APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 2004, p. 5357–5365 Vol. 70, No. 9
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.9.5357–5365.2004
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Localization and Characterization of Two Novel Genes Encoding Stereospecific Dioxygenases Catalyzing 2(2,4-Dichlorophenoxy)propionate Cleavage in *Delftia acidovorans* MC1

Kathleen M. Schleinitz,[1] Sabine Kleinsteuber,[1]* Tatiana Vallaeys,[2,3] and Wolfgang Babel[1]

Department of Environmental Microbiology, UFZ Centre for Environmental Research Leipzig-Halle, Leipzig, Germany,[1] and Institut National de la Recherche Agronomique[2] and Laboratoire de Génomique des Microorganismes Pathogène, Institut Pasteur,[3] Paris, France

Received 11 March 2004/Accepted 20 May 2004

Two novel genes, *rdpA* and *sdpA*, encoding the enantiospecific α-ketoglutarate dependent dioxygenases catalyzing *R,S*-dichlorprop cleavage in *Delftia acidovorans* MC1 were identified. Significant similarities to other known genes were not detected, but their deduced amino acid sequences were similar to those of other α-ketoglutarate dioxygenases. RdpA showed 35% identity with TauD of *Pseudomonas aeruginosa*, and SdpA showed 37% identity with TfdA of *Ralstonia eutropha* JMP134. The functionally important amino acid sequence motif HX(D/E)X_{23-26}(T/S)$X_{114-183}$HX_{10-13}R/K, which is highly conserved in group II α-ketoglutarate-dependent dioxygenases, was present in both dichlorprop-cleaving enzymes. Transposon mutagenesis of *rdpA* inactivated *R*-dichlorprop cleavage, indicating that it was a single-copy gene. Both *rdpA* and *sdpA* were located on the plasmid pMC1 that also carries the lower pathway genes. Sequencing of a 25.8-kb fragment showed that the dioxygenase genes were separated by a 13.6-kb region mainly comprising a Tn*501*-like transposon. Furthermore, two copies of a sequence similar to IS*91*-like elements were identified. Hybridization studies comparing the wild-type plasmid and that of the mutant unable to cleave dichlorprop showed that *rdpA* and *sdpA* were deleted, whereas the lower pathway genes were unaffected, and that deletion may be caused by genetic rearrangements of the IS*91*-like elements. Two other dichlorprop-degrading bacterial strains, *Rhodoferax* sp. strain P230 and *Sphingobium herbicidovorans* MH, were shown to carry *rdpA* genes of high similarity to *rdpA* from strain MC1, but *sdpA* was not detected. This suggested that *rdpA* gene products are involved in the degradation of *R*-dichlorprop in these strains.

Chlorinated phenoxyalkanoic acids such as 2,4-dichlorophenoxyacetate (2,4-D), 2-methyl-4-chlorophenoxyacetate (MCPA), 2(2,4-dichlorophenoxy)propionate (dichlorprop), and 2(2-methyl-4-chlorophenoxy)propionate (mecoprop) have been widely used as broad leaf herbicides since their introduction in the 1940s. The ability to degrade the phenoxyacetate herbicides is widespread among bacteria (16). In contrast, breakdown of the structurally very similar chiral phenoxypropionate derivatives is far less widespread, and only few strains able to mineralize these compounds have been described (9, 21, 30, 35, 42, 50).

Degradation of phenoxyalkanoate herbicides is initiated by the etherolytic cleavage of the side chain yielding the respective phenol (Fig. 1). Subsequently, conversion into intermediates of the tricarboxylic acid cycle takes place via the modified *ortho*-pathway (16). Side chain cleaving specificity is crucial and determines the range of substrates that can be mineralized by a given microorganism.

Thus far, two types of 2,4-D-cleaving enzymes have been described. In *Ralstonia eutropha* JMP134, 2,4-D cleavage is catalyzed by a well-studied Fe(II) and α-ketoglutarate (αKG)-dependent dioxygenase, which is encoded by *tfdA* (13, 20, 39, 45, 59). The presence of the functionally essential motif HX(D/E)X_{23-26}(T/S)$X_{114-183}$HX_{10-13}R/K indicates that TfdA is related to several other (referred to as group II) αKG-dependent dioxygenases (18). Recently, a three-dimensional model of the active site was proposed (10) on the basis of predictions made from site-directed mutagenesis studies (20) and secondary structure predictions (10). The identity of the suggested substrate-binding residues was confirmed by site-directed mutagenesis and inactivation studies (8). The *tfdA* genes are widespread among bacteria (17, 31, 33, 36, 39, 42, 47, 53, 54, 55, 56), also among strains unable to degrade 2,4-D (19). Recently, several α-proteobacterial strains were shown to carry *tfdA*α genes, which are *tfdA*-like, showing between 46 and 60% nucleotide identity to the canonical *tfdA* genes and forming a phylogenetic clade distinct from *tfdA* (22, 23). Three other genes, clustered as *cadABC*, were found to encode a second type of 2,4-D-cleaving enzyme, a putative monooxygenase responsible for 2,4-D cleavage in *Bradyrhizobium* sp. strain HW13 (24).

Studies on *Sphingobium* (formerly *Sphingomonas* [48]) *herbicidovorans* MH and *Delftia acidovorans* MC1 indicated that enantiospecific dichlorprop and mecoprop cleavage is catalyzed by Fe(II)- and αKG-dependent dioxygenases (35, 38). The enzymes have been biochemically characterized (34, 38, 57, 58), but no data regarding the genetic background are yet available. Thus far, the dioxygenases have been assumed to be *tfdA* gene products (39), because some dichlorprop- and mecoprop-degrading strains carry *tfdA* genes (36, 39) and cell extracts of *R. eutropha* strain JMP134 showed some specificity

* Corresponding author. Mailing address: UFZ Centre for Environmental Research Leipzig-Halle, Department of Environmental Microbiology, Permoserstr. 15, 04318 Leipzig, Germany. Phone: 49-341-235-2191. Fax: 49-341-235-2247. E-mail: sabine.kleinsteuber@ufz.de.

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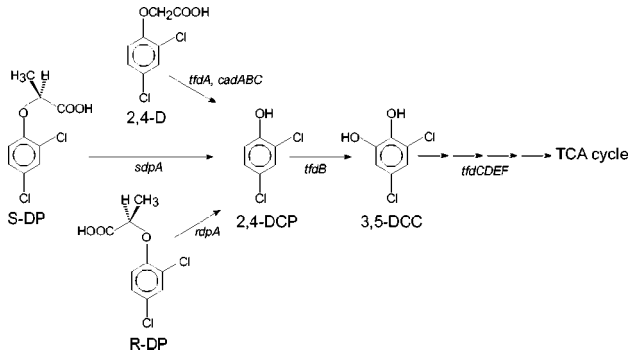

FIG. 1. Modified *ortho*-pathway for the degradation of chlorinated phenoxyalkanoate herbicides. 2,4-D, 2,4-dichlorophenoxyacetate; *R*-DP and *S*-DP, *R*- and *S*-enantiomers of 2(2,4-dichlorophenoxy)propionate, respectively; 2,4-DCP, 2,4-dichlorophenol; 3,5-DCC, 3,5-dichlorocatechol; TCA, tricarboxylate.

toward cleaving *S*-dichlorprop (39). However, no activity for *S*-mecoprop or the *R*-enantiomers was detected in strain JMP134, and the organism was unable to grow on phenoxypropionate herbicides. Recently, available N-terminal and internal amino acid sequences of the enantiospecific dioxygenases from *D. acidovorans* MC1 showed only low similarities to TfdA enzymes (57), and *tfdA* genes were not detected in this strain (36). This suggested that genes other than *tfdA* are involved in dichlorprop degradation in strain MC1.

In *D. acidovorans* MC1 the first step in dichlorprop degradation was found to be highly unstable and easily lost under nonselective conditions (36). Loss of dichlorprop-cleaving activity was correlated with a deletion in the plasmid pMC1, and it was therefore hypothesized that the deleted fragment contains the genes encoding dichlorprop cleavage (36).

Based on the recently available peptide sequences of the *R*- and *S*-dichlorprop-cleaving dioxygenases, we investigated the genetic background of dichlorprop cleavage in *D. acidovorans* MC1. Two novel genes encoding the *R*- and *S*-dichlorprop-cleaving dioxygenases in *D. acidovorans* MC1 were identified and localized. The phylogenetic relationship of the *R*,*S*-dichlorprop-cleaving dioxygenases to other α-ketoglutarate-dependent dioxygenases was analyzed. The organization of these genes within the genome of strain MC1, as well as in a mutant deficient in dichlorprop cleavage, was investigated with regard to implications for the instability of this trait.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. *D. acidovorans* MC1 and *Rhodoferax* sp. strain P230, isolated from contaminated building rubble (9, 35), *D. acidovorans* MC1-DP$^-$ (a mutant of strain MC1 arisen by spontaneous loss of dichlorprop-cleaving ability), and *S. herbicidovorans* MH, isolated from soil (21), were all grown on MM agar (36) containing 0.5 mM dichlorprop at 30°C. *Escherichia coli* TOP10F′ (pCR2.1; Invitrogen) was used for cloning PCR products. The cosmid library was constructed in *E. coli* XL1-Blue MR (Stratagene). *E. coli* XL1-Blue MRF′ (Stratagene) was used for maintaining pScosPA1. The vector pScosPA1 is a new broad-host-range cosmid identical to pSBC1 (43) except for a gentamicin resistance instead of the tetracycline resistance (E. Mahenthiralingam, unpublished data). A shotgun library of a cosmid was constructed by using the vector pcDNA2.1 (Invitrogen). *E. coli* S17-1 (pAG408::*gfp* [46]) was used in transposon mutagenesis experiments. *E. coli* strains were cultivated on Luria-Bertani (LB) agar at 37°C with the addition of appropriate antibiotics. Overnight cultures were prepared in liquid LB medium containing appropriate antibiotics at 37°C (*E. coli* strains) or 30°C (dichlorprop-degrading strains) on a rotary shaker. Antibiotics and dichlorprop were filter sterilized through a 0.2-μm-pore-size Minisart cellulose acetate filter (Sartorius) and added at a concentration of 100 μg/ml.

Mercury resistance was examined on LB agar plates containing 0.1 mM $HgCl_2$ incubated at 30°C for 48 h. *R. eutropha* JMP134(pJP4) and *R. eutropha* JM222 were used as positive and negative controls, respectively.

Construction of gene libraries. A genomic library of *D. acidovorans* MC1 was constructed by using cosmid pScosPA1, as described elsewhere (43). The average insert size of library clones was 34 kb. A total of 2,592 clones were picked into 96-well plates. After overnight growth 8% (vol/vol) dimethyl formamide was added, and the cosmid library was stored at −80°C.

A shotgun library of cosmid pScos-R5 was constructed in pcDNA2.1 according to the method of Buchrieser et al. (4) by using cosmid DNA isolated as described elsewhere (2, 3).

Transposon mutagenesis. The transposon of the suicide vector pAG408, a miniTn*5* derivative, carries a promoterless *gfp* gene (46) and was used for transposon mutagenesis of strain MC1. Mutagenesis was performed according to standard procedures (40). Overnight cultures of the donor and the recipient grown in LB medium supplemented with antibiotics (donor) were centrifuged, washed, and resuspended in LB medium to a final optical density at 600 nm (OD_{600}) of 1.0. Equal volumes of the donor and the recipient were mixed, and 100 μl of the mixture was plated out on LB agar, followed by incubation at 30°C for 6 h. The bacterial lawn was then washed from the plates with MM medium. Dilution series were prepared in MM and 100 μl of the 10^{-3} to the 10^{-5} dilutions was plated on selective MM agar plates containing 100 μg of gentamicin/ml, 27.8 mM fructose, and 0.5 mM dichlorprop. After incubation at 30°C for 6 days green fluorescing mutants were isolated and tested for dichlorprop degradation by high-pressure liquid chromatography as described elsewhere (60). In mutant clones able to degrade one of the enantiomers but not the other the region flanking the transposon was PCR amplified and sequenced.

DNA amplification. Degenerate primers (Table 1) were designed from N-terminal and internal peptide sequences of the enantiospecific dioxygenases of *D. acidovorans* MC1 (57) for PCR amplification of *rdpA* and *sdpA*. According to preliminary alignments of these peptide sequences with TfdA and TauD, the expected PCR fragment sizes were ca. 350 bp (*R*-dichlorprop dioxygenase gene) and 540 bp (*S*-dichlorprop dioxygenase gene). All primers were synthesized by MWG Biotech. Total genomic DNA used as a template was prepared from colonies grown on LB agar as described elsewhere (25). PCR was performed by using the *Taq* PCR Mastermix kit (Qiagen) and a PTC-200 thermal cycler (MJ Research). First, 100 pmol of each degenerate primer was added to 25 μl of PCR sample. The temperature gradient program involved an initial denaturation step at 94°C for 3 min, followed by 36 cycles of 94°C for 45 s, 50 to 70°C for 45 s, and 72°C for 2 min, with a final elongation step of 72°C for 6 min. Amplification of an *rdpA* gene fragment with the primers rdp_fG and rdp_r involved an initial denaturation step at 94°C for 3 min, followed by 36 cycles of 94°C for 45 s, 54°C for 45 s, and 72°C for 1 min, with a final elongation step of 72°C for 6 min. Amplification of an *sdpA* gene fragment with the primers sdp_fT and sdp_rA was the same as for *rdpA* amplification, except that the annealing step was performed at 60°C and that elongation lasted 90 s.

The *rdpA* genes from *S. herbicidovorans* MH and *Rhodoferax* sp. strain P230 were amplified with primers rdp_forw (5′-atg gta ggt ctc aaA TGC ATG CTG CAC TGT CCC CC-3′; lowercase letters denote a linker sequence) and rdp_rev (5′-atg gta ggt ctc agc gct CTA GCG CGC CGG GCG CAC G-3′). Amplification was the same as for the *rdpA* fragment except for the annealing step being performed at 60°C for 30 s.

Regions flanking Tn*5* transposon insertion sites were amplified by ST-PCR (6). To each sample either Tn*5*_outF (5′-GGC AGA TCT GAT CAA GAG-3′ [27]) annealing to the I end of the transposon or Tn*5*_outR (5′-CCG CAC TTG TGT ATA AGA GTC-3′) designed from the O end of the transposon were added as specific primers. Either of the following FP primers (44) was added as a random primer: FP-1, 5′-AGT TCA AGC TTG TCC AGG AAT TC$(N)_7$ GGC CT-3′; FP-2, 5′-AGT TCA AGC TTG TCC AGG AAT TC$(N)_7$ GCG CT-3′; FP-3, 5′-AGT TCA AGC TTG TCC AGG AAT TC$(N)_7$ CCG GT-3′; and FP-4, 5′-AGT TCA AGC TTG TCC AGG AAT TC$(N)_7$ CGC GT-3′.

PCR products for the use as hybridization probes were generated as follows: the *mer* operon was partially amplified with primers mer_f (5′-CAC CAG TTC AGA CAG CAC G-3′) and mer_r (5′-ACC GGT GAA ACC AAG TCC-3′) amplifying a 1,450-bp fragment. The IS*91*-like element was partially amplified by using the primers IS_f (5′-ATG TCA GCC ACT TCC AAG C-3′) and IS_r (5′-GGA GGC AGT ATG CAT TTG G-3′) amplifying a 1,622-bp fragment. The PCR program for both fragments involved an initial denaturation step of 94°C for 3 min, followed by 36 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 2 min, with a final elongation step of 72°C for 6 min. Amplification of *tfdB*, *tfdC*, and

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TABLE 1. Degenerate primers designed for the amplification of *rdpA* and *sdpA*

Peptide or primer	Sequence[a]	Position[b]
RDP_internal sequence 2	W E T L S P T M Q A T I E G L N	162–177
rdp_r	3′-AC GTY CGN TGN TAI CTY CC-5′	
RDP_internal sequence 3	N E I L D A F H T Y Q V I Y F P	43–58
rdp_fA[c]	5′-GCI TTY CAY ACN TAY CA**A** GT-3′	
rdp_fG[c]	5′-GCI TTY CAY ACN TAY CA**G** GT-3′	
SDP_N terminus	M Q T T L Q I T P T G A T L G A	1–16
sdp_fC[c]	5′-ATG CAR ACI ACI CTG CAR AT**C** AC-3′	
sdp_fT[c]	5′-ATG CAR ACI ACI CTG CAR AT**T** AC-3′	
SDP_internal sequence 1	L G H V Q Q A G S A Y I	193–204
sdp_rA[c]	3′-GT**A** CAN GTY GTY CGN CC-5′	
sdp_rG[c]	3′-GT**A** CAN GTY GTY CGN CC-5′	

[a] As described previously (57). IUPAC ambiguity codes: R = A or G, Y = C or T, N = any base, I = inosine.
[b] Alignment is shown in Fig. 2.
[c] To avoid degeneracy near the 3′ end, two pools of primers were designed from some peptides (sequence differences are indicated in boldface).

tfdD gene fragments was performed as described in reference 35. Fragments of *tfdE* and *tfdF* were amplified according to the method of Hoffmann et al. (17).

Sequencing and sequence analysis. For sequencing, PCR products were ligated to pCR2.1 and transformed into *E. coli* TOP10F′ by using the TA cloning kit (Invitrogen). Cycle sequencing was performed by using the ABI Prism BigDye Terminator v2.0 cycle sequencing ready reaction kit (PE Applied Biosystems). M13 primers were used for amplifying and sequencing cloned PCR fragments. After amplification with M13 primers and electrophoretic separation, fragments were extracted with the E.Z.N.A gel extraction kit (Peqlab Biotechnologie). Sequencing reactions (total volume, 10 μl) contained 2 μl of premix, 10 pmol primer, and 10 to 50 ng of template DNA. Primer walking on cosmid DNA was performed with primers designed from the sequences of the *rdpA* and *sdpA* PCR products. Sequencing reactions (total volume, 20 μl) contained 6 μl of premix, 20 pmol of primer, and 500 ng of cosmid DNA. After 25 cycles of 96°C for 30 s, 55°C for 15 s, and 60°C for 4 min, the DNA was precipitated by adding 80 μl of H_2O, 10 μl of 3 M sodium acetate (pH 4.6), and 250 μl of ethanol; washed with 70% ethanol; vacuum dried; and redissolved in 20 μl of template suppression reagent (PE Applied Biosystems). Sequencing from cosmid DNA involved an additional purification step prior to the ethanol precipitation by using Centri-Sep spin columns (Princeton Separations). After denaturation at 90°C/2 min the sample was chilled on ice. Electrophoresis, data collection, and analysis were carried out on an ABI Prism 310 genetic analyzer (PE Applied Biosystems). Both strands were sequenced. Sequencing data were analyzed with the Sequence Navigator software, contigs were assembled by using the Autoassembler software (PE Applied Biosystems), and the BLASTn and BLASTx programs (version 2.2.2. [1]) were used to search for similar sequences in nucleotide and protein sequence databases. Sequences were aligned by using the programs CLUSTAL W (51) or MultAlign (7). Phylogenetic analysis was performed by using the protml tool of the ARB package (29) when the maximum-likelihood method was applied and MEGA2.1 (26) when trees were constructed with the neighbor-joining or maximum-parsimony method.

Recombinant pcDNA2.1 DNA was isolated as described elsewhere (2, 3). A Perkin-Elmer 9700 thermocycler was used for cycle sequencing. Sequencing reactions (total volume, 10 μl) contained ca. 300 ng of template, 1.5 μl of ABI Prism BigDye Terminator v1.1 Cycle Sequencing RR-100 mix, 1.2 μl of 5× sequencing buffer, and 2.4 pmol of primer T7 or M13. The sequencing program involved an initial denaturation step (94°C for 60 s), followed by 70 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Sequencing reactions were precipitated with 40 μl of 76% ethanol for 30 min, washed with 70% ethanol, and resuspended in 10 μl of 0.3 mM EDTA buffer. Electrophoresis, data collection, and analysis were performed by using an ABI Prism 3700 genetic analyzer (PE Applied Biosystems). Automatic sequence assembly was carried out by using PHRED (11, 12), PHRAP (P. Green, unpublished data), and the Autoassembler software. When gaps were closed between contigs, cosmid DNA was used as a template and custom-made oligonucleotides were added as primers. The sequence was edited by using CONSED (15) and annotated by using Vector NTI Advance (InforMax). Similarity searches were carried out as mentioned above.

DNA-DNA hybridization. Screening of the cosmid library was done by replica plating 12 96-well grids onto positively charged nylon membranes (Roche) that had been placed on selective agar. Cells of strain MC1 (positive control) and *E. coli* XL1-Blue MRF′ (pScosPA1) (negative control), as well as strains MH and P230, were smeared onto the nylon membrane placed on LB agar. After overnight growth, DNA was immobilized onto the membranes according to the manufacturer's guidelines.

Genomic DNA of strain MC1 was isolated as described by Sokol et al. (43). The low-copy plasmid pMC1 was isolated with a Nucleobond AX100 kit (Macherey-Nagel). Plasmid and genomic DNA were PstI digested according to standard procedures (40), separated electrophoretically in 0.8% agarose, blotted onto positively charged nylon membrane according to the manufacturer's guidelines, and cross-linked by UV irradiation for 1 min.

Cosmid and plasmid DNA was isolated from 20 ml of LB medium by using the Nucleobond AX100 kit (Macherey-Nagel). Next, 5 μl of cosmid DNA was applied to positively charged nylon membrane and cross-linked by UV irradiation for 1 min. then, 100 μl of plasmid DNA was PstI or EcoRI/XmaI digested according to standard procedures (40), ethanol precipitated, separated, blotted, and UV cross-linked as described above.

PCR fragments were digoxigenin labeled by using the DIG DNA labeling kit (Roche). For hybridization and immunodetection, the DIG Easy Hyb Wash and Block buffer set and the DIG nucleic acid detection kit (Roche) were used according to the manufacturer's guidelines. Hybridizations were performed at 50°C (*tfdC*, *tfdD*, *tfdE*, and *tfdF* probes), 55°C (*rdpA* probe), 60°C (probes against *tfdB*, the *mer* operon, and the IS*91* element), or 62°C (*sdpA* probe). Stripping was carried out according to the manufacturer's instructions.

Nucleotide accession numbers. The nucleotide sequences determined in the present study were deposited in the GenBank, EMBL, and DDBJ databases under accession numbers AY327575, AF516751, and AF516752.

RESULTS

Identification, sequencing, and analysis of dichlorprop-cleaving dioxygenase genes in *D. acidovorans* MC1. Degenerate primers were designed from N-terminal and internal peptide sequences of both enantiospecific dioxygenases of *D. acidovorans* MC1. Primers with a degeneracy near the 3′ end proved to be nonspecific; therefore, two pools of primers were synthesized to avoid degeneracy at such positions (Table 1). PCR with the primer pairs rdp_fG-rdp_r (*rdpA*-specific primers) and sdp_fT-sdp_rA (*sdpA*-specific primers) yielded fragments of 339 and 532 bp, respectively, whereas all other primer combinations did not produce specific fragments. The two PCR fragments were cloned and sequenced. The deduced amino acid sequences of both fragments contained sequences at their N and C termini, respectively, that correctly corresponded to the peptide sequences used for designing the prim-

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TABLE 2. Sequence identities of the dichlorprop-cleaving enzymes RdpA and SdpA to other group II αKG-dependent dioxygenases

α-KG dioxygenase[a]	Strain, accession no.	% Identities[b] to	
		RdpA	SdpA
TfdA	*Ralstonia eutropha*, A27082	32/279	37/244
TauD	*Pseudomonas aeruginosa*, NP_252624	35/279	27/258
AtsK	*Pseudomonas putida*, AAD31784	31/296	31/296
YSD	*Saccharomyces cerevisiae*, Q12358	29/279	27/304
PvcB	*Pseudomonas aeruginosa*, AAC21672	20/298	21/277
SyrI	*Pseudomonas syringea*, AAB63253	17/323	18/308
CarC	*Pectobacterium carotovorum*, AAD38231	16/285	17/275
γ-BBH	*Pseudomonas* sp. strain AK-1, P80193	16/309	17/291
CS2	*Streptomyces clavuligerus*, AAA26723	16/208	17/275

[a] TfdA, 2,4-D/αKG dioxygenase; TauD, taurine/αKG dioxygenase; AtsK, putative αKG-dependent alkylsulfatase; YSD, sulfonate/αKG dioxygenase; PvcB, pyoverdine biosynthesis enzyme; SyrI, syringomycin biosynthesis enzyme I; CarC, carbapenem biosynthesis enzyme C; γ-BBH, γ-butyrobetaine hydroxylase; CS2, clavaminate synthase 2.

[b] That is, the percent sequence identity over the total number of amino acids examined.

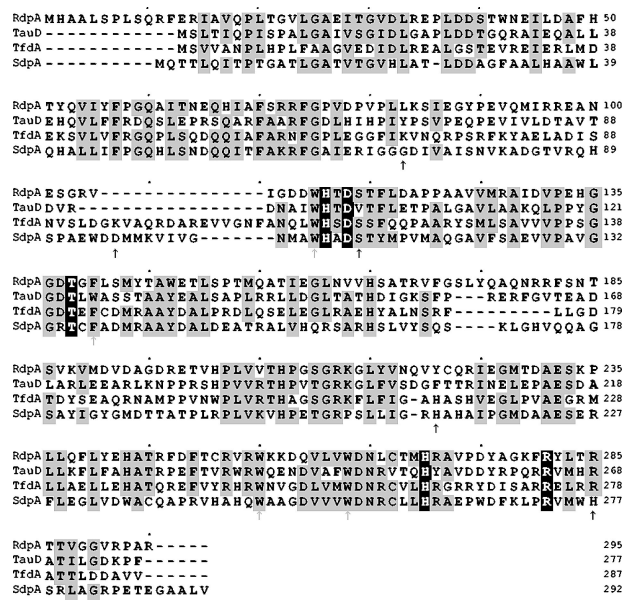

FIG. 2. Sequence alignment of RdpA and SdpA with other group II αKG-dependent dioxygenases. TfdA, *R. eutropha* 2,4-D/αKG dioxygenase (accession no. A27082); TauD, *P. aeruginosa* taurine/αKG dioxygenase (accession no. NP_252624). Conserved Fe(II)- and αKG-binding residues are shaded in black; other conserved regions are shaded in gray. Black arrows indicate 2,4-D binding residues in TfdA; gray arrows indicate residues undergoing posttranslational hydroxylation in TauD or TfdA. Amino acid positions numbers are shown to the right of each line. The alignment was created with CLUSTAL W.

ers. In screens of a genomic cosmid library of strain MC1 10 of 1,152 clones hybridized with the *rdpA* gene probe. Of these 10, 4 hybridized with the *sdpA* gene probe, but PCR with the *sdpA*-specific primers yielded a product with only 1 of these 4 clones, designated pScos-R5. Both dioxygenase genes were then completely sequenced from this clone. The coding regions of the *R*-dichlorprop and *S*-dichlorprop dioxygenase genes were 888 and 879 bp, respectively. No significant sequence similarities to other known genes were found searching nucleotide databases. However, the deduced amino acid sequences of RdpA and SdpA both shared significant similarities to other αKG-dependent dioxygenases ranging between 37 and 16% (Table 2). RdpA and SdpA shared 26% sequence identities. Furthermore, similarities to a number of putative dioxygenases belonging to the genera *Pseudomonas*, *Yersinia*, *Ralstonia*, *Streptomyces*, *Bordetella*, *Bradyrhizobium*, *Azotobacter*, *Shigella*, *Chromobacterium*, *Burkholderia*, and *Mycobacterium* were found and ranged between 31 and 16%.

The amino acid sequences of both dioxygenases were analyzed on the basis of the alignment shown in Fig. 2. It shows that an internal sequence tentatively aligned to positions 257 to 287 of TfdA by Westendorf et al. (57), comprises positions 85 to 131 of RdpA.

Transposon mutagenesis. Of 959 green fluorescing transposon mutants, 2 were found to carry the transposon insertion in *rdpA*. In clone MC1-T270, the insertion was located after bp 627; in clone MC1-T647, the mini-Tn*5* had inserted after bp 814. Both mutants had lost the ability to cleave *R*-dichlorprop but were still able to grow on the *S*-enantiomer.

Organization of *rdpA* and *sdpA* within the genome of *D. acidovorans* MC1. The 25,768-bp sequence of the genomic fragment from strain MC1 containing *rdpA* and *sdpA* in cosmid pScos-R5 is represented in Fig. 3A. The assembly was verified by PCR of selected regions (data not shown). The *rdpA* and *sdpA* genes were separated by 13.6 kb, which mainly comprised an 8.7-kb putative transposon, organized in a way characteristic for the Tn*501* subgroup of the Tn*3* transposon family (28). It was flanked by 38-bp inverted repeats that were flanked by 5-bp direct repeats generated during insertion. The open reading frames (ORFs) for the putative transposase and resolvase genes *tnpA* and *tnpR* had an outward direction with the ORF for *tnpA* proceeding 5 bp into the inverted repeat. The *res* site, at which cointegrate resolution during transposition takes place, was located upstream of *tnpR*. The remaining part of the transposon was occupied by a mercury resistance gene cluster. The *merR* gene had a direction divergent from the *merTP CADE* operon. A putative *urf2M* was located adjacent to the *mer* operon. The putative Tn*501* transposon showed 81% nucleotide sequence identity to a putative Tn*3* family transposon of *Nitrosomonas europaea* ATCC 19718 (accession no. BX321858.1). The deduced amino acid sequences of the identified ORFs showed between 82 and 97% identity to proteins involved in transposition and mercury resistance (Table 3). The functionality and mobility of this transposon were not tested, but strain MC1 was found to be resistant to 0.1 mM $HgCl_2$. Mercury resistance was not lost when dichlorprop-cleaving activity was lost, but these mutants were still able to grow in the presence of 0.1 mM $HgCl_2$. In Southern hybridizations the *mer* operon of the plasmid was localized on a 4.4-kb fragment of pMC1 (Fig. 4C). This copy was deleted in the plasmid of the mutant MC1-DP$^-$. However, in hybridizations against genomic DNA, another signal at 2.8 kb was detected that was present in the wild-type strain as well as in strain MC1-DP$^-$ (data not shown), suggesting that strain MC1 carries a second copy of the *mer* operon on the chromosome that is not lost when the dichlorprop-cleaving ability is lost. It is possible that the chromosomal copy of the *mer* operon confers mercury resistance to strains MC1 and MC1-DP$^-$, whereas the copy on the plasmid may lack functionality.

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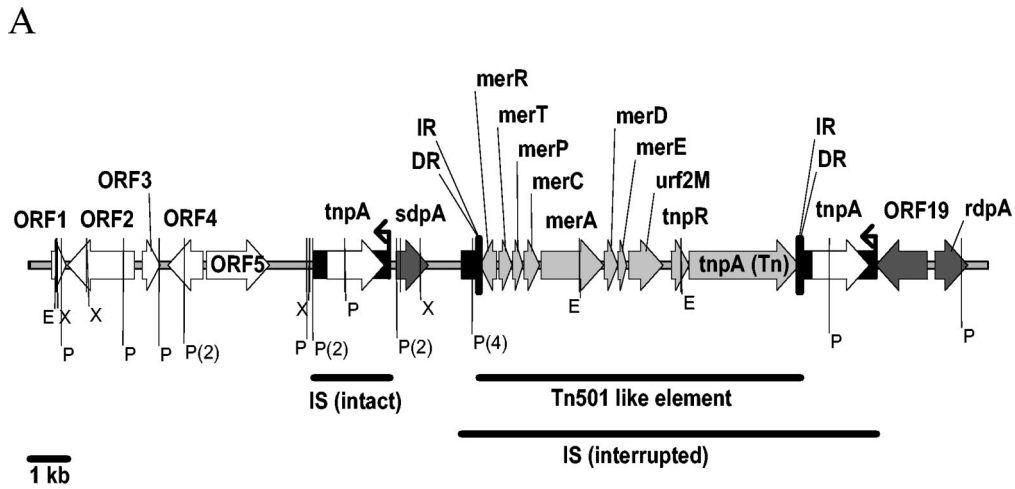

B

IS*91* family: **A**nnn**ATAGGAA**n**TT**n**AA**nnnn

putative IS*91*
element strain MC1: **c**cag**ATAGGAA**a**TT**c**AA**ccgc

FIG. 3. Organization of *rdpA* and *sdpA* within the genome of strain MC1. (A) Genetic map of the genomic fragment containing *rdpA* and *sdpA*. For ORFs showing a high degree of similarity to other genes (>80% identity of the deduced amino acid sequence) the respective names are given. The gene designations are as follows: *tnpA*/*tnpA*(Tn), putative transposases; *sdpA*, *S*-dichlorprop/α-ketoglutarate dioxygenase; *merR*, putative mercury resistance operon transcriptional regulator; *merT*, *merP* and *merC*, putative mercury ion transport genes; *merA*, putative mercury reductase; *merD*, putative secondary regulator; *merE* and *urf2M*, conserved ORF of unknown function; *tnpR*, putative site-specific resolvase; and *rdpA*, *R*-dichlorprop/α-ketoglutarate dioxygenase. ORF1 through ORF5 and ORF19 show significant similarities to genes encoding the proteins as follows: ORF1, carboxymuconolactone decarboxylase; ORF2, potassium/proton antiporter; ORF3, bacterioferritin; ORF4, LysR type regulator; ORF5, dihydroxyacid dehydratase; and ORF19, MFS transporter. The positions of the following features are indicated: IR, 38-bp inverted repeat flanking the Tn*501*-like transposon, DR, 5-bp direct repeat. Two putative IS*91*-like elements are shown as black boxes; the positions of the *ori*IS are indicated as arrows above the sequence. The right IS*91*-like element is interrupted by a Tn*501*-like transposon. Its left partial sequence is not drawn to scale. Single letters denote the following restriction sites: E, EcoRI; P, PstI; and X, XmaI. In the case of multiple PstI restriction sites within less than 100 bp, only the most left site is given and the number of sites is indicated in brackets. (B) Comparison of the sequence at the right end of the putative IS elements with the IS*91* family *ori*IS consensus sequence. Conserved nucleotides are shown in bold uppercase letters.

Furthermore, two copies of a putative IS element resembling the IS*91*/IS*801* family of insertion elements were identified. Members of this family of IS elements do not contain inverted repeats and do not create duplications of the target sequence (14). Insertion of this family of elements is site specific, and replication utilizes the rolling circle mechanism; therefore, an *ori*IS, the origin of replication, and a *ter*IS, the termination sequence, are found at either end of these IS elements (14). The two copies of the putative IS elements in strain MC1 were essentially identical except for a 9-bp deletion and a 47-bp deletion in the left, intact copy. The element next to ORF19 was interrupted by the Tn*501*-like element (Fig. 3A). The elements were 2,037 bp (intact copy) and 2,084 bp (interrupted copy) and contained an IS*801*-like transposase gene showing 99% sequence identity to the homologues on the atrazine catabolic plasmid pADP-1 (Table 3 [32]) and a transposase associated with chlorobenzene degradation genes in *Ralstonia* sp. strain JS705 (37). At the right end of both elements a sequence was identified, that matched the consensus sequence for origin of replication *ori*IS of this group of insertion elements (49) in all but the first nucleotide (Fig. 3B). Neither the termination sequence *ter*IS nor the tetramers GTTC/CTTG for which IS*91* family elements show site specificity were detected.

ORF19, located upstream of *rdpA* showed significant similarities to aromatic compound major facilitator superfamily (MFS) transporters. A 29% identity to the 2,4-D transporters CadK of *Bradyrhizobium* sp. strain HW13 and TfdK of *Achromobacter xylosoxidans* subsp. *denitrificans* and *R. eutropha* JMP134 was detected. A putative Shine-Dalgarno sequence was found 7 bp upstream of the start codon.

Another five ORFs, ORF1 to ORF5, were identified at the left end side of the insert.

Localization of *rdpA* and *sdpA* within the genome of *D. acidovorans* MC1 and comparison of the wild-type plasmid pMC1 with that of the mutant MC1-DP$^-$. Southern hybridizations showed that both *rdpA* and *sdpA* were located on the

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TABLE 3. Localization and predicted function of ORFs in the genomic region of strain MC1 containing *rdpA* and *sdpA*

ORF	Coordinates (bp)	Coding strand[a]	Function of closest match	Source microorganism	% Amino acid identity[b]	No. of amino acids MC1/match[c]	Accession no.
1	574–966	d	Carboxymuconolactone decarboxylase family protein	*Shewanella oneidensis* MR-1	65 (81/124)	130/128	AAN53170.1
2	1006–2799	c	Glutathione-regulated potassium-proton antiporter	*Pseudomonas putida* KT2440	69 (385/556)	597/581	AEO16523.1
3	3018–3494	d	Putative bacterioferritin	*Delftia acidovorans*	70 (112/158)	158/158	CAC83620.1
4	3701–4642	c	Hypothetical protein, similar to LysR-type regulators	*Ralstonia metallidurans*	51 (161/314)	313/360	ZP 00022545.1
5	4745–6438	d	Putative dihydroxy acid dehydratase	*Ralstonia solanacearum*	76 (420/555)	564/557	CAD16119.1
6	7981–9528	d	Putative transposase, similar to IS*801* transposase	*Pseudomonas* sp. strain ADP	99 (512/515)	515/515	AAK50272.1
7	9836–10714	d	2,4-D dioxygenase (TfdA)	*Ralstonia eutropha* JMP134	37 (92/249)	292/287	A27082
8	12107–12541	c	Mercury resistance regulatory protein (MerR)	*Enterobacter aerogenes*	97 (140/144)	144/144	CAA83896.1
9	12613–12963	d	Mercury uptake protein (MerT)	*Alcaligenes* sp.	96 (112/116)	116/116	CAA70196.1
10	12977–13251	d	Mercury uptake protein (MerP)	*Alcaligenes* sp.	94 (86/91)	91/91	CAA70197.1
11	13279–13701	d	Mercury uptake protein (MerC)	*Nitrosomonas europaea* ATCC 19718	86 (121/140)	140/140	CAD84751.1
12	13741–15426	d	Mercury ion reductase (MerA)	*Klebsiella pneumoniae*	88 (494/561)	561/561	AAM44226.1
13	15444–15809	d	Mercury resistance secondary regulatory protein (MerD)	*Pseudomonas* sp. strain ADP	95 (115/121)	121/121	AAK50284.1
14	15806–16042	d	Putative protein (MerE)	*Pseudomonas* sp. strain ADP	91 (71/78)	78/78	AAK50283.1
15	16093–17028	d	Putative protein, similar to Urf2M	*Ralstonia metallidurans*	88 (274/310)	311/329	CAD97548.1
16	17245–17718	d	Resolvase (TnpR)	*Nitrosomonas europaea* ATCC 19718	98 (154/157)	158/186	CAD84747.1
17	17721–20690	d	Tn*3* family transposase (TnpA)	*Nitrosomonas europaea* ATCC 19718	95 (945/989)	989/989	CAD84746.1
18	21024–22571	d	Putative transposase, similar to IS*801* transposase	*Pseudomonas* sp. strain ADP	99 (511/515)	515/515	AAK50727.1
19	22752–24137	c	Putative aromatic compound MFS transporter	*Pseudomonas putida* KT2440	73 (318/433)	461/450	AAN69258.1
20	24330–25217	d	Taurine dioxygenase (TauD)	*Pseudomonas aeruginosa* PA01	35 (100/284)	295/279	NP_252624

[a] c, complementary strand; d, direct strand.
[b] Values in parentheses refer to numbers of identical amino acids over the total number examined.
[c] Number of amino acids in strain MC1 ORF/number in the closest match.

plasmid pMC1 (Fig. 4A). Distinct signals of the expected fragment sizes of 3.5 kb (*rdpA*) and 1.9 kb (*sdpA*) were observed with plasmid DNA (Fig. 4A, lane 1). Much fainter signals of the same fragment sizes were obtained with the genomic DNA, making visible the plasmid content of this. Loss of dichlorprop cleavage in strain MC1-DP$^-$ was associated with a deletion of both dioxygenase genes from pMC1-DP$^-$ (Fig. 4B). Furthermore, the *mer* operon and most of the numerous copies of the IS*91*-like elements were also deleted in the mutant MC1-DP$^-$ (Fig. 4C). In contrast, the lower pathway genes *tfdB-F* remained unaffected (data not shown).

Screening for *rdpA* and *sdpA* in other dichlorprop-degrading bacteria. Southern hybridizations with *rdpA* and *sdpA* probes showed that neither *S. herbicidovorans* MH nor *Rhodoferax* sp. strain P230 carried s*dpA*-like genes, but both strains gave signals with the *rdpA* gene probe (data not shown). The genes were PCR amplified and sequenced. The *rdpA* gene of strain P230 was identical to that of strain MC1; the one of strain MH contained one neutral base substitution.

DISCUSSION

Two novel genes encoding the enantiospecific dichlorprop and mecoprop cleaving dioxygenases RdpA and SdpA in *D. acidovorans* MC1 were identified. The characterization of genes shown to be involved in the cleavage of these chiral herbicides has not previously been reported. To date, *tfdA* genes or genes closely related in sequence to *tfdA* were proposed to be involved in *R*-dichlorprop cleavage in *Alcaligenes denitrificans* (39). This suggestion was based on the observations that a *tfdA* gene is present in *Alcaligenes denitrificans* and that dichlorprop cleavage is catalyzed by an αKG-dependent dioxygenase in this strain as well as in *S. herbicidovorans* MH (38). However, it was not supported by *tfdA* expression studies or amino acid sequences of the *R*-dichlorprop cleaving dioxygenase. The *rdpA* and *sdpA* genes of strain MC1 showed no significant nucleotide similarities to *tfdA*. However, the significant degree of identity of their deduced amino acid sequences to group II αKG-dependent dioxygenases and the distribution of these identities throughout the whole of the primary sequence (Fig. 2) supported the view that RdpA and SdpA are homologous to dioxygenases of this family. The functionally important motif $HX(D/E)X_{23\text{-}26}(T/S)X_{114\text{-}183}HX_{10\text{-}13}R/K$, which is conserved throughout group II αKG-dependent dioxygenases (18), is present in both RdpA and SdpA. In TfdA of *R. eutropha* it includes the three Fe(II)-binding residues His114, Asp116, and His263, and the two αKG-binding residues Thr141 and Arg274 (Fig. 2). All of these residues were conserved in RdpA and SdpA, representing the putative Fe(II) and αKG-binding residues in these enzymes. Arg278, His214, Ser117, Lys95, and Lys71 of TfdA are known to interact with 2,4-D (8, 10). Ser117 homologues were identified in both dichlorprop-cleaving enzymes. A homologue for Arg278 was present only in RdpA, one for His214 was found in SdpA. The tryptophan residues Trp126, Trp238, and Trp246 undergoing posttranslational hydroxylation in TauD and Trp113 undergoing posttranslational hydroxylation in TfdA form a hydrophobic cluster around the active site (10). In RdpA and SdpA all of these residues were conserved or substituted by other hydrophobic amino acids. The basis of the enantiospecificity of

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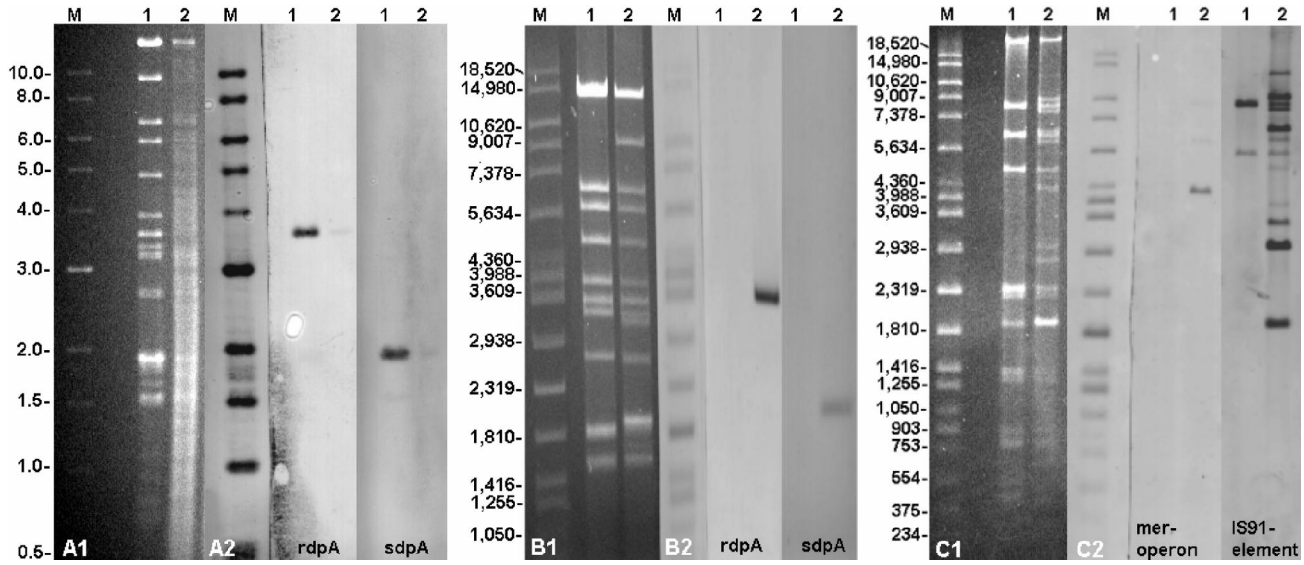

FIG. 4. Localization of *rdpA* and *sdpA* within the genome of *D. acidovorans* MC1 and comparison of plasmid pMC1 with pMC1-DP$^-$, the plasmid of mutant MC1-DP$^-$. (A) Localization of *rdpA* and *sdpA* within the genome of strain MC1. Plasmid DNA (lane 1) and genomic DNA (lane 2) was digested with PstI, separated electrophoretically in 0.8% agarose (A1), and hybridized (A2). Gene probes are given at the bottom. (B and C) Comparison of plasmid pMC1 of the wild-type strain of MC1 (lane 2) with pMC1-DP$^-$, the plasmid of the mutant unable to cleave dichlorprop (lane 1). PstI-digested plasmid DNA was separated electrophoretically in 0.8% agarose (B1) and hybridized against *rdpA* and *sdpA* gene probes (B2). EcoRI/XmaI-digested plasmid DNA was separated electrophoretically in 0.8% agarose (C1) and hybridized against probes for the *mer* operon and the IS*91* element (C2).

the dichlorprop-cleaving enzymes is not known. It is known that the tertiary structure of homologous proteins showing at least 20% identity of amino acid sequence is conserved (41). Among group II αKG dioxygenases a high degree of structural conservation was observed between clavaminate synthase and TauD, which share as little as 7.7% primary sequence identity (10). It is therefore likely that 2,4-D and dichlorprop-cleaving enzymes are structurally very similar and that substrate specificity is determined by only a few amino acids. Site-specific mutagenesis studies are needed to reveal the molecular basis of the substrate specificity and stereospecificity of RdpA and SdpA.

Phylogenetic trees calculated by different methods (data not shown) consistently grouped RdpA in one clade with TauD enzymes, but the assignment of SdpA could not be determined precisely. The primary sequence of SdpA most closely resembled that of TfdA and, like this dioxygenase, SdpA is characterized by a relatively broad substrate specificity (34). TfdA has greatest activity with 2,4-D but shows some activity toward *S*-mecoprop (38). SdpA shows greatest activity with the *S*-enantiomers of mecoprop and dichlorprop but has some activity toward 2,4-D (34). These observations suggested that SdpA is most closely related to 2,4-D-cleaving enzymes, which was also consistent with the position of SdpA in trees constructed without an outgroup. However, when PvcB was used as an outgroup SdpA was represented as a sister group of both the TfdA/TfdAα and the RdpA/TauD clades. At present, the database is too small to determine more precisely the phylogenetic relationship between SdpA and other members of the αKG dioxygenase family.

Transposon mutagenesis inactivation of *rdpA* indicated that it was a single-copy gene and gave a further proof of its functionality and role in strain MC1, besides previous characterization of RdpA and SdpA (34, 57, 58).

An *rdpA* gene identical to that of strain MC1 was identified in *Rhodoferax* sp. strain P230, and one nearly identical was found in *S. herbicidovorans* MH, but neither of these strains carried *sdpA* genes, as indicated by Southern hybridizations allowing ca. 20% mismatches. The *rdpA* genes could be involved in *R*-dichlorprop degradation in strains P230 and MH. Further investigations are necessary to study their role to determine whether *rdpA* genes are generally more widely distributed than *sdpA* genes, as these limited data suggest, and to identify the genes encoding the *S*-dichlorprop-cleaving dioxygenases of strains MH and P230.

In *S. herbicidovorans* MH, three transport proteins specific for 2,4-D, *R*-dichlorprop, and *S*-dichlorprop were identified (60). In strain MC1, ORF19 encodes a putative transport protein (Fig. 4 and Table 3). It was in close proximity to *rdpA*, and the significant similarity of the deduced protein sequence to TfdK and CadK suggested an involvement in *R*-dichlorprop transport. No other potential transporters were detected. Likewise, no genes encoding transcriptional regulators of dichlorprop cleavage were identified. This is in accordance with previous studies which found *rdpA* and *sdpA* to be constitutively expressed (36). ORF4, which shows significant similarities to LysR-type transcriptional regulators, probably regulates the expression of ORF5 rather than that of *sdpA* or *rdpA*.

The ability to degrade dichlorprop in *D. acidovorans* MC1 is spontaneously and irreversibly lost at high frequencies under nonselective conditions, whereas the degradation of dichlorophenol is a stable trait (36). This indicated that the ability to cleave the phenoxyalkanoate herbicides is unstable in strain MC1, which would be consistent with recent acquisition of the

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rdpA and *sdpA* genes. The localization and organization of the initial step in dichlorprop degradation imply that its evolution and recruitment took place independently of the lower pathway. Cases and de Lorenzo (5) postulated that degradative pathways for substrates only very recently found in the environment are often poorly regulated, probably reflecting an early step in the optimization of the corresponding metabolic route, and that biodegradative operons evolve from constitutive expression to substrate-responsive and metabolically controlled transcription. The evolution of the dichlorprop cleavage pathway in strain MC1 is an example supporting this hypothesis, since *rdpA* and *sdpA* are constitutively expressed, whereas the consecutive degradation of the intermediate dichlorophenol is inducible (36).

Previously, the loss of genes involved in etherolytic cleavage of dichlorprop was hypothesized to be caused by a deletion within plasmid pMC1, which occurs concomitantly with loss of dichlorprop cleavage (36). In the present study, both dioxygenase genes were shown to be located on the plasmid and to be deleted in the MC1-DP$^-$ mutant, proving this hypothesis to be correct. Moreover, the *mer* operon was deleted, and the copy number of the IS*91*-like element was found to be considerably reduced. This suggests that recombination between IS*91* elements could lead to the deletion of one or both dioxygenase genes and the Tn*501*-like transposon. Although IS*91*-like elements do not cause deletion of regions adjacent to them, a deletion of a genomic fragment flanked by IS*91*-like elements was observed in *Shigella flexneri* (52). In strain MC1, recombination between the interrupted IS element and the intact IS copy downstream of ORF5 would lead to the deletion of the region between the elements, including *sdpA*. Likewise, the IS elements may have played a role in the acquisition of dichlorprop cleavage in strain MC1. In *Ralstonia* sp. strain JS705 the *clc* genes are flanked by an intact and a partial copy of an insertion element highly similar to the ones identified in strain MC1. These IS elements were proposed to have captured the *clc* genes and made possible the gene transfer from *Ralstonia* sp. strain JS745 to strain JS705 (37). Characterization of the region downstream of *rdpA* and comparison of the MC1-DP$^-$ mutant genotype with the MC1 wild-type will further the understanding of processes having lead to the acquisition and causing instability of the degradative trait.

ACKNOWLEDGMENTS

We thank Esh Mahenthiralingam (Cardiff University) for the gift of pScosPA1. The construction of the cosmid library was carried out by KMS in Andrew Weightman's lab at Cardiff University, and we are very grateful to Andrew Weightman, Esh Mahenthiralingam, and Li Ling Lee for advice and discussions. We thank Ute Lohse and Anett Heidtmann for skillful technical support.

K.M.S. was supported by Deutsche Bundesstiftung Umwelt grant 20000/99.

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