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Stereospecific degradation of the phenoxypropionate herbicide dichlorprop

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Abstract

2,4-Dichlorophenoxyacetic acid (2,4-D)/α-ketoglutarate (α-KG) dioxygenase, TfdA, from *Ralstonia eutropha* JMP134, was purified from recombinant cells and shown by gas chromatographic and colorimetric methods to degrade only the *S* enantiomer of dichlorprop, a phenoxypropionate herbicide. Similarly, cell extracts of *Burkholderia cepacia* RASC, containing a biochemically and genetically related α-KG-dependent dioxygenase, also were shown to oxidize (*S*)-dichlorprop using chiral HPLC and colorimetric methods. In contrast, cell extracts of a mecoprop-degrading strain of *Alcaligenes denitrificans* were shown to catabolize (*R*)-dichlorprop. Although the *A. denitrificans* activity exhibited stereospecificity opposite to that of the JMP134 and RASC strains, its cofactor requirements were found to be characteristic of an α-KG-dependent dioxygenase. A PCR amplification product from the DNA of this strain was shown to encode an amino acid sequence that was 95% and 86% identical to the corresponding region of TfdA in RASC and JMP134, respectively. Thus, closely related herbicide-degrading gene products appear to be capable of exhibiting opposite stereochemical degradative capabilities.

Keywords: Stereospecificity; Herbicide; Dichlorprop; α-Ketoglutarate; TfdA

1. Introduction

Ring-substituted phenoxyacetates and phenoxypropionates are widely used as herbicides to control the growth of broadleaf weeds and woody plants [1]. While phenoxyacetates such as 2,4-dichlorophenoxyacetic acid (2,4-D) lack a chiral carbon atom, phenoxypropionates can exhibit either of two enantiomeric forms because of the methyl group attached to the C-2 carbon atom. Notably, only the *R* enantiomers are active towards plants [2]. A current trend in agriculture is increasing use of only the active enantiomer of chiral herbicides in order to reduce environmental loads of organopesticides [2]. For example, about 75% of dichlorprop (2-(2,4-dichlorophenoxy)propionate) and mecoprop (2-(2-methyl-4-chlorophenoxy)propionate) are sold in their active enantiomeric forms in the European Union, with the expectation that

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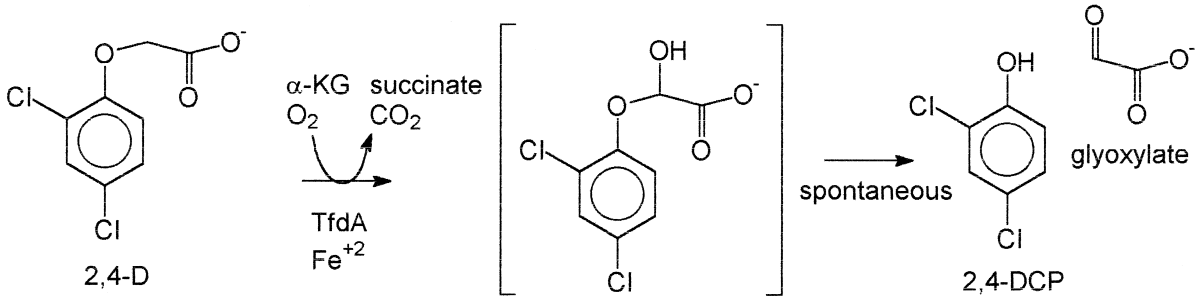
within 5 years only the *R* isomer will be commercially available (R. Akers, personal communication).

Phenoxyacid herbicides are rapidly degraded in soil by bacteria and fungi [1]. One of the best characterized herbicide-degrading organisms is *Ralstonia eutropha* (formerly *Alcaligenes eutrophus* [3]) strain JMP134, capable of using 2,4-D as its sole carbon and energy source. The first step in 2,4-D catabolism is its conversion to 2,4-dichlorophenol (2,4-DCP), catalyzed by the enzyme 2,4-D/α-ketoglutarate (α-KG) dioxygenase [4] or TfdA [5]. This ferrous ion-dependent enzyme couples the oxidative decarboxylation of α-KG to hydroxylation of the substrate at the C-2 carbon to yield a postulated hemiacetal intermediate which spontaneously decomposes to form 2,4-DCP and glyoxylate [4] (Scheme 1). The chlorinated phenol is subsequently metabolized by a series of well-studied enzymes that eventually generate an intermediate of the tricarboxylic acid cycle [6]. It is important to emphasize, however, that not all 2,4-D-degrading microorganisms use this pathway. The biochemistry of phenoxypropionate degradation is much less well understood than that of 2,4-D. Here too, however, side chain removal to release the corresponding phenol has been shown to be an essential step in catabolism of these compounds (e.g., Refs. [7–14]).

Studies to characterize the stereospecificity of enzymes that degrade phenoxypropionate herbicides can have important biotechnological ramifications. For example, a synthetic process could be devised in which a particular herbicide is initially synthesized as the racemic mixture, the *S* enantiomer is degraded by a stereoselective enzyme, and the remaining *R* isomer is purified away from the other reaction products which are recycled. As an alternate illustration, the genes encoding an enzyme specific for the *R* isomer could be used to develop genetically engineered crops that are resistant to the herbicide of interest. Genetic engineering has already been used for crop protection from (achiral) phenoxyacetate herbicides: cotton has shown enhanced tolerance towards 2,4-D when *tfdA* was transformed directly into the plant [15] or into root-associated microbes [16].

Here, we demonstrate that purified *R. eutropha* TfdA specifically degrades the *S* enantiomer of dichlorprop and characterize the kinetics of this process. In addition, we establish that *Burkholderia cepacia* strain RASC, a 2,4-D-degrader containing a TfdA with 80% sequence identity to that from JMP134, has the same stereopreference for the *S* enantiomer. By contrast, we show that cell extracts of a strain of *Alcaligenes denitrificans*, intact cells of which are known to utilize the *R* enantiomer of mecoprop [14], specifically degrade the *R* enantiomer of dichlorprop in an α-KG-dependent process. Although the latter activity exhibits opposite stereospecificity from that found in the 2,4-D-degrading strains, we provide evidence that a *tfdA*-like gene is present in *A. denitrificans*, suggesting that all of these enzymes are closely related to each other.



Scheme 1. Reaction catalyzed by TfdA with 2,4-D.

2. Experimental

2.1. Chemicals

(+)-(*R*)-Dichlorprop was a kind gift from BASF. Racemic and (+)-(*R*)-mecoprop were generously donated by A.H. Marks. Racemic dichlorprop and 2,4-D were purchased from Aldrich.

2.2. Bacterial strains

R. eutropha JMP134 [17] and *B. cepacia* RASC [18] were obtained from the Research on Microbial Evolution laboratory at Michigan State University. A strain of *A. denitrificans* isolated for its ability to degrade mecoprop [14] was obtained from Hilary Lappin-Scott (University of Exeter).

2.3. Activity assay with colorimetric detection of derivatized phenols

The standard assay mix contained 1 mM α-KG, 50 μM each of Fe(II) and ascorbate, 1 mM phenoxyacid substrate in 10 mM imidazole buffer (pH 6.75), and enzyme. In studies of cell extracts, the concentrations of selected reagents were varied in some experiments as noted in the text. The iron and ascorbate were either supplied together as iron(II) ascorbate salt or separately as $(NH_4)_2Fe(SO_4)_2$ and ascorbic acid. Reactions were carried out at 30°C and terminated by addition of EDTA to a final concentration of 5 mM. The phenolic product was detected at 510 nm following addition of 4-aminoantipyrene, pH 10 borate buffer, and potassium ferricyanide, as described [19]. Measured extinction coefficients for the 4-aminoantipyrene adducts were 15,700 M^{-1} cm^{-1} for 2,4-DCP and 11,300 M^{-1} cm^{-1} for 4-chloro-2-methylphenol.

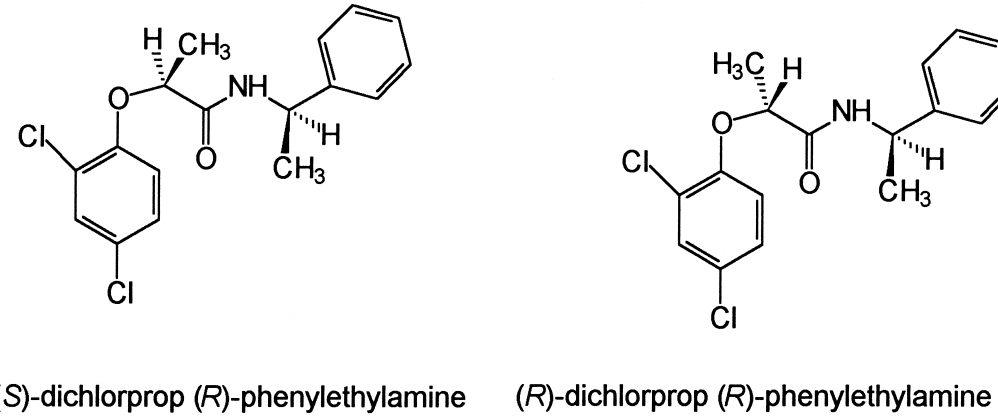
2.4. Preparation of cell extracts and purified enzyme

TfdA was purified from *Escherichia coli* DH5α cells carrying the cloned *R. eutropha* JMP134 *tfdA* gene on plasmid pUS311 by sequential chromatography on DEAE Sepharose and MonoQ (Pharmacia) columns [4,20].

2,4-D and mecoprop degraders were grown aerobically at 30°C in MMO mineral salts medium [21] with 50 ppm yeast extract and the relevant herbicide at initial concentrations between 1 and 5 mM. Cells were harvested when 60–90% of the herbicide was consumed as measured by disappearance of the chromophore at 230 nm. Cell extracts were prepared by resuspending the bacteria in Tris buffer (pH 7.7) with 1 mM EDTA followed by disruption in a French pressure cell and ultracentrifugation (100,000 × *g*, 30 min) or centrifugation in a microcentrifuge (16,000 × *g*, 5 min). Protein concentrations were determined by using the BioRad protein assay with bovine serum albumin as the standard.

2.5. Formation of diastereomers and gas chromatography (GC)

The levels of (*R*)- and (*S*)-dichlorprop in samples were assessed by using a modification of a published GC method [22]. Aqueous reaction mixtures (1 ml) were acidified with 25 μl of HCl (conc.) and extracted with 1 ml methylene chloride. The organic phase (0.5 ml) was dried under a gentle stream of air, 1 drop of thionyl chloride was added, and the sample was incubated 10 min over a steam bath. Following removal of the thionyl chloride under vacuum, the sample was dissolved in 0.1 ml chloroform with 1 drop of (*R*)-phenylethylamine added, allowed to react 25 min at room temperature to form diastereomers (Scheme 2), dried, and transferred to 0.5 ml of ethyl acetate. An aliquot (1 μl) of the sample was injected onto a DB-5 column (polymethylsiloxane with 5% phenyl groups; J&W Scientific), with a temperature program set to increase linearly from 200 to 270°C in 7 min, and the analytes were monitored by electron capture detection (rather than the less sensitive flame ionization detection originally used [22]). Peaks were identified by



(*S*)-dichlorprop (*R*)-phenylethylamine (*R*)-dichlorprop (*R*)-phenylethylamine

Scheme 2. Formation of diastereomers of dichlorprop and (*R*)-phenylethylamine.

comparison with the retention times of the diastereomeric amides of authentic (*R*)-dichlorprop and (*RS*)-dichlorprop.

2.6. High pressure liquid chromatography (HPLC)

Dichlorprop was reacted with cell extracts or purified enzyme as described for the activity assay, and the reactions were stopped with 5 mM EDTA. Aliquots were injected onto a Nucleodex α-PM column (4.0 mm i.d. × 200 mm length; Machery-Nagel, Germany). The eluent was 30% phosphate buffer (50 mM, pH 3) and 70% methanol at a flow rate of 0.7 ml min^{-1}. Species eluting from the column were monitored with UV detection at 230 nm.

2.7. DNA hybridization, amplification and sequencing

Total genomic DNA was isolated from *A. denitrificans* [23] and digested with *Bam*HI. Following separation on a 0.8% agarose gel, the DNA was transferred [23] to a Hybond N membrane (Amersham). Probes for $tfdA_{RASC}$ (a 1-kb *Pst*I fragment [18]) and for $tfdA_{JMP134}$ (an 801-bp *Sty*I fragment [24]) were labeled with digoxigenin according to the Genius™ System instructions (Boehringer-Mannheim Biochemicals). The membrane was blotted with the probes at high stringency (68°C, 50% formamide) or low stringency (68°C, no formamide) using the protocol from the same manual.

A. denitrificans DNA was used as template DNA for PCR amplification with TVU and TVL primers which amplify a 360-bp internal fragment of both RASC and JMP134 *tfdA* [25]. The product was purified by using the GFX PCR DNA and gel band purification kit (Pharmacia Biotech) and was sequenced in both directions by using dye terminator chemistry at the MSU-DOE-PRL Plant Biochemistry DNA Sequencing Facility with TVU and TVL as primers. The nucleotide and amino acid sequences of the amplified product (not including the primer sequence) were aligned to those of JMP134 and RASC using protocols in the GCG suite of programs [26].

2.8. Data analysis

Data were fitted to straight lines, exponential decays, or square hyperbola using the program KaleidaGraph (Synergy software). As well, progress curves of 2,4-DCP production vs. time were fitted to Eq. (1) by non-linear regression using the same program.

$$A_t = A_f(1 - e^{-k(\text{inact})t}) \quad (1)$$

In this equation, A_t is the absorbance at time t, A_f is the final absorbance, and k_{inact} is the rate of inactivation.

3. Results

3.1. TfdA stereospecificity

The stereospecificity of purified TfdA from *R. eutropha* JMP134 was investigated using two methods. The first approach used derivatization with (*R*)-phenylethylamine followed by GC analysis to separate and quantitate the two diastereomers. During the reaction of racemic dichlorprop with TfdA, the percentage of *S* relative to *R* enantiomer decreased over time, suggesting that this enzyme uses the *S* isomer (Fig. 1). The second procedure used to assess stereospecificity involved a colorimetric assay for 2,4-DCP release. 2,4-DCP was produced by TfdA acting on racemic dichlorprop but not on (*R*)-dichlorprop (Fig. 2), a result in agreement with the GC results for this enzyme. The enzyme lost activity over time due to oxidative inactivation events that are minimized by the presence of ascorbic acid [27]. Based on initial rates, (*S*)-dichlorprop utilization (0.81 μmol min^{-1} mg^{-1} protein) was 6% of that observed for 2,4-D decomposition (13 μmol min^{-1} mg^{-1} protein) under saturating substrate concentrations.

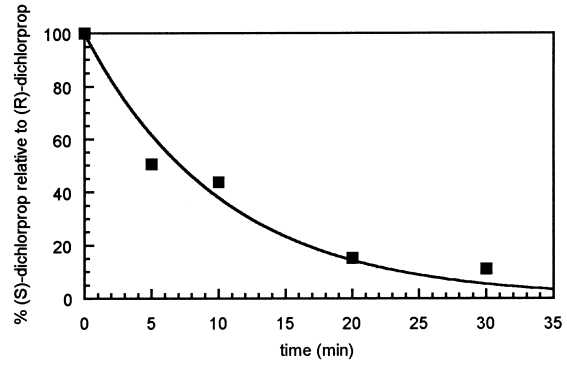


Fig. 1. Stereoselectivity of dichlorprop decomposition catalyzed by TfdA. TfdA from JMP134 (50 μg) was incubated with racemic dichlorprop (100 μM) and ascorbic acid, ferrous ion, and α-KG using standard concentrations for the indicated time periods. Following extraction, the remaining dichlorprop was converted to the (*R*)-phenylethylamine diastereomers and analyzed by GC/electron capture detection. Data were fitted to an exponential decay with a decay rate of 0.096 ± 0.011 min^{-1}.

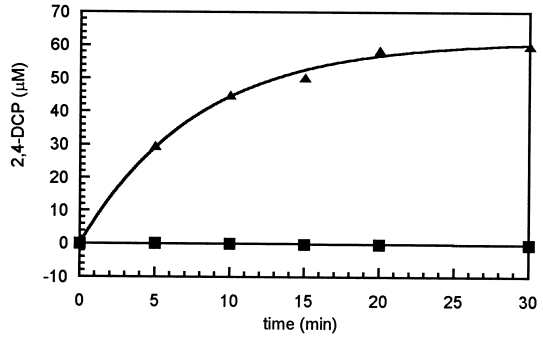


Fig. 2. 2,4-DCP production by TfdA acting on dichlorprop. TfdA from JMP134 (10 μg ml^{-1}) was incubated with a racemic mixture of dichlorprop (▲) at 2 mM or with (*R*)-dichlorprop at the same concentration (■). 2,4-DCP was measured colorimetrically. Data for the racemic dichlorprop were fitted to Eq. (1) ($k_{inact} = 0.131 \pm 0.004$ min^{-1}), while data for the (*R*)-dichlorprop were fitted by linear regression (slope of -0.02 μM min^{-1}).

3.2. Herbicide-degrading activities in cell extracts

Cell extracts were examined from JMP134 and two additional phenoxyacid herbicide degraders: *B. cepacia* RASC, a 2,4-D degrader with a TfdA that is 80% identical to that of *R. eutropha* JMP134 [18], and a strain of *A. deni-*

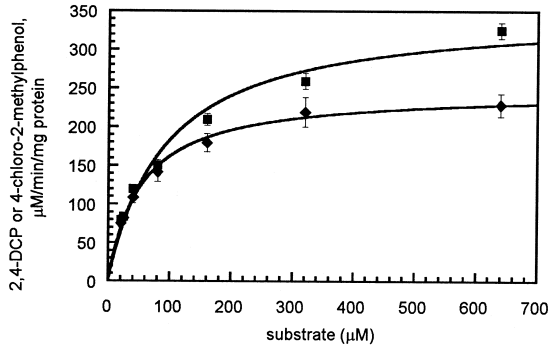


Fig. 3. Kinetic analysis of *A. denitrificans* cell extract activities towards (*R*)-mecoprop and (*R*)-dichlorprop. Cell extracts of *A. denitrificans* were examined for their abilities to decompose (*R*)-dichlorprop (■) and (*R*)-mecoprop (♦). Initial rates calculated from the phenolic product release (based on 6 min time points, triplicate samples) were fitted to the Michaelis–Menten equation to yield values of $K_m = 91 \pm 16$ μM and $V_{max} = 351 \pm 21$ μM min^{-1} mg^{-1} protein for (*R*)-dichlorprop and $K_m = 52 \pm 4$ μM with $V_{max} = 247 \pm 6$ μM min^{-1} mg^{-1} protein for (*R*)-mecoprop. Mecoprop velocity data were corrected for ~1% contamination with a phenolic compound presumed to be 4-chloro-2-methylphenol.

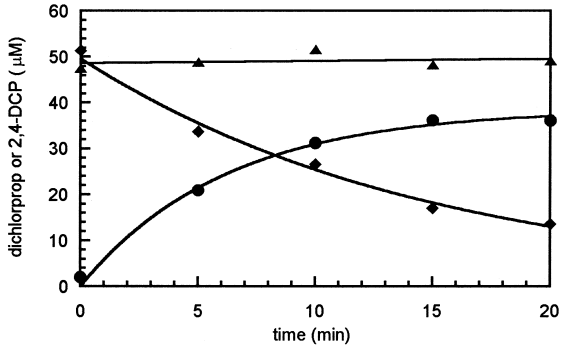


Fig. 4. Stereospecificity of dichlorprop-degrading activity of *A. denitrificans* cell extracts as measured by chiral HPLC. Concentrations of (*S*)-dichlorprop (▲), (*R*)-dichlorprop (♦), and 2,4-DCP (●) were measured at selected time points after incubation of *A. denitrificans* cell extracts (50 μl) with racemic dichlorprop (0.1 mM) and the standard reaction components. Dichlorprop was quantitated by using chiral HPLC and 2,4-DCP was quantitated colorimetrically. Data for the (*S*)-dichlorprop were fit to a straight line of slope 0.05 μM min^{-1}, for (*R*)-dichlorprop to exponential decay with an exponent of -0.067 min^{-1}; and for 2,4-DCP to Eq. (1) with an apparent inactivation rate of 0.16 min^{-1}.

trificans isolated for its ability to degrade (*R*)-mecoprop [14]. Cell extracts of JMP134 and RASC exhibited highest activity towards 2,4-D, but showed ~20% of this activity towards dichlorprop. We attribute the cell extract activity towards phenoxy herbicides in both these strains to TfdA, since Tn5 disruption of the *tfdA* gene in either strain will prevent the conversion of 2,4-D to 2,4-DCP by intact cells [5,18]. By contrast to the specificity of the abovementioned strains, *A. denitrificans* extracts were most active with phenoxypropionates including mecoprop, dichlorprop, 2-(2,4-dibromophenoxy)propionate, and 2-(4-chlorophenoxy)propionate, with only minor activity towards the phenoxyacetates 2,4-D and 2-chloro-4-methylphenoxyacetic acid (~13 fold less than the activity towards dichlorprop). Cell extracts of *A. denitrificans* demonstrated a slightly higher V_{max} towards the dichlorprop than towards mecoprop, the substrate on which it was isolated, but slightly less affinity (higher K_m) (Fig. 3). The *A. denitrificans* activity was dependent on α-KG and ferrous ion, and was rapidly lost (in 2 min) in the absence of ascorbic acid. These cofactor requirements are characteristic of α-KG-dependent dioxygenases.

3.3. Stereospecificity of activity in cell extracts

The stereospecificity of herbicide decomposition by cell extracts was measured using an HPLC column with a chiral stationary phase, which permitted direct separation without prior derivatization of the enantiomers of dichlorprop. In agreement with the results using purified enzyme, cell extracts of JMP134 degraded the *S* enantiomer. Similar results were obtained with

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                150       160       170       180       190       200
JMP134          RAAYDALPRDLQSELEGLRAEHYALNSRFLLGDTDYSEAQRNAMPPVNWPLVRTHAGSGRK
                ||||| ||:|:::||:|||||||||||||:||||||||:||||||||:|||||||||||||
A.denitrificans RAAYDDLPEDFKKELQGLRAEHYALNSRFILGDTDYSESQRNAMPPVSWPLVRTHAGSGRK
                |||||||||||||||||||||||||:||||||||:||||||||||||||:|||||||||
RASC            RAAYDDLPEDFKKELQGLRAEHYALHSRFILGDTEYSESQRNAMPPVSWPLIRTHAGSGRK
                170       180       190       200       210       220

                   210       220       230       240       250
JMP134          FLFIGAHASHVEGLPVAEGRMLLAELLEHATQREFVYRHRWNVGDLVM
                ||||||||:|:|| ||||||||||||||||||||:|||||  |:|||||
A.denitrificans FLFIGAHAGHIEGRPVAEGRMLLAELLEHATQRKFVYRHSWKVGDLVM
                ||||||||:|||||||||||||||||||||||| ||||||||||||||
RASC            FLFIGAHASHIEGRPVAEGRMLLAELLEHATQPKFVYRHSWKVGDLVM
                   230       240       250       260       270
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Fig. 5. Comparison of an *A. denitrificans* TfdA-like sequence with the enzymes from RASC and JMP134. The deduced sequence encoded by the PCR amplification product arising from *A. denitrificans* DNA was compared to the indicated portions of the RASC and JMP134 TfdA sequences. Identical amino acids are indicated by a solid vertical bar and similar amino acids by a dashed bar.

RASC extracts (data not shown). By contrast, *A. denitrificans* extracts degraded (*R*)-dichlorprop (Fig. 4) with concurrent 2,4-DCP production.

3.4. Molecular biological comparison of strains

The similarity in cofactor requirements among the three strains raised the question of whether the *A. denitrificans* activity was due to a gene related in sequence to those which encode the two TfdA enzymes in the other species. *A. denitrificans* genomic DNA hybridized at low stringency to a probe for $tfdA_{JMP134}$ and at high stringency to a segment of $tfdA_{RASC}$ (data not shown). Amplification of *A. denitrificans* DNA with primers specific for *tfdA* yielded a 360-bp DNA fragment with a sequence that was 94% identical to that from RASC and 78% to that from JMP134, in a 327-bp overlap. The translated sequence (109 amino acids long) had 95% identity with TfdA from RASC and 86% identity to that from JMP134 (Fig. 5).

4. Discussion

4.1. R. eutrophus JMP134 TfdA oxidizes the S isomer of phenoxypropionates

α-KG dependent dioxygenases typically exhibit stereoselectivity in the hydroxylations they catalyze (e.g., Refs. [28–31]); thus, it was not surprising to find that *R. eutrophus* JMP134 TfdA oxidizes a single enantiomer (the *S* isomer) of dichlorprop. Based on this result with phenoxypropionate, the enzyme likely catalyzes stereochemically selective oxidation at the *pro-R* position when acting on the prochiral 2,4-D.

4.2. Related gene products can degrade opposite stereoisomers

The above studies demonstrate that the herbicide-degrading activity of *A. denitrificans* exhibits opposite stereochemistry than that found in strains JMP134 or RASC; yet, each of these activities are α-KG-dependent and the enzymes are likely to be genetically related. The present work extends the conclusions derived from studies with a strain of *Sphingomonas herbicidovorans* that is capable of degrading both enantiomers of mecoprop [32]. In that strain, two α-KG-dependent dioxygenases appear to be present, each specific for a separate enantiomer of the substrate [33]. Based on our findings, it is probable that the two *S. herbicidovorans* enzymes are closely related in sequence. The ability for closely related enzymes to recognize opposite stereoisomers has precedent. For example, recent crystallographic characterization of two tropinone reductases demonstrate the presence of almost identical overall protein folding, but the closely related enzymes exhibit opposite substrate stereoselectivities due to differences in a few charged residues at the active site [34].

4.3. Biotechnological implications

The work described here may have biotechnological applications. An *S* isomer selective dichlorprop-degrading enzyme, such as TfdA, may be used to facilitate the preparation of the desired *R* isomer, while a gene encoding an *R* isomer selective enzyme, such as that in *A. denitrificans*, may be useful for development of herbicide-resistant transgenic plants. Also, in understanding the role of microorganisms in the environmental fate of phenoxypropionates, it is useful to have analytical techniques such as those used in this research capable of distinguishing the enantiomers.

Acknowledgements

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use of electron capture detection, and Hilary Lappin-Scott for providing us with the mecoprop-degrading strain of *A. denitrificans*. This work was supported by the National Science Foundation (MCB9603520), the National Science Foundation–Science and Technology Center Grant DEB9120006, and the Michigan State University Agricultural Experiment Station.

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