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Isolation and Characterization of IS*1409*, an Insertion Element of 4-Chlorobenzoate-Degrading *Arthrobacter* sp. Strain TM1, and Development of a System for Transposon Mutagenesis

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A new insertion element of 1,449 bp with 25-bp perfect terminal repeats, designated IS*1409*, was identified in the chromosome of 4-chlorobenzoate-degrading *Arthrobacter* sp. strain TM1 NCIB12013. Upon insertion, IS*1409* causes a target duplication of 8 bp. IS*1409* carries only a single open reading frame of 435 codons encoding the transposase TnpA. Both TnpA and the overall organization of IS*1409* are highly similar to those of some related insertion elements of the ISL*3* group (J. Mahillon and M. Chandler, Microbiol. Mol. Biol. Rev. 62:725–774, 1998). IS*1409* was also found in other 4-chlorobenzoate-degrading *Arthrobacter* strains and *Micrococcus luteus*. Based on IS*1409*, a series of transposons carrying resistance genes for chloramphenicol and gentamicin were constructed. These transposons were used to demonstrate transposition events in vivo and to mutagenize *Arthrobacter* sp. strains.

Insertion elements (IS elements) are small mobile non-self-replicating DNA regions specifying only the gene(s) required for their transposition (16). According to the phylogenetic relationship of the transposases and some features involved in the transposition (e.g., the inverted repeats which constitute the ends of most elements, as well as the recognition sites of the transposase and the target duplication caused by most elements), they can be grouped in different families (16). At least for some IS elements, a target-sequence-dependent site preference has been demonstrated, while members of other families apparently transpose in a random fashion. They are widespread in bacteria and contribute to the plasticity of bacterial genomes due to their transposition ability and to their role as target sites for homologous recombination, which can give rise to deletions, inversions, or more complex rearrangements (20). Some composite transposons derived from IS elements, in addition to the genes required for transposition, harbor genes encoding resistances to antibiotics or degradative pathways.

Arthrobacter, a very common soil bacterium, and related genera like *Micrococcus* form one of the major branches of the actinomycetes, the *Micrococcaceae* (30). Some *Arthrobacter* strains are known to degrade aromatic compounds as well as chlorinated aromatic compounds such as 4-chlorobenzoate (4-CBA). Hydrolytic dehalogenation of 4-CBA by *Arthrobacter* sp. strain SU DSM20407 requires three genes organized in an operon which maps on plasmid pASU1 (25). The same set of genes is also present in *Arthrobacter* sp. strain TM1 (17). In the course of cloning and sequencing of these genes (unpublished data), we identified an IS element upstream of the dehalogenase operon in *Arthrobacter* sp. strain TM1. So far, only one other IS element in *Arthrobacter* and the *Micrococcaceae*, IS*1473* from *Arthrobacter nicotinovorans* (which belongs to the IS*3* family), has been described (19). A growing number of genes specifying degradation of aromatic compounds are known to be located on or associated with transposable elements (for recent reviews see references 31 and 41), with the best known examples being the very large catabolic transposons Tn*4651* and Tn*4653* on the TOL plasmids of pseudomonads. Thus, it seems possible that the genes for the hydrolytic dehalogenation of 4-CBA are located on a transposon.

In order to demonstrate that IS*1409* is a functional transposable element, a series of transposons were constructed based on a slightly modified IS*1409* and carrying antibiotic resistance genes. These transposons are the first which can be used for transposon mutagenesis in *Arthrobacter*.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture media, and antibiotics. Bacterial strains and plasmids used in this study are listed in Table 1. 4-CBA-degrading strains were grown at 28°C in a minimal medium as described by Seiler (27) at pH 8.0, supplemented with 1 g of 4-CBA/liter as the only carbon and energy source and 0.2 ml of trace element solution/liter (9). All other actinomycetes were grown in NBYE medium (8 g of nutrient broth and 5 g of yeast extract/liter; pH 7.5) at 28°C. *Escherichia coli* strains were grown in TBY (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl/liter; pH 7.5) at 37°C. For growing recombinant *E. coli* strains harboring pUC vector derivatives, ampicillin (150 μg/ml) was added to the medium. For *Arthrobacter* spp., chloramphenicol (10 μg/ml) or gentamicin (40 μg/ml) was added to the medium after electroporation of pKGT452 derivatives.

DNA isolation, manipulation, and transfer. Plasmid DNA of *E. coli* was prepared by the alkaline lysis method (22). Plasmid DNA used for sequencing and electroporation was isolated and purified with Qiagen columns as specified by the manufacturer (Qiagen, Hilden, Germany). The endogenous plasmids of *Arthrobacter oxidans* and *Arthrobacter* sp. strain SU were isolated from 1-liter cultures grown overnight in half-strength NBYE containing 1 g of 4-CBA/liter by the method of Kieser (13) using 40°C as the lysis temperature. Preparation of total DNA for hybridization was done as described by Hopwood et al. (9). For total DNA preparation, all strains were grown in rich medium overnight. DNA restriction, ligation, and transformation were carried out by standard procedures (22). Isolation of restriction fragments from agarose gels was done with the JETSORB kit as specified by the manufacturer (Genomed, Bad Oeynhausen, Germany).

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant genotype and/or phenotype	Reference or source[a]
Strains		
Arthrobacter sp. strain SU (DSM20407)	pASU1; 4-CBA$^+$	21
Arthrobacter sp. strain SUX	4-CBA; plasmid-free curing derivative of strain SU	25
Arthrobacter sp. strain TM1 (NCIB12013)	4-CBA$^+$	17
Arthrobacter oxidans CBS2	pAO2; 4-CBA$^+$	14
Arthrobacter nicotinovorans DSM420	pAO1; IS*1473*	DSM
Micrococcus luteus		R. Bergmann, University of Hamburg, Hamburg, Germany
Aureobacterium sp. strain RH025	4-FBA^{+b}	J. Eberspächer, University of Hohenheim, Hohenheim, Germany
Mycobacterium (Rhodococcus) chlorophenolicus PCPI (DSM43286)	PCP^{+b}	DSM
Nocardia sp. strain NCIB10503	Biphenyl$^+$	29
Corynebacterium glutamicum ATCC 13022	IS*C*g1	10
Clavibacter michiganensis subsp. *michiganensis* NCPPB382		NCPPB
Clavibacter michiganensis subsp. *sepedonicus* NCPPB299		NCPPB
Clavibacter michiganensis subsp. *insidiosum* NCPPB1099		NCPPB
E. coli JM109	*recA1 endA1 thi hsdR17 supE44 relA1* Δ*(lac-proAB) gyrA96* F′*(traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15)*	42
Plasmids		
pUC13	Ap^r; cloning vector	39
pUC18	Ap^r; cloning vector	42
pJE258	Mini-F cosmid; Cm^r Tc^r	7
pEZ1	pJE258::25-kb *Sau*3A fragment from strain TM1; 4-CBA$^+$	26
pKGT21	pUC13::1-kb *Sst*I fragment of pEZ1	This work
pKGT451	pUC13::2.3-kb *Eco*RV/*Nru*I fragment of pKGT45; IS*1409*	This work
pKGT452	pUC13::modified IS*1409*; see text	This work
pKGT452Cα	Tn*1409*Cα; *tnpA* and *cmx* in different orientations	This work
pKGT452Cβ	Tn*1409*Cβ; *tnpA* and *cmx* in the same orientation	This work
pKGT452Cα	Tn*1409*Cα; *tnpA* and *aacC1* in different orientations	This work
pKGT452Cβ	Tn*1409*Cβ; *tnpA* and *aacC1* in the same orientations	This work
pKGT1000	pUC18::4.8-kb *Sph*I fragment of mutant A1	This work
pKGT1010	pUC18::5.0-kb *Sph*I fragment of mutant A27	This work

[a] DSM, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, Herts United Kingdom.
[b] 4-FBA, 4-fluorobenzoate; PCP, pentachlorophenol.

Electroporation. Cultures (100 ml) were grown in NBYE to an optical density at 580 nm of about 0.5. The cells were harvested and resuspended in 10 ml of ice-cold deionized water containing 10% glycerol. Lysozyme (100 μl; 4 mg/ml) was added, and the cells were incubated at 28°C for 30 min. After harvesting, cells were washed twice with deionized water–10% (vol/vol) glycerol and then resuspended in 1 to 5 ml of deionized water–10% (vol/vol) glycerol. For electroporation, 1 μg of DNA was added to 200 μl of pretreated cells. Electroporation was performed with a Bio-Rad gene pulser apparatus applying the following parameters: capacity, 25 μF; voltage, 2.5 kV/cm; resistance, 600 to 800 Ω, giving a pulse length of 10 to 13 ms. Cells were mixed immediately with 0.8 ml of SB medium (10 g of tryptone/liter, 5 g of yeast/liter, 4 g of NaCl/liter, 0.5 M sorbitol, 20 mM $MgCl_2$, 20 mM $CaCl_2$ [pH 7.5]) and incubated for 2 h at 28°C before plating on appropriate selective media. Afterwards colonies were restreaked on NBYE plates containing the appropriate antibiotics.

Southern blot analysis. The internal 1-kb *Sst*I fragment of the *tnpA* gene from pKGT21 was labeled using the random primed DNA labeling kit (Boehringer Mannheim, Mannheim, Germany) and used as probe for the transposase gene of IS*1409*. A 2.2-kb *Sph*I/*Bgl*II fragment of pKGT452Cβ was used to detect the *cmx* gene. pUC13 DNA labeled with digoxigenin-11-dUTP by nick translation (22) was used as a probe for integration of the transposon delivery vector. Digested chromosomal and plasmid DNA samples were separated on 0.7 to 1.0% agarose gels and transferred to nylon membranes (Hybond [Amersham, Little Chalfont, United Kingdom] or Nytrans [Schleicher & Schuell, Dassel, Germany]) using a vacuum blotter (Vacugene; Pharmacia). Hybridizations were carried out at 68°C overnight in a buffer containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.02% sodium dodecyl sulfate, 0.1% Na laurylsarcosyl, and 2% blocking reagent (Boehringer Mannheim). The nylon membrane was washed twice with 0.1× SSC–0.1% sodium dodecyl sulfate at 68°C for 15 min.

DNA sequencing and sequence analysis. For DNA sequencing, the dideoxy chain termination method (24) was used with [α-^{32}P]dATP (3,000 Ci/mmol) employing Sequenase 2.0 or Taqquence sequencing kits from U.S. Biochemicals (Cleveland, Ohio) as specified by the manufacturer. For sequence determination, either subclones of pKGT45 were constructed or primer walking with synthetic oligonucleotides (obtained from TIB Molbio, Berlin, Germany, or from Birsner and Grob-Biotech GmbH, Denzlingen, Germany) was conducted. Oligonucleotides ISC2 (5′-GGA ACC TCA CCA ACT ACA TAG C-3′) and ISN2 (5′-CAT GCA GTT GCG CCC ACT ACA C-3′) were used to determine the sequence of insertion sites in Tn*1409* mutants. The oligonucleotides IS1 to IS6 were used to

introduce two base exchanges into the 5′ end of IS*1409* in the construction of pKGT452 (IS1, 5′-AAT TCC GGT ACC ATG GCT CTT CAG AAT TGA GGG TGT-3′; IS2, 5′-AGT GGG CGC AAC TGC ATG CAG CGC CGA GGG CTA GCG GCG T-3′; IS3, 5′-GAT TCA GAC AAG GTG AGG GCC TCG GGA GAG AAT CAG ATT GTC TA-3′; IS4, 5′-GAT CTA GAC AAT CTG ATT CTC TCC CGA GGC CCT CAC CT-3′; IS5, 5′-TGT CTG AAT CAC GCC GCT AGC CCT CGG CGC TGC ATG CAG T-3′; IS6, 5′-TGC GCC CAC TAC ACC CTC AAT TCT GAA GAG CCA TGG TAC CGG-3′).

Sequence assembly and sequence analysis were done with the Microgenie (Beckman, Palo Alto, Calif.) and PCGENE (Intelligenetics, Mountain View, Calif.) programs. The BLAST search programs (1) were used to compare the DNA and deduced protein sequences with database entries at the server of the National Center of Biotechnology Information.

Nucleotide sequence accession number. The sequence reported here has been deposited in GenBank under accession no. AF042490.

RESULTS

Nucleotide sequence of IS*1409*. During the analysis of the nucleotide sequence of the chromosomally borne 4-CBA dehalogenase operon of *Arthrobacter* sp. strain TM1 (NCIB12013) on a 25-kb *Sau*3A fragment inserted into the cosmid pJE258 (pEZ1) (26), an open reading frame (ORF) with significant identities to those encoding transposases was detected. A 2.35-kb *Eco*RV/*Nru*I fragment of pEZ1 was subcloned into the *Sma*I site of pUC13, resulting in plasmid pKGT451 (Fig. 1a). The nucleotide sequence of both strands of pKGT451 was completely determined. The nucleotide sequence and the deduced amino acid sequence are presented in Fig. 1b. The sequence surrounding the transposase gene was found to fulfill the criteria required for an IS element. On both sides of this inserted element a target sequence duplication of 8 nucleotides (nt) was found and confirmed by comparison with the homologous sequence on plasmid pASU1 of *Arthrobacter* sp. strain SU (25), which lacks the IS element. IS*1409* is 1,449 bp long and has perfect terminal inverted repeats of 25 bp. The GC content of the IS element is 61.8%, which is in agreement with the GC content of the *Arthrobacter* host. IS*1409* is inserted about 500 bp upstream of the 4-CBA dehalogenase operon.

Only one ORF of 1,308 bp, spanning almost the entire element, was found. It starts with an ATG codon 110 nt downstream of the left inverted repeat and ends with a TGA stop codon inside the right inverted repeat. The ORF encodes the putative transposase TnpA of IS*1409*, a protein of 435 amino acids (48.9 kDa). It is preceded by a putative ribosome binding site 5 bp upstream of the ATG start codon.

Homology of TnpA from IS*1409* to transposases of other IS elements. Database searches with the deduced TnpA sequence revealed a number of highly similar transposases of bacterial IS elements, as shown in Table 2. The most closely related transposases are found in actinomycetes, with the exception of IS*1411* from a phenol-degrading *Pseudomonas putida* strain (12). For IS*1096*, IS*31831*, and IS*1411* there is very high identity at the DNA level, with 72, 60, and 57% identities, respectively, observed over nearly the whole length of the transposase gene (data not shown). The inverted repeats of these IS elements are also highly conserved (Fig. 2). Based on the homology to these transposases, the 8-nt duplication, and the inverted repeats of 25 bp, IS*1409* is classified as a member of the ISL*3* family (16).

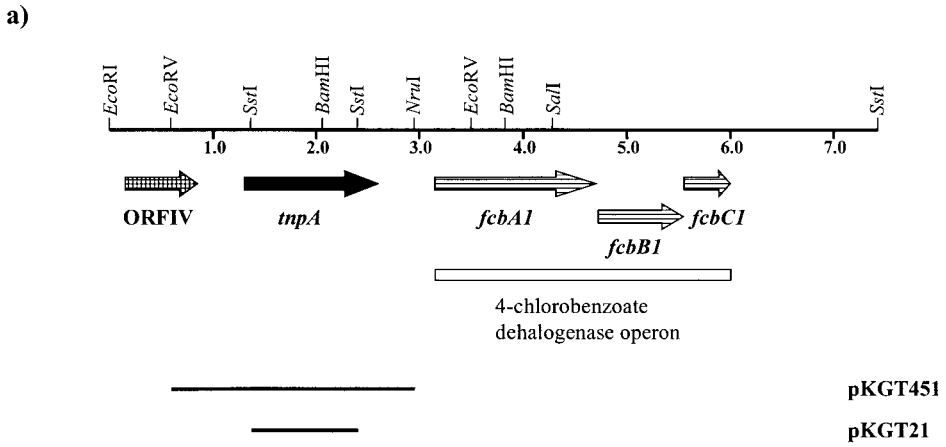
Distribution of IS*1409* among various bacterial strains. Hybridization experiments with an internal 1-kb *Sst*I fragment of IS*1409* as a probe against total or plasmid DNA of various *Arthrobacter* strains were conducted under stringent conditions. For this, DNA was digested with *Bgl*II, which does not cut inside the *tnpA* gene (Fig. 3). In *Arthrobacter* sp. strain TM1, at least eight copies of IS*1409* were found, displaying different signal intensities. The 4-CBA degrading *Arthrobacter* sp. strain SU carries two copies, while only one copy could be identified for the plasmid-cured derivative *Arthrobacter* sp. strain SUX (Fig. 3), indicating that in these strains one copy of IS*1409* is carried by plasmid pASU1. *A. oxidans* CBS2 DNA gave only one and *Micrococcus luteus* DNA gave two positive signals under stringent conditions. IS*1409* was not identified in any of the other actinomycetes tested (Table 1 and Fig. 3) under stringent conditions (data not shown for all strains).

Construction of resistance transposons from IS*1409*. To prove that IS*1409* located on pKGT451 is a functional IS element, derivatives carrying resistance genes were constructed. As the nucleotide sequence of pKGT451 revealed that there are no unique restriction sites present between the left inverted repeat and the start of the putative transposase gene, the 120 bp at the 5′ end of IS*1409* up to the *Bgl*II site was replaced by six overlapping synthetic oligonucleotides (IS1 through IS6) which carried two nucleotide exchanges so that unique *Sph*I and *Nhe*I sites were created. The oligonucleotides were annealed and inserted into *Eco*RI/*Bgl*II-linearized pKGT451, resulting in pKGT452 (Fig. 1b). The changes in the sequence were confirmed by restriction analysis and nucleotide sequence determination.

Then, the chloramphenicol resistance gene *cmx*, encoding an exporter protein of Tn*5564* of *Corynebacterium striatum* (32), and the gentamicin acetyltransferase gene *aacC1* from Tn*1696* (40) were inserted into the *Nhe*I site of pKGT452. Both orientations were obtained after ligation as confirmed by restriction analysis and partial sequencing. The resulting plasmids were designated pKGT452Cα and -β (Fig. 4) and pKGT452Gα and -β, carrying the transposons Tn*1409*Cα and -β and Tn*1409*Gα and -β, respectively, depending on the orientation of the resistance cassette relative to the *tnpA* gene. The size of these transposons is about 3.4 kb.

Transposon mutagenesis of *Arthrobacter* sp. A mutagenesis was conducted with *Arthrobacter* sp. strains SU and SUX by electroporation using covalently closed circular plasmid DNA of pKGT452Cα and -β and pKGT452Gα and -β isolated from *E. coli* JM109. Since pUC13 is not able to replicate in *Arthrobacter*, all resistant clones should arise by transposition or recombination with endogenous copies of IS*1409*. The average rates of antibiotic-resistant clones obtained ranged from 2×10^3 to 1×10^4/μg of DNA in independent experiments. For both pKGT452Cβ and pKGT452Gβ, which contain the resistance gene and the *tnpA* gene oriented in the same direction, about twice as many antibiotic-resistant clones were obtained as for the corresponding plasmids where transposase and resistance genes were transcribed in opposite directions. No significant difference in the number of resistant clones was found between pKGT452C and pKGT452G.

To distinguish between transposition and recombination, chloramphenicol-resistant clones were chosen at random for hybridization. Total DNA was hydrolyzed with *Bgl*II or *Sph*I, both of which leave the *tnpA* gene intact, so that hybridization signals corresponding to sizes larger than 1.4 and 3.4 kb were expected with the *tnpA* probe. With the exception of two clones



b)

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                501  CCCGTCCGGACGATGCAGCCATCCTTTTTTCAGGTGCACTCGTTGTCCGAG  550

          (EcoRI)(KpnI)          IRL                          (SphI)
CAAGTCTGACCATTCGAGATCATTGCCGGCTCTTCAGAATTGAGGGTGTAGTGGGCGCAACTGCTTGCAG  620
          GAATTCCGGATCCAT                                      A

       (NheI)                                                XbaIBglII
CGCCGAGGGGTAGCGGCGTGATTCAGACAAGGTGAGGGCCTCGGGAGAGAATCAGATTGTCTAGATCTTG  690
         C

AATTTTCTACCGCAAGGCCCTCATGTCCAACGCTACTCTCCAGCGCCCTGACCTGACCACATTCTGTCGC  760
                       M  S  N  A  T  L  Q  R  P  D  L  T  T  F  C  R

      SstI
CTCGATGAGCTCGGCCTGGAAGCCACTGGGCAGTGTCTGTACCCTGATCGTGCTGTCATT//--------  820
 L  D  E  L  G  L  E  A  T  G  Q  C  L  Y  P  D  R  A  V  I

                                                      SstI
1771  ------------//CATCAGCACAGGCGTCCCGGCCGCTCTCACCGAGCTCAAGACGCTGGGGC 1820
                       I  S  T  G  V  P  A  A  L  T  E  L  K  T  L  G

GGACCCTCAAACGACGGGCAGAAGACGTGCTGGCGTACTTCGATCGGCCCGGAACATCGAACGGCCCCAC 1890
R  T  L  K  R  R  A  E  D  V  L  A  Y  F  D  R  P  G  T  S  N  G  P  T

AGAAGCAATTAACGGGAGACTGGAACACCTCCGCGGCTCAGCCCTCGGGTTCCGGAACCTCACCAACTAC 1960
  E  A  I  N  G  R  L  E  H  L  R  G  S  A  L  G  F  R  N  L  T  N  Y

                                                      IRR
ATAGCCCGCTCGCTCCTGGAATCCGGCGGATTCAGACCGAAACTACACCCTCAATTCTGAAGAGCCTCAT 2030
 I  A  R  S  L  L  E  S  G  G  F  R  P  K  L  H  P  Q  F  *

TGCCCACCCTCGGCAAGACTACCTATTGCTGTTTATGAGGTTCGTAGCGCGCGTTCGGCCAAGCAGGTCT 2100
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FIG. 1. (A) Physical map of the chromosomal region encoding the 4-CBA dehalogenase genes of *Arthrobacter* sp. strain TM1. Arrows show directions of transcription. Bars indicate regions cloned into plasmids used in this study. (B) Part of the nucleotide sequence of the 2.35-kb *Eco*RV/*Nru*I insert of pKGT451. Relevant restriction sites are underlined and labeled; sites present only in pKGT452 are in parentheses. As the 5′ end of pKGT451 up to the *Bgl*II site was exchanged by synthetic oligonucleotides, pKGT452 starts with an *Eco*RI site 47 and 62 nt upstream of nucleotide exchanges introduced in pKGT451, leading to the unique sites for *Sph*I and *Nhe*I in pKGT452. The 8-bp target duplication is double underlined. The 25-bp perfect inverted repeats (IRL and IRR) of IS*1409* are underlined and boldface. The highly conserved C-terminal region of TnpA, which is characteristic of the ISL*3* family, is underlined.

TABLE 2. Comparison of IS*1409* with some insertion sequences of the ISL*3* family

IS element	Origin	Accession no.	Length of TnpA (aa)	Identity/ similarity (%)	Length of IR[c] (bp)	Target duplication (bp)	Reference
IS*1409*	*Arthrobacter* sp. strain TM1	AF042490	435		25	8	This work
IS*1096*[a]	*Mycobacterium smegmatis*	M76495	413	77/87	25	8	3
IS*B11*	*Brevibacterium linens*	AF052055	436	69/79	25	8	Unpublished data
IS*1411*	*Pseudomonas putida*	M57500	433	60/76	24	8	12
IS*13869*	*Brevibacterium lactofermentum*	Z66534	436	42/57	26	8	4
IS*31831*	*Corynebacterium glutamicum*	D17429	436	42/56	24	8	36
IS*2001*	*Bifidobacterium lactis*	AJ243948	442	43/55	26	?[d]	Unpublished data
IS*204*	*Nocardia asteroides*	U10634	377[b]	30/47	23	8	43
IS*Ps1*	*Pseudomonas stutzeri*	AJ012352	425	27/48	24	8	2
IS*Ae1*	*Alcaligenes eutrophus*	M86608	408	27/41	24	8	15
IS*1165*	*Leuconosioc mesenteroides*	X62617	412	23/38	24	8 or 3	11
IS*1001*	*Bordetella parapertussis*	X66858	406	22/37	28	6 or 8	34
ISL*3*	*Lactobacillus delbrueckii*	X79114	434	20/37	38	8	8
IS*1181*	*Staphylococcus aureus*	L14544	439	19/38	23	8	6

[a] IS1096 is the only one of this group of IS elements which additionally contains a putative resolvase gene
[b] IS204 may contain a frameshift sequencing error or represent a nonfunctional IS element, as the conserved C-terminal domain of the transposase displayed in Fig. 1 occurs in a different reading frame from the *tnpA* gene.
[c] IR, inverted repeat.
[d] ?, data not available.

which gave an identical signal in both digestions, all clones produced signals of different sizes larger than 1.4 or 3.4 kb (Fig. 5). Thus, Tn*1409* seems to transpose in a random fashion. Surprisingly, we observed that in the course of the experiments a copy of the endogenous IS*1409* in strains ASU and ASUX was lost, as indicated by a missing band (compare Fig. 3, lanes 3 and 4, with Fig. 5, lanes 2 and 24). Only 1 out of 20 clones probed gave a signal after hybridization with a digoxigenin-labeled pUC13 probe (data not shown), indicating that integration of the complete pKGT452Cβ by either homologous recombination or cointegrate formation had occurred. In about 10% of the transposon mutants of strain ASU the signal of the endogenous copy of IS*1409* was missing, indicating a recombination event or a more complex rearrangement between the introduced transposon and the endogenous IS*1409* (data not shown). However, this does not impair the usefulness of Tn*1409* for transposon mutagenesis, especially when a strain which does not carry IS*1409* is used.

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Actinomycetes
    IS1409      IRL     GGCTC TTCAG AATTG AGGGT GTAGT
                IRR     GGCTC TTCAG AATTG AGGGT GTAGT
    IS1096      IRL     ----- ---GC -C--- -C--- ----A
                IRR     ----- ---GC -G--C ----- ----A
    ISB11       IRL     ----- ---GC --AC- --A-- ---TA
                IRR     ----- A-CGT --A-A --A-- ----A
    IS13869     IRL     ----- ---C- TT--T --A-- -C-T-G
                IRR     ----- ---C- TT--T --A-- -C-T-G
    IS31831     IRL     ---C- ---C- GT--T G---- AC-T
                IRR     ----- ---CT GT--T --A-- -C-T
    IS204       IRL     ----- ---GC -C--- GTA-G -GC
                IRR     ----- ---GC -G--A GCA-- -GC
Gram-negatives
    IS1411      IRR     ----- ---GC -T--A ----- ----
                IRL     ----- ---GC -T--A ----- ----

    ISAE1       IRL     -C--- --TT- ----T CAA-- -GGT
                IRR     ----- --TT- --AAA T-A-- -GGT
    ISPs1       IRR     --G-A -C-G- ----T CT--- TG-T
                IRL     --G-A -A-G- -T--A -T--- TG-T
    IS1001      IRL     --T-- A--GC GCAAT -AC-- -G--GG
                IRR     --T-- A--GC GCA-T GCC-G -G-AGG
    IS1081      IRL     --T-- ----T CT--T -T--- -GG
                IRR     --T-- -C--C C-AAT GT--- -GG
low GC Gram-positives
    IS1165      IRL     --G-- -AG-A TT--T G-T-- TGGA
                IRR     --AAG --TGT C-AAT G-T-- TG-A
    ISL3        IRL     ----- -AT-A TT--T CTTAC TGATG
                IRR     ----- --TGT C-AAT CTTAG TGATG

    consensus           ggctc ttc-- --ttt ---gt g--
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FIG. 2. Sequence alignment of the terminal inverted repeats (IRL and IRR) of some members of the ISL*3* family. Nucleotides identical to those in the inverted repeats of IS*1409* are shown as dashes.

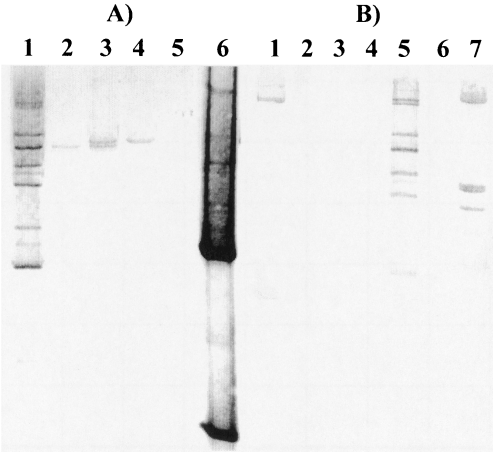

FIG. 3. Southern blot of total DNA of various actinomycete strains digested with *Bgl*II and hybridized with a digoxigenin-labeled internal 1-kb *Sst*I fragment of *tnpA*. (A) Lane 1, *Arthrobacter* sp. strain TM1; lane 2, *A. oxidans*; lane 3, *Arthrobacter* sp. strain SU; lane 4, *Arthrobacter* sp. strain SUX; lane 5, *A. nicotinovorans* DSM420; lane 6, *Sst*I-hydrolyzed pKGT21. (B) Lane 1, *M. luteus*; lanes 2 to 4, *Clavibacter michiganensis* subsp. *michiganensis*, *sepedonicus*, and *insidiosum*; lane 5, *Arthrobacter* sp. strain TM1; lane 6, *A. nicotinovorans* DSM420; lane 7, digoxigenin-labeled λ*Eco*RI/*Hin*dIII.

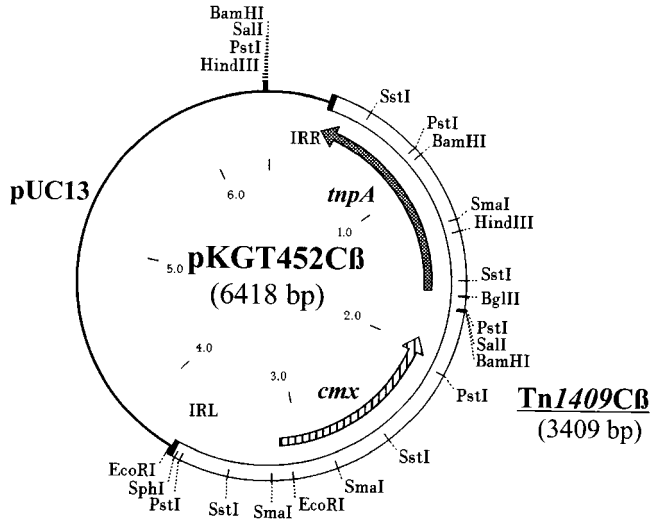

FIG. 4. Physical map of pKGT452Cβ. There are no recognition sites for *Eco*RV and *Stu*I in pKGT452Cβ. The *Sph*I site is located 11 bp inside Tn*1409*Cβ.

Sequence analysis of insertion sites of Tn*1409* in *Arthrobacter*. Total DNA of some mutants generated with Tn*1409*Cβ was isolated and digested with *Sph*I (one site in Tn*1409*Cβ 11 bp downstream of the left inverted repeat), which leaves the *cmx* gene intact. The digested DNA was hybridized with digoxigenin-labeled probes for *tnpA* (a 1-kb *Sst*I fragment from pKGT21), the *cmx* gene (a 2.2-kb *Sph*I/*Bgl*II fragment from pKGT452Cβ), and pUC13. The insertion sites from two mutants (A1 and A27) which did not hybridize with pUC13 were subsequently isolated as *Sph*I fragments giving a signal with the *cmx* probe.

From total DNA of mutant A27, the region containing a 5.0-kb *Sph*I band was cut from the agarose gel, purified, cloned into *Sph*I-linearized pUC18, and used to transform *E. coli* JM109. Colonies resistant to both chloramphenicol and ampicillin were obtained, and one of them was designated pKGT1010 and chosen for sequencing. The sequence obtained was compared to the GenBank database. These data revealed the insertion of Tn*1409*Cβ 59 bp upstream of a gene homologous to branched-chain α-keto acid dehydrogenases (5, 28) in mutant A27. From another mutant *Arthrobacter* strain, SU A1, the hybridizing 4.8-kb *Sph*I fragment was cloned in an analogous procedure (pKGT1000). Preliminary single-stranded sequence determination revealed the insertion of Tn*1409*Cβ into an ORF with homology to the membrane component of ABC transporters involved in iron uptake (23). However, growth of mutant A1 on iron-limited media was not impaired (data not shown). In addition, downstream a small ORF without homologs in the database followed by a third ORF designated *tdk*, similar to thymidine kinase genes (33), was found. The putative target duplication in these mutants confirmed the apparent random transposition, as no preference for a specific insertion site was detected.

DISCUSSION

In this report we describe a novel IS element, IS*1409*, found upstream of the 4-CBA dehalogenase operon of *Arthrobacter* sp. strain TM1. This IS element was found only in members of the *Micrococcaceae*, in 4-CBA-degrading *Arthrobacter* strains, and in *M. luteus*. IS*1409* displays typical features of members of the ISL*3* family, with the highest similarity to IS elements from other actinomycetes. The high extent of identity to IS*1411* from *P. putida* may indicate horizontal transfer of this element from gram-positive to gram-negative bacteria.

The mode of transposition, whether replicative or conservative, for the members of the ISL*3* family is still unknown. The putative resolvase gene occurring in IS*1096* of *Mycobacterium smegmatis* is not necessary for transposition (18). For IS*31831* and IS*Ps1*, the formation of excised transposon circles was reported (2, 38). Integration of the delivery plasmid at low frequency, which was also observed for IS*31831* (37), could arise either by recombination or cointegrate formation.

The insertion sites of IS*1409* and Tn*1409* analyzed so far do not indicate a preference for a specific sequence for insertion. However, both ends of the 8-nt target duplication are rich in GC with an AT-rich central region, for example, TCATTG CCC (endogenous copy), GCCAAAAC (mutant A1), and AC GAAAGT (mutant A27). For the transposons derived from IS*1096* and IS*31831* (18, 37), the same pattern was reported for the insertion sites. This may often lead to insertions within

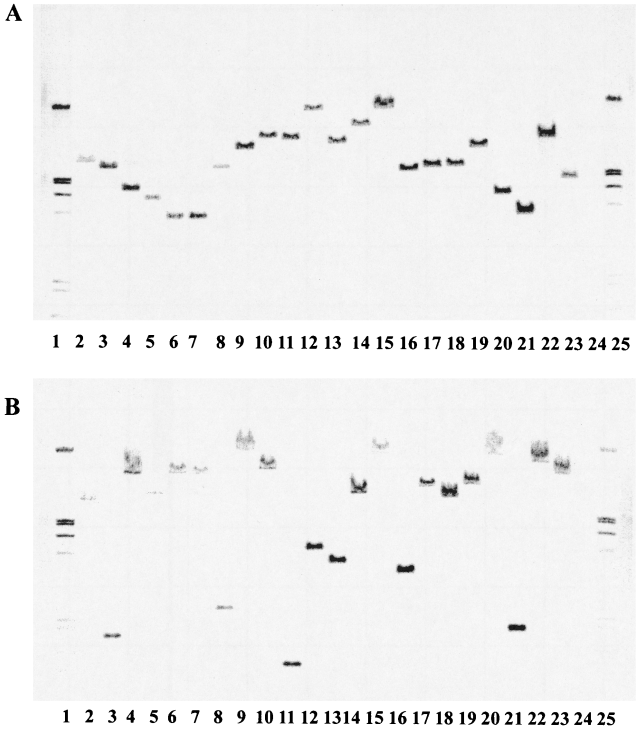

FIG. 5. Southern hybridization of various chloramphenicol-resistant *Arthrobacter* sp. mutants generated with pKGT452Cβ. Total DNA was digested with *Sph*I (A) or *Bgl*II (B) and probed with the internal 1-kb *Sst*I fragment of *tnpA*. As no *Sph*I and *Bgl*II site occurs in the *tnpA* gene, one signal with a minimal size of 3.4 or 1.4 kb (*Sph*I/*Bgl*II) is expected after insertion of Tn*1409*Cβ. Lane 1, digoxigenin-labeled λ*Eco*RI/*Hin*dIII; lane 2 *Arthrobacter* sp. strain SU; lanes 3 to 23, *Arthrobacter* sp. strain SUX mutants D1 to D21; lane 24, *Arthrobacter* sp. strain SUX; lane 25, digoxigenin-labeled λ*Eco*RI/*Hin*dIII. Mutants D4 and D5 (lanes 6 and 7) seem to be identical. All other mutants display different hybridization patterns, indicating a random insertion.

promoter regions in these high-GC gram-positive bacteria; for example, this may have occurred in mutant A27. Such integrations resulting in gene activation and/or inactivation have also been described for pseudomonads (e.g., for IS*1411* and IS*Ps1*) (2, 12).

IS*1409* was used to construct antibiotic resistance gene transposons after the exchange of two nucleotides in the upstream region of the *tnpA* gene. The transposition rates of about 10^3 transpositions/μg of DNA used in electroporation of *Arthrobacter* are in the same range as those obtained with transposons constructed from IS*1096* (3) and IS*31831* (37). Since transposition rates strictly depend on the efficiency of transformation, transposon mutagenesis requires optimization of the conditions for electroporation. Also, the extent of methylation of the DNA used may be important (35).

Although the strains used in transposition experiments possess endogenous copies of IS*1409*, in most cases a transposition of Tn*1409* occurred. Only once did we observe integration of the complete delivery plasmid, and in two cases we found indications of rearrangements as the endogenous IS*1409* was lost. Both phenomena could arise either by recombination or during transposition.

The construction of transposon Tn*1409*, which has a high transposition rate and shows no obvious preference for specific insertion sites, now provides a system for transposon mutagenesis for *Arthrobacter* which may be very useful for genetic investigations in this important actinomycete.

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