Genuine genetic redundancy in maleylacetate-reductase-encoding genes involved in degradation of haloaromatic compounds by *Cupriavidus necator* JMP134

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Maleylacetate reductases (MAR) are required for biodegradation of several substituted aromatic compounds. To date, the functionality of two MAR-encoding genes ($tfdF_I$ and $tfdF_{II}$) has been reported in *Cupriavidus necator* JMP134(pJP4), a known degrader of aromatic compounds. These two genes are located in *tfd* gene clusters involved in the turnover of 2,4-dichlorophenoxyacetate (2,4-D) and 3-chlorobenzoate (3-CB). The *C. necator* JMP134 genome comprises at least three other genes that putatively encode MAR (*tcpD*, *hqoD* and *hxqD*), but confirmation of their functionality and their role in the catabolism of haloaromatic compounds has not been assessed. RT-PCR expression analyses of *C. necator* JMP134 cells exposed to 2,4-D, 3-CB, 2,4,6-trichlorophenol (2,4,6-TCP) or 4-fluorobenzoate (4-FB) showed that $tfdF_I$ and $tfdF_{II}$ are induced by haloaromatics channelled to halocatechols as intermediates. In contrast, 2,4,6-TCP only induces *tcpD*, and any haloaromatic compounds tested did not induce *hxqD* and *hqoD*. However, the *tcpD*, *hxqD* and *hqoD* gene products showed MAR activity in cell extracts and provided the MAR function for 2,4-D catabolism when heterologously expressed in MAR-lacking strains. Growth tests for mutants of the five MAR-encoding genes in strain JMP134 showed that none of these genes is essential for degradation of the tested compounds. However, the role of $tfdF_I$/$tfdF_{II}$ and *tcpD* genes in the expression of MAR activity during catabolism of 2,4-D and 2,4,6-TCP, respectively, was confirmed by enzyme activity tests in mutants. These results reveal a striking example of genetic redundancy in the degradation of aromatic compounds.

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INTRODUCTION

Maleylacetate reductases (MAR) play an important role in degradation of (halo)aromatic compounds in fungi (Gaal & Neujahr, 1980; Jones *et al.*, 1995; Patel *et al.*, 1992; Sparnins *et al.*, 1979), *Actinobacteria* (Huang *et al.*, 2006; Perry & Zylstra, 2007; Seibert *et al.*, 1998; Travkin *et al.*, 1999) and *Proteobacteria* (Armengaud *et al.*, 1999; Daubaras *et al.*, 1996; Endo *et al.*, 2005; Moonen *et al.*, 2008; Nikodem *et al.*, 2003; Seibert *et al.*, 2004; Yoshida *et al.*, 2007). In these micro-organisms, maleylacetate (MA) or 2-chloromaleylacetate (2-CMA) are generated during turnover of halocatechols, hydroxyquinol, chlorohydroxyquinol, hydroquinone and chlorohydroquinone, as ring-cleavage intermediates of a broad array of metabolized compounds.

MA is transformed to 3-oxoadipate through an NAD(P)H-dependent reduction of the carbon–carbon double bond catalysed by MAR (Fig. 1a). In the case of 2-CMA, MAR initially catalyses the NAD(P)H-dependent dechlorination to MA, which is then reduced to 3-oxoadipate (Fig. 1a). Therefore, 2 moles of NAD(P)H per mole of substrate are consumed during the MAR-mediated conversion of 2-CMA to 3-oxoadipate, which is subsequently funnelled to the Krebs cycle (Kaschabek & Reineke, 1992, 1995; Vollmer *et al.*, 1993).

Cupriavidus necator JMP134 is a betaproteobacterium able to grow on a wide range of aromatic and haloaromatic compounds as sole carbon and energy source

Abbreviations: 2-CMA, 2-chloromaleylacetate; 2,4-D, 2,4-dichlorophenoxyacetate; 2,4,6-TCP, 2,4,6-trichlorophenol; 3-CB, 3-chlorobenzoate; 4-FB, 4-fluorobenzoate; MA, maleylacetate; MAR, maleylacetate reductase(s).

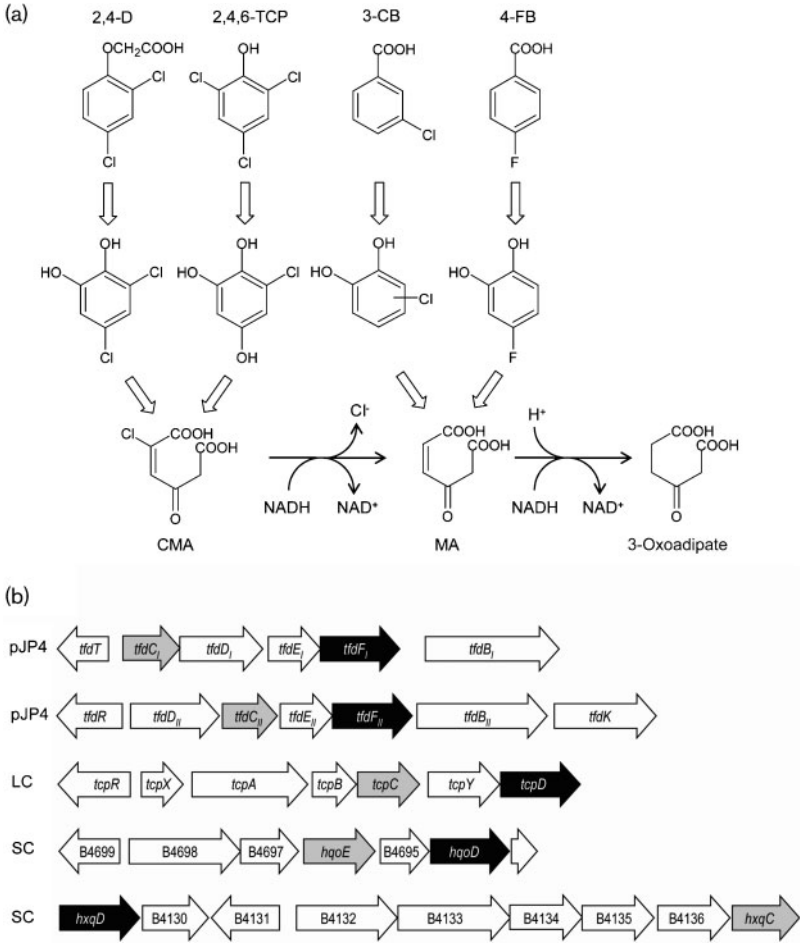

Fig. 1. (a) Haloaromatic compounds channelled to MA in *C. necator* JMP134 and reactions catalysed by MAR. (b) Organization of gene clusters in *C. necator* JMP134 comprising MAR-encoding genes (black arrows). Ring-cleavage dioxygenase-encoding genes are shown as grey arrows. LC, Large chromosome; SC, small chromosome.

(Pérez-Pantoja *et al.*, 2008). The haloaromatic compounds catabolized by *C. necator* JMP134 include 2,4-dichlorophenoxyacetate (2,4-D), 3-chlorobenzoate (3-CB), 2,4,6-trichlorophenol (2,4,6-TCP) and 4-fluorobenzoate (4-FB) which, through diverse catabolic steps, are converted to MA or 2-CMA (Fig. 1a) (Pérez-Pantoja *et al.*, 2008).

The role of MAR in *C. necator* JMP134 has been well established in the turnover of 3,5-dichlorocatechol, 3- (and 4-) chlorocatechol, 4-fluorocatechol and 6-chlorohydroxyquinol, generated during mineralization of 2,4-D (Seibert *et al.*, 1993; Vollmer *et al.*, 1993), 3-CB (Laemmli *et al.*, 2000; Pérez-Pantoja *et al.*, 2000), 4-FB (Schlomann *et al.*, 1990a) and 2,4,6-TCP (Padilla *et al.*, 2000; Sánchez & González, 2007), respectively. Two MAR-encoding genes – $tfdF_I$ and $tfdF_{II}$ – have been identified in *C. necator* JMP134, both belonging to specialized gene clusters for chlorocatechol turnover located on the pJP4 catabolic plasmid (Fig. 1b) (Trefault *et al.*, 2004). The functionality of both genes and their expression during mineralization of 2,4-D and 3-CB has been confirmed (Kasberg *et al.*, 1995; Laemmli *et al.*, 2000, 2004; Pérez-Pantoja *et al.*, 2000; Plumeier *et al.*, 2002). Additionally, the presence of at least one chromosomally encoded MAR has been shown (Padilla *et al.*, 2000; Pérez-Pantoja *et al.*, 2000; Plumeier *et al.*, 2002; Vollmer *et al.*, 1993). Recently, the analysis of the *C. necator* JMP134 genome sequence has revealed the presence of three hypothetical MAR-encoding genes located in chromosomal replicons (Fig. 1b). These additional gene sequences have been termed *tcpD* (Sánchez & González, 2007), *hqoD* and *hxqD*, since they are clustered with genes putatively encoding 2,4,6-TCP, hydroquinone and hydroxyquinol-metabolizing oxygenases, respectively (Pérez-Pantoja *et al.*, 2008); however, their possible function requires experimental confirmation.

In this work, the functionality of the chromosomal MAR-encoding genes of *C. necator* JMP134 was assessed. The results reveal a genuine genetic redundancy for MAR functions in the catabolism of haloaromatic compounds.

METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *C. necator* JMP134(pJP4) and its derivatives were grown at 30 °C in mineral salts medium using 50 mM phosphate buffer (pH 7.5) (Dorn *et al.*, 1974). The medium was supplemented with different haloaromatic compounds – 2,4-D (2.5 mM), 3-CB (2.5 mM), 4-FB (2.5 mM) and 2,4,6-TCP (0.5 mM) – or fructose (10–20 mM) as sole carbon source,

Table 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant phenotype and/or genotype*	Source or reference†
***C. necator* strains**		
JMP134(pJP4)	2,4-D$^+$ 3-CB$^+$ 4-FB$^+$ 2,4,6-TCP$^+$, Hg^R	DSMZ Culture Collection
JMP134(pJP4Δ*tfdF*$_I$)	*tfdF*$_I$ mutant	This study
JMP134(pJP4Δ*tfdF*$_{II}$)	*tfdF*$_{II}$ mutant	This study
JMP134Δ*tcpD*(pJP4)	*tcpD* mutant, Km^R	This study
JMP134Δ*hqoD*(pJP4)	*hqoD* mutant, Km^R	This study
JMP134Δ*hxqD*(pJP4)	*hxqD* mutant, Km^R	This study
JMP134(pJP4Δ*tfdF*$_I$Δ*tfdF*$_{II}$)	*tfdF*$_I$ and *tfdF*$_{II}$ mutant, Gm^R	This study
JMP134Δ*tcpD*(pJP4Δ*tfdF*$_I$Δ*tfdF*$_{II}$)	*tcpD tfdF*$_I$ and *tfdF*$_{II}$ mutant, Km^R Gm^R	This study
JMP134Δ*hqoD*(pJP4Δ*tfdF*$_I$Δ*tfdF*$_{II}$)	*hqoD tfdF*$_I$ and *tfdF*$_{II}$ mutant, Km^R Gm^R	This study
JMP134Δ*hxqD*(pJP4Δ*tfdF*$_I$Δ*tfdF*$_{II}$)	*hxqD tfdF*$_I$ and *tfdF*$_{II}$ mutant, Km^R Gm^R	This study
Other strains		
B. phytofirmans PsJN	2,4-D$^-$	Sessitsch *et al.* (2005)
B. phytofirmans PsJN(pJP4)	2,4-D$^+$, Hg^R	This study
P. putida KT2442	Rf^R	DSMZ Culture Collection
E. coli BW25113(pJP4)	Hg^R	Pérez-Pantoja *et al.* (2003)
Plasmids		
pKD4	*oriR*[R6Kγ], Ap^R Km^R	Datsenko & Wanner (2000)
pKD46	*araC-P*$_{araB}$-λRed recombination genes (γ-β-exo), Ap^R	Datsenko & Wanner (2000)
pBH474	BHR, Flp recombinase expressed constitutively, Gm^R Suc^S	House *et al.* (2004)
pBS1	BHR, *araC-P*$_{BAD}$, Gm^R	Bronstein *et al.* (2005)
pBS1*tcpD*	*tcpD*-expressing pBS1 derivative, Gm^R	This study
pBS1*hqoD*	*hqoD*-expressing pBS1 derivative, Gm^R	This study
pBS1*hxqD*	*hxqD*-expressing pBS1 derivative, Gm^R	This study
pCR2.1-TOPO	Suicide vector in *C. necator* JMP134, Ap^R Km^R	Invitrogen Life Technologies
pTOPOΔ*tcpD*	pCR2.1 derivative with internal fragment of *tcpD*, Ap^R Km^R	This study
pTOPOΔ*hqoD*	pCR2.1 derivative with internal fragment of *hqoD*, Ap^R Km^R	This study
pTOPOΔ*hxqD*	pCR2.1 derivative with internal fragment of *hxqD*, Ap^R Km^R	This study
pLitmusΔ*tfdF*$_{II}$	Gm^R cassette inserted in *tfdF*$_{II}$, Ap^R Gm^R	Ledger *et al.* (2002)

*Ap^R, ampicillin resistance; Gm^R, gentamicin resistance; Km^R, kanamycin resistance; Rf^R, rifampicin resistance; Hg^R, mercury resistance; Suc^S, sucrose sensitivity; BHR, broad host range.
†DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

plus the appropriate antibiotics: kanamycin (100 μg ml^{-1}) and/or gentamicin (20 μg ml^{-1}) (Table 1). *Burkholderia phytofirmans* PsJN(pJP4) and its derivatives were grown under the same conditions but with 2,4-D (2.5 mM) as growth compound plus gentamicin (20 μg ml^{-1}) and L-arabinose (0.25 mM) as inducer of the P_{BAD} promoter in pBS1 derivatives (Table 1). *Pseudomonas putida* KT2442 and its derivatives were grown under the same conditions but with succinate (30 mM) plus gentamicin (20 μg ml^{-1}) and L-arabinose (5 mM) (Table 1). *Escherichia coli* Mach 1 (Invitrogen Life Technologies) was used routinely as plasmid host and was grown at 37 °C in Luria–Bertani (LB) medium plus the appropriate antibiotic: ampicillin (100 μg ml^{-1}), kanamycin (100 μg ml^{-1}) or gentamicin (20 μg ml^{-1}). Growth was determined by measuring the optical density at 600 nm. At least two replicates were performed for each growth measurement.

Detection of transcripts by RT-PCR. Cells of *C. necator* JMP134 were grown overnight in minimal medium with 10 mM fructose as carbon source. This culture was used to inoculate fresh medium and grown until OD_{600}=0.7, subsequently supplemented with 0.25 mM 3-CB, 2,4-D, 4-FB or 2,4,6-TCP and incubated for 1 h. Then, total RNA was obtained from 4 ml of the culture, using RNAprotect Bacteria Reagent and RNeasy Mini kit (Qiagen), according to manufacturer's instructions. The obtained RNA was quantified using a GeneQuant 1300 Spectrophotometer (GE Healthcare) and treated with the Turbo DNase kit (Ambion) to remove any DNA contamination, following the manufacturer's instructions. RT-PCR was carried out using the ImProm-II Reverse Transcription System (Promega) with 1 μg total RNA in 20 μl reactions. After reverse transcription, PCR amplifications were carried out using the primer pairs *tfdF*$_I$intFW/*tfdF*$_I$intRV, *tfdF*$_{II}$intFW/*tfdF*$_{II}$intRV, *tcpD*intFW2/*tcpD*intRV2, *hqoD*intFW/*hqoD*intRV and *hxqD*intFW/*hxqD*intRV (these and all primer pairs used in this work are shown in Table 2) in a 25 μl mixture which contained 1 μl total cDNA, 50 pmol each primer, 50 μM each dNTP, 1 mM $MgCl_2$ and 5 U *Taq* DNA polymerase, prepared in the reaction buffer supplied by the manufacturer. The temperature programme was as follows: initial denaturation at 95 °C for 5 min, then 28 cycles of 30 s at 95 °C, 30 s at 60 °C and 60 s at 72 °C, with a final extension at 72 °C for 10 min. Negative control reactions were performed in the same way, except that reverse transcriptase was omitted in the reaction mixtures.

Construction of plasmid derivatives expressing MAR-encoding genes. *tcpD*, *hqoD* and *hxqD* were PCR-amplified from DNA obtained from strain JMP134, using primer pairs *tcpD*FW/*tcpD*RV, *hqoD*FW/*hqoD*RV and *hxqD*FW/*hxqD*RV, respectively. The PCR products were cloned individually using the pCR8/GW/TOPO TA Cloning kit (Invitrogen Life Technologies), and the resulting plasmids

Table 2. Primer pairs used in this work

Primer	Sequence (5′→3′)*
RT-PCR analysis and inactivation of chromosomal MAR genes	
tfdF$_{I}$intFW	ACGCGAGTTAGCGAAGGATA
tfdF$_{I}$intRV	GAGATAGCAAGCGGCAAATC
tfdF$_{II}$intFW	CTCTACGACCCGCATCACCT
tfdF$_{II}$intRV	GAGCAGATGGCAGAGCTTGT
*tcpD*intFW1	GGCTCGGAGATGACTACGAT
*tcpD*intRV1	CATGGGCGTACAAACCTTCT
*tcpD*intFW2	CACGCAGCAGAAGGTTTGTA
*tcpD*intRV2	AGGATGATCGTGTGCGTTTC
*hqoD*intFW	GGTCTACGATCCCGAACTGA
*hqoD*intRV	GTATGGCACAGCTTGTGGTG
*hxqD*intFW	ATCTACGATCCCGCACTGAG
*hxqD*intRV	ATGGCACAGCTTGTGATGAA
Inactivation of MAR genes encoded in pJP4	
MUT*tfdF*$_{I}$FW	GACCCTTCATGAAGAAGTTCACGCTTGACTACCTGAGCCCGTGTAGGCTGGAGCTGCTTC
MUT*tfdF*$_{I}$RV	GCGGAGTTGCAGGTCACATTATTTGAAATCCGGTCTTCGCCATTCCGGGGATCCGTCGACC
MUT*tfdF*$_{II}$FW	CCGGCGATCTGAATGAATTCGTTGCGCACTTCTGGCCGGTGTGTAGGCTGGAGCTGCTTC
MUT*tfdF*$_{II}$RV	AGAGGTCCATGGGATGTCCGGTTCACGCCGGCATTTCTCCATTCCGGGGATCCGTCGACC
Cloning of MAR genes and verification of gene disruption	
tfdF$_{I}$FW	CTCGAACTGCTTGCAATGTT
tfdF$_{I}$RV	ACCGTACTAAACGCGGAGTG
tfdF$_{II}$FW	ATCTCGAATCGCCGGACA
tfdF$_{II}$RV	TCCTTATCGATAGGTCGGGTCG
*tcpD*FW	TATCGTTTTGACGGGAGACC
*tcpD*RV	CCAACACATACCGCTTCAAA
*hqoD*FW	GAGCAAAACCATGAAATCG
*hqoD*RV	CGATGTCATAGGCGACGA
*hxqD*FW	GACTGAAACGGGAGTTGTCC
*hxqD*RV	TGGTGACTCCTTTCCTCCTG

*The 20 bp priming sequences for pKD4 (Table 1) are underlined.

were electroporated into competent cells of *E. coli* Mach 1. Then, MAR genes were transferred from pCR8/GW/TOPO derivatives to the pBS1 vector (Bronstein *et al.*, 2005) by recombination-based transfer of the PCR product using the Gateway LR Clonase II Enzyme Mix (Invitrogen Life Technologies), according to the manufacturer's instructions. The identity of MAR genes in the recombinant plasmids was confirmed by sequencing. The generated recombinant plasmids were electroporated into *P. putida* KT2442.

Preparation of cell extracts. *P. putida* KT2442 derivatives harbouring MAR genes were grown to late exponential phase in mineral medium with succinate (30 mM) as sole carbon source and in the presence of 5 mM L-arabinose. Mutant derivatives of *C. necator* JMP134 were grown for 18 h in mineral medium with fructose (20 mM) in the presence of 2,4-D (2 mM) or 2,4,6-TCP (0.5 mM) as inducers of MAR activity. *P. putida* and *C. necator* cells were harvested, centrifuged at 8500 r.p.m. for 10 min and washed twice with 1 vol. Tris/acetate (50 mM, pH 7.5). Then, the cells were resuspended in 1 ml Tris/acetate (50 mM, pH 7.5) and subjected to lysis by sonication with three pulses of 10 s at potency level 15 using an ultrasonic cell disruptor (Microson XL2000; Misonic Inc). The lysates were subjected to two successive centrifugations at 16 000 r.p.m. for 45 min at 4 °C. Finally, supernatants were collected and used for enzyme assays.

MAR activity assays. MAR activity was measured spectrophotometrically by the decrease of cofactor NADH at 340 nm (ε_{340}=6300 M^{-1} cm^{-1}). The standard assay mixture contained (ml^{-1}) 35 mM Tris/acetate buffer (pH 7.5), 100 μM MA, 0.2 mM NADH and a suitable quantity of cell extract. After non-specific (in the absence of MA) NADH oxidation at 340 nm had been recorded for 2 min, MA was added and recording of NADH oxidation was continued for 2 min. MAR activity was calculated from the difference between the non-specific and MA-dependent oxidation rates. The protein concentration in the cell extracts was estimated as described by Bradford (1976). BSA was used as protein standard.

Inactivation of *tfdF*$_{I}$ and *tfdF*$_{II}$ in pJP4. *tfdF*$_{I}$ and *tfdF*$_{II}$ were independently inactivated in *E. coli* strain BW25113, harbouring pJP4, using a procedure described by Datsenko & Wanner (2000). Primer pairs MUT*tfdF*$_{I}$FW/MUT*tfdF*$_{I}$RV (for *tfdF*$_{I}$) and MUT*tfdF*$_{II}$FW/MUT*tfdF*$_{II}$RV (for *tfdF*$_{II}$), which contain 20 bp priming sequences for pKD4 (Table 1) and 40 bp homology extensions of the *tfdF*$_{I}$ or *tfdF*$_{II}$ gene sequences, were synthesized. These primer pairs were used with pKD4 as a template to amplify the kanamycin resistance gene flanked by 40 bp of the *tfdF*$_{I}$ or *tfdF*$_{II}$ gene sequences. The following PCR program was used: 95 °C for 5 min, 28 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 90 s, and then 72 °C for 10 min. The PCR products were used to inactivate *tfdF*$_{I}$ or *tfdF*$_{II}$ in *E. coli* strain BW25113(pJP4), harbouring pKD46 that provides the λ Red recombination system from the arabinose-inducible P_{BAD} promoter, by a previously adapted procedure (Pérez-Pantoja *et al.*, 2003). The pJP4 derivatives containing an inactivated *tfdF*$_{I}$ or *tfdF*$_{II}$ gene were

then transferred to strain JMP134 by biparental conjugation as described by Clément *et al.* (2000), and the transconjugants were selected on minimal medium agar plates supplemented with 3 mM benzoate plus kanamycin (100 μg ml^{-1}). The transconjugant colonies were propagated in liquid cultures with kanamycin by five successive transfers until the native wild-type pJP4 plasmid was completely removed and only the corresponding pJP4 derivative containing an inactivated version of $tfdF_I$ or $tfdF_{II}$ was present in *C. necator* JMP134. Primer pairs $tfdF_I$FW/$tfdF_I$RV and $tfdF_{II}$FW/$tfdF_{II}$RV were used to verify by PCR the correct insertion by recombination of the kanamycin resistance cassette in place of the $tfdF_I$ or $tfdF_{II}$ gene sequences. Second, the inserted kanamycin cassettes were eliminated by electroporating a helper plasmid, pBH474 (House *et al.*, 2004), expressing Flp recombinase (Datsenko & Wanner, 2000), generating the respective kanamycin resistance-free mutant strains. Finally, these kanamycin-sensitive mutant derivatives were streaked on 5% sucrose LB agar plates to select for the loss of pBH474, and the deletions in $tfdF_I$ or $tfdF_{II}$ were verified by PCR using the primer pairs $tfdF_I$FW/$tfdF_I$RV and $tfdF_{II}$FW/$tfdF_{II}$RV, to obtain *C. necator* JMP134(pJP4Δ$tfdF_I$) and *C. necator* JMP134(pJP4Δ$tfdF_{II}$), respectively (Table 1).

A double Δ$tfdF_I$ Δ$tfdF_{II}$ mutant derivative of *C. necator* JMP134 was generated from strain JMP134(pJP4Δ$tfdF_I$) by electroporating the suicide pLitmusΔ$tfdF_{II}$ plasmid (Table 1), containing a $tfdF_{II}$ gene sequence disrupted with the insertion of a gentamicin resistance cassette. Mutant derivatives subjected to a double homologous recombination event between $tfdF_{II}$ alleles of pJP4Δ$tfdF_I$ and pLitmusΔ$tfdF_{II}$ were selected on the basis of a gentamicin-resistant and ampicillin-sensitive phenotype. The correct insertion of the gentamicin resistance cassette in $tfdF_{II}$ was verified by PCR using primer pair $tfdF_{II}$FW/$tfdF_{II}$RV to obtain strain JMP134(pJP4Δ$tfdF_I$Δ$tfdF_{II}$) (Table 1).

Chromosomal disruption of gene sequences in *C. necator* JMP134. An internal fragment of each MAR gene was individually amplified by PCR from strain JMP134 DNA, using the primer pairs *tcpD*intFW1/*tcpD*intRV1, *hqoD*intFW/*hqoD*intRV and *hxqD*intFW/*hxqD*intRV. The PCR products were cloned individually using the pCR2.1-TOPO system (Invitrogen Life Technologies) to generate recombinant plasmids pTOPOΔ*tcpD*, pTOPOΔ*hqoD* and pTOPOΔ*hxqD* (Table 1). These suicide plasmids were independently electroporated into cells of *C. necator* JMP134 to obtain a one-recombination-event disruption of the target chromosomal gene. Mutant derivatives were selected on LB agar containing kanamycin, and correct recombinational insertion in the corresponding target gene was confirmed by PCR using primer pairs *tcpD*FW/*tcpD*RV, *hqoD*FW/*hqoD*RV and *hxqD*FW/*hxqD*RV, to obtain derivatives JMP134Δ*tcpD*, JMP134Δ*hqoD* and JMP134Δ*hxqD*, respectively (Table 1).

Triple MAR-encoding gene mutant derivatives of *C. necator* JMP134 were constructed by mobilizing the pJP4Δ$tfdF_I$Δ$tfdF_{II}$ plasmid by biparental conjugation from strain JMP134(pJP4Δ$tfdF_I$Δ$tfdF_{II}$) to each chromosomally encoded MAR mutant derivative, and subsequent selection of transconjugants on minimal medium agar plates supplemented with 3 mM benzoate plus kanamycin (100 μg ml^{-1}) and gentamicin (20 μg ml^{-1}). The transconjugant colonies were propagated in liquid cultures with gentamicin and kanamycin by five successive transfers until the native wild-type pJP4 plasmid of each chromosomally encoded MAR mutant was completely removed, and only the pJP4Δ$tfdF_I$Δ$tfdF_{II}$ derivative was present (checked by PCR). The simultaneous inactivation of three MAR-encoding genes in each triple mutant derivative was verified by PCR using the appropriate primer pairs.

Chemicals. 2,4-D, 3-CB, 4-FB and 2,4,6-TCP were purchased from Sigma-Aldrich. MA was prepared by alkaline hydrolysis from *cis*-dienelactone, which was kindly provided by Walter Reineke (Bergische Universität-Gesamthochschule Wuppertal, Germany). All MA solutions were used on the same day that they were prepared.

RESULTS

RT-PCR analysis of MAR-encoding genes in *C. necator* JMP134

In order to elucidate the role of MAR-encoding genes in *C. necator* JMP134, an RT-PCR analysis of gene expression in cells exposed to haloaromatic compounds channelled to MA was initially performed. *C. necator* JMP134 was exposed to 2,4-D, 3-CB, 4-FB or 2,4,6-TCP during exponential growth on fructose as growth substrate, and total RNA was extracted for RT-PCR analysis. The pJP4-encoded MAR genes ($tfdF_I$ and $tfdF_{II}$) were clearly induced by compounds that produce chlorocatechols as central intermediates (3-CB and 2,4-D) (Fig. 2), in agreement with earlier reports (Laemmli *et al.*, 2004; Leveau *et al.*, 1999). Interestingly, 4-FB turnover, which generates 4-fluorocatechol as intermediate (Schlomann *et al.*, 1990a), also induced $tfdF_I$ and $tfdF_{II}$. 2,4,6-TCP induced *tcpD*, and a slight induction of $tfdF_I$ by 2,4,6-TCP was also observed (Fig. 2), in accordance with an earlier report (Sánchez & González, 2007). In contrast, *hqoD* and *hxqD* showed no induction pattern in response to any haloaromatic compound tested (Fig. 2). Under our assay conditions, only $tfdF_I$ gene transcripts were detected in the absence of haloaromatic inducers (Fig. 2).

Functional assays for chromosomally encoded MAR genes

To assess the functionality of chromosomal MAR-encoding genes of *C. necator* JMP134, *tcpD*, *hxqD* and *hqoD* were cloned downstream of the arabinose-inducible promoter P_{BAD} in pBS1 to generate pBS1*tcpD*, pBS1*hxqD* and pBS1*hqoD*, respectively, and transferred to *P. putida*

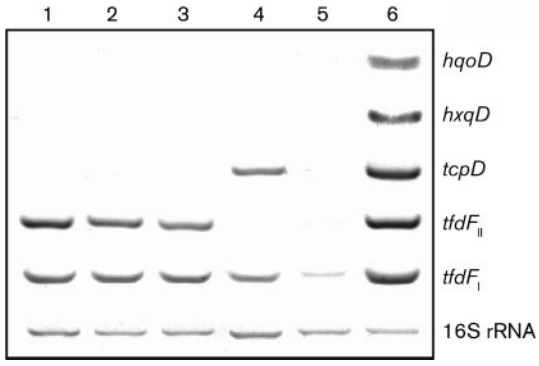

Fig. 2. RT-PCR analysis of MAR-encoding genes in *C. necator* JMP134 following exposure to 2,4-D (lane 1), 3-CB (2), 4-FB (3), 2,4,6-TCP (4) or no inducer (5). Lane 6, PCR products obtained from *C. necator* JMP134 DNA.

KT2442. The genome of this strain does not contain putative MAR-encoding genes (Matus *et al.*, 2003), and no measurable MAR activity was detected in a *P. putida* cell-free extract (data not shown). Cell extracts of *P. putida* KT2442(pBS1*tcpD*) contained 5983 ± 1322 U MAR activity g^{-1}, whereas *hqoD*- or *hxqD*-expressing *P. putida* derivatives showed 1112 ± 247 and 332 ± 128 U MAR activity g^{-1}, respectively. These values are similar to previously reported MAR activity levels in cell extracts of *C. necator* derivatives (Pérez-Pantoja *et al.*, 2000; Plumeier *et al.*, 2002).

The *in vivo* functionality of these chromosomal MAR-encoding genes was assessed in an engineered strain that allowed heterologous complementation for MAR function. *B. phytofirmans* PsJN (Sessitsch *et al.*, 2005) grew on 2,4-D when harbouring an intact pJP4 plasmid (Table 3). However, strain PsJN supplied with a pJP4 derivative lacking both $tfdF_I$ and $tfdF_{II}$ (pJP4Δ$tfdF_I$Δ$tfdF_{II}$) was not able to grow on 2,4-D (Table 3), in agreement with the absence of putative MAR-encoding genes in the genome of this bacterium. Therefore, introduction of a gene sequence encoding a functional MAR should allow growth of this bacterium on 2,4-D. The complementation of *B. phytofirmans*(pJP4Δ$tfdF_I$Δ$tfdF_{II}$) with pBS1*tcpD*, pBS1*hxqD* or pBS1*hqoD* plasmids completely restored the 2,4-D-degrading phenotype (Table 3).

Catabolic phenotype of mutants in MAR-encoding genes of *C. necator* JMP134

In order to evaluate the contribution of MAR-encoding genes to haloaromatic compound catabolism in strain JMP134, each MAR gene was mutated by insertional inactivation. The five *C. necator* JMP134 MAR mutants (Table 1) were tested for growth on haloaromatic substrates (Table 3). The $tfdF_I$ mutant showed slightly slower growth on 2,4-D. However, a polar effect over $tfdB_I$ (located downstream from $tfdF_I$, Fig. 1b) seems to be responsible for the delay on growth, because complementation with a $tfdB_I$-expressing plasmid completely restored efficient growth on 2,4-D (data not shown). The $tfdF_{II}$ mutant did not show significant effects on growth on any of these haloaromatic compounds. These results showed that pJP4 MAR-encoding genes are not essential for growth on haloaromatic compounds metabolized through chlorocatechols in *C. necator* JMP134, in accordance to an earlier report (Laemmli *et al.*, 2004). The *tcpD* mutant showed a significant decrease in growth on 2,4,6-TCP, as reported previously (Sánchez & González, 2007), but growth on this compound was not abolished. Finally, *hqoD* and *hxqD* mutants showed a behaviour similar to the wild-type strain on all haloaromatic compounds. In summary, the inactivation of single MAR-encoding genes in *C. necator* JMP134 did not abolish growth on any of these four haloaromatic compounds, indicating a functional redundancy of MAR-encoding genes.

To study if chromosomal MAR-encoding genes are able to supply the function of the pJP4-located MAR genes in the turnover of chlorocatechols, a $tfdF_I$-$tfdF_{II}$ double mutant of *C. necator* JMP134 was constructed. The *C. necator* JMP134(pJP4Δ$tfdF_I$Δ$tfdF_{II}$) strain still grew on all substrates tested, although severely retarded on 3-CB (Table 3), indicating that some or all the chromosomally encoded

Table 3. Growth of *C. necator* JMP134 mutant derivatives and *B. phytofirmans* PsJN engineered derivatives on haloaromatic compounds

++++, Growth in less than 1 day; +++, growth in 1–3 days; ++, growth in 3–5 days; +, growth in 5–7 days; −, no growth; NA, not applicable.

Strain	Growth on (sole carbon and energy source)			
	2,4-D	3-CB	4-FB	2,4,6-TCP
JMP134(pJP4)	++++	+++	+	++
JMP134(pJP4Δ$tfdF_I$)	+++	+++	+	++
JMP134(pJP4Δ$tfdF_{II}$)	++++	+++	+	++
JMP134Δ*tcpD*(pJP4)	++++	+++	+	+
JMP134Δ*hqoD*(pJP4)	++++	+++	+	++
JMP134Δ*hxqD*(pJP4)	++++	+++	+	++
JMP134(pJP4Δ$tfdF_I$Δ$tfdF_{II}$)	+++	+	+	++
JMP134Δ*tcpD*(pJP4Δ$tfdF_I$Δ$tfdF_{II}$)	+++	+	+	+
JMP134Δ*hxqD*(pJP4Δ$tfdF_I$Δ$tfdF_{II}$)	++	+	+	++
JMP134Δ*hqoD*(pJP4Δ$tfdF_I$Δ$tfdF_{II}$)	+++	+	+	++
PsJN	−	NA	NA	NA
PsJN(pJP4)	+++	NA	NA	NA
PsJN(pJP4Δ$tfdF_I$Δ$tfdF_{II}$)	−	NA	NA	NA
PsJN(pJP4Δ$tfdF_I$Δ$tfdF_{II}$)(pBS1*tcpD*)	+++	NA	NA	NA
PsJN(pJP4Δ$tfdF_I$Δ$tfdF_{II}$)(pBS1*hqoD*)	+++	NA	NA	NA
PsJN(pJP4Δ$tfdF_I$Δ$tfdF_{II}$)(pBS1*hxqD*)	+++	NA	NA	NA

MAR genes are able to replace the mutated pJP4-encoded genes. Triple mutants showed essentially the same growth pattern of *C. necator* JMP134(pJP4Δ$tfdF_I$Δ$tfdF_{II}$) on 2,4-D, 3-CB or 4-FB (Table 3), except for slower growth of *C. necator* JMP134Δ*hxqD*(pJP4Δ$tfdF_I$Δ$tfdF_{II}$) on 2,4-D. On the other hand, *C. necator* JMP134Δ*tcpD*(pJP4Δ$tfdF_I$Δ$tfdF_{II}$) showed a similar impaired growth on 2,4,6-TCP as *C. necator* JMP134Δ*tcpD*. These results indicate that the other chromosomal MAR functions are replacing the inactivated genes, and that only two out of the five MAR-encoding genes are enough to support growth on the four haloaromatic compounds tested.

Induction of MAR activity in MAR-encoding gene mutants of *C. necator* JMP134

To evaluate the role of MAR-encoding genes in the induction of MAR activity by 2,4-D or 2,4,6-TCP, MAR activity levels were measured in *C. necator* JMP134 mutant derivatives (Fig. 3). The induction of MAR activity by 2,4-D or 2,4,6-TCP in the wild-type strain (Fig. 3) was as reported in earlier studies (Padilla *et al.*, 2000; Pieper *et al.*, 1988). The induction of MAR activity by 2,4-D was completely abolished in *C. necator* JMP134(pJP4Δ$tfdF_I$Δ$tfdF_{II}$) (Fig. 3), indicating that chromosomally encoded MAR genes are not turned on in response to this compound, as indicated by RT-PCR analysis (Fig. 2). On the other hand, the induction of MAR activity by 2,4,6-TCP was completely abolished in *C. necator* JMP134Δ*tcpD* (Fig. 3), confirming the main (but not essential) role of *tcpD* in turnover of this compound (Table 3). On the other hand, the basal MAR activity under non-induced conditions was not abolished in any mutant derivative. However, a significant decrease of basal MAR activity levels was observed in *C. necator* JMP134(pJP4Δ$tfdF_I$Δ$tfdF_{II}$) (Fig. 3). This result is also in agreement with the RT-PCR detection of $tfdF_I$ transcripts in the absence of haloaromatic inducers (Fig. 2).

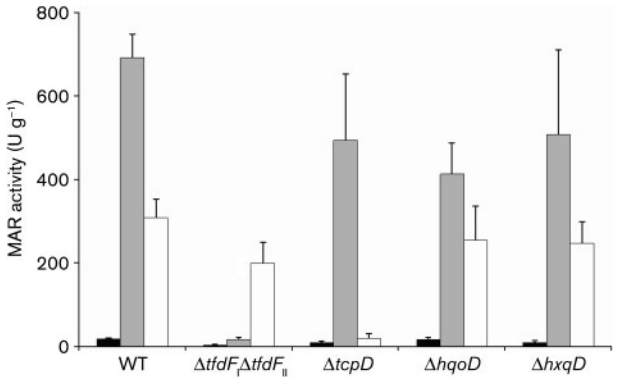

Fig. 3. Summed MAR activity levels in cell extracts of derivatives of *C. necator* JMP134 grown in the absence of haloaromatic compounds (black bars), the presence of 2,4-D (grey), or the presence of 2,4,6-TCP (white bars). Values are the means of three independent determinations. Vertical bars represent SD from the mean.

DISCUSSION

Genetic redundancy means that two or more genes are performing the same function in one organism and that inactivation of one of these genes has little or no effect on the phenotype of the organism. Despite the fact that redundant genes are likely to be eliminated by selective pressure, an analysis of 106 bacterial genomes has revealed that a significant number of apparent genetic redundancies have been maintained in individual genomes (Gevers *et al.*, 2004). However, it should be mentioned that functional support for these apparent genetic redundancies is required for the redundancy to be considered genuine.

In this work we clearly showed that MAR genes of *C. necator* JMP134 constitute a striking example of genuine genetic redundancy. This well-known bacterial model for microbial degradation of aromatic compounds possesses five functional genes encoding MAR, a catabolic function playing a fundamental role in channelling intermediates into the Krebs cycle and, therefore, allowing bacteria to use aromatic compounds as sole carbon and energy sources.

The two plasmid-encoded MAR in *C. necator* JMP134 have been extensively studied (Kasberg *et al.*, 1995; Muller *et al.*, 1996; Seibert *et al.*, 1993; Vollmer *et al.*, 1993). Both pJP4-encoded MAR genes ($tfdF_I$ and $tfdF_{II}$) are functional and contribute to catabolism of chlorocatechols (Plumeier *et al.*, 2002). However, $tfdF_I$ and $tfdF_{II}$ are not essential for degradation of chloroaromatic compounds and are, therefore, redundant (Laemmli *et al.*, 2004). Previous work has suggested the presence of chromosomally encoded MAR in *C. necator*, since MAR activity was reported in cell extracts of *C. necator* JMP222 – a pJP4-cured derivative – grown on 4-FB (Plumeier *et al.*, 2002; Schlomann *et al.*, 1990a), indicating that at least one chromosomally encoded MAR gene can replace the function of pJP4-encoded MAR genes. One candidate to fulfil this role was *tcpD* involved in the degradation of 2,4,6-TCP (Sánchez & González, 2007). However, this MAR activity had not been further evaluated. Two other putative MAR-encoding genes, *hqoD* and *hxqD*, have been proposed to be involved in chlorohydroquinone and hydroxyquinol catabolism, respectively, since they are clustered with putative ring-cleavage dioxygenase-encoding genes (Pérez-Pantoja *et al.*, 2008). In contrast to *tcpD* and the two pJP4 encoded MAR genes, where specific aromatic compounds (2,4,6-TCP, 2,4-D) can be used in growth tests, chlorohydroquinone and hydroxyquinol are too toxic to be assayed as growth substrates in *C. necator* JMP134 (data not shown). Unfortunately, the putative compounds that produce chlorohydroquinone and hydroxyquinol as intermediates in *C. necator* are unknown and the corresponding growth tests cannot be carried out.

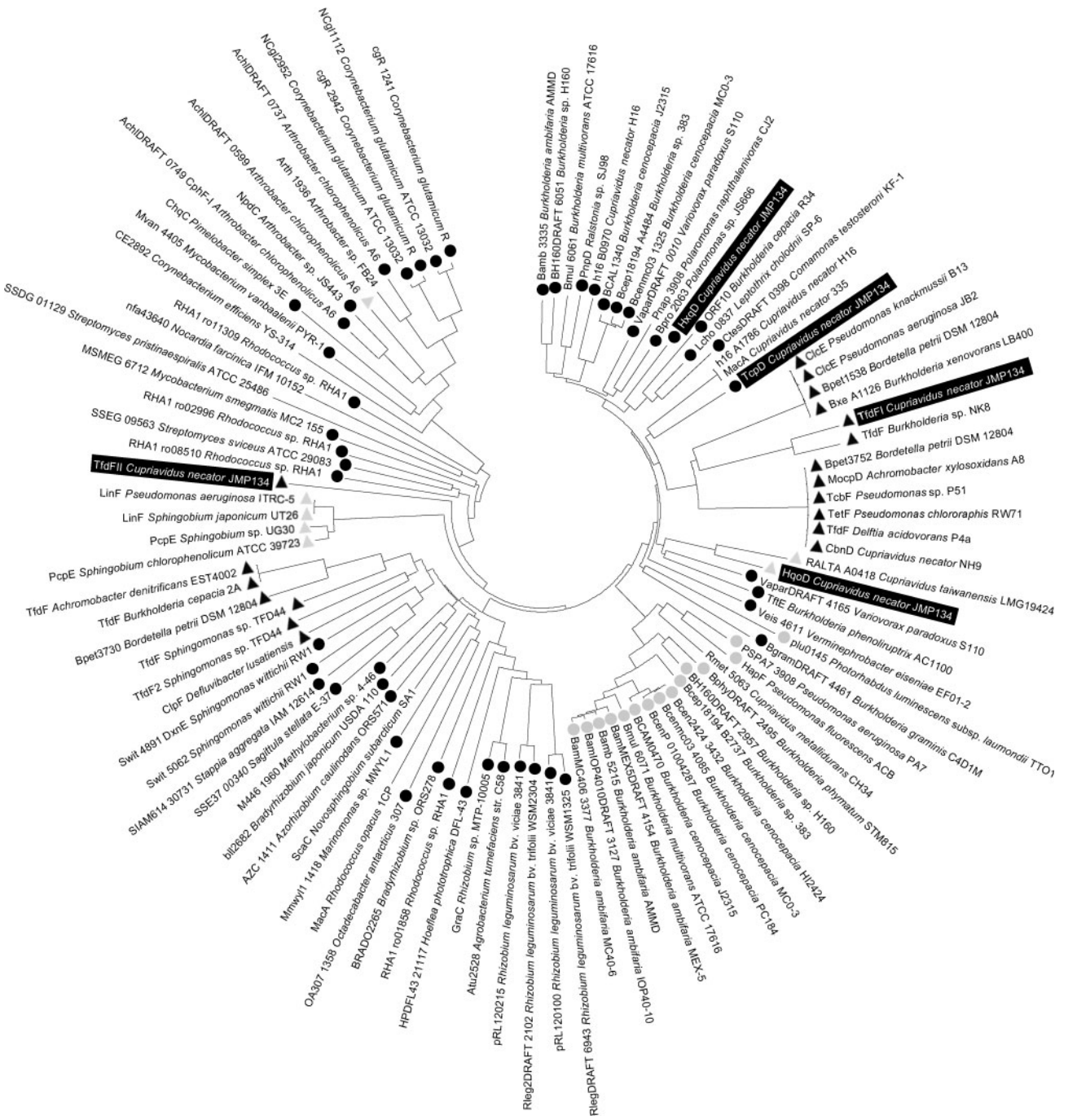

Fig. 4. Dendrogram showing the relatedness of MAR gene products. The dendrogram was obtained by the neighbour-joining method using MEGA 4.0 based on sequence alignments calculated by CLUSTAL W using the default options. Sequences of deduced proteins encoded in the genome of *C. necator* JMP134 are highlighted in black. The positional clustering of MAR-encoding genes with genes putatively encoding chlorocatechol 1,2-dioxygenase (black triangles), (chloro)hydroxyquinol 1,2-dioxygenase (black circles), chlorohydroquinone 1,2-dioxygenase (grey triangles) or hydroquinone 1,2-dioxygenase (grey circles) is shown. Sequences with >30 % amino acid identity to the *tfdF*$_{I}$ gene product were selected from the NCBI database.

In this work, we studied the role of chromosomal MAR-encoding genes in the catabolism of haloaromatics. By using both *in vivo* and *in vitro* approaches, it has been clearly shown that the three chromosomally encoded MAR are functional and provide the required MAR activity for 2,4-D catabolism in the heterologous system of *B. phytofirmans* PsJN. In addition, multiple mutation analysis supported the redundancy of MAR genes, since only two

genes are enough to perform this function in haloaromatic catabolism in *C. necator* JMP134. However, the induction profile of the five MAR genes showed some specificity. As expected, $tfdF_I$ and $tfdF_{II}$ were induced by 2,4-D and 3-CB, which generate the corresponding chloromuconates, inducers of the *tfd* operons (Filer & Harker, 1997). In the same way, 4-FB, which produces the analogue 3-fluoro-*cis*,*cis*-muconate (Schlomann *et al.*, 1990b), also induces $tfdF_I$ and $tfdF_{II}$, indicating that *tfd* genes probably contribute to 4-FB catabolism. However, the presence of *tfd* genes is not essential, since a pJP4-lacking derivative, *C. necator* JMP222, is still able to grow on this substrate (Schlomann *et al.*, 1990a). *hqoD* and *hxqD* did not show induction by any of the haloaromatic compounds tested, suggesting a role in catabolism of as yet unidentified aromatic compounds, probably channelled to chlorohydroquinone and/or hydroxyquinol as ring-cleavage intermediates. The results of MAR activity level tests in mutant derivatives showed that in the absence of the genes induced by 2,4-D and 2,4,6-TCP – $tfdF_I$/$tfdF_{II}$ and *tcpD*, respectively – no induction of the other MAR-encoding genes was observed; this suggests that these genes are not responsive to accumulation of MA or 2-CMA. However, the low background MAR activity level found in these mutant derivatives is enough to support growth on 2,4-D and 2,4,6-TCP.

The findings reported here raise the question of why such genetic redundancy is maintained in the genome of *C. necator* JMP134. We hypothesized that the reason to keep the five MAR-encoding genes is to avoid the intracellular accumulation of MA during haloaromatic catabolism. MA is unstable and at neutral pH is spontaneously decarboxylated to *cis*-acetylacrylate, a dead-end metabolite (Schlomann *et al.*, 1990b). It is interesting to note that each MAR gene in the genome of strain JMP134 is clustered with at least one gene putatively encoding a ring-cleavage dioxygenase producing MA as a downstream metabolite: chlorocatechol 1,2-dioxygenases ($TfdC_I$/$TfdC_{II}$); (chloro)hydroxyquinol 1,2-dioxygenases (TcpC/HxqC) and chlorohydroquinone 1,2-dioxygenase (HqoE) (Fig. 1b) (Pérez-Pantoja *et al.*, 2008). This positional clustering suggests that MAR genes are co-induced with the genes encoding the corresponding upstream step in the pathways, avoiding the possible accumulation of MA.

A dendrogram of MAR gene products, including sequences from complete genomes is shown in Fig. 4. An analysis of the gene sequences flanking MAR-encoding genes included in this dendrogram indicates that the vast majority of MAR genes are associated with genes putatively encoding ring-cleavage dioxygenases that generate MA, as found in strain JMP134. The clustering of MAR gene products belonging to *C. necator* JMP134 indicates that HxqD, putatively involved in hydroxyquinol catabolism, and $TfdF_I$, involved in chlorocatechol catabolism, grouped with several other MAR involved in similar pathways. On the contrary, TcpD and HqoD grouped in small branches with no clear pathway-associated profile, probably indicating that recruitment of these genes in their respective gene clusters is more recent. Finally, it should be noted that $TfdF_{II}$ is not closely related to any other MAR, indicating a completely different evolutionary origin.

It is remarkable that apparent MAR gene redundancy has been found in the genomes of 17 strains included in the dendrogram in Fig. 4, and that the number of MAR genes in these genomes is roughly equal to the number of genes putatively encoding ring-cleavage dioxygenases that generate MA. These observations support the idea that MAR gene redundancy is widespread in bacteria having more than one pathway generating MA as a downstream metabolite.

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