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

Pascale Clément,1 Dietmar H. Pieper2 and Bernardo González1

Author for correspondence: Bernardo González. Tel: +56 2 6862845. Fax: +56 2 2225515. e-mail: bgonzale@genes.bio.puc.cl

Ralstonia eutropha JMP134(pJP4) is able to grow on minimal media containing the pollutants 3-chlorobenzoate (3-CB) or 2,4-dichlorophenoxyacetic (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, tfdK–ISJP4–tfdI–ISJP4–tfdD, giving rise to a 51-kb-long inverted repeat, was also observed. The increase in gene copy number for the tfdCD(DF)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 contains 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, tfdCD(DF)EF and tfdDHICIFII1 (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-chlorobenzoic; 2,4-D, 2,4-dichlorophenoxyacetate.

The GenBank accession numbers for the 3115 nt BamHI-F and 2833 nt EcoRI-F fragments of pJP4 and the 4037 nt EcoRI-E fragment of pJP4-F3 are AF225972, AF225973 and AF225974, respectively.
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 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 HindIII, EcoRI or BamHI and separation on a 0.7% agarose gel) were made using Hybon 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 Styl 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 (GGGTCTCTCGTTTTTCACTGCAA) and IS1071-B (CTTGTGATATAAGCTTGACGCT), and IS1071-A and IS1071-C (GATCCAGAAGCTTCCAGTTGAAG), respectively (Xia et al., 1998). Detection of the intergenic region between tfdA and tfdS was carried out by PCR amplification using primer pairs PR33 (GCGCGGCTATTCTGTTCTTTCCCG, base pairs 984–1008; GenBank accession no. S80112) and RC3 (TGGACCCCTGCGC, 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 1·5 min and 72 °C for 3·5 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 BamHI-F and 2833 nt

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Fig. 1. Schematic diagram of the tfd catabolic region of pJP4. The approximate position of tfd genes, the ISJP4 insertion sequence, and the EcoRI, HindIII, BamHI and KpnI restriction sites are indicated. Black and white bars correspond to previously sequenced and unsequenced regions of the BamHI internal part of the EcoRI-E fragment, respectively. Not to scale.
EcoRI-F fragments of pJP4 and the 4037 nt EcoRI-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 (~95 kb) pJP4 form was detected by gel electrophoresis (data not shown). To study the frequency of this phenomenon, liquid cultures of strain JMP134 in 2,4-D and 3-CB 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 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**

EcoRI, HindIII and BamHI restriction profiles were determined for plasmid pJP4-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). EcoRI fragments C, E, F, H and I, as well as the HindIII-B, -D, -E and -H and BamHI-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. EcoRI-C’, EcoRI-E’, HindIII-B’ and HindIII-D’ (see Fig. 2b for orientation). Ghosal & You (1988) reported that the EcoRI-C band of pYG2 corresponded to two fragments, the wild-type EcoRI-D (10-2 kb), and a distinct 10-2 kb EcoRI fragment, which hybridized with the wild-type EcoRI-E fragment (see Fig. 2a, b for orientation). Similarly, the 10-2 kb band of EcoRI-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 KpnI restriction site, as the wild-type EcoRI-D fragment. The other insert, designated EcoRI-C’ in Fig. 2b, exhibited one KpnI restriction site corresponding to that present in the wild-type EcoRI-E fragment (Fig. 1).

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

The EcoRI-C’ fragment of pJP4-F3 should contain the regulatory genes tfdR and tfdS of the EcoRI-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 EcoRI-E fragment from pJP4 and the 10-2 kb EcoRI-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 EcoRI-E fragment has already been

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**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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth substrate</th>
<th>Relative growth rate</th>
<th>CC 1,2-DO specific activity (U mg⁻¹)\‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>JMP134</td>
<td>2,4-D</td>
<td>1.0</td>
<td>0.63 ± 0.05</td>
</tr>
<tr>
<td>JMP134</td>
<td>3-CB</td>
<td>1.0</td>
<td>0.38 ± 0.09</td>
</tr>
<tr>
<td>JMP134-F3</td>
<td>2,4-D</td>
<td>0.0</td>
<td>NA</td>
</tr>
<tr>
<td>JMP134-F3</td>
<td>3-CB</td>
<td>2.0</td>
<td>1.72 ± 0.26</td>
</tr>
</tbody>
</table>

* 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.
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 BamHI insert in pEF35 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 EcoRI-C′ DNA in pEF102 was compared with the wild-type EcoRI-E sequence, the BamHI-F and BamHI-E′ fragments were found to be identical. Surprisingly, the two sequences flanking BamHI-E′ in pEF102 (BamHI-D′ in Fig. 2b) were found to be identical but inverted, and also identical to DNA corresponding to BamHI-E in pEF3 (Fig. 2a). This indicates that a mirror copy of the tfdR–tfdD1 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 EcoRI-C, -H and -I, and parts of the fragments EcoRI-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-kb PCR product obtained using primer pair PR33 and RC3 could be amplified only from pEF3 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 EcoRI-E, spanning fragments EcoRI-C, -H and -I, and ending in the fragment EcoRI-F, occurred to yield pJP4-F3 (for orientation, see double-headed solid arrow in Fig. 2a). In addition, the observation that the EcoRI insert in pEF102 is an inverted repeat shows that a duplication spanning at least the tfdR, tfdD1 and tfdC111 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 tfdC1,D1,E,F1 module and the tfdD11,C111,E111,F111 module (see double-headed solid arrow in Fig. 2b). Besides EcoRI-C′, the fragment in which the duplication/deletion starts, the only other new EcoRI fragment visible after pJP4-F3 digestion is EcoRI-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 HindIII-D′ fragment of pJP4-F3 (Fig. 2b). The HindIII-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 HindIII, EcoRI and BamHI. Wild-type pJP4 EcoRI-F and HindIII-D fragments, containing sequences that should be partially deleted in pJP4-F3, hybridized with the probe, suggesting that HindIII-D′ contains the deletion/duplication end-point (Fig. 2b). Hybridization was also observed with the wild-type BamHI-A, BamHI-D and EcoRI-A fragments, indicating that sequences within fragments EcoRI-F and EcoRI-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 EcoRI-E, all of the EcoRI-G and -B fragments and part of the -A fragments, and that the duplication end localized in the EcoRI-A fragment joins the deletion end localized in the EcoRI-F fragment, forming the new HindIII-D′ and EcoRI-E′ fragments (Fig. 2a, b).

To verify this assumption, the HindIII-D′ fragment of pJP4 was cloned into pBlueScript, to give pH60 (Fig. 2a), and the HindIII-D′ fragment of pJP4-F3 was cloned to produce pHF64 (Fig. 2b). EcoRI subclones from pH60 and pHF64 were prepared in pBlueScript to give pH30 and pHF41 (Fig. 2a, b). The BamHI-D fragment from the pJP4 plasmid with homology to the HindIII-D′ fragment (Fig. 2b) was also cloned in pBlueScript to give pB54 (Fig. 2a). This plasmid was digested with HindIII and the smallest fragment (0.6 kb) was also cloned in pBlueScript to give pH60 (Fig. 2a). Cloned fragments in plasmids pH30, pHF41 and pHB60 were sequenced (GenBank accession no. AF225973 and AF225974 for the first two clones, respectively). Subclones used for the sequencing of pHF41 and pH30 are shown in Fig. 2(c). As is depicted in Fig. 2(d), 2208 bp from the EcoRI-end of pHF41 (part of the HindIII-D′ fragment) are identical to those of pH30 (part of the HindIII-D fragment); 660 bp at the HindIII-end of pHF41 are identical in sequence to pHB60 (part of the BamHI-D fragment). The ~2 kb of pHF41 between these regions of homology does not match with either of these sequences, and should correspond to the BamHI-A fragment end, next to the BamHI-D fragment in the wild-type (see Fig. 2a).

A search for sequence homologies in pH30 and pHF41 was conducted. A BLASTx analysis of the sequence of pH30 (Fig. 2d) yielded 99% identity for merA (mercury reductase) of Tn501 (Brown et al., 1988), 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 npA 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 EcoRI-A, -F, -H and -I fragments from wild-type pJP4, and the EcoRI-A′ and -E′ fragments from pJP4-F3 hybridized with the IS1071 probe. Fragments BamHI-A and -B, HindIII-A, -D, -E and -H from pJP4, and BamHI-A′, BamHI-C′, HindIII-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 BamHI-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 tfdC1/fD1E1F1 module and the tfdD1C1I1E1F1 module, and ends 1176 bp into the BamHI-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 cleavage pathway are documented for clc genes (Ravatn 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 tfdD1 and tfdD11 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 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., 1999; 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 IncPβ-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 here revealed the presence of determinants for mercury, the search for ORFs in the sequences of pJP4 reported here was also successful. The presence of ORFs between the IS1071 and IS1071-like sequences flanking one pJP4 molecule and the identical, but opposite, tfdS sequence suggests 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.

ACKNOWLEDGEMENTS

We thank Marlene Manzano and Rodrigo de la Iglesias for help with some experiments, Isabelle Himner and Jean Armengaud for advice on sequencing, James Tiedje for providing the tfdA DNA probe, and Rebecca Towers for critical reading of the manuscript. This work was supported by grants 8990004 from FONDECYT-Chile and IFS F/1886-3, and a collaborative grant from CONICYT (Chile) and BMBF-FZK (Karlsruhe, Germany). P.C. was supported by a doctoral FONDECYT grant (no. 2980046).

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Received 16 November 2000; revised 16 March 2001; accepted 3 April 2001.