APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 2007, p. 2769–2776 Vol. 73, No. 9
0099-2240/07/$08.00+0 doi:10.1128/AEM.02584-06

Genetic Characterization of 2,4,6-Trichlorophenol Degradation in *Cupriavidus necator* JMP134[▽]

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Received 6 November 2006/Accepted 13 February 2007

The degradation pathway of 2,4,6-trichlorophenol (2,4,6-TCP), a hazardous pollutant, in the aerobic bacterium *Cupriavidus necator* JMP134(pJP4) (formerly *Ralstonia eutropha* JMP134) is encoded by the *tcp* genes. These genes are located in a genetic context, *tcpRXABCYD*, which resembles a putative catabolic operon. In this work, these gene sequences were individually disrupted and mutant strains were evaluated for their ability to grow on or degrade 2,4,6-TCP. The *tcpX* and *tcpA* mutants completely failed to degrade this compound. Although the *tcpC* mutant was also unable to grow on 2,4,6-TCP, it still transformed this chlorophenol to 6-chlorohydroquinol. In contrast, the *tcpD* mutant grew on 2,4,6-TCP, suggesting the presence of additional maleylacetate reductase-encoding genes. Five other open reading frames encoding maleylacetate reductases, in addition to the *tcpD* gene, were found in the genome of *C. necator*, and two of them provide this function in the *tcpD* mutant. The *tcpR* gene, encoding a putative LysR-type transcriptional regulator, was disrupted, and this mutant strain completely failed to grow on 2,4,6-TCP. Transcriptional fusion studies demonstrated that TcpR activates the expression of the *tcp* genes, responding specifically to 2,4,6-TCP. The transcriptional start of the *tcp* operon was mapped, and a putative σ^{70}-type promoter was identified.

2,4,6-Trichlorophenol (2,4,6-TCP), widely used as a biocide and preservative, is considered a priority environmental pollutant worldwide (14, 32). Aerobic bacteria degrade this pollutant and, in several cases, grow on it as the sole carbon source (3, 5, 15, 20). A catabolic pathway for 2,4,6-TCP (Fig. 1A) has been proposed (21, 26, 33, 37, 39). The pathway is initiated by the conversion of 2,4,6-TCP to 2,6-dichloro-*p*-hydroquinone (2,6-DCHQ) and then to 6-chlorohydroxyquinol (6-CHQ); both steps are catalyzed by flavin adenine dinucleotide (FAD)-dependent 2,4,6-TCP monooxygenase (TCP-MO). 6-CHQ is transformed to 2-chloromaleylacetate (2-CMA) by 6-chlorohydroxyquinol 1,2-dioxygenase (HQDO), and 2-CMA is converted to β-ketoadipate by NADH-dependent maleylacetate reductase (MAR). Recently, the *tcpABC* genes from *Cupriavidus necator* JMP134(pJP4), a well-known chloroaromatic compound-degrading strain (5, 8, 29), were shown to encode enzymes that convert 2,4,6-TCP to 2-CMA (21). However, no evidence about a gene encoding MAR activity was provided. The *tcpABC* genes in *C. necator* are adjacent to four other open reading frames (ORFs) (*tcpY*, *tcpD*, *tcpR*, and *tcpX*), forming a putative catabolic operon (Fig. 1B) (23). The *tcpR* gene has significant identity to the *pcpR* gene, which encodes a LysR-type regulator involved in the degradation of pentachlorophenol in *Sphingobium chlorophenolicum* (4). The TcpX protein is believed to provide $FADH_2$ to TcpA because it has high identity to the TftC protein, which putatively performs this function in the degradation of 2,4,5-TCP in *Burkholderia cepacia* AC1100 (10, 13). This flavin reductase activity would also be encoded by the *tcpB* gene, which shows sequence similarity to genes coding for nitroreductases. However, a *tcpB* mutant still degrades 2,4,6-TCP (21). Whereas the *tcpY* gene does not possess homology to any other gene sequence, the *tcpD* gene shows high sequence identity to maleylacetate reductase genes. In this work, we investigated the function of each of the *tcp* gene sequences in the *tcpRXABCYD* cluster from *C. necator* JMP134(pJP4). We report that this gene cluster encodes all of the functions required for transformation of 2,4,6-TCP to β-ketoadipate and that *tcpR* is the regulatory gene controlling *tcp* gene expression.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids are listed in Table 1. *C. necator* JMP134(pJP4) and its 2,4,6-TCP-mineralizing derivatives were grown at 30°C in minimal medium (16) with 0.4 to 2 mM 2,4,6-TCP as the sole carbon source. To determine growth at higher 2,4,6-TCP levels, *C. necator* JMP134 and its derivatives were initially grown on 0.4 mM 2,4,6-TCP in a 50-ml Erlenmeyer flask, and every 6 days of incubation, the culture was transferred to fresh medium containing a higher concentration (0.2 mM increases), up to 2 mM. Growth was determined by measuring the optical density at 600 nm (OD_{600}). Determination of OD_{600} in cell-free supernatants of these cultures discarded any effect of 2,4,6-TCP or its catabolic intermediates on the OD_{600} values. *C. necator* derivatives not able to proliferate on 2,4,6-TCP were grown on 1 mM phenol plus the appropriate antibiotic (Table 1). *Escherichia coli* strains were maintained on Luria-Bertani (LB) agar plates containing 50 μg of kanamycin ml^{-1} or 50 μg of trimethoprim ml^{-1}.

Analytical methods. The presence of 2,4,6-TCP and its derivatives was determined by UV spectroscopy or by high-performance liquid chromatography (HPLC) using cell-free supernatants from suspensions of cells grown in 8 mM succinate and induced for 3 h with 0.5 mM 2,4,6-TCP. Cells were centrifuged at 6,000 × *g* for 5 min, washed twice, and resuspended in minimal medium to an OD_{600} of 1.0. 2,4,6-TCP (0.3 mM) was added, and the cells were incubated at 30°C in a shaker. Samples of cell-free supernatants were analyzed by UV spectroscopy in a diode array HP8452-A UV-visible spectrophotometer. Samples (20 μl) from cell-free supernatants were taken at different times and injected into a

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▽ Published ahead of print on 23 February 2007.

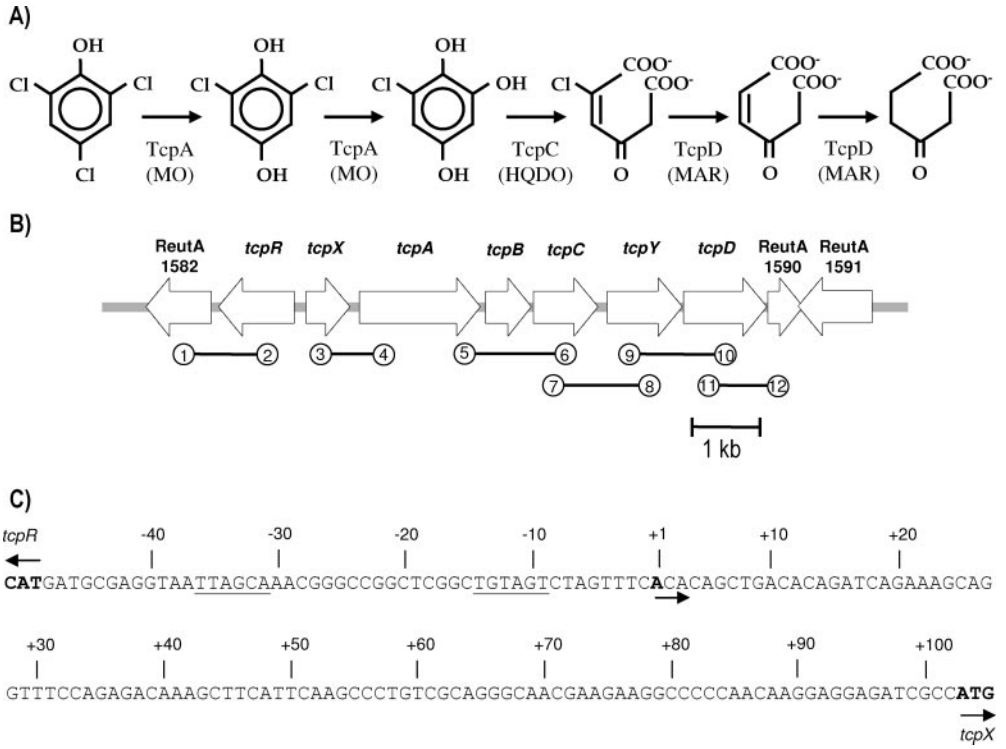

FIG. 1. (A) Degradative pathway proposed for 2,4,6-TCP in *C. necator* JMP134. 2,4,6-TCP-MO, HQDO, and MAR catalyze the conversion of 2,4,6-trichlorophenol to 2,6-dichlorohydroquinone, 6-chlorohydroxyquinol, 2-chloromaleylacetate, maleylacetate, and β-ketoadipate, respectively. (B) Genetic context of the *tcpRXABCYD* gene cluster. The bar represents 1 kb. The numbers in circles correspond to the primers described in Table 2: 1, FAD2; 2, tcpR2; 3, tcpX1; 4, tcpA3; 5, tcpAend; 6, tcpC2; 7, tcpC1; 8, tcpY2; 9, tcpY1; 10, tcpD2; 11, tcpD1; and 12, TCPout. (C) Schematic representation of the organization of the *tcp* promoter region. The arrows indicate the starts of transcription and translation. The transcriptional initiation nucleotide (+1) and the putative −35 and −10 motifs are underlined.

126/166 System Gold Beckman liquid chromatograph equipped with a Waters Symmetry C_{18} 4.6-μm-diameter column (Beckman Instruments, Fullerton, CA). A methanol-H_2O (80:20) mixture containing 0.1% (vol/vol) phosphoric acid was used as the solvent at a flow rate of 1 ml min^{-1}. The column effluent was monitored at 210 nm.

DNA manipulation. Restriction, ligation, and dephosphorylation reactions, purification, and electroporation of DNA were performed by standard procedures (1). Derivatives of the broad-host-range plasmid vector pMLS7 (19) and of pCM132 (22) were mobilized from *E. coli* DH5α to derivatives of *C. necator* JMP134 by triparental mating with the helper strain *E. coli* HB101(pRK600), as previously described (27). Transconjugants were selected on minimal medium agar plates supplemented with 1 mM phenol plus kanamycin or trimethoprim.

Chromosomal disruption of gene sequences in *C. necator* JMP134. An internal fragment (250 to 500 bp) of each gene sequence was PCR amplified from DNA of strain JMP134 using primer pairs listed in Table 2. The PCR products were cloned into pCR2.1-TOPO (Invitrogen Life Technologies, Carlsbad, CA), and each plasmid was electroporated into cells from strain JMP134 to obtain a one-recombination-event chromosomal disruption of each target gene. The electrocompetent cells were obtained as follows: cells from a 50-ml culture (OD_{600} of 0.4 to 0.5) were grown in LB medium and collected by centrifugation at 6,000 × *g* for 5 min at 4°C. The cell pellet was washed three times with cold distilled water, and the cells were collected by centrifugation at 6,000 × *g* and resuspended in 50 μl of distilled water. Recombinant strains were selected on LB agar containing kanamycin. Chromosomal disruptions were checked by the presence/absence of the expected PCR products in the wild-type and the mutant strains, using primer pairs described in Table 2.

Inactivation of *tfdFI* and *tfdFII* genes. The *tfdFI* and *tfdFII* genes were independently inactivated in *E. coli* BW25113(pJP4) cells by the method of Datsenko and Wanner (6). PCR primers MUTF1FW and MUTF1RE (for the *tfdFI* gene) and MUTF2FW and MUTF2RE (for the *tfdFII* gene), which contain 40-bp homology extensions of the *tfdFI* or *tfdFII* gene sequence (Table 2, in boldface) and 20-bp priming sequences for pKD4 (6), were synthesized. These primer pairs were used to amplify the kanamycin resistance gene flanked by 40 bp of the *tfdFI* or *tfdFII* gene sequence, with pKD4 as a template. The PCR program was 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. PCR products were used to inactivate the *tfdFI* or *tfdFII* gene in *E. coli* strain BW25113(pJP4) harboring RecBCD recombinase by a procedure described previously (28). pJP4 derivatives containing the inactivated *tfdFI* or *tfdFII* gene were then transferred to strain JMP134 by biparental conjugation as described previously (27), and the transconjugants were selected on minimal medium agar plates supplemented with 1 mM phenol plus kanamycin. Each transconjugant was transferred five times to the same liquid minimal medium until the pJP4 plasmid was completely removed and only the corresponding pJP4 *tfdFI(II)* mutant was present in *C. necator* JMP134. Primer pairs FORF1/REVF1 and FORF2/REVF2 were used to verify correct recombinational insertion of the kanamycin resistance cassette in place of each *tfdF* gene. This was then confirmed by direct sequencing of the insertion region using these primers.

Constitutive expression of *tcp* gene sequences. To obtain constitutive expression of each *tcp* gene, the corresponding PCR product was cloned into the pMLS7 expression vector (19), using the primers listed in Table 2. The corresponding translation start is included in the primer sequence, and it is located immediately downstream of the EcoRI site. Each PCR product was cloned into pCR2.1 TOPO-TA (Invitrogen Life Technologies, Carlsbad, CA), and the resulting plasmids were digested with EcoRI and HindIII or XbaI to obtain a fragment which was inserted into the pMLS7 plasmid. The identity of the DNA fragments cloned in pMLS7 was confirmed by sequencing.

Detection of transcripts by RT-PCR. Cells from a 50-ml minimal medium culture grown on 8 mM succinate to a OD_{600} of 1.0 were centrifuged at 6,000 × *g* for 5 min, washed twice, and resuspended in the same volume of minimal medium containing 0.5 mM 2,4,6-TCP. When over 50% of chlorophenol degradation had taken place, total RNA was obtained by the Trizol reagent method (Invitrogen Life Technologies, Carlsbad, CA). In brief, 10 ml of culture was centrifuged at 10,000 × *g* for 5 min and the pellet was resuspended in 1 ml of Trizol and incubated for 5 min at room temperature. Two hundred microliters of chloroform was added, and the mixture was agitated vigorously for 15 s. The samples were left for 3 min at room temperature and then centrifuged at 12,000 ×

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype or genotype[a]	Source or reference
Strains		
C. necator		
JMP134	2,4,6-TCP$^+$ 2,4-D$^+$; pJP4	DSMZ[b]
JMP134 *tcpR*	2,4,6-TCP$^-$ Ap^r Km^r	This study
JMP134 *tcpX*	2,4,6-TCP$^-$ Ap^r Km^r	This study
JMP134 *tcpA*	2,4,6-TCP$^-$ Ap^r Km^r	This study
JMP134 *tcpC*	2,4,6-TCP$^-$ Ap^r Km^r	This study
JMP134 *tcpY*	2,4,6-TCP$^+$ Ap^r Km^r	This study
JMP134 *tcpD*	2,4,6-TCP$^+$ Ap^r Km^r	This study
JMP134 ReutA1582	2,4,6-TCP$^+$ Ap^r Km^r	This study
JMP134 *tfdFI*	2,4,6-TCP$^+$ Km^r	This study
JMP134 *tfdFII*	2,4,6-TCP$^+$ Km^r	This study
JMP134 ReutB4129	2,4,6-TCP$^+$ Ap^r Km^r	This study
JMP134 ReutB4694	2,4,6-TCP$^+$ Ap^r Km^r	This study
JMP134 ReutC5982	2,4,6-TCP$^+$ Ap^r Km^r	This study
E. coli BW25113	pKD46; red recombinase	6
Plasmids		
pRK600	Cm^r	12
pCR2.1 TOPO-TA	Ap^r Km^r	Invitrogen
pMLS7	P_{S7} promoter; Tp^r	19
pS7X	*tcpX*; pMLS7 derivative	This study
pS7XA	*tcpX-tcpA*; pMLS7 derivative	This study
pS7A	*tcpA*; pMLS7 derivative	This study
pS7AB	*tcpA-tcpB*; pMLS7 derivative	This study
pS7C	*tcpC*; pMLS7 derivative	This study
pS7D	*tcpD*; pMLS7 derivative	This study
pCM132	*lacZ* promoter-probe vector; Km^r	22
pPRTCP	*tcpR-*P_{tcpX}*-tcpX′*; pCM132 derivative	This study
pPTCP	P_{tcpX}*-tcpX′*; pCM132 derivative	This study
pJP4-*tfdFI* mutant	*tfdFI* Km^r	This study
pJP4-*tfdFII* mutant	*tfdFII* Km^r	This study

[a] 2,4,6-TCP$^+$ and 2,4-D$^+$, able to grow in 2,4,6-TCP or 2,4-D, respectively; *tcp*, catabolic genes involved in 2,4,6-TCP degradation; FAD, FAD synthase; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Tp^r, trimethoprim resistance; Km^r, kanamycin resistance.

[b] DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

g for 15 min at 4°C. The liquid phase was transferred to a clean tube, and 500 μl of isopropanol was added. After 10 min of incubation at room temperature, the samples were centrifuged at 12,000 × *g* for 10 min at 4°C, and the pellet was washed out with 1 ml of cold 70% ethanol. The samples were centrifuged at 7,500 × *g* for 5 min at 4°C and dried for 10 min. The pellets were resuspended in 20 μl of distilled water and 10 μl of 10× MOPS (1× MOPS buffer is 20 mM morpholinepropanesulfonic acid [pH 7.0], 8 mM sodium acetate, and 1 mM EDTA). To remove any DNA contamination, the RNA was treated and cleaned with TURBO DNase kit (Ambion The RNA Company, Austin, TX). The reverse transcription-PCR (RT-PCR [20 μl of reaction mixture]) was carried out using the ImProm-II reverse transcription system (Promega Corporation, Madison, WI) with 1 μg of total RNA. PCRs (25 ul) were performed using specific primer pairs (Table 2) with mixtures that contained 1 μl of total cDNA, 50 pmol of each primer, 50 μM deoxynucleoside triphosphates (dNTPs), 1 mM $MgCl_2$, and 5 U of *Taq* DNA polymerase, prepared in the reaction buffer supplied by the manufacturer. After RT, the mixtures were treated to 95°C for 2 min and subjected to 30 cycles of 30 s at 95°C, 30 s at 54°C, and 1 min at 72°C. Negative control reactions were performed in the same way, except that the RT addition was omitted.

Construction of *tcpR-tcpX′-lacZ* fusions. The vector pCM132 containing the promoterless *lacZ*-Km^r cassette was used (22). A DNA fragment including the complete *tcpR* gene sequence and the first 403 bp of the *tcpX* gene sequence was amplified by PCR with primer pairs tcpRlacZ and tcpX2 (Table 2), using *Pfx* Platinum DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA). The PCR product was cloned into pTOPO-BLUNT (Invitrogen Life Technologies, Carlsbad, CA), and the resulting plasmid was digested with EcoRI to release a 1.7-kb fragment that was then cloned into pCM132. This procedure created a *tcpR-*P_{tcpX}*-tcpX′-lacZ* gene fusion in pCM132 named pPRTCP. Using the same procedure, a P_{tcpX}*-tcpX′-lacZ* gene fusion in pCM132 was created, named pPTCP. The identity of the DNA fragments cloned in pCM132 was confirmed by sequencing. The expression of *lacZ* was determined by β-galactosidase assays performed as follows. Cells were grown in 10 ml of minimal medium containing 8 mM succinate and 50 μg of kanamycin ml^{-1} until an OD_{600} of 0.5. At this point, a 0.1 mM concentration of the putative inducer compound was added and the incubation was prolonged by 3 h. Cells were lysed with chloroform and sodium dodecyl sulfate, and β-galactosidase activities were determined as described previously (24).

Transcription start mapping. The transcription start of the *tcpX* gene was mapped using a protocol for rapid amplification of cDNA ends (RACE). Total RNA (2 μg in a 20-μl reaction) was submitted to RT, as described above, using the primer X1 (Table 2), which anneals 183 bp downstream of the *tcpX* translational start. Clean up of the resulting cDNA fragments was done using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany). A homopolymeric tail was added to the 3′ end of the cDNA (corresponding to the 5′ end of mRNA that was reverse transcribed) using terminal deoxytransferase (Invitrogen Life Technologies, Carlsbad, CA) and dCTPs and incubated at 37°C for 10 min. The dC-tailed cDNA was amplified by PCR using the abridged anchor primer and primer X2 (Table 2), which anneals 127 bp downstream of the *tcpX* translational start. To eliminate any nonspecific PCR products from the first reaction, this PCR product was then PCR amplified with primer X3 (Table 2), which anneals 103 bp downstream of the *tcpX* translational start, and the abridged universal amplification primer. The amplified products were purified using a Qiaquick PCR clean up column (QIAGEN, Hilden, Germany) and were cloned into the pCR 2.1 vector (Invitrogen Life Technologies, Carlsbad, CA).

DNA sequencing and sequence analysis methods. Nucleotide sequences of both DNA strands were determined with an ABI PRISM 377 DNA sequencer (Perkin-Elmer, Foster City, CA). PCR primers were designed with the aid of the Lasergene software package (DNAStar, Inc., Madison, WI).

RESULTS AND DISCUSSION

The *tcpRXABCYD* gene cluster encodes key functions involved in 2,4,6-TCP degradation. A 2,4,6-TCP degradation pathway (Fig. 1A) has been determined in several bacteria, including *C. necator* JMP134 (21, 26, 33, 39). The genetic context related to 2,4,6-TCP degradation has been identified in the *C. necator* JMP134 genome (23), and the functions of the *tcpA*, *tcpB*, and *tcpC* genes have been described (21). To assess the role in 2,4,6-TCP degradation of all the genes from the *tcpRXABCYD* cluster of *C. necator* JMP134, insertional inactivation of each putative gene was carried out. The *tcpA* mutant did not grow on 2,4,6-TCP (not shown). Degradation tests performed with resting cells of this mutant indicated that inactivation completely prevented removal of the chlorophenol (Fig. 2A). The *tcpA* mutant harboring the *tcpA* gene cloned in pS7A degraded 2,4,6-TCP to an extent similar to that of the wild-type strain (Fig. 2A), but was still not able to grow on this compound. A reddish orange metabolite, possibly derived from 6-CHQ, was observed in the culture of the *tcpA* (pS7A) mutant, suggesting a polar effect on the *tcpC* gene encoding HQDO. Such a polar effect is caused by the insertion of a complete copy of the plasmid containing the sequence of the inactivated gene.

As with the *tcpA* mutant, the *tcpX* mutant was also unable to grow on or degrade 2,4,6-TCP (Fig. 2A). The pS7X plasmid, containing the *tcpX* gene, was introduced into the *tcpX* mutant. The resulting strain was still unable to grow on or degrade

TABLE 2. PCR primers used in this work

Primer and function(s)	Sequence (5′→3′)	Primer	Sequence (5′→3′)
Chromosomal disruption, inactivation of *tfdFI* and *tfdFII* genes,[a] and overlapping RT-PCR			
tcpR1	TCAGCTTCTTCCTGCTCGAT	tcpR2	GGATACCAGCACATGCTTCTC
tcpX1	TCACGTGCTCGGCTGTTTGT	tcpX2	ACCATGGCGGACTTGCTGTA
tcpA1	CCAAGAAGCACGACCTGA	tcpA2	CTTGTTGGTGCCGATATG
tcpC1	AGGAGTTCATCCTGCTCAG	tcpC2	CGCACGCCGAACACGGCATC
tcpY1	CGTGGTACCGATCGTGTCT	tcpY2	GATATCGACCTTGGGAGTCG
tcpD1	GGCTCGGAGATGACTACGAT	tcpD2	CATGGGCGTACAAACCTTCT
ReutB4129 1	ATCTACGATCCCGCACTGAC	ReutB4129 2	ATGGCACAGCTTGTGATGAA
ReutB4694 1	GGTCTACGATCCCGAACTGA	ReutB4694 2	GTATGGCACAGCTTGTGGTG
ReutC5982 1	ACCTGCACCAACAGCAGAC	ReutC5982 2	TCTAACACGGCGAAAAATCG
FAD1	TCGATGGTGTACATTGCGGG	FAD2	AAGTCATCCGCACTGGCTTC
MUTF1FW	**GACCCTTCATGAAGAAGTTCACGCTT GACTACCTGAGCCCG**TGTAGGCT GGAGCTGCTTC	MUTF1RE	**GCGGAGTTGCAGGTCACATTATTTGA AATCCGGTCTTCG**CCATTCCGGG GATCCGTCGACC
MUTF2FW	**CCGGCGATCTGAATGAATTCGTTGCG CACTTCTGGCCGGTG**TGTAGGCT GGAGCTGCTTC	MUTF2RE	**AGAGGTCCATGGGATGTCCGGTTCA CGCCGGCATTTCT**CCATTCCGGG GATCCGTCGACC
FORF1	ATGAAGAAGTTCACGCTTGACTAC CTGAGC	REVF1	ACCGTACTAAACGCGGAGTG
FORF2	GCACTAGTAGTGACCGGCGAT	REVF2	CTTATCGATAGGTCGGGTCG
TcpA3	GTGCAGGTCGTAGAAGTCG	tcpAend	GAGGGCCACGACAGCGAATAC
TCPout	AACCTTCCACATTTTGTGCC		
RT-PCR MARs			
RTtfdFI1	ACGCGAGTTAGCGAAGGATA	RTtfdFI2	GAGATAGCAAGCGGCAAATC
RTtfdFII1	GAATTCGTTGCGCACTTCTG	RTtfdFII2	GGCAAGGAGGTCAGGTGAT
RTtcpD1	TCGCGCACGCAGCAGAAGGTTTGT	RTtcpD2	ACGCGGGTTCGGGTACTGGTTCTG
RT ReutB4129 1	ATCTACGATCCCGCACTGAC	RT ReutB4129 2	ATGGCACAGCTTGTGATGAA
RT ReutB4694 1	GGTCTACGATCCCGAACTGA	RT ReutB4694 2	GTATGGCACAGCTTGTGGTG
RT ReutC5982 1	ACCTGCACCAACAGCAGAC	RT ReutC5982 2	TCTAACACGGCGAAAAATCG
RT358F	CCTACGGGAGGCAGCAG	RT907R	CCGTCAATTCTTTTRAGTTT
Transcription start mapping (RACE)			
X1	CGCTCACGGAACAAACAGC	X2	GTGGCGATGACGGTCACG
X3	ACTGCACGCGACAAGGCG	AUAP	GGCCACGCGTCGACTAGTAC
AAP	GGCCACGCGTCGACTAGTACGGGI IGGGIIGGGIIG		
Constitutive expression of *tcp* gene sequences[b]			
tcpRlacZ	ATCGAAGGTCAGCAATACGG	tcpB-XbaI	ATGCTGTCTAGAACTCTTGCATCAC TGGACTCC
tcpR-EcoRI	GCTAATGAATTCGCATCATGGACA CTATTCCC	tcpR-HindIII	CCGCGGAAGCTTATCGAAGGTCA GCAATACGG
tcpX-EcoRI	GGAGGAGAATTCCATGTCGTCCGC AGTCTTC	tcpX-XbaI	CGCGGGTCTAGACGCTTCAAGTCG CGCTAGG
tcpA-EcoRI	AGGAGAGAATTCGATGATTCGCAC TGGCAAGC	tcpA-XbaI	CTGCGTTCTAGACGGAAGATCTTG TCAAGCAG
tcpC-EcoRI	CGGGAGGAATTCGATGCAAGAGT ATGACCAGC	tcpC-HindIII	GACCTTAAGCTTATCTGTCGAACC CATTTGCC
tcpD-EcoRI	TTTGACGAATTCCCCTACGATGAA AGCATTCC	tcpD-HindIII	GTATTGAAGCTTAACCTTCCACAT TTTGTGCC

[a] Primers to inactivate either *tfdFI* or *tfdFII* genes (MUTF1FW, MUTF1RE, MUTF2FW, and MUTF2RE) contain nearly 40-bp homology extensions of the *tfdFI* or *tfdFII* gene sequence (in boldface) and nearly 20-bp priming sequences for pKD4 (6).
[b] Primers include a restriction site which is underlined.

2,4,6-TCP (Fig. 2A). A polar effect on the *tcpA* gene may prevent recovery of the wild-type phenotype. This inactivation approach does not allow us to know if the *tcp*X gene is essential or not. The *tcpX* gene may code for the FAD reductase activity required by TcpA originally ascribed to the *tcpB* gene, but without experimental support (21). The *tcpX* gene putatively encodes a protein with a 54% amino acid identity to the TftC protein from the 2,4,5-TCP degradation pathway in *B. cepacia* AC1100 (10, 13). Partner flavin reductases are usually located in the same operon or physically linked (2, 9, 13, 36), a condition that is fulfilled by both *tcpB* and *tcpX* genes. To elucidate which is the cognate flavin reductase of the TcpA

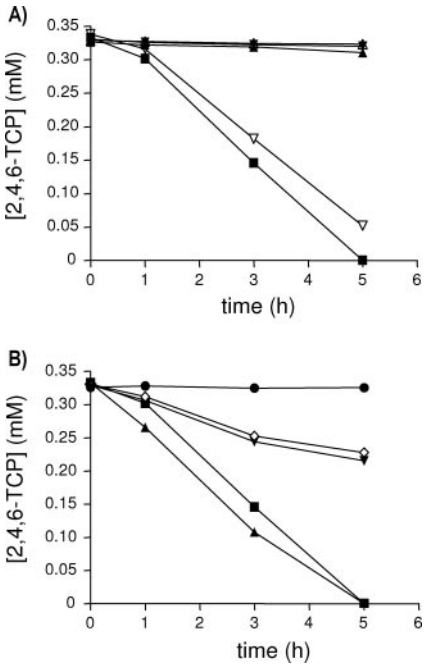

FIG. 2. Degradation of 2,4,6-TCP by *tcpX* and *tcpA* mutant strains of *C. necator* JMP134 and some of its derivatives. (A) Removal of 2,4,6-TCP was detected by high-performance liquid chromatography using samples of supernatants after incubation of preinduced cell suspensions (OD_{600} of 1.0). ■, strain JMP134; ▲, JMP134 *tcpX*; ▼, JMP134 *tcpX* (S7X) constitutively expressing the *tcpX* gene; △, JMP134 *tcpA*; ▽, JMP134 *tcpA* (S7A) constitutively expressing the *tcpA* gene. (B) Removal of 2,4,6-TCP by strains constitutively expressing the *tcpX*, *tcpA*, or *tcpB* genes. ■, strain JMP134; ▲, JMP134 *tcpR* (S7XA) constitutively expressing the *tcpXA* genes; ▼, JMP134 *tcpR* (S7A) constitutively expressing the *tcpA* gene; ◇, JMP134 *tcpR* (S7AB) constitutively expressing the *tcpAB* genes; ●, JMP134 *tcpR*. The values are averages from three or four replicates, with standard deviations of less than 5%.

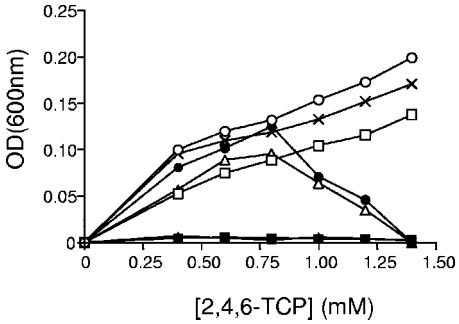

FIG. 3. Growth yields obtained after growing *C. necator* JMP134 and some of its derivatives on different 2,4,6-TCP concentrations: *C. necator* JMP134 (X), JMP134 *tcpR* (■), JMP134 *tcpR* (pPRTCP) (□), JMP134 *tcpC* (▲), JMP134 *tcpC* (pS7C) constitutively expressing *tcpC* (△) JMP134 *tcpD* (●), and JMP134 *tcpD* (pS7D) constitutively expressing the *tcpD* gene (○). Values shown are means of three or four independent experiments, with standard deviations of less than 5%. An OD_{600} of 1.0 corresponds to about 5×10^8 CFU per ml.

protein (protein TcpX or TcpB), we compared the degradation rates of 2,4,6-TCP among different derivatives of the *tcpR* mutant, which is completely unable to grow on or degrade 2,4,6-TCP (see below). This background avoids the effect of any flavin reductase function associated with *tcp* functions. To provide to the *tcpR* mutant the functional monooxygenase and its putative reductase pair, the *tcpX-tcpA* or the *tcpA-tcpB* sequences were cloned into pMLS7, generating the plasmids pS7XA and pS7AB, respectively. The pS7A plasmid was also tested in the *tcpR* mutant. *tcpR* mutants containing both pS7A and pS7AB were able to transform 0.3 mM of 2,4,6-TCP, but at low rate (Fig. 2B). This observation is in agreement with a previous report (21) and indicates that TcpB is either not required for 2,4,6-TCP degradation or is replaced by other reductases in *C. necator*. In contrast, the *tcpR* (pS7XA) mutant transformed this compound at a high rate (Fig. 2B), strongly indicating the interdependence between the TcpX and TcpA proteins as a functional pair: monooxygenase-reductase.

6-CHQ is the central intermediate of the 2,4,6-TCP degradation pathway (11, 18, 21, 26), and the TcpC protein catalyzes the ring cleavage that produces 2-CMA. The *tcpC* mutant failed to grow on 2,4,6-TCP (Fig. 3) and only transformed this chlorophenol to an oxidized product of 6-CHQ, as previously reported (21). The *tcpC* mutant complemented with the *tcpC* gene cloned in pS7C grew on 2,4,6-TCP (Fig. 3). This result also indicates that the *tcpY* and *tcpD* genes, located downstream to the *tcpC* gene, are not affected by a potential polar effect of the *tcpC* gene inactivation or are not essential for growth on 2,4,6-TCP. Both, *tcpY* and *tcpD* mutants were able to degrade and to grow on 2,4,6-TCP. The *tcpY* mutant was able to grow at the same concentrations of 2,4,6-TCP as the wild-type strain, and no differences in the removal rate were detected (data not shown). The *tcpY* gene does not present homology to any known gene, and its product contains the cluster of orthologous groups (COG) 4313. This COG has been found in proteins from different bacterial strains, but mainly β- and γ-proteobacteria. The function of this COG has been related to regulation of phenolic compounds degradation in *Azoarcus* sp. strain EbN1 (30) or the *meta*-pathway of phenol degradation in *Acinetobacter calcoaceticus* (35), but not experimentally proved. In contrast to the *tcpY* mutant, the *tcpD* mutant grew on 2,4,6-TCP but at lower concentrations (up to 1.2 mM) than the wild-type strain (Fig. 3). Complementation of this mutant with the pS7D plasmid restored the wild-type phenotype (Fig. 3).

***tfdFI* and ReutB4129 genes, two other maleylacetate reductase genes carried in *C. necator* JMP134, contribute to degradation of higher levels of 2,4,6-TCP.** The catabolic phenotype of the *tcpD* mutant suggests that other MAR-encoding genes in *C. necator* may provide such function. In addition to the *tcpD* gene, which has 63% amino acid identity to the MAR-encoding gene *macA* from *C. necator* 335, strain JMP134 harbors five other ORFs encoding MAR in its genome. The *tfdFI* and *tfdFII* genes located in the catabolic plasmid pJP4 are involved in the turnover of chlorocatechols (28). Three other ORFs, ReutB4129, ReutB4694, and ReutC5982, are scattered on the chromosomes of *C. necator* JMP134 (unpublished data), showing an amino acid identity of 69% to the *pnpD* gene of *Ralstonia* sp. strain SJ98, 62% identity to the *tftE* gene of *B. cepacia* AC1100, and 29% identity to the *tfdFI* gene of *C. necator* JMP134(pJP4), respectively. Several microorganisms have been reported to have two or more functional MAR activities (17, 31). Interestingly, one of these cases is observed in the

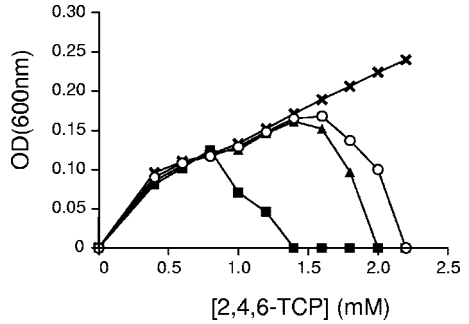

FIG. 4. Involvement of MARs in 2,4,6-TCP degradation. (A) Growth yields of *C. necator* JMP134 derivatives at different concentrations of 2,4,6-TCP. Strain JMP134 and mutant strains (JMP134 *tfdFII*, JMP134 ReutB4694, and JMP134 ReutC5982) were without difference with respect to the wild type (X), JMP134 *tfdFI* (▲), JMP134 *tcpD* (■), and JMP134 ReutB4129 (○). Values shown are means of three or four independent experiments, with standard deviations of less than 5%. An OD_{600} of 1.0 corresponds to about 5×10^8 CFU ml^{-1}.

polychlorophenol-degrading bacterium *Sphingobium chlorophenolicum* (4). The corresponding mutants in *C. necator* JMP134 were obtained by insertional inactivation and were evaluated for growth on 2,4,6-TCP. All mutant strains were able to grow on 2,4,6-TCP; *tfdFII*, ReutB4694, and ReutC5982 mutants did not show any difference with respect to the wild-type strain (data not shown). In contrast, at higher concentrations of 2,4,6-TCP, the ReutB4129 and *tfdFI* mutants reached lower yields than the wild-type strain (Fig. 4). The RT-PCR analysis of the RNA purified from 2,4,6-TCP-grown *C. necator* JMP134 cells showed that only *tfdFI*, *tcpD*, and ReutB4129 sequences were induced (data not shown). This indicates that these genes can contribute to the MAR function during growth on 2,4,6-TCP. The role of the *tfdFI* gene in degradation of 2,4,6-TCP is further supported by the lower growth yields in 2,4,6-TCP observed in strain JMP222 (5), a pJP4-cured derivative of strain JMP134 lacking the two plasmid-encoded MAR (26).

***tcpR* gene encodes a putative LysR-type transcriptional activator that controls *tcp* gene expression using 2,4,6-TCP as an inducer.** The *tcpR* mutant did not grow on 2,4,6-TCP at any concentration tested (Fig. 3), and resting cells of the *tcpR* mutant were also unable to transform 2,4,6-TCP. Furthermore, complete lack of transcription of any of the *tcpXABCYD* genes in the *tcpR* mutant was determined by RT-PCR (data not shown). The *tcpR* mutant harboring the *tcpR* gene cloned in pPRTCP grew on 2,4,6-TCP, supporting the role of the TcpR protein in expression of the *tcp* genes (Fig. 3). To look for putative inducers required by the TcpR protein, the levels of expression of the *tcpR-*P_{tcpX}*-tcpX′-lacZ* fusion in cells exposed to several chlorophenols or related compounds were determined. Cells of the *tcpR* (pPRTCP) mutant were grown until an OD_{600} of 0.5, exposed to a 0.1 mM concentration of each compound for 3 h, and assayed for β-galactosidase expression. A significant induction of the *tcpX* expression was only observed with 2,4,6-TCP and 2,4,6-tribromophenol (Fig. 5). 2,4,6-TCP was able to promote *tcpX* expression in a range of concentrations (Fig. 5). The levels of expression of the P_{tcpX}*-tcpX′-lacZ* construct in the pPTCP plasmid into *C. necator* JMP134 *tcpR* were not significant (Fig. 5). 2,4,5-TCP and pentachlorophenol, two compounds that produce catabolic intermediates similar to those produced in the degradation of 2,4,6-TCP, did not act as inducers for the *tcp* system. Curiously, 2,4,6-tribromophenol is a better inducer of the *tcp* genes than 2,4,6-TCP. It is possible to speculate that the *tcp* genes would be recruited initially as a pathway for degradation of bromophenols, which are widespread in soils and oceans (34). It is also possible that bromophenols simply bind to promoting elements more readily than chlorophenols.

***tcpRXABCYD* genes constitute a catabolic operon in *C. necator*.** Based on sequence analysis (23), it has been previously proposed that the *tcp* genes in *C. necator* JMP134 are expressed as a single operon. To test if the six ORFs located divergently from *tcpR* were transcribed in only one transcript, an RT-PCR analysis of RNA purified from strain JMP134 cells grown on 2,4,6-TCP was conducted, using primer pairs from overlapping *tcp* gene sequences (Fig. 1B). The expected sizes

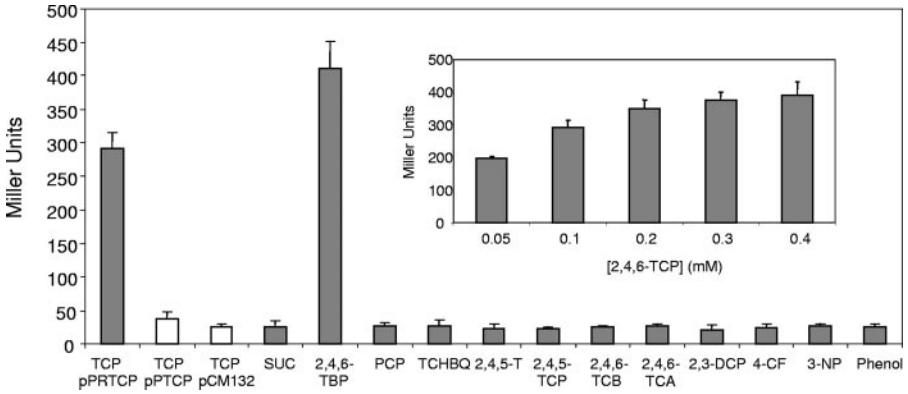

FIG. 5. β-Galactosidase activities from *tcpR-tcpX′-lacZ* fusions with different aromatic compounds. *C. necator* JMP134 *tcpR* (pPRTCP) was grown until the OD_{600} was 0.5 and exposed to 0.1 mM aromatic compounds for 3 h. Abbreviations: SUC, succinate; 2,4,6-TBP, 2,4,6-tribromophenol; PCP, pentachlorophenol; TCHBQ, tetrachlorohydroxibenzoquinone; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; 2,4,5-TCP, 2,4,5-trichlorophenol; 2,4,6-TCB, 2,4,6-trichlorobenzoate; 2,4,6-TCA, 2,4,6-trichloroanisole; 2,3-DCP, 2,3-dichlorophenol; 4-CP, 4-chlorophenol; and 3-NP, 3-nitrophenol. The insert shows activity of the *tcpR-tcpX′-lacZ* fusion at different concentrations of 2,4,6-TCP. The values are averages for three independent tests conducted in duplicate.

and DNA sequences for all of the PCR products were obtained with these primer pairs (data not shown), indicating that *tcp* genes are transcribed as one transcript. Since TcpA requires FAD for catalysis, it is worth noting that ReutA1582, encoding a putative FAD synthetase (amino acid identity of 39% to SCO5711 of *Streptomyces coelicolor* A3) (3), is located downstream of the *tcpR* gene (Fig. 1B). Use of the overlapping RT-PCR primer-pair approach showed that this ORF is not cotranscribed with *tcpR*. Consistently, the ReutA1582 mutant grew on 2,4,6-TCP like the wild-type strain (not shown). In addition, no PCR product was obtained with primer pairs 11 and 12 (Fig. 1B), targeting ReutA1590 putatively encoding a formiminoglutamase function (amino acid identity of 66% with Rmet_5045 of *Ralstonia metallidurans* CH34), further supporting that the *tcpD* gene is the last gene of the operon.

To determine the transcriptional initiation site of the *tcp* operon, putatively located in the *tcpR-tcpX* intergenic region, 5′ RACE PCR experiments were carried out with RNA extracted from cells of *C. necator* JMP134 grown on 2,4,6-TCP. The nucleotide sequence of the 5′ RACE PCR product showed that transcription starts at the adenine located 103 bases upstream of the *tcpX* gene translational start site (Fig. 1C). A putative −10 sequence, TGTAGT, separated by 16 bp from a putative −35 sequence, TTAGCA, is located upstream of this transcriptional start site (Fig. 1C). These sequences are good candidates for the *tcp* gene promoter, since they resemble the −10 and −35 promoter consensus sequences, TATAAT and TTGACA, respectively (7). More than 100 bp between the +1 site and the ATG site are also found for the *tftC* gene in *B. cepacia* AC1100 (13). However, the −10 and −35 consensus sequences do not show significant similarities.

The organization of the *tcp* genes has similarities to and differences from those of other gene clusters involved in chlorophenol degradation. The *tcpXA* genes are homologous to and are located in the same order as the corresponding *tftCD* genes of the 2,4,5-TCP pathway encoded in *B. cepacia* AC1100 (10). However, these genes in strain AC1100 are in two identical copies and in different replicons (13). Furthermore, the *tftEFGH* genes are located in an unrelated genetic context compared with the other *tft* functions (13, 38). *Ralstonia pickettii* DTP0602 also harbors homologues to the *tcp* genes. In this case, the *hadABC* genes have the same genetic organization of the corresponding *tcpABC* genes (15). Unfortunately, no information on the *hadABC* gene flanking sequences is available. In contrast, the 4-chlorophenol pathway in *Arthrobacter chlorophenolicus* (25), encoded by genes localized in two clusters [*cphF(I)C(I)SRBA(I)X* and *cphC(II)F(II)A(II)*], and the PCP pathway in *S. chlorophenolicum* (4), encoded by the transcriptional units *pcpE*, *pcpMA*, *pcpC*, and *pcpBDR*, have an organization completely different from that of *tcp* genes. These observations suggest that the genetic abilities to degrade chlorophenols in bacteria have different evolutionary origins.

ACKNOWLEDGMENTS

This work was financed by FONDECYT 1030493 and Millennium Nucleus Microbial Ecology and Environmental Microbiology and Biotechnology grant P/04-007-F. M.A.S. is a CONICYT and DIPUC Ph.D. fellow.

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