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Uptake of the herbicide 2,4-dichlorophenoxyacetate (2,4-D) by *Delftia acidovorans* MC1 - complex kinetic characteristics in dependence of pH and growth substrate

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Abstract

The uptake of the herbicide 2,4-dichlorophenoxyacetate (2,4-D) by the bacterial strain *Delftia acidovorans* MC1 was studied using ^{14}C-labeled compound. Implication of active transport was suggested due to the effect of carbonylcyanide *m*-chlorophenylhydrazone (CCCP) as an uncoupler of the proton motive force, the presence of which reduced the uptake rate by up to 90%. Kinetic characteristics revealed a complex pattern that was strongly affected by the external pH. With 2,4-D-grown cells, the uptake characteristics followed a hyperbolic shape at pH 6.8 showing an intermediary plateau at ca. 20-100 µM 2,4-D. In contrast, the kinetics at pH 7.5 and 8.5 revealed a sigmoidal pattern. The maximum rate was obtained at around 400 µM 2,4-D, and amounted to 15-20 nmol/min*mg protein. Higher substrate concentrations led to inhibition. With cells grown on (RS)-2-(2,4-dichlorophenoxy)propionate [(RS)-2,4-DP], the uptake rate increased to about 65 nmol/min*mg protein which hints at substrate-dependent induction of specific carrier(s). With 2,4-D-grown cells, such a high rate was obtained only after cloning and expression of the *tfdK* gene, which encodes a specific transporter for 2,4-D. The uptake pattern of 2,4-D changed with mutant strains of MC1 that were phenotypically deficient of cleavage activity to ether bond in phenoxyalkanoate herbicides. Apparently, genes coding for proteins in uptake function were in addition deleted. With strains that lacked (R)-2,4-DP cleavage, the plateau disappeared and the kinetics followed a more continuous pattern. Strains that lacked (S)-2,4-DP cleavage showed faint 2,4-D uptake at all. The present picture hints at three proteins that are involved in 2,4-D uptake by active transport. With degradation-negative mutants of strain MC1, the influx of 2,4-D proceeded at a low rate. It was linearly dependent on the 2,4-D concentration corresponding to a micro-molar rate constant of $1.08*10^{-5}$ min^{-1} mg protein^{-1}. The latter hints at 2,4-D influx into the cell by diffusion via cytoplasmic membrane at a low rate.

Key words: 2,4-dichlorophenoxyacetate, microbial degradation, *Delftia acidovorans*, active transport, complex kinetics, pH effect, influence of growth substrate

1. Introduction

Phenoxyalkanoate herbicides are in general degradable but bacterial strains that carry this property show selective characteristics. Strain selected for the capability to utilize phenoxyacetate herbicides like 2,4-dichlorophenoxyacetate (2,4-D) and 4-chloro-2-methylphenoxyacetate (MCPA) are in general restricted to the productive degradation of this spectrum of herbicides. This is mainly attributed to the substrate specificity of the first degradation step, which is typically catalyzed by 2,4-dichlorophenoxyacetate/α-ketoglutarate-dioxygenase (TfdA) [1–3]. Strains that were selected for their capability to utilize racemic phenoxypropionate compounds like (RS)-2-(2,4-dichlorophenoxy)propionate (2,4-DP, dichlorprop) and (RS)-2-(4-chloro-2-methylphenoxy)propionate (MCPP, mecoprop) show in general a broader spectrum of productive degradation that include, in addition, e.g. 2,4-D and MCPA. This capability is attributed to the presence of two α-ketoglutarate-dependent dioxygenases, called RdpA and SdpA, which have enantio- and substrate specific properties [4-6]. After cleavage of the ether bond, the original substrate becomes anonymous as the further metabolism is in general common. This includes hydroxylation of the liberated phenol derivative to the corresponding catechol, e.g. by TfdB, which is then channeled into the general metabolism via the modified ortho cleavage pathway, i.e. by TfdC-F [3, 7]

Besides ether bond cleavage, penetration across the cytoplasmatic membrane has the potential to selectively control herbicide utilization. This was convincingly shown in the case of *Sphingomonas herbicidovorans* MH. During growth under selective conditions on 2,4-D, (R)-2,4-DP and (S)-2,4-DP, respectively, the induction of substrate-specific transporters was indicated as a prerequisite for the uptake of these compounds. Penetration by passive diffusion was ineffective and apparently not stimulated by the presence of catalytic activity for ether bond cleavage in this strain. This follows from the fact that cells grown on non-selective substrates showed only faint accumulation of the radiolabel during incubation with either of these herbicides, despite the fact that catalytic activity for cleaving (S)-2,4-DP and 2,4-D were constitutively expressed [8, 9]. This is in contrast to the experience with *Ralstonia eutropha* JMP134. Uptake of 2,4-D was studied and shown also in this strain to be an energy-dependent process. It was inducible and followed saturation-like pattern up to a concentration of 60 μM. The latter was attributed to the action of a hydrophobic protein, called TfdK, which showed resemblance to transport proteins of the MFS major facilitator superfamily[10]. Knockout of *tfdK* resulted in reduced uptake of 2,4-D in the micromolar concentration range [11]. It was surprising, however, to find that uptake of 2,4-D was almost not impaired with the *tfdK* mutant at a substrate level amounting up to 2 mM. This was attributed by the authors to the penetration of this compound by a passive process being coupled to the catalytic action of TfdA. Complete lack of energy-driven uptake of 2,4-D was ascribed to a strain of *Burkholderia cepacia*, for which the transport of this compound across the cytoplasm membrane was suggested to function solely by diffusion of the non-dissociated acid [12]. However, there was no information given about how this deficit influenced productive degradation in this strain.

We have previously investigated the growth of *D. acidovorans* MC1 on various phenoxyalkanoate herbicides [13]. The results indicated a deficit for 2,4-D utilization under slightly alkaline conditions: dilution rates at chemostat cultivation as low as 0.033 h^{-1} were accompanied by very high stationary 2,4-D concentration, and the maximum

dilution rate which enabled steady state conditions was limited to about 0.05 h^{-1}. At more neutral pH, the restrictions of using 2,4-D were not observed to that extent: the stationary substrate concentration was distinctly lower over a wide range of dilution rates, and the maximum growth rate was three-fold higher at pH 6.8 than at pH 8.5 [13]. The various pH values were not *per se* a cause of these differences. During growth of strain MC1 on (RS)-2,4-DP we did not observe a strong pH effect on substrate consumption characteristics. Any deficit in α-ketoglutarate-dependent dioxygenase activity could also be ruled out. More likely, the lack of 2,4-D utilization is due to deficits in the uptake of this compounds by strain MC1. Preliminary investigations with 2,4-D showed indeed low uptake rates at alkaline pH. Cloning of *tfdK* resulted in a significant rise in 2,4-D influx into the cell. Consequently, the transconjugant strain showed improved growth on 2,4-D at pH 8.5 [13].

The present study was aimed at elucidating the uptake characteristics of 2,4-D by *D. acidovorans* MC1 in a more detail. Studies were carried out with cells grown on 2,4-D, (RS)-2,4-DP or mixtures of both substrates. The impact of the external pH was taken into account. The uptake patterns obtained with strain MC1 were compared to those of the transconjugant strain TK62 equipped with *tfdK*. The investigation was extended to mutant strains that were phenotypically devoid of RdpA and/or SdpA activity. It was speculated that the deletions in the genome of these mutants comprised genes that encode proteins relevant to uptake function. Resolution of this pattern should indicate the contribution of putative uptake proteins and lead to a platform, from which the molecular basis of the uptake process might be targeted in future investigations.

2. Experimentals and Methods

Bacterial strains and cultivation. Cloning the *tfdK* gene from *D. acidovorans* P4a into *D. acidovorans* MC1 was performed as described elsewhere [13].

Strain MC1 and herbicide-positive derivative strains were 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; strain MC1100 was stored on pyruvate agar plates. Components of MSS comprised (in mg/l): NH_4Cl, 760; KH_2PO_4, 340; K_2HPO_4, 485; $CaCl_2$ * 6 H_2O, 27; $MgSO_4$ * 7 H_2O, 71.2; components of TES comprised (in mg/l): $FeSO_4$ * 7 H_2O, 4.98; $CuSO_4$ * 5 H_2O, 0.785; $MnSO_4$ * 4 H_2O, 0.81; $ZnSO_4$ * 7 H_2O, 0.44; Na_2MoO_4 * 2 H_2O, 0.25 (30).

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 Biostat MD fermenter (Braun Biotech International, Melsungen, BRD) operating at a working volume of 0.8 L containing MSS and TES. 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 the various substrates as indicated in Table 1 at a concentration of 9 mM, or mixtures of 6 mM (RS)-2,4-DP and 6 mM 2,4-D. The pH value was held constant at 7.5. 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 (MSS + TES medium). The dilution rate was D = 0.06 h^{-1}, cultures

were harvested from the fermenter after at least 5 volume exchanges had occurred, i.e. when steady state was reached.

Chemicals. [U-^{14}C]2,4-D was purchased from Sigma (5.17 mCi/mmol). The stock solution was prepared in and diluted with 0.05 N NaOH. The uncoupler carbonylcyanide *m*-chlorophenylhydrazone (CCCP) was purchased from Sigma and added at a concentration of 50 µM. 2,4-D was obtained from Merck and (R/S)-2,4-DP from Sigma.

Uptake experiments. Biomass was obtained from the effluent suspension of the fermenter, collected on ice, washed and concentrated in MSS + TES medium at pH 6.8, and in the same medium buffered with 100 mM sodium carbonate/bicarbonate at pH 7.5 and 8.5.

A 990 µl suspension (routinely at about 0.2 mg dry mass/ml) was incubated at 30°C in an open tube with shaking for 2 min. The experiment was started by adding 10 µl of labeled 2,4-D to achieve the desired concentration. Samples were taken at intervals of 10 s and the cells immediately separated from the medium by filtration through cellulose nitrate membranes (25 mm, pore width 0.2 µM; Schleicher and Schüll, Dassel, Germany), pre-wetted with the respective buffer containing 100 µM non-labeled 2,4-D. The membranes were flushed 3 times with 1 ml quantities of the respective buffer, containing 100 µM non-labeled 2,4-D and transferred into scintillation vials. These contained 6 ml of Filter Count solution (Perkin Elmer, Boston, USA). The uncoupler carbonylcyanide *m*-chlorophenylhydrazone (CCCP) was added at a concentration of 50 µM as indicated, and the suspension was pre-incubated in the presence of this compound for 1 min before starting the uptake experiments.

Scintillation. After solubilization of the filters, radioactive counts (depletions per minute, dpm) were determined over 10 min in a Packard TRI CARB Liquid Scintillation Analyzer (2300 TR). Determination was performed as single sets of measurements with the greater series of substrate concentrations or as duplicates. In the latter case, deviations of the individual rates ranged between 5-15%. Uptake rates were determined from linear regression of the data sets.

Analytical methods. Phenoxyalkanoate herbicides were measured by HPLC on a Nucleosil C18 column (Knauer, Berlin, BRD) with 40% acetonitril and 60% 0.132 M sodium phosphate solution pH 2.8 as the mobile phase. Protein was determined according to Bradford using bovine serum albumin as the standard.

3. Results

2,4-D uptake with strains MC1 and TK62 (MC1::*tfdK*). In order to validate the experimental protocol, the uptake characteristics were followed as a function of substrate concentration and pH. Fig 1 shows an example obtained with an external 2,4-D concentration of 19.5 µM; similar patterns were found with the other concentrations tested. The progress curves for 2,4-D accumulation followed a saturation-like shape for strains MC1 and TK62 as determined over a time interval of 440 s (Fig. 1). Initial uptake rates were determined from 4 - 6 samples taken within the first 40-60 s. Linearity of the profile slope during this period was confirmed by confidence intervals, which were in general ≤ 10%; most values were < 5%. The uptake rates were significantly higher for strain TK62 carrying the *tfdK* gene than for strain MC1. The rate was increased by lowering the medium pH. For MC1 (and TK62), the rates were 0.5 (1.3) nmol/min*mg protein at pH 8.5, and 1.3 (3.2) and 5.3 (7.4) nmol/min*mg protein, respectively, at pH 7.5 and pH 6.8.

Radio-labeled material accumulated within both wild type and transconjugant strains, indicating that active transport of 2,4-D across the cell membranes occurred. After incubation for 1 min at 3.9 µM 2,4-D, there was about 20-fold and 70-fold excess of the intracellular over the extracellular concentration, respectively, with strains MC1 and TK62 (Fig. 2). By increasing the extracellular concentration, the quotient decreased and approached a value of 2-3 at the higher 2,4-D levels (i.e., at 1500 µM). We used the specific uptake rate (nmol/min*mg protein) to calculate the intracellular accumulation. This guaranteed a common basis in the various experiments, as leveling off of 2,4-D uptake was not observed in general during the time of measurement (Fig. 1). This short time, moreover, restricted the effect of distributing carbon over consecutive metabolic steps. The total intracellular volume was derived by taking into account protein concentration of 0.27 mg per 10^9 cells, being rods of 1x2 µM. The presence of the uncoupler CCCP lead to a drastic decrease in 2,4-D accumulation. At a concentration of 50 µM, the influx rate was reduced by more than 90% with strain TK62 and by about 50% with strain MC1 (data not shown).

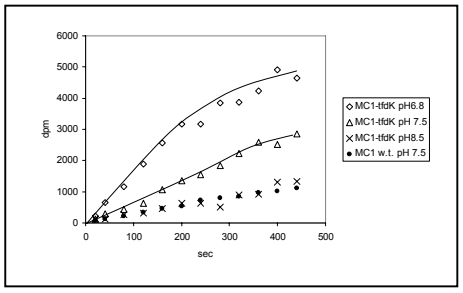

FIG. 1. Uptake characteristics of ^{14}C-2,4-D by *D. acidovorans* MC1 (w.t.) and TK62 (MC1::*tfdK*). The strain was cultivated on 2,4-D at pH 7.5 at a dilution rate of D=0.05 h^{-1}. Incubation was performed in the presence of 19.5 µM 2,4-D. Aliquots of 100 µl were taken at respective times for determining the intracellular content of the radiolabel.

Kinetic properties. The kinetic characteristics of the wild type and the transconjugant strain were determined to identify in greater detail the impact of *tfdK* on 2,4-D uptake. Fig. 3 shows the results after incubation at pH 7.5. The results from several experiments were overlaid in this figure which was aimed at documenting the reliability of the experimental data by using suspension from different samples. It is clear that concentration-dependent 2,4-D uptake followed a saturation-like shape in both cases, which emphasized the active nature of substrate penetration. The presence of *tfdK* led to a significant increase of affinity and rate of accumulation for 2,4-D, the maximum specific uptake rate was 65 nmol/min*mg protein at a 2,4-D concentration of 1-1.5 mM (not shown). To illustrate the effect of *tfdK* in the transconjugant strain, the putative kinetic characteristics of TfdK expressed in strain TK62 was derived from the experimental graphs by subtracting the rate figures of strain MC1 from those of TK62 (Fig. 3).

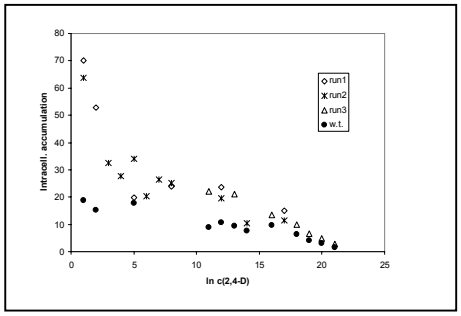

FIG. 2. Accumulation characteristics of ^{14}C-2,4-D by *D. acidovorans* MC1 and TK62. The intracellular concentration of the radiolabel was determined from the specific accumulation rate. In the case of transconjugant strain TK62 (MC1::*tfdK*), the data of different experiments (run 1 to 3) were included.

From the resultant graph we calculated V_{max} = 14.6 nmol/min*mg protein and K_M = 315 µM; an inhibition term was not considered.

The uptake kinetics was determined as a function of external pH. The results for MC1 are shown in Fig. 4. The data reveal significant differences in kinetic patterns at different pH values. The most evident feature at pH 6.8 is the high affinity for 2,4-D uptake. This resulted in 75% of the effective maximum rate at 20 µM 2,4-D. The uptake leveled off at this rate to form a pronounced plateau. A further increase in the rate required 2,4-D concentrations greater than 100 µM. At pH 8.5, in contrast, 2,4-D uptake behaved quite different. At the lower substrate range, the data followed a sigmoidal shape (Fig.4). Uptake was almost insignificant up to 30 µM 2,4-D (Fig. 4, inset). Accordingly, about 10-fold higher concentration of 2,4-D was required under these conditions to reach a rate as high as that obtained at pH 6.8 at the low 2,4-D. Sigmoidal shape was also observed for uptake at pH 7.5 (Fig. 3, Fig. 4 inset). Sigmoidal dependency was confirmed by Hill coefficients of >1 in these cases. The overall kinetic characteristics showed excess substrate inhibition at all pH values tested. This decrease was most pronounced at pH 8.5, where the rate was reduced to 1/3 of the effective maximum at the highest concentration tested.

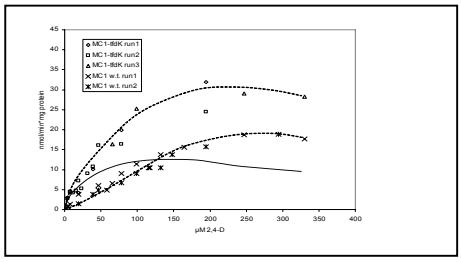

FIG. 3. Kinetics of 2,4-D uptake by *D. acidovorans* MC1 and TK62. The strains were chemostatically grown on 2,4-D at D = 0.05 h^{-1} and pH 7.5. Uptake experiments were performed at pH 7.5.

Influence of the growth substrate on 2,4-D uptake. High affinity and fast utilization of (RS)-2,4-DP was observed during chemostat growth of strain MC1 [13]. This is in contrast to the behavior with 2,4-D alone and suggests effective uptake systems for both of these enantiomers. The uptake of 2,4-D was tested with cells of strain MC1 grown on (RS)-2,4-DP; the results are shown in Fig. 5. The kinetic characteristics of 2,4-D uptake were quite different. At pH 6.8, the rate pattern still followed a hyperbolic shape at the lower substrate level. However, at external 2,4-D concentrations that exceeded 300 µM, the rates drastically increased and corresponded to about 60 nmol/min*mg protein (Fig. 5). This indicates most likely the induction of an additional uptake protein during growth on (RS)-2,4-DP which might be involved in the utilization of this substrate. Interestingly, double reciprocal plots of the rate data obtained at the low substrate range resulted in identical K_M values of 41 µM with 2,4-D- and (RS)-2,4-

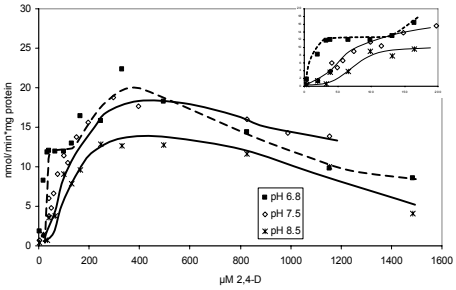

FIG. 4. Kinetics of 2,4-D uptake by *D. acidovorans* MC1 after growth on 2,4-D in dependence of pH. The strain was chemostatically grown on 2,4-D at D = 0.05 h^{-1} and pH 7.5. Uptake experiments were performed at pH values as indicated.

-DP-grown cells. This means that the induced protein did not show significant uptake activity at the lower concentration range. This is most likely explained by a sigmoidal dependency, a property that seems to be a general trait in the uptake kinetics of this strain. At pH 8.5, similarity to 2,4-D-grown only cells was observed; the kinetics followed a sigmoidal shape at lower substrate concentrations (Fig. 5). At higher concentrations, the inhibition term was apparently compensated for by the activity of the putatively induced uptake protein, and remained at a higher level.

Due to the pronounced stimulation of 2,4-D influx into the cell by (RS)-2,4-DP-grown MC1, uptake properties were studied with cells grown chemostatically on equimolar concentrations of both herbicides. The results were surprising and revealed a converse effect (Fig. 6). Clearly evident, the uptake characteristics showed that the highly affine uptake system was functional at pH 6.8 with cells grown under these conditions. However, we did not obtain any rise in the uptake rate by further increasing the 2,4-D concentration as observed for (RS)-2,4-DP-grown only MC1. Instead, a strong reduction in the rate was found that tended to zero. At pH 8.5, this deficit was even more pronounced; we did not observe significant uptake at all up to 1.5 mM 2,4-D (not shown).

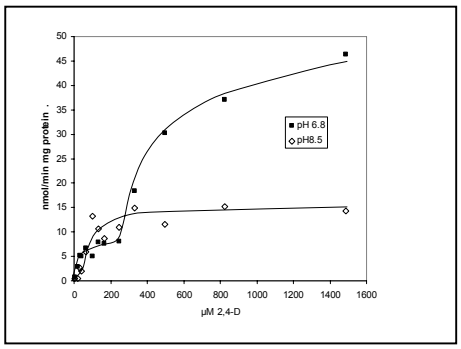

FIG. 5. Kinetics of 2,4-D uptake by *D. acidovorans* MC1 after growth on (RS)-2,4-DP. The strain was chemostatically grown on (RS)-2,4-D at D = 0.05 h^{-1} and pH 7.5. Uptake experiments were performed at pH values as indicated.

Uptake properties with mutant strains deficient in phenoxyalkanoate cleavage. The complex kinetic patterns, and the relative rate changes (Fig. 4-6) were considered an indication that different catalysts contribute to the overall rate of uptake. At present, we have no detailed information on the genes and the respective proteins responsible for this behavior. However, we have isolated mutant strains that were devoid of distinct catalytic activity, i.e. were lacking either *rdpA* and/or *sdpA*. We are not aware at present which further genes were affected by the putative deletions in the genome. The physiological responses observed in this investigation, however, indicate that the deletions extended to genes that encode uptake proteins. Strain MC1010 (RdpA negative) behaved completely different to strain MC1 regarding 2,4-D uptake (Fig. 7). Most remarkably, the complexity of the kinetic characteristics was lost. At pH 6.8, we observed instead continuous kinetic characteristics with pronounced substrate inhibition. The behavior at pH 8.5 was typical, referring to

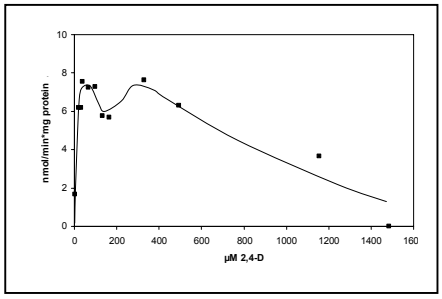

FIG. 6. Kinetics of 2,4-D uptake by *D. acidovorans* MC1 after growth on 2,4-D and 2,4-DP. The strain was chemostatically grown on 2 mM (RS)-2,4-DP and 2 mM 2,4-D at D = 0.05 h^{-1} and pH 7.5. Uptake experiments were performed at pH 6.8.

the pattern of the wild type strain; uptake was sigmoidal (Fig. 7). The activity remained at a very low level over the whole concentration range.

Strain MC1073 (SdpA negative) was grown on (R)-2,4-DP (as growth on 2,4-D is not possible with this deletion mutant), and no significant uptake of 2,4-D occurred over the concentration range of 1.5 mM. With mutant strain MC1100, which is characterized by the loss of a 40 kb fragment of the plasmid and which lacks RdpA and SdpA activity [4, 14], the uptake rates were very low as determined after growth on fructose. Measurement over a period of up to 5 min at pH 7.5, and a substrate range of 39 to 825 µM 2,4-D, revealed a linear dependence of the specific uptake rate q_s on external 2,4-D (µM) concentration with $q_s = 1.08 * 10^{-5} * c_{2,4}$ (min^{-1} * mg $protein^{-1}$). The linear plot hints to a passive, i.e. diffusion-controlled process in this case.

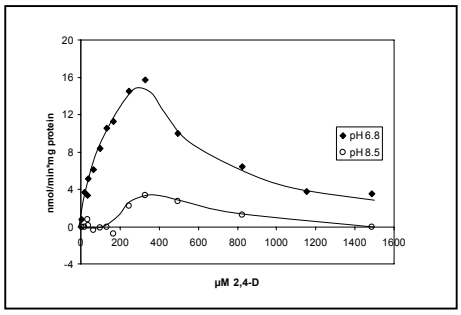

FIG. 7. Kinetics of 2,4-D uptake by *D. acidovorans* MC1010 (RdpA negative) after growth on 2,4-D. The strain was chemostatically grown on 2,4-D at D = 0.05 h^{-1} and pH 7.5. Uptake experiments were performed at pH values as indicated.

4. Discussion

The results indicate that uptake of 2,4-D by strain MC1 is carried out and controlled by active transport. This follows from i) the uptake and accumulation characteristics (Fig. 1, 2) which led to a several-fold increase of the intracellular over the extracellular concentration of 2,4-D, ii) the saturation-like kinetics in distinct ranges of substrate concentration and iii) the finding that the influx of 2,4-D was significantly reduced in the presence of uncouplers like CCCP. Compounds of the latter type are known to elicit trans-membrane proton gradients, and thus, energy generation in general. Active transport might be specified as proton symport in this case, if a similar mechanism is responsible in strain MC1 as was attributed to the uptake of 2,4-D in *R. eutropha* JMP134. In the latter strain, the TfdK protein was shown to be involved in 2,4-D uptake [11]. TfdK is a member of the aromatic acid:proton symporter (AAHS) family of the major facilitator superfamily (MFS) of uptake proteins [10]. The fact that the uptake of 2,4-D was significantly increased in strain TK62, that harbor the *tfdK* gene supports in general the impact of active transport on 2,4-D influx. The results with TK62 moreover indicate that TfdK was effectively expressed in the transconjugant strain of *C. acidovorans* MC1 and arranged in the membrane in a functional manner. As a consequence of expressing *tfdK*, strain MC1 was released from growth restrictions on 2,4-D as was evident at pH 8.5 [13].

The kinetic patterns obtained with the wild type and mutant strains revealed essential details on 2,4-D transport. Evidently, several systems are involved in uptake. This follows from observations that i) the kinetic characteristics are of complex shape, ii) induction and repression phenomena occurred in relation to the various growth substrates, and iii) deletion mutants with degradation deficient phenotypes (RdpA- or SdpA-negative) showed different kinetics. The complex kinetic profiles observed with 2,4-D-grown cells suggests the presence of at least two proteins. One of the transporters has a

high affinity for 2,4-D. It is characterized – as verified by a plateau or trough region - by a pronounced inhibition term (Figs. 4, 6). It was a characteristic feature of deletion mutant MC1010 (RdpA negative), that it was apparently deficient of this type of transporter. This forced us to question if an open reading frame, designated ORF19 [14], which is located just upstream to *rdpA* and which has similarity to an MFS transporter, is the gene encoding protein in question. This remains to be answered by further investigations. Strain MC1010 seems in general to express only one transporter during growth on 2,4-D, which follows from the continuous shape of the kinetic characteristic (Fig. 7). The shape and position of the maximum coincides in this case with a second phase in the activity profile in MC1 after plateau formation (Figs. 4, 6).

The existence of a third uptake mechanism became evident after growth on (RS)-2,4-DP. This is likely to result from induction during growth on this substrate and should be connected to the uptake of one of the enantiomers of 2,4-DP. It showed activity to 2,4-D at the higher substrate level and resulted in a three-fold increase of uptake rate compared to 2,4-D grown only cells. This resembles the pattern observed with *S. herbicidovorans* MH [8, 9]. The expression of two enantiospecific transporters was described with this strain during growth on racemic 2-phenoxypropionate herbicides. These proteins showed activity also to the uptake of 2,4-D. During growth on 2,4-D, in contrast, a protein was induced, that was highly specific for the uptake of 2,4-D and did not support the penetration of either (R)- or (S)-2,4-DP. This is similar to the behavior of strain MC1, from which strong regulation of 2,4-D uptake became evident due to the activity profiles at various growth conditions. When supplied in combination with (RS)-2,4-DP, e.g., the presence of 2,4-D did apparently repress proteins that were involved in the utilization of (RS)-2,4-DP. As these proteins support, in addition, the uptake of 2,4-D, this effect should contribute to deficits in simultaneous herbicide utilization in strain MC1 [13].

The patterns of 2,4-D uptake revealed in general some unusual properties. The shape of the kinetics was drastically changed at pH 7.5 and 8.5 in comparison to pH 6.8. Most evidently, the high affine uptake was no longer functional. Instead, the activity profile followed a sigmoidal shape (Figs. 3, 4, 5, 7). It seems that the various proteins involved in 2,4-D uptake change their characteristics in dependence on the external pH. A likely explanation is that conformational changes of membrane proteins or rearrangements of the membrane structure occurred with changing pH. Conformational changes are common and intrinsic to the function of transport proteins [15, 16]. Excess substrate inhibition is another feature and might contribute to the restricted use of 2,4-D by this strain. Taking into account enzyme inhibition by, e.g., a two-site mechanism as described by Webb (50), the sharp transition to the inhibited state as was observed with MC1 (Fig. 6) is of typical shape with this type of substrate interaction [18, 19]. Whether multi-drug (efflux) transport systems are involved and contribute to the overall accumulation characteristic of 2,4-D - as a further explanation of the uptake characteristics - remains open. Several types of efflux transporters are known [20] to function as a defense mechanism in diverse bacteria [21-23]. Their presence is even considered as a general trait in biological membranes [16]. However, pronounced inhibition effects were not observed in the case of *R. eutropha* JMP134 [11] and *S. herbicidovorans* MH [9], which makes the role of such mechanisms acting on 2,4-D accumulation rather unlikely.

When we consider physiology of substrate uptake from a more integrated perspective, the impact of the overall metabolism has to be taken into account. Accordingly, the influx

of a compound usually results from a coupling of the penetration properties to consecutive metabolism. Irreversible metabolic steps are particularly decisive to channel the compound into general metabolism [24, 25]. With strain MC1, the α-ketoglutarate-dependent dioxygenases, i.e. RdpA and above all SdpA, which initiate the degradation of 2,4-D, can play such a role (for details of the enzyme data see Westendorf et al., [5, 6]). The cleavage reaction depends on the presence of α-ketoglutarate, the supply of which is strongly coupled to the general metabolism and to the physiological state of the population [26, 27]. Thus the kinetics of these enzymes in combination with the kinetics of the uptake proteins will determine 2,4-D utilization rates. This is the essence of the model presented by Leveau et al. [11] on 2,4-D utilization by *R. eutropha* JMP134: uptake in the higher concentration range was assumed by these authors to take place to a main extent by diffusion of the non-dissociated acid. The high rates that were observed with the *tfdK* mutants strain should accordingly result from a coupling of 2,4-D diffusion to its catalytic conversion by TfdA. This interpretation has merits; however, it contradicts the experience made on phenoxyalkanoate uptake with *S. herbicidovorans* MH [9]. The presence of α-ketoglutarate-dependent cleavage activity was not sufficient in this strain to obtain any relevant uptake rate or intracellular accumulation of radio-labeled substrate. This means, that diffusion did not play a significant role in this case. Please note, that the pKa values of 2,4-D and 2,4-DP are 2.6 and 3.0, respectively, i.e. the non-dissociated acid was present at only about 0.01-0.1% at neutral pH. The activity profile exhibits a strong decrease of the uptake rate at increasing 2,4-D concentrations in the case of *D. acidovorans* MC1. This does not support the assumption of a significant passive penetration. It rather indicates a mediator-coupled process with distinct kinetic properties and regulation. The fact that the SdpA-negative mutant strains *D. acidovorans* MC1071 did not show significant 2,4-D uptake may, however, be explained by both, i.e. lack of specific uptake proteins and/or lack of catalytic activity. A final answer will be expected after characterizing and treating the specific uptake proteins in distinct models.

5. Conclusion

The results have shown that the uptake of the herbicide 2,4-dichlorophenoxyacetate (2,4-D) by *C. acidovorans* MC1 proceeds mainly by an active, energy-driven process. Elimination of the trans-membrane proton gradients was accompanied by a drastic reduction of the uptake rate.

Several putative proteins seemed to be involved in the uptake of 2,4-D by this strain. Their simultaneous action resulted in the formation of complex kinetic characteristics. The various uptake proteins showed induction and repression patterns in dependence of the growth substrate.

The external pH exhibited a strong influence of the uptake kinetics. This followed a hyperbolic shape at neutral to slightly acidic pH, and a sigmoidal shape at the more alkaline pH values.

Cells incubated at pH 8.5 did show only faint uptake of 2,4-D. This seems to correlate to the deficit of the strain to effectively grow on this substrate under these conditions. The deficit could be overcome by cloning of a specific uptake protein for 2,4-D which resulted in improved 2,4-D utilization and emphasizes the role of active transport in the uptake of this compound.

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