

Genetic characterization of insertion sequence ISJP4 on plasmid pJP4 from *Ralstonia eutropha* JMP134

Johan H.J. Leveau, Jan Roelof van der Meer *

Swiss Federal Institute for Environmental Science and Technology (EAWAG) and Swiss Federal Institute for Technology (ETH),
CH-8600 Dübendorf, Switzerland

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Abstract

Directly adjacent to the (*tfdT*-)*tfdCDEF* gene cluster for chlorocatechol breakdown on plasmid pJP4 of *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) JMP134, we identified a 0.9-kb DNA element, designated ISJP4, with the typical features of a bacterial insertion sequence. ISJP4 occurs as a single complete copy on plasmid pJP4. About 9 kb away from this copy, in the *tfdA*-*tfdS* intergenic region, we found a 71-bp duplication of the ISJP4 right-hand extremity. In addition, we discovered a complete copy of ISJP4 on the chromosome of the *R. eutropha* JMP134 strain that we use routinely in our laboratory. We suppose that this copy resulted from a recent transposition of the plasmid-borne ISJP4, since it was shown to be lacking from the chromosomes of *R. eutropha* JMP222 and JMP289, two previously pJP4-cured derivatives of JMP134. By comparing both complete copies and their flanking regions, we could establish that element ISJP4 has a size of 915 bp and is bordered by 18-bp inverted repeats with one mismatch. Based on sequence similarity of its coding regions, ISJP4 could be classified into the IS5 group of the IS4 family of bacterial insertion sequences, where it is mostly related to IS402 of *Burkholderia cepacia*. A TAA direct repeat, presumably resulting from a duplication of the target site, flanked the chromosomal copy of ISJP4. We could demonstrate that a piece of DNA that is flanked by two complete copies of ISJP4 can be transposed. Even more so, one complete ISJP4 plus its *tfdA*-*tfdS* intergenic remnant were sufficient to mediate transposition of intervening DNA. A possible role of ISJP4 in the formation of the *tfd* pathway genes will be discussed. © 1997 Elsevier Science B.V.

Keywords: Formerly *Alcaligenes eutrophus* JMP134; 2,4-Dichlorophenoxyacetic acid; *tfd* genes; IS4-family

1. Introduction

Plasmid pJP4 harbours the genetic information that allows its host strain *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) JMP134 to use 2,4-dichlorophenoxyacetic acid (2,4-D) as sole carbon and energy source (Don and Pemberton, 1981). The *tfdA* (Streber et al., 1987) and *tfdB* (Perkins et al., 1990) genes encode the enzymes for conversion of 2,4-D via 2,4-dichlorophenol into 3,5-dichlorocatechol. The latter compound is further degraded to 3-oxoadipate by the gene products of the *tfdCDEF* operon (Perkins et al., 1990).

The *tfdT* gene, which is located upstream and transcribed divergently from *tfdCDEF*, was investigated previously for its presumed role in the regulation of *tfdCDEF* expression (Leveau and van der Meer, 1996). Despite its homology to other regulators of chlorocatechol degradation, the TfdT protein was incapable of activating *tfdCDEF*. Sequence analysis of the region directly downstream of the *tfdT* gene revealed the presence of a large DNA element with typical features of a bacterial insertion sequence (IS). We postulated that this element, designated ISJP4, disrupted the reading frame of the *tfdT* gene and led to the synthesis of a truncated, non-functional protein. Interestingly, the *tfdCDEF* genes were still expressed, probably because another regulatory protein, very similar to TfdT, could successfully cross-activate the *tfdCDEF* promoter. This regulator is encoded by the identical genes *tfdR* and *tfdS*, which are located elsewhere on plasmid pJP4 (Matrubutham and Harker, 1994; You and Ghosal, 1995).

* Corresponding author. Tel.: +41 1 8235438. Fax: +41 1 8235547;
e-mail: vdmeer@eawag.ch

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; bp, base pair(s); IS, insertion sequence; kb, kilobase(s); ORF, open reading frame; cfu, colony forming unit.

In this paper, we present the complete sequence of ISJP4 and an analysis of its prominent features. We demonstrate that ISJP4 has recently been active, can mobilize neighbouring DNA, and might have been involved in shaping the present day's organization of the *tfd* genes on pJP4.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth media

The bacterial strains and plasmids used in this study are listed in Table 1. *R. eutropha* strains were grown on NB or on *Pseudomonas* mineral medium (MM) (Gerhardt et al., 1981) with the addition of 5 mM 2,4-D or fructose as sole carbon and energy source. *E. coli* strains were cultivated on LB medium (Sambrook et al., 1989). Where appropriate, antibiotics were added in the following final concentrations: ampicillin, 100 µg/ml; kanamycin, 50 µg/ml; rifampicin, 10 µg/ml; streptomycin, 250 µg/ml.

2.2. DNA isolation, manipulation and sequencing

Total DNA from *R. eutropha* strains was isolated according to the method of Marmur (Marmur, 1961). Plasmid pJP4 was isolated from *R. eutropha* JMP134 (pJP4) as described by Hansen and Olsen (1978). Standard recombinant DNA techniques were carried out according to Sambrook et al. (1989). Restriction enzymes and other DNA-modifying enzymes were obtained from GIBCO/BRL Life Technologies (Gaithersburg, MD, USA), Pharmacia LKB Biotechnology (Uppsala, Sweden), or Appligene (Illkirch, France) and used according to the specifications of the manufacturer. Oligonucleotides for the polymerase chain reaction (PCR) were obtained from MWG-BIOTECH AG (Ebersberg, Germany). DNA sequencing was performed as described by Sanger et al. (1977), using a Sequenase kit (version 2.0; US Biochemical, Cleveland, OH, USA) with [α -³²P]dATP (3000 Ci/mmol; Amersham International, Amersham, UK). Computer analysis of DNA and amino-acid sequences was performed with the programs GCG (ver-

Table 1
Bacterial strains and plasmids^a

	Relevant characteristics	Reference
Bacterial strains		
<i>Ralstonia eutropha</i> JMP134 (pJP4)	pJP4 ⁺ ; 2,4-D ⁺ ; fructose ⁺	Don and Pemberton (1981)
<i>Ralstonia eutropha</i> JMP134 (pJP4)	Same, but with a chromosomal copy of ISJP4 on the chromosome	This work
<i>R. eutropha</i> JMP222	pJP4 ⁻ ; 2,4-D ⁻ ; Sm ^r ; fructose ⁺	Don and Pemberton (1981)
<i>R. eutropha</i> JMP289	pJP4 ⁻ ; 2,4-D ⁻ ; Rif ^r ; fructose ⁺	Don and Pemberton (1981)
<i>R. eutropha</i> JMP134 (RP1)	pJP4 ⁻ ; RP1 ⁺ ; 2,4-D ⁻ ; Km ^r ; fructose ⁺ ; chromosomal copy of ISJP4	This work
<i>R. eutropha</i> JMP289::cba106-1 to -6	Km ^r ; obtained by transformation of JMP289 with pCBA106	This work
<i>R. eutropha</i> JMP289::cba108-1 to -6	Km ^r ; obtained by transformation of JMP289 with pCBA108	This work
<i>Escherichia coli</i> LE392	Fructose ⁻ ; used as host for RP1	Murray et al. (1977)
<i>E. coli</i> DH5 α	Supported propagation of plasmid pCBA70	Sambrook et al. (1989)
<i>E. coli</i> TG1	Supported propagation of M13mp18/19	Sambrook et al. (1989)
Plasmids		
M13mp18/19	Cloning vehicle for sequencing	Sambrook et al. (1989)
pT7Blue(R) T-vector	Ap ^r ; linear with T-overhanging ends to facilitate cloning of PCR-products	Novagen
pJP4	Harbors <i>tfdA</i> , <i>tfdB</i> and <i>tfdCDEF</i> genes coding for a 2,4-D degradative pathway; belongs to the IncP incompatibility group	Don and Pemberton (1985)
RP1	Broad host range R-plasmid; confers resistance to kanamycin; belongs to the IncP incompatibility group	Datta et al. (1971)
pKT230	Km ^r ; can replicate in <i>R. eutropha</i>	Bagdasarian et al. (1981)
pCBA70	pT7Blue(R) T-vector carrying a 3.5-kb PCR product with the flanking regions of the chromosomally located copy of ISJP4; Ap ^r	This work
pCBA100	Contains Km cassette with a copy of ISJP4 downstream on pUC	This work
pCBA106	Contains Km cassette flanked by two direct repeats of ISJP4	This work
pCBA107	Contains Km cassette flanked by two inverted repeats of ISJP4	This work
pCBA108	Contains Km cassette flanked downstream by ISJP4 and upstream by the <i>tfdA-tfdS</i> intergenic region	This work
pCBA109	Contains Km cassette flanked downstream by ISJP4 and upstream by the <i>tfdA-tfdS</i> intergenic region in the opposite direction	This work

^aAp^r, ampicillin resistance; Km^r, kanamycin resistance; Sm^r, streptomycin resistance; Rif^r, rifampicin resistance; 2,4-D[±], (in)ability to grow on 2,4-D as sole carbon and energy source; fructose[±], (in)ability to grow on fructose as sole carbon and energy source.

sion 7.0, J. Devereux, University of Wisconsin) and DNASTAR (Lasergene, Madison, WI, USA). The nucleotide sequence of element ISJP4 presented in this study has been deposited in the GenBank database under accession number U16782.

2.3. Construction of strain *R. eutropha* JMP134 (RP1)

R. eutropha JMP134 (RP1) is a derivative of the *R. eutropha* JMP134 (pJP4) strain, which we use routinely in our laboratory. It was derived by exchanging plasmid pJP4 with RP1. Both plasmids belong to the IncP1 incompatibility group. *R. eutropha* JMP134 (pJP4) and *E. coli* LE392 (RP1) were grown to an OD₆₀₀ of 1.0. One-millilitre samples of each bacterial culture were mixed and concentrated on a filter, which was incubated on an LB-plate overnight at 30°C. After incubation, the bacterial cells were resuspended from the filter in 1 ml MM, and dilutions were plated out on MM-plates containing 5 mM fructose and 50 mg/ml kanamycin. Colonies that appeared were transferred to fresh plates, and six transconjugants were verified for the loss of plasmid pJP4 by establishing their inability to grow on 2,4-D and by hybridizing the total DNA of these strains to a pJP4-specific probe (results not shown).

2.4. Hybridization experiments

For the hybridization experiments, plasmid or chromosomal DNA was digested to completion with different restriction enzymes, after which the DNA fragments were separated on a 0.8% agarose gel in TAE. The DNA fragments were then blotted on to Qiabran (Qiagen, Chatsworth, CA, USA) using standard vacuum-blotting procedures. Hybridization of the membrane to radioactively labeled probes was done according to Sambrook et al. (1989). As probes, we used the *Sma*I–*Mlu*I internal portion of element ISJP4 copy A, the *Pst*I–*Bam*HI fragment of pCBA70 harboring a DNA segment that flanked ISJP4 copy B, or the *Bam*HI fragment of pCBA100 with the kanamycin cassette. These fragments were radioactively labeled with [α -³²P]dATP (Amersham) using a Random Primed DNA Labeling Kit (Boehringer-Mannheim, Mannheim, Germany). After hybridization, the membrane was washed twice for 30 min in a high stringency buffer at 62°C, and subsequently exposed to autoradiographic film. After exposure to film, probes were washed off again by a 2 × 30 min treatment at 95°C in 0.1 × SSC plus 1.5% SDS. After verifying complete removal of the probes, the membrane was rehybridized with a second probe.

2.5. Isolation of ISJP4 copy B and its flanking DNA

We designed a digestion/religation/PCR protocol to isolate the extremities of ISJP4 copy B and its flanking region. A graphic presentation of this protocol is given

in Fig. 4A. Total DNA from *R. eutropha* JMP134 (pJP4) was cut to completion with *Bam*HI. After phenol/chloroform extraction, the digested DNA was religated with T4 DNA ligase in an overnight incubation at 15°C. The ligation mixture was used directly as template DNA in a PCR. Besides the DNA, PCR mixtures contained 0.5 mM of both primers, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2 mM MgCl₂, 0.05% W1, 0.2 mM of each deoxynucleoside triphosphate, 0.2 mg/ml BSA and 20 U/ml of *Taq* DNA polymerase (Life Technologies). As primers, we used ISJP4-1 (5'-CGATCAGTGCCACAGTTCATCATCAATGATCG-3') and ISJP4-2 (5'-ACGTACTCACGCGTGCTTCACAACCTTTCGTCG-3'), which were complementary to internal sequences of ISJP4 and outward-facing (Fig. 1). Amplification was carried out in a Crocodile II thermocycler (Appligene) with the following settings: after denaturing for 2 min at 94°C, 10 cycles of 10 sec at 94°C, 30 s at 65°C and 10 min at 68°C were carried out. After this, 15 cycles of 10 s at 94°C, 30 s at 65°C plus an extension period at 68°C for a time span that was 20 s longer than in the previous cycle, followed. A 7-min incubation at 68°C completed the reaction. The 3.5-kb PCR product was purified on an agarose gel, isolated using a GeneClean Kit (Bio101, La Jolla, CA, USA), and subsequently cloned into pT7Blue(R)T-vector (Novagen, Madison, WI, USA). The resulting plasmid was designated pCBA70 (Fig. 4B).

2.6. ISJP4-mediated mobilization of DNA

To demonstrate ISJP4-mediated mobilization of neighbouring DNA segments, we constructed pUC-based plasmids that contained different copies of ISJP4 flanking a kanamycin (Km) cassette (for restriction maps, see Fig. 5). Plasmid pCBA100 was made by the insertion of a Km cassette on a 1.7-kb *Sal*I fragment of pUT/Km (Herrero et al., 1990) into the *Xho*I site of the 2.7-kb *Pst*I–*Hind*III fragment of pJP4 containing ISJP4 in pUC19. Insertion of a 1.6-kb *Nco*I–*Hind*III fragment of pJP4 with ISJP4 on it into *Pst*I-cut pCBA100 resulted in plasmid pCBA106. Plasmid pCBA107 is identical to pCBA106, but with the inserted ISJP4 in the opposite orientation. For cloning of the piece of ISJP4 DNA residing in the *tfdA*–*tfdS* intergenic region, we first amplified this region in a PCR using a set of specific primers (5'-CCTCCGCTGCAGCAACGAAATAGC-3', located in the coding region of *tfdS* and 5'-AACACCAGCACCGAATTCTCGTCC-3' in the coding region of *tfdA*). The 0.4-kb PCR product was cloned into pT7Blue(R)T-vector, and its DNA sequence was verified. The region containing the ISJP4 border sequence was recovered from this plasmid as a *Pst*I fragment and ligated into *Pst*I-cut pCBA100 to yield plasmid pCBA108. pCBA109 is the same as pCBA 108, but with the ISJP4 border region in the opposite orientation. These plasmids were transformed to *R. eutropha*

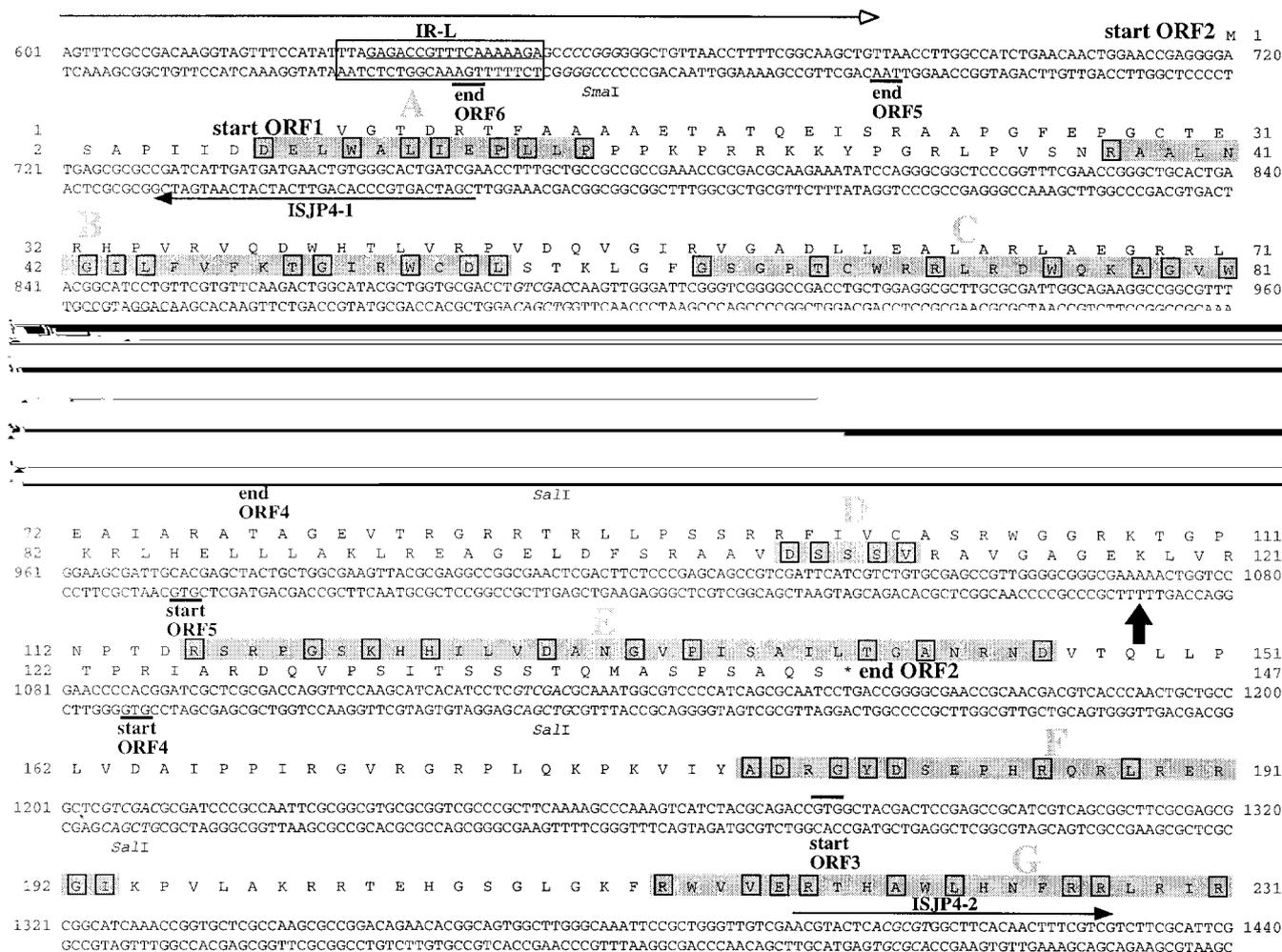


Fig. 1. Nucleotide sequence of ISJP4 copy A and its flanking regions on plasmid pJP4. Bases are numbered relative to the ATG start codon of the *tfdT* gene (Leveau and van der Meer, 1996), whose 3'-end is marked on the sequence with an open-pointed arrow. Restriction sites are marked in italics in and below the sequence. The 21-bp inverted repeats flanking the central core of ISJP4 are boxed; the 18-bp inverted repeat sequences (IR-L, IR-R), which probably represent the true boundaries of ISJP4 (see Discussion), are underlined. The 71-bp stretch that is marked with a grey line at the right-hand extremity is identical to the piece of ISJP4 in the *tfdA-tfdS* intergenic region. The position and orientation of primers ISJP4-1 and ISJP4-2 that were used to isolate ISJP4 copy B are indicated with arrows. The amino-acid sequences of the ORF1 and ORF2 translation products are given above the nucleotide sequence. Also shown are the starts and ends of ORFs 3 to 6. Segments A through G represent regions of the ORF1 and ORF2 products that are conserved (highly conserved residues are highlighted with boxes) in the translation products of two IS5 subclusters of the IS4 family of bacterial insertion sequences (Rezsöhazi et al., 1993). These include IS402 from *Burkholderia cepacia* (Ferrante and Lessie, 1991), IS1237 from *Clavibacter xyli* subsp. *cynodontis* (Laine et al., 1994), IS427 from *Agrobacterium tumefaciens* (pUCD800) (de Meirsmen et al., 1990), IS869 from *A. tumefaciens* (Paulus et al., 1991), IS1301 from *Neisseria meningitidis* (Hammerschmidt et al., 1996), IS1248 from *Paracoccus denitrificans* (van Spanning et al., 1995), IS*Rm4* from *Rhizobium meliloti* GR4 (Soto et al., 1992), IS1031D from *Acetobacter xylinum* (Coucheron, 1993) (representative of IS1031A and IS1031C as well), IS1032 from *A. xylinum* (Iversen et al., 1994), an IS-element from *Mycobacterium tuberculosis* (Mariani et al., 1993) and Tn4811 from *Streptomyces lividans* 66 (Chen et al., 1992). The black arrow at the AAAAA sequence indicates the position of the putative -1 translational frameshift signal (see Discussion).

JMP289 by electroporation as described elsewhere (Taghavi et al., 1994). Aliquots of transformation mixtures were plated on NB plus 50 mg/ml kanamycin after *c.* 1, 3, 17 and 22 h of phenotypic expression at 30°C, and the number of Km^r colonies was determined. Of the transformants with pCBA106 and pCBA108, six were selected (designated JMP289::cba106-1 to -6 and JMP289::cba108-1 to -6, respectively) from which total genomic DNA was isolated and hybridized with probes for ISJP4 and for the Km-cassette.

3. Results

3.1. ISJP4 is related to IS5 group members of the IS4 family of bacterial insertion sequences

Directly downstream of and partially overlapping with the *tfdT* gene on plasmid pJP4 (Leveau and van der Meer, 1996), we identified a 0.9-kb DNA sequence that revealed substantial homology with IS-elements. This sequence was designated ISJP4 and is presented in

Fig. 1. Two 21-bp inverted repeats seemed to form the ends of the element, although the outermost three nucleotides of each repeat could actually have been caused by a complex target site duplication (see Discussion). Six possible open reading frames (ORFs)—with a size of at least 50 codons, and starting with an ATG or GTG—could be identified on the ISJP4 sequence (Fig. 1). Of these, two (i.e., ORF1 and ORF2) encode polypeptides with significant homology to proteins encoded by members of two previously recognized subclusters within the so-called IS5 group of the IS4 family of bacterial insertion sequences (Rezsöházy et al., 1993) (results not shown). A database search with the translation products of ORFs 3 to 6 produced no notable similarity scores. The ISJP4 ORF1 and ORF2 translation products were most similar to those of ORF1 (72% in a 210-aa overlap) and ORF2 (75% in 147 aa), respectively, of IS402 from *Pseudomonas (Burkholderia) cepacia* (Ferrante and Lessie, 1991). The other members of the two IS5 subclusters were clearly less similar to ISJP4 than IS402. We could identify seven stretches of amino-acid sequences (segments A to G) in ORF1 and ORF2 that are relatively well conserved between members of the IS5 subgroups (Fig. 1). Interestingly, whereas some IS-elements had all or most of the seven conserved regions combined in one single protein (ORF832 of IS1032, and the transposases of IS1031D, an IS-element from *Mycobacterium tuberculosis* and Tn4811), for ISJP4, these stretches occurred either in the N-terminal part of the ORF2 protein or in the C-terminal portion of the ORF1 product. This was also the case for IS402 (ORF2 and ORF1), IS1237 (ORF2 and ORF1), IS427 (ORF4 and ORF3), IS869 (ORF3 and ORF1), IS1301 (ORF1 and ORF2), IS1248 (ORF3 and ORF1) and ISRm4 (two transposases) (for references, see legend of Fig. 1).

3.2. ISJP4 occurs as a single complete copy on plasmid pJP4

The newly discovered element ISJP4 on plasmid pJP4 is situated directly next to a cluster of genes coding for chlorophenol degradation, i.e., (*tfdT*-) *tfdCDEF* and *tfdB* (Perkins et al., 1990; Leveau and van der Meer, 1996) (Fig. 2B). We checked whether more copies of ISJP4 existed on plasmid pJP4, perhaps forming a composite transposon. To this end, we hybridized a radioactively labeled *SmaI*–*MluI* internal fragment of ISJP4 to purified plasmid pJP4 DNA that was digested with *Bam*HI, *Eco*RI and *Hind*III. The locations of these sites on the physical map of plasmid pJP4 are known (Don and Pemberton, 1985). In all three cases, the ISJP4 probe hybridized to a single fragment (Fig. 2A): the 12-kb *Bam*HI-3 fragment (lane 1), the 15-kb *Eco*RI-2 fragment (lane 2) and the 22-kb *Hind*III-2 fragment (lane 3). This showed that the region of pJP4

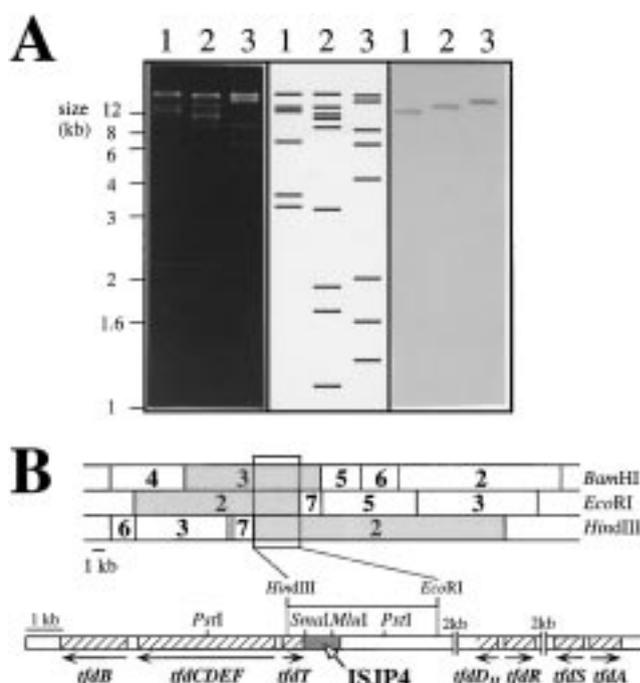


Fig. 2. Hybridization of purified plasmid pJP4 DNA digests with the ISJP4 probe. (A) The left panel shows an agarose gel with different digests of plasmid pJP4. The middle panel is a schematic drawing of all restriction fragments appearing on agarose gel. The right panel shows the resulting autoradiogram after hybridization. Lanes: 1, digested with *Bam*HI; 2, *Eco*RI; 3, *Hind*III. (B) Part of the restriction map of plasmid pJP4 for *Bam*HI, *Eco*RI and *Hind*III [modified from Don and Pemberton (1985)]; the numbering of the *Bam*HI, *Eco*RI and *Hind*III restriction fragments of pJP4 was according to the latter reference. Shaded boxes represent the fragments hybridizing with the ISJP4 probe. A close-up of the approximately 4-kb *Eco*RI–*Hind*III fragment and a genetic map of the *tfd* region are presented at the bottom. The *Sma*I and *Mlu*I sites that were used to generate the ISJP4 probe and the position of the *Pst*I restriction sites directly surrounding ISJP4 are indicated.

that contained ISJP4 was limited to an approximately 4-kb *Eco*RI–*Hind*III fragment (Fig. 2B). According to the map of pJP4, this fragment lies directly upstream of (*tfdT*-) *tfdCDEF*, which is where we discovered ISJP4 originally. In other hybridizations, we found that the ISJP4 probe hybridized to a single 4.8-kb *Pst*I fragment of plasmid pJP4 (Fig. 3A, lanes 16 and 17). This fragment partly overlaps the 4-kb *Eco*RI–*Hind*III fragment of pJP4 (Fig. 2B). DNA sequence analysis downstream of ISJP4 up to the first *Pst*I site did not reveal any additional ISJP4-like sequences (not shown). This indicated that there exists only one complete copy of ISJP4 on pJP4. This copy will be referred to as ISJP4 copy A from here on.

Interestingly, at least one short sequence (71 bp), identical to ISJP4 copy, exists elsewhere on plasmid pJP4 (copy C hereafter). We found a DNA sequence identical to the right-hand extremity of ISJP4 between the *tfdS* and *tfdA* genes on plasmid pJP4 (Figs. 1 and 2B). The sequences surrounding this DNA stretch

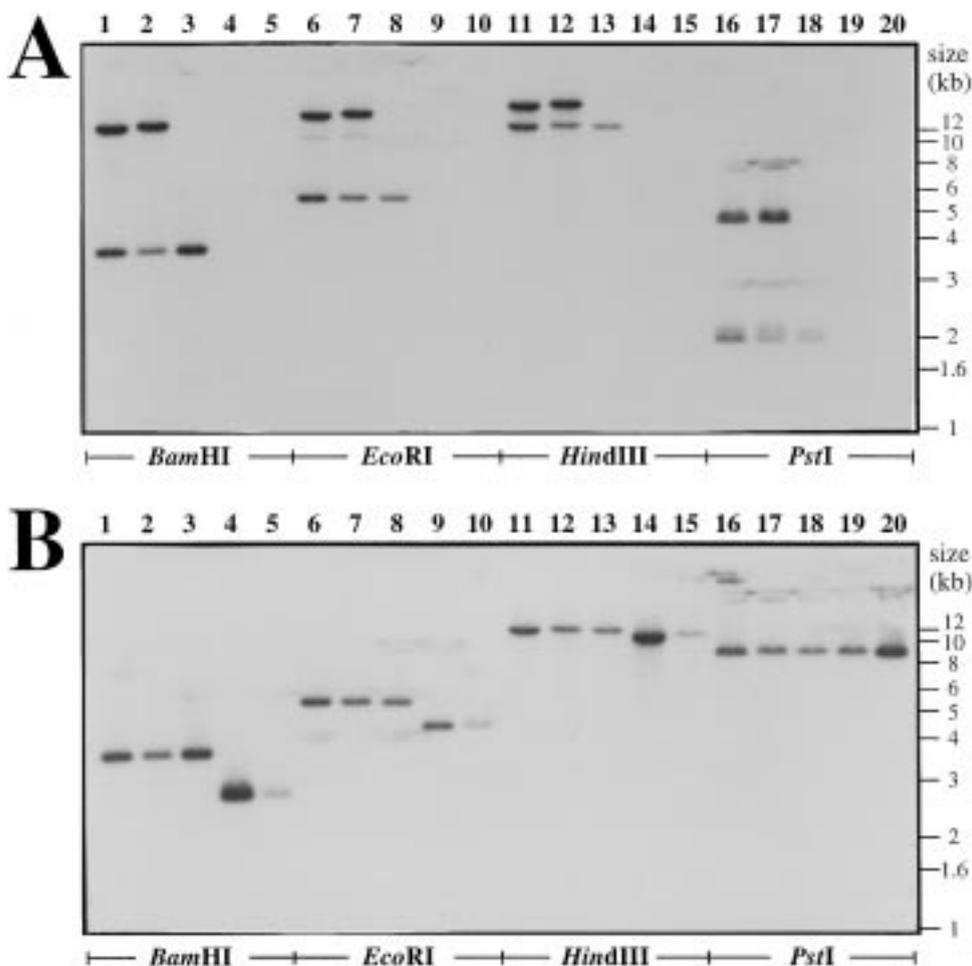


Fig. 3. Hybridization of total DNA digests from different *R. eutropha* strains with the ISJP4 probe (A) or the pCBA70-*PstI*/*BamHI* probe (B). Details on the hybridization procedure are given in Materials and methods. The migration distance of standard-sized DNA fragments is given on the right. Lanes: 1, 6, 11 and 16, total DNA of *R. eutropha* JMP134 (pJP4) grown on 2,4-D; 2, 7, 12 and 17, total DNA of *R. eutropha* JMP134 (pJP4) grown on fructose; 3, 8, 13 and 18, total DNA of *R. eutropha* JMP134 (RP1) grown on fructose; 4, 9, 14 and 19, total DNA of *R. eutropha* JMP222 grown on fructose; 5, 10, 15 and 20, total DNA of *R. eutropha* JMP289 grown on fructose. The DNA in lanes 1 through 5 was digested with *BamHI*, in lanes 6 through 10 with *EcoRI*, in lanes 11 through 15 with *HindIII* and in lanes 16 through 20 with *PstI*.

revealed no resemblance to ISJP4 copy A or to sequences flanking it (not shown).

3.3. A second copy of ISJP4 exists in *R. eutropha* JMP134

We also checked the total genomic DNA of *R. eutropha* JMP134 (pJP4) for the presence of additional ISJP4 copies. This might give us a clue as to whether ISJP4 was able to transpose. In contrast to the plasmid hybridizations, we found two DNA fragments hybridizing for each of the three restriction enzymes when we used the internal ISJP4 probe on digested total DNA from *R. eutropha* JMP134 (Fig. 3A). The 12-kb *BamHI* (upper band in lanes 1 and 2), 15-kb *EcoRI* (upper band in lanes 6 and 7) and 22-kb *HindIII* (upper band in lanes 11 and 12) fragments were restriction fragments of plasmid pJP4 that hybridized with the probe (see

Fig. 2A). The same explanation held for the hybridizing 4.8-kb *PstI* fragment (Fig. 3A, upper band in lanes 16 and 17). However, the additional hybridizing fragments, i.e., 4.0-kb *BamHI* (Fig. 3A, lower band in lanes 1 and 2), 5.8-kb *EcoRI* (lower band in lanes 6 and 7), 13-kb *HindIII* (lower band in lanes 11 and 12) and 2.0-kb *PstI* (lower band in lanes 16 and 17), could not originate from pJP4, since we had not detected them in hybridizations with purified plasmid pJP4 (Fig. 2A). The second copy of ISJP4 (copy B) was then isolated by applying the PCR with ISJP4-outward facing primers (ISJP4-1 and ISJP4-2, Fig. 1). As template, we took *BamHI*-digested and subsequently religated total DNA of *R. eutropha* JMP134 (pJP4). Only recircularized *BamHI* fragments that contained a complete copy of ISJP4 would serve as a template and render a PCR product (Fig. 4A). The PCR product that we obtained had a size of 3.5 kb (not shown), which was what we expected

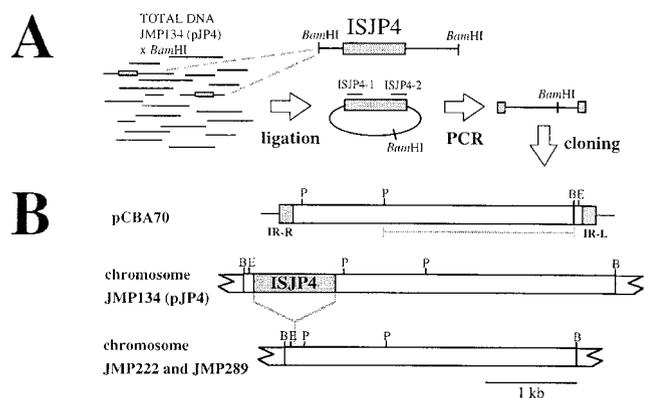


Fig. 4. (A) Graphical presentation of the strategy that was followed to obtain the flanking DNA of the second ISJP4. Details are given in the text. (B) Map of plasmid pCBA70 containing the PCR-product with part of the ISJP4 copy B plus its flanking regions. Relevant restriction sites are indicated (B, *Bam*HI; E, *Eco*RI; P, *Pst*I). The *Pst*I–*Bam*HI fragment that was used as a chromosomal probe is marked with a grey line. Also shown is a reconstruction of the 4.0-kb *Bam*HI chromosomal fragment of *R. etrophia* JMP134 that contained copy B of ISJP4. Depicted below is the same fragment, but lacking ISJP4 copy B, as it occurs in *R. etrophia* JMP222 and JMP289.

for amplification of the recircularized 4.0-kb *Bam*HI fragment containing ISJP4 copy B (Fig. 3A, lanes 1 and 2). Theoretically, we also expected a PCR product from the circularized 12-kb *Bam*HI-3 fragment of plasmid pJP4, but the size of that expected product (11 kb) most probably exceeded the capacity of the *Taq* DNA polymerase in the PCR. The obtained 3.5-kb PCR product was cloned (plasmid pCBA70) and from its restriction map we were able to reconstruct the topography of the original 4.0-kb *Bam*HI fragment (Fig. 4B). DNA sequence analysis of pCBA70 revealed that the expected parts of an ISJP4 copy were indeed present. The amplified ends of this ISJP4 copy B were identical to those of the original copy A on plasmid pJP4, including the inverted repeats. The regions directly outside the inverted repeats were different from those on plasmid pJP4 (partly shown in Fig. 6A). These regions were searched for homologies, and we found a coding region *c.* 475 bp downstream of the IR-R of copy B with significant similarities to methyltransferases of various prokaryotic and eukaryotic organisms (not shown). Next, the 2.1-kb *Pst*I–*Bam*HI fragment of pCBA70 (Fig. 4B), which carried part of the DNA region into which ISJP4 copy B was inserted, to total DNA of *R. etrophia* JMP134. To distinguish between hybridization signals from chromosomally located and plasmid-borne fragments, we also probed the total DNA of three derivative strains of *R. etrophia* JMP134 (pJP4) that had been cured of plasmid pJP4. Two of these had already been isolated previously, i.e., *R. etrophia* JMP222 and JMP289 (Don and Pemberton, 1981). As a third strain, we constructed a pJP4⁻ derivative of the *R. etrophia* JMP134 (pJP4) strain that we use routinely

in our laboratory, by employing the principle of plasmid exclusion. To this end, we mated *R. etrophia* JMP134 (pJP4) with *E. coli* LE392 harbouring plasmid RP1. Plasmids pJP4 and RP1 belong to the same incompatibility group IncP1. Therefore, by selecting for the ability to grow on fructose (held only by *R. etrophia* JMP134) and kanamycin resistance (encoded by plasmid RP1), we were able to isolate an *R. etrophia* JMP134 strain that was cured of plasmid pJP4, but contained RP1.

The total DNA of *R. etrophia* JMP134 (pJP4) that was digested with either *Bam*HI, *Eco*RI, or *Hind*III, showed only a single hybridization signal with the pCBA70 probe in each case (Fig. 3B, lanes 1 and 2, 6 and 7, 11 and 12, respectively). This signal could be attributed to the 4.0-kb *Bam*HI, 5.8-kb *Eco*RI and 13-kb *Hind*III fragments that had hybridized before with the ISJP4 probe and that carry ISJP4 copy B (see Fig. 3A). An identical hybridization pattern was found for *R. etrophia* JMP134 (RP1) (Fig. 3B, lanes 3, 8 and 13). Since this strain was devoid of plasmid pJP4, these hybridization signals must have come from chromosomally located fragments.

3.4. ISJP4 is absent in pJP4-cured strains *R. etrophia* JMP222 and JMP289

The total DNAs of *R. etrophia* JMP222 and JMP289 also gave single hybridizing bands for each of the restriction enzymes *Bam*HI, *Eco*RI and *Hind*III (Fig. 3B). Rather unexpectedly, however, the sizes of these fragments (3.1-kb *Bam*HI in lanes 4 and 5, 4.9-kb *Eco*RI in lanes 9 and 10 and 12-kb *Hind*III in lanes 14 and 15) were smaller than those observed for the other two strains. Interestingly, the difference in size was always 0.9 kb, which is about the size of element ISJP4. It therefore appeared to us that strains JMP222 and JMP289, unlike *R. etrophia* JMP134 (pJP4) and its direct derivative *R. etrophia* JMP134 (RP1), did not have a copy of ISJP4 on their chromosome. Hybridization of *Pst*I-digested total DNAs to the pCBA70 probe revealed an identical 9-kb fragment for all four strains (Fig. 3B; lanes 16 through 20), indicating that this region on the chromosomes of all *R. etrophia* derivatives was not disturbed. This would be expected, since this probe was part of a chromosomal *Pst*I fragment outside ISJP4 of *R. etrophia* JMP134 (pJP4) or JMP134 (RP1) (Fig. 4B).

Hybridizations of total DNAs from *R. etrophia* JMP222 and JMP289 to the ISJP4 probe showed no signal (Fig. 3A, lanes 4 and 5, 9 and 10, 14 and 15, 19 and 20), whereas the ISJP4-containing fragments of *R. etrophia* JMP134 (RP1) [namely the 4.0-kb *Bam*HI (Fig. 3A, lane 3), 5.8-kb *Eco*RI (lane 8), 13-kb *Hind*III (lane 13) and 9-kb *Pst*I (lane 18) fragments] hybridized positively.

3.5. ISJP4 can mobilize neighbouring DNA sequences as a composite transposon

The finding of ISJP4 copy C suggested that perhaps at one time, a complete ISJP4 existed at this position on pJP4, which, together with copy A, might have formed a composite transposon that mediated the mobilization of the intervening pJP4 DNA. Since copy C contains an intact right-hand repeat (IR-R) sequence, it may actually still be part of such a composite transposon. To test ISJP4-mediated mobility of DNA sequences, we transformed strain JMP289 with a set of suicide plasmids (Fig. 5) that contained a kanamycin resistance cassette flanked by either one copy of ISJP4-A (pCBA100), two directly oriented copies of ISJP4-A (pCBA106), two invertedly oriented copies of ISJP4-A (pCBA107), one copy A and one copy C in the orientation as on pJP4 (pCBA108), or one copy A and one copy C in the opposite orientation (pCBA109). Only by transposition of the cassette to the chromosome of JMP289, could Km resistance be conferred. Fig. 5 summarizes the results of a typical transformation experiment (see legend for details). We obtained a fair number of Km^r colonies for pCBA106, pCBA107 and pCBA108. With plasmid pCBA109, no transformants were obtained, whereas only one colony appeared after transformation with pCBA100. A control transformation without the addition of DNA gave no colonies, whereas as positive control transformation pKT230 was applied. By plating on non-selective NB plates, we found that the rate of survival of JMP289 after electrotransformation was the same for all the pCBA constructs (results not shown). With the Km cassette trapped between two direct copies of ISJP4 (pCBA106), the number of trans-

formants was slightly higher than when the two ISJP4 copies occurred as an inverted repeat (pCBA107), but this was not considered to be significant. With pCBA108, which contains only one intact copy of ISJP4 plus copy C on the *tfdA-tfdS* intergenic region, the transformation frequency was clearly higher. The proper orientation of copy C was crucial for transposition: with plasmid pCBA109, no transformants were obtained. The single transformant with pCBA100 may be the result of a simple transposition of ISJP4 upstream of the Km cassette, followed by a complex transposition of the whole DNA region.

To verify the nature of these transpositions, total genomic DNA from six colonies of the pCBA106-transformation (referred to as JMP289::cba106-1 to -6) and six colonies of the pCBA108-transformation (JMP289::cba108-1 to -6) was *Pst*I- or *Eco*RI-digested and hybridized with radioactively labeled probes for ISJP4 and for the Km cassette (Fig. 6). DNA from JMP289::cba106 strains, which was cut with either *Pst*I or *Eco*RI, gave two hybridization signals with the ISJP4 probe for strain numbers 1, 3, 4, 5 and 6 (Fig. 6A), and one with the Km-probe, as expected (Fig. 6B). This band corresponded to one of the ISJP4-hybridizing fragments, either the one with the right copy of ISJP4 (*Pst*I-digestion) or the one with the left copy (*Eco*RI-digestion) (left and right according to Fig. 5). The sizes of hybridizing fragments were different between transformants, indicating that transposition had occurred at different locations. Furthermore, the hybridizing fragments had a size larger than the expected minimal sizes of 1.3 and 3.8 kb for the left and right *Pst*I fragments, and 3.5 and 1.6 kb for the left and right *Eco*RI fragments, respectively (Fig. 5), if one assumes the borders

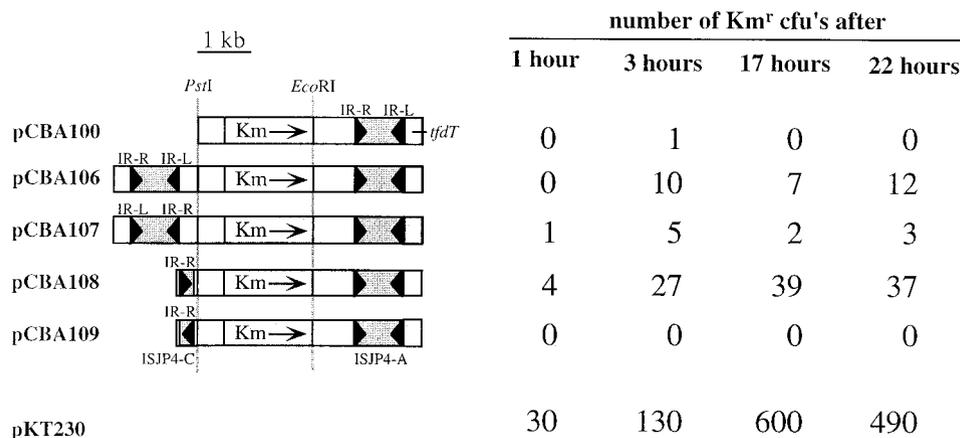


Fig. 5. Transformation of *R. eutropha* JMP289 with plasmids containing a Km cassette and different conformations of ISJP4. On the left, the restriction maps of plasmid pCBA100, pCBA106, pCBA107, pCBA108, and pCBA109 are given. 0.5 mg of purified plasmid DNA was used to electrotransform 5×10^8 JMP289 cells (see Materials and methods). After 1, 3, 17, and 22 h of incubation at 30°C, a 1/4 volume aliquot of the electrotransformation mixtures was plated on NB plates supplemented with 50 µg/ml of kanamycin. The number of Km^r colonies that arose from a particular time aliquot is given in the table on the right. As a control, we transformed JMP289 with 0.5 mg of plasmid pKT230, which confers kanamycin resistance to, and replicates in, JMP289 (bottom line).

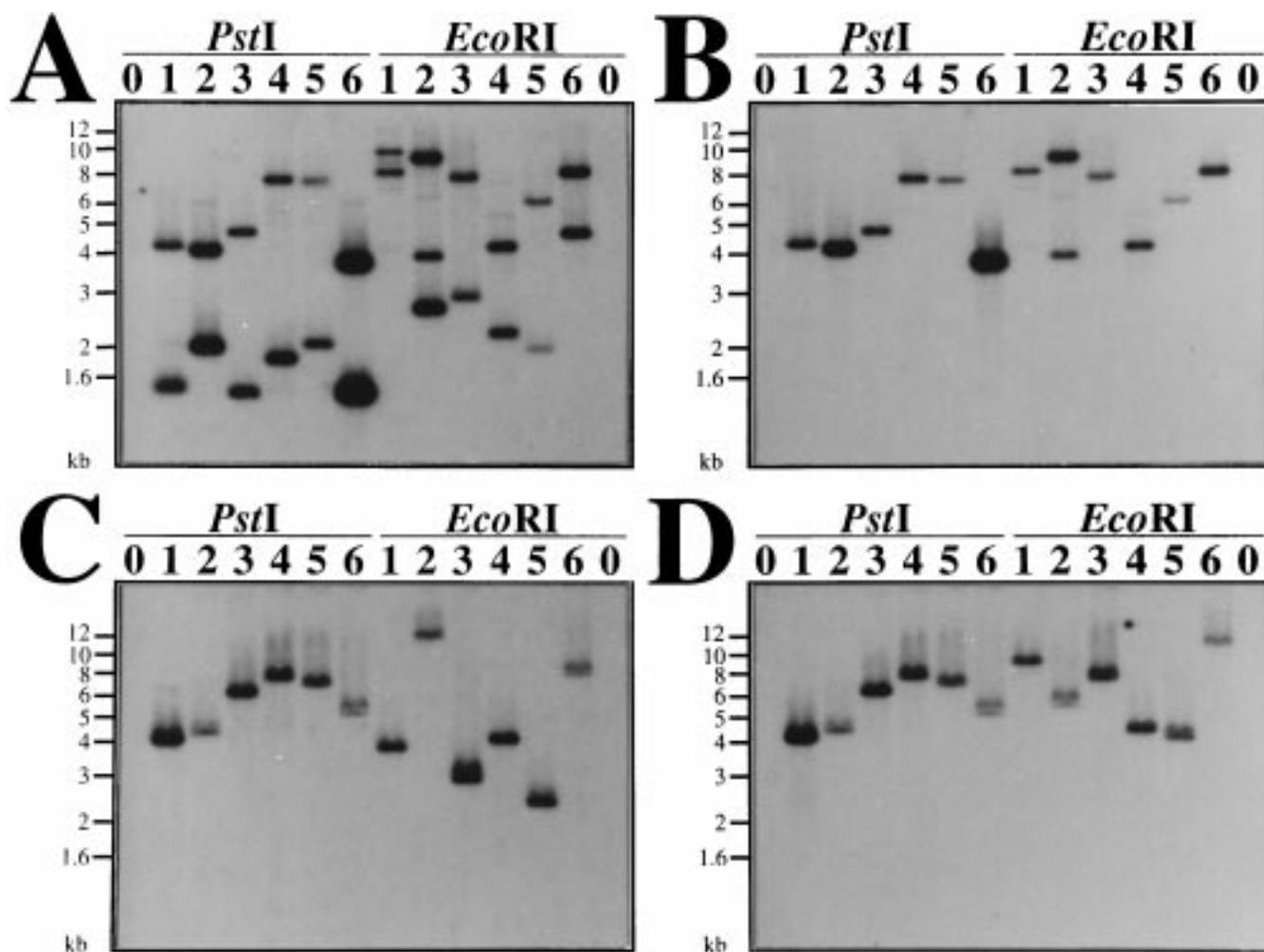


Fig. 6. Hybridization of *Pst*I- or *Eco*RI-digested total DNA from JMP289::cba106-1 to -6 (A and B, 1 to 6) or JMP289::cba108-1 to -6 (C and D, 1 to 6) with a probe for ISJP4 (A and C) or the Km cassette (B and D). The migration distance of standard-sized DNA fragments is given. Lanes that are labeled with 0 contain DNA from host strain JMP289.

of the transposed DNA to be contained within IR-R of the left copy and IR-L of the right copy of ISJP4. It seemed that a multiple transposition had taken place in JMP289::cba106-2. *Eco*RI-digested total DNA gave three hybridizing bands with the ISJP4 probe, two of which also hybridized with the Km probe. As for the JMP289::cba108 strains, hybridization of *Pst*I- or *Eco*RI-digested DNA with the ISJP4 probe gave only one hybridization signal (Fig. 6C). The corresponding fragment should include the right, complete copy of ISJP4 (Fig. 5) and indeed had sizes larger than the minimal size of 3.8 (for *Pst*I) or 1.6 (for *Eco*RI) kb. For all strains, the *Pst*I fragment that hybridized with the ISJP4 probe also did so with the Km probe, again as expected (Fig. 5). With the *Eco*RI-digested DNAs, a different pattern was found with the Km probe (Fig. 6D); the corresponding fragments had sizes larger than the expected minimal size of 2.5 kb. As for pCBA106, transposition from pCBA108 seemed to have occurred at different locations on the chromosome.

4. Discussion

In this paper, we report the discovery of element ISJP4 on plasmid pJP4 from *R. eutropha* JMP134. Its sequence bears the typical features of a prokaryotic IS-element. The translation products of the overlapping ORF2 and ORF1 on ISJP4 indicate seven stretches of amino-acid sequences that are conserved in transposases encoded by various members of the IS5 group. Two of these regions, segments F and G, actually correspond to the N3 and C1 signature sequences for the IS4 family (Rezsöhazy et al., 1993). Furthermore, the spacing between regions N3 and C1 of the ISJP4 ORF1 translational product is typical of members of the IS5 group within this family. Both N3 and C1 have been shown to be similar to regions of the integrases of many retroelements, and are presumed to be essential for the transposition reaction (Rezsöhazy et al., 1993). Segments A, B and E have previously been recognized to be conserved between some IS5 group members

(Iversen et al., 1994). However, no function has yet been ascribed to these regions, or to segments C and D. Interestingly, segment B encompasses a stretch of amino acids (GILVVFKT) similar to the ATP/GTP binding site motif A ([A,G]X₄GK[S,T]) of several bacterial helicases (Hodgman, 1988).

ORF2 and ORF1 of ISJP4 are overlapping in a large region, yet placed in different reading frames. For some IS5 group members with a similar organization, like IS402, IS427 and IS869, it has been proposed that translational frameshifting would fuse the two ORFs and render one single protein in which all conserved regions are present (Iversen et al., 1994). Translational frameshifting is considered to be an important mechanism by which the transposition frequency of IS-elements is controlled (Galas and Chandler, 1989), and was shown to occur for several IS-elements and

viruses (reviewed in Chandler and Fayet, 1993). We did not find a frameshift signal such as the proposed hepta-nucleotide of the type Y YYX XXZ (with Y, X and Z being any nucleotide) (Chandler and Fayet, 1993) on the DNA sequence of ISJP4 ORF2. However, there is a stretch of 5 A-residues (GAA AAA CTG, whose position on the ISJP4 DNA is marked in Fig. 1), which resembles a so-called non-heptanucleotide frameshift signal (Chandler and Fayet, 1993).

ISJP4 is active and has recently undergone a simple transposition event: our laboratory strain of *R. eutropha* JMP134 (pJP4) possessed an extra, chromosomally located copy of ISJP4, whereas strains *R. eutropha* JMP222 and JMP289, two pJP4-cured derivatives of JMP134, lacked such a copy. Presumably, the originally isolated *R. eutropha* JMP134 (pJP4) (Don and Pemberton, 1981) contained no chromosomal copy of

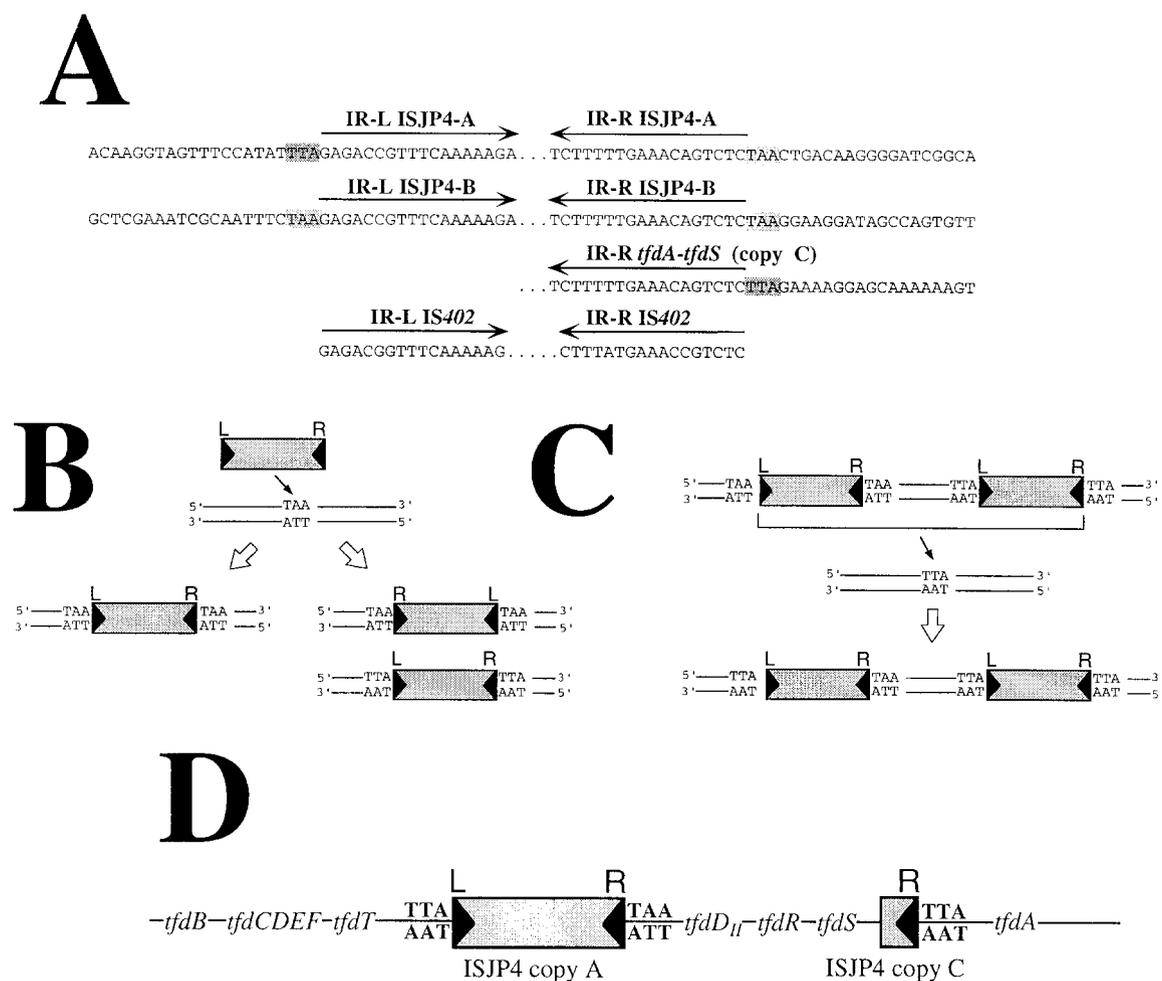


Fig. 7. (A) Comparison of the flanking sequences of ISJP4 copies A and B, and the ISJP4-duplicated region in the *tfdA-tfdS* intergenic region. Inverted repeats are indicated by arrows. Possible target site duplications, as discussed in the text, are shaded. Also given are the IR-L and IR-R boundaries of IS402 (Ferrante and Lessie, 1991). (B) Single insertion event of ISJP4. The proposed target site TAA (or complementary TTA) appears either as a TAA or as a TTA direct repeat after insertion. ISJP4 DNA is represented by grey boxes. Inverted repeats IR-L and IR-R are drawn as black triangles. (C) Transposition and target duplication by a hypothetical composite transposon consisting of two copies of ISJP4. Note that after transposition, the left copy of ISJP4 is flanked by divergently oriented TAA trinucleotides (as was found for ISJP4 copy A on plasmid pJP4). (D) Location and orientation of ISJP4 DNA and their flanking trinucleotides on plasmid pJP4 relative to the *tfd* genes (not drawn to scale).

ISJP4; only after strains JMP222 and JMP289 were derived by curing JMP134 of plasmid pJP4, did ISJP4 on pJP4 in *R. eutropha* JMP134 undergo transposition, inserting a copy of itself into the chromosome of its host strain. Consequently, this event must have taken place recently during storage or cultivation of strain JMP134 in the laboratory.

By aligning the outside ends of copies A and B and the flanking DNA regions, we found that the largest possible inverted repeat (with one mismatch) that is shared between the two copies is 19 bp in size and starts with 5'-AGAGAC...-3' (Fig. 7A). This would be in agreement with the first three 5' nucleotides of other IRs from IS5 group members (Rezsöházy et al., 1993), and especially those of element IS402 (Fig. 7A). The TAA direct repeats flanking copy B and the TTA-TAA inverted repeats flanking copy A would be the evidence for the target site duplication, either by a single insertion event or by multiple transpositions (Galas and Chandler, 1989). So far, most IS5 members have a preference for TAA, TTA or TGA targets (Coucheron, 1993; de Meirsmen et al., 1990; Ferrante and Lessie, 1991; Iversen et al., 1994; Laine et al., 1994; van Spanning et al., 1995)

ISJP4, as it resides on plasmid pJP4, is probably part of a composite transposon, formed by copy A and, 9 kb away from copy A in the *tfdS-tfdA* intergenic region, a 71-bp incomplete copy of ISJP4. This copy still has the IR-R sequence, flanked by a 5'-TTA-3' trinucleotide, just like IR-L of copy A (Fig. 7A and D). Perhaps it arose from a previous insertion of copy A, which resulted in the 5'-TTA-3' repetition, as predicted from a double insertion (Fig. 7C). We provided evidence that ISJP4 copy A and C are actually still functional as a composite transposon. However, the orientation of copy C is very important. Composite transposition only occurred when the IR-R of copy C was located in the opposite direction from the IR-L of ISJP4 copy A. The putative transposase protein encoded by copy A may act on the IR-L of copy A, and use the properly oriented IR-R of copy C instead of that of copy A (Fig. 7D). A somewhat similar situation is known for transposon Tn10, which is about 9.3 kb in size and flanked by a functional (IS10-Right) and non-functional (IS10-Left) IS copy (Kleckner, 1989). The non-functionality of IS10-Left is the result of an inactivated transposase gene. However, the functional transposase specified by IS10-Right can act on the extremity of IS10-Left to promote transposition of the c. 6.7-kb DNA fragment in between the two elements.

Was ISJP4 involved in shaping the 2,4-D catabolic gene organization on plasmid pJP4? DNA rearrangements, such as duplications and deletions, are common features of IS-elements (Galas and Chandler, 1989) and their involvement in horizontal gene transfer is undebatable (van der Meer et al., 1992). A previous comparison of plasmids pJP4 and pEMT1 (Top et al., 1995) showed

that the latter plasmid harbours similar *tfd* genes to pJP4, and in a similar organization, except that pJP4 seems to have approximately 9 kb of additional DNA between the *tfdA* and *tfdCDEF* genes (Top et al., 1995). This seems exactly the size of the fragment encompassed by ISJP4 copy A and IR-R of copy C (Fig. 7D). It may indicate that ISJP4 was involved in the transposition of this DNA to pJP4. Perhaps, in the process of doing so, it became partially deleted, leaving but a shred of itself in the *tfdS-tfdA* intergenic region.

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