translation initiation signal of the transposase gene (Fig. 4). As described for IS*10* (14), once transcribed from an external promoter these sequences were able to form a hairpin RNA structure which sequesters translation initiation signals and results in termination of transcription. This mechanism of "protection from outside transcription" avoids overexpression of the transposase and consequently an excess of transposition events that may be detrimental to the bacterial host. Analysis of the IS*1999* sequence revealed that such a potential structure could be formed when transcribed from an external promoter. The mechanism of protection from outside activation is likely to occur in IS*1999*, as suggested by our data. In pIS*1999*.Kan, the transcription of the IS*1999* transposase seems to be protected from the P_{lac} promoter. Indeed, the orientation of the tagged IS*1999* (IS*1999*.Kan, sense; IS*1999*as.Kan, antisense) compared to the strong P_{lac} promoter from the cloning vector did not lead to significant change of IS*1999* transposition frequency.

(iii) The consensus DNA target of IS*1999* (NGCNNNGCN) resembled that of IS*10* (NGCTNAGCN) (9). The consensus sequence for IS*1999* insertions was determined by alignment of target sequences of randomly chosen insertion sites (hotspots and non-hotspots). IS*10* insertion sites that are not hotspots deviate more from the consensus sequence, especially at positions 4 and 6 (9). By extension, this fact may explain why no obvious consensus sequence was detected at positions 4 and 6 in the consensus target sequence of IS*1999*. However, regarding all the insertion sites analyzed (Fig. 2), the T is favored at position 4 (6T, 3A, 4C, 2G) and the A is favored at position 6 (5A, 4G, 4C, 2T).

Moreover, several base pairs of the DNA sequences flanking the target sites of IS*1999*.Kan were conserved (Fig. 2). Indeed, homology seemed to extend several base pairs beyond and to one side of the target sequence, suggesting that flanking sequences may influence the target site recognition, as is known to occur for IS*10* (2).

The target site located in the *attI1* site of class 1 integrons (Fig. 2) is located downstream of promoter sequences (P_{ant} promoter located in the 5′ conserved sequence region of class 1 integrons [25]), and insertion of transposons such as Tn*10* has been shown to be inhibited by heavily transcribed target sequences (3). Thus, we hypothesize that the *attI1* site may be less efficient for IS*1999* insertion.

(iv) IS*1999* and IS*10* possess a similar IHF binding site adjacent to the IRL end (Fig. 4). Tn*10* is flanked by two identical IRL ends, both of which have IHF binding sites. The DNA bending activity of IHF stimulates assembly of an intermediate with folded transposon ends in which the transposase has additional subterminal contacts. These contacts are required to activate the chemical steps during the cleavage reaction (28).

We have shown that two copies of IS*1999* were also capable of mobilizing intervening DNA sequence in the form of many composite transposons. However, a transposon made of two copies of IS*1999* in outward and opposite orientations (Tn*RR*) (involving the action of the transposase at two IRR ends, both ends lacking an IHF binding site) was less efficient for transposition. Transposition seems to be more efficient when at least one IRL end (containing an IHF binding site [Tn*LR*, Tn*LL*]) is present at the outside boundaries of the transposon. Thus, we hypothesize that IHF may also play an important role in IS*1999* transposition and may explain the difference of transposition frequencies measured between Tn*RR*, Tn*LR*, and Tn*LL*.

As Tn*1999* and Tn*RR* had similar structures, both were expected to have similar transposition frequencies. However, transposition of Tn*1999* was not detected in our assay. Our study revealed that IS*1999*.2 elements were less efficient in transposition than IS*1999*. Compared to IS*1999*, the IS*1999*.2 sequence contained only one nucleotide substitution occurring outside of the transposase gene (G instead of T; bp 81), which corresponds to the +1 transcriptional initiation site of the P_{in} promoter that is responsible for transcription of the transposase gene (30). The P_{in} promoter is a weak promoter, and single changes occurring at the −35 or −10 promoter sequence of P_{in} have been shown to influence the IS*10* transposition frequency (10, 30). However, sequences located in the immediate region of the transcription initiation site have been shown to influence the transcriptional initiation (11). This could be the case for IS*1999*.2, since the promoter is less efficient. An alternative explanation might be that the silent mutations replace frequently used codons by rarely used codons, thus slowing down the translation of *tnpA* (6). However, codon usage is likely to contribute very moderately, if at all.

The duplication of the 9-bp (CGTTCAGCA) sequence that is located on each side of the Tn*1999* strongly suggested that it was a transposon that may be able to transpose. If this structure is indeed a transposon, the combination of the presence of two IS*1999*.2 elements, the lower level of IS*1999-tnpA* expression, and their outward and opposite orientations might be a part of the factors responsible for the low-transposition frequency of Tn*1999*. However, further experiments will be necessary to clearly demonstrate this issue.

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