

Purification and Characterisation of the Enantiospecific Dioxygenases from *Delftia acidovorans* MC1 Initiating the Degradation of Phenoxypropionate and Phenoxyacetate Herbicides

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Summary

After cultivation on (*R,S*)-2-(2,4-dichlorophenoxy)propionate, two α -ketoglutarate-dependent dioxygenases were isolated and purified from *Delftia acidovorans* MC1, catalysing the cleavage of the ether bond of various phenoxyalkanoate herbicides. One of these enzymes showed high specificity for the cleavage of the *R*-enantiomer of substituted phenoxypropionate derivatives: the K_m values were 55 μM and 30 μM , the k_{cat} values 55 min^{-1} and 34 min^{-1} with (*R*)-2-(2,4-dichlorophenoxy)propionate [(*R*)-2,4-DP] and (*R*)-2-(4-chloro-2-methylphenoxy)propionate, respectively. The other enzyme predominantly utilised the *S*-enantiomers with K_m values of 49 μM and 22 μM , and k_{cat} values of 50 min^{-1} and 46 min^{-1} with (*S*)-2-(2,4-dichlorophenoxy)propionate [(*S*)-2,4-DP] and (*S*)-2-(4-chloro-2-methylphenoxy)propionate, respectively. In addition, it cleaved phenoxyacetate herbicides (i.e. 2,4-dichlorophenoxyacetate: $K_m = 123 \mu\text{M}$, $k_{\text{cat}} = 36 \text{ min}^{-1}$) with significant activity. As the second substrate, only α -ketoglutarate served as an oxygen acceptor for both enzymes. The enzymes were characterised by excess substrate inhibition kinetics with apparent K_i values of 3 mM with (*R*)-2,4-DP and 1.5 mM with (*S*)-2,4-DP. The reaction was strictly dependent on the presence of Fe^{2+} and ascorbate; other divalent cations showed inhibitory effects to different extents. Activity was completely extinguished within 2 min in the presence of 100 μM diethylpyrocarbonate (DEPC).

Introduction

The degradation of chlorinated/methylated phenoxyalkanoate herbicides is usually initiated by the cleavage of the ether bond of these compounds leading to the formation of respective phenolic moieties and the oxidised alkanoates. The prevailing reaction is catalysed by an α -ketoglutarate-dependent dioxygenase as studied with the purified enzyme from *Ralstonia eutropha* JMP134; α -ketoglutarate is oxidatively decarboxylated

to succinate in this step [1, 2]. The enzyme from the above strain was determined to be highly specific for the phenoxyacetate structure. Genes encoding this type of enzyme, i.e. *tfdA*, were found to be widespread in microbial communities [3–9]. By contrast, there are only a few examples of axenic strains that are able to productively utilise phenoxypropionate herbicides: *Sphingomonas herbicidovorans* (*Flavobacterium* sp.) MH [10, 11], *Rhodospirillum rubrum* P230 [12] and *Delftia (Comamonas) acidovorans* MC1 [13]. Remarkably, these strains utilise a broader spectrum of phenoxy herbicides; in addition to their ability of degrading the racemic phenoxypropionates, they similarly attack phenoxyacetate derivatives. Again, this reaction proved to be catalysed by an α -ketoglutarate-dependent dioxygenase [14–16].

Preliminary investigations revealed enantioselective properties of the enzymes catalysing the cleavage of the ether bond of the racemic phenoxypropionates as holds for *S. herbicidovorans* MH [14, 17] and *D. acidovorans* MC1 [15]. The enantioselectivity of this reaction is also supported by the fact that a strain of *Alcaligenes denitrificans* is only able to utilise the *R*-enantiomer of 2-(4-chloro-2-methylphenoxy)propionate [18]. These enzymes have, however, not been studied in detail.

The present investigation is aimed at elucidating the enzymatic basis of the broad herbicide consumption profile including the phenoxyacetate derivatives in *D. acidovorans* MC1. Enzymes carrying ether-cleaving activity with respect to the various phenoxyalkanoate herbicides were purified and characterised with regard to their catalytic properties.

Materials and Methods

Cultivation

Strain MC1 was inoculated from individual colonies grown on (*R,S*)-2,4-DP into PYE medium (3 g/l of each peptone and yeast extract and 1.8 g/l of fructose) and propagated in an overnight culture; then it was incubated for a further day in the presence of 100 mg/l of (*R,S*)-2,4-DP. One litre of this culture was used to inoculate a fermenter (INFORS, ALGU 503) operating at a working volume of 4 l. Cultivation proceeded under aerobic conditions at pH 8.0 and 30 °C. The medium was composed of [mg/l]: NH_4Cl , 1520; KH_2PO_4 , 680.5; K_2HPO_4 , 870.9; $\text{CaCl}_2 \times 6 \text{H}_2\text{O}$, 11; $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$, 142; $\text{FeSO}_4 \times 7 \text{H}_2\text{O}$, 10; $\text{CuSO}_4 \times 5 \text{H}_2\text{O}$, 1.57; $\text{MnSO}_4 \times 4 \text{H}_2\text{O}$, 1.23; $\text{ZnSO}_4 \times 7 \text{H}_2\text{O}$, 0.88; $\text{Na}_2\text{MoO}_4 \times 2 \text{H}_2\text{O}$, 0.5. (*R,S*)-2,4-DP was supplied as the sole carbon and energy source in a fed batch regime by adapting the feed rate to the substrate consumption capacity of the culture. Excess substrate in the medium did not exceed 0.5 g/l. In addition, a trace element solution containing the respective salts in the concentration specified above was continuously fed at a rate of 0.77 ml/h. Biomass increase (OD_{700}) and substrate consumption were monitored off-line. After the cell density had reached an $\text{OD}_{700} = 2$, about 50% of the suspension was harvested. The remaining culture in the fermenter was supplied with an adequate portion of the mineral salt solution and cultivation continued.

Cells were harvested by centrifugation at 4 °C at $8875 \times g$. The pellet was washed and re-suspended in 10 mM Tris/HCl buffer, pH 7.5 (buffer A). The suspension was stored at –20 °C pending further use.

Purification

Purification was performed by application of a GradiFrac System including pump P-1 and monitor UV-1 (AMERSHAM PHARMACIA BIOTECH). Unless otherwise indicated, all steps were carried out at 4 °C.

Step 1: Cells were disintegrated using a French Pressure Cell (AMINCO, Silver Spring, USA) by three passages at 140 MPa. Particle-free supernatants were obtained by centrifugation at 4 °C for 25 min at $20,000 \times g$.

Step 2: Protamine sulphate (1%, pH 7.0) was added to the supernatant to give a final concentration of 0.15%. The solution was stirred for 20 min and separated from the precipitate by centrifugation for 20 min at $20,000 \times g$.

Step 3: Ammonium sulphate was added by stirring the supernatant from step 2 to give a final concentration of 1.25 M. After stirring for 20 min, the precipitate was removed (20 min centrifugation at $20,000 \times g$). A further quantity of ammonium sulphate was added to the supernatant to give a final concentration of 2.5 M. After stirring for a further 20 min, the pellet was collected by centrifugation and used for further steps.

Step 4: The pellet was solubilised in a small quantity of buffer A and applied to a Hi Prep 26/10 desalting column (PHARMACIA, Sweden) filled with Sephadex G-25. The column was equilibrated with buffer A. Application of the sample and elution proceeded at a rate of 5 ml/min. The eluate was sampled in fractions of 10 ml, which were monitored for conductivity and protein content.

Step 5: Ion exchange chromatography (IEX) was carried out on a Source30Q (PHARMACIA, Sweden) column (25/14). The column was equilibrated with buffer A. The sample was applied with a rate of 1 ml/min and further treated with buffer A to remove non-bound proteins. The proper elution was started at the same rate by linearly increasing NaCl gradients of varying steepness. The total volume of the gradient solutions amounted to 120–180 ml. Eluates were collected in fractions of 6 ml.

Step 6: Hydrophobic interaction chromatography (HIC) was performed on a Butyl-Sepharose (PHARMACIA, Sweden) column with a bed volume of 20 ml. The column was equilibrated with buffer A containing different initial concentrations of ammonium sulphate. The protein samples derived from each purification step were supplied with ammonium sulphate to reach the desired initial concentration corresponding to the column equilibrium concentration. Desorption was performed by using a linearly decreasing ammonium sulphate gradient at a rate of 1 ml/min. The total volume of the gradient solutions amounted to 180 ml. Eluates were collected in fractions of 6 ml.

Step 7: Gel filtration was performed on a Superdex200 prep grade (PHARMACIA, Sweden) column (100/10). It was equilibrated with buffer A containing 0.15 M NaCl. Before application, the protein solutions were concentrated by precipitation with 3.4 M ammonium sulphate, re-solubilised in a small volume and applied and eluted at a rate of 0.5 ml/min. The eluates were collected in fractions of 10 ml.

Electrophoresis

One-dimensional electrophoresis was carried out with a PowerEase 500 system (NOVEX, San Diego, USA) on 12% Tris glycine precast acrylamide gels (NOVEX). The samples contained 1–2 μg of protein. The running buffer was composed of 2.9 g/l Tris base, 14.4 g/l glycine and 1.0 g/l SDS. The sampling buffer contained 2 ml glycerol, 4 ml 10% SDS [w/v], 0.5 ml 1% bromophenol blue and 2.5 ml 0.5 M Tris/HCl, pH 6.8, in 10 ml distilled water. The gels were treated by 10 mA. Mark 12™ Wide Range Standard (NOVEX) was applied as a reference.

Gels were stained by using the SilverXpress kit (NOVEX) following the instructions of the supplier, or by using Coomassie brilliant blue.

Enzyme Measurement

Ether-cleaving dioxygenase activity was routinely measured according to FUKUMORI and HAUSINGER [1] by determining the phenolic intermediates liberated in this enzymatic reaction after their reaction with 4-aminoantipyrine. The assay for the enzyme reaction contained 1 mM herbicide (sodium salt), 1 mM α -ketoglutarate, 200 μM ascorbic acid, 200 μM ammonium iron(II) sulphate in 10 mM imidazole buffer (pH 6.75). The buffer was gassed with air at 30 °C for 30 min before adding the individual components. The reaction was performed at 30 °C, started by adding the enzyme at a

concentration of 0.05–0.3 μM (with respect to the monomer). After respective times, usually within a period of 10 min, samples (up to 5) were taken and the enzyme reaction was stopped by adding 50 μl of 20 mM EDTA to 1 ml of the reaction mixture. The phenolic products were determined by adding 100 μl of borate buffer, pH 10 (3.09 g H_3BO_4 ; 3.73 g KCl; 44 ml 1 N NaOH ad 1 l), 10 μl 2% 4-aminoantipyrin and 10 μl 8% potassium hexacyanoferrate III. After 5 min of incubation at 30 $^\circ\text{C}$, the extinction of the samples was measured at 510 nm (U-2000, HITACHI, Tokyo, Japan). The progress curves proved almost linear under these conditions as followed from linear regression giving a measure of confidence of > 0.98 with the substrate concentrations tested. The standard deviation of the individual points derived from triplicates amounted to $\leq 8\%$. A comparison of the colorimetric assay with a direct measurement of the reaction by following substrate disappearance and product accumulation via HPLC gave identical rates.

Analytical Methods

Phenoxyalkanoates were determined by HPLC according to [19]. The biomass concentration was measured on the basis of the optical density at 700 nm. An $\text{OD}_{700} = 1$ corresponds to a biomass concentration of about 0.5 g/l dry mass.

Results

Enzyme Purification

Treatment of crude extract in the presence of 0.15% protamine sulphate and precipitation of proteins in the range of 1.25–2.5 M ammonium sulphate kept almost all of the ether-cleaving enzymes of the crude extract detected by measuring activities in the presence of the individual enantiomers of 2,4-DP (Tab. 1) and of 2,4-D (not shown). Application of IEX chromatography separated two protein fractions from each other, which exhibited distinct activity to the various substrates. The fraction eluted at low salt concentrations of around 0.12 M exhibited pronounced activity toward (*S*)-2,4-DP and, in addition, activity with respect to 2,4-D which coincided with the peak at low NaCl concentration. A second fraction found at around 0.35 M NaCl exhibited selective activity by only converting the *R*-enantiomer. Pooled fractions carrying the respective activities were further treated by HIC chromatography. This resulted in a significant purification of the two enzymes (Tab. 1), which were consecutively named the *R*-specific RdpA and the *S*-specific SdpA enzyme, respectively. Again, activity with respect to 2,4-D cleavage coincided with the activity profile for (*S*)-2,4-DP. Characterisation by SDS-PAGE revealed that this purification protocol resulted in a rather pure product with the *S*-specific enzyme (Fig. 1). In contrast, significant impurities were still observed with the *R*-specific enzyme (Fig. 2). Densitometric analysis revealed an enrichment of 80% of the *R*-specific enzyme at this preparation step. Use of gel filtration to remove these impurities was less successful as the activity was drastically diminished (Tab. 1). This step was therefore omitted in further preparations. These impurities did, however, not disturb consecutive kinetic investigations as it was proven that any reaction using the phenolic intermediate as the substrate (chlorophenol hydroxylase) was absent after IEX chromatography. This was verified by keeping product, i.e. dichlorophenol, unutilised within 12 h of incubation in the presence of the respective enzyme

fractions. From the electrophoretic patterns (electropherograms), molecular weights of the (subunits of the) *S*-specific and the *R*-specific enzyme of about 32 kD and 36 kD, respectively, were derived.

Tab. 1. Purification of 2,4-DP/ α -KG dioxygenases from *D. acidovorans* MC1

Purification step	<i>(R)</i> -specific enzyme			<i>(S)</i> -specific enzyme		
	Specific activity [mU/mg]	Purification [-fold]	Total activity [%]	Specific activity [mU/mg]	Purification [-fold]	Total activity [%]
Crude extract	7.2	1	100	6.8	1	100
PS-P	6.7	0.93	93	5.8	0.85	95
AS-P	13.8	1.9	92	11.3	1.7	86
Source 30Q	25.4	3.5	66	101	14.9	65
Butyl-sepharose	172.8	24	45	259	38	8
Gel filtration	154	21.4	1	205	30.2	0.8

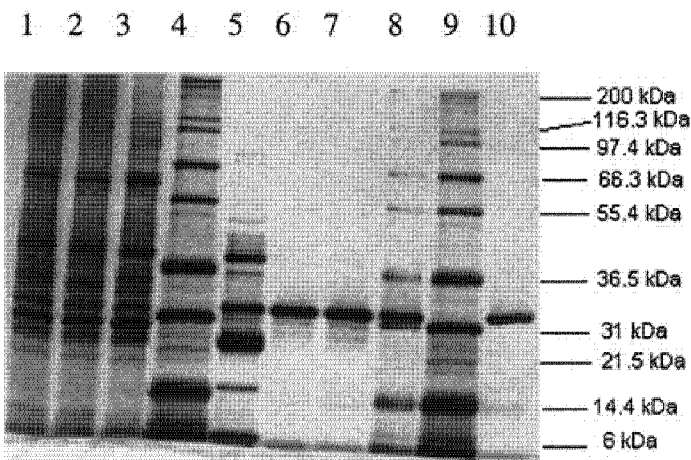


Fig. 1. Purification of *(S)*-2,4-DP/ α -KG dioxygenase from *D. acidovorans* MC1
Protein samples were analysed by gel electrophoresis on a 12% polyacrylamide gel and visualised by silver staining.
1: Crude extract, 2: Supernatant after the treatment with 0.15% protamine sulphate, 3: Fraction precipitated between 1.25–2.5 M ammonium sulphate, 5: Pooled fractions with *(S)*-2,4-DP-cleavage activity after IEX, 6+7: Fractions with *(S)*-2,4-DP-cleavage activity after HIC, 8+10: Pooled fractions with *(S)*-2,4-DP-cleavage activity after GF, 4+9: Molecular weight marker.

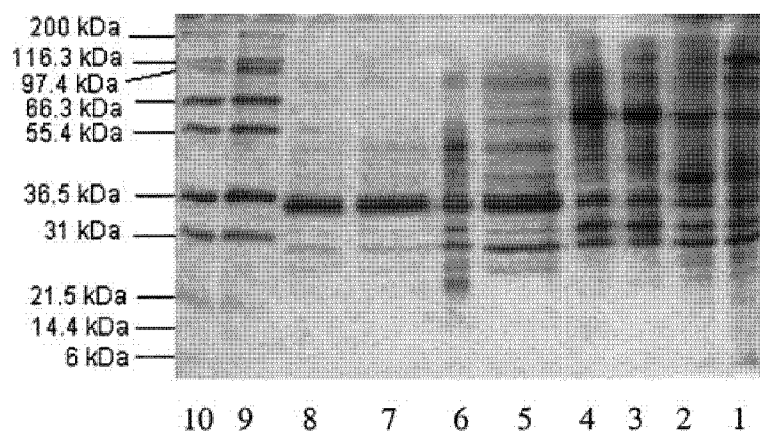


Fig. 2. Purification of *(R)*-2,4-DP/ α -KG dioxygenase from *D. acidovorans* MC1. Protein samples were analysed by gel electrophoresis on a 12% polyacrylamide gel and visualised by silver staining.
1: Crude extract, 2: Supernatant after treatment with 0.15% protamine sulphate, 3+4: Pooled fractions with *(R)*-2,4-DP-cleavage activity after IEX, 5+6: Pooled fractions with *(R)*-2,4-DP-cleavage activity after HIC, 7+8: Pooled fractions with *(R)*-2,4-DP-cleavage activity after GF, 9+10: Molecular weight marker.

The *R*-specific enzyme was found to be stable and did not require supplements when stored at -20° in buffer A. Storage at 4°C resulted in a loss of activity within about 20 days; the enzyme was significantly stabilised under these conditions by adding 1% BSA or 3.4 M ammonium sulphate (Fig. 3). A similar pattern with respect to stability was observed with the *S*-specific enzyme (not shown).

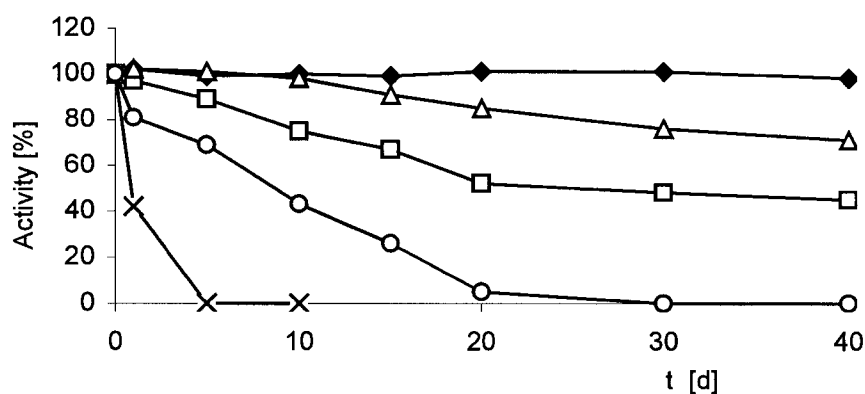


Fig. 3. Stability of the *(R)*-2,4-DP/ α -KG dioxygenase (after HIC) at different storage conditions
(○) 4°C without addition, (×) 4°C after the addition of $50\ \mu\text{M}$ ascorbic acid, (□) 4°C after the addition of 1% BSA, (Δ) 4°C after the addition of 3.4 M ammonium sulphate, (◆) -20°C without addition.

Kinetic Properties

Preliminary investigations were performed in order to elucidate the optimum conditions for the enzyme reaction. The activity of the *R*- and *S*-specific enzyme depended on the presence of both Fe^{2+} and ascorbic acid, as is shown in Figs. 4 and 5. The concentration-dependent activity profiles were characterised by a complex shape (see Discussion). Consecutive kinetic investigations were performed at a Fe^{2+} and an ascorbic acid concentration of $200\ \mu\text{M}$. With regard to the physical parameters, the temperature optimum was determined as $30\ ^\circ\text{C}$ with the *R*-specific and $25\ ^\circ\text{C}$ with the *S*-specific enzyme; the optimum pH value was around 6 with both enzymes. The activity patterns depending on the latter parameters were of similar complexity as indicated above.

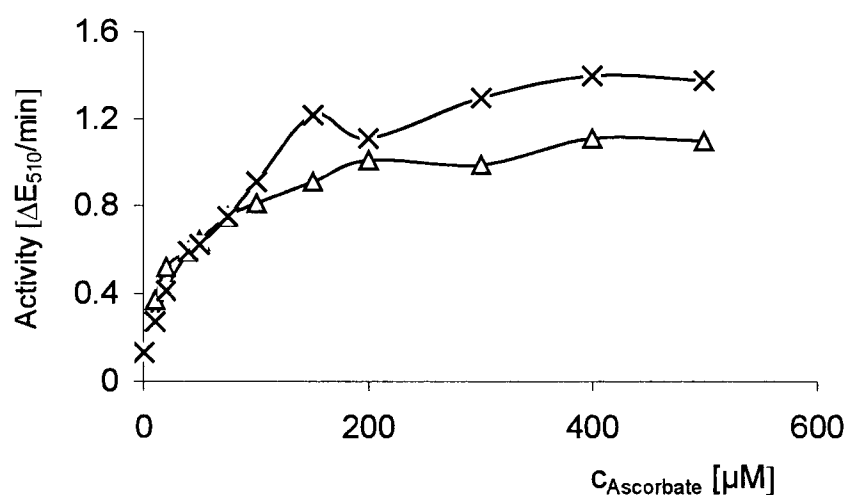


Fig. 4. Effect of ascorbic acid on the activity of 2,4-DP/ α -KG dioxygenases ($\times$) *S*-specific enzyme, (Δ) *R*-specific enzyme.

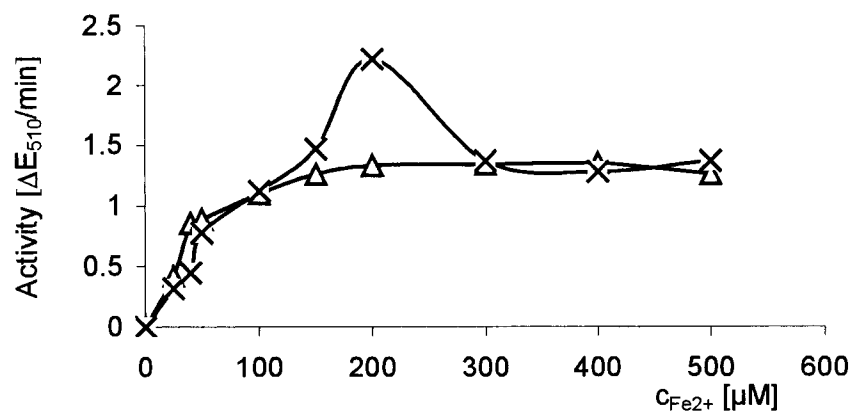


Fig. 5. Activation of 2,4-DP/ α -KG dioxygenases by ferrous ions ($\times$) *S*-specific enzyme, (Δ) *R*-specific enzyme. The enzyme activity was determined using standard assay conditions.

Tab. 2. Substrate specificity and kinetic parameters of (*R*)-2,4-DP/ α -KG dioxygenase

Substrate (concentration range studied)		K_m [μ M]	k_{cat} [min^{-1}]	k_{cat}/K_m	[%]
(<i>R</i>)-2,4-DP	(20–200 μ M)	54.9 ± 4.4	55.2	1	/ (100)
(<i>S</i>)-2,4-DP	(100–5000 μ M)	–	–	–	
(<i>R</i>)-2,4-MCPP	(10–200 μ M)	30 ± 1.2	34.4	1.15	/ (115)
(<i>S</i>)-2,4-MCPP	(20–600 μ M)	261 ± 40	3.8	0.02	/ (2)
2,4-D	(20–2000 μ M)	1305 ± 120	7.6	0.006	/ (0.6)
2,4-DB	(100–2000 μ M)	–	–	–	
3-Phenoxypropionic acid	(100–2000 μ M)	–	–	–	
(<i>R,S</i>)-2-(2,4,5-Trichlorophenoxy)propionic acid	(10–200 μ M)	130.8 ± 9.3	50	0.382	/ (38)
(<i>R,S</i>)-2-(<i>m</i> -Chlorophenoxy)propionic acid	(20–400 μ M)	176.2 ± 4.2	34.4	0.195	/ (19)
(<i>R,S</i>)-2-(4-Chlorophenoxy)propionic acid	(10–200 μ M)	131 ± 0.4	65.2	0.498	/ (50)
α -Ketoglutarate	(2.5–50 μ M)	27.8 ± 3.8	20	0.719	/ (100)
α -Ketobutyrate	(10–5000 μ M)	–	–	–	
α -Keto adipate	(10–5000 μ M)	–	–	–	
α -Ketoisovalerate	(10–5000 μ M)	–	–	–	
α -Ketovalerate	(10–5000 μ M)	–	–	–	
Pyruvate	(10–10000 μ M)	–	–	–	

(– = no activity detected)

All experiments were performed at 30 °C in 10 mM imidazol buffer (pH 6) containing 200 μ M ascorbate, 200 μ M $(\text{NH}_4)_3\text{Fe}(\text{SO}_4)_2$, the respective substrates with the concentration ranges indicated, 1 mM of the second substrate [α -KG at variable herbicides; (*R*)-DP at variable keto acids] and enzyme.

Kinetic investigations were aimed at revealing the enzymatic basis of substrate specificity and diversity with respect to the herbicides. The results obtained with the *R*-specific enzyme are summarised in Tab. 2. As expected, the highest activity was obtained with the *R*-enantiomers of the dichlorinated and chloro/methyl-substituted phenoxypropionates taking into account k_{cat} and K_m and the quotient of both parameters. By contrast, very low or no activity was detected in the presence of the respective *S*-enantiomers; 2,4-D was not cleaved at all. This is reflected by both k_{cat} and K_m . Considerable activity was also found with other derivatives of 2-phenoxypropionates including the trichlorinated compound. Although these were applied as the racemates, it is most likely the *R*-enantiomers that were predominantly attacked. (Some effects exerted by the presence of the respective *S*-enantiomers cannot, however, be ruled out under these

conditions. Hence these values are more indicative of the consumption of these compounds rather than usable kinetic constants.) As the second substrate, only α -ketoglutarate served as an oxygen acceptor; alternative substrates were of no effect in a wide range of concentrations tested. It should be noted that by applying variable substrate concentrations similarly complex characteristics were observed as shown in Figs. 4 and 5, the reasons for which are discussed later. Nevertheless, the LINEVEAVER-BURK, HANES and EADIE-HOFSTEE plots exhibited fairly linear dependencies with confidence intervals in most cases of $\geq 95\%$. The kinetic constants derived from such complex characteristics should, however, only be considered as apparent (overall) constants.

The picture was rather different with the *S*-specific enzyme. As expected, pronounced activities were found with the *S*-configuration of 2,4-DP and MCPP. Due to the K_m value, (*S*)-MCPP was the preferred substrate. Most important, however, is the fact that this enzyme is also able to cleave 2,4-D with significant activity (Tab. 3). Again, only α -ketoglutarate served as the second substrate.

Tab. 3. Substrate specificity and kinetic parameters of (*S*)-2,4-DP/ α -KG dioxygenase

Substrate (concentration range studied)		K_m [μ M]	k_{kat} [min^{-1}]	k_{kat}/K_m	[%]
(<i>R</i>)-2,4-DP	(50–500 μ M)	–	–	–	
(<i>S</i>)-2,4-DP	(20–200 μ M)	49 ± 9.1	50	1.02	/ (100)
(<i>R</i>)-2,4-MCPP	(20–1000 μ M)	–	–	–	
(<i>S</i>)-2,4-MCPP	(10–100 μ M)	21.8 ± 5	46	2.11	/ (207)
2,4-D	(50–200 μ M)	122.8 ± 2	36	0.29	/ (29)
2,4-DB	(100–2000 μ M)	–	–	–	
3-Phenoxypropionic acid	(100–2000 μ M)	–	–	–	
(<i>R,S</i>)-2-(2,4,5-Trichlorophenoxy)propionic acid	(100–2000 μ M)	–	–	–	
(<i>R,S</i>)-2-(<i>m</i> -Chlorophenoxy)propionic acid	(20–200 μ M)	11.5 ± 6.4	15	0.13	/ (13)
(<i>R,S</i>)-2-(4-Chlorophenoxy)propionic acid	(10–100 μ M)	68.3 ± 9.8	17	0.25	/ (25)
α -Ketoglutarate	(2.5–50 μ M)	24.1 ± 7.6	1.7	34	/ (100)
α -Ketobutyrate	(10–5000 μ M)	–	–	–	
α -Ketoadipate	(10–5000 μ M)	–	–	–	
α -Ketoisovalerate	(10–5000 μ M)	–	–	–	
α -Ketovalerate	(10–5000 μ M)	–	–	–	
Pyruvate	(10–10000 μ M)	–	–	–	

(– = no activity detected)

All experiments were performed at 25 °C in 10 mM imidazol buffer (pH 6) containing 200 μ M ascorbate, 200 μ M $(\text{NH}_4)_3\text{Fe}(\text{SO}_4)_2$, the respective substrates with the concentration ranges indicated, 1 mM of the second substrate [α -KG at variable herbicides; (*R*)-DP at variable keto acids] and enzyme.

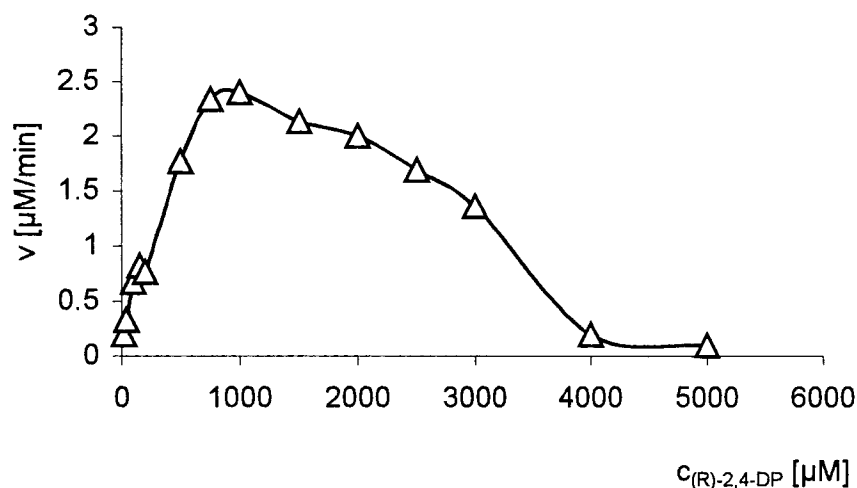


Fig. 6 a. Effect of (*R*)-2,4-DP on the activity of (*R*)-2,4-DP/ α -KG dioxygenase
The assay contained 0.055 μM enzyme.

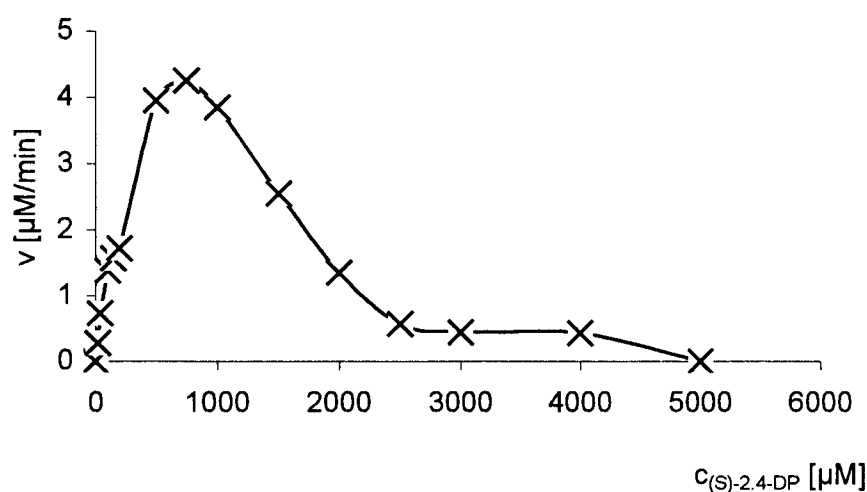


Fig. 6 b. Effect of (*S*)-2,4-DP on the activity of (*S*)-2,4-DP/ α -KG dioxygenase
The assay contained 0.055 μM enzyme.

Both enzymes exhibited excess substrate inhibition. According to the data in Figs. 6a and b, K_i values of about 3 mM with the *R*-specific and of about 1.5 mM with the *S*-specific enzyme can approximately be taken from these data. However, this is only formal and does not reflect the real inhibition pattern, again caused by the complex dependencies on substrate concentration.

Fe^{2+} is essential for the enzyme reaction (Fig. 5), other divalent cations tested such as Cu^{2+} , Mn^{2+} , Mg^{2+} , Zn^{2+} , Ni^{2+} and Co^{2+} could not substitute this ion.

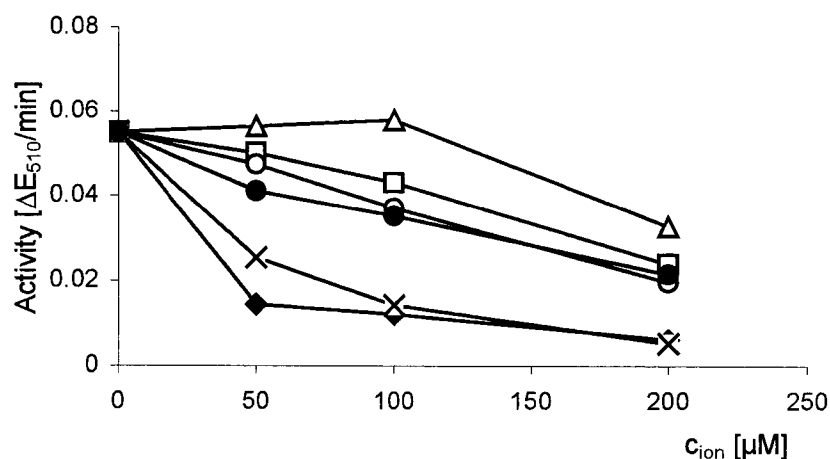


Fig. 7 a. Metal ion-dependent inactivation of (*R*)-2,4-DP/ α -KG dioxygenase
The enzyme activity was determined by using standard assay condition in the presence of various metal ions.

(Δ) Mg^{2+} , ($\square$) Ni^{2+} , ($\circ$) Mn^{2+} , ($\bullet$) Co^{2+} , ($\times$) Zn^{2+} , ($\blacklozenge$) Cu^{2+} .

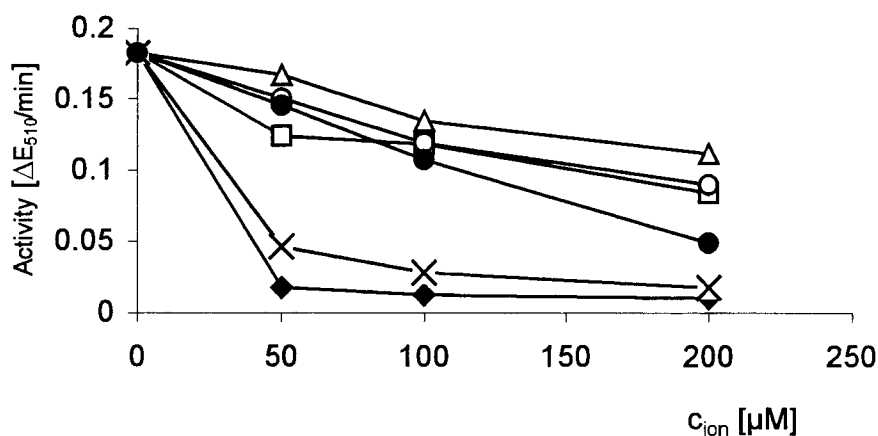


Fig. 7 b. Metal ion-dependent inactivation of (*S*)-2,4-DP/ α -KG dioxygenase

The enzyme activity was determined by using standard assay condition in the presence of various metal ions.

(Δ) Mg^{2+} , ($\square$) Ni^{2+} , ($\circ$) Mn^{2+} , ($\bullet$) Co^{2+} , ($\times$) Zn^{2+} , ($\blacklozenge$) Cu^{2+} .

Moreover, they even behaved in an inhibitory manner. This was most significantly observed with copper and nickel, both of which exhibited, according to the properties shown in Figs. 7 a and b, K_i values of around 50 μ M with the *R*-specific but far less than 50 μ M with the *S*-specific enzyme. The other ions tested exerted weaker effects. In addition, the enzyme was inhibited by DEPC. The application of 100 μ M led to an almost complete loss of activity within 2 min with both enzymes; lower concentrations behaved more gradually (Figs. 8 a,b).

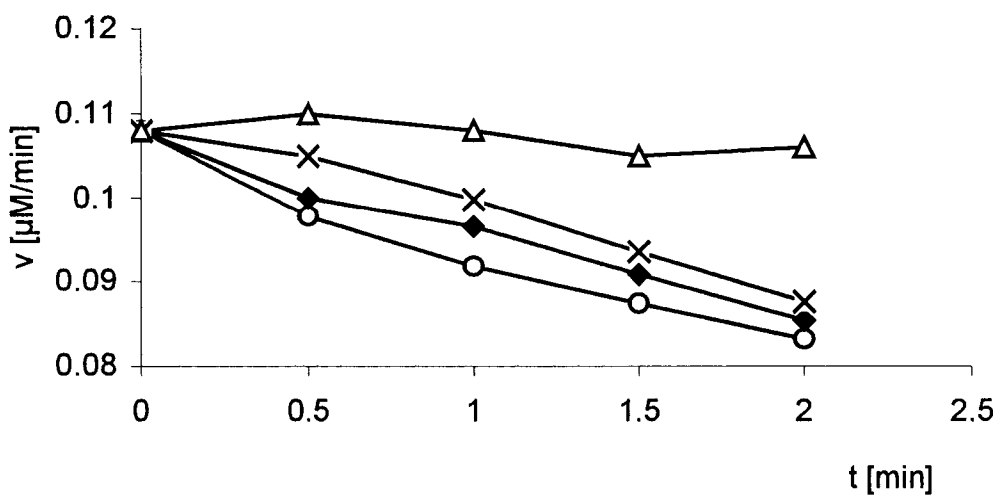


Fig. 8 a. Inactivation of (*R*)-2,4-DP/ α -KG dioxygenase with DEPC
(○) 100 μ M DEPC, (◆) 50 μ M DEPC, (×) 20 μ M DEPC, (Δ) 0 μ M DEPC.

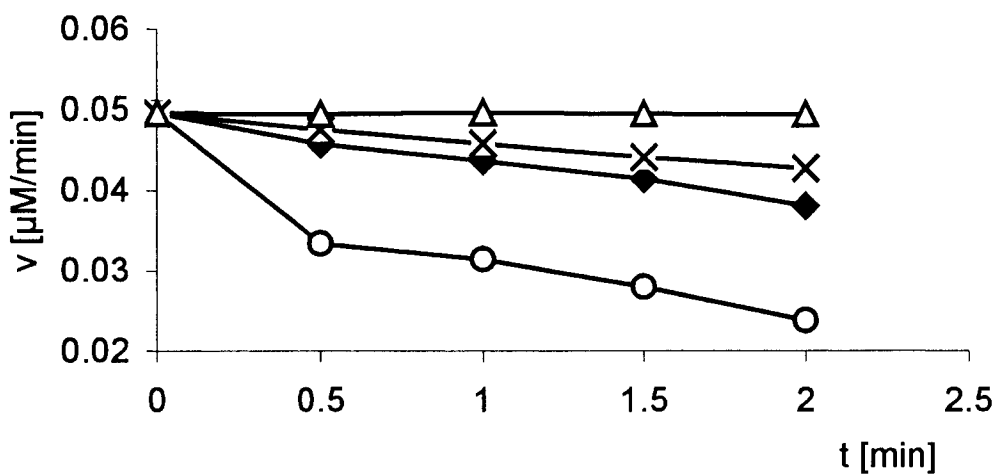


Fig. 8 b. Inactivation of (*S*)-2,4-DP/ α -KG dioxygenase with DEPC
(○) 100 μ M DEPC, (◆) 50 μ M DEPC, (×) 20 μ M DEPC, (Δ) 0 μ M DEPC.

Discussion

The present investigation has clearly elucidated the enzymatic basis for the degradation of enantiomeric phenoxypropionate and the phenoxyacetate herbicides. Two different enzymes with different activity profiles were isolated. One of the enzymes is very specific to the *R*-configuration of the phenoxypropionates, it neither cleaved the *S*-enantiomer

mers to a significant extent nor did it utilise phenoxyacetate derivatives (2,4-D). The other enzyme isolated has predominant activity towards the *S*-configuration of the phenoxypropionates; in addition, it exhibits significant activities against 2,4-D. Due to their activities and the predominant substrates utilised, these enzymes should be called (*R*)-phenoxypropionate/ α -ketoglutarate-dioxygenase (RdpA) and (*S*)-phenoxypropionate/ α -ketoglutarate-dioxygenase (SdpA), respectively. The reaction type proved similar to the 2,4-D/ α -ketoglutarate dioxygenase from *Ralstonia eutropha* JMP134(pJP4) [1, 2]: the activity was strongly dependent on Fe²⁺ and ascorbate [16] as was the general case with α -ketoglutarate-dependent dioxygenases [20, 21]. It was inhibited by DEPC, a compound known to be reactive to histidine [22]. Two histidine residues have been shown to be essential components in the active centre of TfdA [24].

Besides the enzymes of strain MC1, there are strong indications of enantioselective enzymes with respect to 2-phenoxypropionates in the case of *S. herbicidovorans* MH. The separation of crude extracts of this strain by SDS electrophoresis revealed distinct protein bands of 32 kD and 34 kD after growth on (*S*)-MCP or (*R*)-MCP, respectively, and of both of these bands after growth on the racemate [14]. This pattern tallies with the molecular weights found for the respective ether-cleaving proteins (subunits) of strain MC1. Moreover, a molecular weight of 32 kD is found with the canonical TfdA protein from *R. eutropha* JMP134 [2], cleaving 2,4-D with high specificity. The application of primers, derived from conserved regions of *tfdA* genes [7], did not result in a specific amplification product in PCR with the genome of strain MC1 [12, 16] and strain MH as the template. This might be expected in agreement with the different substrate specificity found with the various strains (i.e. JMP134 vs. MC1). But by comparing strain MC1 and MH, significant differences became obvious, too: whereas strain MH prefers 2,4-D as derived from the maximum growth rate on the various herbicides [17], strain MC1 has a four-fold higher rate on (*RS*)-2,4-DP in comparison to 2,4-D [16]. This means that a similar herbicide consumption profile in these strains must be provoked by a divergent genetic background. This deviates from the experience with *tfdA*: this gene determining 2,4-D degradative specificity is distributed in the microbial world in a highly conserved manner [7]. To complete this picture, a third strain, *Rhodospirillum rubrum* sp. P230, has the same profile of herbicide utilisation as strains MH and MC1. This strain carries TfdA (as followed from sequencing of a respective PCR product), but is nevertheless unable to productively degrade 2,4-D [12, 16]. Very recently, two further strains were described [24] as carrying phenoxypropionate cleavage activity and also giving positive response to *tfdA* probes.

Investigations are in progress to elucidate the molecular structure of both enzymes from strain MC1 and the genes they derive from. It is furthermore apparent from the present results that both enzymes are characterised by complex kinetic behaviour, indicating isoforms of the enzyme. Proteolytic modifications should be ruled out as the cause of this pattern as this was also observed in enzyme preparations carried out in the presence of protease inhibitors. However, two-dimensional electrophoretic separation of the respective enzyme preparations or applying crude extracts revealed protein spots which are indicative of isoforms differing in charge rather than molecular weight [25]. This has to be verified in order to elucidate the structure and catalytic properties of these isoenzymes.

Abbreviations

BSA	Bovine serum albumin
2,4-D	2,4-Dichlorophenoxyacetate
2,4-DB	4-(2,4-Dichlorophenoxy)butyrate
2,4-DP	2-(2,4-Dichlorophenoxy)propionate
DEPC	Diethylpyrocarbonate
HIC	Hydrophobic interaction chromatography
IEX	Ion exchange chromatography
MCCP	2-(4-Chloro-2-methylphenoxy)propionate
GF	Gel filtration

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References

- [1] FUKUMORI, F., HAUSINGER, R. P.: *Alcaligenes eutrophus* JMP134 “2,4-dichlorophenoxyacetate monooxygenase” is an α -ketoglutarate-dependent dioxygenase. *J. Bacteriol.* **175** (1993), 2083–2086.
- [2] FUKUMORI, F., HAUSINGER, R. P.: Purification and characterization of 2,4-dichlorophenoxyacetate/ α -ketoglutarate dioxygenase. *J. Biol. Chem.* **268** (1993), 24311–24317.
- [3] NELSON, J. W., JOSEPHSON, K. L., PEPPER, I. L., ARNOLD, R. B., DIGIOVANNI, G. D., SINCLAIR, N. A.: Frequency of horizontal gene transfer of a large catabolic plasmid (pJP4) in soil. *Appl. Environ. Microbiol.* **60** (1994), 4053–4058.
- [4] HONG, S. M., AHN, Y. J., PARK, Y. K., MIN, K. H., KIM, C. K., KA, J. O.: Effects of genetically different 2,4-D-degradative plasmids on degradation phenotype and competitiveness of soil microorganisms. *J. Microbiol.* **33** (1995), 208–214.
- [5] FULTHORPE, R. R., RHODES, A. N., TIEDJE, J. M.: Pristine soils mineralise 3-chlorobenzoate and 2,4-dichlorophenoxyacetate via different microbial populations. *Appl. Environ. Microbiol.* **62** (1996), 1159–1166.
- [6] HOGAN, D. A., BUCKLEY, D. H., NAKATSU, C. H., SCHMIDT, T. M., HAUSINGER, R. P.: Distribution of the *tfdA* gene in soil bacteria that do not degrade 2,4-dichlorophenoxyacetic acid (2,4-D). *Microb. Ecol.* **34** (1997), 90–96.
- [7] VALLAEYS, T., FULTHORPE, R. R., WRIGHT, A. M., SOULAS, G.: The metabolism of 2,4-dichlorophenoxyacetic acid degradation involves different families of *tfdA* and *tfdB* genes according to PCR-RFLP analysis. *FEMS Microbiol. Ecol.* **20** (1996), 163–172.
- [8] VALLAEYS, T., COURDE, L., MCGOWAN, C., WRIGHT, A. D., FULTHORPE, R. R.: Phylogenetic analyses indicate independent recruitment of diverse gene cassettes during assemblage of the 2,4-D catabolic pathway. *FEMS Microbiol. Ecol.* **28** (1999), 373–382.
- [9] HOFFMANN, D., KLEINSTEUBER, S., MÜLLER, R. H., BABEL, W.: Development and application of PCR primers for the detection of the *tfd* genes in *Delftia acidovorans* P4a involved in the degradation of 2,4-D. *Acta Biotechnol.* **21** (2001), 321–331.
- [10] HORVATH, M., DITZELMÜLLER, M., LOIDL, M., STREICHSBIER, F.: Isolation and characterisation of a 2-(2,4-dichlorophenoxy)propionic acid-degrading soil bacterium. *Appl. Microbiol. Biotechnol.* **33** (1990), 213–216.
- [11] ZIPPER, C., NICKEL, K., ANGST, W., KOHLER, H.-P. E.: Complete microbial degradation of both enantiomers of the chiral herbicide mecoprop [(*RS*)-2-(4-chloro-2-methylphenoxy)propionic acid] in an enantioselective manner by *Sphingomonas herbicidovorans* sp. nov. *Appl. Environ. Microbiol.* **62** (1996), 4318–4322.

- [12] EHRIG, A., MÜLLER, R. H., BABEL, W.: Isolation of phenoxy herbicide-degrading *Rhodospirillum rubrum* species from contaminated building material. *Acta Biotechnol.* **17** (1997), 351–356.
- [13] MÜLLER, R. H., JORKS, S., KLEINSTEUBER, S., BABEL, W.: *Comamonas acidovorans* MC1: a new isolate capable of degrading the chiral herbicides dichlorprop and mecoprop and the herbicides 2,4-D and MCPA. *Microbiol. Res.* **154** (1999), 241–246.
- [14] NICKEL, K., SUTER, M. J. F., KOHLER, H.-P. E.: Involvement of two α -ketoglutarate-dependent dioxygenases in enantioselective degradation of (*R*)- and (*S*)-mecoprop by *Sphingomonas herbicidovorans* MH. *J. Bacteriol.* **179** (1997), 674–6679.
- [15] MÜLLER, R. H., BABEL, W.: Separation of two dichlorprop/ α -ketoglutarate dioxygenases with enantiospecific properties from *Comamonas acidovorans* MC1. *Acta Biotechnol.* **19** (1999), 349–355.
- [16] MÜLLER, R. H., KLEINSTEUBER, S., BABEL, W.: Physiological and genetic characterisation of two bacterial strains exhibiting phenoxyacetate and phenoxypropionate-utilizing activity. *Microbiol. Res.* **156** (2001), 121–131.
- [17] ZIPPER, C., BUNK, M., ZEHNDER, A. J. B., KOHLER, H.-P. E.: Enantioselective uptake and degradation of the chiral herbicide dichlorprop [(*RS*)-2-(2,4-dichlorophenoxy)propanoic acid] by *Sphingomonas herbicidovorans* MH. *J. Bacteriol.* **180** (1998), 3368–3374.
- [18] TETT, V. A., WILLETS, A. J., LAPPIN-SCOTT, H. M.: Enantioselective degradation of the herbicide mecoprop [2-(2-methyl-4-chlorophenoxy) propionic acid] by mixed and pure cultures. *FEMS Microbiol. Ecol.* **14** (1994), 191–200.
- [19] OH, K. H., TUOVINEN, O. H.: Degradation of 2,4-dichlorophenoxyacetic acid by mixed cultures of bacteria. *J. Ind. Microbiol.* **6** (1990), 275–278.
- [20] CHIOU, Y. M., QUE, L.: Models of α -keto acid-dependent non-heme iron enzymes: structure and reactivity of $[\text{Fe}^{\text{II}}(\text{L})(\text{O}_2\text{CCOPh})](\text{ClO}_4)$ complexes. *J. Am. Chem. Soc.* **117** (1995), 3999–4013.
- [21] QUE, L., HO, R. Y. N.: Dioxygen activation by enzymes with mononuclear non-heme iron active sites. *Chem. Rev.* **96** (1996), 2607–2624.
- [22] WHITLING, A. K., QUE, L., SAARI, R., HAUSINGER, P. R., FREDRICK, M. A., MCCracken, J.: Metal coordination of a Cu(II)-substituted α -keto acid-dependent dioxygenase that degrades the herbicide 2,4-D. *J. Am. Chem. Soc.* **119** (1997), 3413–3414.
- [23] HOGAN, D. A., SMITH, S. R., SAARI, E. A., MCCracken, J., HAUSINGER, R. P.: Site-directed mutagenesis of 2,4-dichlorophenoxyacetic acid/ α -ketoglutarate dioxygenase. *J. Biol. Chem.* **17** (2000), 12400–12409.
- [24] SMEJKAL, C. W., VALLAEYS, T., SEYMOUR, F. A., BURTON, S. K., LAPPIN-SCOTT, H. M.: Characterization of (*R/S*)-mecoprop [2-(2-methyl-4-chlorophenoxy)propionic acid]-degrading *Alcaligenes* sp. CS1 and *Ralstonia* sp. CS2 isolated from agricultural soils. *Environ. Microbiol.* **3** (2001), 288–293.
- [25] WESTENDORF, A., BENNDORF, D., MÜLLER, R. H., BABEL, W.: The two enantiospecific dichlorprop/ α -ketoglutarate-dioxygenases from *Delftia acidovorans* MC1 – protein and sequence data of RdpA and SdpA. *Microbiol. Res.* **157** (2002), in press.