

Pseudo-recalcitrance of Chlorophenoxyalkanoate Herbicides – Correlation to the Availability of α -Ketoglutarate

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Summary

Cleavage of the ether bond of chlorophenoxyalkanoate herbicides is catalyzed by an α -ketoglutarate-linked dioxygenase (TfdA). In this step, α -ketoglutarate is decarboxylated to succinate and must be regenerated for continual substrate cleavage. Limitations in herbicide degradation are to be expected in the case of a shortage of α -ketoglutarate. Such a situation was simulated and studied with *Delftia* (formerly *Comamonas*) *acidovorans* MC1 and *Rhodospirillum rubrum* sp. P230, which constitutively express etherolytic dioxygenase activity by excreting 2,4-dichlorophenol (DCP) as a dead-end product. The results showed that 2,4-dichlorophenoxyacetate (2,4-D) could hardly be cleaved under these conditions which is attributed to the inability to regenerate α -ketoglutarate from the cleavage products, i.e. succinate and glyoxylate [1]. With pyruvate, in contrast, liberated as the oxidized alkanic acid from the cleavage of (RS)-2-(2,4-dichlorophenoxy)propionate (2,4-DP), the regeneration of α -ketoglutarate seems to be guaranteed from succinate as resulted from the utilization of 2,4-DP to a considerable amount under these conditions. The extent was limited, however, which was apparently caused by the accumulation of DCP. Continual cleavage of 2,4-DP could be demonstrated in the presence of *Ochrobactrum* sp. K2-14, which functions as a DCP-consuming strain. Addition of extra metabolites, i.e. α -ketoglutarate or other readily metabolizable substrates, improved the cleavage of the herbicides. This was most pronounced with 2,4-D that was found now to be also utilized to a considerable extent. Conversely, the cleavage of the herbicides (2,4-DP) was reduced and ultimately ceased with cells depleted by starvation of the pool of metabolites. Again, this deficit could be restored by adding α -ketoglutarate. The limitations in utilizing phenoxyalkanoate herbicides are discussed in terms of pseudo-recalcitrance owing to deficits in metabolites (α -ketoglutarate) rather than enzyme activity (TfdA).

Introduction

The initial step of the degradation of 2,4-D encoded by the gene *tfdA* from *Ralstonia eutropha* JMP134 has been elucidated and shown to follow an α -ketoglutarate-depend-

ent dioxygenase mechanism [2, 3]. As a result of this catalytic step, the phenoxyalkanoates are cleaved into the phenolic derivative and the oxidized alkanic acid, whereas α -ketoglutarate yields succinate and CO_2 . Further metabolism of the chlorophenolic intermediate proceeds via a modified *ortho* cleavage pathway [4, 5]. TfdA-like activity is widespread in nature [6–8]. Moreover, etherolytic activity being catalyzed by an α -ketoglutarate-dependent dioxygenase has been shown to also function with other phenoxyalkanoates, such as the enantiomeric phenoxypropionates. This holds true with *Sphingomonas herbicidovorans* MH [9] or *Delftia acidovorans* MC1 and *Rhodoferrax* sp. P230 [10]. The specificity of the degradation of the different phenoxyalkanoates is based accordingly on the specificity of the enzymes initiating the metabolic route of decomposing the various phenoxyalkanoates. The fact that the cleavage of these herbicides requires α -ketoglutarate (which is oxidatively decarboxylated to succinate in this reaction) necessitates its regeneration. In the case of complete degradation of the ring structure, no limitations should arise in view of the fact that C2 (acetyl-CoA) and C4 (succinate) compounds were derived in the primary assimilatory route besides succinate and the oxidized alkanic acid (glyoxylate, pyruvate) resulting from the etherolytic cleavage reaction. In the case of incomplete degradation, by contrast, in which the phenolic intermediate may form a dead-end product, restrictions concerning continual cleavage are to be expected [1]. Thus despite the expression of TfdA or analogous enzymes, substrate turnover via these enzymes may be limited. Consequently, this property should influence and decide the fate of chlorophenoxy herbicides in biotopes in which, for example, consortia are responsible for the complete degradation of these compounds, i.e., in which the individual enzymatic steps or partial sequences are harboured in different strains.

In this investigation, the ability to attack the phenoxyalkanoate herbicides under such conditions and the limitations resulting from the necessity of regenerating α -ketoglutarate are considered. Such strains were used which exhibit constitutive expression of TfdA-like cleavage activity without further metabolizing the phenolic intermediates. Effects resulting from DCP accumulation were taken into account

Materials and Methods

Strains and Cultivation

The investigations were carried out with *Rhodoferrax* sp. P230, which was isolated from herbicide-contaminated building rubble [11], and *Delftia acidovorans* MC1 obtained from a fixed-bed reactor operated for the degradation of a mixture of chlorophenoxy herbicides [12].

The mineral salt solution (MM) used for growth under minimal conditions contained [in mg/l]: NH_4Cl , 760; KH_2PO_4 , 340; K_2HPO_4 , 485; $\text{CaCl}_2 \times 6 \text{ H}_2\text{O}$, 27; $\text{MgSO}_4 \times 7 \text{ H}_2\text{O}$, 71.2; $\text{FeSO}_4 \times 7 \text{ H}_2\text{O}$, 4.98; $\text{CuSO}_4 \times 5 \text{ H}_2\text{O}$, 0.785; $\text{MnSO}_4 \times 4 \text{ H}_2\text{O}$, 0.81; $\text{ZnSO}_4 \times 7 \text{ H}_2\text{O}$, 0.44; $\text{Na}_2\text{MoO}_4 \times 2 \text{ H}_2\text{O}$, 0.25. Strain MC1 is able to grow prototrophically; in the case of Strain P230, the medium was supplemented with either 0.1 g/l yeast extract or a vitamin mixture containing biotin (3.8 mg/l), thiamine (9 mg/l) and vitamin B12 (0.14 mg/l). For growth on complex carbon source, the medium contained 3 g/l of each peptone and yeast extract and 10 mM fructose (PYE + F) or 1 g/l of each peptone, yeast extract and α -ketoglutarate (PYE + α -KG). The pH values were adjusted to 8.5.

Ochrobactrum sp. K2-114 [13] was grown chemostatically on 2 g/l dichlorophenol at a dilution rate of 0.04 h^{-1} . The cells were harvested, washed and resuspended into MM containing 50% [v/v] glycerol at an $\text{OD}_{700} = 30$ and stored at -20°C . Before use, the cells were centrifuged and washed two times with MM.

Degradation Experiments

The strains were routinely grown as an overnight culture on complex medium. Strain MC1 was inoculated from colonies grown under selective conditions on (*RS*)-2-(2,4-dichlorophenoxy)propionate; Strain P230 was obtained from a sequentially transferred culture grown on PYE+F or PYE+ α -KG. The cells were collected by centrifugation, washed in nitrogen-free MM and routinely re-suspended into nitrogen-free MM to an optical density of OD₇₀₀ = 0.25. Streptomycin was routinely added to the suspensions at a concentration of 50 mg/l if not otherwise indicated. At respective times, samples were withdrawn, the biomass separated by centrifugation and the supernatant used for analysis.

Analyses

The chlorophenoxyalkanoate compounds and the chlorophenols were determined by HPLC using a SHIMADZU chromatograph with diode array detection according to a method described elsewhere [14]. The biomass concentration was determined by using the optical density at 700 nm (OD₇₀₀). An OD₇₀₀ of 1 corresponds to a bacterial dry mass of 0.54 g/l with Strain MC1 and to 0.46 g/l with Strain P230.

Chemicals

(*R*)-2,4-DP was procured from RIEDEL-DEHAEN. (*S*)-2,4-DP was prepared following the method of ZIPPER [15]. (*R*)-2-(4-chloro-2-methylphenoxy)propionate and (*S*)-2-(4-chloro-2-methylphenoxy)propionate (MCP) were synthesized according to MATELL [16]. The other phenoxyalkanoates were obtained from SIGMA with the highest purity available.

Results

Cleavage Pattern

Application of 0.45 mM (*RS*)-2,4-DP to non-induced cells of *Rhodospirillum rubrum* sp. P230 resulted in etherolytic cleavage of this compound the extent being limited. DCP resulting from this reaction behaved as a dead-end product in the presence of streptomycin and was excreted into the medium (Fig. 1). The degradation pattern was completely paralleled by supplying pure (*R*)-2,4-DP which is in accordance with a constitutive expression of *R*-specific cleavage activity: 0.15–0.17 mM of this enantiomer were converted under these conditions leading to the accumulation of approximately equimolar concentrations of DCP (Fig. 1). Applying lower initial substrate concentrations (0.18 mM), again about 0.15 mM of (*R*)-2,4-DP were cleaved, whereas only half of the molar concentration of the racemic substrate disappeared (Fig. 1). This was attributed to the *S*-enantiomer that was not utilized by the non-induced cells of this strain [10]. 2,4-D was also not degraded to a significant extent under these conditions (not shown). The presence of streptomycin did not essentially influence the cleavage pattern of the herbicides as was derived from the data obtained after different periods of pre-incubation with this antibiotic.

In contrast, Strain MC1 shows constitutive activity for the cleavage of both (*R*)- and (*S*)-2,4-DP. The cleavage activity of the *R*-enantiomer was high with cells grown overnight on complex medium. The substrate routinely supplied (0.45 mM) was completely utilized with the given concentration of biomass of 0.1–0.15 g/l. In contrast, some of

the substrate remained unutilized after incubation with the racemic mixture under these conditions (Fig. 2). This was probably caused by the *S*-enantiomer, which was utilized to only a limited extent under the prevailing conditions, and at a lower rate. In general, the extent of cleavage was found to be a function of the biomass concentration with the various herbicides. The graphs of concentration vs. extent, obtained after 24 h of incubation (substrate consumption characteristics almost followed the progress curves as shown in Fig. 2) exhibited approximate linearity (Fig. 3). This means that the specific conversion rate is a constant with the various substrates, i.e., substrate consumption was not dependent on the concentration of DCP accumulated in the time run. 2,4-D, again, was hardly degraded by Strain MC1 under these conditions (Fig. 2). Nevertheless, the disappearance of a very small but distinct amount of this substrate was observed in most experiments. The significance of this property became obvious from the data in Fig. 3 according to which this behaviour became more evident the higher the initial biomass concentration was. Due to these data, 2,4-D was cleaved to an extent of about 3% in comparison to (*R*)-2,4-DP.

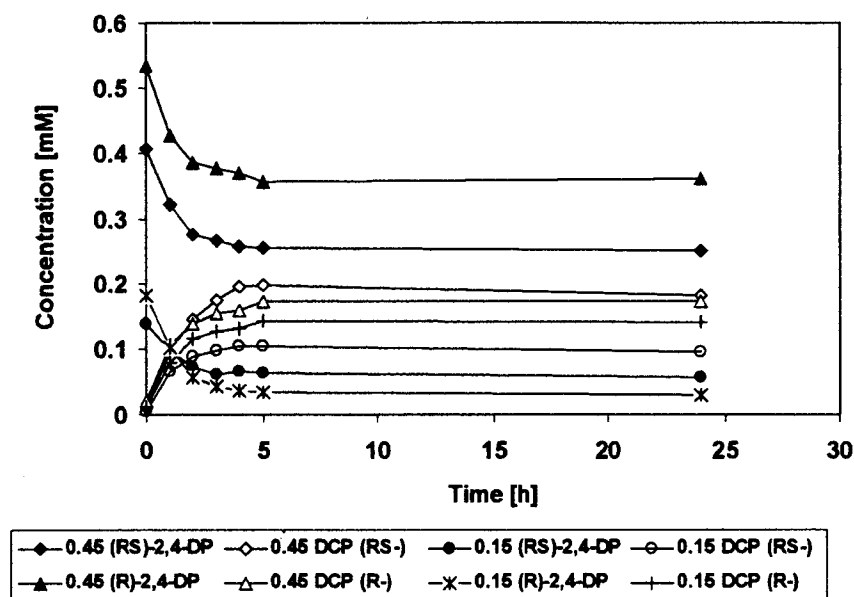


Fig. 1. Degradation of various phenoxyalkanoates by non-induced cells of *Rhodoferrax* sp. P230

The samples contained 0.15 to 0.45 ± 0.04 mM of the respective herbicides (see legend). The references regarding DCP in the legend correspond to the original substrate used.

Influence of α -Ketoglutarate

The data shown above revealed essential differences in the cleavage characteristics of the different herbicides. Whereas 2,4-D remained almost inert by these cultures, the enantiomers of 2,4-DP were utilized to a considerable extent in accordance with the expression profile of the respective etherolytic dioxygenases [10].

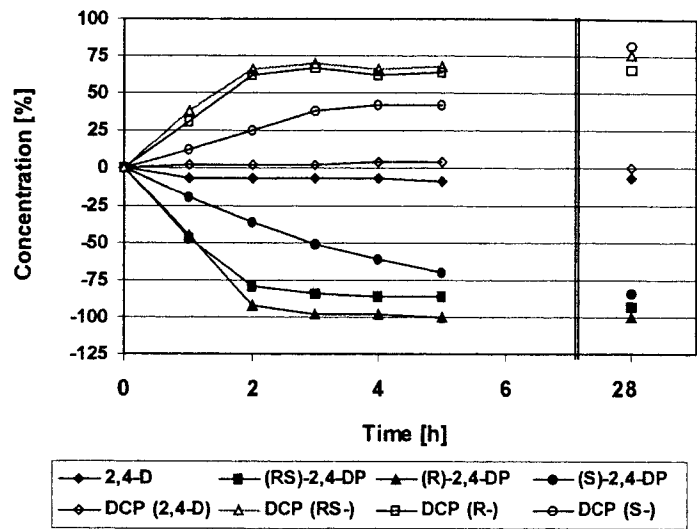


Fig. 2. Degradation of various phenoxyalkanoates by non-induced cells of *Delftia acidovorans* MC1
The samples contained 0.45 ± 0.04 mM of the respective herbicides; the biomass concentration corresponded to $OD_{700} = 0.1$.

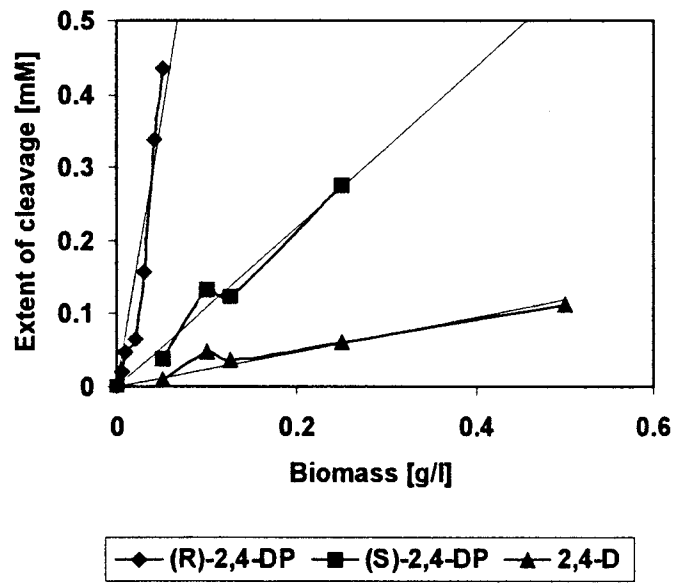


Fig. 3. Influence of the biomass concentration on the extent of cleavage of various phenoxyalkanoates by non-induced cells of Strain MC1
Strain MC1 was grown overnight on PYE + F. The cells were harvested and re-suspended to the respective biomass concentrations. The degradation experiments were performed in the presence of 0.45 ± 0.05 mM of the various phenoxyalkanoates.

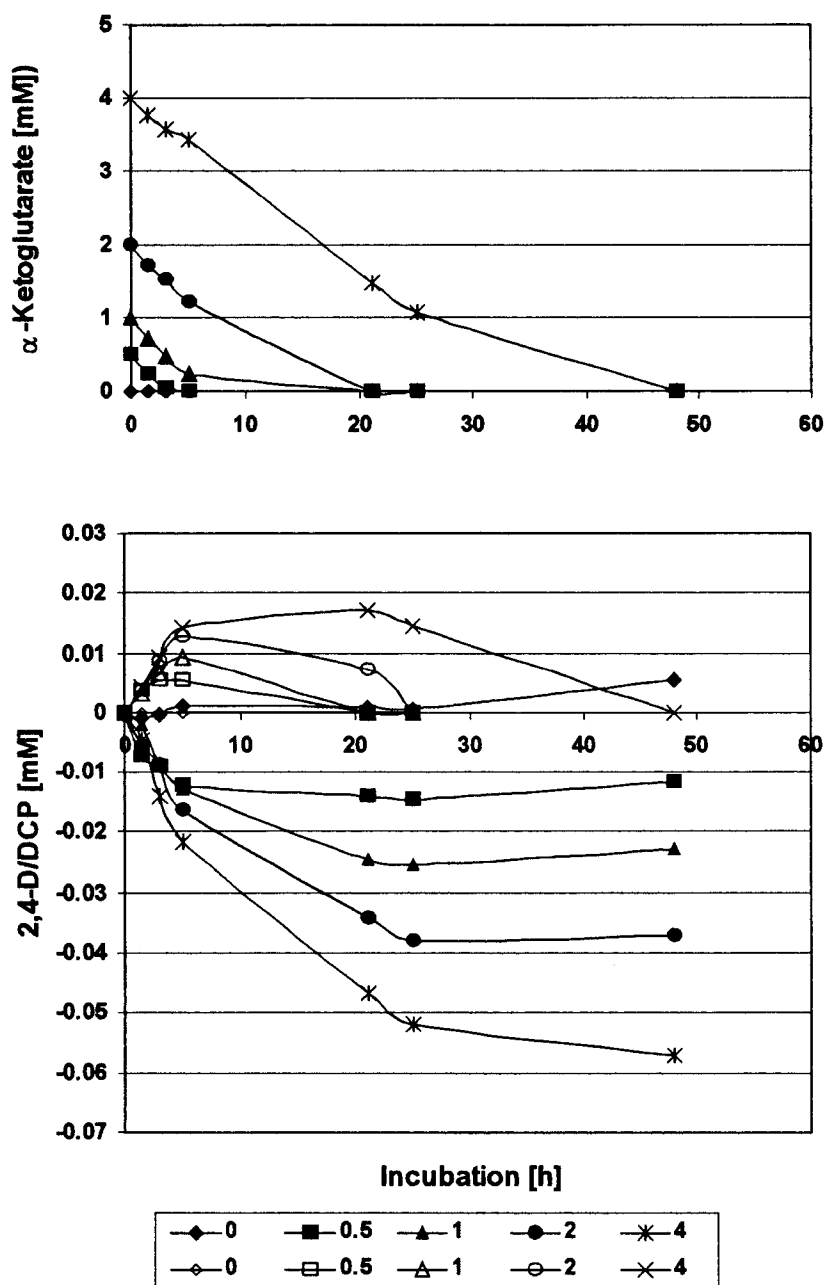


Fig. 4. Degradation of 2,4-D by non-induced cells of *Rhodoferrax* sp. P230. The strain was grown on PYE + α -KG medium. Washed cells were incubated in nitrogen-free medium containing 0.4–0.45 mM 2,4-D in the absence and presence of different concentrations of α -ketoglutarate (see figures in the legend [in mM]). Streptomycin was omitted.
Full symbols: 2,4-D concentration; open symbols: DCP concentration.

The consumption of the latter substrates, however, proceeded at different rates and showed limitations in the extent under the respective conditions. Different effects, described as follows, were shown to be responsible for this behaviour.

The availability of α -ketoglutarate as the second oxygen acceptor is a pre-requisite for continual herbicide utilization initiated by this special dioxygenase mechanism. Consequently, the supply of α -ketoglutarate should stimulate the cleavage of the herbicides by this two-substrate reaction. This, indeed, holds true. Stimulation was most pronounced with 2,4-D (Fig. 4). The extent of cleavage of this herbicide was dependent on the α -ketoglutarate concentration, after exhaustion of this compound no further consumption of 2,4-D was observed (Fig. 4). As the decrease of 2,4-D was faint in the absence of α -ketoglutarate, this compound plays the role of "start assistance" [17] under these conditions. In contrast, the phenoxypropionates served as suitable substrates for non-induced cells, i.e. these herbicides were also remarkably degraded without any addition. Nevertheless, the presence of α -ketoglutarate drastically increased the rate of herbicide consumption (Fig. 5). This was the case – referring to the lower α -ketoglutarate concentrations – as long as α -ketoglutarate was present. After its depletion, the rate decreased and attained such a value as observed in the initial phase of herbicide conversion in the absence of α -ketoglutarate. A further characteristic of herbicide consumption under these conditions was that the cleavage rate was drastically decreased to about 20% of its initial value after about 5 h. It is a remarkable fact that this property did apply also to the sample that yet contained α -ketoglutarate. It is another remarkable fact that substrate cleavage continued with a constant rate (irrespective of the actual DCP concentration) in the various samples.

This abrupt shift in the degradation rate should be caused by the accumulation of DCP known to be an inhibitor (uncoupler) and may consequently interfere with substrate uptake. An inhibitory pattern of this compound was demonstrated by pre-incubating with DCP Strains MC1 and P230; this affected substrate consumption (Fig. 6). According to these characteristics, extracellular application of DCP reduced the initial cleavage activity of the herbicides immediately. The effect was dependent on the DCP concentration and was even enforced with time, especially in the presence of 0.3 mM of DCP. In the presence of 0.06 mM of DCP, inhibition was moderate and paralleled more or less the shape of the activity profile observed in the absence of DCP. Similar inhibition was obtained by adding dinitrophenol: An incubation of 4 h in the presence of 1 mM completely prevented cleavage of (R)-2,4-DP by Strain MC1 (not shown). The inhibition characteristics observed when extracellular DCP was used, however, were in contrast to the behaviour of the decline of activity when DCP was accumulated due to the action of the dioxygenases. Here inhibitory effects were noticed only after a period of approx. 5 hours irrespective of the concentration of DCP accumulated during this time (Fig. 5). This means that the cells suffer from DCP derived from intracellular or extracellular sources in a different manner. Referring to the activity/DCP-accumulation properties, the cleavage pattern should be altered by avoiding the accumulation of DCP. Consequently, the picture was completely changed by incubating Strain P230 in the presence of *Ochrobactrum* sp. K2-14, the latter being induced for the complete degradation of DCP [13]. In the presence of this strain that did not carry etherolytic activities [13], the amount of (R)-2,4-DP supplied was completely converted (Fig. 7).

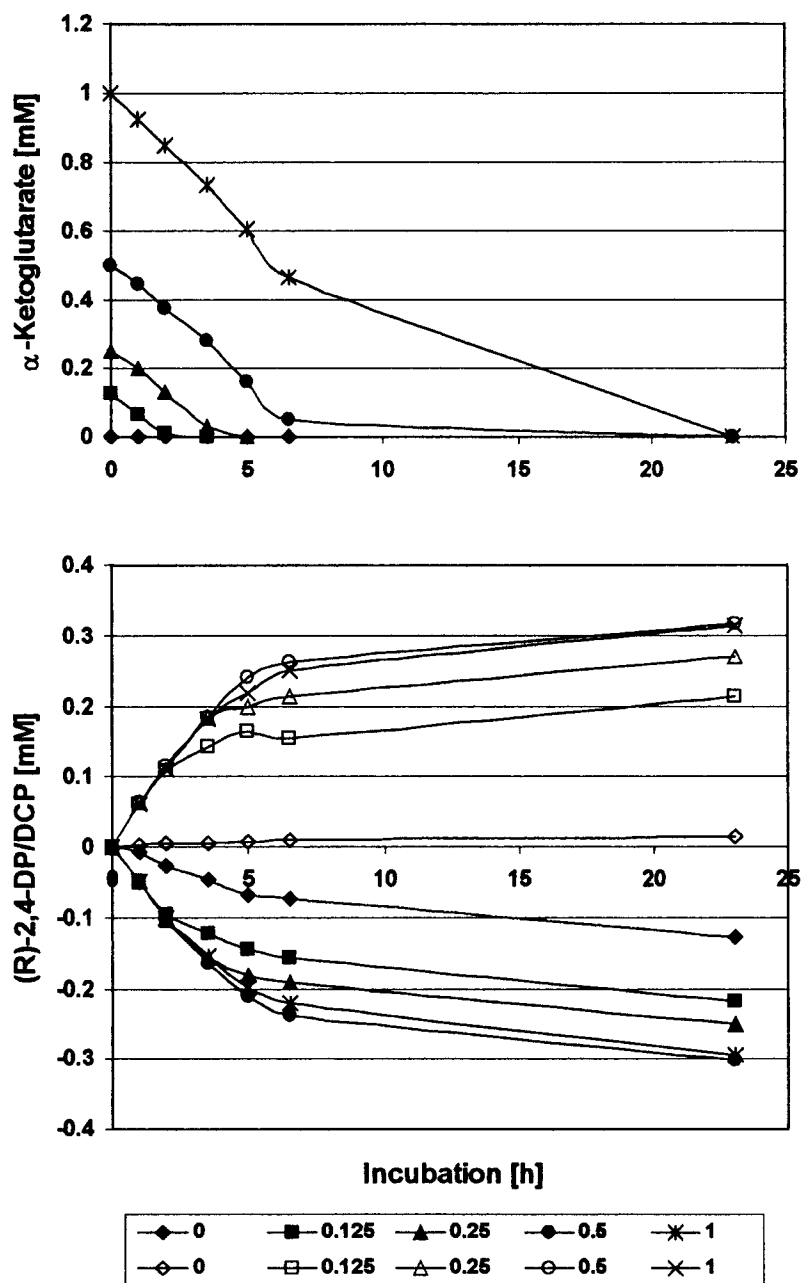


Fig. 5. Degradation of (R)-2,4-DP by non-induced cells of *Rhodospirillum rubrum* sp. P230. The strain was grown on PYE + α -KG medium. Washed cells were incubated in nitrogen-free medium containing 0.4–0.45 mM (R)-2,4-DP in the absence and presence of different concentrations of α -ketoglutarate (see figures in the legend [in mM]). Streptomycin was omitted.
Full symbols: (R)-2,4-DP concentration; open symbols: DCP concentration.

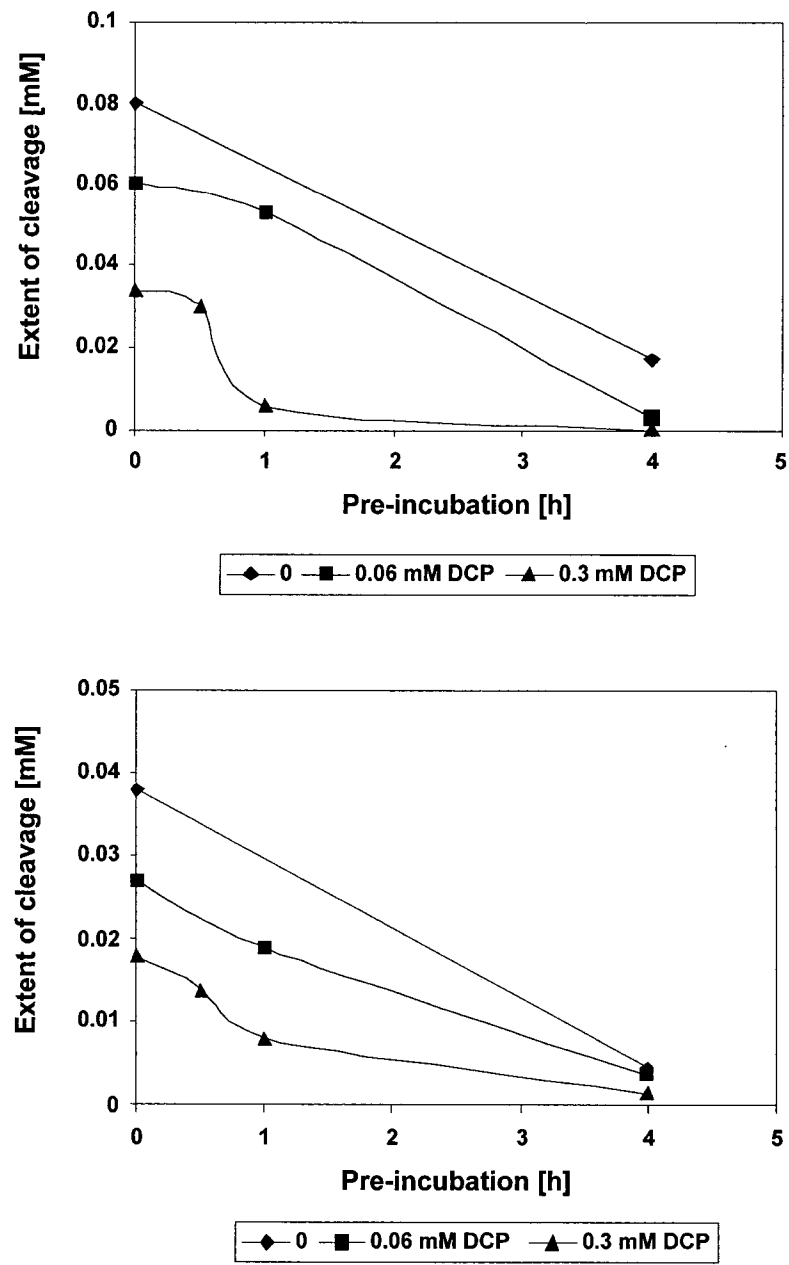


Fig. 6. Effect of pre-incubation with DCP on the cleavage of herbicides
Strains MC1 (upper part) and P230 (lower part) were pre-incubated in the absence and presence of different concentrations of DCP. After a respective time of pre-incubation, (*S*)-2,4-DP (Strain MC1) or (*R*)-2,4-DP (Strain P230) were added and the progress of herbicide consumption was recorded. The values correspond to the final amounts of substrate cleaved after 24 h of incubation. For both strains, the cell density corresponded to $E_{700} = 0.1$.

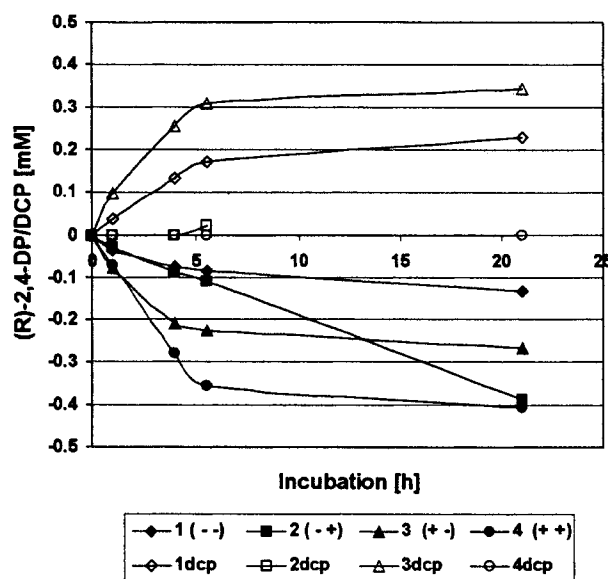


Fig. 7. Effect of *Ochrobactrum* sp. K2-14 on the degradation of R-2,4-DP by *Rhodoferrax* sp. P230

The cells were prepared as shown in the legend to Fig. 5. The incubation was performed in the absence or presence of α -ketoglutarate (– or +, first position in the brackets of the legend) and in the absence or presence of *Ochrobactrum* sp. P230 (– or +, second position in the brackets of the legend). The cells of this strain induced for DCP degradation were added to the suspension to give a final density of $E_{700} = 0.1$.

The presence of α -ketoglutarate stimulated the rate of herbicide consumption by a factor of 5, in a manner similar to the results shown in Fig. 5 regarding the initial phases. Even the absolute rates of substrate consumption were similar in these two experiments (Fig. 5 and Fig. 7). This means that DCP accumulated during the first 5 hours did not essentially influence the cleavage properties. This fact was supported by directly comparing the initial rates shown in Fig. 7, which are almost identical over the first 5 hours, but suddenly declined in those cases where DCP accumulated. The further rates of substrate consumption were again nearly identical irrespective of the concentration of DCP (Curve 1 vs. 3).

The results in Fig. 6 indicated that cleavage activity decreased during incubation also in those cases where DCP was absent during pre-incubation. This should be attributed to starvation effects as is shown in more detail in Fig. 8. According to these data, cleavage activity declined over time and was almost nil already after 4 hours of incubation in the absence of any carbon source. This effect could be raised by supplying α -ketoglutarate: In the presence of an amount of 100 mg/l, the effect as provoked after 4 hours of starvation could completely be restored. Some additive behaviour in the extent of the effect became evident when feeding α -ketoglutarate immediately or subsequently to starvation or both (Fig. 8). The stimulation of cleavage of phenoxy herbicides was, however, not solely dependent on α -ketoglutarate but was also found when

using other substrates, some of these results are listed in Tab. 1. Again, the effect was most pronounced with 2,4-D. In addition to the effects concerning the extent of cleavage, the presence of these substrates increased also the rate of cleavage by a factor of up to 3 (not shown).

Tab. 1. Influence of various carbon/energy sources on the cleavage of phenoxyalkanoates in non-induced cells

Substrate	Degradation (-fold)		
	Strain MC1		Strain P230
	2,4-D	(S)-2,4-DP	(R)-2,4-DP
Without	1 (42) ¹⁾	1 (240) ¹⁾	1 (48) ¹⁾
α -Ketoglutarate	4.6	1.2	1.9
Pyruvate	3.3	1.3	1.3
Fructose	5.9	1.35	1.15
Yeast extract	2.2	1.2	3.3

The cells were derived from overnight cultures on PYE + F medium. The incubation was performed in nitrogen-free minimal medium in the presence of streptomycin. The substrates were supplied (as the sodium salts with the organic acids) at a concentration of 100 mg/l.

¹⁾ The values show the relative extent of the increase in cleavage by adding various substrates; at a value of 1, obtained in the absence of an additional carbon source, the total amounts of substrate (μ M) cleaved within 24 h are given in the brackets.

Discussion

Various effects may cause phenoxyalkanoate herbicides to behave recalcitrant. At a first glance and as an experience made already formerly [18, 19], the presence of DCP, the first intermediate in the degradation of these compounds, is one factor that seems to interfere with utilization of phenoxyalkanoates despite the expression of the respective degradative enzymes. This may be attributed to DCP function as an uncoupler [20] and may interfere with the uptake of the herbicides shown to a large extent to be an energy-dependent process [21, 22]. Such effects accompanying the accumulation of DCP were considered, but on the basis of the present results only as secondary. This especially holds true as some substrate turnover may be obtained also in the presence of (uncoupling) DCP concentrations due to passive substrate uptake (Fig. 7; cf. [22]). The primary effect causing recalcitrance is attributed to the α -ketoglutarate dependent nature of the initial cleavage reaction and might be caused by a shortage of intracellular carbon metabolites, finally α -ketoglutarate. Cleavage of phenoxyalkanoates by an α -ketoglutarate-linked dioxygenase is a two-substrate reaction. One component, i.e. the phenoxyalkanoate herbicide, is externally offered as the proper substrate, whereas the other component, α -ketoglutarate, must be replenished by the cell's metabolism itself. No limitations are to be expected in this reaction if the phenoxyalkanoates are

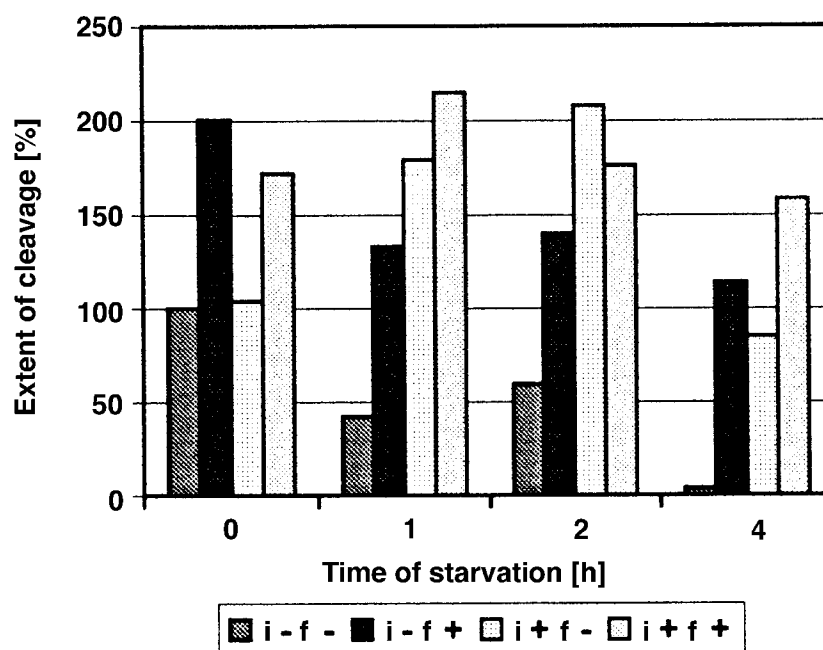


Fig. 8. Effect of starvation and addition of α -ketoglutarate on the extent of cleavage of (S)-2,4-DP with non-induced cells of Strain MC1

Strain MC1 was grown overnight on PYE + α -KG. The cells were harvested, washed and resuspended into MM to an $OD_{700} = 0.25$.

i: Addition of (S)-2,4-DP after the time of starvation without (–) or with (+) 0.5 mM α -ketoglutarate.

f: Pre-incubation of 4 h on 0.5 mM α -ketoglutarate after the time of starvation before adding (S)-2,4-DP without (–) or with (+) a second quantity of α -ketoglutarate.

completely degraded. The primary substrate assimilation of the various phenoxyalkanoate herbicides results in a pool of C2, C3 and C4 intermediates [4, 23] that are suitable precursors for the regeneration of α -ketoglutarate [1]. With incomplete degradation, however, releasing DCP or any further intermediate as a dead-end product, difficulties may arise because a potential source of α -ketoglutarate (i.e. acetyl-CoA and succinate) connecting the modified *ortho* pathway to the central metabolism will not become available. The flow of carbon considered under these conditions makes it plain that, with phenoxyacetate herbicides, the regeneration of α -ketoglutarate would not be possible (taking into account succinate and glyoxylate as the available metabolites in this case) and no continual cleavage would proceed [1]. This was the predominant feature in the present experiments, which showed that 2,4-D resided almost unutilized under the given conditions. The application of a second carbon source ought to substitute for the depletion of the intracellular pool of metabolites relaxing the constraints with respect to the availability of α -ketoglutarate. As a result, both the rate and extent of cleavage were increased indeed. With 2,4-D, this additive may even provide start assistance [17]. Considered from another angle, the TfdA-like reactions

may waste intracellular metabolites under these conditions. Consequently, these cells might suffer not only from an accumulation of DCP but also from a shortage of energy impairing maintenance effort and thus endangering survivability [24].

By contrast, the same cultures showed a significant degree of degradation in the case of phenoxypropionates. Here, the regeneration of α -ketoglutarate from succinate, implying pyruvate as the cleavage product from these herbicides, is more easily enabled for stoichiometric reasons [1]. This finding became evident from the pattern in Fig. 5. A high initial rate of herbicide cleavage was observed which indicates a self-sufficient regeneration of α -ketoglutarate. The presence of external α -ketoglutarate nevertheless behaved stimulatory. Remarkably, the initial rate suddenly declined to $1/5$ after about 5–6 hours, an effect not dependent on the presence of α -ketoglutarate; rather an almost constant rate was finally observed, independently of the presence of this metabolite. This shift should be caused by DCP being accumulated (as it was prevented in the presence of a DCP-consuming strain; cf. Fig. 7). The consecutive substrate consumption characteristics should be an expression of the rate of (passive) transport of the herbicide across the membrane. Besides, non-specific uptake of phenoxy herbicides was found to occur as was shown with non-induced cells of *S. herbicidovorans* [22] or with mutants of *Ralstonia eutropha* JMP134(pJP4) lacking the specific transport protein [21].

Properties of recalcitrance should be of significance to natural biotopes. 2,4-D degradation is most often found to be associated with consortia; this follows from a global survey on pristine biotopes [25]. It is interesting to note that indications of 2,4-D degradation became evident in this study only after at least 2 weeks' incubation, whereas 3-chlorobenzoate utilization proceeded immediately. Localization of the degradative capabilities on different strains is one explanation although the respective genes were often found to be harboured on the same plasmid. Consequently, and in accordance with our results, however, it will be harder to spot strains exhibiting incomplete degradation, as this potential is masked due to the metabolic peculiarities. With the same consequence, a range of strains has been isolated in the past that shows complete degradation of 2,4-D [23, 26, 27]. For this, the strains applied in this paper represent a suitable model for studying this property due to the constitutive expression of cleavage activities. In contrast, but not unexpectedly from the present results (for further details see [1]), complete degradation by pure cultures has been questioned in the past for phenoxypropionate herbicides because this property was exclusively found to be associated with consortia [28–30]; complete degradation has only recently been attributed to individual strains. Besides the two bacterial species considered in this publication [11, 12], a third strain, *Sphingomonas herbicidovorans* MH, has been reported as being capable to perform the complete degradation of these compounds [31, 32]. The experience that phenoxypropionate degradation is more likely to be found in consortia might be caused by the fact that the cleavage reaction could be detected more easily in these cases.

Due to the metabolic peculiarities, the phenoxyalkanoate herbicides may prove apparently inert despite the existence of TfdA activity. The present experiments should contribute to an understanding of the fate of these compounds in natural habitats. If consortia play a role in degradation, especially with phenoxyacetates, the cleavage reaction requires the presence of a carbon source in addition to the herbicides or the

interspecies exchange of carbon compounds in order to proceed; induction and repression mechanisms may modulate the effects. Under selective conditions, however, genetic information can rather easily be acquired to complete the degradative performances. Localization on plasmids of these genes is one aspect of ease distribution. Another aspect, the modular arrangement of the complete degradative pathway of phenoxyalkanoates found in various strains by separating the genes encoding for TfdA (phenoxyalkanoate/ α -ketoglutarate dioxygenase), TfdB (chlorophenol hydroxylase) and TfdC-F (enzymes of the modified *ortho* cleavage pathway) into separate operons [33], should be favourable. Capture of respective genes has indeed been demonstrated [34]. Sometimes such mechanisms might be correlated to the effects observed in enrichment cultures in which, after a rather long period of incubation, degradability suddenly started, freeing the culture (strain) from metabolic limitations as discussed here. These observations were also made by TIEDJE *et al.* [25] because most samples obtained from pristine environments revealed 2,4-D degradation only after a long time of incubation.

In conclusion, these results have shown that recalcitrance observed with phenoxyalkanoate herbicides need not necessarily originate from deficits in degradative enzymes, particularly in the initial etherolytic enzymes, but can be caused by gaps or shortages in metabolism, affecting the α -ketoglutarate dependency of this initial degradative step. This property has to be taken into account when describing the phenoxy herbicide degradation potential in natural biotopes and when treating them in technical systems.

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