Molecular characterization of a deletion/duplication rearrangement in *tfd* genes from *Ralstonia eutropha* JMP134(pJP4) that improves growth on 3-chlorobenzoic acid but abolishes growth on 2,4-dichlorophenoxyacetic acid

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***Ralstonia eutropha* JMP134(pJP4) is able to grow on minimal media containing the pollutants 3-chlorobenzoate (3-CB) or 2,4-dichlorophenoxyacetate (2,4-D). *tfd* genes from the 88 kb plasmid pJP4 encode enzymes involved in the degradation of these compounds. During growth of strain JMP134 in liquid medium containing 3-CB, a derivative strain harbouring a ~95 kb plasmid was isolated. This derivative, designated JMP134(pJP4-F3), had an improved ability to grow on 3-CB, but had lost the ability to grow on 2,4-D. Sequence analysis of pJP4-F3 indicated that the plasmid had undergone a deletion of ~16 kb, which included the *tfdA*–*tfdS* intergenic region, spanning the *tfdA* gene to a previously unreported IS1071 element. The loss of the *tfdA* gene explains the failure of the derivative to grow on 2,4-D. A ~23 kb duplication of the region spanning *tfdR*–*tfdD*_{II}–*E*_{II}–*F*_{II}–*tfdB*_{II}–*tfdK*–ISJP4–*tfdT*–*tfdC*_I–*D*_I–*E*_I–*F*_I–*tfdB*_I, giving rise to a 51-kb-long inverted repeat, was also observed. The increase in gene copy number for the *tfdCD(DC)EF* gene cluster may provide an explanation for the derivative strain's improved growth on 3-CB. These observations are additional examples of the metabolic plasticity of *R. eutropha* JMP134, one of the more versatile pollutant-degrading bacteria.**

Keywords: chloroaromatics, catabolic plasmid, IS1071 insertion sequence, chlorocatechol pathway

INTRODUCTION

Bacteria play a fundamental role in the degradation of chloro-organic pollutants. *Ralstonia eutropha* JMP134(pJP4) degrades several chloroaromatic compounds through chromosomal (Clément *et al.*, 1995) or plasmid (pJP4)-encoded functions (Don & Pemberton, 1981; Pieper *et al.*, 1988). Plasmid pJP4 is an 88 kb, self-transmissible, broad-host-range, catabolic plasmid belonging to the IncPβ incompatibility group (Don & Pemberton, 1981; Smith & Thomas, 1987). pJP4 con-

tains genes (*tfd*) encoding enzymes involved in the catabolism of 2,4-dichlorophenoxyacetate (2,4-D) and 3-chlorobenzoate (3-CB), which have been intensively studied. The *tfd* genes are located in a 20-kb-long region surrounded by a conserved IncPβ plasmid backbone (Burlage *et al.*, 1990). The organization of the *tfd* genes is complex (Fig. 1). The *tfdA* gene encodes a dioxygenase that converts 2,4-D to 2,4-dichlorophenol (Fukumori & Hausinger, 1993). The *tfdB* gene encodes a 2,4-dichlorophenol monooxygenase that hydroxylates 2,4-dichlorophenol to 3,5-dichlorocatechol (Perkins *et al.*, 1990). Two different gene clusters, *tfdC*_I*D*_I*E*_I*F*_I and *tfdD*_{II}*C*_{II}*E*_{II}*F*_{II} (Fig. 1), encode enzymes for the catabolism of chlorocatechols to 3-oxoadipate. The enzymes from both modules are active during degradation of 2,4-D (Leveau *et al.*, 1999; Laemmli *et al.*, 2000) and 3-CB (Pérez-Pantoja *et al.*, 2000). Two

Abbreviations: 3-CB, 3-chlorobenzoate; 2,4-D, 2,4-dichlorophenoxyacetate.

The GenBank accession numbers for the 3115 nt *Bam*HI–F and 2833 nt *Eco*RI–F fragments of pJP4 and the 4037 nt *Eco*RI–E' fragment of pJP4-F3 are AF225972, AF225973 and AF225974, respectively.

Fig. 1. Schematic diagram of the *tfd* catabolic region of pJP4. The approximate position of *tfd* genes, the ISJP4 insertion sequence, and the *Eco*RI, *Hind*III, *Bam*HI and *Kpn*I restriction sites are indicated. Black and white bars correspond to previously sequenced and unsequenced regions of the *Bam*HI internal part of the *Eco*RI-E fragment, respectively. Not to scale.

identical regulatory genes, *tfdR* and *tfdS*, have also been described (Matrubutham & Harker, 1994).

During the course of a study of the role of *tfd* genes in the degradation of 3-CB (Pérez-Pantoja *et al.*, 2000), a derivative of strain JMP134 was isolated that exhibited improved growth on 3-CB but failed to grow on 2,4-D. This derivative (JMP134-F3) harbours a larger ~95 kb form of pJP4, designated pJP4-F3, the molecular characterization of which is reported here.

METHODS

Bacterial strains and growth conditions. *R. eutropha* JMP134(pJP4) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and was routinely grown on solid minimal medium containing 2 mM 2,4-D or 3-CB (Kröckel & Focht, 1987). *Escherichia coli* XL-1 Blue (Bullock *et al.*, 1987) was used for cloning and sequencing. Cultures of *R. eutropha* and its derivatives were grown at 30 °C. *E. coli* cultures were incubated at 37 °C. Bacterial matings were performed on Luria-Bertani (LB) plates as described by Clément *et al.* (2000). Growth rates were calculated from the slopes of the corresponding growth (OD₆₆₀ versus time) curves.

DNA manipulation. Screening for plasmid pJP4 was carried out by the procedure described by Kado & Liu (1981). Plasmid DNA was visualized after electrophoresis on agarose gel (0.8%, w/v, in electrophoresis running buffer containing 40 mM Tris/acetate and 1 mM EDTA, pH 8.0) for 5 h at a constant voltage of 70 V. pJP4 DNA suitable for restriction and Southern analysis was prepared from *E. coli* XL-1 Blue derivatives obtained after conjugative transfer of the wild-type plasmid or its derivative, pJP4-F3, from *R. eutropha* JMP134 (Clément *et al.*, 2000), using the Spin Miniprep Kit (Qiagen). Total cellular DNA preparations were made using the Wizard genomic DNA purification kit (Promega). Restriction, ligation and dephosphorylation reactions and the electroporation of DNA were performed by standard procedures (Ausubel *et al.*, 1992).

Southern blots of pJP4 DNA fragments (obtained after digestion with *Hind*III, *Eco*RI or *Bam*HI and separation on a 0.7% agarose gel) were made using Hybond N+ membranes (Amersham Pharmacia Biotech). Probes were labelled using the BioNick labelling system and hybridization was detected with the PhotoGene nucleic acid detection system (Gibco-BRL), as recommended by the supplier. An 801 bp *Sty*I fragment of the *tfdA* gene was used as a probe (Holben *et al.*,

1992). The remaining probes were prepared from the PCR amplification products cloned in pGem-T Easy (Promega). The IS1071-AB and IS1071-AC probes were obtained with primer pairs IS1071-A (GGGGTCTCCTCGTTTTTCAGTCAA) and IS1071-B (CTTTGAGATATAAAGCTTGACAGCT), and IS1071-A and IS1071-C (GATCCAGAAAGCTGCCAGTTGAAG), respectively (Xia *et al.*, 1998). Detection of the intergenic region between *tfdA* and *tfdS* was carried out by PCR amplification using primer pairs PR33 (GCCGCGCTATTCTGTCCCTTCCCG, base pairs 984–1008; GenBank accession no. S80112) and RC3 (TCGACCCCTGCGGCG, base pairs 783–797; GenBank accession no. M16730). The conditions for the PCR were as follows: 95 °C for 5 min, 52 °C for 1.5 min and 72 °C for 3.5 min; 32 cycles of 95 °C for 1.5 min, 55 °C for 1 min and 72 °C for 3 min; and finally 72 °C for 10 min. The final concentrations were 0.5 pM for the primers, 250 µM for the dNTPs, 2 mM MgCl₂ and 0.5 U *Taq* polymerase per 10 µl reaction. PCR amplification for the generation of IS1071 probes was performed as described by Xia *et al.* (1998). The reactions were carried out in a MiniCycler (MJ Research).

Sequence analysis. Subcloning of fragments for sequencing was carried out in pBlueScript II SK(+/-). Nucleotide sequencing of both DNA strands was carried out using a PRISM sequencing kit (Perkin-Elmer) with double-stranded DNA templates in the presence of 5% DMSO. Samples containing fluorescence-labelled dideoxynucleotide terminators were processed on a 373 stretch automated sequencer (Applied Biosystems). Sequences were compiled and analysed using DNASTAR software (DNASTAR). The computational resource of the National Centre of Biotechnology Information was used through the BLASTX software facilities.

Enzyme assays. *R. eutropha* JMP134 and its derivatives were grown for 24 h on liquid minimal medium containing 2 mM 2,4-D or 3-CB. A 100 ml aliquot of each culture was harvested at the end of the exponential growth phase by centrifugation for 15 min at 7000 r.p.m. in a Beckman J2-21 centrifuge, washed twice in minimal medium and resuspended in 5 ml 50 mM Tris/acetate, pH 7.5. Cells were disrupted by sonication (Sonics & Materials) four times for 30 s at 90% of the maximum output, and the soluble protein fraction was obtained after 1 h centrifugation at 130000 g in a Beckman L-80 ultracentrifuge. Cell extracts (0.1–5.0 mg protein ml⁻¹) were assayed without further purification. Chlorocatechol 1,2-dioxygenase activity was assayed and quantified as described elsewhere (Pérez-Pantoja *et al.*, 2000). Protein determinations were performed as described by Bradford (1976).

Nucleotide sequence accession numbers. The GenBank accession numbers for the 3115 nt *Bam*HI-F and 2833 nt

*Eco*RI-F fragments of pJP4 and the 4037 nt *Eco*RI-E' fragment of pJP4-F3 are AF225972, AF225973 and AF225974, respectively.

RESULTS

Derivatives of *R. eutropha* JMP134 growing on 3-CB have an enlarged pJP4 plasmid

R. eutropha JMP134 is routinely transferred on solid medium containing 2,4-D or 3-CB without there being any change in the electrophoretic mobility of the pJP4 plasmid. However, when strain JMP134 was grown in liquid minimal medium containing 3 mM 3-CB, an enlarged (~95kb) pJP4 form was detected by gel electrophoresis (data not shown). To study the frequency of this phenomenon, liquid cultures of strain JMP134 in minimal medium containing 3-CB or 2,4-D were subcultured to the same fresh medium every 2 d, then plated on LB agar media to isolate colonies. After three subcultures in 3 mM 3-CB, 30 % of colonies (of 50 analysed each transfer) showed the presence of the enlarged plasmid. After five subcultures, more than 90 % of colonies possessed the enlarged plasmid. When strain JMP134 was subcultured to liquid medium containing 2 mM 3-CB, 5 and 25 % of colonies showed the enlarged plasmid after three and five subcultures, respectively. In contrast, no colonies harbouring the enlarged pJP4 were detected after eight subcultures to liquid medium containing 3 mM 2,4-D. Interestingly, all colonies possessing the enlarged plasmid were unable to grow on 2,4-D, either in liquid medium containing 1–3 mM 2,4-D or on solid medium containing 2 mM 2,4-D. All such colonies gave no PCR product when tested for the presence of an *IS1071* sequence (see below), using primer pair *IS1071A/C*. One of the colonies harbouring the enlarged pJP4 form was designated JMP134-F3 and was selected for further analysis. All cells harbouring the enlarged pJP4 plasmid were unable to grow on 2,4-D medium, but grew two times faster than the wild-type on liquid cultures with 3 mM 3-CB, and showed higher chlorocatechol 1,2-dioxygenase (TfdC) specific activity in crude extracts (Table 1).

Restriction profiles: comparison of the wild-type and enlarged pJP4 plasmids

*Eco*RI, *Hind*III and *Bam*HI restriction profiles were determined for plasmid JP4-F3 from strain JMP134-F3, and were observed to be different from those for wild-type pJP4, and strikingly similar to those reported previously for plasmid pYG2, another pJP4 derivative (Ghosal *et al.*, 1985). *Eco*RI fragments C, E, F, H and I, as well as the *Hind*III-B, -D, -E and -H and *Bam*HI-B fragments of pJP4, were absent in pJP4-F3 (see Fig. 2a for orientation). Some new fragments were clearly visible in pJP4-F3, i.e. *Eco*RI-C', *Eco*RI-E', *Hind*III-B' and *Hind*III-D' (see Fig. 2b for orientation). Ghosal & You (1988) reported that the *Eco*RI-C band of pYG2 corresponded to two fragments, the wild-type *Eco*RI-D (10.2 kb), and a distinct 10.2 kb *Eco*RI fragment, which hybridized with the wild-type *Eco*RI-E fragment (see Fig. 2a, b for orientation). Similarly, the 10.2 kb band of *Eco*RI-digested pJP4-F3 DNA was shown to consist of two fragments. Cloning of the 10.2 kb DNA band into pUC19 resulted in two kinds of clones. One of these contained an insert without a *Kpn*I restriction site, as the wild-type *Eco*RI-D fragment. The other insert, designated *Eco*RI-C' in Fig. 2b, exhibited one *Kpn*I restriction site corresponding to that present in the wild-type *Eco*RI-E fragment (Fig. 1).

Comparison of the sequences of *Eco*RI fragments from strains JMP134 and JMP134-F3, containing the *tfdR/S* regulatory region

The *Eco*RI-C' fragment of pJP4-F3 should contain the regulatory genes *tfdR* and *tfdS* of the *Eco*RI-E fragment of pJP4 (Fig. 1). As strain JMP134-F3 exhibited different growth characteristics and chlorocatechol 1,2-dioxygenase activity with respect to wild-type strain JMP134 (Table 1), a more detailed study of the wild-type and the pJP4-derivative fragments containing the regulatory genes was carried out. The 8.3 kb *Eco*RI-E fragment from pJP4 and the 10.2 kb *Eco*RI-C' fragment from pJP4-F3 were cloned in pUC19, yielding plasmids pE83 and pEF102, respectively (Fig. 2a, b). Since a significant part of the wild-type *Eco*RI-E fragment has already been

Table 1. Relative growth rate and chlorocatechol 1,2-dioxygenase (CC 1,2-DO) specific activity for *R. eutropha* strains JMP134 and JMP134-F3 growing on 2,4-D or 3-CB

Strain	Growth substrate	Relative growth rate*	CC 1,2-DO specific activity (U mg ⁻¹)†
JMP134	2,4-D	1.0	0.63 ± 0.05
JMP134	3-CB	1.0	0.38 ± 0.09
JMP134-F3	2,4-D	0.0	NA
JMP134-F3	3-CB	2.0	1.72 ± 0.26

* Growth was tested for up to 5 d. Values are means of two independent experiments. Growth rates for strain JMP134 in 2,4-D and 3-CB were 0.24 h⁻¹ and 0.31 h⁻¹, respectively.

† Values are means of three independent experiments carried out with crude extracts from cells grown on 2,4-D or 3-CB as sole carbon and energy source. NA, Not applicable.

Fig. 2. (a, b) Comparison of the *EcoRI*, *BamHI* and *HindIII* physical and genetic maps (not to scale) of pJP4 (a) and pJP4-F3 (b), based on published maps (Don & Pemberton, 1985; Ghosal *et al.*, 1985) and this work. The approximate positions of the *tfd* and *mer* genes and the ISJP4, IR-R-ISJP4, IS1071 and IS1071-like elements are shown. The double-headed, solid arrow on the pJP4 map corresponds to sequences that are not present in pJP4-F3. The double-headed, solid arrow on the pJP4-F3 map corresponds to duplicated sequences. The relevant clones used in this study from the wild-type (a) or the rearranged pJP4 derivative (b) are shown as black bars. (c) Strategy used to sequence relevant subclones from pEB35, pEF102, pHEF41 and pHE30. Abbreviations: B, *BamHI*; E, *EcoRI*; H, *HindIII*; Bg, *BglII*; S, *SacI*; K, *KpnI*; C, *Clal*; N, *NotI*; Sm, *SmaI*; X, *XhoI*; Xb, *XbaI*; P, *PstI*. (d) Enlargement of part of the *HindIII*-D' fragment of pJP4-F3 containing the junction region. The black and white bars correspond to pJP4-F3 and pJP4 sequenced DNA, respectively. The hatched region in pHE30 corresponds to the region where the homology to pHEF41 is lost. The positions of the ORFs are depicted.

sequenced (Leveau & van der Meer, 1996; You & Ghosal, 1995), only the *BamHI*-F internal fragment of the *EcoRI*-E fragment, containing sequences not yet

reported (Fig. 1), was analysed. The *BamHI*-F DNA fragment was cloned into pBlueScript, giving pEB35 (Fig. 2a), and a number of subclones for sequencing were

obtained from that plasmid, as well as from plasmid pEF102 (Fig. 2c). The sequence of the *Bam*HI insert in pEB35 showed the presence of part of the two inverted repeats containing the *tfdS* and *tfdR* genes, and a 1277-bp-long non-repeated sequence (GenBank accession no. AF225972). When the sequence of the *Eco*RI-C' DNA in pEF102 was compared with the wild-type *Eco*RI-E sequence, the *Bam*HI-F and *Bam*HI-E' fragments were found to be identical. Surprisingly, the two sequences flanking *Bam*HI-E' in pEF102 (*Bam*HI-D' in Fig. 2b) were found to be identical but inverted, and also identical to DNA corresponding to *Bam*HI-E in pE83 (Fig. 2a). This indicates that a mirror copy of the *tfdR*-*tfdD*_{II} region in pJP4-F3 had replaced the *tfdA*-*tfdS* region of the wild-type, producing a large inverted repeat. Further details of this duplication are given below. This information, along with the data from the restriction profile of pJP4-F3 (Fig. 2b), indicates that the deletion includes the wild-type fragments *Eco*RI-C, -H and -I, and parts of the fragments *Eco*RI-E and -F (double-headed solid arrow in Fig. 2a). The absence of the *tfdA*-*tfdS* intergenic region in pJP4-F3 was further confirmed by the fact that the expected 1.1 kb PCR product obtained using primer pair PR33 and RC3 could be amplified only from pE83 and not from pEF102, and, correspondingly, from pJP4 but not from pJP4-F3. Southern analysis of the total DNA of strain JMP134-F3, using *tfdA* as the probe, showed that the sequence of this gene was not present. The absence of *tfdA* in strain JMP134-F3 explains its failure to grow on 2,4-D (Table 1).

Determination of the ends of the large inverted repeat in pJP4-F3

As mentioned above, a deletion starting in the pJP4 fragment *Eco*RI-E, spanning fragments *Eco*RI-C, -H and -I, and ending in the fragment *Eco*RI-F, occurred to yield pJP4-F3 (for orientation, see double-headed solid arrow in Fig. 2a). In addition, the observation that the *Eco*RI insert in pEF102 is an inverted repeat shows that a duplication spanning at least the *tfdR*, *tfdD*_{II} and *tfdC*_{II} genes had occurred. This duplication may be significantly longer and may exceed the length of the deletion since pJP4-F3 is larger than the parent pJP4 plasmid. Thus, the duplication may possibly span both the *tfdC*_I*D*_I*E*_I*F*_I module and the *tfdD*_{II}*C*_{II}*E*_{II}*F*_{II} module (see double-headed solid arrow in Fig. 2b). Besides *Eco*RI-C', the fragment in which the duplication/deletion starts, the only other new *Eco*RI fragment visible after pJP4-F3 digestion is *Eco*RI-E'. Therefore, it may be assumed that the second duplication/deletion junction lies within this fragment (Fig. 2b). Correspondingly, this junction should also lie within the *Hind*III-D' fragment of pJP4-F3 (Fig. 2b). The *Hind*III-D' fragment from pJP4-F3 was cloned in pBlueScript to give pHF64 (Fig. 2b), and was used as a probe against pJP4 and pJP4-F3 plasmid DNA digested with *Hind*III, *Eco*RI and *Bam*HI. Wild-type pJP4 *Eco*RI-F and *Hind*III-D fragments, containing sequences that should be partially deleted in pJP4-F3, hybridized with the

probe, suggesting that *Hind*III-D' contains the deletion/duplication end-point (Fig. 2b). Hybridization was also observed with the wild-type *Bam*HI-A, *Bam*HI-D and *Eco*RI-A fragments, indicating that sequences within fragments *Eco*RI-F and *Eco*RI-A have some homology and might flank the large inverted repeat (Fig. 2a). Thus, it can be assumed that the duplication occurring in pJP4-F3 encompasses part of the wild-type *Eco*RI-E, all of the *Eco*RI-G and -B fragments and part of the -A fragments, and that the duplication end localized in the *Eco*RI-A fragment joins the deletion end localized in the *Eco*RI-F fragment, forming the new *Hind*III-D' and *Eco*RI-E' fragments (Fig. 2a, b).

To verify this assumption, the *Hind*III-D fragment of pJP4 was cloned into pBlueScript, to give pH60 (Fig. 2a), and the *Hind*III-D' fragment of pJP4-F3 was cloned to produce pHF64 (Fig. 2b). *Eco*RI subclones from pH60 and pHF64 were prepared in pBlueScript to give pHE30 and pHEF41 (Fig. 2a, b). The *Bam*HI-D fragment from the pJP4 plasmid with homology to the *Hind*III-D' fragment (Fig. 2b) was also cloned in pBlueScript to give pB54 (Fig. 2a). This plasmid was digested with *Hind*III and the smallest fragment (0.6 kb) was also cloned in pBlueScript to give pBH06 (Fig. 2a). Cloned fragments in plasmids pHE30, pHEF41 and pBH06 were sequenced (GenBank accession nos AF225973 and AF225974 for the first two clones, respectively). Subclones used for the sequencing of pHEF41 and pHE30 are shown in Fig. 2(c). As is depicted in Fig. 2(d), 2208 bp from the *Eco*RI-end of pHEF41 (part of the *Hind*III-D' fragment) are identical to those of pHE30 (part of the *Hind*III-D fragment); 660 bp at the *Hind*III-end of pHEF41 are identical in sequence to pBH06 (part of the *Bam*HI-D fragment). The ~1.2 kb of pHEF41 between these regions of homology does not match with either of these sequences, and should correspond to the *Bam*HI-A-fragment end, next to the *Bam*HI-D fragment in the wild-type (see Fig. 2a).

A search for sequence homologies in pHE30 and pHEF41 was conducted. A BLASTX analysis of the sequence of pHE30 (Fig. 2d) yielded 99% identity for *merA* (mercury reductase) of Tn501 (Brown *et al.*, 1983), 99% identity with *merD* from *Pseudomonas stutzeri* (Reniero *et al.*, 1998), 99% identity with ORF2 of the mercury-resistance transposon from *P. stutzeri* (Reniero *et al.*, 1998), and 100% identity with *tnpA* of IS1071 in Tn5271 (Nakatsu *et al.*, 1991). Since IS1071 has been localized in catabolic transposons and has been involved in DNA rearrangements, the presence of IS1071 sequences in pJP4 and pJP4-F3 was further studied by Southern analysis. Probes IS1071-AB and IS1071-AC, which correspond to the left and right halves of IS1071, respectively (Xia *et al.*, 1998), gave the same hybridization pattern. The *Eco*RI-A, -F, -H and -I fragments from wild-type pJP4, and the *Eco*RI-A' and -E' fragments from pJP4-F3 hybridized with the IS1071 probe. Fragments *Bam*HI-A and -B, *Hind*III-A, -D, -E and -H from pJP4, and *Bam*HI-A', *Bam*HI-C', *Hind*III-A' and -D' from pJP4-F3 also hybridized (see maps in Fig. 2a, b for orientation). This evidence, along with

sequence data, strongly suggests that there is at least one copy of an IS1071 in pJP4. As the *EcoRI*-A, *HindIII*-A and *Bam*HI-A fragments of pJP4 also hybridized with both IS1071 probes, it can be suggested that an IS1071-like element, or part of IS1071, is also present.

During the rearrangement, the part of the IS1071 sequence (containing two *EcoRI* recognition sites) that corresponds to fragments *EcoRI*-H, -I and -F was deleted (Fig. 2a). As a consequence, primer IS1071-AC could not anneal; this meant that it was no longer possible to amplify this sequence with primers IS1071A/C. The difference between the wild-type and rearranged plasmids was used to screen isolated colonies, and to quantify the rearrangement frequency (see above).

The data indicate that pJP4-F3 is the result of a duplication event involving a ~23 kb section that starts in the *tfdS* gene or the *tfdA*-*tfdS* intergenic region belonging to the *tfdR*/*tfdS* small inverted repeat, spans both the *tfdC_ID_IE_IF_I* module and the *tfdD_{II}C_{II}E_{II}F_{II}* module, and ends 1176 bp into the *Bam*HI-A fragment. Plasmid pJP4 also underwent a deletion that starts in the *tfdS* gene or the *tfdA*-*tfdS* intergenic region and ends 637 bp (corresponding to the hatched region of pHE30 shown in Fig. 2d) into the wild-type *EcoRI*-F fragment, leaving only the right portion of the IS1071. The absence of *tfdA* explains the inability of strain JMP134-F3 to grow on 2,4-D. On the other hand, the duplication that forms a 51-kb-long inverted repeat containing two copies of both *tfdCD*(DC)EF gene clusters is responsible for the increased TfdC activity, and supports the improved growth of this strain on 3-CB.

DISCUSSION

In this work, we have reported a deletion/duplication rearrangement of pJP4 produced after subculture of *R. eutropha* JMP134 in liquid medium containing 3-CB. The restriction enzyme profile and the presence of a very long inverted repeat (~51 kb, including the loop) in the enlarged pJP4 are very similar (if not identical) to that previously reported by Ghosal *et al.* (1985). In that case, however, the enlarged pJP4 form was obtained after conjugative transfer of pJP4 from *R. eutropha* to a *Pseudomonas putida*, followed by selection for growth on 3-CB. Although strain JMP134 is capable of growth both on solid and in liquid cultures containing 3-CB without rearrangements of plasmid pJP4, repeated subculturing in liquid medium readily selects for bacteria containing the rearranged plasmid. These observations suggest that the driving force for this rearrangement was the selective pressure for efficient growth on 3-CB. Accordingly, one of the outcomes of this rearrangement is the duplication of most of the region containing the *tfdCD*(DC)EF gene clusters. It has been shown recently that a *tfd* gene dosage effect is important for efficient growth on 3-CB of *R. eutropha* JMP134 derivatives (Pérez-Pantoja *et al.*, 2000). Additional examples of the effect of gene dosage on growth on chloroaromatics by the modified *ortho* ring

Fig. 3. Scheme for the intermolecular homologous recombination in pJP4 resulting in pJP4-F3. Two single crossovers take place (a) between the identical *tfdR*/*S* sequences (1) and the homologous IS1071 elements (2), respectively, to give rise to two products (b). One corresponds to the rearranged plasmid characterized in this work. Note that no complete copy of the IS1071 is left in this plasmid, and that two copies of the *tfd_I* and *tfd_{II}* gene modules are present. The other recombination product should be lost upon transfer to liquid cultures with 3-chlorobenzoate.

cleavage pathway are documented for *clc* genes (Ravatt *et al.*, 1998) and *tcb* genes (Klemba *et al.*, 2000). Accumulation of 2-chloromuconate during growth of *R. eutropha* JMP134 on 3-CB has also been observed (Pieper *et al.*, 1993), which can be explained by poor activity of chloromuconate cycloisomerases encoded by *tfdD_I* and *tfdD_{II}* genes (Pérez-Pantoja *et al.*, 2000). If this is the rate-limiting step for growth on 3-CB, it can be partially overcome by a gene-dosage effect.

The simplest explanation for the rearrangement reported in pJP4-F3 is a double crossover, homologous recombination between two pJP4 molecules (Fig. 3). A single crossover occurs between the *tfdR* sequence from

one pJP4 molecule and the identical, but opposite, *tfdS* sequence from the second pJP4 molecule. The second crossover takes place between the IS1071 sequence from one pJP4 molecule and the IS1071-like sequence from the other pJP4 molecule. This recombination gives rise to two rearranged plasmids (Fig. 3): the first, pJP4-F3, is reported here, while the other one, lacking most of the *tfd* genes, is selected against during growth in the presence of 3-CB.

The available data suggest that the *tfd* catabolic region is flanked by one complete copy of IS1071 and one end of this element, or an IS1071-like element (Figs 2 and 3). The IS1071 insertion sequence plays a role in several gene rearrangements (Tan, 1999; Wyndham *et al.*, 1988), suggesting that this element may be involved in the pJP4 rearrangement reported here. Large deletions in pJP4 during conjugative transfer (Clément *et al.*, 2000) or during transposon mutagenesis with Tn1771 (Don & Pemberton, 1985) have been reported. In the latter case, deletion of an approximately 40 kb region encompassing *tfd* catabolic genes was observed. That deletion corresponds roughly to the region framed by the IS1071 and the IS1071-like sequences reported here (Fig. 2a). As there are several examples of catabolic transposons containing IS1071 (Nakatsu *et al.*, 1991; Peel & Wyndham, 1999; Xia *et al.*, 1998; Vedler *et al.*, 2000), it is interesting to speculate that pJP4 originated from the insertion of an IS1071-based composite transposon (containing determinants for chloroaromatic metabolism) into an R751 IncP β -like element.

The search for ORFs in the sequences of pJP4 reported here revealed the presence of determinants for mercury resistance. Since DNA hybridization analyses of pJP4 with probes for Tn501 *mer* genes mapped these determinants to the EcoRI-F and EcoRI-D fragments, it has been proposed that the resistance to mercury in pJP4 is related to the presence of a Tn501 element (Burlage *et al.*, 1990; Smith & Thomas, 1987). Our results show that such ORFs are effectively present in this region of the pJP4 plasmid. The high level of homology suggests that these ORFs are actively involved in the resistance to mercury.

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