Biodegradation (2006) 17: 263–273
DOI 10.1007/s10532-005-6894-8

2,4-Dichlorophenoxyacetic acid (2,4-D) utilization by *Delftia acidovorans* MC1 at alkaline pH and in the presence of dichlorprop is improved by introduction of the *tfdK* gene

Doreen Hoffmann & Roland H. Müller*
*Department for Environmental Microbiology, UFZ Centre for Environmental Research, Permoserstr. 15, D-04318, Leipzig, Germany (*author for correspondence: e-mail: rmueller@umb.ufz.de)*

Accepted 4 May 2005

Key words: Delftia acidovorans MC1, 2,4-dichlorophenoxyacetic acid (2,4-D), 2-(2,4-dichlorophenoxy) propanoic acid (2,4-DP), effect of tfdK gene, simultaneous utilization of 2,4-D and 2,4-DP, uptake characteristics

Abstract

Growth of *Delftia acidovorans* MC1 on 2,4-dichlorophenoxyacetic acid (2,4-D) and on racemic 2-(2,4-dichlorophenoxy)propanoic acid ((*RS*)-2,4-DP) was studied in the perspective of an extension of the strain's degradation capacity at alkaline pH. At pH 6.8 the strain grew on 2,4-D at a maximum rate (μ_{max}) of 0.158 h^{-1}. The half-maximum rate-associated substrate concentration (K_s) was 45 μM. At pH 8.5 μ_{max} was only 0.05 h^{-1} and the substrate affinity was mucher lower than at pH 6.8. The initial attack of 2,4-D was not the limiting step at pH 8.5 as was seen from high dioxygenase activity in cells grown at this pH. High stationary 2,4-D concentrations and the fact that μ_{max} with dichlorprop was around 0.2 h^{-1} at both pHs rather pointed at limited 2,4-D uptake at pH 8.5. Introduction of *tfdK* from *D. acidovorans* P4a by conjugation, coding for a 2,4-D-specific transporter resulted in improved growth on 2,4-D at pH 8.5 with μ_{max} of 0.147 h^{-1} and K_s of 267 μM. Experiments with labeled substrates showed significantly enhanced 2,4-D uptake by the transconjugant TK62. This is taken as an indication of expression of the *tfdK* gene and proper function of the transporter. The uncoupler carbonylcyanide *m*-chlorophenylhydrazone (CCCP) reduced the influx of 2,4-D. At a concentration of 195 μM 2,4-D, the effect amounted to 90% and 50%, respectively, with TK62 and MC1. Cloning of *tfdK* also improved the utilization of 2,4-D in the presence of (*RS*)−2,4-DP. Simultaneous and almost complete degradation of both compounds occurred in TK62 up to $D = 0.23\ h^{-1}$ at pH 6.8 and up to $D = 0.2\ h^{-1}$ at pH 8.5. In contrast, MC1 left 2,4-D largely unutilized even at low dilution rates when growing on herbicide mixtures at pH 8.5.

Introduction

Phenoxyalkanoate herbicides are easily degraded by microorganisms that are capable of cleaving the ether bond as the initial reaction. This was most extensively studied with 2,4-dichlorophenoxyacetate (2,4-D) where this catalytic step was ascribed to the 2,4-D/α-ketoglutarate dioxygenase (TfdA). This enzyme was shown to effectively cleave phenoxyacetate herbicides (Fukumori & Hausinger 1993 a, b; Pieper et al. 1988; Saari et al. 1999). Knowledge on the substrate range was extended by studies on the degradation of racemic phenoxypropionate herbicides, carried out e.g. by *Alcaligenes denitrificans* (Tett et al. 1994), *Sphingobium* (*Sphingomonas*) *herbicidovorans* MH (Nickel et al. 1997; Zipper et al. 1996, 1998), *Rhodoferax* sp. P230 (Ehrig et al. 1997), *Delftia acidovorans* MC1 (Müller et al. 1999a, 2001) and *Stenotrophomonas maltophilia* PM (Mai et al.

2001). In *Delftia acidovorans* MC1, two enzymes which differed from TfdA were detected. They catalyze in a stereospecific manner the cleavage of the *R*- and *S*-enantiomers of 2-(2,4-dichlorophenoxy)propionate [(*RS*)-2,4-DP; dichlorprop] and 2-(4-chloro-2-methylphenoxy)propionate [(*RS*)-MCPP; mecoprop]. These enzymes are designated as *(R)*-phenoxypropionate/α-ketoglutarate-dioxygenase (RdpA) and *(S)*-phenoxypropionate/α-ketoglutarate-dioxygenase (SdpA), respectively (Westendorf et al. 2002, 2003). Strain MC1 was also shown to utilize 2,4-D and 4-chloro-2-methylphenoxyacetate (MCPA). Cleavage of the latter compounds is predominantly catalyzed by SdpA, whereas RdpA contributed with less activity. Because of its broad substrate spectrum, strain MC1 promised to be applicable for the simultaneous degradation of mixtures of herbicides that commonly occur in polluted sites. However, when we studied herbicide degradation under alkaline conditions typical for contaminated building rubble of former production sites (Müller et al. 1999b, 2000), we observed a relatively low utilization rates of 2,4-D which seemed to be discrepant to *in vitro* activity for 2,4-D cleavage. In contrast, utilization rates of racemic phenoxypropionate herbicides were high regardless of the pH.

We suspect that the limited use of 2,4-D under alkaline conditions was due to restrictions in the uptake of this compound. The deficit in the transport of this compound across the cell membrane might result from the lack of a specific carrier protein. The role of specific carrier proteins for the uptake of phenoxyalkanoate herbicides was shown in various strains. With *Ralstonia eutropha* JMP134(pJP4), 2,4-D uptake could be ascribed to a distinct protein, called TfdK (Leveau et al. 1998). The induction of a specific protein, involved in the uptake of 2,4-D was reported with *S. herbicidovorans* MH (Zipper et al. 1998). The latter strain carries moreover proteins with enantiospecific properties that are involved in the uptake of the enantiomers of dichlorprop and mecoprop.

The goal of this study is to elucidate the impact of uptake on 2,4-D utilization by *D. acidovorans* MC1. This was achieved by comparing the kinetics of herbicide consumption by strain MC1 with those of the derivative strain TK62 that was equipped with *tfdK*. Growth studies with strain MC1 and TK62 were performed on 2,4-D and on mixtures of 2,4-D and (*RS*)-2,4-DP as the substrate(s). The effect of TfdK was tested in uptake studies using labeled 2,4-D.

Materials and Methods

Bacteria

Investigations were performed with *D. acidovorans* MC1 (Ehrig et al. 1997). Transconjugant strain *D. acidovorans* MC1-*tfdK* (TK62) was obtained by conjugating *tfdK* from *D. acidovorans* P4a (Hoffmann et al. 2003) into strain MC1.

Cloning of the tfdK gene

A DNA-fragment (2 kb) of strain P4a carrying the *tfdK* gene (1.4 kb) and comprising the flanking regions inclusively the putative promoter region was amplified by PCR using genomic DNA as template and primers *tfdK*1f 5′-TGA-TCC-AGA-GGT-TGC-CCA-CG-3′ and *tfdK*2r 5′-GAT-CTG-GAC-GTG-CAC-ATC-ATC-G-3′ derived from the DNA sequence described by Hoffmann et al. (2003). The PCR reaction was performed with Pfu polymerase (Promega), PCR conditions were 2 min at 95 °C followed by 35 cycles of 45 s at 95 °C, 30 s at 62 °C and 4 min at 72 °C and a final extension step of 5 min at 72 °C (Mastercycler 5330, Eppendorf). The *tfdK* PCR product was cloned into the *SmaI* site of the suicide plasmid vector pAG408 (Suarez et al. 1997) and transformed into *E. coli* S17-1 (λ-pir) *via* electroporation (Easyject Plus Elektroporator, Peqlab Biotechnologie GmbH, Erlangen) according to the instruction manual. The recombinant plasmid was transferred from *E. coli* S17-1 (λ-pir) into *D. acidovorans* MC1 by conjugation. As a result, the miniTn5 cassette of pAG408 carrying the kanamycin and gentamycin resistence genes, the promotorless *gfp* gene and the cloned *tfdK* fragment of strain P4a within the *SmaI* site integrated into the genome of *D. acidovorans* MC1. Transconjugants were screened on mineral agar plates pH 8.5 with 0.5 g/l 2,4-D, 30 μg/ml gentamycin and 50 μg/ml kanamycin; the function of the green fluorencent protein was ignored. Clones which were characterized by strong colony growth were tested for 2,4-D degradation in liquid cultures. The degradation rates were determined in an assay with 0.1 g/l 2,4-D in minimal medium pH 8.5 and

compared to the wild type strain which served as a control. Clones selected due to improved degradative activity were tested for the *tfdK* gene by PCR using the above primers. The expression of the *tfdK* gene was not followed up on a molecular level.

Cultivation

Strain MC1 was grown and stored at pH 8.5 on agar plates containing mineral salts solution (MSS), trace elements solution (TES), 1.5% agar and 400 mg/l of (*RS*)-2-(2,4-dichlorophenoxy)propanoic acid [(*RS*)-2,4-DP] as the sole carbon and energy source. MSS was composed of (in mg/l): NH_4Cl, 760; KH_2PO_4, 340; K_2HPO_4, 485; $CaCl_2 \cdot 6H_2O$, 27; $MgSO_4 \cdot 7H_2O$, 71.2; TES was composed of (in mg/l): $FeSO_4 \cdot 7H_2O$, 4.98; $CuSO_4 \cdot 5H_2O$, 0.785; $MnSO_4 \cdot 4H_2O$, 0.81; $ZnSO_4 \cdot 7H_2O$, 0.44; $Na_2MoO_4 \cdot 2H_2O$, 0.25. Colonies from agar plates were used to inoculate shaking flasks with PYE medium at pH 8.5 containing (in g/l): peptone, 3.0; yeast extract, 3.0; and fructose, 1.8. The culture was grown overnight on a rotary shaker (200 rpm) at 30 °C. This culture was used to inoculate a fermenter with one liter working volume containing MSS and TES; the pH was 7.5 during pre-cultivation. The fermenter was aerated and operated discontinuously for 12 h at 30 °C in the presence of 0.1 g/l (*RS*)-2,4-DP. Continuous cultivation was performed by feeding MSS containing sodium pyruvate, (*RS*)-2,4-DP or 2,4-D respectively at a concentration of 9 mM, or mixtures of (*RS*)-2,4-DP and 2,4-D as specified in the descriptions of the individual experiments. The pH value was adjusted to and held constant at 6.8 or 8.5. A stock of TES (pH 2.0) was fed separately to the fermenter at a rate adjusted to give the final concentration as indicated above.

In the case of chemostat experiments, cultures were harvested from the fermenter after at least five volume exchanges had occurred, i.e. when steady state was reached. In order to determine the exact stationary substrate concentration at the time of sampling, the biomass was immediately separated from the culture broth by filtration on 0.2 μm membrane filters using under-pressure (Müller et al. 1995a). In order to determine the substrate consumption characteristics and maximum growth rates on the various herbicides, transient state cultivation with dilution rate gradients was performed as outlined elsewhere (Müller et al. 1995b). This allows derivation of the desired parameters in an effective way. Starting from steady state cultures, the dilution rate D was steadily increased by increments ΔD at time intervals Δt as given in the results. Maximum growth rate was derived from the accumulation characteristic of residual substrate; details are indicated in the individual results. In order to determine substrate consumption in herbicide mixtures, transient state experiments with substrate gradients were performed in some cases. The culture was in this case pre-grown on 6 mM (*RS*)-2,4-DP. After reaching steady state at the respective dilution rate, 2,4-D was fed *via* a linear gradient (cf. Müller et al. 1985) in addition to (*RS*)-2,4-DP. The 2,4-D concentration extended from 0 to 6 mM in two reservoir flasks which had a total volume of 6.8 l. The experiments were performed at dilution rates in a range of 0.06 – 0.17 h^{-1}.

Analyses

2,4-D and (*RS*)-2,4-DP concentrations were determined by HPLC on a Nucleosil 100 reverse phase column according to Oh & Tuovinen (1990), the enantiomers were selectively gathered on a Nucleodex-α-PM column with permethylated α-dextrin as the chiral stationary phase (Macherey-Nagel, Düren, FRG). Biomass concentration was measured as optical density at 700 nm and as dry mass after drying to weight constancy at 105 °C.

Enzyme assay

Cells were disintegrated by a Dyno mill (Retsch, Haan, FRG) operated for 20 min at 4 °C at maximum frequency using glass beads (150–212 microns, Sigma). The density of the suspension was about 15 g dry mass per litre. Crude cell-free extracts were obtained by centrifugation at 10,000 × g for 20 min at 4 °C.

Rdpa- and SdpA-activity was assayed colorimetrically after condensation of the phenolic products of the cleavage reaction with aminoantipyrine. The standard assay contained (1 ml final volume) 200 μM Fe(II)SO_4, 200 μM ascorbate, 1 mM α-ketoglutarate, and 1 mM of the respective phenoxyalkanoate in 10 mM imidazol buffer pH 6 (the buffer was gassed with air before use). The reaction mixture was treated in a thermomixer

Comfort (Eppendorf) by shaking at 950 rpm at optimum temperature of the respective enzymes, i.e. 30 °C (RdpA) and 25 °C (SdpA), respectively. Reaction was started by adding the enzyme and stopped by adding 1 mM EDTA. Reaction was followed over a period of up to 2 min, and four samples were sequentially taken during this time.

Product formation was assayed by adding 100 μl borate buffer (3.09 g borate, 3.73 g KCl, 44 ml 1 N NaOH per litre), 10 μl 2% 4-aminoantipyrine (w/v) and 10 μl 8% potassium hexacyanoferrat (III) (w/v) to 1 ml samples. After 5 min shaking at 30 °C (950 rpm), the absorbance at 510 nm was measured. The extinction coefficients of 14.2 $M^{-1}\,cm^{-1}$ and 11.2 $M^{-1}\,cm^{-1}$ for the 4-aminoantipyrine adducts of 2,4-DCP and 2,4-MCP, respectively were derived from calibration curves. For deriving the cleavage rates, the data sets of the individual progress curves were treated by linear regression analysis.

Substrate uptake experiments

Biomass was obtained from fermenter effluent. Suspension was collected on ice, centrifuged, washed and resuspended as a concentrate in MSS/TES at pH 6.8 or MSS/TES buffered with 100 mM sodium carbonate/bicarbonate at pH 7.5 or 8.5. Uptake experiments were started immediately after biomass preparation.

Uniform-labeled ^{14}C-2,4-D (5.17 mCi/mmol) was purchased from Sigma. Stock solution was prepared and diluted in 0.05 N NaOH. Nine hundred ninety μl of cell suspension corresponding to about 0.2 mg dry mass was incubated at 30 °C in open tubes and shaken for 3 min. The experiment was started by adding 10 μl of ^{14}C-2,4-D solution. Samples were taken at intervals (of usually 10 s) and the cells collected by immediate filtration through cellulose nitrate filters (25 mm, pore width 0.2 μM; Schleicher and Schüll, Dassel, FRG), pre-wetted with the respective buffer containing 100 μM non-labeled 2,4-D. The filters were flushed three times with 1 ml quantities of the respective buffer, containing 100 μM non-labeled 2,4-D and transferred into scintillation vials containing 6 ml of Filter Count solution (Perkin Elmer, Boston, USA). After the filters had dissolved, radioactive counts (decays per minute, dpm) were determined over 10 min in a Packard TRI CARB Liquid Scintillation Analyzer (2300 TR). Uptake rates were derived from linear regression of data sets of the progress curves; the R^2 values ranged in general between 0.85 and 0.95. Rates of duplicate runs were averaged in general to calculate the uptake rates. The uncoupler carbonylcyanide *m*-chlorophenylhydrazone (CCCP) was used at a concentration of 50 μM added to the cell suspensions 1 min before the addition of the ^{14}C-2,4-D solution.

Results

Strains MC1 and TK62 were continuously grown at pH 6.8 or 8.5. The kinetic parameters obtained at steady state are shown in Table 1. According to these data, growth of MC1 on 2,4-D at pH 8.5 was considerably slower than at pH 6.8 in that steady state was only reached up to a dilution rate D of 0.045 h^{-1}. Chemostat cultivation at pH 8.5 was accompanied by high stationary substrate concentrations that amounted to 0.65 mM at $D = 0.025\ h^{-1}$ and 1.6 mM at $D = 0.033\ h^{-1}$, continuous growth was difficult to stabilize at higher D. This indicated a low affinity for 2,4-D at pH 8.5. At pH 6.8, growth on 2,4-D was improved, the K_s was 45 μM and μ_{max} 0.16 h^{-1} (Table 1). Maximum growth with the enantiomers of 2,4-DP at pH 8.5 was significantly higher and similar at both pH values. No attempts were made to exactly determine K_s with this substrate as the residual substrate concentration was below or around the detection limit of 10 μM over a wide range of dilution rates. Rather, the μ_{max} values were roughly estimated by transient state techniques as indicated in the method section. The figures derived in this way showed, that (*RS*)-2,4-DP utilization proceeded effectively in this strain on either of both pH values. In principle, the racemic substrate can be considered as a mixed substrate and the consumption characteristics of the individual enanatiomers followed separately. Growth with the *R*-enantiomer was faster than with the *S*-enantiomer. At a D of around 0.15 h^{-1}, *(S)*-2,4-DP started to significantly accumulate over a low and almost constant level obtained up to this dilution rate. This was indicative of approaching the maximum consumption rate on this enantiomer. From the accumulation characteristic of *(S)*-2,4-DP at transient state regime, a μ_{max} of 0.17 h^{-1} was derived by extrapolating the linear part of the

Table 1. Growth kinetic parameters of *Delftia acidovorans* MC1 on phenoxyalkanoate herbicides

Substrate	TfdK	pH 6.8		pH 8.5	
		μ_{max} (h^{-1})	K_s (μM)	μ_{max} (h^{-1})	K_s (μM)
2,4-D	–	0.158	45.2	0.045[a]	n.a.
	+	0.176	9.5	0.147	267.0
(S)−2,4-DP [b]	–	0.17 [c]	n.d.	0.17 [c]	n.d.
(R)-2,4-DP [b]	–	> 0.19 [c,d]	n.d.	0.21 [c,e]	n.d.

[a]From Müller et al. 2001; unstable growth at higher dilution rates.
[b]The enantiomers were considered and analytically followed as individual substrates after application of the racemate as the growth substrate (see text).
[c]Steady state up to $D = 0.15\ h^{-1}$ during growth on the racemic substrate; reference to the maximum growth rate on *(S)*-2,4-DP and *(R)*-2,4-DP during growth on the racemate were derived from the accumulation characteristics of the individual enantiomers that occurred during transient state cultivation when applying a dilution rate gradient with a ΔD of 0.05 h^{-1} at time intervals Δt of 2 h in a range of $D = 0.15 - 0.19\ h^{-1}$.
[d]The experiment was stopped at $D = 0.19\ h^{-1}$; the concentration of *(R)*-2,4-DP did not accumulate essentially but remained low at 0.085 mM.
[e]Growth of the culture on (*RS*)-2,4-DP at steady state at $D = 0.19\ h^{-1}$; shift to $D = 0.23\ h^{-1}$ and derivation of μ_{max} from wash-out characteristic of biomass (Esener et al. 1981).
n.a., not applicable due to too few steady state values.
n.d., not determined with transient state technique during consumption of the racemate.

accumulation characteristic to the abscise (per definition: the tangent through the inflection point; Müller et al. 1995b). In contrast, the *(R)*-2,4-DP concentration remained at a low level and behaved almost unchanged up to $D = 0.19\ h^{-1}$. Due to this pattern, the maximum rate on *(R)*-2,4-DP will apparently exceed 0.2 h^{-1}. Exact determination of the respective rates will however require growth on the individual enantiomers which was not intended in the present study.

Looking for potential limiting steps in 2,4-D utilization, we measured the α-ketoglutarate-dependent dioxygenase activity involved in the ether bond cleavage. 2,4-D was cleaved at an average rate of 0.1 ± 0.02 μmol/min mg protein as determined in crude extracts of cultures after growth on either pH value. The external pH applied during growth did not show significant impact on 2,4-D cleavage activity. With *(R)*-2,4-DP and *(S)*-2,4-DP, dioxygenase activities amounted to 0.3–0.44 μmol/min mg protein and 0.08–0.11 μmol/min mg protein, respectively, in cells grown at pH 6.8, and to 0.22–0.27 μmol/min mg protein and 0.12–0.14 μmol/min mg protein, respectively, in cells grown at pH 8.5.

Based on the above results, it appeared likely that transport across the cell membrane did limit the utilization of 2,4-D at pH 8.5. To check on this, 2,4-D uptake was measured (Table 2). The specific uptake rate for 2,4-D was lower by strain MC1 than by *Ralstonia eutropha* JMP134. The latter strain is known as an efficient 2,4-D utilizer and was used for reason of comparison. The rates rose with higher 2,4-D concentrations and dropped at higher pH. Cloning of the gene *tfdK* into MC1 had a pronounced effect on 2,4-D uptake. The specific uptake rates of strain TK62 were above those of MC1 in most cases. At pH 7.5 these equalled those of strain JMP134. The presence of CCCP reduced the substrate uptake by more than 90% in TK62 (Table 2), and by roughly 50% in MC1. This hints at active processes involved in the uptake of 2,4-D.

The presence of *tfdK* improved 2,4-D utilization at pH 8.5. Strain TK62 showed stable growth up to a maximum dilution rate of 0.15 h^{-1}. In accompany, the affinity of the cell for 2,4-D was increased as indicated by the K_s value of 267 μM at pH 8.5 (Table 1). This is in clear contrast to a stationary concentration of 1.6 mM that was obtained in MC1 at dilution rates as low as 0.033 h^{-1}. At pH 6.8, the presence of *tfdK* reduced the K_s by a factor of 6. The external pH was of impact on the affinity for 2,4-D even after cloning of *tfdK*: the K_s with TK62 was 30 times higher at pH 8.5 than at pH 6.8 (Table 1).

The growth parameters of MC1 on (*RS*)-2,4-DP suggested effective uptake of both of the enantiomers. The question was, if the expression of putative proteins involved in the uptake of these

Table 2. Uptake rates of 2,4-D with *Delftia acidovorans* MC1, derivative TK62 (MC1 + *tfdK*) thereof, and of strain *Ralstonia eutropha* JMP134

Strain (grown on)	Uptake rate (nmol/min mg protein)								
^{14}C-2,4-D	3.9 μM[a]			39 μM[a]			195 μM[a]		
pH	6.8	7.5	8.5	6.8	7.5	8.5	6.8	7.5	8.5
MC1 (DCPP)	0.69	0.33	0.23	3.6	1.54	0.61	9.4	5.3	2.9
MC1 (2,4-D)	1.8	0.73	0.44	8.3	1.37[b]	1.43	12.0	12.9	3.5
TK62 (2,4-D)	3.3	3.0	0.25	12.9	10.1[b]	4.2	20.9	32.0	18.9
JMP134 (2,4-D)		1.66			10.00			36.1	

[a]Deviations between duplicates were $<15\%$ and $<30\%$, respectively, at 2,4-D concentrations ≤ 39 μM and >39 μM.
[b]In the presence of 50 μM CCCP the rate was about 0.7 nmol/min mg protein.

enantiomers would support the consumption of 2,4-D. Accordingly, both substrates were simultaneously supplied to MC1. A substrate gradient technique was used in this case in order to follow 2,4-D utilization as a function of the substrate ratio. For this, 2,4-D was fed by a linearly increasing concentration gradient to a culture growing at steady state on (*RS*)-2,4-DP. At the end of this gradient, the mixing proportion due to wash-in of 2,4-D into the fermenter amounted to 6 mM (*RS*)-2,4-DP and 4.2 mM 2,4-D. Much 2,4-D remained unutilized during its wash-in as shown in Figure 1 for growth at $D = 0.17$ h^{-1}, it finally reached 3.22 mM. The low affinity of strain MC1 for 2,4-D seemed to retain also under these conditions and inhibited the simultaneous utilization with (*RS*)-2,4-DP at pH 8.5. In contrast to pH 8.5, simultaneous utilization of 2,4-D and (*RS*)-2,4-DP was quite effective at pH 6.8 (Figure 2).

The presence of *tfdK* improved the utilization of 2,4-D provided in a mixture with (*RS*)-2,4-DP. At pH 8.5, both substrates were almost completely utilized by TK62 at dilution rate $D = 0.1$ h^{-1}, the stationary 2,4-D concentration was as low as about 0.1 mM (Figure 3a). To follow the utilization of 2,4-D in dependence of growth rate, a

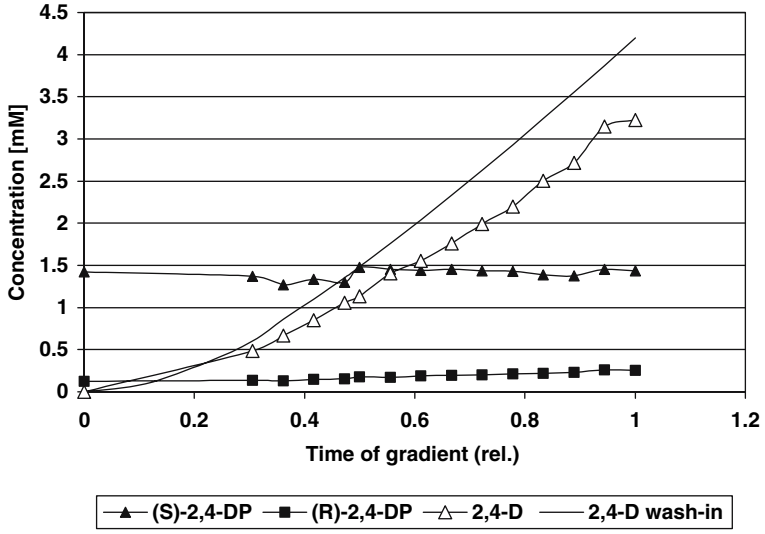

Figure 1. Herbicide consumption characteristics of *D. acidovorans* MC1 after wash-in of 2,4-D to a culture growing continuously on (*RS*)-2,4-DP at pH 8.5. Growth was performed at $D = 0.17$ h^{-1}; wash-in of 2,4-D proceeded by a linear gradient with concentrations of $S_1 = 0$, $S_2 = 6$ mM. The calculation of the actual 2,4-D concentration was performed according to $S(t) = a(t - 1/D) + S_1 + (a/D - S_1)\exp(-Dt)$ with $a = (S_2 - S_1)F/Vg$. S_1 and S_2, initial and final substrate concentration in the gradient; F, feeding rate; Vg, total volume of the gradient system (6.8 l in the present case); *D*, dilution rate.

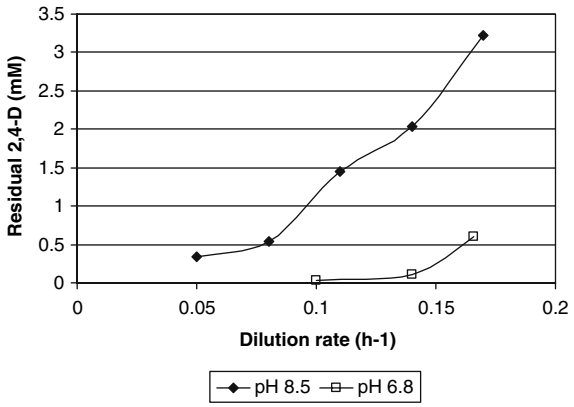

Figure 2. Residual 2,4-D concentration during growth of *D. acidovorans* MC1 on mixtures with (*RS*)-2,4-DP. Growth on the substrate mixture was performed by feeding 2,4-D by a linear gradient to a culture which was at steady state on (*RS*)-2,4-DP (see legend to Figure 1). The figures indicate the residual 2,4-D concentration at the mixing ratio of 6 mM (*RS*)-2,4-DP and 4.2 mM 2,4-D which was finally obtained during substrate feed with the 2,4-D gradient.

transient state technique with step-wise increase of dilution rate was applied in these cases. The residual 2,4-D remained at a low level over a wide range of dilution rate. The effective use of this substrate was documented by the specific rate of 2,4-D consumption which correspondingly increased and amounted to 1.2 mmol 2,4-D (h g dry mass)$^{-1}$ at the highest dilution rate (Figure 3b). Evidently, there was some flattening in the specific consumption rate over a dilution rate. This seemed to follow more or less distinct phases. To emphasize this pattern, the individual date were grouped and indicated by individual slopes. We assume, that this behavior is caused by the participation of two different enzymes in 2,4-D cleavage, i.e. SdpA and RdpA (for details to the enzymes see Westendorf et al. 2002). At a D of about 0.16 h^{-1} the overall capacity of degrading 2,4-D was apparently exhausted (Figure 3b). A brief note is made on the effect which becomes apparent at $D = 0.185$ h^{-1} (Figure 3a) Here, the transient cultivation regime, i.e. the step-wise increase of the dilution rate was interrupted and continuous cultivation pursued up to reaching steady state. This was done for evaluating the experimental procedure with respect to residual substrate concentration (for details to this method see Müller et al. 1995b). Expectedly, this stop resulted in a certain increase of the stationary substrate concentration. This was in an order however to show that the transient-state procedure was appropriate to reveal relevant patterns of substrate consumption.

At pH 6.8, the residual substrate concentration indicated an even more effective use of 2,4-D by TK62 in the presence of (*RS*)-2,4-DP. Up to dilution rates of $D = 0.2$ h^{-1}, the residual substrate concentration remained below 0.08 mM (Figure 4a). This low level of 2,4-D indicated the metabolic capacity and was convincing evidence that simultaneous utilization of both substrates will be proceeded without pronounced competitive effects. This held despite the fact that common degradative enzymes were used for cleaving both 2,4-D and the enantiomers of 2,4-DP. Only at $D > 0.2$ h^{-1} residual 2,4-D steeply increased and reached 1.2 mM at $D = 0.23$ h^{-1}. The latter rate seemed to approximate the maximum capacity of phenoxyalkanoate utilization in this strain as became evident from the accumulation of dichlorophenol.

Discussion

The utilization of the various phenoxyalkanoate herbicides proceeds in general by a similar scheme: the cleavage of the ether bond is followed by the degradation of the liberated phenol moiety *via* the modified ortho cleavage pathway after this compound had been hydroxylated to the corresponding catechol. Specificity in substrate utilization should, for this reason, be exerted by the cleavage step. This is in most cases catalyzed by an α-ketoglutarate-dependent dioxygenase. Indeed, the initial dioxygenases showed more or less pronounced substrate specificity and strong enantioselectivity (Fukumori & Hausinger 1993b; Nickel et al. 1997; Westendorf et al. 2003). A further discriminating step is to be expected in the uptake of these herbicides. This step is above all selective, if an active process is involved.

The consumption of (*RS*)-2,4-DP with MC1 proceeds at high rates with respect to both enantiomers whereas the concentration of residual substrates remained low over a wide range of dilution rates. This is in general in agreement with the catalytic activity of the two dioxygenases, i.e. SdpA and RdpA, which enantioselectively cleave the *S*-enantiomers and the *R*-enantiomers, respectively. Moreover, the affinity of the enzymes

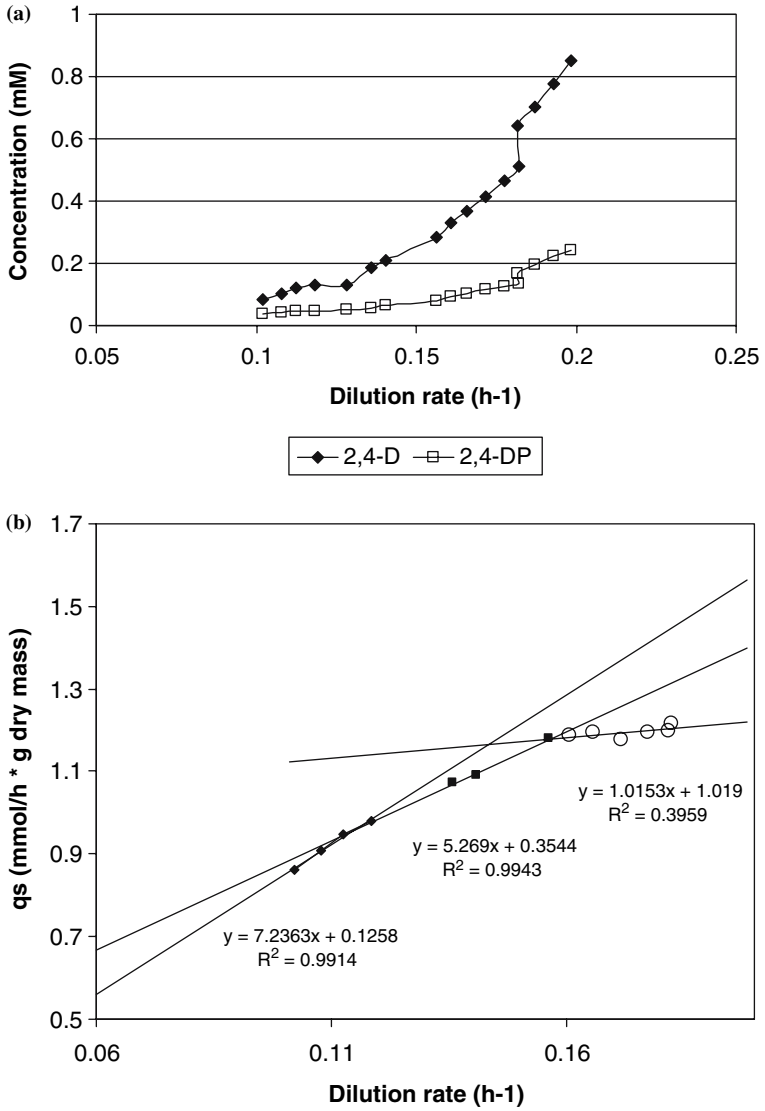

Figure 3. (a) Characteristics of the stationary herbicide concentration during continuous growth of *D. acidovorans* MC1-*tfdK* (TK62) on mixtures of (*RS*)-2,4-DP and 2,4-D at pH 8.5. The strain was continuously cultivated up to steady state on a mixture of 6 mM (*RS*)-2,4-DP and 3 mM 2,4-D. Then the dilution rate was increased by increments of $\Delta D = 0.03\ h^{-1}$ at time intervals $\Delta t = 2$ h. At $D = 0.185\ h^{-1}$ the dilution rate gradient was interrupted and growth proceeded up to steady state; thereafter, stepwise increase in the dilution rate was continued. (b) Specific consumption rates of 2,4-D during growth of strain TK62 on 6 mM (*RS*)-2,4-DP and 3 mM 2,4-D at pH 8.5. The data of linear regression of the various phases of the graph, arbitrarily indicated by different symbols, are included.

is high exhibiting K_M values of 33 μM with *(S)*-2,4-DP (SdpA) and 37 μM with *(R)*-2,4-DP (RdpA). With respect to 2,4-D, the K_M values were 107 μM and 900 μM, respectively, with SdpA and RdpA, (Westendorf et al. 2003; Westendorf 2004). The dioxygenase activity can be correlated to the growth properties. For instance, an activity of 0.1 μmol/min mg protein is sufficient to enable growth rates of about 0.15 h^{-1} taking into account a yield coefficient of 0.044 g/mmol (Müller & Babel 1994) and a protein content of 60%. This figure indicates that the 2,4-D degradation at pH 8.5 was not limited by the dioxygenase activity. The kinetic properties of SdpA and RdpA should

also be responsible for the different phases by which the specific 2,4-D consumption rate was characterized over dilution rate (Figures 3b and 4b). Resolution of this pattern on the level of enzyme kinetics would require however knowledge on the intracellular concentration of 2,4-D which was out of the scope of the present investigation.

Proper kinetic parameters of the dioxygenases are a pre-condition but apparently not sufficient to explain the characteristics of herbicide utilization by strain MC1. It will require moreover effective uptake. This seems to be guaranteed in MC1 with respect to the enantiomers of 2,4-DP which were effectively utilized on either pH tested. The low residual substrate concentrations during chemostat cultivation demand for active uptake. Such favorable parameters were not observed during growth of MC1 on 2,4-D, which showed deficits in utilizing this herbicide at alkaline conditions. As the dioxygenase activities were apparently not responsible for this lack, the deficits for growing on 2,4-D under alkaline conditions were attributed to uptake properties. This was verified by introducing a specific transporter. Cloning of *tfdK* into strain MC1 liberated the transconjugant strain TK62 from the growth restriction. Maximum growth rates on 2,4-D of 0.147 h^{-1} were found with strain TK62 at pH 8.5. This equalled the rates obtained at pH 6.8. We are not aware at present of a plasmidal or chromosomal of integration of *tfdK* gene. It seems to be however a stable trait as loss of this feature was not observed after cultivation on complex medium. Although the expression of the *tfdK* gene was not verified on an mRNA or protein level, the kinetic effects indicate the function and functioning of TfdK in strain TK62. Support came moreover from the experiments with labeled 2,4-D. The accumulation characteristic of 2,4-D revealed a 35-fold increase of the intracellular ^{14}C-label with TK62 after incubation at pH 7.5 for 1 min on 19.5 μM 2,4-D. It should be mentioned that active transport of 2,4-D was also observed with MC1 under these conditions, although to a less extent. The intracellular label increased 15-fold. This indicates that active transport should play some role also in the wild type strain, the effect was apparently not sufficient to effectively support growth at pH 8.5 (see also Table 2).

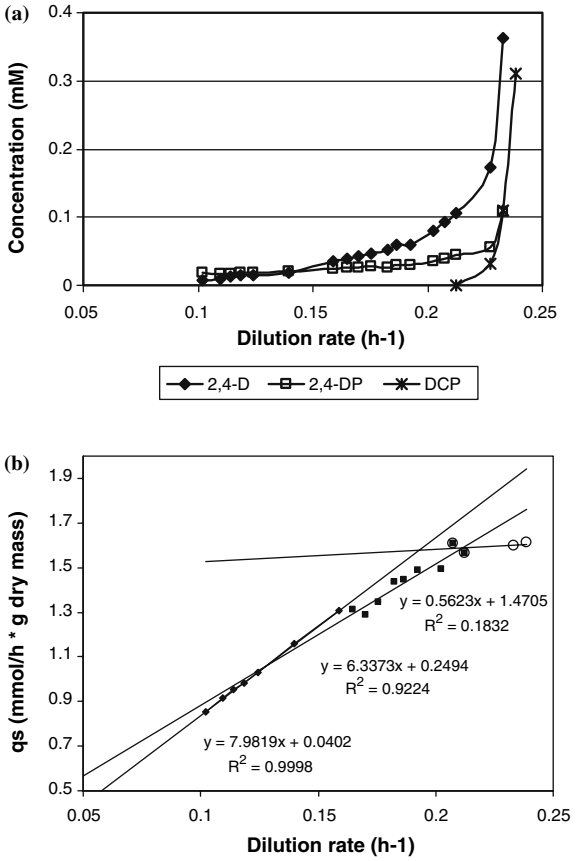

Figure 4. (a). Characteristics of the stationary herbicide concentration during continuous growth of *D. acidovorans* MC1-*tfdK* (TK62) on mixtures of (*RS*)-2,4-DP and 2,4-D at pH 6.8. The strain was continuously cultivated up to steady state on a mixture of 6 mM (*RS*)-2,4-DP and 3 mM 2,4-D. Then the dilution rate was increased by increments of $\Delta D = 0.03\ h^{-1}$ at time intervals $\Delta t = 2$ h. (b) Specific consumption rates of 2,4-D during growth of strain TK62 on 6 mM (*RS*)-2,4-DP and 3 mM 2,4-D at pH 6.8. The data of linear regression of the various phases of the graph, arbitrarily indicated by different symbols, are included.

At present we have no detailed knowledge on potential proteins involve in the uptake of the herbicides by MC1. Recently, sequence analyses of cosmid clones from the MC1 plasmid indicated an open reading frame (ORF) which was located upstream to the *rdpA* gene. This has similarity by 29% to *tfdK* (Schleinitz et al. 2004). The TfdK protein, in turn, is a member of the Major Facilitator Superfamily (MFS) transporter and belongs to the sub-family of aromatic acid:H^+ symporter (AAHS) (Pao et al. 1998). This similarity makes it likely that the protein encoded from this ORF has a function in the uptake of herbicides in MC1.

Although not further substantiated by data, the action of specific transporters in MC1 is postulated with respect to the enantiomers of 2,4-DP. The stationary substrate concentration was as low as the detection limit (<10 μM) at growth rates covering about 60–80% of μ_{max}. As the concentration of free acid at given pH is similar for 2,4-DP (pK_a = 3.1) and 2,4-D (pK_a = 2.9), the extent of uptake by passive diffusion should also be similar. Hence, an active process is mandatory in the case of both enantiomers of 2,4-DP. Whether these putative uptake proteins are as unspecific to carry also 2,4-D or if there is a distinct carrier with specificity for 2,4-D remain open questions. It is known from *S. herbicidovorans* MH, that growth on 2,4-D resulted in the induction of a carrier, that is specific for this substrate and that did not show affinity to any enantiomer of the phenoxypropionate herbicides. In contrast, induction by either of these enantiomers enabled the uptake of 2,4-D in addition to the inducer itself (Zipper et al. 1998). This is indicative for complex enzymatic and regulatory properties with respect to active herbicide transport. The role of TfdA in the uptake of 2,4-D was shown in strain *R. eutropha* JMP134. The effect was limited however, as a *tfdK* knockout mutant showed deficit in the uptake of 2,4-D only at the lower substrate level. The deficit was nearly abolished at the higher substrate concentrations (Leveau et al. 1998). The present data with strain MC1 are not sufficient to reveal details on the specificity and activity of putative transporters of the various phenoxyalkanoate herbicides. This would require more sophisticated investigations with respect to the transport kinetics and to the proteins involved.

The results made in general evident that appropriate catalytic activity is not sufficient for effective degradation of phenoxyalkanoate herbicides but will likewise require effective uptake of the pollutants. The deficit in 2,4-D transport that became evident in strain MC1 was overcome by cloning the *tfdK* gene. The transconjugant strain that resulted from this manipulation was shown to effectively exploit the broad degradative spectrum inherent in this strain. This makes this bug or strains with likely properties a potential tool for application in bioremediation processes. This applies particularly to adverse environmental conditions, e.g. alkaline milieu as was relevant to former production sites.

References

Esener AA, Roels JA, Kossen NWF & Roozenburg JWH (1981) Description of microbial growth behaviour during wash-out phase; determination of the maximum growth rate. J. Appl. Microbiol. Biotechnol. 13: 141–144

Ehrig A, Müller RH & Babel W (1997) Isolation of phenoxy herbicide-degrading *Rhodoferax* species from contaminated building material. Acta Biotechnol. 17: 351–356

Fukumori F & Hausinger RP (1993a) *Alcaligenes eutrophus* JMP 134 "2,4-dichlorophenoxyacetate monooxygenase" is an α-ketoglutarate dependent dioxygenase. J. Bacteriol. 175: 2083–2086

Fukumori F & Hausinger RP (1993b) Purification and characterization of 2,4-dichlorophenoxyacetate/α-ketoglutarate dioxygenase. J. Biol. Chem. 268: 24311–24317

Hoffmann D, Kleinsteuber S, Müller RH & Babel W (2003) Structural organization and localization of the chlorocatechol pathway in the 2,4-D degrading alkalitolerant strain *Delftia acidovorans* P4a. Microbiology 149: 2545–2556

Leveau JHJ, Zehnder AJB & Van der Meer JR (1998) The *tfdK* gene product facilitates uptake of 2,4-dichlorophenoxyacetate by *Ralstonia eutropha* JMP 134(pJP4). J. Bacteriol. 180: 2237–2243

Mai P, Jacobsen SO & Aamand J (2001) Mineralization and cometabolic degradation of phenoxyalkanoic acid herbicides by a pure bacterial culture isolated from an aquifer. Appl. Microbiol. Biotechnol. 56: 486–490

Müller RH, Markuske KD & Babel W (1985) Formate gradients as a means for detecting the maximum carbon conversion efficiency: correlation between formate utilization and biomass increase. Biotechnol. Bioeng. 17: 1599–1602

Müller RH & Babel W (1994) Phenol and its derivatives as heterotrophic substrates for microbial growth – an energetic comparison. Appl. Microbiol. Biotechnol. 42: 446–451

Müller RH & Babel W (1995a) Determination of Ks-values during growth of *Alcaligenes eutrophus* JMP 134 on phenol, 2,4-dichlorophenoxyacetic acid and fructose. Acta Biotechnol. 15: 347–353

Müller RH, Bley T & Babel W (1995b) Transient state cultivation as a means for determining maximum growth rates of microorganisms in inhibition kinetics. J. Microbiol. Meth. 22: 209–219

Müller RH, Jorks S, Kleinsteuber S & Babel W (1999a) *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: 241–246

Müller RH, Müller RA, Jahn Y & Babel W (1999b) A biotechnological approach of detoxifying herbicide-contaminated building rubble. In: Alleman BC and Leeson A (eds) Bioremediation of Nitroaromatic and Haloaromatic Compounds, (pp 143–148). Vol 5(7) Battelle Press, Columbus, Richland

Müller RH, Müller RA, Jahn Y & Babel W (2000) Bioremediation of building material contaminated with herbicides. In: Wise DL, Trantolt DJ and Cichon EJ (eds) Remediation of Hazardous Waste Contaminated Soils, (pp 121–131). 2nd edition, Marcel Dekker, New York

Müller RH, Kleinsteuber S & Babel W (2001) Physiological and genetic characteristics of two strains utilizing phenoxypropionate

and phenoxyacetate herbicides. Microbiol. Res. 156: 121–131

Nickel K, Suter MJF & Kohler HPE (1997) Involvement of two α-ketoglutarate-dependent dioxygenases in enantioselective degradation of (*R*)- and (*S*)-mecoprop by *Sphingomonas herbicidovorans* MH. J. Bacteriol. 179: 6674–6679

Oh KH & Tuovinen OH (1990) Degradation of 2,4-dichlorophenoxyacetic acid by mixed cultures of bacteria. J. Ind. Microbiol. 6: 275–278

Pao A, Paulsen IT & Saier MH Jr (1998) Major facilitator superfamily. Microb. Mol. Biol. Rev. 62: 1–14

Pieper DH, Reineke W, Engesser KH & Knackmuss HJ (1988) Metabolism of 2,4-dichlorophenoxyacetic acid, 4-chloro-2-methylphenoxyacetic acid, and 2-methylphenoxyacetic acid by *Alcaligenes eutrophus* JMP 134. Arch. Microbiol. 151: 365–371

Saari ER, Hogan DA & Hausinger RP (1999) Stereospecific degradation of the phenoxypropionate herbicide dichlorprop. J. Mol. Catal. B: Enzymatic–6: 421–428

Schleinitz KM, Kleinsteuber S, Vallaeys T & Babel W (2004) Localization and characterization of two novel genes encoding stereospecific 2(2,4-dichlorophenoxy)propionate cleavage in *Delftia acidovorans* MC1. Appl. Environ. Microbiol. 70: 5357–5365

Suarez A, Güttler A, Strätz M, Staendler LH, Timmis KN & Guzmán CA (1997) Green fluorescent protein-based systems for genetic analysis of bacteria including monocopy application. Gene 196: 69–74

Tett VA, Willetts AJ & Lappin-Scott HM (1994) Enantioselective degradation of the herbicide mecoprop [2-(2-methyl-4-chlorophenoxy)propionic acid] by mixed and pure bacterial cultures. FEMS Microbiol. Ecol. 14: 191–200

Westendorf A, Müller RH & Babel W (2002) Purification and characterization of the enantiospecific dioxygenases from *Delftia acidovorans* MC1 initiating the degradation of phenoxypropionate and phenoxyacetate herbicides. Acta Biotechnol. 23: 3–17

Westendorf A, Müller RH & Babel W (2003) The two enantioselective dichlorprop/α-ketoglutarate-dioxygenases from *Delftia acidovorans* MC1 – protein data of RdpA and SdpA. Microbiol. Res 157: 317–322

Westendorf A, (2004) Enantioselektive Verwertung von Phenoxyalkanoat-Herbiziden durch Bakterien – Biochemische Charakterisierung der Dioxygenasen des initialen Abbau-Schrittes. Dissertation, Univ. Leipzig

Zipper C, Nickel K, Angst W & Kohler HPE (1996) 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: 4318–4322

Zipper C, Bunk M, Zehnder AJB & Kohler HEP (1998) Enantioselective uptake and degradation of the chiral berbicide dichlorprop [(*RS*)-2-(2,4-dichlorophenoxy)propionic acid] by *Sphingomonas herbicidovorans* MH. J. Bacteriol. 180: 3368–3374