Molecular and Population Analyses of a Recombination Event in the Catabolic Plasmid pJP4

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Cupriavidus necator JMP134(pJP4) harbors a catabolic plasmid, pJP4, which confers the ability to grow on chloroaromatic compounds. Repeated growth on 3-chlorobenzoate (3-CB) results in selection of a recombinant strain, which degrades 3-CB better but no longer grows on 2,4-dichlorophenoxyacetate (2,4-D). We have previously proposed that this phenotype is due to a double homologous recombination event between inverted repeats of the multicopies of this plasmid within the cell. One recombinant form of this plasmid (pJP4-F3) explains this phenotype, since it harbors two copies of the chlorocatechol degradation *tfd* gene clusters, which are essential to grow on 3-CB, but has lost the *tfdA* gene, encoding the first step in degradation of 2,4-D. The other recombinant plasmid (pJP4-FM) should harbor two copies of the *tfdA* gene but no copies of the *tfd* gene clusters. A molecular analysis using a multiplex PCR approach to distinguish the wild-type plasmid pJP4 from its two recombinant forms, was carried out. Expected PCR products confirming this recombination model were found and sequenced. Few recombinant plasmid forms in cultures grown in several carbon sources were detected. Kinetic studies indicated that cells containing the recombinant plasmid pJP4-FM were not selectable by sole carbon source growth pressure, whereas those cells harboring recombinant plasmid pJP4-F3 were selected upon growth on 3-CB. After 12 days of repeated growth on 3-CB, the complete plasmid population in *C. necator* JMP134 apparently corresponds to this form. However, wild-type plasmid forms could be recovered after growing this culture on 2,4-D, indicating that different plasmid forms can be found in *C. necator* JMP134 at the population level.

Cupriavidus necator (formerly *Ralstonia eutropha*) JMP134 (pJP4) was first isolated due to its ability to grow on chloroaromatic pollutants, such as 2,4-dichlorophenoxyacetate (2,4-D) or 3-chlorobenzoate (3-CB). The key catabolic functions are encoded by the *tfd* genes in the catabolic plasmid pJP4 (8, 12, 14). Among the *tfd* functions are two similar gene modules (Fig. 1): *tfdC_ID_IE_IF_I* and *tfdD_{II}C_{II}E_{II}F_{II}*, which both code for the enzymes of the modified *ortho* ring cleavage pathway for chlorocatechols (17, 25, 28), performing the key steps in 3-CB and 2,4-D degradation, and *tfdA*, an α -ketoglutarate-dependent dioxygenase involved in the first step of phenoxyalkanoic acid degradation (10, 11) (Fig. 1). Previous studies have determined that the *tfd* gene dosage is important for degradation of these chloroaromatic substrates (15, 16, 26) and that plasmid pJP4 is found in approximately five copies within the cell (30). This gene dosage factor has also been observed in other catabolic models, for instance, in duplication of the *clc* element from *Pseudomonas* sp. strain B13 (29).

When *C. necator* JMP134(pJP4) is grown on 3-CB for prolonged periods of time, a new phenotype appears with improved degradation of 3-CB but loss of the ability to grow on 2,4-D (6). The analysis of the DNA sequence of pJP4 (31) suggests that this catabolic phenotype can be explained by a double homologous recombination between a complete and a

truncated copy of IS1071 and the identical *tfdR* and *tfdS* gene sequences (21). One of the recombinant plasmids, pJP4-F3, harbors two copies of the *tfdC_ID_IE_IF_I* and *tfdD_{II}C_{II}E_{II}F_{II}* gene modules but loses the *tfdA* gene (Fig. 2). This plasmid has been isolated and previously characterized (6). The other recombinant plasmid form, named pJP4-FM, should harbor two copies of *tfdA* but no copies of the *tfdC_ID_IE_IF_I* and *tfdD_{II}C_{II}E_{II}F_{II}* gene modules (Fig. 2). Up to this point, this plasmid, pJP4-FM, had not been studied. On the basis of the presence or absence of catabolic genes, the presence of the pJP4-F3 plasmid explains the improved growth on 3-CB (Fig. 1B), whereas pJP4-FM should allow *C. necator* JMP134 to grow on the 2,4-D derivative 2-methylphenoxyacetic acid (2-MPA), but not on 3-CB or 2,4-D (Fig. 1B). Initial degradation of 2-MPA requires the *tfdA* gene, but subsequent catabolism needs functions that are encoded in the chromosome of the strain. In this case, the missing *tfdB* gene functions can be carried out by a chromosomally encoded phenol hydroxylase for nonhalogenated phenols (13, 16) (Fig. 1B). This led to the hypothesis that pJP4 can normally undergo homologous recombination events that modify the catabolic phenotype of the cell host and that recombinant forms can be isolated after growth under selective pressure, namely, use of a specific aromatic compound as the sole carbon source. To test this hypothesis, we designed a multiplex PCR approach to target strategic plasmid sites that should provide different PCR products and banding patterns for wild-type pJP4 and its rearranged derivatives. We report here the molecular and population analyses of this recombinant event in *C. necator* JMP134(pJP4) under different growth conditions.

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FIG. 1. Genes and pathways involved in degradation of chloroaromatic compounds by *C. necator* JMP134(pJP4). (A) Schematic representation of the organization of *tfd* genes in plasmid pJP4 (not drawn to scale). IR, inverted repeat. (B) Genes involved in catabolism of 3-CB, 2,4-D, and 2-MPA. *benABCD* genes are encoded on the chromosome.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. necator* JMP134(pJP4) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and was grown in minimal medium liquid cultures, prepared as described previously (1), containing 3 mM 2,4-D, 3-CB, 2-MPA, 4-chloro-2-methylphenoxyalkanoic acid (MCPA) (Aldrich Chemical Company Inc., Milwaukee, WI), benzoate, or 10 mM fructose (Merck AG, Darmstadt, Germany) or in Luria-Bertani (LB) medium (3) at 30°C for 24 to 48 h. *C. necator* JMP134(pJP4-F3) and JMP134(pJP4-FM) strains were grown in minimal medium containing 3 mM 3-CB or 2-MPA, respectively.

Catabolic phenotype tests. Liquid cultures of *C. necator* JMP134(pJP4) grown for 2 days at 30°C on 2,4-D, 3-CB, 2-MPA, MCPA, benzoate, fructose, or LB were serially diluted and plated on minimal medium agar plates containing the corresponding carbon source. Then, about 230 colonies from each condition, except for growth on 2-MPA where 340 colonies were tested, were picked and simultaneously plated on agar plates containing 2 mM 3-CB or 2,4-D to test for the pJP4-F3 phenotype or 2-MPA or 3-CB to test for the pJP4-FM phenotype. Colonies that grew only on the 3-CB plates were defined as belonging to the catabolic phenotype of strain JMP134(pJP4-F3), whereas those colonies that grew only on 2-MPA displayed the catabolic phenotype of strain JMP134(pJP4-FM). Approximately 10 colonies of each catabolic phenotype were analyzed, and finally one colony of each group was used as a representative of the catabolic phenotype.

Kinetics of the appearance of the catabolic phenotype of *C. necator* JMP134(pJP4-F3) and JMP134(pJP4-FM). Cells of strain JMP134(pJP4) previously grown on 3 mM 2,4-D were transferred to 2-ml minimal medium cultures containing 3 mM or 5 mM 3-CB and grown at 30°C. After 2 days of incubation, 20- μ l aliquots from cultures growing in 3 mM 3-CB were transferred to fresh 2-ml minimal medium cultures containing 3 mM 3-CB or 3 mM 2-MPA. Successive transfers from 3-CB cultures to fresh 3 mM 3-CB medium and from 2-MPA cultures to fresh 3 mM 2-MPA medium were performed every 2 days for up to

14 days for 3-CB and up to 6 days for 2-MPA. The same procedure for transferring to fresh 5 mM 3-CB was followed for cultures containing 5 mM 3-CB. Every culture was serially diluted and plated on minimal medium agar plates containing the corresponding carbon source. Then, about 120 colonies from each condition, i.e., transfer number and growth source, were picked and simultaneously plated on agar plates containing 2 mM 3-CB or 2,4-D or containing 2-MPA or 3-CB to determine the number of colonies belonging to each phenotype. Aliquots (20 μ l) of selected end point cultures in 3 mM 3-CB were transferred to liquid cultures containing 3 mM 2,4-D and then transferred to fresh 2,4-D cultures every 2 days for up to 8 days. To analyze the immediate effect of 3-CB on growth, *C. necator* JMP134(pJP4) was grown on 10 mM fructose in a BioFlo 110 chemostat (New Brunswick Scientific, Edison, NJ) and then fed with 3 mM 3-CB as the sole carbon source. Chemostat parameters were as follows: culture volume of 700 ml, dilution rate of 0.069 h⁻¹, airflow rate of 0.16 liter h⁻¹, agitation of 250 rpm, and a temperature of 30°C. Samples were taken every hour for the first 6 h and every 12 h for the next 2 days.

Design and use of a multiplex PCR procedure. The multiplex PCR uses 10 oligonucleotide sequences (F1R1 to F5R5), which were targeted to amplify five key regions in the native pJP4 plasmid (Fig. 2) and carefully designed and optimized to generate unique and differentiable patterns for the native and recombinant forms of the pJP4 plasmid (Table 1). The design of the multiplex PCR was carried out using a computer cluster of 16 Pentium IV processors running LINUX operating system and based on a proprietary software written in ANSI C language, which was developed by one of the authors of this work (F. Melo, unpublished data). Access to this software is possible and should be requested by sending an e-mail to the author at fmelo@bio.puc.cl). The computer software receives a list of template DNA sequences in FASTA format as input, along with a series of constraints that must be fulfilled to produce a successful set of oligonucleotides for multiplex PCR. The best set of oligonucleotides is produced by a genetic-algorithm-based optimization. The following oligonucleotide constraints were considered: length range (18- to 30-mers), melting temperature

FIG. 2. Double homologous recombination in pJP4 plasmid. The crossover points and the positions of the multiplex PCR primer pairs in pJP4 (above) and expected derivatives (below) are shown. New combinations of primer pairs are enclosed in boxes.

range (60 to 65°C), energy of intramolecular interactions (>−4 kcal/mol), total number of contiguous intermolecular interactions (<6 bp), total number of contiguous intermolecular interactions at the 3′ end (<5 bp), total number of noncontiguous intermolecular interactions (<9 bp), and specificity or maximum number of contiguous 3′ interactions with other regions in the template sequences (8 bp). In addition to this, the following constraints for any possible PCR oligonucleotide pair were considered: minimum length difference between any

two amplified fragments (100 bp) and maximum melting temperature difference (3°C). An accurate prediction of melting temperatures for oligonucleotides was carried out with the consensus nearest-neighbor method (23), using dnaMATE software (24). A total monovalent salt concentration equivalent to 150 mM was considered in the final reaction mix for the calculations. The energy of intramolecular interactions was calculated with MFOLD software (33). In addition to this, the specificity of the optimal sets of oligonucleotides for multiplex PCR

TABLE 1. Expected sizes for PCR products from the pJP4, pJP4-F3, and pJP4-FM plasmid DNA templates by the multiplex PCR procedure

Zone(s)	Expected size (bp) for PCR product from plasmid:		
	pJP4	pJP4-F3	pJP4-FM
1	648	648	
2	2,198	2,198	
3	1,774		1,774
4	985		985
5	1,369	1,369	1,369
1 to 4		757	877
2 and 3		1,823	2,148

produced by the software was finally checked against the complete genome of *C. necator* JMP134 (GenBank accession numbers NC_007347, NC_007336, and NC_007348) in order to verify that the selected oligonucleotides were specific for the pJP4 plasmid (accession number NC_007337).

The final and optimal primer pairs for multiplex PCR defined for different zones of the native pJP4 plasmid (Fig. 2) were as follows: for zone 1, F1 (5'-CCGACTTGTCGCGAACTG TCCA-3') and R1 (5'-TCAGGCAACAAA CCGATTGACGTTTC-3'); for zone 2, F2 (5'-CCTTC GTAGGAGACGGATG AGCAC-3') and R2 (5'-CTGCAGCTGGATGGTCGGATAGA-3'); for zone 3, F3 (5'-GACGATCACGTTGGGATGCAGC-3') and R3 (5'-AAAAGAG GATG AAGGGGATTGCGA-3'); for zone 4, F4 (5'-GATCTTTGCCCGCG GTTGAAGG-3') and R4 (5'-CAGATGCGTGACTGGCGAATGC-3'); and for zone 5, F5 (5'-CGGGTCTTCACGGAAGAACAAGGTATT-3') and R5 (5'-ACAAGCTCATTTTCTGGATCGTGGC-3'). These optimal primer pairs produced distinct PCR patterns for the recombinant plasmids by the negative amplification of four native regions and the positive amplification of four additional regions generated by a new combination of primers through the loss or generation of specific priming sites after the double homologous recombination event of the native plasmid (Fig. 2). The expected sizes of PCR bands produced for each plasmid variant are shown in Table 1.

Each multiplex PCR was performed in a total volume of 25 μ l containing 0.25 μ l *Taq* polymerase (Invitrogen, Carlsbad, CA), 2.0 μ l of 10 $\times$ Invitrogen Pfx PCR buffer, 0.5 μ l dimethyl sulfoxide (Merck AG, Darmstadt, Germany), 0.5 μ l Invitrogen Pfx enhancer, 1.5 μ l of 3.0 mM MgSO₄, 1.25 μ l of 10 mM deoxynucleoside triphosphate, 0.5 μ l of each primer (200 μ M) for zones 1 and 4, 1.2 μ l of each primer (200 μ M) for zone 2, 0.8 μ l of each primer (200 μ M) for zone 3, 0.3 μ l of each primer (200 μ M) for zone 5, and 2.5 μ l of supernatant obtained from a liquid culture or an individual colony resuspended in water after denaturation for 10 min at 95°C and centrifugation for 5 min at 6,000 $\times$ g in an Eppendorf 580R centrifuge as a DNA template. Reactions were carried out in Perkin-Elmer GeneAmp PCR system 2400 (Perkin-Elmer, Wellesley, MA) as

follows: (i) 95°C for 10 min; (ii) 30 cycles, with 1 cycle consisting of 95°C for 45 s, 65°C for 2 min 30 s, and 72°C for 1 min; and (iii) 10 min at 72°C.

Electrophoretic conditions. PCR products obtained from the multiplex PCR were separated in 0.8% ultrapure agarose (Invitrogen, Carlsbad, CA) in sodium borate buffer (4, 5) with 1 μ l/ml SYBR green (Invitrogen, Carlsbad, CA). Electrophoresis was carried out using sodium borate buffer at 120 V for 1.5 h or 5 h with a Sigma Aldrich Chemical Group PS500-2 electrophoresis power supply (Aldrich Chemical Company, St. Louis, MO). The DNA standard was O'Range-Ruler DNA ladder (200 bp; Fermentas International Inc., Ontario, Canada).

Cloning and sequencing of PCR products. New DNA bands obtained from recombinant forms were isolated from the gels and purified using a QIAGEN gel extraction kit (QIAGEN, Hilden, Germany) and then cloned into a TOPO-TA vector (Invitrogen, Carlsbad, CA) as specified by the supplier. Each construction was electroporated at 2.5 mA into electrocompetent *Escherichia coli* DH5 α cells as described previously (2), and cells were recovered in LB medium for 1 hour before plating. Plates were prepared with LB medium which contained 50 μ g/ml kanamycin. 5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) and isopropyl- β -D-thiogalactopyranoside (IPTG) (Invitrogen, Carlsbad, CA) were included in the plates at concentrations of 20 μ g/ml in both cases. The plates were incubated overnight at 37°C. White colonies were picked and grown in liquid cultures in LB containing kanamycin (50 μ g/ml) and checked for the presence of the corresponding insert by using the multiplex PCR protocol. Two positive colonies from each PCR product were submitted to plasmid extraction using QIAprep Miniprep kit (QIAGEN, Hilden, Germany) and sequenced at MacroGen (MacroGen Online Sequencing System, Korea). Alignment of the sequences was done using AlignX (InforMax-Invitrogen, Carlsbad, CA), against expected pJP4-F3 and pJP4-FM plasmid sequences.

RESULTS AND DISCUSSION

Phenotypic derivatives of plasmid pJP4 are obtained by a double homologous recombination. Phenotypic and genetic evidence indicates that a variant of *C. necator* JMP134 containing a derivative of plasmid pJP4 (pJP4-F3) appears after prolonged growth on 3-CB (6, 12). The possibility of a double-crossover homologous recombination to form plasmid pJP4-F3 has been proposed (6). If this proposal is correct, pJP4-FM, another pJP4 derivative, containing two copies of the *tfdA* gene and no *tfd* gene modules encoding chlorocatechol degradation, would also be formed after the homologous recombination event (Fig. 2). Although previous studies identified the existence of strain JMP134(pJP4-F3) (6, 31), there are no studies that verify the double-recombination hypothesis molecularly. Furthermore, to date, strain JMP134(pJP4-FM) had not been

FIG. 3. Multiplex PCR product profile for plasmids pJP4, pJP4-F3, and pJP4-FM. The profiles for products from multiplex PCR for wild-type plasmid pJP4 (WT), pJP4-F3 (F3), and pJP4-FM (FM) are shown. Gels (0.8% agarose) were prepared in SB buffer (4, 5) and run at 120 V for 1 h (left) or 5 h (right). Only the boxed PCR products are shown in the right panel. Black arrows correspond to PCR products found in pJP4 and one of its derivatives. Thin arrows are unresolved PCR product bands. Hatched and white arrows are PCR products found only in pJP4-F3 and pJP4-FM derivatives, respectively. std, molecular size standards.

FIG. 4. Sequence analysis of new PCR products for wild-type and recombined forms of pJP4. Sequences of PCR products from zones 1 to 4 (A) or zones 2 and 3 (B). Sequences in normal type correspond to zone 1 or 2, whereas sequences in bold type and underlined correspond to zone 3 or 4. Overlapping sequences corresponding to inverted repeats where the crossover took place are boxed. Only the sequences adjacent to inverted repeats are shown.

isolated or studied. To carry out a molecular and population study of this double homologous recombination event, we designed a multiplex PCR procedure to target strategic sites in the wild-type plasmid and its proposed derivatives (Fig. 2). Zone 1 primer pairs target the truncated *IS1071* sequence; primer pairs for zone 2 target the *tfdR* and *tfdD_{II}* gene sequences, primer pairs for zone 3 target the *tfdS* and *tfdA* gene sequences, primer pairs for zone 4 target the complete copy of *IS1071*, and zone 5 primer pairs target the *mer* genes. The *mer* genes are involved in mercury resistance (31) and were used as a control for a nonrecombining site on pJP4. These primer pairs should produce a different set of PCR products, using the multiplex PCR procedure, for pJP4, pJP4-F3, and pJP4-FM, except the primer pair for the *mer* genes, which should be the same for all of the plasmid variants. If recombination occurs between the inverted repeated sequences of the complete and

truncated copies of *IS1071* and the inverted identical *tfdR* and *tfdS* gene sequences, combinations of the same primer pairs should give rise to new recombinant PCR products, while other PCR products should disappear (Fig. 2). New PCR products arise because of combinations of primer pairs for zones that are too distant in pJP4 but are near enough in recombined forms to produce an amplification product. Expected PCR product sizes for pJP4, pJP4-F3, and pJP4-FM derivatives were calculated using the double-crossover homologous recombination model (Table 1).

The wild-type strain and the isolated derivatives of *C. necator* JMP134 obtained in this study, strains JMP134(pJP4-F3) and JMP134(pJP4-FM), were grown on 2,4-D, 3-CB, or 2-MPA, respectively, and used as a template for multiplex PCR to check for the pattern of PCR products. Each pJP4 plasmid derivative gave different PCR products that are characteristics

FIG. 5. Basal recombination of pJP4 in *C. necator* JMP134 grown on different carbon sources. (A) Percentage of colonies of *C. necator* JMP134(pJP4-F3) (white bars) or *C. necator* JMP134(pJP4-FM) (black bars) after growth on LB medium or minimal medium containing fructose, benzoate, 2,4-D, MCPA, 3-CB, or 2-MPA. A total of 230 colonies (340 in the case of growth on 2-MPA) were analyzed in each case. The values are averages $\pm$ standard deviations (error bars) from two independent experiments. (B) Multiplex PCR products from cultures of *C. necator* JMP134(pJP4) grown on LB medium or minimal medium containing fructose (Fruc), benzoate (Benz), 2,4-D, MCPA, 3-CB, or 2-MPA. Symbols and electrophoresis conditions are as described in the legend to Fig. 3.

FIG. 6. Kinetics of appearance and disappearance of the JMP134(pJP4-F3) strain phenotype. (A) Percentages of colonies of *C. necator* JMP134(pJP4-F3) after successive transfers of strain JMP134(pJP4) on 3 mM 3-CB (squares) or 5 mM 3-CB (triangles). (B) Multiplex PCR products from cultures of *C. necator* JMP134(pJP4) repeatedly grown on 3 mM 3-CB. The white numbers at the bottom of the lanes indicate the number of days of growth on 3-CB. (C) Percentages of colonies of *C. necator* JMP134(pJP4-F3) after successive transfers of end point 3 mM 3-CB culture on 3 mM 2,4-D. (D) Multiplex PCR products from cultures of *C. necator* JMP134(pJP4-F3) repeatedly grown on 3 mM 2,4-D. The white numbers at the bottom of the lanes indicate the number of days of growth on 2,4-D. (E) Percentages of colonies of *C. necator* JMP134(pJP4-F3) after growth of strain JMP134(pJP4) in a chemostat fed with 3 mM 3-CB. (F) Multiplex PCR products from *C. necator* JMP134(pJP4) grown on a chemostat fed with 3-mM 3-CB. The white numbers at the bottom of the lanes indicate the number of hours of growth. Symbols and electrophoresis conditions in panels B, D, and F are as described in the legend to Fig. 3. The values in panels A, C, and E are averages $\pm$ standard deviations (error bars) from two independent experiments.

of the recombinant zones (Fig. 3). To verify whether the new bands effectively correspond to recombinant products, the bands were isolated from the agarose gel, cloned, and sequenced. These sequences were aligned against the pJP4 sequence and the pJP4-F3 and pJP4-FM sequences, matching

only the latter two, with identities higher than 99% (Fig. 4). The zones for other combinations of primers were too far apart to be detected under these conditions.

These results indicate that pJP4, due to the presence of the inverted identical sequences (complete and truncated

FIG. 7. Kinetics of appearance of the JMP134(pJP4-FM) strain phenotype after successive transfers on 3-CB or 2-MPA. (A) Percentages of colonies of *C. necator* JMP134(pJP4-FM) after successive transfers of strain JMP134(pJP4) on 3 mM 3-CB and then repeatedly grown on 3-mM 2-MPA for 2 (squares), 4 (triangles), or 6 (circles) days. A total of 120 colonies were analyzed for each condition. The values are averages $\pm$ standard deviations (error bars) from two independent experiments. (B) Multiplex PCR products from cultures of *C. necator* JMP134(pJP4) previously grown on 3-CB and then grown in 2-MPA for 2 days, which correspond to squares in panel A. The white numbers at the bottom of the lanes indicate the number of days of initial growth on 3-CB. Symbols and electrophoresis conditions are as described in the legend to Fig. 3.

IS1071 and *tfdR* and *tfdS* gene sequences), is capable of producing two recombinant forms: pJP4-F3 and pJP4-FM. Though other groups have studied recombination events in vivo in plasmids using artificial selection (9) and described genetic rearrangements in plasmids due to inverted repeats (22), this is the first time that an in vivo double homologous recombination is characterized molecularly and kinetically (see below).

Recombinant forms of pJP4 are found at a low basal frequency and without selection. We studied whether recombination of pJP4 and appearance of strains JMP134(pJP4-F3) and JMP134(pJP4-FM) can be detected in the absence of selective growth pressure. To do so, we grew *C. necator* JMP134 in different substrates and then selected 230 colonies (340 in the case of growth on 2-MPA) from each culture and tested for growth on 3-CB or 2,4-D to detect the JMP134(pJP4-F3) strain phenotype and on 2-MPA or 3-CB to search for strain JMP134(pJP4-FM) phenotype. The growth substrates were LB, fructose, benzoate, 2-MPA, 3-CB, 2,4-D, or MCPA. LB and fructose are carbon sources that do not require *tfd* genes for their catabolization, whereas MCPA, 2,4-D, 2-MPA, and 3-CB strictly require *tfd* functions for initial degradation (25, 27, 28). The degradation of benzoate is chromosomally encoded, although some *tfd* functions may use benzoate intermediates (27). After 2 days of growth, a low percentage of these colonies analyzed for each carbon source showed recombinant phenotypes, with the exception of colonies from a 3-CB culture (Fig. 5A). The percentages of strain JMP134(pJP4-F3) recombinants were around 1 for colonies from cultures grown on LB, fructose, benzoate, or 2,4-D and below the detection limit for 2-MPA or MCPA but more than 40 for colonies obtained from a 3-CB culture. Strain JMP134(pJP4-FM) recombinants were detected (around 1%) only in colonies from cultures grown on fructose, benzoate, 2-MPA, or MCPA. When 2-day liquid cultures grown in the different carbon sources were analyzed by the multiplex PCR procedure, faint bands corresponding to both recombinant plasmid forms (around 750 bp and 900 bp for pJP4 F3 and pJP4-FM, respectively) were found for all substrates (Fig. 5B). The presence of these faint bands was also

observed after several transfers to 2,4-D, fructose, and 2-MPA (Fig. 6C and E and 7B).

These results indicate that low levels of pJP4 recombination take place when cells of *C. necator* JMP134 are proliferating. In the case of growth on 3-CB, on the other hand, the phenotype of strain JMP134(pJP4-F3) is strongly selected against the wild type, and this can be explained by a *tfd* gene dosage effect (6, 26).

Time course of the appearance of the JMP134(pJP4-F3) strain phenotype. To study the appearance of pJP4-F3 in depth, we repeatedly grew *C. necator* JMP134(pJP4) on 3-CB liquid cultures. Every 2 days, an aliquot of each 3-CB culture was transferred to a fresh 3-CB liquid culture and so on for up to 14 days. A total of 120 colonies were obtained from plates inoculated with serial dilutions from the different 3-CB liquid cultures and checked for growth in 3-CB and 2,4-D. After the first transfer to a 3-CB liquid culture, we found that at a concentration of 3 mM 3-CB, 41% of the colonies displayed the JMP134(pJP4-F3) strain phenotype and that at a concentration of 5 mM 3-CB, this percentage increased to 65 (Fig. 6A). One hundred percent of the colonies had the JMP134(pJP4-F3) strain phenotype after 12 days of growth on 3 mM 3-CB and after 10 days of growth on 5 mM 3-CB (Fig. 6A). Samples from cultures transferred at 3 mM 3-CB were analyzed using the multiplex PCR procedure. After successive transfers to 3-CB liquid cultures, the *C. necator* JMP134 plasmid population shifted from the wild-type pJP4 PCR product pattern towards the pJP4-F3 one (Fig. 6B). Apparently, 100% of the cells in the culture grown for 14 days on 3 mM 3-CB were strain JMP134(pJP4-F3). However, when an aliquot of this culture was transferred to 2,4-D liquid cultures, the population of cells from strain JMP134(pJP4-F3) decreased to 30% after only 2 days of incubation and returned to the wild-type percentages after the fourth transfer, i.e., 8 days of culture (Fig. 6C). Selected culture samples were analyzed using the multiplex PCR procedure, which allowed detection of a rapid disappearance of the PCR product pattern of pJP4-F3 plasmid, after 2 days (Fig. 6D). The latter indicates that the wild-type pJP4 form was still present in a low fraction of the cells in the

population of strain JMP134(pJP4-F3) obtained after 14 days of growth on 3-CB. It should be noted that reversion to the wild type is never observed in cell populations obtained from a strain JMP134(pJPF3) clone (6; this work).

Due to the high percentage of cells from strain JMP134(pJP4-F3) found after only 2 days of growth on 3-CB (Fig. 5 and 6), we explored whether recombination occurs at earlier times. Since it takes 2 days to detect growth of strain JMP134 in 3-CB liquid batch cultures, we used a chemostat to monitor earlier states of exposure to 3-CB by growing cells first in fructose as the sole carbon source and then changing the sole carbon source in the medium to 3-CB. During the first 5 hours of exposure to 3-CB, the percentage of recombinant plasmid forms remained at basal levels, but after 6 h, the recombinant forms increased rapidly (Fig. 6E). The multiplex PCR procedure supported the phenotypic pattern (Fig. 6F). These results indicate that, upon selection on 3-CB, the cells of strain JMP134(pJP4-F3) rapidly outnumber those of the wild-type strain JMP134(pJP4), which is not surprising if we consider the lag effect produced by the medium change, supported by a drop in the stable culture optical density at 600 nm from 2.7 to 0.7 after the culture medium was changed.

Appearance of the JMP134(pJP4-FM) strain phenotype. The double homologous recombination event that produces the pJP4-F3 plasmid form (Fig. 2) should simultaneously generate the pJP4-FM derivative containing two copies of the *tfdA* gene, encoding the first step in degradation of phenoxyalkanoic acids. Theoretically, cells harboring the pJP4-FM plasmid should be able to grow efficiently on 2-MPA, which after the ether cleavage catalyzed by the TfdA protein (11, 27), is transformed to 2-methylphenol. This substituted phenol, like several other phenols, can then be transformed to a substituted catechol by the TfdBI and TfdBII proteins on plasmid pJP4. In the case of substitutions that are not halogens, such as in the case of 2-MPA, the compounds can be degraded by chromosomally encoded phenol hydroxylase functions and the chromosomal *meta* ring cleavage pathway encoded in *C. necator* JMP134 (16, 27). Therefore, cells harboring pJP4-FM should have a selective advantage in culture media containing 2-MPA as the sole carbon and energy source. Although 2-MPA did not select for strain JMP134(pJP4-FM) in basal recombination tests (Fig. 5), we tested whether several transfers to 2-MPA cultures after recombination events selected by growth on 3-CB would allow selection of strain JMP134(pJP4-FM). Aliquots of all liquid cultures transferred to 3 mM 3-CB were transferred to 2-MPA liquid cultures and incubated for 2 days. Afterwards, an aliquot of each culture grown on 2-MPA was transferred to a fresh 2-MPA liquid culture and so on for up to 6 days. A total of 120 colonies were picked from all these cultures and checked for growth in 2-MPA and 3-CB. No selective enrichment for strain JMP134(pJP4-FM) on 2-MPA cultures was found (Fig. 7A), which was coincident with the PCR product pattern detected in these cultures (Fig. 7B). This result is consistent with the low level of recombinants found in cultures grown on 2-MPA after 2 days (Fig. 5), indicating that strain JMP134(pJP4) is selected over strain JMP134(pJP4-FM), even though the latter harbors two copies of the *tfdA* gene. Gene dosage effects for growth on 2,4-D and MCPA have been recently demonstrated (16), which can be explained by the formation of the toxic compounds 2,4-dichlorophenol

and 4-chloro-2-methylphenol as intermediates. The turnover of these chlorophenols depends on the activities of the TfdA and TfdB proteins (16, 27). The latter are encoded by the *tfdB* genes, which are located in the pJP4 plasmid and absent in pJP4-FM (Fig. 1 and 2). It is possible that either 2-methylphenol is less toxic than 2,4-dichlorophenol or 4-chloro-2-methylphenol or it is efficiently metabolized by chromosomal functions present in both strains JMP134(pJP4) and JMP134(pJP4-FM). An alternative explanation is that 2-methylphenol is toxic and that efficient formation in strain JMP134(pJP4-FM), because of the two copies of the *tfdA* gene, selects against this strain. The latter possibility may explain why very few cells from strain JMP134(pJP4-FM) could be obtained after transfer to liquid cultures containing 2-MPA as the sole carbon source (Fig. 5).

In summary, this work demonstrates recombination of a catabolic plasmid leading to different catabolic phenotypes in the absence of obvious selective growth pressures. This phenomenon may also take place in other catabolic plasmids containing inverted repeats, providing a mechanism for metabolic plasticity. For example, these types of elements have been found in catabolic plasmids, such as pCAR1 (19), pWW100 (18), and pADP-1 (7, 20), and have been proposed to contribute to plasmid structuring and recombination events, but they have not been studied in depth. Another example is plasmid pEST4011 (32), which is a spontaneous deletion/duplication derivative of plasmid pD2M4 in which various inverted repeated elements are present. We suggest that this kind of recombination event is probably more common than thought and may play an important role in the appearance of catabolic (and other) plasmids containing repeated elements. It is interesting to note that if isolation of *C. necator* JMP134(pJP4) had been carried out by growth on 3-CB instead of 2,4-D, when isolated 25 years ago (8), the catabolic plasmid harbored by this strain would not be pJP4 as it is known today, but the pJP4-F3 plasmid form we have been reported (6, 31; this work).

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