Doctoral Thesis

Metabolism of phenoxyalkanoic acid herbicides in Sphingomonas herbicidovorans MH
cloning and characterization of two enantiospecific α-ketoglutarate-dependent dioxygenases and degradation pathway analysis

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Metabolism of Phenoxyalkanoic Acid Herbicides in
*Sphingomonas herbicidovorans* MH:
Cloning and Characterization of Two Enantiospecific
α-Ketoglutarate-Dependent Dioxygenases and
Degradation Pathway Analysis

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SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

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presented by

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Zurich, 2004
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SUMMARY

The bacterium *Sphingomonas herbicidovorans* MH is able to degrade phenoxyalkanoic acid herbicides, such as the chiral mecoprop (2-(R,S)-2-methyl-4-chlorophenoxypropanoic acid) and dichlorprop (2-(R,S)-2,4-dichlorophenoxypropanoic acid). Strain MH degrades mecoprop and dichlorprop enantiomeric, whereby the (S) enantiomer is preferentially converted. This thesis work was carried out to (i) elucidate the genetic background of the mecoprop and dichlorprop degradation pathway and to (ii) characterize the two α-ketoglutarate-dependent dioxygenases that catalyze the initial degradation step and to study the features of enantioselective enzyme reactions.

To elucidate the genetic background of the phenoxyalkanoic acid degradation pathway in *S. herbicidovorans* MH, PCR- and hybridization experiments were carried out with primers and probes derived from related genes. Strain MH expressed two distinct α-ketoglutarate dependent dioxygenases, designated (R)- and (S)-dichlorprop dioxygenases (RdpA and SdpA, respectively) that converted phenoxypropanoic acid herbicides to the corresponding achiral phenols. Further breakdown of the phenol was proposed to proceed analogously to the well-studied pathway of 2,4-D (2,4-dichlorophenoxyacetic acid) in *Ralstonia eutropha* JMP134(pJP4) through a phenol hydroxylase forming a substituted catechol and further through a modified ortho-cleavage pathway. Several genes of this pathway were isolated: a putative phenol hydroxylase gene (*tfdB*), a putative regulatory gene (*cadR*), two genes for dichlorocatechol 1,2-dioxygenases (*dccA_{HH})*, a complete and an incomplete gene copy for a dienelactone hydrolase (*dccD_{HH})*, a gene for a part of a maleylacetate reductase (*dccE*) and a gene for a potential phenoxyalkanoic acid permease (*tfdK*). No gene for a chloromuconate cycloisomerase was found. In contrast to the 2,4-D pathways in other microorganisms, the *sdp*, *rdp*, and *dcc* genes of *S. herbicidovorans* MH were not located in one regulon and their expression was not tightly regulated. No coherent pattern was derived on the possible origin of the *sdp*, *rdp*, and *dcc* genes. *rdpA* from the *Alphaproteobacterium S. herbicidovorans* MH was 99% identical to *rdpA*
Summary

from the *Betaproteobacterium Delftia acidovorans* MC1. Such close resemblance is evidence for a recent gene exchange between *Alpha-* and *Betaproteobacteria*. On the contrary, DccA1 and DccA9 did not group within the known chlorocatechol 1,2-dioxygenases from *Beta-* and *Gammaproteobacteria*, but formed a separate branch in clustering analysis. This suggests a different reservoir of the genes of the modified ortho-cleavage pathway in *Alphaproteobacteria* from the ones in *Beta-* and *Gammaproteobacteria* and a reduced gene transfer between these bacterial groups.

We intensively investigated the two α-ketoglutarate-dependent dichlorprop-dioxygenases RdpA and SdpA, which were 30% identical to each other. The amino acid sequence of RdpA was 100% identical to the one of RdpA from the dichlorprop degrader *D. acidovorans* MC1, whereas the amino acid sequence of SdpA was only 63% identical to the one of SdpA from *D. acidovorans* MC1. With TfdA and TauD, the identities lay in the range of 30–37%. A conserved motif of α-ketoglutarate-dependent dioxygenases, HXDX₂₄TX₁₃₁HX₁₀₁R for RdpA and HXDX₂₄TX₁₂₇HX₁₀₁R for SdpA, was recognized in both proteins. In RdpA, His-111, Asp-113 and His-270 are predicted to form the 2-His-1-carboxylate facial triad involved in iron(II) binding and the residues Thr-138 and Arg-281 were putative α-ketoglutarate binding sites. The predicted iron-binding residues in SdpA were His-102, Asp-104 and His-257; and the predicted α-ketoglutarate binding residues were Thr-129 and Arg-268.

We expressed and purified both enzymes as 6xHis-tagged proteins and characterized them with respect to their physicochemical properties, their substrate, cosubstrate and cofactor specificities and enzyme kinetics. RdpA turned out to be a trimer with a subunit size of 36 kDa whereas SdpA was a monomer with a size of 32 kDa. RdpA exclusively converted the (R) enantiomers of dichlorprop and mecoprop whereas SdpA was specific for the (S) enantiomers. Both dioxygenases preferred substituted phenoxypropanoic acids to unsubstituted ones. Furthermore, RdpA and SdpA were only slightly or not active at all with phenoxyacetic and phenoxybutyric acids as substrates. RdpA activity was linearly dependent on oxygen concentration whereas SdpA showed Michalis-Menten behavior with O₂ as
the substrate with an apparent $K_m$-value of 159 μM. Both dioxygenases were quite specific for their cosubstrate α-ketoglutarate. The only other cosubstrate that supported activity was 2-oxoadipate. High specificity was also observed for Fe(II) which could not be replaced by any of the tested divalent cations. In the absence of ascorbate, RdpA and SdpA exhibited lower activity suggesting uncoupled decarboxylation of α-ketoglutarate.

*S. herbicidivorans* MH grows on 2,4-D, but neither RdpA nor SdpA catalyzed 2,4-D to a significant extent indicating the existence of a third phenoxyalkanoic acid converting enzyme. However, *tfdA*-like, *tfdAα*-like or *cadAB*-like genes were not detected in strain MH with PCR- and hybridization experiments.

Finally, we modeled the native structure of RdpA and SdpA with TauD as a template and speculated about the active sites of the two enzymes. The overall structures of RdpA and SdpA could be modeled on the TauD structure and showed the typical jellyroll motif. The model confirmed the predicted iron(II)- and α-ketoglutarate-binding residues in both dioxygenases. In both proteins, a cavity was formed by several amino acid residues, which had the potential to bind the aromatic ring of the phenoxypropanoic acids. The most striking difference between RdpA and SdpA was the replacement of the neutral Gly-107 in RdpA by the basic Asn-95 in SdpA at the active site. This indicates an important role of this residue in interacting with the alkyl- or the carboxy-moiety of the substrates and hence also in determining the stereospecificity of the enzyme. Since these are only model predictions based on an existing template structure, further direct crystallization studies are needed to obtain a more appropriate view on the native fold and the active sites of RdpA and SdpA. The work presented here gives the basis for such experiments.
ZUSAMMENFASSUNG

Das Bakterium *Sphingomonas herbicidivorans* MH baut Phenoxyalkansäure-Herbizide wie die chiralen Verbindungen Mecoprop (2-((R,S)-2-Methyl-4-chlorophenoxypropionsäure) und Dichlorprop (2-((R,S)-2,4-Dichlorphenoxypropionsäure) ab. Der Stamm MH baut Mecoprop und Dichlorprop enantioselektiv ab, wobei das (S) Enantiomer bevorzugt umgewandelt wird. Diese Doktorarbeit wurde ausgeführt um (i) den genetischen Hintergrund des Mecoprop- und Dichlorprop-Abbauweges zu untersuchen und um (ii) die zwei α-Ketoglutarat abhängigen Dioxygenasen zu charakterisieren, die den ersten Abbauabschnitt katalysieren und um die Eigenschaften dieser enantioselektiven Enzymreaktion zu studieren.

Zusammenfassung

Im Gegensatz dazu können DccA$_{j}$ und DccA$_{ii}$ nicht mit den bekannten Chlorocatechol 1,2-Dioxygenasen von *Beta*- und *Gammaproteobakterien* gruppiert werden, sondern bilden einen eigenen Ast in einer Klusteranalyse. Daher wird vorgeschlagen, dass verschiedene Reservoirs von Genen des modifizierten *ortho*-cleavage Abbauweges in *Alphaproteobakterien* und *Beta*- und *Gammaproteobakterien* existieren und dass die Gene zwischen diesen Bakteriengruppen nur begrenzt ausgetauscht werden.

Im weiteren untersuchten wir die zwei *α*-Ketoglutarat abhängigen Dichlorprop Dioxygenasen, die 30% identisch zueinander sind. Die Aminosäuresequenz von RdpA ist 100% identisch mit derjenigen von RdpA vom Dichlorprop-Abbauer *D. acidovorans* MC1, während die Aminosäuresequenz von SdpA nur 63% identisch ist mit derjenigen von SdpA von Stamm MC1. Die Identität zu TfdA und TauD liegt im Bereich zwischen 30–37%. Ein in *α*-Ketoglutarat abhängigen Dioxygenasen konserviertes Motiv, HXDX$_{24}$TX$_{13}$HX$_{10}$R in RdpA und HXDX$_{24}$TX$_{12}$HX$_{10}$R in SdpA, wurde in beiden Proteinen gefunden. Für RdpA wurde vorhergesagt, dass His-111, Asp-113 und His-270 die 2-His-1-carboxylate facial triad bilden, die in die Eisen(II)-Bindung involviert ist, und Thr-138 und Arg-281 bilden mögliche *α*-Ketoglutarat bindende Aminosäuren. In SdpA wurden His-102, Asp-104 und His-257 als Eisen(II)-bindende und Thr-129 und Arg-268 als *α*-Ketoglutarat bindende Aminosäuren vorhergesagt.

S. herbicidovorans MH wächst auf 2,4-D, aber weder RdpA noch SdpA wandeln 2,4-D mit einer signifikanten Umsatzrate um. Dies deutet darauf hin, dass ein drittes, Phenoxyalkansäure umsetzendes Enzym existiert. Dennoch wurden mit PCR- und Hybridisierungsexperimenten keine tfdA-ähnliche, tfdAα-ähnliche oder cadAB-ähnliche Gene im Stamm MH gefunden.

CHAPTER 1

General Introduction
OUTLINE OF THE THESIS

Herbicides are applied in large amounts and are thereby introduced into the environment. Besides the desired effects, they are designed for, they contaminate and may harm ecosystems. Because of their synthetic structure and recalcitrance, it is important to know, if and how herbicides are degraded. In particular, many herbicides (such as mecoprop and dichlorprop) are chiral compounds, which can effect degradability, but is often not taken into account. We propose that the herbicide enantiomers have to be treated as individual molecules and that degradation has to be studied for each of them separately.

Microbial degradation is an important route for mineralization of phenoxyalkanoic acid herbicides. Many pure bacterial cultures have been described that metabolize phenoxyalkanoic acid herbicides, among which Sphingomonas herbicidovorans MH. Strain MH grows on mecoprop, dichlorprop and 2,4-dichlorophenoxyacetic acid as sole carbon and energy source. Interestingly, S. herbicidovorans MH transforms both enantiomers, but prefers (S)-mecoprop and (S)-dichlorprop over the (R) enantiomers. Enantioselective degradation of such herbicides is due to different uptake systems and two distinct α-ketoglutarate-dependent dioxygenases involved in the initial degradation step of mecoprop and dichlorprop to the achiral phenol. Despite the knowledge on the uptake and dioxygenase activities, the genes for the mecoprop and dichlorprop degradation pathway had not been isolated and studied so far in S. herbicidovorans MH.

The first goal of this thesis was to investigate the genetic background of phenoxyalkanoic acid herbicide degradation in S. herbicidovorans MH. Specific research questions to be answered were: (i) Which genes are involved in the degradation pathway of the (R)- and (S)-enantiomer of phenoxypropanoic acid herbicides? (ii) How are the genes organized? (iii) How are they regulated? Secondly, we focused on the biochemical characterization of the two α-ketoglutarate-dependent dioxygenases, which are involved in the initial, enantioselective degradation step of mecoprop and dichlorprop. Our specific
interest was to compare the two enzymes to each other and to other dioxygenases of this family in terms of sequence similarity, substrate specificities, and physicochemical characteristics. Furthermore, models of the two dioxygenases were compared to gain insights into the molecular details of chiral catalysis.

The thesis is presented as follows. The introduction (chapter I) consists of two different parts. In the first part, the general problems with chiral substances in the environment and the state of the art on mecoprop and dichlorprop degradation are described. In the second part of the introduction, the family of α-ketoglutarate-dependent dioxygenases is introduced. Chapter II describes an analysis of the genes coding for the enzymes involved in phenoxyalkanoic acid degradation in *S. herbicidovorans* MH. In Chapter III and IV, we focus on the two enantioselective α-ketoglutarate-dependent dioxygenases and their biochemical characterization is presented. Chapter V will discuss the results obtained during this thesis work in a more general perspective and will present structure models of the two dioxygenases. Finally, the appendix contains the data from several growth studies and partial protein purifications of the (S)-dichlorprop dioxygenase.
General Introduction:
Chirality of Pollutants – Effects on Metabolism and Fate

In most cases, enantiomers of chiral compounds behave differently in biochemical processes. Therefore, the effects and the environmental fate of the enantiomers of chiral pollutants need to be investigated separately. In this review, the different fates of the enantiomers of chiral phenoxyalkanoic acid herbicides, acetamides, organochlorines, and linear alkylbenzenesulfonates are discussed. The focus lies on the biological degradation, which may be enantioselective in contrast to non-biotic conversions. The data show that it is difficult to predict which enantiomer may be enriched and that accumulation of an enantiomer is dependent on the environmental system, the species, and the organ. Racemization and enantiomerization processes occur and make the interpretation of the data even more complex. Enantioselective degradation implies that enzymes involved in the conversion of such compounds are able to differentiate between the enantiomers. ‘Enzyme pairs’ have evolved which exhibit an almost identical overall fold. Only subtle differences in their active site determine their enantioselectivities. On the other extreme, there are examples of non-homologous ‘enzyme pairs’ that have developed through convergent evolution to enantioselectively turn over the enantiomers of a chiral compound. For a better understanding of enantioselective reactions, more detailed studies of enzymes involved in enantioselective degradation need to be performed.

Chapter 1

INTRODUCTION

A chiral (Greek *cheir*, hand) object or molecule is not superimposable on its mirror-image whereas an achiral object can be superimposed on its mirror-image. An alternative definition is the lack of symmetrical elements: a chiral object lacks reflectional symmetry (123). Common known chiral objects are a person’s right and left hands, snail shells and clockwise- or counterclockwise-threaded screws. For chiral molecules, a tetrahedral C-atom bound to four different substituents is most common. The carbon atom is the stereogenic center and the two possible structures behave like the image and mirror-image of each other and are not superimposable. These structures are called enantiomers (Greek *enantios*, opposite). Amazingly, chirality is more the rule than the exception in our living world; and the important building blocks of life, such as DNA, RNA, and proteins, are all composed of chiral molecules. Moreover, they are homochiral – proteins consist of L-amino acids, and DNA and RNA consist of the D-enantiomers of deoxyribose and ribose, respectively.

The phenomenon of molecular chirality was first observed in the middle of the nineteenth century. Pasteur prepared sodium ammonium salts of the optically active (+)-tartaric acid and the optically inactive racemic acid. He observed that both crystals comprised hemihedral facets and that the hemihedral facets of the crystals of the optically active (+)-tartaric acid all lay in the same direction, whereas some of the hemihedral facets of the crystals of the optically inactive racemic acid inclined to the left and some to the right. He separated the crystals and found that the solution of crystals with hemihedral facets to the right deviated the plane of polarized light to the right and vice versa. He had discovered that the optical inactivity of racemic tartaric acid is due to the fact that it is a mixture of right- and left-handed tartaric acid. Later, van’t Hoff and Le Bel introduced the model of the tetrahedral carbon atom to explain enantiomerism.

Although pure enantiomers of chiral compounds have identical physico-chemical properties – for a more thorough discussion about racemates and their enantiomer compounds, see Eliel and Wilen (26) – their behavior in biochemical
processes might be strikingly different. The effects of differently acting stereoisomeric drugs can be categorized basically as follows (5).

a) The stereoisomers of a chiral drug may have similar modes of action, but may differ in their affinity to a receptor or an enzyme resulting in different reaction rates.

b) The inactive stereoisomer may act as a competitive antagonist.

c) Enantiomers may have opposite or different effects as is the case for barbiturates, where the (–)-enantiomer is a sedative and the (+)-enantiomer has convulsive effects.

d) There are many chiral drugs for which one or both enantiomers have the desired effect and only one enantiomer causes unwanted side-effects. Well known is the Contergan tragedy. Contergan was a sedative that contained racemic thalidomide. Both enantiomers had the desired therapeutic effects, whereas only the (S)-enantiomer had teratogenic effects and caused severe malformations of human babies (7).

e) Side-effects may be non-stereoselective and both isomers may cause them, but only one isomer may have the desired effect.

f) In contrast to this, the inactive enantiomer may antagonize the side-effects of the active isomer. In such cases, an enantiomerically pure compound is not preferred.

As this list shows, the relationships between the effects of active and inactive stereoisomers in a pharmacological context can get quite complicated and certainly cannot be easily predicted, but needs to be empirically established. Therefore, stereoisomers should be treated as separate drugs and developed accordingly (32).

It is important to note that the dispositions mentioned above not only hold for pharmacologically active compounds but equally apply to all bioactive compounds that are chiral. Chiral pesticides, for instance, are introduced into the environment in large amounts as racemic mixtures. In many cases, the effects of the enantiomers of herbicides on plants have been investigated, but not the fates of
the enantiomers after application. Furthermore, not much is known about the degradation potential of the enantiomers. The application of racemic mixtures leads often to ‘isomeric ballast’, thereby unnecessarily polluting the environment (6, 143).

In the environment, abiotic transformations of chiral compounds are mostly non-enantioselective. This is in contrast to biological processes, which usually proceed with high stereo- or enantioselectivity. Therefore, changes in enantiomeric ratios (ER) and enantiomeric fractions (EF) are good indicators of biological degradation. The ER is defined as the ratio of the concentration of one of the enantiomers of a chiral compound divided by the concentration of the other enantiomer; and the EF is defined as the ratio of ER divided by ER+1 (42, 44, 63, 144, 147).

Table 1: Chiral environmental pollutants discussed in this review

<table>
<thead>
<tr>
<th>Class</th>
<th>Representatives</th>
<th>Main use</th>
<th>Environmental systems discussed in this review</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenoxypropanoic acids</td>
<td>Mecoprop, dichlorprop</td>
<td>Herbicide</td>
<td>Soil, aquatic systems, wastewater treatment plants, sewage sludge, landfill leachates, plants</td>
</tr>
<tr>
<td>Acetamide pesticides</td>
<td>Acetochlor, metalaxyl, metolachlor, dimethenamid</td>
<td>Pesticide</td>
<td>Soil, aquatic systems, sewage sludge, plants</td>
</tr>
<tr>
<td>Organochlorines</td>
<td>α-Hexachlorocyclohexane, chlordane</td>
<td>Contaminant of technical lindane (insecticide)</td>
<td>Soil, sediment, aquatic systems, sewage sludge, methanogenic conditions, biota</td>
</tr>
<tr>
<td>Linear alkylbenzene-</td>
<td></td>
<td>Detergent</td>
<td>Soil</td>
</tr>
<tr>
<td>sulfonates (LAS), linear</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>alkylbenzenes (LAB)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Here, we review the literature on the metabolism and environmental fate of chiral pollutants (Table 1). We focus on differences in the environmental fate of the enantiomers, i.e. on enantioselective biotic conversions. We intend to show how important it is to differentiate between enantiomers and stereoisomers and to treat enantiomers as distinct compounds with respect to their degradation potential. Furthermore, we describe enzymes involved in enantioselective processes. We
discuss stereoselectivity and the similarities and differences between such enzyme pairs that act on the enantiomers of chiral substrates.

**CHIRAL POLLUTANTS AND THEIR FATE IN THE ENVIRONMENT**

**Phenoxypropanoic acid herbicides.** Representatives of phenoxyalkanoic acid herbicides are 2,4-dichlorophenoxyacetic acid (2,4-D), 2-methyl-4-chlorophenoxyacetic acid (MCPA), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and the chiral compounds mecoprop (2-((R,S)-2-methyl-4-chlorophenoxypropanoic acid) and dichlorprop (2-(R,S)-2,4-dichlorophenoxypropanoic acid; Fig. 1). They are systemic and post-emergence herbicides and act as synthetic auxins (1, 2, 77). They were introduced in the 1940s and 1950s to control broadleaf weeds in agriculture, lawn pastures and industry. Racemic mecoprop is also used to control growth of weeds in building material such as bituminous seals, insulators for flat roofs and rubber seals (9, 10). Phenoxyalkanoic acid herbicides, often applied in formulation with other herbicides, are among the most widely used herbicides in the world (145). In 1999, 12.7–15.0×10^6 kg active ingredients (a.i.) of 2,4-D were used to control broadleaf weeds in agriculture, 7.7–9.0×10^6 kg a.i. in the industrial/commercial/government market and 3.2–4.1×10^6 kg a.i. in the home and garden sector in the United States (22). Mecoprop was applied in a range of 1.4–2.3×10^6 kg a.i. in the United States (22).

The chiral herbicides mecoprop and dichlorprop each have one stereogenic center and, therefore, two enantiomers exist (Fig. 1). Since 1953, it has been known that only the (R) enantiomers show herbicidal activity (83). Nevertheless, the racemic mixtures were and still are applied, thereby introducing large amounts of isomeric ballast into the environment. In many countries, mecoprop and dichlorprop are nowadays also sold as enantiomerically pure compounds (named mecoprop-P and dichlorprop-P, respectively; 143).
Fig. 1. Chemical structure of the phenoxyalkanoic acid herbicides 2,4-dichlorophenoxyacetic acid (2,4-D, A), which is achiral, and the two enantiomers of dichlorprop, which is chiral (B). C Proposed degradation pathway of (R)- and (S)-mecoprop by *Alcaligenes denitrificans* and *Sphingomonas herbicidovorans* MH. Note that *A. denitrificans* exclusively degrades (R)-mecoprop, whereas *S. herbicidovorans* MH degrades both enantiomers (modified from 105, 134, Chapter 2).

As outlined above, the enantiomers of chiral compounds behave differently in biochemical processes. Therefore, to study the fate of chiral herbicides, it is important to differentiate between the enantiomers and to monitor the different degradation patterns. Today, GC, GC-MS, and HPLC methods are available to
Chirality of pollutants

separate and quantitate enantiomers and to selectively investigate the environmental fate of the enantiomers of chiral pollutants (90).

The environmental fate of phenoxyalkanoic acid herbicides is determined by their physico-chemical properties and by their biodegradability. They are water soluble (up to 620 mg/l) and are strong acids (145). Since they are mostly present in the dissociated (anionic) form in the environment, they do not adsorb onto soil and have a low tendency to accumulate in organic matter (33, 149). The half-life in soil after application is from one to several weeks (Table 2) and, due to their mobility, there is a risk of contaminating aquatic systems (46). Indeed, phenoxyalkanoic acid herbicides are often found in subsurface and groundwater samples (33, 38). Concentrations in surface soils were in the range of milligrams per kilogram of soil, whereas concentrations were lower in groundwater samples, i.e., 10–250 µg/l downstream of landfills (80, 149) and less than 1 µg/l in groundwater aquifers polluted due to agricultural use (120).

The effect of environmental conditions on the photodecomposition of dichlorprop and mecoprop in soil surfaces was investigated by Romero et al. (116). They found that, on dry soil surfaces, photolysis may dominate other transformation pathways. However, in moist soils, photodecomposition played an important role only during the first 2 days of exposure. Afterwards, when microbial degradation became dominant, photodecomposition was much less important. In a study using different types of calcareous soils, mecoprop and dichlorprop dissipation was investigated (117). Generally, degradation was slower in clay loam soils than in silt and sandy loam soils (Table 2). Dissipation was enantioselective indicating biological degradation and was dependent on the soil type. The (S) enantiomer persisted for a longer time in silt and sandy loam soils than in clay loam soils. The addition of peat had intriguing effects as the persistence of mecoprop and dichlorprop increased in silt and sandy loam soils, but decreased in clay loam soils. Lewis et al. (73) studied the effect of environmental changes on the enantioselective degradation of mecoprop and dichlorprop in Brazilian forest and pasture soils. The pasture samples preferentially degraded (S)-dichlorprop, whereas both enantiomers were equally transformed in forest
samples. Enrichments in organic nutrients shifted the enantioselectivity for methyl-
dichlorprop towards the preferential degradation of the (S) enantiomer. Other
studies showed, that the (S) enantiomer was degraded significantly faster in soil
than the herbicidally active (R) enantiomer (37, 90). Only two studies reported
enantiomerization and/or racemization (13, 90). Both studies showed that
enantiomerization was biologically mediated. The authors drew the important
conclusion that, due to enantiomerization, the measured enantiomeric composition
of residues does not reflect that of the applied products. Additionally, two-phase
degradation kinetics with initially slower rates was observed in one of these studies
(90). The authors suggested that two or more different enzyme systems are
involved in the degradation.

In marine systems polluted with racemic dichlorprop, (R)-dichlorprop is
preferentially degraded by microorganisms (78, 79). In aerobic aquifer samples
incubated with different concentrations of mecoprop in laboratory experiments,
mecoprop was degraded within 200 days (46). (R)- and (S)-mecoprop was found in
equal concentrations in the landfill leachate of Kölliken (Switzerland), indicating a
racemic mixture of mecoprop in the landfill itself. But in groundwater samples
downstream of the landfill, the enantiomeric ratio of mecoprop increased, i.e., (R)-
mecoprop was in excess. As sorption to organic material was of minor importance
and should be non-enantioselective, this finding indicates in-situ biodegradation
(149). Williams et al. (144) showed in a recent study that degradation of mecoprop
in a limestone aquifer downstream of a landfill depended on the redox conditions.
Under methanogenic, sulphate-reducing or iron-reducing conditions, mecoprop
was not degraded. In nitrate-reducing microcosms, (R)-mecoprop was degraded,
whereas the (S) enantiomer was stable. In contrast, (S)-mecoprop degraded faster
than (R)-mecoprop under aerobic conditions. In a field experiment in Denmark,
mecoprop and dichlorprop were degraded in an aerobic aquifer within a distance of
1 m from the source within a period of 120 days. But in contrast to the reports
mentioned beforehand, no enantioselective degradation was observed (118).
Table 2: Degradation rates for dichlorprop and mecoprop found in environmental samples

<table>
<thead>
<tr>
<th>Compound</th>
<th>System</th>
<th>Sample</th>
<th>Rate constants (t_{1/2}; \text{days}) ((R,S)-)</th>
<th>Degradation time ((\text{days})^a), ((\text{lag phase}; \text{days}))</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dichlorprop</td>
<td>Brazilian soil</td>
<td>Forest</td>
<td>((11)^{bc})</td>
<td></td>
<td>(73)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pasture</td>
<td>((16)^{bd})</td>
<td></td>
<td>(73)</td>
</tr>
<tr>
<td></td>
<td>Calcareous soils</td>
<td>Silt loam</td>
<td>55 (13)$^c$</td>
<td>41 (17)$^e$</td>
<td>(117)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sandy loam</td>
<td>53 (13)$^c$</td>
<td>47 (15)$^e$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Clay loam</td>
<td>11 (62)$^c$</td>
<td>18 (38)$^e$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Garden soil</td>
<td>Sandy loam</td>
<td>32 (21.9), 150 ((4.6)^{e})</td>
<td>98 (7.1), 176 ((3.9)^{e})</td>
<td>(90)</td>
</tr>
<tr>
<td></td>
<td>Soil</td>
<td></td>
<td>((6.6)^b)</td>
<td>((8.7)^b)</td>
<td>(37)</td>
</tr>
<tr>
<td></td>
<td>Aquatic system</td>
<td>Aerobic aquifer (in situ)</td>
<td>213±41, 352±92$^i$</td>
<td>225±79, 188±3$^i$</td>
<td>(118)</td>
</tr>
<tr>
<td></td>
<td>Sewage sludge</td>
<td>Aerobic</td>
<td></td>
<td></td>
<td>(4.33-6.2) (146)</td>
</tr>
<tr>
<td>Mecoprop</td>
<td>Calcareous soils</td>
<td>Silt loam</td>
<td>43 (15)$^c$</td>
<td>33 (21)$^c$</td>
<td>(117)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sandy loam</td>
<td>63 (11)$^c$</td>
<td>56 (12)$^c$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Clay loam</td>
<td>14 (50)$^c$</td>
<td>22 (32)$^c$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Soil</td>
<td>Sandy loam</td>
<td>((9±1)^c)</td>
<td></td>
<td>(126)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heavy loam</td>
<td>((8±2)^c)</td>
<td></td>
<td>(126)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Clay loam</td>
<td>((7±2)^c)</td>
<td></td>
<td>(126)</td>
</tr>
<tr>
<td></td>
<td>Garden soil</td>
<td>Sandy loam</td>
<td>69 (10), 174 (4)$^{c,e}$</td>
<td>102 (6.8), 189 ((3.7)^{c,e})</td>
<td>(90)</td>
</tr>
<tr>
<td></td>
<td>Aquatic system</td>
<td>Aerobic aquifer (lab)</td>
<td></td>
<td></td>
<td>(46)</td>
</tr>
</tbody>
</table>

Chirality of pollutants
<table>
<thead>
<tr>
<th>Compound System</th>
<th>Sample</th>
<th>Rate constants (t_{1/2}; days)</th>
<th>Degradation time (days)^a, (lag phase; days)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(R,S)- (R)- (S)-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aerobic aquifer (in situ)</td>
<td></td>
<td>106-132 (60-80)^a</td>
<td></td>
<td>118</td>
</tr>
<tr>
<td>Limestone aquifer: aerobic</td>
<td></td>
<td>1.32^i 1.9^i</td>
<td></td>
<td>144</td>
</tr>
<tr>
<td>Limestone aquifer: nitrate-reducing</td>
<td></td>
<td>0.65^i not degraded</td>
<td></td>
<td>144</td>
</tr>
<tr>
<td>Limestone aquifer: Fe-reducing (NO₃⁻ added)</td>
<td></td>
<td>225 (21)^m</td>
<td></td>
<td>144</td>
</tr>
<tr>
<td>Limestone aquifer: methanogen/sulphatreducing</td>
<td></td>
<td>not degraded</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sewage sludge Aerobic</td>
<td></td>
<td>176±28, 304±88^i</td>
<td>174±52, 273±47^j (4.33-6.2)</td>
<td>146</td>
</tr>
</tbody>
</table>

^a Degradation time is defined as the time until dichlorprop or mecoprop was degraded to a concentration below the detection limit.
^b First-order kinetic.
^c In 38% of the samples, the (S) enantiomer was degraded faster, in 13% the (R) enantiomer.
^d In 93% of the samples, the (S) enantiomer was degraded faster, in 6.7% the (R) enantiomer.
^e Data obtained from experiments with the racemate. First-order kinetic, unit of the rate constant: 10⁻³ day⁻¹.
^f Data obtained from experiments with the single pure enantiomer. First-order kinetic, unit of the rate constant: 10⁻³ day⁻¹.
^g The two rate constants were determined during two degradation phases: slow phase 0-16 days, fast phase 16-35 days.
^h Non-enantioselective degradation.
^i Data obtained from experiments with the racemate (first number) and the single enantiomer (second number). Zero-order kinetic (x μmol·h⁻¹·g⁻¹ dry weight).
^j The concentrations were too low to measure enantioselective degradation. However, a second lag phase after removal of 50% of the initial concentration strongly indicated enantioselective degradation.
^k Data obtained from experiments with the racemate. Zero-order kinetic, units expressed as x μmol·h⁻¹·g⁻¹ dry weight.
^l No degradation was observed for 119 days. Nitrate was then added to the microcosm and, after a lag-phase of 21 days, mecoprop degraded suggesting that nitrate acted as an electron-acceptor.
Experiments with activated and digested sludge showed that phenoxypropanoic acid herbicides are degraded aerobically but not anaerobically. Mecoprop and dichlorprop (10–40 mg/l) were degraded aerobically within 7 days, the \((S)\) enantiomers being preferentially degraded, while degradation was not observed during 49 days of incubation under anaerobic conditions (146). An experiment with 1 mM mecoprop in a simulated wastewater treatment plant showed 100% removal after 40 days (106).

All these studies showed that biological degradation is the most important process by which these herbicides are eliminated from the environment. Furthermore, biological degradation was enantioselective in most cases, emphasizing the importance of investigating the environmental fate of each enantiomer separately. In these cases, the observed changes in enantiomeric fractions or ratios gave conclusive evidence for natural attenuation of the herbicides (144, 149). However, racemization may occur and therefore, careful experiments and measurements are needed for solid interpretations.

In most cases, microorganisms enantioselectively degrade mecoprop and dichlorprop. A particular strain might degrade only one enantiomer or it might sequentially degrade the two enantiomers. Degradation was well investigated for the achiral phenoxyalkanoic acid herbicide 2,4-D in \textit{Ralstonia eutropha} JMP134(pJP4). 2,4-D is degraded by an \(\alpha\)-ketoglutarate-dependent dioxygenase (TfdA) by ether-bond cleavage to 2,4-dichlorophenol (35, 36, 130). Then, 2,4-dichlorophenol is hydroxylated to 3,5-dichlorocatechol by a phenol-hydroxylase (TfdB; 31, 76, 111). The catechol undergoes ortho-ring fission catalyzed by a chlorocatechol 1,2-dioxygenase (TfdC) yielding 2,4-dichloro-\(\textit{cis},\textit{cis}\) muconate (8, 111), which then is metabolized to \(\textit{cis}\)-2-chlorodiene lactone and further to 2-chloromaleylacetate by a muconate cycloisomerase and a diene lactone hydrolase (TfdD and TfdE, respectively; 66, 111). It was suggested that the chiral phenoxyalkanoic acid herbicides are degraded accordingly. Table 3 lists all known bacterial strains in pure culture that are able to grow with mecoprop and dichlorprop as the sole carbon and energy source. The earliest reported investigations were made with \textit{Alcaligenes denitrificans} (Table 3), which was isolated from a consortium and
exclusively degrades the \( (R) \) enantiomer using it as sole carbon and energy source (133, 134). \( (R) \)-mecoprop degradation was shown to proceed in a manner analogous to 2,4-D degradation, i.e., degradation to the achiral 4-chloro-2-methylphenol and then to 5-chloro-3-methylcatechol (Fig. 1C). Subsequently, 5-chloro-3-methylcatechol is degraded through the modified ortho-cleavage pathway, yielding 2-methylfural, 2-methylmaleylacetate, and finally 5-methyl-3-oxoadipate. In a further study, the question was addressed whether the genes encoding the enzymes involved in mecoprop degradation were similar to those encoding the enzymes for 2,4-D degradation. A fragment of a \( tfdA \)-like gene was amplified by PCR from \( A. \) denitrificans and sequenced. It was 86% identical to the corresponding region of \( tfdA \) from \( R. \) eutropha JMP134(pJP4) (119). However, the \( \alpha \)-ketoglutarate-dependent dioxygenase and the other enzymes involved in degradation have not been further characterized in this strain.

In 1990, \( Sphingomonas \) hericidivorans MH (formerly \( Flavobacterium \) sp.) was isolated from soil samples polluted with dichlorprop (49, 148). In contrast to \( A. \) denitrificans, \( S. \) hericidivorans MH is able to degrade both enantiomers of dichlorprop and mecoprop to completion (148). Growth experiments with either the racemate or single isomers showed that \( S. \) hericidivorans MH degraded the substrates enantioselectively. The \( (S) \) enantiomer was degraded before the \( (R) \) enantiomer in all growth experiments performed so far. When \( S. \) hericidivorans MH was incubated with the single enantiomer, a lag phase of about 3 days for \( (S) \)- and one of about 7 days for \( (R) \)-mecoprop was observed. However, when grown on the racemate, degradation is sequential, the \( (S) \) enantiomer being used first (148). Enantioselectivity was also found for substrate uptake. When \( S. \) hericidivorans MH grew on the single enantiomers, only the enantiomer that served as the substrate was taken up. These data and further experiments indicated that \( S. \) hericidivorans MH harbors two inducible transport systems involved in enantioselective uptake (105, 147).
<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenoxyalkanoic acid substrates</th>
<th>Isolation source</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Sphingomonas herbicidovorans</em> MH</td>
<td>(R,S)-Mecoprop, (R,S)-Dichlorprop, 2,4-D, MCPA</td>
<td>Soil (dichlorprop)</td>
<td>(49, 63, 148)</td>
</tr>
<tr>
<td><em>Delftia</em> (formerly <em>Comamonas</em>) <em>acidovorans</em> MC1</td>
<td>(R,S)-Mecoprop, (R,S)-Dichlorprop, 2,4-D, MCPA</td>
<td>Herbicide contaminated building rubble (2,4-D)</td>
<td>(93, 94)</td>
</tr>
<tr>
<td><em>Rhodoferax</em> sp. strain P230</td>
<td>(R,S)-Mecoprop, (R,S)-Dichlorprop, 2,4-D, MCPA</td>
<td>Concrete rubble of a demolished herbicide production plant (2,4-dichlorophenol)</td>
<td>(25)</td>
</tr>
<tr>
<td><em>Alcaligenes denitrificans</em></td>
<td>(R)-Mecoprop</td>
<td>Garden soil</td>
<td>(134)</td>
</tr>
<tr>
<td><em>Alcaligenes</em> sp. CS1</td>
<td>(R,S)-Mecoprop, (R,S)-Dichlorprop, 2,4-D, MCPA</td>
<td>Agricultural soil (mecoprop)</td>
<td>(125)</td>
</tr>
<tr>
<td><em>Ralstonia</em> sp. CS2</td>
<td>(R,S)-Mecoprop, (R,S)-Dichlorprop, 2,4-D, MCPA</td>
<td>Agricultural soil (mecoprop)</td>
<td>(125)</td>
</tr>
</tbody>
</table>
Two strains, *Alcaligenes* sp. CS1 and *Ralstonia* sp. CS2, were isolated from agricultural soils. They are able to degrade racemic mecoprop and dichlorprop in addition to the achiral herbicides 2,4-D and MCPA (125). The genomes of both isolates were screened under low-stringency conditions with *tfd*-gene probes in hybridization experiments. The experiments demonstrated that both strains harbored *tfdABC*-like genes on plasmids. *Rhodoferax* strain P230, another strain that is able to degrade chiral phenoxyalkanoic acid herbicides was isolated from contaminated building material. According to preliminary PCR experiments, this strain also harbors *tfdA*-like genes (25). *Delftia acidovorans* MC1 (formerly *Comamonas acidovorans* MC1) was isolated from a herbicide-contaminated building site (93, 94). The strain is able to degrade racemic dichlorprop but, unfortunately, enantiospecific degradation was not investigated. To isolate the genes coding for the enzymes, PCR experiments were carried out with primers for *tfdA*, *tfdB*, and *tfdC* (62, 136) revealing the presence of *tfdBC* genes. In contrast to these genes, a *tfdA*-like gene could not be amplified with the applied primers although enzyme activities dependent on ferrous ions and α-ketoglutarate could be detected in cell-free extracts.

**Acetamide pesticides.** The group of acetamide pesticides comprises a large number of herbicides and fungicides (Fig. 2). The activity is dependent on the acyl moiety. In the case of herbicides, the substituent is often –CH$_2$Cl, whereas in the case of fungicides the substituent is often –CH$_2$OCH$_3$. Acetamide pesticides are used to control annual grasses and certain broadleaf weeds in corn, soybeans, and peanuts. They are also used to control phytopathogenic fungi, such as *Peronosporales* in potatoes, sugar beets, and other crops (72, 122, 145). Acetamide pesticides act as protein synthesis inhibitors (18) and RNA-polymerase I inhibitors (11). The compounds metolachlor [2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)acetamide], metolachlor-s and alachlor [2-chloro-N-(2,6-diethylphenyl)-N-(methoxymethyl)acetamide] are among the most commonly used pesticides in the world and in 1999, 11.8–13.6×10$^6$ kg, 7.3–8.6×10$^6$ kg, and 3.2–4.5×10$^6$ kg million a.i., respectively, were applied in the United States (22).
Other commonly applied acetamide pesticides are acetochlor [2-chloro-N-(2-ethyl-6-methylphenyl)-N-(ethoxymethyl)acetamide], metalaxyl [N-(2,6-dimethyl-phenyl)-N-(methoxyacetyl)alanine methyl ester] and dimethenamid [2-chloro-N-(2,4-dimethylthien-3-yl)-N-(2-methoxyl-1-methylethyl)acetamide]. Alachlor is achiral, acetochlor is axial-chiral and metalaxyl is C-chiral, giving two enantiomers. Dimethenamid and metolachlor are axial and C-chiral and, therefore, four stereoisomers exist. For metolachlor, herbicidal activity is exclusively associated with the 1’(S) isomers. In the case of metalaxyl, the (R) enantiomer is about 3–10 times more fungicidally active than the (S) enantiomer (34).

Degradation of different acetamides in soil and sewage sludge proceeds in the order alachlor > acetochlor > dimethenamid > metolachlor > metalaxyl, with half-lives ranging from 4–32 days in soil and from 10–86 hours in sewage sludge (14, 91). Dimethenamid and metolachlor degradation showed low to moderate enantio- and/or stereoselectivity, whereas metalaxyl was degraded highly enantioselectively. Enantioselectivity was dependent on the environmental system: in soil, 1’(R)-(−)-Metalaxyl was degraded faster than the (S) enantiomer, whereas reversed enantioselectivity was found in sewage sludge (14, 91). Different rates and opposite enantioselectivity were also found in a study in which the degradation in soil was compared to the one in plants. The (R) enantiomer was degraded faster in soil, the (S) enantiomer faster in plants (82). In a recent study (16), the faster degradation of the herbicidal active (R) enantiomer in soil was confirmed. It was also found, that only 40–50% of metalaxyl was degraded to the chiral intermediate metalaxyl carboxylic acid (MX-acid; 2-[(2,6-dimethylphenyl)methoxyacetyl-amino]propanoic acid). In this step, the configuration of the chiral C-atom was retained. Further degradation of MX-acid is also enantioselective, the (S) enantiomer being converted more rapidly. The remaining metalaxyl is metabolized through other pathways. Both enantiomers of metalaxyl and MX-acid are configurationally stable and no indications of biological mediated racemization or enantiomerization have been found (16, 82). Monkiedje and coworkers (89) investigated the degradation and persistence of racemic and enantiopure forms of metalaxyl in temperate and tropical soils. All forms of metalaxyl had lower degra-
dation rates in Cameroonian soils than in German soils. Interestingly, an opposite enantioselectivity was observed in the two regions. The \((R)\) enantiomer was faster degraded than the \((S)\) enantiomer in German soils, but slower than the \((S)\) enantiomer in Cameroonian soils. This finding suggests that different microbial populations are involved in the degradation process. In another recent study, the enantioselectivity correlated with the soil pH (12). In aerobic soils with pH>5, the \((R)\) enantiomer was faster degraded than the \((S)\) enantiomer. In aerobic soils with pH 4–5, both enantiomers were degraded similarly, whereas in aerobic soils with pH<4 and in anaerobic soils, the enantioselectivity was reversed. The authors reevaluated published kinetic data from dichlorprop and mecoprop studies and found indications of similar correlations. However, no correlation between enantioselectivity and soil pH was observed for MX-acid, the chiral intermediate.

**Fig. 2.** Chemical structures of acetamide pesticides. **A** General structure. \(R\) and \(R’\) represent \(\text{CH}_3\) and/or \(\text{C}_2\text{H}_5\). **B-F** Structures of the five acetamide pesticide alachlor (achiral), metolachlor, acetochlor, metalaxyl, and dimethenamid. The chiral centers and the axial-chiral elements are indicated by asterisks. Note that, in dimethenamid, phenyl is replaced by 2,4-dimethylthien-3-yl.
In the case of metolachlor, there is currently a chiral switch from racemic metolachlor to (S)-metolachlor, which is highly enriched in the herbicidally active enantiomer. Most likely, this results in lower overall concentrations and a changed enantiomeric ratio in environmental samples. Indeed, in Switzerland, a rapid response in terms of the enantiomeric composition was observed in surface waters after the replacement of the racemic metolachlor by the highly enriched enantiomer (17).

**Organochlorines: hexachlorocyclohexane and chlordane.** Hexachlorocyclohexane (HCH) belongs to the organochlorine compounds and used to be one of the most widely applied broad-spectrum insecticides. It was introduced during world war II (92) and was used mainly in forestry, agriculture, and as a wood preservative. HCH comprises eight isomers, the chiral α-, and the achiral β-, γ-, δ-, ε-, η-, υ-, and ι-isomers, of which only the γ-HCH has insecticidal properties. Technical grade lindane (Fig. 3A) consists typically of 60–70% α-HCH, 5–12% β-HCH, 10–15% γ-HCH and 6–10% δ-HCH (15, 56), whereas the commercial insecticide marketed today comprises 99% of the γ-isomer. In Europe, the use of technical grade lindane was banned in the 1970s and in 2000, lindane was completely banned for all agricultural and gardening applications. Although the total amount of applied HCH was reduced globally, it is still a contaminant of great concern in many countries (e.g., in India). HCH is semivolatile and has a low tendency to accumulate in soils. It is transported by water and air and, today, HCH has accumulated in regions where HCH never was used, such as the Arctic and the Baltic Sea (see for instance (42, 84, 141). Chlordane is another representative of the organochlorine pesticides and was widely applied in the United States from 1945 until it was banned in 1988 (20, 43). Technical chlordane comprises 140 compounds and was used to control pests in lawn, garden, and crops and as a termiticide (43). The organochlorine representatives cis- and trans-chlordane (Fig. 3B) and heptachlor epoxide, a metabolite of heptachlor, are chiral and each enantiomer has different biological properties and environmental fate (112, for a review see 44).
Today, the Arctic Ocean is a source of HCH. HCH is eliminated from the Arctic Ocean by water outflow, volatilization, and degradation (72). A study of the removal of α- and γ-HCH in the eastern Arctic ocean found that the rate constants for microbial degradation were about 3–10 times higher than those for hydrolysis (42). Enantioselective degradation was observed, the (+)-α-HCH being converted...
preferentially. The calculated half-lives for (+)-α-HCH, (–)-α-HCH, and γ-HCH were 5.9, 23.1, and 18.8 years, respectively. However, reversed enantioselectivity was found in other marine environments, such as the Bering Sea and the Chukchi Seas (57). Enantioselective degradation was also investigated in other aquatic environments. It was found that enantioselective degradation was greatest in small Arctic lakes with enantiomeric ratios between 0.3 and 0.7, although the nutrient availability in such lakes is very low (29, 71). Law et al. suggested (71) that this was due to biofilm formation and environmental conditions in which the contact between α-HCH and the microbial population was maximized by either the path length, as in streams, or the long water residence time, as in lakes. In the food web, no preference for the enrichment of one enantiomer in higher trophic levels could be observed. Cetaceans, dolphin species, showed preferential accumulation of (+)-α-HCH (54). (+)-α-HCH was also more abundant than the (–) enantiomer in the blubber of harbor seals, grey seals, and harp seals and in the liver, kidney, and muscle tissues of ducks (54). In contrast to this, an ER of <1 was found in hooded seals (54). In invertebrates, mussels, and fishes, the ratio reflected that of the surrounding water, indicating no preferential accumulation (52, 88). Also, for chlordane compounds, no uniform trend for ER changes and increasing trophic levels could be found (142). These findings suggest that it will be difficult to predict which enantiomer may be enriched and that accumulation is dependent on both the organ and the species.

In soils, organochlorines are also enantioselectively degraded. In soils from the United States corn belt, (+)-trans chlordane was preferentially degraded, whereas for cis-chlordane, the (–)-enantiomer was converted faster except in four soils, in which non-enantioselective degradation was found (3). α-HCH was enantioselectively degraded in three muck soils, but not in silt loam soils from British Columbia (30). In soils from near a former HCH factory, only low enantioselectivity was found (ER = 1.099; 92). In archived UK soils, statistically significant enantioselective degradation was not observed for cis- or trans-chlordane, nor for α-HCH (85). The reported half-lives for α-HCH and other organochlorines in soils were ~7 years (α-HCH) to 25 years (dieldrin; 85).
In anaerobic sewage sludge, HCH is degraded with half-lives of 20–178 h, in the order of $\gamma$-HCH > (+)-$\alpha$-HCH > (−)$\alpha$-HCH > $\delta$-HCH > $\beta$-HCH. Indications are given that degradation is 80–95% biologically mediated, although abiotic degradation in sterilized sewage sludge is much higher than hydrolysis in water (15, 104). $\alpha$-HCH is degraded enantioselectively, whereby the (+)-enantiomer is degraded faster. This leads to enrichment of (−)$\alpha$-HCH in sewage sludge (15). Middeldorp et al. (87) described a bacterial consortium which was able to degrade $\beta$-HCH as well as $\alpha$-, $\gamma$- and $\delta$-HCH under methanogenic conditions.

A microbial community able to degrade HCH was isolated from marine environments (53). It degrades the (+)$\alpha$-HCH and the corresponding $\beta$-penta-chlorocyclohexene ($\beta$-PCCH) faster than the respective enantiomers. Another consortium comprising eight bacterial strains and a fungus was isolated from soil and sewage. It preferentially degraded $\alpha$-HCH, but nothing is reported about enantioselectivity (81). Two Bacillus strains, B. circulans and B. brevis, isolated from contaminated soil, are able to degrade $\alpha$-, $\beta$-, $\gamma$- and $\delta$-HCH at high rates (40) and two Pseudomonas strains isolated from agricultural soils are able to degrade $\gamma$-HCH (102). S. paucimobilis UT26 is able to grow on $\gamma$-HCH as sole carbon- and energy source (55, 96, 98). The lin-genes were sequenced and shown to code for the enzymes involved in HCH-metabolism (97-99).

**Linear alkylbenzenesulfonates and linear alkylbenzenes.** Linear alkylbenzenesulfonates (LAS) and linear alkyl benzenes (LAB) and their degradation products are environmentally relevant chiral substances (Fig. 4). LAS are used as detergents and LAB are the precursors in LAS synthesis and are found in low amounts in commercial LAS (19, 48). LAS and LAB are biodegradable under aerobic conditions (132). It was suggested that degradation starts with $\omega$-oxidation of the alkyl side chain (108) and chain-shortening proceeds with $\beta$-oxidation, which is hindered when the side-chain is cut back to four or five carbon atoms from the point of attachment to the benzene ring (23, 50, 139). Rhodococcus rhodochrous PB1 enantioselectively metabolizes the LAB-intermediate 3-phenylbutyric acid. R. rhodochrous is able to grow on (R)-3-phenylbutyric acid, whereas
the (S) enantiomer is only cometabolically transformed to presumably (S)-3-(2,3-dihydroxyphenyl)butyric acid (124). This compound is then abiotically transformed to reactive and potentially toxic quinones (64, 124). Recently, *D. acidovorans* SPB1 was isolated from an enrichment culture (121) which degrades 2-(4-sulfophenyl)butyrate (SPB) sequentially (Fig. 4B). The (R) enantiomer is degraded first and only when it is exhausted does the (S) enantiomer start degrading. Metabolism converges at the achiral 4-sulfocatechol. 4-sulfocatechol undergoes *ortho* cleavage via 3-sulfo-cis,cis-muconate.

Fig. 4. Linear alkyl benzenes (LAB) and derivatives. A Chemical structure of a LAB isomer and the metabolite 3-phenylbutyric acid. Chain shortening is supposed to be non-enantioselective, as the stereogenic center is far away from the reaction point. B Chemical structure of 2-(4-sulfophenyl)butyrate.
ENANTIOSELECTIVE ENZYMES

Enantioselective degradation of chiral pollutants by microorganisms is rather the rule than the exception. For enantioselective metabolism, one or more enzyme reactions involved in the uptake or in the different degradation steps must be enantioselective. The three-point model (24, 107) is often used to explain the phenomenon of stereoselectivity in enzymatic reactions. As shown in Fig. 5, the model postulates that the active enantiomer binds more tightly to the active site of the enzyme because the sequence of the three groups around the asymmetric carbon atom, ABC, forms the triangular face of a tetrahedron that matches the complementary triad of the chiral binding site, A’B’C’, of the active site. The less active enantiomer binds ineffectively, since it has a mirror-image sequence of the three groups, CBA, which leads to a mismatch with the active site (65). In some cases, this model needs to be expanded to a so-called four-location model (86; Fig. 5, III). When isocitrate dehydrogenase is provided with the substrate racemate, L-isocitrate is exclusively bound to the protein crystals in the absence of Mg$^+$ but, in the presence of Mg$^+$, the D-isomer binds. The crystal structure revealed that three of the four groups of the C2-atom of isocitrate bind to the same three residues, but not the fourth group. In other words, the protein needs not three but four locations in the active site to differentiate between the two enantiomers. In general, the three-point model works as long as it is assumed that the binding site can be approached only from one direction. But if the active site is in a cleft or on protruding residues, only binding or direction to the fourth group enables the protein to distinguish between the enantiomers.
Chirality of pollutants

Fig. 5. Three-point attachment model modified from Easson and Stedman and Ogston (24, 107; diagram I and II) and the four-location model by Mesecar and Koshland (86); diagram III). A’, B’, C’ binding sites in the active site of the enzyme. For one enantiomer of the chiral substrate, the three ligands (A, B, C) are oriented counterclockwise and coincide with the binding sites of the enzyme. It can be seen that the ligands of the other enantiomer (diagram II) bind ineffectively to the enzyme. If the active site can be approached from both sides (diagram III), both enantiomer will bind. In such a case, an additional binding site – a fourth location (D’ and D’’, respectively) – is necessary for selective recognition of an enantiomer.

Degradation of the enantiomers of chiral pollutants may proceed along different avenues:

a) Two enantioselective enzymes exist, each converting only one substrate enantiomer.

b) Both enantiomers are simultaneously converted by one enzyme, but at different rates.

c) Sequential conversion of the substrate enantiomers by one enzyme, i.e., the enzyme preferentially degrades one enantiomer. The other enantiomer is
eventually also degraded, but only when the former one has been (com-
pletely) degraded.
d) Enantioselective conversion of one enantiomer by one enzyme and
isomerization of the other enantiomer by an isomerase.

Not many of the enzymes involved in the degradation of chiral pollutants
are well studied in terms of their stereoselectivity. Recently, two genes, \textit{rdpA} and \textit{sdpA}, were isolated and sequenced from \textit{S. herbicidovorans} MH (Chapter 2). The
genes encode two distinct \(\alpha\)-ketoglutarate-dependent dioxygenases, are involved in
enantioselective degradation of dichlorprop and mecoprop in this strain and cleave
the ether bond to the corresponding phenol with concomitant release of pyruvate
and succinate. \(\alpha\)-Ketoglutarate and oxygen are required as cosubstrates, iron(II) as
a cofactor, and ascorbate as a reducing agent. It was shown that SdpA is constitu-
tively expressed whereas RdpA is induced during growth on the (\(R\)) enantiomer or
the racemate. Interestingly, SdpA was repressed when \textit{S. herbicidovorans} MH was
grown on (\(R\))-mecoprop (105). \textit{rdpA} and \textit{sdpA}-genes were also isolated and sequenced from the dichlorprop degrader \textit{D. acidovorans} MC1. RdpA and SdpA
were partially purified and it was found that RdpA converts (\(R\))-dichlorprop, (\(R\))-
and (\(S\))-mecoprop, but not (\(S\))-dichlorprop (137, 138). SdpA from this strain is
enantioselective and converts only the (\(S\)) enantiomer of the two phenoxypro-
panoic acid herbicides. The amino acid residues, which are involved in substrate-
and cofactor-binding, have not yet been determined. However, from alignments
with other \(\alpha\)-ketoglutarate-dependent dioxygenase and inhibitor studies, it is likely
that there are two histidines and one aspartate involved in Fe(II)-binding (the
2-His-1-carboxy-facial triad; 45). At the moment, nothing is known about the
stereospecificity of the substrate-binding sites and whether the substrate-binding
sites of the two enzymes are similar or not. \(\alpha\)-Ketoglutarate-dependent
dioxygenases involved in 2,4-D degradation were also able to utilize phenoxy-
propanoic acids as substrates. Interestingly, they are highly enantioselective and
convert only one enantiomer. TfdA from \textit{R. eutropha} JMP134(JP4) and
\textit{Burkholderia cepacia} RASC oxidize only (\(S\))-dichlorprop, whereas TfdA from \textit{A.}
denitrificans exclusively converts the (R) enantiomer (119). This indicates that closely related enzymes may exhibit different enantioselectivities.

Another example of an enzyme that enantioselectively catalyzes a key reaction in the degradation of a recalcitrant pollutant is γ-hexachlorocyclohexane (HCH) dehydrochlorinase (LinA) from S. paucimobilis UT26 (97, 99). LinA catalyzes two reactions. The first reaction is the dechlorination of γ-HCH to γ-pentachlorocyclohexene (PCCH) and the second reaction is the dechlorination of γ-PCCH to 1,3(R),4,6-(R)-tetrachlorocyclo-hexa-1,4-diene (CDN), a compound that is presumed to spontaneously rearrange to 1,2,4-trichlorobenzene (TCB). For these reactions, no cofactors are needed. Beside γ-HCH, LinA also converts α- and δ-HCH, but not β-HCH, which indicates that the enzyme requires a biaxial HCl-pair on the substrate molecule. δ-HCH is only converted to δ-PCCH. Interestingly, LinA differentiates between 1,3(S),4(R),5(R),6(S)-PCCH and 1,3(R),4(S),5(S),6(R)-PCCH, whereby only the former enantiomer is a metabolite in the degradation of γ-HCH. When provided with the racemate obtained by chemical alkaline dehydrochlorination, LinA converts the former enantiomer to 1,2,4-TCB and the latter one to 1,2,3-TCB. Therefore, the first step in the degradation of γ-HCH is highly enantioselective and gives rise to only one product enantiomer. Site-directed mutagenesis experiments allowed a closer look at the active site and it was found that the catalytic dyad, His-73 and Asp-25, is involved in stereoselectivity. It was suggested that the topological differentiation is caused by this catalytic dyad, whereas the enantiomeric differentiation is due to noncovalent interaction of the double-bond substituents with noncatalytic residues in the active site (135).

Ring-hydroxylating dioxygenases are important enzymes in the degradation of many aromatic pollutants. In most cases, they convert their achiral substrate to chiral products (cis-dihydrodiols), a process which is regio- and enantioselective; and many conversions lead to enantiomerically pure products (51). Naphthalene dioxygenase (NDO) belongs to this class of enzymes. Because the structure of NDO was solved (59, 60), it is the model enzyme for studying molecular aspects of enantioselectivity in such reactions. NDO is a multicomponent enzyme and
Chapter 1

catalyzes a wide range of reactions, such as cis-hydroxylations, mono-oxygenations, and desaturations. It consists of three components: an iron-sulfur-flavoprotein reductase, an iron-sulfur-ferredoxin, and the oxygenase itself, which is built of a small $\alpha$- and a large $\beta$-subunit with the overall structure $\alpha_3\beta_3$ (27, 60). NDO is NAD(P)H dependent and the reductase and ferredoxin component transfer the electron from NAD(P)H to the oxygenase (27, 28, 41). Each subunit of the oxygenase component contains a Riesketype [2Fe-2S] center and a mononuclear nonheme iron. Electrons are transferred from the Rieskecenter to the mononuclear iron of an adjacent $\alpha$-subunit, which is the site of oxygen activation and catalysis. Phenylalanine residue 253 (F253) has been shown to play an important role in controlling regio- and enantioselectivity in phenanthrene, biphenyl and naphthalene oxidation. By site-directed mutagenesis, F352 was altered into different amino acids. NDO variant F352W, in which phenylalanine was replaced by tryptophan, exhibited the most pronounced changes in stereochemistry (109, 110). Other variants of F352 also showed altered regioselectivity with biphenyl and phenanthrene. The combination of a F352V variant of NDO and the enantioselective toluene cis-dihydrodiol dehydrogenase from P. putida F1 was used to produce the enantiomerically pure (−)-biphenyl cis-(3S,4R)-dihydrodiol and (−)-phenanthrene cis-(1S,2R)-dihydrodiol from biphenyl and phenanthrene, respectively (110). The enantioselective oxidation of dihydrodiols was also investigated for chlorobenzene cis-dihydrodiol dehydrogenase (TcbB) from Pseudomonas sp. strain P51 and it was found that the enantioselectivity of the conversion is highly dependent on the substrate (114). TcbB exclusively oxidizes (+)-cis-(1R,2S)-indandiol, whereas both enantiomers of cis-1,2-dihydroxy-1,2,3,4-tetrahydronaphthalene are converted.

Racemases (isomerases) catalyze racemization of their substrates and are thereby involved in enantioselective degradation pathways. One well studied example is mandelic acid racemase (MR). MR converts D- to L-mandelic acid and vice versa. P. putida biotype A degrades both enantiomers of mandelic acid by means of a racemase. L-Mandelic acid is the substrate of a L-(+)-mandelic acid dehydrogenase, which is highly enantioselective. Further metabolism proceeds
through the so-called mandelate pathway to benzoic acid (61), which is further metabolized via catechol and through the ortho-cleavage pathway. D-Mandelic acid is metabolized by the same enzymes except that it is initially converted to the L-enantiomer by MR. Interestingly, MR itself is nonenantioselective, i.e., D-mandelic acid is converted to the L-enantiomer at the same rate as the L-enantiomer is converted to the D-enantiomer (140). The racemization proceeds by a two-base mechanism (113). This means that two basic amino acid residues, which are juxtaposed on either side of the chiral substrate carbon, are involved. One base abstracts the proton of the substrate whereas the conjugate acid of the other base protonates the intermediate from the other side. For the opposite reaction, the roles are reversed. For MR from *P. putida* F1, the two basic residues were identified as lysine residue 166 (K166) and histidine residue 297 (H297; 58, 69, 70, 103). K166 abstracts the $\alpha$-proton from the $(S)$ enantiomer whereas H297 deprotonates the $(R)$ enantiomer.

An interesting example of an enzyme pair which metabolizes the enantiomers of a chiral compound is the paired tropinone reductases (TR-I, TR-II) from *Datura stramonium*. Many plants produce tropane alkaloids and, in this synthesis, the TRs reduce a carbonyl group of an alkaloid intermediate, tropinone, to hydroxygroups with different diastereometric configurations: tropine (3$\alpha$-hydroxytropane) and P-tropine (3$\beta$-hydroxytropane). The crystal structures of both TRs were determined (100). The two enzymes share 64% amino acid identity and the overall folding is almost identical. The binding sites for the cofactor NADH and the positions of the active site residues are well conserved. The active sites are composed mainly of hydrophobic amino acids but differently charged amino acids cause different electrostatic environments. In TR-I, there is a positively charged histidine (H112), whereas in TR-II a polar tyrosine (Y100) occupies this position. In TR-II, the hydrophobic valine residue 168 of TR-I is replaced by the negatively charged glutamate residue 156. These differences are of importance for the positioning of tropinone. The nitrogen atom of tropinone is negatively charged under physiological pH conditions and, thereby, tropinone is oriented in different ways in the active site by the different electrostatic environments.
These examples demonstrate how single amino acid residues are responsible for highly enantioselective reactions and enable the enzymes to differentiate between enantiomers. Just by changing a few amino acid residues involved in regio- and enantioselective substrate recognition, the stereoselectivity of an enzymatic reaction can be changed or even reversed.

Convergent evolution is another approach nature has chosen to metabolize enantiomers of chiral compounds. Many enzyme pairs, each turning over one enantiomer of a chiral compound, have evolved independently and are not related to each other. Examples are the D- and L-lactate dehydrogenases (D-LDH, L-LDH), which belong to the D- and L-ketoacid dehydrogenase families, respectively. Sequence comparisons show that these two enzyme families are not related to each other evolutionarily. L- and D-LDH catalyze the reduction of pyruvate to lactate, with the concomitant consumption of NADH, producing enantiomerically pure products. By crystal structure analysis, it was found, that the overall folding of the D-LDH is completely different from that of L-LDH. It was hypothesized that, despite the different folding, the active sites are mirror-images of each other (39, 68). This is true insofar as the same amino acid residues are involved in substrate-binding and catalysis and they are in structurally equivalent positions. However, their exact roles in binding and catalysis may not be the same (129). Convergent evolution was also found for the D-amino acid transferases (D-AAT) and the L-aspartate aminotransferases (L-Asp-AT). D-AAT and L-Asp-AT do not share any identity at the sequence level and their overall folding is different, but the enzymatic mechanism is similar. Both enzymes contain a pyridoxal phosphate (PLP) and catalyze a transamination of the respective amino acid enantiomer. Sugio et al. (131) showed that there are striking similarities between the active sites of the two enzymes, especially concerning the binding of the PLP and its intermediates. The α-amino- and α-carboxyl group of the substrate amino acid are bound in the same orientation in relation to the pyridoxal phosphate ring and the protein. The side-chain is therefore, due to the inverted chirality, oriented in the opposite direction.

Other interesting enzymes in terms of their enantioselectivity are the 2-haloacid dehalogenases (DEXs) which convert chiral 2-haloacids into 2-hydroxyacids.
DEXs are involved in the degradation of halogenated organic compounds and are classified into four groups based on their substrate and stereochemical specificities (128). L-DEXs convert L-haloacids into D-hydroxyacids with inversion of the configuration at the C2-carbon atom of the substrate. D-DEX acts specifically on the D-enantiomer to produce L-hydroxyacids. DL-DEX, dehalogenates both enantiomers with inversion of the configuration at the C2-carbon atom; and DL-DEX, converts both enantiomers to the corresponding hydroxyacids with retention of the configuration at the C2-carbon atom. L-DEXs are well studied, both in terms of reaction mechanisms and in terms of substrate- and stereo-specificity (67, 74, 75, 127). Additionally, crystal structures of two representative enzymes, L-DEX from *Pseudomonas* sp. YL and L-DEX from *Xanthobacter autotrophicus* GJ10 have been solved (47, 74, 115). The normal reaction mechanism proceeds via an ester intermediate. First, Asp-10 acts as a nucleophile and attacks the C2-carbon atom of the substrate forming an ester-intermediate and a halide ion. A water molecule is activated and hydrolyzes the intermediate from the back giving the D-hydroxyacid. From mutagenesis studies and the crystal structure of L-DEX YL, several amino acid residues (Tyr-12, Leu-45, Phe-60, Trp-179, Gln-44, Lys-151, Asn-177) were identified which built a hydrophobic pocket. While the carboxylic moiety of the substrate is bound to Asp-10, the alkyl group is located in the hydrophobic pocket. This hydrophobic pocket is responsible for the stereo- and substrate-specificity of the enzyme. It is not possible to accommodate an alkyl group at the place of the hydrogen atom due to steric hindrance by the main chain and the side chain atoms of Leu-11, Tyr-12, and their neighbors in this pocket. This determines the enantioselectivity (and substrate specificity) of the enzyme. In contrast to L-DEX, DL-DEX, converts both enantiomers. DL-DEX from *Pseudomonas* sp. strain 113 (DL-DEX 113) has a significant sequence homology with D-DEXs, e.g. 23% with D-DEX from *P. putida* AJ1, but little with L-DEXs (101). To study the reactive site, several polar and charged amino acid residues conserved among DL- and D-DEX were mutated. When the enzymatic activity was lower than in the wild type, the effect was always equal for both enantiomers. The results suggest that DL-DEX 113 has one single
active site for D- and L-2-haloacids. In other words, DL-DEX 113 does not – in contrast to L-DEX – discriminate between the alkyl group and the hydrogen atom at the C2-atom of 2-haloacids (101).

L-DEX are examples of enzymes that discriminate between enantiomers by the spatial arrangement of the active site rather than by specific interactions of single amino acid residues with the substrate. It would be interesting to know, how the substrates are bound to the active sites in D- and DL-DEX in order to compare the mechanism, but crystallographic data are not yet available.

We have described enzymes with overall conserved folding, where just a few amino acid residues determine stereospecificity together with examples of enzymes that express opposite stereospecificity and have completely different folds and surprising similar active sites that are mirror-images. Theoretically, two enzymes with identical sequences but built from enantiomeric amino acid monomers, i.e., one built from L-amino acids and the other from D-amino acids, should have opposite stereospecificities for chiral substances. Such an enzyme pair was studied by Milton et al. (21). They synthesized the HIV-1 protease completely with D-amino acids. The D-HIV-1 protease indeed showed the opposite substrate specificity. Inversed specificity was also observed with enantiomeric inhibitors. These data imply that the L- and the D-forms of the enzyme are exactly image and mirror-image of each other, resulting in opposite substrate specificity.

CONCLUSION

The environmental fate of the enantiomers of chiral compounds differs not only with regards to unwanted side-effects but also with regard to degradation. As the examples show, there is no rule to decide which enantiomer is preferably degraded. It depends on the specific compounds, the environmental compartment, the environmental conditions, and the microbial community. As other authors have emphasized, it is important to consider both stereochemistry and chirality when studying the effects and degradation potential of chiral compounds (e.g. 4, 5, 64, 65). This is also true when enantiomerically pure compounds are used and therefore, only one enantiomer is introduced into the environment. As was pointed out,
enantiomers may undergo racemization or enantiomerization processes and therefore, the environmental fate of each enantiomer always needs to be investigated.

Studies on enantioselective enzymes have helped and will help to broaden our understanding of the effects of chirality on the living world. It will be important to study such enzymes more intensively to learn how nature deals with chiral objects. The following questions need to be addressed. Does an enzyme change its stereospecificity easily? How do enzymes which are evolutionarily not related to each other adapt to convert enantiomers of a chiral compound? Evolution has not preferred one particular mechanism, as far as we know, but further studies need to be carried out to answer such questions in more detail.
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Chirality of pollutants


Chapter 1


Chirality of pollutants


Chapter 1


General Introduction:

$\alpha$-Ketoglutarate-Dependent Dioxygenases
α-Ketoglutarate-dependent dioxygenases are a large group of non-heme iron enzymes and have been identified in many organisms ranging from prokaryotes, eukaryotes and even viruses. They catalyze a wide range of oxidative processes such as hydroxylations, epoxidations, desaturations, ring formation, and expansion reactions. These enzymes are involved in the biosynthesis of a variety of important molecules such as antibiotics, plant hormones, carnitine and collagen (4, 14, 24, 34, 43, 53). α-Ketoglutarate-dependent dioxygenases do not show high sequence similarity, but are grouped according to their biochemical features. Besides the prime substrate and oxygen, they require α-ketoglutarate as a cosubstrate, iron(II) as a cofactor and ascorbate as a reducing agent. In the course of the reaction, one oxygen atom is incorporated into the prime substrate and the other one ends up in succinate. Despite their sequence diversity, α-ketoglutarate dependent dioxygenases have in common a 2-His-1-carboxylate motif, which is involved in iron binding (15). This motif is part of a rather conserved region, which has been used to classify the members in three subgroups. Subgroup I members share the motif HXDX_{50-70}HX_{10}(R/K)XS, subgroup II members share the motif HX(D/E)X_{138-207}HX_{10-13}R and subgroup III members share the motif HXDX_{72-101}HX_{10}(R/K)XS (18).

Here, I would like to give details on the functioning of a few α-ketoglutarate-dependent dioxygenases, which is indicative of the diversity of the reactions they catalyze, and of biosynthetic pathways they are involved in. In addition, table 1 lists the most important α-ketoglutarate-dependent dioxygenases and Fig. 1 depicts reactions catalyzed by α-ketoglutarate-dependent dioxygenases.
Table 1: Overview of representative α-ketoglutarate-dependent dioxygenases

<table>
<thead>
<tr>
<th>Dioxygenase(^a)</th>
<th>Organism</th>
<th>Structure</th>
<th>Involved in</th>
<th>Reaction (Fig. 1)</th>
<th>Reaction type</th>
<th>Crystal structure</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prolyl-4-hydroxylase</td>
<td>Mammals, plants, insects, nematodes, virus-1</td>
<td>tetramer (α(_2)β(_2)), dimer, monomer</td>
<td>collagen and elastin biosynthesis</td>
<td>1</td>
<td>hydroxylation</td>
<td></td>
<td>(21, 29)</td>
</tr>
<tr>
<td>Lysyl-hydroxylase</td>
<td>Humans</td>
<td>dimer</td>
<td>collagen biosynthesis</td>
<td></td>
<td>hydroxylation</td>
<td></td>
<td>(22)</td>
</tr>
<tr>
<td>IPNS</td>
<td><em>Cephalosporium acremonium</em>, <em>Penicillium chrysogenum</em></td>
<td>tetramer</td>
<td>penicillin biosynthesis</td>
<td>2</td>
<td>cyclization</td>
<td></td>
<td>(50, 52)</td>
</tr>
<tr>
<td>DAOCS/DACS</td>
<td><em>Cephalosporium acremonium</em>, <em>Streptomyces clavuligerus</em>(^b)</td>
<td>monomer</td>
<td>cephalosporin biosynthesis</td>
<td>3</td>
<td>expansion hydroxylation</td>
<td>yes</td>
<td>(1, 61)</td>
</tr>
<tr>
<td>CAS</td>
<td><em>Streptomyces clavuligerus</em></td>
<td>monomer</td>
<td>clavulanic acid biosynthesis</td>
<td>4</td>
<td>hydroxylation cyclization desaturation</td>
<td>yes</td>
<td>(19, 26)</td>
</tr>
<tr>
<td>AlkB</td>
<td><em>Escherichia coli</em>, homologs in human</td>
<td>monomer</td>
<td>DNA and RNA repair</td>
<td>5</td>
<td>demethylation</td>
<td></td>
<td>(60)</td>
</tr>
<tr>
<td>γ-Butyrobetaine hydroxylase</td>
<td><em>Pseudomonas</em> sp. AK1, calf liver, humans</td>
<td>heterodimer</td>
<td>L-carnitine biosynthesis (Lipid metabolism)</td>
<td>6</td>
<td>hydroxylation</td>
<td></td>
<td>(25)</td>
</tr>
<tr>
<td>ANS</td>
<td>Plants</td>
<td>monomer</td>
<td>anthocyanin biosynthesis</td>
<td>7</td>
<td>desaturation</td>
<td>yes</td>
<td>(56, 66)</td>
</tr>
<tr>
<td>TauD</td>
<td><em>E. coli</em></td>
<td>dimer</td>
<td>use of taurine as alternative sulfur source</td>
<td>8</td>
<td>hydroxylation</td>
<td>yes</td>
<td>(7, 9, 32)</td>
</tr>
<tr>
<td>YLL057c</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>monomer</td>
<td>use of alternative sulfur sources</td>
<td></td>
<td>hydroxylation</td>
<td></td>
<td>(17)</td>
</tr>
</tbody>
</table>
Table 1 (cont.): Overview of representative α-ketoglutarate-dependent dioxygenases

<table>
<thead>
<tr>
<th>Dioxygenase&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Organism</th>
<th>Structure</th>
<th>Involved in</th>
<th>Reaction (Fig. 1)</th>
<th>Reaction type</th>
<th>Crystal structure</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtsK</td>
<td><em>Pseudomonas putida</em> S-313</td>
<td>tetramer</td>
<td>use of alternative sulfur sources</td>
<td>9</td>
<td>hydroxylation</td>
<td></td>
<td>(20, 28)</td>
</tr>
<tr>
<td>TfdA</td>
<td><em>Ralstonia eutropha</em> JMP134(pJP4)</td>
<td>dimer</td>
<td>2,4-D degradation</td>
<td>10</td>
<td>hydroxylation</td>
<td>model</td>
<td>(11, 12, 18)</td>
</tr>
<tr>
<td>RdpA</td>
<td><em>Delftia acidovorans</em> MC1</td>
<td>trimer</td>
<td>(R)-dichlorprop degradation</td>
<td>11</td>
<td>hydroxylation</td>
<td></td>
<td>(62, 63)</td>
</tr>
<tr>
<td>SdpA</td>
<td><em>Delftia acidovorans</em> MC1</td>
<td>monomer</td>
<td>(S)-dichlorprop degradation</td>
<td>12</td>
<td>hydroxylation</td>
<td></td>
<td>(62, 63)</td>
</tr>
<tr>
<td>HtxA</td>
<td><em>Pseudomonas stutzeri</em> WM88</td>
<td>dimer</td>
<td>use of hypophosphate as P-source</td>
<td>13</td>
<td>hydroxylation</td>
<td></td>
<td>(64)</td>
</tr>
</tbody>
</table>

<sup>a</sup> See text for explanation of abbreviations.

<sup>b</sup> In *C. acremonium*, one enzyme (DAOC/DACS) catalyzes the two subsequent reactions (from penicillin N to deacetoxycephalosporin and then to deacetylcephalosporin C). In *S. clavuligerus*, two related enzymes exist (DAOCS and DACS) of which each catalyzes one reaction.
Fig. 1. Reactions catalyzed by α-ketoglutarate-dependent dioxygenases. 1 prolyl 4-hydroxylase. 2 isopenicillin N synthase. 3 Deacetoxycephalosporin C synthase and Deacetylcephalosporin C synthase.
Fig. 1 (cont.) 4 Clavaminate synthase. 5 AlkB.
Fig. 1 (cont.) 6 γ-butyrobetaine hydroxylase. 7 anthocyanidin synthase. 8 taurine dioxygenase. 9 alkylsulfonate dioxygenase. 10 2,4-D dioxygenase.
Representative $\alpha$-ketoglutarate-dependent dioxygenases

Prolyl-4-hydroxylase represents $\alpha$-ketoglutarate-dependent dioxygenases that are involved in protein modification. It catalyzes the post-translational hydroxylation of proline residues to trans-4-hydroxyprolyl in many organisms such as humans, chickens and nematodes (Fig. 1.1) (see reviews 21, 37). In mammals, hydroxylated prolines are essential for the formation of the triple helix
structure of collagen and elastin, because they stabilize the structure by forming inter- and intrachain hydrogen bonds (29).

Deacetoxycephalosporin C synthase (DAOCS), deacetylcephalosporin C synthase (DACS), isopenicillin N synthase (IPNS) and clavaminate synthase (CAS) are well known α-ketoglutarate-dependent dioxygenases involved in antibiotic formation. DAOCS and DACS from *Streptomyces clavuligerus* catalyze sequential reactions in the biosynthesis of the antibiotic cephalosporin. DAOCS carries out the ring expansion of penicillin N to deacetoxycephalosporin C whereas DACS hydroxylates deacetoxycephalosporin C to deacetylcephalosporin C (Fig. 1.3) (2). IPNS catalyzes the formation of isopenicillin N from δ- \((L-\alpha\text{-amino-adipoyl})-L\text{-cysteiny}-L\text{-valine}\) in cephalosporin biosynthesis (Fig. 1.3). IPNS was the first well characterized α-ketoglutarate-dependent dioxygenase and possesses the conserved motif common to the subgroup I dioxygenases. However, the primary amino acid sequence is only distantly related to this enzyme family, and IPNS does not require α-ketoglutarate as cosubstrate (50, for reviews, see 51, 52, 54). Nevertheless, this enzyme is particularly interesting, because extensive crystallographic data and data of other studies about binding of cofactors and cosubstrates are available. Since IPNS carries a similar native enzyme fold as other α-ketoglutarate-dependent dioxygenases, it serves as an important model for understanding the enzymatic mechanism of this family (23, 27, 40, 41, 52, 58, 59).

CAS represents the diversity of α-ketoglutarate-dependent dioxygenases ‘par excellence’. It catalyzes three reactions, a hydroxylation, an oxidative cyclization and a desaturation all involved in the biosynthesis of the β-lactamase inhibitor clavulanic acid (Fig. 1.4) (10, 49).

The α-ketoglutarate-dependent dioxygenase AlkB from *E. coli* is involved in DNA and RNA repair. AlkB couples the oxidative decarboxylation of α-keto-glutarate to the hydroxylation of 1-methyladenine and 3-methylcytosine resulting in the release of formaldehyde and the unmodified base (Fig. 1.5) (60).

α-Ketoglutarate-dependent dioxygenases also occur in plants and participate in many biosynthetic pathways such as those for flavonoids, gibbellerins and alkaloids (for a review, see 14). One example is anthocyanidin synthase (ANS)
which catalyzes the desaturation of anthocyanidin to anthocyanin in the flavonoid pathway (Fig. 1.7) (56).

Taurine dioxygenase (TauD) oxidizes taurine to sulfite and aminoacet-aldehyde in *E. coli* (Fig. 1.8) (7). Another subgroup of α-ketoglutarate-dependent dioxygenases are the phenoxyalkanoic acid herbicide converting enzymes 2,4-D dioxygenase (TfdA), *(R)*-dichlorprop dioxygenase (RdpA) and *(S)*-dichlorprop dioxygenase (SdpA; 11, 12, 33, 48, 57, 62, 63). They catalyze the first step in the degradation of herbicides such as 2,4-D, mecoprop and dichlorprop by oxidative cleavage of the ether-bond of the phenoxyalkanoic acid, a reaction that yields the respective phenol as a product (Fig. 1.10-1.12). Just recently, the first α-ketoglutarate-dependent dioxygenase that acts on an inorganic substrate was isolated. Hypophosphite dioxygenase (HtxA) from *Pseudomonas stutzerii* WM88 converts hypophosphite to hypophosphate, which then can be further metabolized. The reaction enables the bacterium to use hypophosphite as inorganic phosphorus source (Fig. 1.13) (64).

In the past few years, enzyme mechanisms and possible intermediates of α-ketoglutarate-dependent dioxygenases catalyzed reactions were intensively investigated. The crystal structures of IPNS, DAOCS, CAS, TfdA, TauD and AlkB and subsequent biochemical studies revealed how the different substrates, iron(II) and dioxygen are positioned at the active sites (6, 8, 9, 16, 18, 26, 32, 40, 41, 60, 61, 67, 68). Despite their low sequence identity, α-ketoglutarate-dependent dioxygenases possess several common structural and mechanistic characteristics. Results from transient kinetic studies coupled with the structural data have led to a proposal for a common reaction mechanism (4, 35, 36, 38, 42, 46, 53, 55).

Here, I want to focus on subgroup II α-ketoglutarate-dependent dioxygenases and will therefore discuss the enzyme mechanism of TauD and TfdA in more details. Additionally, I want to shortly introduce the phenoxypropanoic acid converting enzymes RdpA and SdpA.
**α-Ketoglutarate-dependent taurine dioxygenase (TauD)**

TauD converts taurine to sulfite and aminoacetaldehyde while decomposing α-ketoglutarate to succinate and CO₂ and enables its host to use taurine as a sulfur source (7). TauD is 30% similar to TfdA and 38% to AtsK, the alkylsulfatase from *Pseudomonas putida* (11, 12, 20). TauD has a relative relaxed substrate specificity with respect to the sulfonic acid but shows stricter specificities for the cosubstrate α-ketoglutarate. This can only be replaced by α-ketoacidipate but with a loss up to 4-10% of the original activity (7). Titration studies demonstrated that each TauD subunit binds one ferrous iron molecule, one α-ketoglutarate, and one taurine (46).

The X-ray crystal structure of TauD complexes with Fe(II), α-ketoglutarate and taurine has been solved (9, 32). The native fold of TauD resembles the one of other α-ketoglutarate dependent dioxygenases such as IPNS and is most similar to the one of CAS (40, 61, 68). The jellyroll motif found in all native structures of α-ketoglutarate-dependent dioxygenases so far is formed by the β-strands β5-8 and β14-17. The Fe(II) binding residues were determined to be His-99, Asp-101 and His-255. α-Ketoglutarate is bound to Fe(II) in a bidentate manner through the C1 carboxylate and the C2 oxo-group, whereas the C5 carboxylate forms a salt bridge to Arg-266 and a hydrogen bond with Thr-126 (Fig. 2). The substrate binding residues for the taurine amine group are likely to be Tyr-73, Asn-95, and Ser-158. They form hydrogen bonds with the amine moiety and thereby build a distorted tetrahedral environment. The sulfonate ion interacts with Arg-270, His-70 and the backbone of Val-102. These hydrogen-bonds allow TauD to select for a tetrahedral substrate anion, which is consistent with the finding that His-70 is conserved in alkylsulfonate and alkylsulfate α-ketoglutarate-dependent dioxygenases such as AtsK.
Based on crystallization and spectroscopic studies, an enzyme mechanism for TauD was proposed (9, 32, 35, 36, 46), which significantly contributed to further understanding of the common enzyme mechanism for this class of enzymes (Fig. 3). It was proposed that in the resting enzyme, a sixcoordinate iron is in the centre of the active site and three iron ligands are occupied by water molecules. α-Ketoglutarate binds to Fe(II) in a bidentate manner displacing two water molecules, which restores the sixcoordinate metal center. In general, an ordered binding mechanism was suggested for α-ketoglutarate-dependent dioxygenases, in which the α-ketoglutarate binds prior to either substrate or dioxygen. In taurine dioxygenase, taurine binds prior to oxygen in contrast to other α-ketoglutarate dependent dioxygenases such as prolyl-hydroxylase in which the binding order is reversed (22). The binding of taurine leads to a conformational change from six-coordinate to pentacoordinate Fe(II) by loss of the third water molecule. This conformational change was confirmed by crystallization experiments with apo and holoforms of TauD (32). O₂ then binds opposite of His-255 restoring a sixcoordinate iron again. The oxygen-binding results in the formation of a species with iron(III) superoxide radical anion character. It has further been postulated that the attack of O₂ at the carboxylate moiety of α-ketoglutarate leads to an iron(IV)
peroxo species and then an iron(II) peracid product which in turn is transformed by heterolytic cleavage of the O–O bond to an iron(IV)–oxo species. Substrate hydroxylation is supposed to occur in a two step-process involving hydrogen atom abstraction followed by binding of the oxygen at the C1 of taurine. Unfortunately, no experimental evidence was found for any of these intermediates, but an analogous non-heme iron complex that consisted of Fe(IV) and a nonheme macrocyclic ligand has been characterized spectroscopically. This crystal structure revealed that an iron(IV)-oxo species can exist in a nonporphyrin ligand environment (42). Just recently, Price et al. (36) characterized a high-spin Fe(IV) complex in TauD. They propose intermediates, which exhibit resonance hybrid characteristics between Fe(III) and Fe(IV). One of these intermediates is the above mentioned ferryl-oxo species, another a peroxyhemiketal complex. However, none of these structures has been known among other inorganic and protein complexes.

α-Ketoglutarate-dependent dioxygenases are inactivated in the absence of a reducing agent because uncoupling of oxidative α-ketoglutarate decomposition from substrate oxidation leads to the oxidation of iron(II) to iron(III) at the active site (30, 31, 39). In the presence of a reducing agent such as ascorbate, this inactivation is reversible. TauD is also inactivated by oxygen in the absence of taurine in a process called self-hydroxylation. Self-hydroxylation leads to a transient tyrosyl radical (Tyr-73), that undergoes hydroxylation to a catechol (44, 45). This modification causes an irreversible inactivation of TauD.
Fig. 3. Proposed reaction mechanism of TauD (9, 14, 32, 35, 36, 46). A. Resting TauD, in which all three ligand places are occupied by water molecules. B. Binary complex with α-ketoglutarate bound in a bidentate manner (complex absorbs at 530 nm). C. Ternary complex upon binding of taurine, i.e. conformational change to an unsaturated Fe(II) center. The complex absorbs at 520 nm. D. Species with iron(III) superoxide radical anion character E. Iron(IV) peroxo species. F. Fe(IV)oxospecies. G. Iron(II) peracid species.

**α-Ketoglutarate-dependent 2,4-D dioxygenase (TfdA)**

TfdA, the α-ketoglutarate-dependent 2,4-D dioxygenase from *Ralstonia eutropha* JMP134(pJP4) converts 2,4-D and several other phenoxyalkanoic acid to the corresponding phenol and the ketoalkanoic acid (11-13). TfdA exhibits best catalytic efficiency with 2,4-D, but also converts other substituted phenoxyacetic acids, (S)-dichlorprop and cinnamic acids (5, 11-13). Ferrous iron at the active site cannot be replaced by another divalent cation such as Co(II), Cu(II), Li(II), Mg(II),
Mn(II), Ni(II) and Zn(II); whereas the cosubstrate specificity is more relaxed since α-ketoadipate and pyruvate can substitute α-ketoglutarate, although with the result of a much lower enzymatic rate.

Inactivation as for other α-ketoglutarate-dependent dioxygenases is also observed for TfdA. TfdA is rapidly inactivated in the absence of 2,4-D (5, 47). A charge-transfer to oxygen leads to a Fe(III) in the active center, which can be reactivated by ascorbate. The iron-oxidation of TfdA is not due to uncoupling of α-ketoglutarate decarboxylation and substrate hydroxylation as it is for other α-ketoglutarate dependent dioxygenases (30, 31, 39). The generation of an inactive TfdA species that could not be reversed by ascorbate was observed in the presence of poor substrates such as thiophenoxyacetic acid. This inactivated TfdA arose from an oxidative reaction which is likely to involve hydroxyl radical reactions (47).

The TfdA structure has not been resolved by crystallography, but has been modeled by using the crystal structure of TauD (9). The modeling suggested that the overall fold of the two enzymes must be very similar. The metal ligands are, consistent with spectroscopic and mutagenesis studies (18), His-114, Asp-116 and His-263, whereas Arg-274 forms a salt bridge with C5 of α-ketoglutarate. In contrast to the proposed structural model, the involvement of Thr-141 in a hydrogen bond with the C5 of α-ketoglutarate could not be confirmed by mutagenesis (6). Arg-278, Lys-71, His-214 and the backbone amide of Ser-117 interact with the carboxy group of 2,4-D and Lys-95 was shown to interact with the ether oxygen of 2,4-D. It was further hypothesized that Arg-78 acts as a gate for 2,4-D entry into the active site and that His-213 and His-216 facilitate 2,4-D binding. Since these residues are not conserved among α-ketoglutarate dependent dioxygenases, this reflects differences in the substrate binding sites (18).

In addition to modeling the structure, several studies were directed to obtain more information about the metal-ligand residues. X-ray absorption and electron paramagnetic resonance/electron spin-echo envelop modulation studies with Cu(II)-TfdA suggested that two equatorially oriented histidines interact with the copper and that one histidine is replaced or reoriented upon binding of 2,4-D,
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α-ketoglutarate and O$_2$ (3, 65). However, Cu(II)-TfdA does not seem to be identical to Fe(II)-TfdA since α-ketoglutarate is bound in a monodentate manner to the copper center, but in a bidentate manner to the ferrous iron. A significant change in the metal environment upon formation of the Cu(II)- and Fe(II)-NO-TfdA-α-ketoglutarate-2,4-D complex suggested first a reorientation of the active site, i.e., the loss of a His-ligand. This model was not consistent with findings of other α-ketoglutarate-dependent dioxygenases and therefore, the metal environment change may more likely be explained by the following model: α-ketoglutarate binds in a bidentate manner and 2,4-D is located in a pocket near the Fe(II). The 2-His-1-Asp facial triad is maintained upon binding of 2,4-D, but a water molecule is lost. Thereby, the metal environment changes from a six- to a five-coordination, which agrees with the results obtained with TauD.

**α-Ketoglutarate-dependent (R)- and (S)-dichlorprop dioxygenase (RdpA and SdpA) from *Delftia acidovorans* MC1**

Two dichlorprop dioxygenase genes *rdpA* and *sdpA* have been isolated from the dichlorprop degrading microorganism *D. acidovorans* MC1 (62). Sequence information showed that RdpA and SdpA must belong to the α-ketoglutarate-dependent dioxygenases. They catalyze the enantioselective conversion of (R)- and (S)-dichlorprop to the achiral phenol. RdpA and SdpA are strictly specific for the respective enantiomer, but SdpA also converts 2,4-D. Other ketoacids do not support enzymatic activity. Both enzymes were also strictly dependent on ferrous iron and other divalent cations showed an inhibitory effect (63). No further investigations concerning amino acids involved in catalysis have been carried out so far.
REFERENCES:


CHAPTER 2

Genetic Analysis of Phenoxyalkanoic Acid Degradation in Sphingomonas herbicidovorans MH

Phenoxyalkanoic acid degradation is well studied in Beta- and Gammaproteobacteria, but the genetic background has not been elucidated so far in Alphaproteobacteria. We report the isolation of several genes involved in dichlor- and mecoprop degradation from the Alphaproteobacterium Sphingomonas herbicidovorans MH and propose that the degradation proceeds analogously to that previously reported for 2,4-D. Two genes for α-ketoglutarate-dependent dioxygenases, sdpAMH and rdpAMH, were found which both were adjacent to sequences with potential IS-elements. Furthermore, a gene for a dichlorophenol hydroxylase (tfdB), a putative regulatory gene (cadR), two genes for dichlorocatechol 1,2-dioxygenases (dccAIII), two for dienelactone hydrolases (dccDI/II), part of a gene for maleylacetate reductase (dccE) and one gene for a potential phenoxyalkanoic acid permease were isolated. In contrast to other 2,4-D degraders, the sdp, rdp, and dcc genes were scattered over the genome and their expression was not tightly regulated. No coherent pattern was derived on the possible origin of the sdp, rdp, and dcc pathway genes. rdpAMH was 99% identical to rdpAMC1, a (R)-dichlorprop/α-ketoglutarate dioxygenase from Delftia acidovorans MC1, which is evidence for a recent gene exchange between Alpha- and Betaproteobacteria. Conversely, DccAIII and DccAII did not group within the known chlorocatechol 1,2-dioxygenases, but formed a separate branch in clustering analysis. This suggests a different reservoir and reduced transfer for the genes of the modified ortho-cleavage pathway in Alphaproteobacteria than the ones in Beta- and Gammaproteobacteria.

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INTRODUCTION

Phenoxyalkanoic acid herbicides such as 2,4-dichlorophenoxyacetic acid (2,4-D), chiral mecoprop [(\(R\),\(S\))-2-(4-chloro-2-methyl-phenoxypropanoic acid], and chiral dichlorprop [(\(R\),\(S\))-2-(2,4-dichlorophenoxypropanoic acid)] (Fig. 1) are widely used against broadleaved weeds in agriculture, lawn pastures and industries. They were introduced in large amounts into the environment in the 1940s and 1950s (1, 10, 55). 2,4-D, mecoprop and dichlorprop are synthetic compounds and have only been applied for some decades. However, microorganisms able to use them as sole carbon and energy source have been isolated from different environments (21, 50, 51, 58, chapter 1). 2,4-D degrading strains from the \(\text{Beta}\)- and \(\text{Gammaproteobacteria}\) groups mostly harbor similar genes for 2,4-D degradation (17, 24, 33, 48, 49), which is supportive that frequent gene exchange has occurred and hence their adaptation and wide distribution (17).

In many cases, the first step in the degradation of phenoxyalkanoic acids is the enzymatic conversion of the acids to the corresponding phenols, then via the catechols and through the modified \textit{ortho}-cleavage pathway (21, 41). The best-studied degradation pathway is that of 2,4-D in the soil bacterium \textit{Ralstonia eutropha} JMP134(pJP4) (Fig. 1). Both the genetic and the enzymatic details of the pathway have been established in this bacterium. \textit{R. eutropha} JMP134(pJP4) degrades 2,4-D by an \(\alpha\)-ketoglutarate-dependent dioxygenase that cleaves the ether-bond to produce 2,4-dichlorophenol (15, 16, 43). 2,4-Dichlorophenol in turn is hydroxylated to 3,5-dichlorocatechol by a phenol-hydroxylase (TfdB\(_{\text{I/II}}\)) (13, 29, 38). 3,5-Dichlorocatechol undergoes \textit{ortho}-ring fission to 2,4-dichloro-\textit{cis},\textit{cis}-muconate, a reaction catalyzed by chlorocatechol 1,2-dioxygenase (TfdC\(_{\text{I/II}}\)) (29, 37-39). 2,4-Dichloro-\textit{cis},\textit{cis}-muconate is then converted to \textit{cis}-2-chlorodiene-lactone and further to 2-chloromaleylacetate by chloromuconate cycloisomerase (TfdD\(_{\text{I/II}}\)) and dienelactone hydrolase activity (TfdE\(_{\text{I/II}}\)), respectively (27, 38). Finally, chloromaleylacetate is reduced to 3-oxoadipate via maleylacetate by maleylacetate reductase (TfdF\(_{\text{I/II}}\)) (38, 39). Tett \textit{et al.} (46) showed that (\(R\))-mecoprop degradation in \textit{Alcaligenes denitrificans} proceeds analogously to 2,4-D degradation in \textit{R. eutropha} JMP134(pJP4). Similarly, phenol hydroxylase, chloro-
catechol 1,2-dioxygenase and chloromuconate cycloisomerase activities are induced in *Delftia acidovorans* MC1 upon exposure to dichlorprop. This suggests that dichlorprop is also metabolized through a modified *ortho*-cleavage pathway (6).

**Fig. 1.** Pathway proposed for dichlorprop degradation in *S. herbicidovorans* MH and for 2,4-D degradation in *R. eutropha* JMP134(pJP4). The enzymes of the pathway in *S. herbicidovorans* MH are printed in bold. The enzymes of the genes, which have not yet or partially been isolated from *S. herbicidovorans* MH are displayed within brackets.
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*Sphingomonas herbicidovorans* MH is a versatile phenoxyalkanoic acid degrader and grows on mecoprop and dichlorprop as sole carbon and energy sources (22, 26, 58). Although strain MH metabolizes both enantiomers, it preferentially uses the (S) enantiomer. Nickel *et al.* (36) showed that *S. herbicidovorans* MH harbors two distinct α-ketoglutarate-dependent dioxygenase activities that are involved in the enantioselective degradation of mecoprop and dichlorprop to achiral phenols. However, nothing is known about the genetic background of the mecoprop and dichlorprop degradation pathway in strain MH. *S. herbicidovorans* MH belongs to the *Alphaproteobacteria* whereas other well-studied phenoxyalkanoic acid degraders such as *R. eutropha* JMP134(pJP4) and *D. acidovorans* MC1 belong to the *Betaproteobacteria* and the *Gamma-proteobacteria*. Total DNA from phenoxyalkanoic acid degrading *Alphaproteobacteria* did not hybridize to *tfdA* gene probes encoding α-ketoglutarate-dependent 2,4-D dioxygenases (17, 24, 33, 48, 49). PCR-experiments aimed at amplifying DNA fragments from *Sphingomonas* strains with *tfdA*-primers also failed (42, 48). Therefore, the hypothesis was formulated that *Alphaproteobacteria* might harbor different dioxygenases catalyzing the initial step of phenoxyalkanoic acid degradation. Only recently, the first *tfdA*-homologous gene, *tfdAα*, was isolated from a 2,4-D degrading bacterium belonging to the *Bradyrhizobium-Agromyces-Nitrobacter-Afipia* (BANA)-cluster of *Alphaproteobacteria* (23). On the other hand, fragments similar to phenol hydroxylase (e.g., *tfdB*-like) and chlorocatechol 1,2-dioxygenase genes (e.g. *tfdC*-like) have also been amplified from *Sphingomonas* strains with degenerate primers in the PCR (30, 48). Whereas the *tfdB*-like gene fragments from *Sphingomonas* strains were more than 60% similar to *tfdB* genes from *Beta- and Gammaproteobacteria*, *tfdC*-like gene fragments showed little similarity to other known chlorocatechol 1,2-dioxygenase genes and formed a rather coherent group among themselves (48). Hence, *Alphaproteobacteria* might not only harbor different dioxygenases but also different chlorocatechol degradation genes and it was thus proposed that gene flow might be less common between *Alpha- and Beta- or Gammaproteobacteria* than among *Beta- and Gammaproteobacteria* themselves (17, 18).
In order to test this hypothesis and to elucidate the phenoxyalkanoic acid degradation pathway, we isolated and characterized several genes from *S. herbicidovorans MH* by PCR amplification with degenerated primers, DNA:DNA hybridization, cosmid library construction and DNA sequencing. We propose that these genes are involved in phenoxyalkanoic acid degradation in strain MH and are equivalent to those known from the 2,4-D degradation pathway of *R. eutropha* JMP134(pJP4). The expression of the characterized genes was analyzed in hybridization experiments with mRNA isolated from *S. herbicidovorans MH* cultures that were induced with mecoprop or 2,4-D. The two chlorocatechol 1,2-dioxygenases were expressed in *Escherichia coli* and characterized in terms of their substrate specificities. Finally, the phylogenetic relationships of the *S. herbicidovorans MH* chlorocatechol pathway genes were compared to those of other phenoxyalkanoic acid degradation pathways.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** *S. herbicidovorans MH* was grown in baffled Erlenmeyer flasks shaking at 30°C either in complex medium (57) or in mineral medium supplemented with the appropriate carbon- and energy source. The mineral medium was prepared as described in Nickel *et al.* (36), and was modified by reducing the amount of added peptone to 10 mg/l and by adding 10 mg/l yeast extract. *E. coli* strains were grown either at 30°C or at 37°C in Luria-Bertani (LB) medium (40) with the appropriate antibiotic. Ampicillin and kanamycin were added to final concentrations of 50 µg/ml, chloramphenicol to 25 µg/ml. If necessary, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Biosynth AG, Staad, Switzerland) was added to a final concentration of 50 µg/ml.

**Standard molecular techniques.** Cloning and digestions were done according to established procedures (4, 40). Restriction enzymes and other DNA modifying enzymes were purchased from Promega (Wallisellen, Switzerland) and Fermentas (Nunningen, Switzerland). Plasmids and cosmids were isolated by the boiling miniprep method, the alkaline lysis method according to Sambrook *et al.* (40) or with the E.Z.N.A. plasmid miniprep kit II (Peqlab Biotechnologies GmbH, Baden-Dättwil, Switzerland) as suggested by the manufacturer. Gel extraction was carried out with the MinElute Gel extraction Kit (Qiagen AG, Basel, Switzerland) according to the protocol of the supplier.
Chapter 2

Construction of a cosmid library of S. herbicidovorans MH. The SuperCos1 Cosmid Vector Kit (Strategene, Amsterdam, The Netherlands) was used to construct a cosmid library. S. herbicidovorans MH was grown in 500 ml mineral medium containing 200 mg/l (R,S)-mecoprop until a turbidity of 0.15 (measured at 546 nm) was reached. The culture was harvested by centrifugation for 30 min at 6000 × g. The pellet was resuspended in 15 ml lysis buffer (10 mM NaCl, 20 mM Tris-HCl, pH 8, 1 mM EDTA, 100 µg/ml proteinase K and 0.5% (w/v) SDS) and incubated overnight at 50°C. The DNA was isolated by phenol-chloroform extraction (40), precipitated with ethanol and dissolved in 4 ml TE (10 mM Tris-HCl, pH 8, 1 mM EDTA) and stored at 4°C. Partial digestion of the DNA was done with Sau3AI using different enzyme concentrations for 15 min at 37°C. The digestion was stopped by adding EDTA to a final concentration of 1 mM. The preparation of the host strain E. coli XL-1 BlueMR, the packaging and the titering were carried out according to the protocol of the supplier (Stratagene). Finally, about thousand clones were picked and grown overnight in microtiter plates in LB supplemented with kanamycin at 30°C. Glycerol was added to a final concentration of 15% and the cosmid library was stored at –80°C.

Screening for genes involved in phenoxyalkanoic acid degradation by PCR. To isolate rdpA, we amplified a fragment with the PCR-primers RDPin_fG and RDPin_r (53) from chromosomal DNA of S. herbicidovorans MH. Oligonucleotides for PCR were obtained from Microsynth GmbH (Balgach, Switzerland). The following conditions were applied: initial denaturation for 5 min at 93.5°C; denaturing for 30 sec at 93.5°C; annealing for 30 sec at 52°C, 49°C, 46°C, 43°C, 41°C, and 39°C; extension for 45 sec at 72°C (5 cycles at each annealing temperature; 10 cycles at 39°C) followed by a final extension for 4 min at 72°C. A chlorophenol hydroxylase gene fragment (tfdB) was amplified with the tfdBup and tfdBlow primers from total DNA of S. herbicidovorans MH as described by Vallaeys et al. (48).

To screen for a chlorocatechol 1,2-dioxygenase gene, the degenerate PCR-primers CCDb and CCDe described by Leander et al. (30) were used. The PCR-protocol was as follows: initial denaturation for 6 min at 93.5°C, 35 cycles denaturation for 1 min at 93.5°C, 35 cycles denaturation for 1 min at 95°C, annealing for 1 min at 48°C and extension for 1 min at 72°C followed by one extension cycle for 6 min at 72°C. PCR-fragments were cloned in pGEM®-T Easy (Promega) and sequenced to check the insert (Table 1).

Hybridization of the cosmid library and construction of plasmids harboring genes involved in phenoxyalkanoic acid degradation. For colony blotting of the cosmid library, the colonies were grown overnight on LB agar plates at 37°C. Colony material was transferred to Hybond™-XL membrane (Amersham Biosciences, Dübendorf, Switzerland) by direct contact. The colony material on the membrane was lysed twice with a solution of 0.5 M NaOH for 2 min and washed twice with a solution of 1 M Tris-HCl (pH 8). The DNA was fixed on the membrane by UV light (254 nm, 120 ml/cm²) in a Stratalinker model 1800 (Stratagene).
Phenoxyalkanoic acid genes in *S. herbicidovorans MH*

The cosmid library of *S. herbicidovorans MH* was hybridized with the inserts of pMec1 (*dccA*-fragment) and pMec5 (*rdpA*-fragment). The fragments were radioactively labeled with [α-\(^{32}\)P]dATP using the Random Primed DNA Labeling Kit according to the supplier’s protocol (Roche Applied Science, Rotkreuz, Switzerland). Positively reacting cosmids were isolated, digested and checked by Southern hybridization. Additionally, all positive reacting cosmids were hybridized in the same way with the insert of pMec23 to screen for the *tfdB*-gene. Suitable fragments were then subcloned in pUC-vectors (5) yielding the plasmids listed in Table 1.

Table 1. Plasmids constructed in this work

<table>
<thead>
<tr>
<th>plasmid</th>
<th>relevant characteristics</th>
<th>source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMec1</td>
<td>0.3-kb PCR-fragment obtained with CCDb and CCDe primers cloned into pGEM®-T Easy; contains part of <em>dccA</em></td>
<td>total DNA</td>
</tr>
<tr>
<td>pMec2</td>
<td>1.5-kb <em>SalI</em>-fragment in pUC28 (5), contains part of the chlorocatechol degradation cluster II</td>
<td>cosmid 9H11</td>
</tr>
<tr>
<td>pMec4</td>
<td>2.3-kb <em>SalI</em>-fragment in pUC28, contains part of the chlorocatechol degradation cluster I</td>
<td>11D11</td>
</tr>
<tr>
<td>pMec5</td>
<td>0.3-kb PCR-fragment obtained with RDPin_f and RDPin_r primers; contains part of <em>rdpA</em></td>
<td>total DNA</td>
</tr>
<tr>
<td>pMec7</td>
<td><em>dccA</em> in pGEM®-T Easy</td>
<td>pMec4</td>
</tr>
<tr>
<td>pMec9</td>
<td><em>dccA</em> in pRSET6α (<em>NdeI-BamHI cloned</em>) (44)</td>
<td>pMec7</td>
</tr>
<tr>
<td>pMec10</td>
<td>2.8-kb <em>NcoI</em>-fragment in pUC28; contains <em>rdpA</em> and a transposase</td>
<td>10A12</td>
</tr>
<tr>
<td>pMec12</td>
<td>4.5-kb <em>BamHI</em>-fragment in pUC28; contains part of the chlorocatechol degradation cluster II</td>
<td>9H11</td>
</tr>
<tr>
<td>pMec14</td>
<td>0.7-kb PCR-fragment in pGEM®-T Easy, contains <em>rdpA</em></td>
<td>pMec10</td>
</tr>
<tr>
<td>pMec16</td>
<td>5-kb <em>KpnI</em>-fragment in pUC28; contains <em>sdpA</em></td>
<td>10A12</td>
</tr>
<tr>
<td>pMec18</td>
<td>0.7-kb PCR-fragment in pGEM®-T Easy, contains <em>sdpA</em></td>
<td>pMec16</td>
</tr>
<tr>
<td>pMec20</td>
<td><em>dccA</em> in pGEM®-T Easy</td>
<td>pMec12</td>
</tr>
<tr>
<td>pMec21</td>
<td><em>dccA</em> in pET8c (44)</td>
<td>pMec20</td>
</tr>
<tr>
<td>pMec23</td>
<td>1.1-kb PCR-fragment obtained with primers <em>tfdBup</em> and <em>tfdBlow</em> cloned into pGEM®-T Easy, contains part of <em>tfdB</em></td>
<td>total DNA</td>
</tr>
<tr>
<td>pMec25</td>
<td>5.5-kb <em>SalI</em>-fragment in pUC18Not; contains <em>tfdB</em></td>
<td>11D11</td>
</tr>
</tbody>
</table>

**Transposon insertion and sequencing.** The EZ::TN<KAN-2> Tnp Transposome™ Kit (Epicentre Technologies, Madison, USA) was used for the insertion of a kanamycin resistance marker and sequencing priming sites into the plasmids with cloned *S. herbicidovorans MH* DNA. The insertion reaction was carried out according to the protocol of the supplier. DNA sequencing was performed on double stranded DNA templates with the Thermo Sequenase Primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Biosciences) and M13 primers (5) or KAN-2 FP-
Chapter 2

1/RP-1 primers (Epicentre Technologies). Sequencing reactions were analyzed on an automated DNA sequencer model 4200 IR\(^2\) (LI-COR Inc., Lincoln, USA). Primers for sequencing were labelled at the 5'-end with IRD-700 or IRD-800 and were purchased from MWG Biotech AG (Ebersberg, Germany). Sequencing data were analyzed with DNASTAR software (DNASTAR Inc., Madison, USA) and compared to the databases by BLAST searches (www.ncbi.nlm.nih.gov/Blast; 45). Sequences were deposited in the EMBL database under accession numbers AJ628859-AJ628863.

**Construction of expression plasmids pMec9 and pMec21, expression of DccA\(_{II}\) in E. coli and preparation of cell extract.** The \(dccA_I\) gene was reamplified from a cosmid fragment by PCR with the primer pair CCD2/CCD3 (5'-GAGAGGTCATATGAGCAATCGT-3', 5'-AACGCGAGGATCCGCAGACCAT-3') thereby introducing a \(NdeI\)- and a \(BamHI\)-restriction site (underlined). The \(dccA_{II}\)-gene was amplified by PCR with the forward primer ClcA2_for (5'-AGGTTCCATGGGCAATCG-3') to introduce a \(NcoI\)-site and the reverse primer ClcA2_rev (5'-AGGATCCGTGCCTGTCAG-3') to create a \(BamHI\)-site. The fragments were first cloned into pGEM®-T Easy and verified by sequencing. They were then digested with the respective enzymes and cloned in the same restriction sites in pRSET6\(\alpha\) and pET8c, respectively (44) (Table 1).

To express the proteins, a preculture of \(E.\ coli\) BL21 (DE3)(pLysS) harboring pMec9 or pMec21 was grown overnight at 37°C. A volume of 1% (v/v) of the preculture was transferred to fresh medium and grown at 30°C until a turbidity of 0.4 – 0.6 (measured at 546 nm) was reached, after which isopropyl-\(\beta\)-D-thiogalactopyranoside was added to a final concentration of 0.05 mM. The culture was incubated for another 3 hours at 30°C and the cells were harvested by centrifugation. A pellet of approximately 1 g wet weight was resuspended in 2.5 ml of a solution of 40 mM Tris-HCl, pH 7.4 containing 0.3 mM EDTA. The cells were broken by one passage through a French Pressure cell at 1 MPa. The lysate was centrifuged at 16'000 \(\times\) g for 30 min at 4°C, the supernatant recovered and stored at –20°C until use as cell extract in enzyme activity measurements.

**Enzyme assay.** Enzymatic activity of chlorocatechol 1,2-dioxygenase with all substrates was measured spectrophotometrically at 260 nm in a quartz cuvette for 30 sec except for protocatechuate, which was monitored at 290 nm. To 485 \(\mu\)l buffer (containing 40 mM Tris-HCl, 0.3 mM EDTA and with a pH of 7.4), 10 \(\mu\)l cell-extract of \(E.\ coli\) BL21 (DE3)(pLysS) harboring pMec9 or pMec21 containing between 15 and 30 \(\mu\)g total protein was added. The reaction was started by adding the substrate from a 20 mM stock in methanol to a final concentration of 0.4 mM. To calculate activity, extinction coefficients were taken from Broderick and O’Halloran and Dorn et al. (7, 11).

**Induction experiment.** S. herbicidovorans MH was grown on mineral medium with 20 mM pyruvate to a turbidity of 0.4–0.6 (measured at 546 nm). The culture was induced by the
addition of (R)-, (S)-mecoprop or 2,4-D dissolved in DMSO (10 g/l) to a final concentration of 100 mg/l and incubated for another 30 min at 30°C. A culture to which only DMSO was added was used as a negative control. To stop induction and prevent RNA degradation, one volume of culture was added to two volumes of RNA-protector immediately after sampling (Qiagen AG). The mixture was incubated at room temperature for 5 min, centrifuged at 13’000 × g for 1 min and the supernatant was decanted. The pellets were stored at –20°C.

**RNA-extraction.** RNA was extracted with the RNeasy® Mini Kit from Qiagen AG according to the supplier’s protocol. The RNA, solved in RNase free water, was then stored by adding two volumes of ethanol and 0.1 volume of 5 M sodium acetate (pH 5.2) at –20°C. RNA-concentrations were determined spectrophotometrically at 260 nm.

**In vitro synthesis of antisense mRNA.** The pGEM®-T Easy plasmids pMec14, 18 and 20 harboring rdpA, sdpA, and dccAII, respectively, were linearized by restriction enzyme digestion in order to allow insert-specific transcription from either the T7 or the SP6 promoter. In vitro transcription reactions were carried out with biotin-16-UTP and T7 or SP6 RNA polymerase with the Riboprobe® in vitro Transcription Systems (Promega) according to the protocol of the supplier. The size and yield of all antisense mRNA probes were checked on 1% agarose gels.

**Dot blot hybridization of mRNA.** Total RNAs were washed with 70% ethanol and dissolved in 50 μl RNase-free water. Equal volumes of RNA dilutions (0.5 μg/50 μl, 0.1 μg/50 μl, 0.05 μg/50 μl) were blotted onto positively charged nylon membranes (Qiagen GmbH) in a dot-blot manifold (Invitrogen Life Technologies, Basel, Switzerland) containing a 96-well 3 mm gasket. The samples were washed with an equal volume of 1 × SSC (0.15 M NaCl, 15 mM sodium citrate) directly after their application. On each blot, a series of DNA standards were included (not shown). The standards contained 100, 50, 10, 1, 0.1 ng DNA/50 μl of the plasmid pMec14, 18 or 20.

**Phylogenetic Analysis.** Amino acid sequences were aligned with the software tool CLUSTALX (47). Phylogenetic analysis was done with the maximum likelihood method by using the software Tree-Puzzle (http://www.tree-puzzle.de). The dendrogram was drawn with the program TreeViewPPC (http://taxonomy.zoology.gla.ac.uk/ rod/rod.html).

**Chemicals.** (R,S)-, (R)-mecoprop and 2,4-D were purchased at Riedel-de Haén (Buchs, Switzerland; PESTANAL®, 99% purity). (S)-mecoprop was synthesized by C. Zipper (1998) (56). Catechol, 4-chloro-, 3,5-dichloro-, 4,5-dichloro-, tetrachloro-, 3-methyl- and 4-nitrocatechol were purchased at Sigma-Aldrich (Buchs, Switzerland; 97-99% purity). 3-Chlorocatechol was bought at Promochem GmbH (Wesel, Germany; 99% purity). All other chemicals were either obtained from Fluka (Buchs, Switzerland) or from Merck AG (Dietikon, Switzerland).
RESULTS

Sequence determination of *sdpA*, *rdpA* and *tfdB*. Degradation of phenoxyalkanoic acid herbicides usually begins with an ether cleavage to the corresponding phenol by $\alpha$-ketoglutarate dependent dioxygenases (15, 16, 43, 53, 54). By using a primer set previously developed for isolating a gene fragment encoding a (R)-dichlorprop dioxygenase from *D. acidovorans* MC1 (53), a 0.3-kb fragment was amplified from total DNA of *S. herbicidovorans* MH. This PCR-amplified fragment was cloned (pMec5) and sequenced to confirm its identity to the dichlorprop dioxygenase gene. It was subsequently used to screen a cosmid library of *S. herbicidovorans* MH. Several cosmids were positively hybridizing with the pMec5 insert and a 2.8-kb NcoI-fragment was identified from cosmid 10A12 to contain the region of identity to pMec5. This fragment was subcloned (pMec10), sequenced and analyzed in detail (Table 2). The sequence revealed two open reading frames (ORFs), one of which was almost identical to the (R)-dichlorprop dioxygenase gene *rdpA* from *D. acidovorans* MC1 (*rdpAMC1*; Table 2 and Fig. 2A). At the nucleotide level, the 888-bp *rdpA* ORF from *S. herbicidovorans* MH contained one neutral mutation compared to *rdpAMC1* whereas the predicted RdpA$_{MH}$ protein had a 100% identical amino acid sequence to RdpA$_{MC1}$. RdpA belonged to the class of $\alpha$-ketoglutarate dependent dioxygenases and showed a 35% identical amino acid sequence to TauD from *Pseudomonas aeruginosa*, and percentages of identities below 33% to other members of this enzyme class (Table 3). Upstream of *rdpA$_{MH}$*, a 1200-bp region was found which was similar to an insertion element from *Ralstonia* sp. strain JS705 (Table 2). The transposase ORF comprised 1149 nucleotides.
Table 2. Genes and corresponding products isolated from *S. herbicidovorans* MH

<table>
<thead>
<tr>
<th>plasmid</th>
<th>ORF&lt;sup&gt;a&lt;/sup&gt;</th>
<th>position (nt)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>probable function or product</th>
<th>homologous protein&lt;sup&gt;c&lt;/sup&gt;</th>
<th>source</th>
<th>reference accession number</th>
<th>% amino acid identity&lt;sup&gt;d&lt;/sup&gt;</th>
<th># amino acids&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMec10</td>
<td><em>tnpA</em></td>
<td>226-1374</td>
<td>transposase</td>
<td>TnpA</td>
<td><em>Ralstonia</em> sp. JS705</td>
<td>CAD60927</td>
<td>87 (352/403)</td>
<td>403/414</td>
</tr>
<tr>
<td></td>
<td><em>rdpA</em></td>
<td>1733-2620</td>
<td>(R)-dichlorprop/α-ketoglutarate dioxygenase</td>
<td>RdpA</td>
<td><em>D. acidovorans</em> MC1</td>
<td>AAM90962</td>
<td>100 (295/295)</td>
<td>295/295</td>
</tr>
<tr>
<td>pMec16</td>
<td><em>(tnpA)</em></td>
<td>(1-447)</td>
<td>transposase (C-terminus)</td>
<td>transposase</td>
<td><em>Ralstonia</em> sp. JS705</td>
<td>CAD60927</td>
<td>79 (117/148)</td>
<td>-/414</td>
</tr>
<tr>
<td></td>
<td><em>sdpA</em></td>
<td>750-1613</td>
<td>(S)-dichlorprop/α-ketoglutarate dioxygenase</td>
<td>SdpA</td>
<td><em>D. acidovorans</em> P4a</td>
<td>AAM90963</td>
<td>58 (171/290)</td>
<td>287/292</td>
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<tr>
<td>pMec25</td>
<td><em>(cadR)</em></td>
<td>(1-972)</td>
<td>transcriptional regulator</td>
<td>CadR</td>
<td><em>Bradyrhizobium</em> sp. HW13</td>
<td>BAB78520</td>
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<td>-/336</td>
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<td></td>
<td>ORF2</td>
<td>1062-2627&lt;sup&gt;f&lt;/sup&gt;</td>
<td>outer membrane receptor protein</td>
<td>COG1629</td>
<td><em>Novosphingobium aromaticivorans</em></td>
<td>ZP_00096178</td>
<td>26 (129/488)</td>
<td>521/743</td>
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<tr>
<td></td>
<td><em>tfdB</em></td>
<td>2654-4453</td>
<td>dichlorophenol hydroxylase</td>
<td>TfdB</td>
<td><em>D. acidovorans</em> MC1</td>
<td>AAM76774</td>
<td>57 (334/585)</td>
<td>600/586</td>
</tr>
<tr>
<td>pMec4</td>
<td><em>(dccE)</em></td>
<td>(1-247)</td>
<td>(partial) maleylacetate reductase</td>
<td>TfdF</td>
<td><em>Burkholderia cepacia</em></td>
<td>AAK81685</td>
<td>53 (42/78)</td>
<td>-/357</td>
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<td>(cluster I)</td>
<td><em>dccA&lt;sub&gt;i&lt;/sub&gt;</em></td>
<td>514-1350</td>
<td>chlorocatechol 1,2-dioxygenase</td>
<td>TfdC</td>
<td><em>R. eutropha</em> JMP134(pJP4)</td>
<td>P05403</td>
<td>53 (134/255)</td>
<td>278/255</td>
</tr>
<tr>
<td></td>
<td><em>dccD&lt;sub&gt;i&lt;/sub&gt;</em></td>
<td>1293-2015</td>
<td>dienelactone hydrolase</td>
<td>TfdE&lt;sub&gt;II&lt;/sub&gt;</td>
<td><em>R. eutropha</em> JMP134(pJP4)</td>
<td>P94136</td>
<td>47 (106/222)</td>
<td>240/235</td>
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<tr>
<td>plasmid</td>
<td>ORF&lt;sup&gt;a&lt;/sup&gt;</td>
<td>position&lt;sup&gt;b&lt;/sup&gt; (nt)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>probable function or product</td>
<td>homologous protein&lt;sup&gt;c&lt;/sup&gt;</td>
<td>source</td>
<td>reference accession number</td>
<td>% amino acid identity&lt;sup&gt;d&lt;/sup&gt;</td>
<td># amino acids&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>pMec2+12</td>
<td>tfdK</td>
<td>595-1968</td>
<td>putative transport protein</td>
<td>TfdK</td>
<td><em>R. eutropha</em> JMP134(pJP4)</td>
<td>AAC44725</td>
<td>36 (159/440)</td>
<td>457/463</td>
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<td>(cluster II)</td>
<td>(orfB)</td>
<td>2494-2865&lt;sup&gt;g&lt;/sup&gt;</td>
<td>IS&lt;sup&gt;c&lt;/sup&gt;c3 transposase orfB (C-terminus)</td>
<td>IS&lt;sup&gt;c&lt;/sup&gt;c3 transposase OrfB</td>
<td><em>Caulobacter crescentus</em> CB15</td>
<td>NP_419441</td>
<td>65 (81/123)</td>
<td>-/326</td>
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<tr>
<td></td>
<td>dccA&lt;sub&gt;II&lt;/sub&gt;</td>
<td>3320-4087</td>
<td>chlorocatechol 1,2-dioxygenase</td>
<td>TfdC</td>
<td><em>R. eutropha</em> JMP134(pJP4)</td>
<td>BAA74530</td>
<td>51 (125/242)</td>
<td>256/251</td>
</tr>
<tr>
<td></td>
<td>(dccD&lt;sub&gt;II&lt;/sub&gt;)</td>
<td>4100-(4705)</td>
<td>(interrupted dienelactone hydrolase)</td>
<td>TfdE&lt;sub&gt;II&lt;/sub&gt;</td>
<td><em>R. eutropha</em> JMP134(pJP4)</td>
<td>P94136</td>
<td>50 (88/173)</td>
<td>-/235</td>
</tr>
<tr>
<td></td>
<td>(tnpA)</td>
<td>5161-4677</td>
<td>transposase (C-terminus)</td>
<td>TnpA of IS&lt;sub&gt;6100&lt;/sub&gt;</td>
<td><em>Salmonella enterica</em> subsp.</td>
<td>AAG03007</td>
<td>100 (156/156)</td>
<td>-/264</td>
</tr>
</tbody>
</table>

<sup>a</sup> Incomplete ORFs are set in parentheses.

<sup>b</sup> The position is given respectively to the nucleotides of the plasmid insert.

<sup>c</sup> Given is the most similar protein identified by BLASTX.

<sup>d</sup> The values in parentheses refer to the number of identical amino acids over the aligned region.

<sup>e</sup> The number of amino acids of the protein in *S. herbicidovorans* MH is given first, the number of the reference protein second.

<sup>f</sup> The exact start position of the ORF could not be determined. Other possible start codons are at nucleotides 969, 1185, and 1290.

<sup>g</sup> The nucleotide region represents the part, which is similar to the C-terminus of outer membrane proteins, since no start codon was found upstream of position 2494 and at nucleotide 2389, a stop codon is encoded.
The cosmid 10A12 was subsequently hybridized under low stringency conditions with a 0.7-kb EcoRI-fragment of pMec10 containing the rdpA<sub>MH</sub> gene which revealed a weak signal in addition to the rdpA containing fragments itself (not shown). The signal could be located to a 5-kb KpnI-fragment, which was cloned (pMec16) and partially sequenced. An ORF of 864 nucleotides was identified whose predicted amino acid sequence showed significant similarity to the group of α-ketoglutarate dependent dioxygenases and highest similarity to the α-ketoglutarate-dependent (S)-dichlorprop dioxygenase from <i>D. acidovorans</i> MC1 (Table 2). For this reason, this ORF was designated sdpA<sub>MH</sub>. The predicted amino acid sequence for SdpA<sub>MH</sub> was 63% identical to SdpA from <i>D. acidovorans</i> MC1 (SdpA<sub>MC1</sub>) and 37% to TfdA from <i>R. eutropha</i> JMP134(pJP4) (Table 3). Upstream of sdpA<sub>MH</sub>, a similar insertion element was identified as the one in the region upstream of rdpA<sub>MH</sub>. However, the complete ORF of this element was not present on the insert of pMec16.

With the primer pair tfdBup and tfdBlow, a 1.1-kb PCR-fragment was amplified from total DNA of <i>S. herbicidovorans</i> MH, cloned (pMec23) and sequenced. The insert was then used to hybridize cosmids 10A12, 9H11 and 11D11, which harbor other genes of phenoxyalkanoic acid degradation. A 5.5-kb SalI fragment from 11D11 was subsequently cloned (pMec25) and partially sequenced. The sequence revealed two complete and one incomplete ORF whose deduced amino acid sequences showed similarity to known proteins in the databank (Table 2). The deduced amino acid sequence of the incomplete ORF at the left end of the insert was similar to CadR from <i>Bradyrhizobium</i> sp. HW13 and was therefore tentatively designated <i>cadR</i> (25). The ORF downstream showed low similarity (<26%) to the C-terminal region of outer membrane receptor proteins. The ORF starting at nucleotide 2654 and comprising 1800 bp showed similarity to a dichlorophenol hydroxylase gene and was designated <i>tfdB</i> (Table 2).
Table 3. Identity of RdpA and SdpA to other α-ketoglutarate dependent dioxygenases (determined by BlastP two sequences)

<table>
<thead>
<tr>
<th>protein</th>
<th>Strain</th>
<th>amino acids</th>
<th>accession number</th>
<th>substrate</th>
<th>% identity</th>
<th>RdpA</th>
<th>SdpA</th>
</tr>
</thead>
<tbody>
<tr>
<td>RdpA</td>
<td><em>S. herbicidovorans</em> MH</td>
<td>295</td>
<td>AJ628859</td>
<td>(R)-dichlorprop</td>
<td>-</td>
<td>30 (79/262)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Delftia acidovorans</em> MC1</td>
<td>295</td>
<td>AAM90962</td>
<td>(R)-dichlorprop</td>
<td>-</td>
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</tr>
<tr>
<td>SdpA</td>
<td><em>S. herbicidovorans</em> MH</td>
<td>287</td>
<td>AJ628860</td>
<td>(S)-dichlorprop</td>
<td>30 (79/262)</td>
<td>-</td>
<td></td>
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<td>TauD</td>
<td><em>E. coli</em> K-12</td>
<td>283</td>
<td>AAC73471</td>
<td>taurine</td>
<td>33 (94/281)</td>
<td>29 (82/280)</td>
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<td>TauD</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>277</td>
<td>NP 252624</td>
<td>taurine</td>
<td>35 (99/282)</td>
<td>32 (91/277)</td>
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<tr>
<td>AtsK</td>
<td><em>Pseudomonas putida</em></td>
<td>301</td>
<td>AAD31784</td>
<td>alkyl sulfate esters</td>
<td>31 (93/293)</td>
<td>27 (77/277)</td>
<td></td>
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<tr>
<td>TfdA</td>
<td><em>Burkholderia cepacia</em> (pIJB)</td>
<td>288</td>
<td>AAB47567</td>
<td>2,4-D</td>
<td>28 (82/290)</td>
<td>35 (90/252)</td>
<td></td>
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<tr>
<td>TfdA</td>
<td><em>Burkholderia</em> sp. RASC</td>
<td>297</td>
<td>AAB17363</td>
<td>2,4-D</td>
<td>26 (79/293)</td>
<td>37 (107/284)</td>
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<tr>
<td>TfdA</td>
<td><em>Ralstonia eutropha</em> JMP134(pJP4)</td>
<td>287</td>
<td>AAA21983</td>
<td>2,4-D</td>
<td>30 (88/288)</td>
<td>37 (107/287)</td>
<td></td>
</tr>
<tr>
<td>RdpA</td>
<td><em>Delftia acidovorans</em> P4a</td>
<td>287</td>
<td>AAM76772</td>
<td>2,4-D</td>
<td>28(81/288)</td>
<td>36 (105/286)</td>
<td></td>
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<tr>
<td>SdpA</td>
<td><em>Delftia acidovorans</em> MC1</td>
<td>292</td>
<td>AAM90963</td>
<td>(S)-dichlorprop</td>
<td>27 (75/268)</td>
<td>63 (183/290)</td>
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<tr>
<td>TfdA</td>
<td>strain RD-5-C2 (member of BANA-cluster)</td>
<td>295</td>
<td>BAB92964</td>
<td>2,4-D</td>
<td>28 (83/287)</td>
<td>36 (106/288)</td>
<td></td>
</tr>
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<td>TfdA</td>
<td><em>Achromobacter xylosoxidans subsp. Denitrificans</em></td>
<td>287</td>
<td>AAF59421</td>
<td>2,4-D</td>
<td>27 (80/288)</td>
<td>37 (106/286)</td>
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Sequence determination and organization of two chlorocatechol degradation gene clusters. To determine whether genes for chlorocatechol degradation through an ortho-cleavage pathway were present in the genome of strain MH, a PCR was carried out with the conserved primer set CCDb and CCDe (30). Amplified fragments were cloned and sequenced to determine the nature of the insert. One clone (pMec1) was found to contain a part of a putative chlorocatechol 1,2-dioxygenase gene (Table 1). The cosmid library was then hybridized with the pMec1 insert to retrieve the complete gene for chlorocatechol 1,2-dioxygenase. Interestingly, two different fragments of cosmids 9H11 and 11D11 were hybridizing, which were both subcloned and sequenced (pMec2 and pMec4). The insert of pMec4 revealed three ORFs, the deduced amino acid of one of which sequence had significant similarity to chlorocatechol 1,2-dioxygenases (Table 2). This ORF was tentatively designated \( dccA_I \) for dichlorocatechol 1,2-dioxygenase. The exact start of \( dccA_I \) could not be determined completely, since several alternative possible start codons occurred in sequential position (i.e., at positions 265, 367, 400, 472 and 514). However, on the basis of the position of a putative Shine-Dalgarno sequence (AGGAGA at position 500), we propose the \( dccA_I \) gene start at position 514. Interestingly, flanking the \( dccA_I \) gene were ORFs putatively encoding a dienelactone hydrolase (designated \( dccD_I \)) (Fig. 2A, Table 2) and a maleylacetate reductase. The maleylacetate reductase ORF was not completely recovered on the insert of pMec4.

The left end of the insert of pMec2 contained a part of a chlorocatechol 1,2-dioxygenase gene. To retrieve the complete ORF, a 4.5-kb \( BamHI \)-fragment from cosmid 9H11, which was partially overlapping to the insert of pMec2, was cloned (pMec12) and sequenced. The whole ORF encoding the putative chlorocatechol 1,2-dioxygenase was obtained and tentatively designated \( dccA_{II} \) since it was not identical to \( dccA_I \). Flanking the \( dccA_{II} \) on the inserts of pMec2 and pMec12 were ORFs for another dienelactone hydrolase and a putative transport facilitator protein (Table 2, Fig. 2A). However, the second possible ORF for dienelactone hydrolase (\( dccD_{II} \)) was interrupted by a part of an insertion element identical to IS6100. The ORF upstream of \( dccA_{II} \) most likely codes for a transporter protein similar to the
TfdK 2,4-D transporter. Between *dccA*II and *tfdK*, the sequence revealed part of another insertion element (ISC*c*3). The sequence information thus indicated that *S. herbicidovorans* MH harbored at least two complete genes for a chlorocatechol 1,2-dioxygenase, which both were located upstream of a (partial) dienelactone hydrolase gene. Evidence was also found for a chloromaleylacetate reductase upstream of *dccA*II. Except for a gene for chloromuconate cycloisomerase, this would provide an almost complete modified ortho cleavage pathway to strain MH.

**Fig. 2.** (A) Configuration of phenoxyalkanoic acid degradation genes in *S. herbicidovorans* MH, (B) Comparison of the gene clusters for the modified ortho-cleavage degradation pathway in different bacteria. pJP4, *R. eutropha* JMP134 (M35097, U16782); pAC27, *P. putida* (M16964); pP51, *Pseudomonas* sp. (M57629). The arrows indicate the localization, the size and the direction of the transcription of the genes. Dashed lines around boxes represent incompletely sequenced open reading frames. Similar patterns of hatching and shading between genes indicate DNA homology or amino acid similarity of the translated gene products. α-ketoglutarate dependent dioxygenases, 2,4-D transport protein, (chloro)phenol hydroxylases, chlorocatechol 1,2-dioxygenases, chloromuconate cycloisomerases, dienelactone hydrolases, maleylacetate reductases, transposases.
Expression of DccA\textsubscript{I} and DccA\textsubscript{II} in \textit{E. coli} BL21(DE3)(pLysS). The \textit{dccA}\textsubscript{I} and \textit{dccA}\textsubscript{II} ORFs were expressed in \textit{E. coli} BL21(DE3)(pLysS) harboring pMec9 and pMec21. In both cell extracts, bands of the expected sizes 30.9 and 27.9 kDa, respectively, were seen on SDS-PAGE gels (not shown), which verified the translation of both genes in \textit{E. coli}. Incubation of the cell extracts with 4-methylcatechol clearly showed the appearance of 4-methylmuconate with a maximal absorption at 255 nm (Fig. 3). The substrate range for both enzymes was determined in cell extracts from \textit{E. coli} BL21(DE3)(pLysS) by incubation with different substituted catechols (Table 4). DccA\textsubscript{I} and DccA\textsubscript{II} exhibited essentially the same substrate range (Table 4). Both enzymes converted chloro- and methyl-substituted catechols; the highest activity was detected with 4-methylcatechol whereas 3,5-dichlorocatechol was preferred to monosubstituted catechols and catechol. 4,5-Dichlorocatechol was also converted, although at very low rates. No activity was measured with tetrachlorocatechol, \textit{p}-nitrocatechol, protocatechuate, and hydroquinone.

![Graph](image)

**Fig. 3.** UV-spectrum of 4-methylcatechol turnover by cell-extracts of \textit{E. coli} BL21(DE3)(pLysS) harboring pMec9 induced with isopropyl-\textit{p}-thiogalacto-pyranoside. The different lines represent different incubation times in min. The arrow points to the increase in absorption at 255 nm ($\lambda_{\text{max}}$ of 3-methylmuconate).
Table 4. DccA\textsubscript{I} and DccA\textsubscript{II} activities with different substrates measured in cell-extract of \textit{E. coli} BL21(DE3)(pLysS) harboring pMec9 or pMec21

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DccA\textsubscript{I}</td>
<td>DccA\textsubscript{II}</td>
</tr>
<tr>
<td>Catechol</td>
<td>22.66\textsuperscript{a} (±1.59)\textsuperscript{b}</td>
<td>18.86\textsuperscript{a} (±3.23)\textsuperscript{b}</td>
</tr>
<tr>
<td>3-Chlorocatechol</td>
<td>41.02 (±1.93)</td>
<td>34.43 (±2.01)</td>
</tr>
<tr>
<td>4-Chlorocatechol</td>
<td>24.59 (±1.55)</td>
<td>22.13 (±0.69)</td>
</tr>
<tr>
<td>3-Methylcatechol</td>
<td>61.69 (±3.05)</td>
<td>47.16 (±3.40)</td>
</tr>
<tr>
<td>4-Methylcatechol</td>
<td>100 [1.48-2.59]\textsuperscript{c}</td>
<td>100 [1.12-2.15]\textsuperscript{c}</td>
</tr>
<tr>
<td>3,5-Dichlorocatechol</td>
<td>56.52 (±3.91)</td>
<td>49.11 (±2.88)</td>
</tr>
<tr>
<td>4,5-Dichlorocatechol</td>
<td>0.66 (±0.34)</td>
<td>0.64 (±0.31)</td>
</tr>
<tr>
<td>Tetrachlorocatechol</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>\textit{p}-Nitrocatechol</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Protocatechuate</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>0</td>
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</tr>
</tbody>
</table>

\textsuperscript{a} Enzyme activities in \% compared to the value obtained with 4-methylcatechol. The data represent means of three independent cell-extracts of which each was measured three times with all substrates except for tetrachloro- and \textit{p}-nitrocatechol, protocatechuate and hydroquinone, which represent means of two independent cell-extracts.

\textsuperscript{b} Standard deviations

\textsuperscript{c} The values in square brackets represent the range of specific activity in the cell extracts (in U/mg protein).

**Expression of \textit{rdpA}, \textit{sdpA}, \textit{dccA\textsubscript{I}} and \textit{dccA\textsubscript{II}}.** To investigate whether \textit{rdpA}, \textit{sdpA} and \textit{dccA\textsubscript{II}} were involved in phenoxyalkanoic acid degradation in \textit{S. herbicidovorans} MH, we grew the strain in batch cultures with pyruvate and pulsed the culture with (\textit{R})-, (\textit{S})-mecoprop or 2,4-D. The formation of specific mRNAs was then determined by dotblot hybridizations (Fig. 4). Unexpectedly, mRNAs for \textit{sdpA} and \textit{rdpA} were always present, irrespective of the addition of mecoprop and 2,4-D, but mRNA for \textit{dccA\textsubscript{II}} appeared specifically after exposure to both mecoprop and 2,4-D (Fig. 4). It has to be noted, however, that the hybridization experiments cannot distinguish between \textit{dccA\textsubscript{I}} and \textit{dccA\textsubscript{II}} because of their high similarity. These results strongly suggest that mecoprop and 2,4-D induce a modified ortho cleavage pathway involving \textit{dccA\textsubscript{I}} and/or \textit{dccA\textsubscript{II}}.
Phenoxyalkanoic acid genes in *S. herbicidovorans* MH

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**DISCUSSION**

**Phenoxyalkanoic acid degradation in *S. herbicidovorans* MH.** Here we studied the genetic background of phenoxyalkanoic acid degradation in *S. herbicidovorans* strain MH. Several candidate genes were isolated, which showed similarity on amino acid level to other proteins involved in phenoxyalkanoic acid and chlorocatechol degradation. Strain MH harbors two genes encoding two α-ketoglutarate dependent dioxygenases (named RdpA<sub>MH</sub> and SdpA<sub>MH</sub>), which were rather different to the archetypal TfdA enzyme (Table 3) but very similar to two dioxygenases from *D. acidovorans* MC1. Enzymes from *D. acidovorans* MC1 catalyze the enantioselective reaction of dichlorprop to dichlorophenol (53). RdpA<sub>MH</sub> and SdpA<sub>MH</sub> have been expressed in *E. coli* and indeed catalyze the enantioselective conversion of mecoprop and dichlorprop (Chapters 3 and 4). This strongly suggests that RdpA<sub>MH</sub> and SdpA<sub>MH</sub> catalyze the first step in the degradation pathway of mecoprop and dichlorprop in strain MH (Fig. 1). Both genes were constitutively expressed and not specifically induced upon exposure to mecoprop or 2,4-D. Previously, however, it was observed that the (R)-specific enzyme is only present when strain MH is grown on (R)-mecoprop, (R)-

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**Fig. 4.** Dot blot hybridization of total RNA isolated from batch cultures of *S. herbicidovorans* MH after induction with (R)-, (S)-mecoprop or 2,4-D. Antisense mRNA probes used for hybridization are indicated on the left. (R)-, (S)-mecoprop and 2,4-D on the top show the induction substrate; DMSO represents the uninduced state. a, b, c stand for individual replicates.
dichlorprop or the racemate whereas (S)-activity is always present except when the strain is grown on the (R)-enantiomer (36). Therefore, it might be that expression of \( \text{rdp}_{\text{MH}} \) and \( \text{sdp}_{\text{MH}} \) is further regulated on the translational or posttranslational level.

*S. herbicidovorans* MH was shown to harbor two distinct chlorocatechol 1,2-dioxygenase genes, designated \( \text{dccA}_1 \) and \( \text{dccA}_2 \). Both genes were induced when strain MH was exposed to mecoprop or 2,4-D. When expressed in *E. coli*, DccA\(_1\) and DccA\(_2\) showed activity towards different substituted catechols but preferred 3,5-dichlorocatechol to monosubstituted catechols. Both enzymes showed essentially the same substrate specificities. From these results we conclude that DccA\(_1\), DccA\(_2\) or both are involved in dichlorprop, mecoprop and 2,4-D degradation in *S. herbicidovorans* MH. However, we do not know whether the two dichlorocatechol 1,2-dioxygenases have different roles.

We hypothesize that also the \( \text{tfdB} \)-like gene is involved in mecoprop and dichlorprop degradation in strain MH, although this was not specifically tested here. The finding of other genes from the modified *ortho*-cleavage pathway (i.e., dienelactone hydrolase and maleylacetate reductase) is further evidence to propose that 3,5-dichlorocatechol and 3-methyl-5-chlorocatechol are processed through a metabolic pathway similar to the one for 2,4-D degradation in *R. eutropha* JMP134 (pJP4) (Fig. 1). Currently, no information is available concerning the location of a chloromuconate cycloisomerase gene, the product of which would be needed to complete the pathway.

**Genetic organization of phenoxyalkanoic degradation.** The genes for phenoxyalkanoic acids degradation in *S. herbicidovorans* MH were not organized in one or two operons but scattered over the genome. The \( \text{rdp}_{\text{MH}} \) and \( \text{sdp}_{\text{MH}} \) gene were located on the same cosmid but separated by at least 10 kb. The genes for chlorophenol and chlorocatechol degradation were organized in three clusters, which were located on different cosmids. Such a gene organization is in contrast to that of the \( \text{tfd} \) genes for 2,4-D degradation in *R. eutropha* JMP134(pJP4), which are present in one compact region of 22 kb (29). A dispersed gene organization seems to be common for *Sphingomonas* strains. For instance, the \( \text{pcp} \) genes coding
Phenoxyalkanoic acid genes in *S. herbicidovorans* MH for pentachlorophenol degradation in *Sphingobium chlorophenolicum* ATCC 39723 are organized on non-contiguous parts of the DNA (8). A scattered gene organization was also found for the dioxin-degradative genes in *Sphingomonas* sp. strain RW1 (2). The *lin* genes for lindane degradation in *Sphingomonas paucimobilis* are present in five different operons and unlinked clusters (28). This contrasting gene organization in sphingomonads may have consequences for regulation of pathway expression. Expression of *rdpA*<sub>MH</sub> and *sdpA*<sub>MH</sub> in strain MH was constitutive whereas *dccA*<sub>III</sub> were inducibly expressed. This scheme is similar to the one for expression of the lindane pathway in *S. paucimobilis*, where the first three genes are constitutively expressed and only the genes for chlorohydroquinone conversion are inducible (34). In contrast, the 2,4-D pathway in *R. eutropha* JMP134 (pJP4) is very tightly regulated (31, 32). It might be that the organization observed in *Sphingomonas herbicidovorans* MH is not optimized yet and that regulatory networks controlling *rdpA* and *sdpA* expression will evolve when the strain remains being exposed to phenoxyalkanoic acid containing environments. On the other hand, the absence of inducible expression might just be a different strategy found in sphingomonads compared to pseudomonads, which nonetheless results in functional catabolic pathways. When considering that inducible catabolic pathways often seem to need a threshold concentration before induction takes place, it may even be an advantageous strategy to continuously produce the first enzymes necessary for the pathway.

Potentially two clusters might be involved in chlorocatechol degradation in strain MH. Both clusters clearly have a different gene order than the typical operons for chlorocatechol such as the *clcABD* genes from *P. putida* (pAC27) (14), the *tfdCDEF* genes in *R. eutropha* JMP134(pJP4) (9, 19, 20, 38) and the *tcbCDEF* genes in *Pseudomonas* sp. P51 (52) (Fig. 2B). It was noteworthy, that a gene for a chloromuconate cycloisomerase was not present within the regions sequenced from strain MH. Furthermore, cluster II was flanked by sequences with clear homology to (parts of) IS-elements that even interrupted *dccD*<sub>II</sub>; this points at (recent) genetic rearrangements.
Evolutionary aspects of the phenoxyalkanoic acid degradation pathway in *S. herbicidovorans* MH. It has been proposed that phenoxyalkanoic acid degradation genes are highly mobile and thus are easily transferred among bacteria (17, 24, 33, 48, 49). On the other hand, gene flow is assumed to be less frequent between *Alphaproteobacteria* and *Beta*- and *Gammaproteobacteria* than among *Beta*- and *Gammaproteobacteria* (17). The unique combination and organization of the phenoxyalkanoic acid degradation genes in *S. herbicidovorans* MH indicate that a recent gene exchange between *Alpha*- and *Betaproteobacteria* must have taken place. This can be concluded from the finding that RdpA<sub>MH</sub> is 100% identical to RdpA<sub>MC1</sub> from *D. acidovorans* MC1, a member of the *Betaproteobacteria*. The transposase genes located next to *rdpA<sub>MH</sub>* and *sdpA<sub>MH</sub>* also support the hypothesis of gene mobilization. The transposase gene located nearby *rdpA<sub>MH</sub>* is very similar to a presumably promiscuous IS element which was implicated in transferring the toluene dioxygenase from *Ralstonia* sp. strain JS745 to *Ralstonia* sp. strain JS705 (35).

RdpA<sub>MH</sub> and SdpA<sub>MH</sub> are only modestly similar to TfdA<sub>α</sub>, the *tfdA*-like representatives of other *Alphaproteobacteria*, but more similar to the respective genes in the *Betaproteobacteria* *D. acidovorans* MC1. This contradicts the hypothesis that the *tfdA*-like representatives of *Alphaproteobacteria* and *Beta*- and *Gammaproteobacteria* have arisen by divergent evolution (23). However, all known *Alphaproteobacteria* harboring *tfdA<sub>α</sub>* have been isolated from pristine environments whereas *S. herbicidovorans* MH was isolated from a soil column percolated with dichlorprop (22). These conditions might have been more favorable for selection of *S. herbicidovorans* MH transconjugants which harbor a *rdp* gene than those carrying a *tfdA<sub>α</sub>* gene.
Fig. 5. Dendrogram illustrating sequence similarities of chlorocatechol 1,2-dioxygenases. The dendrogram was constructed as described in the material and methods section. CatA from *Burkholderia* sp. NK8 was used as an outgroup. The dccAI/II-genes from *S. herbicidovorans* MH are the only completely sequenced chlorocatechol 1,2-dioxygenases from a *Sphingomonas* strain. The partial PCR-sequences deposited in the database and their conceptual translations for other *Sphingomonas* strains were used to perform the dendrogram since the classification was not affected by the shortened sequences. The numbers at the branches stand for quartet puzzling (QP) reliability. Branches with values in the range of 90-100% are very strongly supported and with values >70% are principally reliable. Accession numbers for the sequences: TfdC *R. eutropha* JMP134(pJP4) P05403; TfdCII *R. eutropha* JMP134(pJP4) AAC44730; TfdC *Burkholderia* LMG9824; MocpA *Achromobacter xylosoxidans* A8 CAD56206; TcbB *Pseudomonas* sp. P51 P27098; ClcA *Pseudomonas aeruginosa* AAF00195; ClcA *P. putida* (pAC27) P11451; ClcA *Ralstonia* sp. JS705 CAA06947;
Chapter 2

In contrast to the α-ketoglutarate dependent dioxygenase genes, chlorocatechol 1,2-dioxygenases from *Beta- and Gammaproteobacteria* present in the database are less related to DccA\_III than they are among themselves. The phylogenetic relationships of strain MH chlorocatechol 1,2-dioxygenases became apparent in a dendrogram that includes several (chboro)catechol 1,2-dioxygenase and places the one from *Burkholderia* sp. NK8 as an outgroup (Fig. 5). The chlorocatechol 1,2-dioxygenases from Gram-positive bacteria clearly grouped together and formed a separate branch, which is in agreement with the results obtained by Eulberg et al. (12). Six different groups of chlorocatechol 1,2-dioxygenase could be defined: (i) ClpC, (ii) *Sphingomonas* homologs, (iii) TfdC\_pJP4 homologs, (iv) the TcbC-TetC-group, (v) TfdC\_II\_pJP4 homologs and (vi) the ClcA homologs. Subgroups (i) and (ii) build new branches whereas the classification of subgroups (iii) to (vi) agrees with the one described by Armengaud et al. (3). The two novel groups comprise sequences from *Alphaproteobacteria*, which were included for the first time in a dendrogram. The analysis shows a separate cluster of *Sphingomonas* chlorocatechol 1,2-dioxygenases and supports our hypothesis that *Sphingomonas* strains have not exchanged their different chlorocatechol 1,2-dioxygenase genes recently with those from *Beta- and Gammaproteobacteria*. The phenoxyalkanoic acid degradation pathway of strain MH turned out to be unique in its composition, genetic organization and regulation and it will be important to further study its characteristics in order to broaden our understanding of pathways different to the canonical 2,4-D degradation pathways in *Beta- and Gammaproteobacteria*.

Fig. 5 (cont.) ClpC *Desulvibacter lusatiensis* CAD60254; TfdC *Burkholderia* sp. R172 AAP92136; ClcA *Rhodococcus erythropolis* JC6206; ClcA *Rhodococcus opacus* 1CP AAC38251; CatA *Burkholderia* sp. NK8 BAB21462. For the partial sequences, the conceptual translation supplied by the authors was used and the accession numbers refer to the protein databases. *tfdC Sphingomonas* sp. tfd44 AAC23513; *tfdC D. acidovorans* MC1 AAD55082; *tfdC Sphingomonas* sp. EML 146 AAC23511; *tfdC Sphingomonas* sp. AW5 AAC23512.
REFERENCES


Phenoxyalkanoic acid genes in *S. herbicidovorans* MH


CHAPTER 3

Purification and Characterization of α-Ketoglutarate-dependent (R)-Dichlorprop Dioxygenase RdpA from *Sphingomonas herbicidovorans* MH

*Sphingomonas herbicidovorans* MH harbors two distinct α-ketoglutarate-dependent dichlorprop dioxygenases involved in phenoxyalkanoic acid herbicide degradation. Here, we report the purification and biochemical characterization of the (R)-dichlorprop dioxygenase (RdpA). RdpA was expressed and purified as a 6xHis-tagged fusion protein from *E. coli* BL21(DE3)(pLysS). RdpA belongs to subgroup II of α-ketoglutarate-dependent dioxygenases and has the specific common motif HXDX_{24}TX_{13}HX_{10}R. His-111, Asp-113 and His-270 build the 2-His-1-carboxylate facial triad and are predicted to be involved in iron binding. RdpA enantiospecifically transformed only the (R) enantiomers of mecoprop and dichlorprop with α-ketoglutarate as cosubstrate to the achiral phenols under concomitant release of succinate and pyruvate. Kinetic investigations showed highest affinity to (R)-mecoprop [2-(R)-2-methyl-4-chlorophenoxypropanoic acid] (\(K_{m}\)-value = 98.8 µM) and highest turnover rates for (R)-4-chlorophenoxypropanoic acid (\(k_{cat}\) = 241.9 min⁻¹). RdpA preferred phenoxypropanoic acids to other phenoxyalkanoic acids indicating that the alkyl moiety attached to α-carbon atom plays an important role for optimal orientation of the substrate at the active site. The apparent \(K_{m}\)-value for α-ketoglutarate was 2.8 µM. RdpA did not exhibit Michaelis-Menten kinetics with oxygen as a substrate, but was linearly dependent on O₂ in the measured range of air-saturated water (0-0.23 mM). Only 2-oxoadipate could replace α-ketoglutarate as cosubstrate, but activity was reduced to 2% of the activity on mecoprop with α-ketoglutarate. Ferrous iron was necessary for full activity of the enzyme and other divalent cations could not replace it.

Chapter 3

INTRODUCTION

Phenoxyalkanoic acid herbicides are widely used to control broadleaf weeds in agriculture, lawn pastures and industries. They are systemic and post emergence herbicides and act as synthetic auxins (1, 2, 26). Representatives are mecoprop \((2-(R,S)-2\text{-methyl-4-chlorophenoxypropanoic acid})\), dichlorprop \((2-(R,S)-2,4\text{-dichloro-phenoxypropanoic acid})\) and 2,4-D \((2,4\text{-dichlorophenoxyacetic acid})\).

Mecoprop and dichlorprop are chiral, have one stereogenic center and therefore, exist as two enantiomers. Already in the fifties, it was shown that only the \((R)\) enantiomers have herbicidal effects (27). However, both enantiomers were and still are applied and thereby, large amounts of isomeric ballast are introduced into the environment (11, 46, 47). *Sphingomonas herbicidovorans* MH, an *Alphaproteobacterium*, is able to grow on both enantiomers of mecoprop and dichlorprop and on 2,4-D as sole carbon and energy source (20, 22, 49). In batch cultures, it degrades the two enantiomers sequentially, the \((S)\) enantiomer being used first. (23, Chapter 1).

In *S. herbicidovorans* MH, the chiral mecoprop or dichlorprop is converted to the achiral phenol in an enantioselective reaction (Fig. 1). Nickel *et al.* (33) showed in experiments with cell-extracts, that two distinct enzymes are involved, which are highly specific for the respective enantiomer and that both enzymes belong to the enzyme family of \(\alpha\)-ketoglutarate-dependent dioxygenases. \(\alpha\)-Ketoglutarate-dependent dioxygenases build a heterologous group of enzymes, which are classified according to their biochemical characteristics. They are non-heme iron dioxygenases and require oxygen and \(\alpha\)-ketoglutarate as cosubstrates, ferrous ion as cofactor and ascorbate as a reducing agent. They catalyze a wide range of oxidative processes such as hydroxylations, epoxidations, desaturations, ring formation and expansion reactions (for reviews, see 8, 17, 25, 36, 41). Despite the diversity of their primary sequence, all of these dioxygenases have a 2-His-1-carboxylate facial triad (for a review, see 18) at the catalytic center. Furthermore, they have a motif in common according to which they are grouped into three subgroups (19). Important here is subgroup II, of which the representatives have the motif \(\text{HX}(D/E)X_{23-26}(T/S)\text{X}_{114-183}\text{HX}_{10-13}\text{R}\). Well known members are the \(\alpha\)-
ketoglutarate-dependent taurine dioxygenase (TauD) from *Escherichia coli* involved in taurine utilization (12) and 2,4-D dioxygenase (TfdA) from *R. eutropha* JMP134(pJP4) involved in 2,4-D degradation (14-16). TfdA catalyzes the oxidative cleavage of the ether-bond in 2,4-D to 2,4-dichlorophenol.

Fig. 1. Initial degradation step of chiral phenoxypropanoic acids in *S. herbicidovorans* MH catalyzed by the α-ketoglutarate-dependent (R)-dichlorprop dioxygenases (RdpA) and α-ketoglutarate-dependent (S)-dichlorprop dioxygenases (SdpA).

Recently, the genes coding for the enzymes involved in phenoxypropanoic acid degradation in *S. herbicidovorans* MH have been identified and isolated (Chapter 2). The genes for two α-ketoglutarate-dependent dioxygenases named RdpA and SdpA were discovered and are likely to be responsible for the initial degradation steps of mecoprop and dichlorprop. At amino acid level, RdpA is 30% identical to TfdA but 100% to RdpA from *Delftia acidovorans* MC1, another phenoxypropanoic acid degrader (28, 44). The primary sequence of SdpA, on the other hand, only showed 30% amino acid identity with RdpA.

In this study, we expressed and purified RdpA as a 6xHis-tagged fusion protein. Enzyme activity measurements with the purified protein verified the
classification as an α-ketoglutarate-dependent dioxygenase and primary sequence alignment showed that RdpA belonged to subgroup II. We further characterized RdpA in terms of kinetic behavior for different substrates and cosubstrates, determined the (co)substrate specificities, and provided evidence that RdpA exclusively converts the \((R)\) enantiomer of phenoxypropanoic acid herbicides.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** *Escherichia coli* DH5α was used as a host for cloning experiments and *E. coli* BL21(DE3)(pLysS) as a host for expression studies with constructs in pET-15b (Novagen, Darmstadt, Germany). *E. coli* strains were grown at 30°C or 37°C in Luria-Bertani (LB) medium (40). When appropriate, ampicillin and chloramphenicol were added at 50 µg/ml and 25 µg/ml final concentrations, respectively. Solid media were prepared by the addition of 1.5% (w/v) agar.

**Standard molecular techniques.** Cloning and digestions were done according to established procedures (4, 40). Restriction enzymes and other DNA modifying enzymes were purchased from Promega (Wallisellen, Switzerland) and Fermentas (Nunningen, Switzerland). Plasmids and cosmids were isolated by the boiling miniprep method, the alkaline lysis method according to Sambrook et al. (40) or with the E.Z.N.A. plasmid miniprep kit II (Peqlab Biotechnologies GmbH, Baden-Dättwil, Switzerland) as suggested by the manufacturer. Gel extraction was carried out with the MinElute Gel Extraction Kit (Qiagen AG, Basel, Switzerland) according to the protocol of the supplier.

**Construction of N-terminal His-tagged recombinant RdpA expression plasmid.** Expression plasmid pMec15 was constructed in the PCR by reamplification of the *rdpA* gene from pMec10 (Chapter 2) with the following primers: 5’-CGC TCA TAT GCA TGC TGC ACT-3’ and 5’-AGC GGG GAT CCG CGT CGC C-3’. Thereby, a Ndel restriction site at the translational start and a BamHI restriction site just past the stop codon were introduced (underlined nucleotides). The fragment was first cloned into pGEM®-T Easy (Promega) generating pMec14 and verified by sequencing. The plasmid was then digested with Ndel and BamHI and ligated into the same sites of vector pET-15b yielding pMec15.

**Expression and purification of recombinant RdpA.** *E. coli* BL21(DE3)(pLysS) harboring pMec15 was grown in Erlenmeyer flasks containing LB-medium complemented with ampicillin and chloramphenicol at 30°C with constant shaking. To express the proteins, a preculture of *E. coli* BL21(DE3)(pLysS) harboring pMec15 was grown overnight at 37°C. A volume of 1% (v/v) of the preculture was transferred to fresh medium and grown at 30°C until an optical density of 0.4–0.6 (measured at 546 nm) was reached, after which isopropyl-β-D-
thiogalactopyranoside was added to a final concentration of 1 mM. The culture was incubated for another 3 hours at 30°C and the cells were harvested by centrifugation at 7'500 × g for 30 min at 4°C and the pellet was stored at –20°C until further use. For the preparation of cell extracts, 1 g of cells (wet weight) was resuspended in 3 ml of lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH adjusted to 8) and the cells were broken by one passage through a French press at 1 MPa. The lysate was centrifuged for 45 min at 16'000 × g at 4°C and aliquots were stored at –20°C.

RdpA was purified in a one-step procedure. Up to one mg of protein was loaded on a 2-ml Ni-NTA superflo-flow-agarose column (Qiagen) at a flow rate of 0.5 ml/min. Unbound protein was removed stepwise with three times five column volumes of wash-buffer (50 mM NaH₂PO₄, 300 mM NaCl) containing 20 mM, 50 mM, and 100 mM imidazole (pH adjusted to 8), respectively, at the same flow rate. RdpA was then eluted in 1-ml fractions with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH adjusted to 8) at a flow rate of 0.8 ml/min. The active fractions were pooled and desalted into 20 mM Tris-HCl, pH 7.5, using PD-10 columns (Amersham Biosciences, Dübendorf, Switzerland) and stored at 4°C. Thrombin protease (Amersham Biosciences) was used to cleave the N-terminal His-tag. The N-terminal His-tag of approximately 10 µg of RdpA was completely cleaved by 0.5 U of thrombin protease within 2 h at room temperature.

**End-point assay.** Analysis of alternative substrate use was done in an end-point assay by measuring the formation of the product by HPLC. The standard assay mixture contained 50 mM
imidazole, pH 6.75, 1 mM substrate, 1 mM α-ketoglutarate, 100 µM (NH₄)₂Fe(SO₄)₂ and 1 mM ascorbic acid. The assay mixtures were incubated at 30°C for 1 min and inactivated in a boiling waterbath for 2 min. Negative controls without RdpA were treated in the same manner. The products were analyzed on a Nucleodex α-PM column (200 by 4 mm, Macherey-Nagel, Düren, Germany) as described previously (33). Specific activity was defined as the amount of phenol (µmol) which was formed per min and per mg of protein in the presence of the phenoxalkanoic acids. Oxidation rates were corrected for non-specific formation of the product in the absence of the enzyme.

Oxygen electrode measurements. Kinetic measurements with oxygen were done in a Rank brothers oxygen electrode at 30°C. The standard assay mixture contained 50 mM imidazole, pH 6.75, 2 mM substrate, 2 mM α-ketoglutarate, 100 µM (NH₄)₂Fe(SO₄)₂ and 1 mM ascorbic acid in a 1-ml volume. The assays were started by the addition of the substrate and O₂-concentration was monitored in 2 sec intervals for 15 min. An assay without substrate showed that inherent O₂-consumption was small and could be neglected for the calculation of specific activities. Specific activity was defined as the amount of oxygen (µmol) that the enzyme oxidized per min and per mg of protein in the presence of all substrates.

Kinetic Measurements. The kinetic parameters $K_m$ and $k_{cat}$ were calculated with a computer program (IgorPro, WaveMetrics Inc., Lake Oswego, OR). The calculation was based on weighted nonlinear regression analysis of the Michaelis-Menten model. To calculate $K_m$ and $k_{cat}$ of RdpA with respect to oxygen, we used the first derivative, i.e. the velocity (µmol × min⁻¹), of the monitored oxygen concentration in dependence of time.

Protein concentration determination and SDS-PAGE. Protein concentrations were measured using the Bradford-method (7) with the Bio-Rad reagent dye concentrate reagens following the instructions of the manufacturer (Bio-Rad, Reinach, Switzerland). Bovine serum albumin fraction V (Sigma-Aldrich) was used as a reference protein. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 12% acrylamide slab gels according to established procedures (24).

Native size determination of RdpA. Molecular weight analysis of purified RdpA was determined on a Superdex 200 preparation grade HiLoad 16/60 size exclusion column (Amersham Biosciences). Approximately 0.25 mg of protein were loaded on the HiLoad column and eluted at a flow rate of 0.2 ml/min in 50 mM Na₂HPO₄-KH₂PO₄-buffer (pH 7) containing 150 mM NaCl. The calibration curve was done with the gel filtration standard from Bio-Rad containing thyroglobulin (bovine, 670 kDa), Gamma globulin (bovine, 158 kDa), Ovalbumin (chicken, 44 kDa), Myoglobin (horse, 17 kDa) and Vitamin B-12 (1.35 kDa). Blue dextran was used to determine the void volume.
**Sequencing.** DNA sequencing was performed on double stranded DNA templates with the Thermo Sequenase Primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Biosciences) and M13 primers (5). Sequencing reactions were analyzed on an automated DNA sequencer model 4200 IR² (LI-COR Inc., Lincoln, USA). Primers for sequencing were labelled at the 5'-end with IRD-700 or IRD-800 and were purchased from MWG Biotech AG (Ebersberg, Germany). Sequencing data were analyzed with DNASTAR software (DNASTAR Inc., Madison, USA) and compared to the databases by BLAST searches (www.ncbi.nlm.nih.gov/BLAST, (42)).

**Chemicals.** Phenoxyalkanoic acids were purchased at Riedel-de Haën (Buchs, Switzerland; PESTANAL®, 99% purity) or Sigma-Aldrich (97-99% purity). (S)-mecoprop and (S)-dichlorprop were synthesized by C. Zipper (48). α-Ketoglutarate and other (di)carboxyacids were bought from Sigma-Aldrich. All other chemicals were obtained from Fluka (Buchs, Switzerland) or from Merck AG (Dietikon, Switzerland).

**RESULTS**

**Expression and purification of recombinant RdpA.** The rdpA gene was expressed in *E. coli* BL21 (DE3)(pLysS) harboring pMec15 as a N-terminal His-tag fusion protein. The expression produced a moderate level of recombinant RdpA in the soluble fraction amounting to approximately 16% of total protein. Decrease of incubation temperature during expression in *E. coli* did not increase the amount of the 6xhis-tagged RdpA in cell-free extract. 6xHis-tagged RdpA was purified about 16-fold in a single step procedure to apparent homogeneity by Ni-NTA-affinity chromatography (Table 1 and Fig. 2). The calculated mass of RdpA was 33.2 kDa and that of the 6xHis-tagged RdpA fusion protein 34.9 kDa, which was consistent with the size estimated by SDS-PAGE of approximately 36 kDa. The recombinant RdpA was treated with thrombin protease to cleave the 6xHis-tag and enzyme activity of the native and the recombinant protein was measured. Both enzymes were equally active towards (R)-mecoprop suggesting that the 6xHis-tag had no influence on the activity. Therefore, all experiments were carried out with the recombinant protein, if not stated otherwise.
Table 1: Representative purification table of α-ketoglutarate-dependent (R)-dichlorprop dioxygenase

<table>
<thead>
<tr>
<th></th>
<th>volume (ml)</th>
<th>total protein (mg)</th>
<th>total activity (U)</th>
<th>specific activity (U/mg protein)</th>
<th>recovery %</th>
<th>purification -fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>cell extract</td>
<td>10</td>
<td>133.1</td>
<td>30.9</td>
<td>0.2</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ni-NTA superflow</td>
<td>7</td>
<td>1.8</td>
<td>7</td>
<td>3.8</td>
<td>22.7</td>
<td>16.5</td>
</tr>
</tbody>
</table>

Fig. 2. Purification of α-ketoglutarate-dependent (R)-dichlorprop dioxygenase from *S. herbicidovorans* MH expressed in *E. coli* BL21(DE3)(pLysS)(pMec15) as a 6xHis-tagged fusion protein. Protein samples were analyzed on denaturing gel electrophoresis on a 12% polyacrylamide gel and stained with Coomassie Brilliant Blue. Lane M, molecular weight markers (indicated in kDa), lane 1, extract of induced cells, lane 2, apparently pure RdpA (obtained after Ni-NTA-superflow chromatography).

The native structure of the protein was determined by size exclusion chromatography after cleaving the 6xHis-tag by thrombin treatment. A native weight of 104±7 kDa was found which was consistent with a homotrimeric structure assuming a subunit weight of 33.2 kDa.

**RdpA is an α-ketoglutarate-dependent dioxygenase catalyzing an enantioselective reaction.** (R)-dichlorprop dioxygenase was classified as an α-ketoglutarate-dependent dioxygenase according to its amino acid sequence (Chapter 2). This finding was verified by enzyme assays. RdpA activity was completely abolished in the absence of α-ketoglutarate, strongly reduced in assays without ferrous iron and slightly reduced in the absence of ascorbate (Table 2).
Table 2: Dependence of α-ketoglutarate-dependent (R)-dichlorprop dioxygenase activity on substrate, cosubstrate and cofactors.

<table>
<thead>
<tr>
<th></th>
<th>specific activity (U/mg protein)</th>
<th>% activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>complete assay</td>
<td>3.4</td>
<td>100</td>
</tr>
<tr>
<td>- (R)-mecoprop(^a)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>- α-ketoglutarate(^a)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>- Fe(II)(^a)</td>
<td>0.6</td>
<td>12.7</td>
</tr>
<tr>
<td>- ascorbate(^a)</td>
<td>2.9</td>
<td>85.3</td>
</tr>
</tbody>
</table>

\(^a\) Complete assay without the specified component.

Enzymatic activity of RdpA was measured with three different assays: (i) the coupled continuous assay, (ii) the end-point assay and (iii) \(\text{O}_2\)-measurements. In all these assays, different substrate or product concentrations of the enzymatic reaction were measured. Hence, the specific activity was calculated upon the production of pyruvate in the coupled continuous assay, the formation of the phenolic compound in the end-point assay and the consumption of oxygen with the oxygen electrode. All calculations resulted in similar specific activities demonstrating that oxygen consumption, pyruvate and phenol production had a 1:1 stoichiometry as expected for this reaction (Fig. 1). It has to be noted that specific activity was reproducible for a single purified RdpA batch, but that between replicates, i.e., RdpA purified from independently induced \(\text{E. coli}\) cultures, varied by a factor of 2 and ranged between 2 – 4 U/mg protein.

We measured enzyme activity of RdpA with the racemate and the single enantiomers of mecoprop and dichlorprop. RdpA only converted the (\(R\)) enantiomer of both compounds but not the (\(S\)) enantiomers. When incubated with the racemate, no inhibition of the enzyme by the (\(S\)) enantiomer was observed (not shown).
Fig. 3. Temperature (panel A) and pH (panel B) dependence of α-ketoglutarate-dependent (R)-dichlorprop dioxygenase. (A) The black diamonds represent data obtained with the end-point assay, the black circles data obtained with the coupled continuous assay. The measurements were carried out with two independently purified (R)-dichlorprop dioxygenase samples. (B) The diamonds show the mean value of six measurements of two independent experiments. Enzyme activity was determined with the coupled continuous assay.

**Physicochemical characterization.** RdpA was stable at 4°C for up to eight weeks and at room temperature for up to seven days, but was inactivated by several freezing and thawing cycles. Therefore, RdpA was stored at 4°C. The pH-optimum was investigated in 50 mM imidazole in a range from pH 6 to 7.75 and the optimum was found to be between 6.5-6.75 (Fig. 3A). Temperature dependence
was also studied, but no optimum could be determined (Fig. 3B). The measurements were carried out with two independently purified RdpA batches and specific activity was measured once with the coupled continuous assay, and then repeated with the end-point assay in order to avoid misleading results due to inactivation of the reporter enzyme L-lactate dehydrogenase in the coupled-continuous assay. RdpA-activity was stable over a wide range of 20 to 55°C. A slightly higher activity was observed at 10°C and 20°C, but only in the end-point assay. However, the general trend for both enzyme activity measurements agreed and showed no Arrhenius-like behaviour.

**Substrate, cosubstrate and cofactor specificities.** Substrate specificities of RdpA were determined using the coupled-continuous assay for phenoxypropanoic acids and using the end-point assay for all other substrates. On one hand, we examined the influence of the alkanoic acid group attached by an ether bond to the phenoxyring by testing activities with phenoxyacetic, phenoxypropanoic, and phenoxybutyric acids as substrates. On the other hand, the effect of different substituents such as hydroxy-, methyl- and chloride groups at the aromatic ring was investigated. The phenoxypropanoic acids are chiral and as mentioned earlier, RdpA<sub>H</sub> only converted \((R)\)-dichlorprop and \((R)\)-mecoprop. RdpA exclusively converted phenoxypropanoic acids and none of the phenoxyacetic or –butyric acids, with the exception of 3-methyl-5-chlorophenoxyacetic acid (MCPA), which was oxidized at 10% of the highest activity. 2-(\(R,S\))-4-chlorophenoxypropanoic acid was turned over with the highest rate followed by dichlorprop and mecoprop. Interestingly, also the hydroxylated and the unsubstituted phenoxypropanoic acids were converted (Table 3).
Chapter 3

Table 3: Substrate specificities of α-ketoglutarate-dependent (R)-dichlorprop dioxygenase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activitya (%) ± std. dev.</th>
<th>Activity (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(R,S)-Mecoprop</td>
<td>100</td>
<td>3.1 / 4.2c</td>
</tr>
<tr>
<td>(R,S)-Dichlorprop</td>
<td>115.5 ± 3.6</td>
<td>3.6</td>
</tr>
<tr>
<td>2-(R,S)-Phenoxypropanoic acid</td>
<td>39.7 ± 7.6</td>
<td>1.2</td>
</tr>
<tr>
<td>2-(R,S)-3-Chlorophenoxypropanoic acid</td>
<td>14.8 ± 2.0</td>
<td>0.5</td>
</tr>
<tr>
<td>2-(R,S)-4-Chlorophenoxypropanoic acid</td>
<td>152.1 ± 9.0</td>
<td>4.9</td>
</tr>
<tr>
<td>2-(R,S)-2,4,5-Trichlorophenoxypropanoic acid</td>
<td>49.4 ± 4.3</td>
<td>1.6</td>
</tr>
<tr>
<td>2-(R,S)-4-Hydroxyphenoxypropanoic acid</td>
<td>51.3 ± 7.3</td>
<td>1.6</td>
</tr>
<tr>
<td>2,4-D</td>
<td>&lt;1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>MCPA</td>
<td>7.7 ± 4.3</td>
<td>0.3</td>
</tr>
<tr>
<td>2,4-Dichlorophenoxybutyric acid</td>
<td>&lt;1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>4-Chloro-2-methylphenoxybutyric acidc</td>
<td>&lt;1</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

a The substrate range was determined with RdpA\textsubscript{H} purified from two independent batches and enzyme activity of each batch was measured three times with all substrates.
b The standard deviation is given in %.
c The first number is the activity determined in the coupled continuous assay, the second in the end-point-assay.
d Enzyme activity was determined with the end-point-assay.
e In one measurement, very low activity could be determined. However, the measured 2,4-dichlorophenol concentration was below the detection limit of 1 µM and no increase of the amount of 2,4-dichlorophenol converted per time was observed when the amount of RdpA\textsubscript{H} in the assay was increased. Therefore, the activity was set to <1%.

We also tested whether several carboxylic- and dicarboxylic acids could replace α-ketoglutarate as a cofactor. Since the reporter enzyme in the coupled continuous assay, L-lactate dehydrogenase, showed activity with some co-substrates, RdpA activity was determined with the end-point assay. RdpA exhibited a quite narrow cosubstrate range (Table 4). Only with 2-oxoadipate, the C\textsubscript{6}-dicarboxylic acid, slight activity was observed whereas the other tested co-substrates, oxaloacetate, 2-oxobutyrate and 2-oxovalerate, could not replace α-ketoglutarate.
Table 4: Cosubstrate specificities of α-ketoglutarate-dependent (R)-dichlorprop dioxygenase

<table>
<thead>
<tr>
<th>Cosubstrate</th>
<th>% Activity</th>
<th>Specific activity (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Ketoglutarate</td>
<td>100</td>
<td>3.8</td>
</tr>
<tr>
<td>Oxaloacetate</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2-Oxobutyrate</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2-Oxovalerate</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2-Oxoadipate</td>
<td>1.9</td>
<td>0.1</td>
</tr>
</tbody>
</table>

In the absence of ferrous iron, RdpA activity was reduced to 12% of its maximal activity. Activity was independent of the iron-salt, but other divalent cations could not support activity in the same range as Fe(II) (Table 5). Activity was diminished with CaCl$_2$ and MgCl$_2$ to about the same level as in the absence of ferrous ion and was totally abolished with Co(II), Mn(II), and Ni(II), indicating an inhibition of the enzyme by these cations.

Table 5: Cofactor specificities of α-ketoglutarate-dependent (R)-dichlorprop dioxygenase

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>% Activity</th>
<th>Specific activity (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe(NH$_4$)$_2$(SO$_4$)$_2$</td>
<td>100</td>
<td>2.8</td>
</tr>
<tr>
<td>Fe(SO$_4$)$_2$</td>
<td>97.5</td>
<td>2.7</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>15.9</td>
<td>0.4</td>
</tr>
<tr>
<td>CoCl$_2$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>9.4</td>
<td>0.3</td>
</tr>
<tr>
<td>MnCl$_2$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NiCl$_2$</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Kinetic analysis.** We determined the kinetic parameters for the (R) enantiomers of mecoprop, dichlorprop, and 2-(R,S)-4-chlorophenoxypropanoic acid, the cosubstrates α-ketoglutarate and oxygen. For the phenoxypropanoic acids and α-ketoglutarate, the coupled continuous assay was used whereas the O$_2$-consumption was measured in an oxygen electrode. Michaelis-Menten kinetic was observed for the (R)-phenoxypropanoic acids and α-ketoglutarate. RdpA exhibited the lowest $K_m$-value and highest efficiency for (R)-mecoprop, whereas $k_{cat}$ was
highest for (R,S)-4-chlorophenoxypropanoic acid (Table 6). The apparent $K_m$-value for $\alpha$-ketoglutarate was 2.8 µM which was below the lowest tested $\alpha$-ketoglutarate concentration, but the obtained data fitted well to a Michaelis-Menten curve and the value seems reliable.

Table 6: Kinetic parameters of $\alpha$-ketoglutarate-dependent (R)-dichlorprop dioxygenase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (min⁻¹)</th>
<th>$k_{cat}/K_m$ (µM⁻¹ × min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(R)-Mecoprop (10 – 2000 µM)</td>
<td>98.8±6.4</td>
<td>114.2</td>
<td>1.16</td>
</tr>
<tr>
<td>(R)-Dichlorprop (50 – 2000 µM)</td>
<td>163.7±13.5</td>
<td>170.1</td>
<td>1.04</td>
</tr>
<tr>
<td>(R)-4-Chlorophenoxypropanoic acid (120 – 2000 µM)</td>
<td>262.4±10.1</td>
<td>241.9</td>
<td>0.92</td>
</tr>
<tr>
<td>$\alpha$-Ketoglutarate (7-1900 µM)</td>
<td>2.8±0.8</td>
<td>135.6</td>
<td>48.82</td>
</tr>
</tbody>
</table>

Oxygen kinetic parameters were measured by monitoring the O₂-consumption in an oxygen electrode. The reaction rate at the different O₂-concentrations was then calculated by taking the first derivative of the O₂-consumption curve. The dependence on oxygen kinetic did not follow Michaelis-Menten kinetics in the oxygen concentration range of air-saturated water at 30°C (Fig. 4) and therefore, a $K_m$-value could not be calculated.

![Fig. 4. Kinetics of $\alpha$-ketoglutarate-dependent (R)-dichlorprop dioxygenase towards oxygen. The open triangles and black circles represent two independent experiments.](image-url)
**DISCUSSION**

**RdpA is an α-ketoglutarate-dependent dioxygenase.** α-Ketoglutarate-dependent dioxygenases are grouped according to their cosubstrate and cofactor requirement. Enzyme activity measurements demonstrated that RdpA belonged to the heterologous enzyme family of α-ketoglutarate-dependent dioxygenases, since its activity was strictly dependent on oxygen and α-ketoglutarate. Initial activity was reduced without ascorbate to about 85% of the highest activity. Ascorbate is formally not needed in the reaction and it is generally thought that it keeps Fe(II) in the reduced state. Two other α-ketoglutarate-dependent dioxygenases, prolyl 4-hydroxylase and lysyl hydroxylase, are almost completely inactivated within one minute in the absence of ascorbate (10, 30, 31, 37). The inactivation is thought to involve a side reaction in which α-ketoglutarate is decarboxylated. This reaction is coupled to the oxidation of Fe(II) to Fe(III). Ascorbate reduces the ferryl iron thereby reactivating the enzyme. TfdA, on the other hand, is not as sensitive to the absence of ascorbate and is only inactivated after 30-40 minutes (15). However, the reduced activity of RdpA when ascorbate was absent indicates that the side-reaction of α-ketoglutarate decarboxylation takes place although inactivation is not as fast as it is for the peptide hydroxylases.

An alignment of α-ketoglutarate-dependent dioxygenases with sequence homology to RdpA revealed the 2-His-1-carboxylate facial triad and several other conserved amino acid residues (Fig. 5). RdpA possesses the motif of subgroup II of the α-ketoglutarate-dependent dioxygenase family, which is HXDX_{24}TX_{13}HX_{10}R. Based on the crystal structure of TauD and on the structure model of TfdA and on mutagenesis studies, the role of some conserved amino acid residues in RdpA were predicted (13, 34, 38). His-111, Asp-113 and His-270 were recognized to build the facial triad and are likely to ligands for iron-binding, whereas Thr-138 and Arg-281 are putative α-ketoglutarate binding sites.

The (R)-dichlorprop dioxygenases from *S. herbicidovorans* MH and *D. acidovorans* MC1 are, to our knowledge, the only representatives of the class of α-ketoglutarate-dependent dioxygenases with a homotrimeric structure (43). Most
enzymes of this class are mono- or homodimers such as SdpA from *D. acidovorans* MC1 and TfdA, tetramers such as the alkylsulfatase from *Pseudomonas putida* are also known (12, 15, 43).

RdpA is surprisingly insensitive to temperature. No Arrhenius-like activity increase was observed and RdpA<sub>MM</sub> was stable up to 55°C. This findings partially agree with those for an α-ketoglutarate-dependent 2,4-D dioxygenase from *Burkholderia cepacia* 2a, which also showed an unexpectedly low activity increase with increasing temperature and at 10°C, still showed 77% activity of the one obtained at 25°C (35). In contrast, TfdA from *R. eutropha* JMP134(pJP4), exhibits a more typical activity increase with higher temperature, is only stable up to 30°C, and is completely inactivated at 40°C within 5 min (15).

**RdpA catalyzes the enantioselective reaction from (R)-phenoxypropanoic acids to the achiral phenol.** RdpA converts only the (R)-enantiomer of phenoxypropanoic acid herbicides, but not the (S) enantiomer. Also after prolonged incubation, we could not find any activity with the (S)-enantiomer of either dichlorprop or mecoprop. We propose therefore that RdpA from *S. herbicidovorans* MH catalyzes an enantioselective reaction. This result agrees with the results obtained with the identical (R)-dichlorprop dioxygenase RdpA from *D. acidovorans* MC1 which did not convert (S) enantiomers of phenoxypropanoic acids to a significant extent (44). α-Ketoglutarate-dependent 2,4-D dioxygenases are also able to oxidize mecoprop and dichlorprop and they also convert only one enantiomer. Surprisingly, they differ in the preference of the enantiomer despite their high nucleotide sequence similarity. TfdA from *R. eutropha* JMP134(pJP4) and *Burkholderia cepacia* RASC cleave the ether-bond of only (S)-dichlorprop, whereas TfdA from *A. denitrificans* exclusively converts the (R) enantiomer (39). It can be concluded that none of these α-ketoglutarate-dependent dioxygenases is able to convert both enantiomers of phenoxypropanoic acids with a significant catalytic rate. To oxidize both enantiomers, two distinct dioxygenases are required in strains such as *S. herbicidovorans* MH or *D. acidovorans* MC1 (33, 43, 44, Chapters 2 and 4).
Fig. 5. Alignment of amino acid sequences of RdpA from strain MH and other group II α-ketoglutarate-dependent dioxygenases. The sequence names and accession numbers are shown in parentheses: 2,4-D dioxygenase from *R. eutropha* JMP134(pJP4) (TfdA_pJP4, AAA21983), (R)-dichlorprop dioxygenase from *S. herbicidovorans* MH (RdpA_MH, CAF32811), (S)-dichlorprop dioxygenase from *S. herbicidovorans* MH (SdpA_MH, CAF32813), (S)-dichlorprop dioxygenase from *D. acidovorans* MC1 (SdpA_MC1, AAM90963), taurine dioxygenase from *E. coli* (TauD, AAC73471), alkylsulfate dioxygenase from *Pseudomonas putida* (AtsK, AAD31784); clavaminate synthase from *Streptomyces clavuligeres* (CAS1, A44241). Highly conserved residues (dark grey boxes) and residues conserved in six out of the seven sequences (light grey boxes) are highlighted. Asterisks mark the (conserved) Fe(II)-binding residues, triangles the α-ketoglutarate binding residues of TauD and TfdA (13, 34). They are aligned to the proposed residues of RdpA_MH. Amino acid numbers for each sequence are indicated at the right of each line.
Substrate and cosubstrate specificities of RdpA. RdpA exhibited narrow substrate specificities with respect to the alkyl moiety at the alkanoic acid side chain since only phenoxypropanoic acids were converted, but not 2,4-D and phenoxybutyric acids. This is in contrast to RdpA from *D. acidovorans* MC1, which reacted with 2,4-D at 14% activity of the activity obtained with *(R)*-dichlorprop (44). Also TfdA from *R. eutropha* JMP134(pJP4) exhibited a broader substrate range and converts, besides phenoxyacetic acids, also *(S)*-phenoxypropanoic acids. However, neither RdpA nor TfdA react with phenoxybutyric acids (15, 44). All these results indicate that the active sites of RdpA and of TfdA might be specific with regard to the alkanoic acid substituent and are indifferent with regard to the substituents of the aromatic ring. Additionally, RdpA is not inhibited by the *(S)* enantiomers of phenoxypropanoic acids, which indicates no binding of these enantiomers at the active site. It can therefore be proposed that only the *(R)* enantiomers fit into the active site and that the other substrates are excluded either by steric hindrance or due to repulsion of charged amino acid residues.

α-Ketoglutarate-dependent dioxygenases show different cosubstrate specificities. AtsK, an alkylsulfatase from *Pseudomonas putida* S-313, and TfdA, for instance, exhibit a broad cosubstrate range and many oxoacids substitute for α-ketoglutarate (15, 21). TauD and the inorganic α-ketoglutarate-dependent hypophosphite dioxygenase HtxA exhibited stricter specificities and besides α-ketoglutarate, only 2-oxoadipate supported (reduced) activity in the range of 4 – 23% (12, 15, 45). RdpA showed the same cosubstrate specificities as the latter group, but the remaining activity with 2-oxoadipate was only 2%. Many α-ketoglutarate-dependent dioxygenases including RdpA are well adapted to α-ketoglutarate and have high affinity to α-ketoglutarate with apparent *K*<sub>m</sub>-values in the range of 3 – 20 µM.

Activity linearly dependent on oxygen. The oxygen kinetics for RdpA did not follow the Michaelis-Menten model. Initial velocities were determined in the range of 0 – 0.23 mM O<sub>2</sub>, of which the upper value represents maximum oxygen solubility of air-saturated water at 30°C. A linear relationship between O<sub>2</sub>-concen-
(R)-dichlorprop dioxygenase
tration and $V_{\text{max}}$ was observed and hence, a $K_m$-value could not be calculated. The linear dependence can be explained by the fact, that the enzyme is only fully saturated at concentrations above 0.23 mM O$_2$ as it was determined for the 3,5-dichlorocatechol dioxygenase from *Pseudomonas cepacia* CSV90 with a $K_m$-value of 652 µM O$_2$ (6). To observe Michaelis-Menten kinetics, it would be necessary to carry out enzyme activity measurements with pure oxygen dissolved in water. In contrast to the low oxygen affinity of RdpA, apparent $K_m$-values of 40 – 50 µM O$_2$ were observed for lysyl hydroxylase and an α-ketoglutarate-dependent 4-hydroxylase from *Catharanthus roseus* which is involved in vindoline biosynthesis (9, 37). Flavonol dioxygenase exhibits even higher affinity and a $K_m$-value of 5.26 µM O$_2$ was determined (3). This indicates that, in comparison to other α-ketoglutarate-dependent dioxygenases, RdpA exhibits unusual low affinity to oxygen and that its activity is highly dependent on oxygen concentration under natural conditions. Furthermore, it has to be noted that oxygen concentrations in ‘normal’ laboratory enzymatic measurements are not saturating for this enzyme.

We presented here the characteristics of the α-ketoglutarate-dependent (R)-dichlorprop dioxygenase from *S. herbicidovorans* MH that catalyzes the enantioselective reaction from (R)-phenoxypropanoic acids to the achiral phenol. Interestingly, *S. herbicidovorans* MH harbors a second dioxygenase which is specific for the (S) enantiomers (32, Chapter 4). These two dioxygenases are a good example of an enzyme pair of which each enzyme converts one enantiomers of a chiral substrate. It will be important to further elucidate the enzyme mechanisms, how they discriminate between the enantiomers, and how they evolved in one strain.
REFERENCES


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\(\alpha\)-Ketoglutarate Dependent (S)-Dichlorprop Dioxygenase (SdpA) from *Sphingomonas herbicidovorans* MH: Purification and Characterization of an Enantiospecific Enzyme

*Sphingomonas herbicidovorans* MH harbors two enantiospecific \(\alpha\)-ketoglutarate-dependent dichlorprop dioxygenases involved in phenoxyalkanoic acid degradation. Here, we characterize (S)-dichlorprop dioxygenase (SdpA), which was expressed and purified as a 6xHis-tagged fusion protein from *E. coli* BL21(DE3)(pLysS). SdpA belongs to sub-group II of \(\alpha\)-ketoglutarate-dependent dioxygenases. It enantiospecifically transformed the (S) enantiomers of phenoxypropanoic acids and was slightly inhibited in the presence of the (R) enantiomer. It showed highest activity for (R,S)-mecoprop [2-(R,S)-2-methyl-4-chlorophenoxypropanoic acid] and preferred substituted phenoxy-propanoic acids to substituted phenoxybutyric acids and –acetic acids. Kinetic investigations showed Michaelis-Menten kinetics with all substrates and cosubstrates. SdpA had the highest affinity and the highest turnover rate for (S)-mecoprop \((K_m \text{-value} = 132 \mu M, k_{cat} = 304 \text{ min}^{-1})\). For oxygen and \(\alpha\)-ketoglutarate, the apparent \(K_m\)-values were 159 \(\mu M\) and 19.6 \(\mu M\), respectively. Cosubstrate specificity was narrow since only with 2-oxoadipate, apart from \(\alpha\)-ketoglutarate, slight activity was measured. In the absence of ferrous iron, enzyme activity was strongly decreased and other divalent cation could not replace Fe(II). Although results from growth experiments suggest that strain MH should harbor another more specific 2,4-D converting enzyme, *tfdA*-like, a *tfdAα*-like or *cadAB*-like genes were not discovered in a screening by heterologous hybridization and PCR.
INTRODUCTION

Phenoxyalkanoic acid herbicides such as 2,4-dichlorophenoxyacetic acid (2,4-D), chiral mecoprop (2-(R,S)-2-methyl-4-chlorophenoxypropanoic acid) and chiral dichlorprop (2-(R,S)-2,4-dichlorophenoxy-propanoic acid) have been widely used against broadleaf weeds in agriculture, industries and lawn pastures since the 1940ies (1, 10). A number of bacteria was isolated from the environment which are able to degrade these herbicides. One representative is the Alphaproteobacterium Sphingomonas herbicidovorans MH, which is able to grow on several phenoxy-alkanoic acid herbicides as sole carbon and energy source (20, 25, 46). S. herbicidovorans MH degrades mecoprop and dichlorprop enantioselectively, the (S)-enantiomer being used first (47). The chiral substrates are converted to the achiral phenols, which are then transformed to the respective catechols (Chapter 2). The catechols are metabolized through the modified ortho-cleavage pathway to intermediates of the tricarboxylic acid cycle. This pathway is analogous to the well-studied degradation pathway of 2,4-D in Ralstonia eutropha JMP134(pJP4). However, the genetic background of the pathway differs in these two strains despite the analogy of the reactions (9, 13, 14, 32, 39, Chapter 2).

α-Ketoglutarate-dependent dioxygenases are non-heme iron enzymes, which are involved in various reactions such as hydroxylations, epoxidations, ring formations, and expansion reactions (17, 33). They are grouped according to their cosubstrate requirement since most members need dioxygen and α-ketoglutarate as cosubstrates, ferrous iron as cofactor and ascorbate as a reducing agent. α-Ketoglutarate dependent-dioxygenases exhibit low amino acid sequence similarity, but they share a 2-His-1-carboxylate facial triad (18). Phenoxyalkanoic acid herbicide degraders such as R. eutropha JMP134(pJP4) and the (R)-mecoprop degrader Alcaligenes denitrificans harbor an α-ketoglutarate-dependent 2,4-D dioxygenase, called TfdA (13, 14, 36). Two distinct dioxygenases, named (R)- and (S)-dichlorprop dioxygenases (RdpA and SdpA, respectively), were isolated from S. herbicidovorans MH. They are thought to catalyze the oxidative ether-cleavage of the respective enantiomer of phenoxypropanoic acids to the achiral phenol (Fig. 1) (30, Chapter 3). RdpA and SdpA share 30% and 37% amino acid identity with
TfdA from *R. eutropha* JMP134(pJP4), respectively (13, 14, Chapter 2). Sequence comparisons of RdpA and SdpA to other α-ketoglutarate-dependent dioxygenases showed, that RdpA is identical to *(R)*-dichlorprop dioxygenase from the dichlorprop degrader *Delftia acidovorans* MC1 (here designated RdpA<sub>MC1</sub>), whereas SdpA is 60% identical to *(S)*-dichlorprop dioxygenase (here designated SdpA<sub>MC1</sub>) from the same strain (43). RdpA<sub>MC1</sub> and SdpA<sub>MC1</sub> were enantiospecific and converted only the respective enantiomer of phenoxypropanoic acids (44).

![Chemical Structures](image)

Fig. 1. Initial degradation step of chiral phenoxypropanoic acids in *S. herbicidovorans* MH catalyzed by the α-ketoglutarate-dependent *(R)*-dichlorprop dioxygenases (RdpA) and α-ketoglutarate-dependent *(S)*-dichlorprop dioxygenases (SdpA).

Phenoxyalkanoic acid degraders have been classified in three groups according to their genetic background (15, 23). Group I comprises 2,4-D degrading *Beta* - and *Gammaproteobacteria* which harbor *tfd*-gene homologs and were isolated from contaminated sites. Fast growing *Alphaproteobacteria*, mostly *Sphingomonas* strains, build group II and it was postulated that they do not possess *tfdA*-homologs. The third group is composed of slow-growing *Alphaproteobacteria*, which are associated with members of the *Bradyrhizobium-Agromonas-Nitrobacter-Afipia* (BANA)-cluster isolated from pristine environments. These
strains harbor a distinct group of 2,4-D degrading genes, the *cad*-genes. The *cadAB*-genes code for the large and small subunit of a 2,4-D oxygenase and share homology to the 2,4,5-trichlorophenoxyacetic acid oxygenase *tftAB*-genes from *Burkholderia cepacia* AC1100, but not to *tfdA* (21, 22, 24). It was hypothesized that members of group II and III do not possess *tfdA*-homologs but recently, such gene homologs were detected in several *Alphaproteobacteria*. The *tfdAα*-genes, as they were named, showed 56-60% nucleotide identity to *tfdA* (21, 22).

Here, we report the characterization of the α-ketoglutarate-dependent (S)-dichlorprop dioxygenase from *S. herbicidovorans* MH. We have expressed SdpA as a 6xHis-tagged fusion protein and determined physicochemical parameters, the substrate, cosubstrate and cofactor specificities and verified its classification. Additionally, we screened for another phenoxyalkanoic acid converting enzyme specific for 2,4-D but no *tfdA*-like, *tfdAα*-like or a *cadAB*-like gene was detected.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** *Escherichia coli* DH5α was used as a host for cloning experiments and *E. coli* BL21(DE3)(pLysS) as a host for expression vector pET-15b (Novagen, Darmstadt, Germany). *E. coli* strains were grown at 30°C or 37°C in Luria-Bertani (LB) medium (37). When appropriate, ampicillin and chloramphenicol were added at 50 µg/ml and 25 µg/ml final concentrations, respectively. Solid media were prepared by the addition of 1.5% (w/v) agar.

**Standard molecular techniques.** Cloning and digestions were done according to established procedures (3, 37). Restriction enzymes and other DNA modifying enzymes were purchased from Promega (Wallisellen, Switzerland) and Fermentas (Nunningen, Switzerland). Plasmids and cosmids were isolated by the boiling miniprep method, the alkaline lysis method according to Sambrook *et al.* (37) or with the E.Z.N.A. plasmid miniprep kit II (Peqlab Biotechnologies GmbH, Baden-Dättwil, Switzerland) as suggested by the manufacturer. Gel extraction was carried out with the MinElute Gel extraction Kit (Qiagen AG, Basel, Switzerland) according to the protocol of the supplier.

**Sequencing.** DNA sequencing was performed on double stranded DNA templates with the Thermo Sequenase Primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Biosciences) and M13 primers (4). Sequencing reactions were analyzed on an automated DNA sequencer model 4200 IR² (LI-COR Inc., Lincoln, USA). Primers for sequencing were labelled at
the 5′-end with IRD-700 or IRD-800 and were purchased from MWG Biotech AG (Ebersberg, Germany). Sequencing data were analyzed with DNASTAR software (DNASTAR Inc., Madison, USA) and compared to the databases by BLAST searches (www.ncbi.nlm.nih.gov/BLAST, 40).

Screening for other α-ketoglutarate-dependent phenoxyalkanoic acid dioxygenases.

To screen for tfdA- and tfdAα-like genes, we carried out the PCR with the tfdA-derived primers tfdAfor 5′-TGG CAI AGC GAC AGI TCC TTT-3′, tfdArev 5′-CIG CGG TTG TCC CAI ATC IC-3′, tfdAfor2 5′-ACG GAG TTC TG(C/T) GA(C/T) AT-3′ and tfdArev2 5′-AAC GCA GCG (G/A)T(G/T) ATC CCA-3′ (42) in all possible combinations with chromosomal DNA of S. herbicidovorans MH. Oligonucleotides for PCR were obtained from Microsynth GmbH (Balgach, Switzerland). The following conditions were applied: initial denaturation for 2 min at 93.5°C; denaturing for 30 sec at 93.5°C; annealing for 30 sec at 63°C, 59°C, 56°C, 53°C, 50°C, and 46°C; extension for 45 sec at 72°C (5 cycles at each annealing temperature) or as an alternative, 35 cycles at an annealing temperature of 35°C; followed by a final extension for 4 min at 72°C. As a positive control, total DNA of R. eutropha JMP134(pJP4) was used.

For southern hybridization, total DNA of S. herbicidovorans MH fractionated on agarose gel was blotted on a Hybond™-XL membrane (Amersham Biosciences, Dübendorf, Switzerland) with a vacuum blower (VacuGene XL, Amersham Biosciences) by the procedure described by Ravatn et al. (35). Total DNA was hybridized with a 0.7-kb EcoRI-fragment from pCBA101 (0.7-kb EcoRI-fragment with tfdA in pGEM7, J. R. van der Meer, unpublished) and with a 1.1-kb SalI-fragment of pBHS1 harboring a part of cadAB (24). The fragments were radioactively labeled with [α-32P]dATP using the Random Primed DNA Labeling Kit or with digoxigenin-dUTP using the DIG High Prime DNA labeling and Detection Starter Kit II according to the protocol of the manufacturer (Roche Applied Science, Rotkreuz, Switzerland). Hybridization experiments were performed in SDS/BSA hybridization buffers (0.5 M sodium-phosphate [pH7], 7% sodium dodecyl sulfate, 1% bovine serum albumin) at 55°C for 16 hours. The membranes were washed twice with a solution of 5 × SSC containing 1 mM EDTA for 2 min at room temperature and twice with a solution of 2 × SSC plus 0.1% SDS for 30 min at 55°C. Binding of digoxigenin-labeled probes was immunologically detected according to the supplier’s protocol (Roche Applied Science).

Construction of N-terminal 6xHis-tagged recombinant SdpA expression plasmid.

Expression plasmid pMec19 was constructed in the PCR by reamplification of the SdpA gene from pMec16 (Chapter 2) with the following primers: 5′-CAG GAG GAT TCA TAT GTC A-3′ and 5′-GCC AGC TGG ATC CGC CGA TGA-3′. Thereby, a NdeI restriction site at the translational start codon and a BamHI restriction site just past the translational stop codon were introduced (underlined nucleotides). The fragment was first cloned into pGEM®-T Easy
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(Promega), generating pMec18, and verified by sequencing. The plasmid was then digested with NdeI and BamHI and ligated into the same sites of vector pET-15b yielding pMec19.

**Expression and purification of recombinant SdpA.** *E. coli* BL21(DE3)(pLysS) harboring pMec19 was grown in Erlenmeyer flasks containing LB-medium complemented with ampicillin and chloramphenicol at 30°C with constant shaking. To express the protein, a preculture of *E. coli* BL21 (DE3)(pLysS) harboring pMec19 was grown overnight at 37°C. A volume of 1% (v/v) of the preculture was transferred to fresh medium and grown at 30°C until an optical density of 0.4–0.6 (measured at 546 nm) was reached, after which isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM. The culture was incubated for another 3 hours at 30°C and the cells were harvested by centrifugation at 7'500 × g for 30 min at 4°C and the pellet was stored at −20°C until further use. For the preparation of cell extracts, 1 g cells (wet weight) was resuspended in 3 ml lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH adjusted to 8) and the cells were broken by one passage through a French press (Hypramag, Zurich, Switzerland) at 1 MPa. The lysate was centrifuged for 45 min at 16’000 × g at 4°C and aliquots were stored at −20°C.

6xHis-tagged SdpA was purified in a two-step procedure. Unless stated otherwise, all steps were carried out at 4°C. Up to one mg protein was loaded on a 5-ml Ni-NTA superflow-agarose column (Qiagen) at a flow rate of 0.3 ml/min. Unbound protein was removed stepwise with five column volumes of wash-buffer (50 mM NaH₂PO₄, 300 mM NaCl) containing 20 mM imidazole (pH adjusted to 8) and five column volumes of wash buffer containing 50 mM imidazole (pH adjusted to 8). SdpA₉ was then eluted in 1-ml fractions with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 200 mM imidazole, pH adjusted to 8) at a flow rate of 0.6 ml/min. The active fractions were pooled and desalted into 20 mM Tris-HCl (pH 7.5) using PD-10 columns (Amersham Biosciences, Dübendorf, Switzerland) at room temperature and loaded on a Q2 Bio-Scale column (BioRad) equilibrated in the same buffer. The enzyme was eluted with a linear concentration gradient from 20 mM Tris-HCl (pH 7.5) to 20 mM Tris-HCl (pH 7.5) plus 600 mM NaCl in six column volumes. SdpA was found to elute at approximately 200-250 mM NaCl. The active fractions were pooled again and stored at 4°C. Thrombin protease (Amersham Biosciences) was used to cleave the N-terminal His-tag. The N-terminal His-tag of approximately 10 µg SdpA was completely cleaved by 0.5 U thrombin protease within 2 hrs at room temperature.

**Enzyme Activity Assays.** Enzyme activity measurements were carried out using the three following different assays (Chapter 3): (i) coupled continuous assay, (ii) end-point assay and measuring of product formation by HPLC, and (iii) oxygen electrode measurements. The coupled continuous assay was used to determine specific activities of SdpA towards phenoxypropanoic acids, the end-point assay for all other substrates. In all assays, 50 mM imidazole (pH 6.75), 1
mM substrate, 1 mM α-ketoglutarate, 100 μM (NH₄)₂Fe(SO₄)₂ and 1 mM ascorbic acid were used and the reaction was started by the addition of the enzyme.

**Protein concentration determination and SDS-PAGE.** Protein concentrations were measured using the Bradford-method (6) with the Bio-Rad reagent dye concentrate reagens following the instructions of the manufacturer (Bio-Rad, Reinach, Switzerland). Bovine serum albumin fraction V (Sigma-Aldrich) was used as a reference protein. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 12% acrylamide slab gels according to established procedures (26).

**Native size determination of SdpA.** Molecular weight analysis of purified 6xHis tagged SdpA was determined on a Superdex 200 preparation grade HiLoad 16/60 size exclusion column (Amersham Biosciences). Approximately 0.25 mg protein were loaded on the HiLoad column and eluted at a flow rate of 0.2 ml/min in 50 mM Na₂HPO₄-KH₂PO₄-buffer (pH 7) containing 150 mM NaCl. The calibration curve was done with the gel filtration standard from Bio-Rad containing thyroglobulin (bovine, 670 kDa), Gamma globulin (bovine, 158 kDa), Ovalbumin (chicken, 44 kDa), Myoglobin (horse, 17 kDa) and Vitamin B-12 (1.35 kDa). Blue dextran was used to determine the void volume.

**Chemicals.** Phenoxyalkanoic acids were purchased at Riedel-de Haën (Buchs, Switzerland; PESTANAL®, 99% purity) or Sigma-Aldrich (97-99% purity). (S)-mecoprop and (S)-dichlrorprop were synthesized by C. Zipper (45). α-Ketoglutarate and other (di)carboxyacids were purchased from Sigma-Aldrich. All other chemicals were obtained from Fluka (Buchs, Switzerland) or from Merck AG (Dietikon, Switzerland).

**RESULTS**

**Expression and purification of recombinant SdpA.** The *sdpA* gene was expressed in *E. coli* BL21 (DE3)(pLysS) harboring pMec19. The expression produced low levels of 6xHis-tagged SdpA amounting to less than 10% of total protein concentration in the soluble fraction of the cell-extract as determined on SDS-PAGE. Reduction of the expression temperature did not increase the amount of soluble SdpA and therefore, all expression experiments were carried out at 30°C. Recombinant SdpA was purified in a two-step procedure to apparent homogeneity as summarized in Table 1 and Fig. 2. The calculated subunit size of the 6xHis-tagged SdpA was 33.7 kDa, which was consisted with a size of approximately 32 kDa determined by SDS-PAGE. Gel filtration chromatography was used to estimate a native size of approximately 32 kDa suggesting a
monomeric structure. The 6xHis-tag was cleaved by thrombin protease treatment from the recombinant SdpA. The native and the recombinant protein were equally active in enzyme activity measurements. Hence, the 6His-tagged α-ketoglutarate-dependent (S)-dichlorprop dioxygenase was used in all further experiments.

Table 1: Representative purification table of α-ketoglutarate-dependent (S)-dichlorprop dioxygenase

<table>
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<th></th>
<th>volume (ml)</th>
<th>total protein (mg)</th>
<th>total activity (U)</th>
<th>specific activity (U/mg protein)</th>
<th>recovery %</th>
<th>purification -fold</th>
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<td>18.5</td>
<td>0.4</td>
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<td>1</td>
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<td>6.3</td>
<td>8.9</td>
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</table>

Fig. 2. Purification of *Sphingomonas herbicidovorans* MH 6xHis tagged recombinant α-ketoglutarate-dependent (S)-dichlorprop dioxygenase from *E. coli* BL21(DE3)(pLysS) harboring (pMec19). Protein samples were analyzed on denaturing gel electrophoresis on a 12% polyacrylamide gel and stained with Coomassie Brilliant Blue. Lane M, molecular weight markers (indicated in kDa), lane 1, extract of induced cells, lane 2, partially purified SdpA (obtained after Ni-NTA-superflow chromatography), lane 3, apparently pure SdpA (obtained after anion-exchange chromatography).

**SdpA is an α-ketoglutarate-dependent dioxygenase catalyzing an enantioselective reaction.** SdpA had been grouped into the family of α-ketoglutarate-dependent dioxygenases according to its amino acid sequence (Chapter 2). Enzyme activity measurements confirmed this classification. SdpA
activity was abolished in the absence of α-ketoglutarate and strongly reduced in the absence of Fe(II) (Table 2). Without ascorbate, activity was decreased to less than 40% of the one in the presence of all components. Additionally, the three different activity measurements, the coupled-continuous assay, the end-point assay and oxygen-electrode measurements, demonstrated that SdpA converted mecoprop stoichiometrically to 2-methyl-4-chlorophenol and pyruvate under con-comitant consumption of molecular oxygen (Fig. 1).

SdpA converted only the (S) enantiomers of phenoxypropanoic acids and no activity was observed with the (R) enantiomers. In the presence of (R)-mecoprop, i.e., when the racemate was used, activity reduced to approximately 85% indicating slight inhibition (data not shown).

Table 2: Dependence of α-ketoglutarate-dependent (S)-dichlorprop dioxygenase activity on substrate, cosubstrate and cofactor

<table>
<thead>
<tr>
<th></th>
<th>Specific activity (U/mg protein)</th>
<th>% Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete assay</td>
<td>7.0</td>
<td>100</td>
</tr>
<tr>
<td>– (R)-mecoprop</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>– α-ketoglutarate</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>– Fe(II)</td>
<td>0.6</td>
<td>8.2</td>
</tr>
<tr>
<td>– ascorbate</td>
<td>2.7</td>
<td>37.8</td>
</tr>
</tbody>
</table>

a Complete assay without the specified component.

**Physicochemical characterization.** SdpA did not lose measurable activity at room temperature for several hours and lost less than 10% of its activity when stored at 4°C within 3 days. Storage on ice and freezing-thawing cycles reduced activity to about 80%. It appeared important to prewarm SdpA for several minutes at room temperature prior to activity measurements. The temperature optimum was established to be between 35°C and 40°C (Fig. 3A). The pH-optimum was determined in 50 mM imidazole buffer with the respective pH and was found to be between pH 6.5 and 6.75 (Fig. 3B).
Fig. 3. Temperature (panel A) and pH (panel B) dependence of α-ketoglutarate-dependent (S)-dichlorprop dioxygenase. (A) The black squares represent data obtained with the end-point assay, the black diamonds data obtained with the coupled continuous assay. The measurements were carried out with two independently purified (S)-dichlorprop dioxygenase samples. (B) The diamonds show the mean value of six measurements of two independent experiments. Enzyme activity was determined with the coupled continuous assay.

**Substrate, cosubstrate and cofactor specificities.** For several substrates, it was tested whether they support activity of SdpA. In one series including besides the phenoxypropanoic acids the phenoxyacetic and phenoxybutyric acids, the influence of the alkanoic acid attached to the phenoxygroup by an ether-bond was tested. In other experiments, the effects of having different substituents such as methyl-, hydroxy- and chloride groups on the aromatic moiety were studied.
Substrate specificities of SdpA for phenoxypropanoic acid were determined by using the continuous-coupled assay whereas the end-point assay was applied for all other substrates. When single enantiomers of the tested phenoxypropanoic acids were not commercially available, racemic mixtures were used at a final concentration of the (S) enantiomers of 1 mM. SdpA showed the highest turnover rate for substituted phenoxypropanoic acids. Of those, mecoprop was transformed at the highest rate (Table 3). Unsubstituted phenoxypropanoic acid was converted at only 11.6\% of the activity with mecoprop. 3-Methyl-5-chlorophenoxyacetic acid was oxidized at a rate of 20\% of the activity with mecoprop. 2,4-D supported activity only poorly at approximately 5\% of the highest activity and phenoxybutyric acids were not converted at considerable rates.

Table 3: Substrate Specificities of α-ketoglutarate-dependent (S)-dichlorprop dioxygenase\(^a\)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity(^b) (% ± std. dev.)</th>
<th>Activity (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(R,S)-Mecrop(^c)</td>
<td>100</td>
<td>8.19 / 6.25(^d)</td>
</tr>
<tr>
<td>(R,S)-Dichlorprop(^c)</td>
<td>65.3±9.7</td>
<td>5.08</td>
</tr>
<tr>
<td>2-(R,S)-Phenoxypropanoic acid(^c)</td>
<td>11.6±2.6</td>
<td>0.94</td>
</tr>
<tr>
<td>2-(R,S)-3-Chlorophenoxypropanoic acid</td>
<td>33.2±2.5</td>
<td>2.88</td>
</tr>
<tr>
<td>2-(R,S)-4-Chlorophenoxypropanoic acid(^d)</td>
<td>66.1±3.2</td>
<td>5.92</td>
</tr>
<tr>
<td>2-(R,S)-2,4,5-Trichlorophenoxypropanoic acid(^d)</td>
<td>71.5±6.6</td>
<td>1.39</td>
</tr>
<tr>
<td>2-(R,S)-4-Hydroxyphenoxypropanoic acid(^e)</td>
<td>19.6±3.2</td>
<td>1.80</td>
</tr>
<tr>
<td>2,4-D(^e)(^f)</td>
<td>5.6±5.2</td>
<td>0.33</td>
</tr>
<tr>
<td>3-Methyl-5-chlorophenoxyacetic acid(^e)</td>
<td>20.3±6.2</td>
<td>1.25</td>
</tr>
<tr>
<td>2,4-Dichlorophenoxybutyric acid(^e)</td>
<td>&lt;1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>4-Chloro-2-methylphenoxybutyric acid(^e)</td>
<td>2.3±1.2</td>
<td>0.14</td>
</tr>
</tbody>
</table>

\(^a\) The substrate range was determined with two independently purified batches of SdpA and enzyme activity of each batch was measured three times with all the substrates.

\(^b\) The standard deviation is given in %.

\(^c\) Enzyme activity was determined with the coupled continuous assay.

\(^d\) The first number is specific activity measured in the coupled continuous assay, the second in the end-point-assay.

\(^e\) Enzyme activity was determined with the end-point-assay.

\(^f\) Only one SdpA sample showed slight activity with 2,4-D. The data represent a mean value and might be overestimated.
To investigate, if and which other carbonic acids could substitute α-ketoglutarate, the end-point assay had to be used since the reporter enzyme of the coupled-continuous assay, D-lactate dehydrogenase, showed activity with some of the tested carbonic acids. SdpA appeared to be quite specific for α-ketoglutarate. The only tested cosubstrate able to support activity (slightly) was 2-oxoadipate (Table 4).

We further examined whether divalent cations could substitute Fe$^{2+}$ in the enzyme reaction. The alternative cations Ca$^{2+}$, Co$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, and Ni$^{2+}$ could not substitute for Fe$^{2+}$ and even completely abolished SdpA$_{H}$ activity indicating an inhibitory effect (Table 5).

Table 4: Cosubstrate specificities of α-ketoglutarate-dependent (S)-dichlorprop dioxygenase

<table>
<thead>
<tr>
<th>cosubstrate</th>
<th>specific activity (U/mg protein)</th>
<th>% activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-ketoglutarate</td>
<td>6.7</td>
<td>100</td>
</tr>
<tr>
<td>oxaloacetate</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2-oxobutyrate</td>
<td>0.03</td>
<td>0</td>
</tr>
<tr>
<td>2-oxovalerate</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2-oxoadipate</td>
<td>0.3</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 5: Cofactor specificities of α-ketoglutarate-dependent (S)-dichlorprop dioxygenase

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>Specific activity (U/mg protein)</th>
<th>% Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe(NH$_4$)$_2$(SO$_4$)$_2$</td>
<td>7.2</td>
<td>100</td>
</tr>
<tr>
<td>Fe(SO$_4$)$_2$</td>
<td>6.8</td>
<td>92.8</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CoCl$_2$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MnCl$_2$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NiCl$_2$</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
**Kinetic analysis.** We examined the kinetic behaviour of SdpA towards phenoxypropanoic acids, α-ketoglutarate and oxygen (Table 6). For the measurements of the prime substrates and α-ketoglutarate, the coupled continuous assay was carried out; O₂-kinetic was determined in the oxygen-electrode. Generally, SdpA exhibited Michaelis-Menten kinetic with all tested substrates and cosubstrates. (S)-Mecoprop was the best prime substrate, i.e., the apparent $K_m$-value was lowest and the catalytic efficiency was the highest. SdpA had high affinity to α-ketoglutarate with an apparent $K_m$-value of 19.6 µM whereas it had quite low affinities to O₂; a $K_m$-value of 159 µM was calculated for oxygen.

Table 6: Kinetic parameters of α-ketoglutarate-dependent (S)-dichlorprop dioxygenase

<table>
<thead>
<tr>
<th>substrate</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (min⁻¹)</th>
<th>$k_{cat}/K_m$ (µM⁻¹ × min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(S)-mecoprop (50 – 2000 µM)</td>
<td>132±17.7</td>
<td>303.9</td>
<td>2.3</td>
</tr>
<tr>
<td>(S)-dichlorprop (120 – 2000 µM)</td>
<td>494.8±43.9</td>
<td>284.6</td>
<td>0.58</td>
</tr>
<tr>
<td>O₂ (0.02 – 0.2 mM)</td>
<td>159.32</td>
<td>116⁴</td>
<td>0.754</td>
</tr>
<tr>
<td>α-ketoglutarate (8 – 1000 µM)</td>
<td>19.6±2.5</td>
<td>229.7</td>
<td>11.72</td>
</tr>
</tbody>
</table>

⁴ The kinetic values for O₂ were determined with material from two different purified SdpA, which both were less active than the ones used for other kinetic experiments. However, the stoichiometry of the reaction was verified with material from a third purification batch (data not shown).

**Screening for other 2,4-D degrading genes.** We screened total DNA of *S. herbicidovorans* MH for a *tfdA*-like dioxygenase and for the aromatic ring hydroxylating dioxygenase CadAB by hybridization and PCR experiments. The *tfdA*- and the *cadAB*-derived probes did not hybridize to total DNA of *S. herbicidovorans* MH and no *tfdA*-like gene could be amplified with the PCR-primers derived from the conserved regions of *tfdA*- and *tfdAα*-genes.

**DISCUSSION**

**SdpA is an α-ketoglutarate-dependent dioxygenase.** A Blast search had already suggested that SdpA belongs to the diverse family of α-ketoglutarate-dependent dioxygenases. These enzymes are classified in three groups according to...
a conserved motif containing the 2-His-1-carboxylate facial triad. By sequence alignment, the motif of group II \( \alpha \)-ketoglutarate-dependent dioxygenases (HXDX_{24}TX_{127}HX_{10}R) was detected in the primary sequence as well. According to the crystal structure of \( \alpha \)-ketoglutarate-dependent taurine dioxygenase (TauD) from \textit{E. coli} K-12 (12, 31), a structure model and mutagenesis studies of TfdA (12, 19), His-102, Asp-104 and His-257 are predicted to be the iron(II) binding sites and build the 2-His-1-carboxylate facial triad. Thr-129 and Arg-268 are putative \( \alpha \)-ketoglutarate binding sites.

Enzyme activity measurements confirmed the classification of SdpA as an \( \alpha \)-ketoglutarate-dependent dioxygenase. Enzyme activity was completely abolished in the absence of \( \alpha \)-ketoglutarate and strongly reduced in the absence of ferrous iron. Interestingly, SdpA showed only 38% initial activity in the absence of ascorbate, although ascorbate is formally not needed for the conversion of phenoxypropanoic acids. Ascorbate is thought to keep the iron(II) at the catalytic center in the reduced state and also both prolyl-4-hydroxylase and lysyl-hydroxylase were inactivated rapidly in the absence of ascorbate (8, 27, 28, 34). Even more strikingly, AlkB, a DNA base repair enzyme from \textit{E. coli}, did not exhibit any activity at all in the absence of ascorbate (41). The inactivation mechanism was proposed to involve decarboxylation of \( \alpha \)-ketoglutarate in a side-reaction, which is coupled to the oxidation of Fe(II) to Fe(III). The inactivation is reversible in the presence of ascorbate, because the iron is reduced again.

**Enantioselective conversion of phenoxypropanoic acid herbicides involves the enzyme pair RdpA and SdpA.** SdpA exclusively converted (S)-dichlorprop and (S)-mecoprop but not the (R) enantiomers. This confirms previous enzyme activity experiments with cell-free extracts in which the existence of two distinct \( \alpha \)-ketoglutarate-dependent dioxygenases in strain MH was proposed. Only recently, the \( \alpha \)-ketoglutarate-dependent (R)-dichlorprop dioxygenase, specific for the (R) enantiomers, was isolated and characterized (30, Chapter 3). Apparently, \textit{S. herbicidivorans} MH harbors two distinct enzymes with opposite enantiospecificities. The existence of two enantiospecific enzymes would make an interesting model for understanding the basis of enantioselectivity. In some cases,
like TfdA from *R. eutropha* JMP134(pJP4), from *Burkholderia cepacia* RASC and from *A. denitrificans*, one could assume that only the active site must be different, since TfdA<sub>pJP4</sub> and TfdA<sub>RASC</sub> only transform (S)-dichlorprop whereas TfdA from *A. denitrificans* cleaves the ether-bond of (R)-mecoprop and all three use 2,4-D as substrates as well (36). This was also shown for the enzyme pair tropinone reductase I and II from *Daturam stramonium* involved in the enantioselective biosynthesis of tropane alkaloids. They share 64% amino acid identity and their native structure is almost identical, but their active sites differ (29). Another possibility for differing enantiospecificity is a completely different overall enzyme fold, but a similar active site. This is apparently the case for the enzyme pair L- and D-lactate dehydrogenase which do not share sequence similarity to each other, have a completely different fold, but possess a surprisingly similar active site (16, 38).

Since RdpA and SdpA have very little primary sequence identity to TauD, useful predictions on their overall structure about the similarity of their native folds, the substrate binding sites and the active sites cannot be answered by primary sequence alignment alone, but the determination of a crystal structure or appropriate modeling is required.

**Substrate, cosubstrate and cofactor specificities of SdpA.** SdpA exhibited activity with most tested phenoxyalkanoic acids. High activity was observed for chloro- and methylsubstituted derivatives of phenoxypropanoic acids and the catalytic efficiency was highest for (S)-mecoprop. This indicates that SdpA has adapted to methyl- and chlorophenoxyalkanoic acids. This agrees with the results obtained with SdpA<sub>MC1</sub> but is in contrast to TfdA<sub>α</sub>, which prefers unsubstituted phenoxyacetic acids to chlorophenoxyacetic acids (21). Furthermore, it indicates that the substituents are important to accommodate the substrate at the catalytic center. Surprisingly, 2,4,5-trichlorophenoxypropanoic acid was the second best substrate of all acids tested and was converted at rates of 71% of highest activity obtained with mecoprop. The third chloride atom seems to also fit into the active site and apparently does not hinder the conversion. Despite their close relatedness, SdpA from *D. acidovorans* MC1 showed no activity at all with 2,4,5-trichlorophenoxypropanoic acid (44). In general, substituted phenoxy-
propanoic acids were better substrates than substituted phenoxyacetic and phenoxybutyric acids. This suggests that the alkyl moiety of the alkanoic acid side chain is more important than the substituent at the aromatic ring for activity. Similar observations were made for TfdA, which has 2,4-D as the best substrates, but also hydroxylates propionic acids although with much lower catalytic efficiency (13, 14).

SdpA exhibited Michaelis-Menten kinetics for oxygen, but the calculated apparent $K_m$-value of 159 µM was high when compared to the ones of other $\alpha$-ketoglutarate-dependent dioxygenases, but not as high as the one of RdpA, for which even no Michaelis-Menten kinetics could be observed in the range of 0-0.2 mM O$_2$ (Chapter 3). For example, the $\alpha$-ketoglutarate-dependent dioxygenases lysyl-hydroxylase and deacetoxycephalosporin C synthase have affinity constants in the range of 40 – 50 µM for O$_2$ (7, 34) and flavonol dioxygenase has an apparent $K_m$-value of 5.26 µM O$_2$ (2). In contrast to these $\alpha$-ketoglutarate-dependent dioxygenases, 3,5-dichlorocatechol dioxygenase from *Pseudomonas cepacia* CSV90 has a $K_m$-value of 652 µM for O$_2$ (5).

SdpA has high affinity for $\alpha$-ketoglutarate (apparent $K_m$-value of 19.6 µM). This agrees well with the results obtained for other $\alpha$-ketoglutarate-dependent dioxygenases such as RdpA, SdpAMC1, TfdA and TauD (11, 14, 44, Chapter 3). In contrast to TfdA and TfdA$\alpha$, but in agreement with RdpA, SdpA had a narrow cosubstrate specificity and only 2-oxoadipate could substitute for $\alpha$-ketoglutarate at low rates (14, 21, Chapter 3). The cofactor range was limited to ferrous iron and other divalent cations could not substitute. This concurs with the findings from other $\alpha$-ketoglutarate-dependent dioxygenases (11, 14, 44, Chapter 3).

**Existence of another dioxygenase involved in phenoxyalkanoic acid degradation in strain MH.** RdpA and SdpA do not convert 2,4-D at a significant rate. Yet previously, it had been shown that *S. herbicidovorans* MH grows on 2,4-D with a higher specific growth rate than on mecoprop and dichlorprop (46). This would suggest, that another phenoxyalkanoic acid converting enzyme, specific for 2,4-D, must exist in *S. herbicidovorans* MH. Despite our attempts to detect genes for other phenoxyalkanoic acid converting enzymes in *S. herbicidovorans* MH by
hybridization and PCR-experiments, we found no conclusive evidence for a \textit{tfdA}-like, a \textit{tfdA\alpha}-like, or a \textit{cadAB}-like gene(s). The reason for this failure might be that the unknown gene is too dissimilar to be detected with the known \textit{tfdA}, \textit{tfdA\alpha}- or \textit{cadAB} consensus primers or hybridization probes. Therefore, it still remains unclear what kind of enzyme is responsible for the high growth rates of \textit{S. herbicidovorans} MH with 2,4-D.

Many questions concernings the substrate binding sites, the active site, the protein folding and perhaps even the possibility to change the enantiospecificity of RdA to that of SdpA (or vice versa) remain. The answers to these questions will shed light on the interactions of enzymes and their chiral substrates and may allow us to identify, which features dictate enantiospecificity. Rational mutagenesis studies based on enzyme structures or models are planned to answer such questions in the future.
REFERENCES


CHAPTER 5

General Discussion & Outlook
This study focused on the genetics of phenoxyalkanoic acid degradation in *Sphingomonas herbicidovorans* MH and on the biochemical characterization of the two α-ketoglutarate-dependent dioxygenases involved in the initial, enantioselective degradation step. In the final chapter of this thesis, I will try to discuss how the results contribute to a broader understanding of phenoxyalkanoic acid degradation and of enantioselective enzymatic reactions.

**Degradation pathway of phenoxypropanoic acids in *S. herbicidovorans* MH.** One of our aims was to unravel the complete degradation pathway of phenoxyalkanoic acid herbicides in strain MH. In chapter 2, we showed that *S. herbicidovorans* MH degrades mecoprop, dichlorprop and 2,4-D through a modified-ortho-cleavage pathway, i.e., a pathway analogous to 2,4-D degradation in *Ralstonia eutropha* JMP134(pJP4). Surprisingly, however, the isolated genes and the genetic organization for the mecoprop pathway from *S. herbicidovorans* MH were not similar to the canonical *tfd*-genes found in typical 2,4-D degraders such as *R. eutropha* JMP134(pJP4) and *Variovorax paradoxus*. *S. herbicidovorans* MH harbored the *rdpA*- and the *sdpA*-gene encoding two α-ketoglutarate-dependent dichlorprop dioxygenases. Further, a phenol-hydroxylase gene (*tfdB*) and most of the genes of chlorocatechol degradation were discovered: two dichlorocatechol 1,2-dioxygenase genes (*dccA_{I/II}*), two dienelactone hydrolase genes *dccD_{I/II}*, one gene *dccE* coding for a maleylacetate reductase, a putative transport gene (*tdfdK*) and a putative regulator gene (*cadR*). Four aspects were remarkable for the phenoxypropanoic acid degradation pathway in *S. herbicidovorans* MH: (i) the genes were flanked by IS elements; (ii) the homology of the dichlorprop genes in strain MH to other phenoxyalkanoic acid genes pointed to different evolutionary origins; (iii) the genes were ‘scattered’ over the genome (i.e., not organized in one or two long polycistronic operons) and for several genes two complete copies were found; (iv) expression of the genes of the dichlorprop pathway was not tightly regulated. These points indicate a different evolutionary background for the dichlorprop pathway genes from the one in
General discussion

Beta- and Gammaproteobacteria and suggest that S. herbicidovorans MH might be able to acquire DNA from other sources by horizontal gene transfer relatively easily. With regard to the expression of new DNA, our results would point to a strategy employed by the organism to express new genes constitutively and perhaps later in evolutionarily development to control them by inducible expression. Similar results have been found with regard to catabolic pathways in other Sphingomonas strains (1-3), and might indicate a more general phenomenon for Sphingomonads to enable them to use new carbon sources and to adapt to new ‘ecological niches’. From a scientific point of view, Sphingomonas strains are ideal for isolating novel genes and for studying evolutionary processes. It would be interesting to try to determine whether the IS elements in S. herbicidovorans MH are sufficiently active to mobilize the gene cassettes and transfer them to a new host. Recently, for example, it was shown that Sphingomonas paucimobilis has very active IS elements, which rearrange and delete the genes for the lindane degradation pathway (3). It would also be fascinating to study whether strain MH is carrying vehicles for horizontal gene transfer (e.g., plasmids, phages, or genomic islands) or even might be able to take up DNA by transformation. Many interesting genetic and regulatory questions regarding the dichlorprop pathway still remain unanswered. Does the isolated cadR-gene code for a regulatory protein of this pathway? Do other regulatory genes exist for this pathway? Where is the gene for the chloromuconate cycloisomerase? Is the isolated tfdK-gene a 2,4-D or perhaps a mecoprop or a dichlorprop transporter protein? Do other transport proteins exist for phenoxypropanoic acids? The results obtained so far indicate that Sphingomonas strains either are at a different evolutionary stage than the canonical 2,4-D degraders or apply other strategies for gene organization and regulation to survive in a ‘contaminated’ environment.

Phenoxyalkanoic acid converting dioxygenases. In chapter 3 and 4, we studied the two α-ketoglutarate-dependent dioxygenases RdpA and SdpA, which catalyze the enantioselective conversion of (R)- and (S)-phenoxy-
propanoic acids to the achiral phenol. We purified and characterized both enzymes in terms of physicochemical parameters, substrate, cosubstrate, and cofactor specificities and determined the enzyme kinetic parameters. We demonstrated that both enzymes are enantiospecific and that they do not convert 2,4-D at a significant rate. Since *S. herbicidovorans* MH grows on 2,4-D, it would be plausible to presume the existence of a third phenoxyalkanoic acid dioxygenase. Indeed, we screened for a 2,4-D converting enzyme, but no *tfdA*-like, *tfdAα*-like or *cadAB*-like gene could be isolated (Chapter 4). Two possible explanations can be given at the moment: either we did not detect the gene with the chosen methods or *S. herbicidovorans* MH harbors another unknown 2,4-D converting dioxygenase. I suggest that no other *tfdA*-like gene exists in strain MH, since neither hybridization nor PCR-experiments led to positive results. The occurrence of a *tfdAα* or a *cadAB*-like gene is, to my opinion, also unlikely, since in the PCR, no *tfdAα* fragment was amplified and in hybridization experiments with *cadAB*-derived probes, no positive results were obtained. However, I would not totally rule out the possibility that *S. herbicidovorans* MH harbors one of these genes due to the following reasons. *tfdAα* genes have been isolated from many *Sphingomonas* strains and we might just have failed to amplify it in the PCR. The same argument may hold for the *cadAB*-like gene. Furthermore, *S. herbicidovorans* MH harbors a *cadR*-like gene, which may indicate to the existence of other genes from the *cad*-pathway. Unfortunately, we had neither time nor money to sequence additional large regions from our cosmids to screen for other *cad*-genes. Therefore, additional genetic experiments or large scale sequencing will be required to further elucidate the pathway for 2,4-D and phenoxypropanoic acid degradation in strain MH.

The enantiospecific *(R)- and *(S)-dichlorprop dioxygenases. Chirality plays an important role in biochemical processes and enantioselective degradation of chiral substances is rather the rule than the exception (Chapter 1). This study about phenoxypropanoic acid degradation in *S. herbicidovorans* MH elucidated one aspect of the biochemical background of enantioselective
General discussion

The two enantiospecific α-ketoglutarate dioxygenases RdpA and SdpA are responsible for the enantioselective degradation of these herbicides in strain MH. We demonstrated that both dioxygenases are strictly enantiospecific. Interestingly, *S. herbicidovorans* MH harbors two enzymes catalyzing the opposite enantioselective reaction and these enzymes are only 30% identical. One hypothesis for enantioselectivity is that the enzymes share similar primary amino acid sequences and have a similar native fold, but differ in their active sites (13). Such a difference might arise in divergent evolution. One example are the 2,4-D dioxygenases, which also convert phenoxypropanoic acids. The amino acid sequences of a PCR-fragment of 2,4-D dioxygenases from *R. eutropha* JMP134(pJP4) and from *Alcaligenes denitrificans* are 86% identical, but the enzymes exhibit opposite enantioselectivity for phenoxypropanoic acid herbicides in cell-free extract (15). Contrasting examples for enantioselectivity exist, in which the enzymes have little primary sequence similarity resulting in a different protein fold. Such enzyme pairs have arisen by convergent evolution (5, 10, 17, 19, chapter 1). The low sequence similarity of SdpA and RdpA is suggestive for this hypothesis, although the results obtained so far do not allow further speculation about the evolution of the two dioxygenases.

Modeling of the structure of RdpA and SdpA. The existence of two enzymes that catalyze opposite enantioselective reactions assures that both enzymes are well adapted to their substrate and convert it with high specificity. What exactly is causing the difference in enantioselectivity? Only a few enzyme pairs have been examined in details, although enantioselective conversions are very important in biocatalysis to produce enantiomerically pure chemicals in the pharma, agro and food industrial sector (11, 18). Many production processes are based on enzymes that have been modified by random techniques such as DNA shuffling, mutagenesis and others (8, 9). For instance, the enantiospecificity of the D-specific hydantoinase from *E. coli* was converted by several mutagenesis steps leading to a L-specific hydantoinase, which is used for the production of L-methionine (12). Unfortunately, the
molecular details for the enantiospecificities are not completely understood in many cases. The characterization of additional enzyme pairs with opposite enantiospecificities such as RdpA and SdpA will help to increase our understanding of the structural and mechanistic features dictating enantiospecificity and may be valuable for future engineering of enzymes with tailored chiral synthesis or degradation activities.

To understand at least some of the structural differences between RdpA and SdpA, I tried to model both enzymes’ fold on an existing structure of a similar protein. For RdpA and SdpA, it was possible to use the structure of the related α-ketoglutarate-dependent dioxygenase TauD. Modeling was done with the Swiss Model, an automated Comparative Protein Modelling Server, at Expasy (http://swissmodel.expasy.org/) using the so-called ‘first approach mode’ (6, 14, 16). When SdpA was subsequently modeled on the apo-form of TauD (PDB 1otj), a ‘misfolded’ active site was the result. RdpA could not be modeled directly without the complexed template. Hence, the TauD sequence complexed to Fe(II), α-ketoglutarate and taurine was entered into the program (database coordinates: PDB 1os7, ExPDB 1os7B).

With this templates, the models of RdpA and SdpA predicted an almost identical overall fold (Fig. 1), which was very similar to the one of TauD. The typical jellyroll motif of α-ketoglutarate-dependent dioxygenase was identified in both proteins. His-111, Asp-113, His-270 in RdpA and His-102, Asp-104 and His-257 in SdpA are positioned well to function in binding of the iron(II) (Fig. 2A and B), which agrees with the prediction of the 2-His-1-carboxylate facial triad made according to the alignment (Fig. 5, chapter 3). The same holds true for Thr-138 and Arg-281 in RdpA and Thr-129 and Arg-268 in SdpA, which are proposed to interact with α-ketoglutarate. The model shows that the iron(II)- and α-ketoglutarate binding sites of RdpA and SdpA were located at almost identical positions in the modeled fold (Fig. 2C). We may also speculate about possible substrate binding residues. In the ‘front’ of the active sites, Ser-114, Thr-115, Tyr-216, Val-220, Tyr-221 in RdpA and Ser-105, Thr-106, Arg-207, His-208 in SdpA build similar cavities, which seem to leave enough space
to position the aromatic ring of phenoxypropanoic acids. One residue, which could be involved in phenoxypropanoic acid binding in SdpA, is His-208. It is located near the active site in the cavity and it corresponds to His-214 of TfdA, which participates in 2,4-D positioning or catalysis (4, 7).

Fig. 1. Models of the native structures of RdpA (on the left) and SdpA (on the right). Helices are shown in red, β-strands in yellow. The β-strands of the conserved jellyroll motif are colored separately in green. The pictures were constructed with the program Swiss-PdbViewer (http://swissmodel.expasy.org/spdbv/).

Another interesting residue is Ser-161 of SdpA of which the amide backbone may also play a role in substrate binding. The corresponding residue in TauD, Ser-158, is involved in binding of the amine of taurine. The most striking difference between RdpA and SdpA is the ‘replacement’ of the neutral Gly-107...
in RdpA by the basic Asn-98 in SdpA. In TauD, the corresponding amino acid residue Asn-95 binds to the amine of taurine. This indicates that the two residues may also play an important role in orienting the respective enantiomer of the phenoxypropanoic acid in RdpA and SdpA. Two opposed mechanism of interactions between the substrate propanoic acid moiety and the asparagine are plausible. The side-chain of asparagine is positively charged at a biological pH and hence, hydrogen bonds may be formed with the carboxylic group of the propanoic acid moiety of the substrate. On the contrary, the placement of the carboxylic group of the phenoxypropanoic acid might be sterically hindered due to the ‘large’ side chain of asparagine, which is bigger than the H-atom of glycine. At the moment, none of these mechanisms can be favored and therefore, we cannot make exact predictions about the placement of the substrate.

Fig. 2. A. Model of the active site of RdpA. The predicted iron(II)- and \(\alpha\)-ketoglutarate binding residues are shown in white-red-blue; the predicted substrate binding residues are in orange. \(\alpha\)-Ketoglutarate and iron(II) are in grey. The iron(II)-binding residues of RdpA are shown in yellow, the ones of SdpA in dark blue. The \(\alpha\)-ketoglutarate binding residues are in light yellow (RdpA) and light green (SdpA); the substrate binding residues in orange (RdpA) and light blue (SdpA). The pictures were constructed with the program Swiss-PdbViewer.
Fig. 2 (cont.) B. Model of the active site of SdpA. The predicted iron(II)- and α-ketoglutarate binding residues are shown in white-red-blue; the predicted substrate binding sites in light blue. α-ketoglutarate and iron(II) are in grey. C. Superposition of the modeled active sites of RdPA and SdpA.
Nevertheless, this simple model provides interesting insights into the active sites of the two dioxygenases. One has to be aware though that the applied procedure most likely biases the outcome because both proteins were modeled with the same template. The model might be useful to make reasonable predictions about the enantiospecificity, which could then be tested in mutagenesis and spectroscopic studies. Finally, direct crystallization will be necessary to elucidate the native structure, to fully understand the enzyme features, which dictate enantiospecificity, and to gain more details about the mechanistics of these reactions. The work presented here gives the basis for such experiments.
REFERENCES:


CHAPTER 6

Appendix
INTRODUCTION

One aim of this thesis was to isolate and characterize the two α-ketoglutarate-dependent dioxygenases from Sphingomonas herbicidovorans MH, which are involved in the initial enantioselective degradation step of phenoxypropanoic acids. Two strategies were applied to reach this goal: a ‘genetic one’, i.e., isolation of the genes, and a ‘biochemical one’, i.e., purification of the protein. As described in chapter 2, we isolated the rdpA-gene by using PCR-primers developed on the basis of the related rdpA-gene from Delftia acidovorans MC1. At the beginning of this study, both approaches were followed up and we intended to purify the (S)-dichlorprop dioxygenase from crude extract and to screen for the gene by applying reverse genetics. For the reverse genetics approach, three ‘subgoals’ were set:

a) Growth studies to determine optimal growth conditions for obtaining cells with as high as possible (S)-dichlorprop dioxygenase activity
b) Purification of (S)-dichlorprop dioxygenase from cell-free extract
c) Screening for the gene by reverse genetics

Although – in the end – we did not succeed completely to purify the (S)-dichlorprop dioxygenase from native S. herbicidovorans MH cells, I would like to describe some of the results here because a lot of time was spent on this approach. At that moment, the biochemical approach was the most promising, since PCR and hybridization experiments with tfdA-derived primers and tfdA-probes had not led to positive results (chapter 4).

MATERIALS AND METHODS

Growth experiments. Standard growth experiments were carried out as described in chapter 2. To obtain higher cell mass, glucose, yeast extract and peptone were added to complex medium at the final concentrations of 0.44 g/l, 1.2 g/l and 6 g/l, respectively. To obtain large amounts of cells for the protein purification, S. herbicidovorans MH was cultivated on complex medium in a 700-l reactor at ETH Hönggerberg to an OD₅₄₆ of approximately 1.5. Cells were harvested by centrifugation at 7’500 × g at 4°C for 15 min.
Preparation of cell-free extracts. For the preparation of cell-free extracts, 1 g cells (wet weight) was washed once in 20 mM NaHPO₄-KH₂PO₄, pH adjusted to 6.5, centrifuged and resuspended in 3 ml cell-extract buffer (20 mM Tris-HCl, 1 mM dithiothreitol, 0.095 mg/ml AEBSF, pH adjusted to 7.5). The cells were broken by two passages through a French press at 1 MPa, the lysate was centrifuged at 16’000 × g at 4°C for 40 min and the aliquots were stored at –20°C.

Partial Purification of (S)-dichlorprop dioxygenase. Anion exchange chromatography. Approximately 50 mg cell-free extract was loaded on a Q10 Bio-Scale column (Bio-Rad, Reinach, Switzerland) in 20 mM Tris-HCl, pH 7.5. The enzymes were eluted with a linear concentration gradient from 20 mM Tris-HCl (pH 7.5) to 20 mM Tris-HCl (pH 7.5) containing 1 M NaCl in ten column volumes. (S)-dichlorprop dioxygenase was found to elute at approximately 200-250 mM NaCl. The active fractions were pooled, concentrated with YM-10 columns according to the instructions of the manufacturer (Amersham Biosciences, Dübendorf, Switzerland) and stored at –20°C until further use.

Hydroxyapatite chromatography. Active anion-exchange fractions were loaded on a hydroxyapatite column (Macro-Prep® Ceramic Hydroxyapatite, Type I, 40 µm, 20 ml column volume, Bio-Rad). The fractions were loaded in 1 mM K₂HPO₄×3 H₂O/PO₄³⁻ buffer (pH adjusted to 6.8) and eluted with a linear concentration gradient to 400 mM K₂HPO₄×3 H₂O/PO₄³⁻ buffer (pH adjusted to 6.8) in ten column volumes. The active fractions eluted at approximately 5-10 mM K₂HPO₄×3 H₂O/PO₄³⁻. They were again pooled and concentrated as after the anion-exchange chromatography step and stored at –20°C until further use.

It has to be noted that the use of hydrated potassium phosphate (K₂HPO₄×3 H₂O) and PO₄³⁻ was important, because the pyrophosphate, contained in the commercially available anhydrous K₂HPO₄, bound irreversibly to the ceramic hydroxyapatite material and prevented binding of the proteins to the column material.

Gel filtration. Gel filtration was carried out on a Superdex 200 preparation grade HiLoad 16/60 size exclusion column (Amersham Biosciences). Active protein fractions were loaded on the HiLoad column and eluted at a flow rate of 0.2 ml/min in 50 mM Na₂HPO₄-KH₂PO₄-buffer (pH 7) containing 150 mM NaCl. The calibration curve was done with the gel filtration standard from Bio-Rad consisting thyroglobulin (bovine, 670 kDa), Gamma globulin (bovine, 158 kDa), Ovalbumin (chicken, 44 kDa), Myoglobulin (horse, 17 kDa) and Vitamin B-12 (1.35 kDa). Blue dextran was used to determine the void volume.

SDS-PAGE and Electroblotting. SDS-PAGE was carried out as described in chapter 2. Electroblotting of proteins to a PVDF protein sequencing membrane (Bio-Rad) was carried out according to standard procedures (1).
Standard molecular procedures and construction of the cosmid library. Cloning, plasmid isolations and sequencing were done according to established procedures (1, 4). The construction of the cosmid library was carried out as described in Chapter 2.

Hybridization experiments. For southern hybridization, total DNA of *S. herbicidovorans* MH fractionated on an agarose gel was blotted on a Hybond™-XL membrane (Amersham Biosciences, Dübendorf, Switzerland) with a vacuum blower (VacuGene XL, Amersham Biosciences) by the procedure described by Ravatn et al. (3). Colony blotting of the cosmid library was carried out as described in chapter 2. Total DNA and the colony blots were hybridized with oligonucleotides obtained by backtranslation of the N-terminal sequences (table 1). The oligonucleotides were radioactively labeled with \[^{32}\text{P}\]ATP using T4 polynucleotide kinase according to the protocol of the manufacturer (Promega, Wallisellen, Switzerland). For non-radioactive labeling, the oligonucleotides were labeled with biotin 16-ddUTP using terminal dideoxyribonucleotide transferase also according to the manufacturer’s instructions (Promega). Hybridization experiments were performed in SDS/BSA hybridization buffers (0.5 M sodium-phosphate [pH7], 7% sodium dodecyl sulfate, 1% bovine serum albumin) at 40, 45, and 48°C for 16 hours. The membranes were washed twice with a solution of 5 × SSC containing 1 mM EDTA for 2 min at room temperature and twice with a solution of 2 × SSC plus 0.1% SDS for 30 min at the hybridization temperature. Chemiluminescent detection of biotinylated DNA-probes was carried out according to the protocol ‘Southern-Light™ & Southern Star™ Systems’ (Applied Biosystems, Rotkreuz, Switzerland).

Enzyme activity measurements. Enzyme activity measurements were done with the end-point assay measuring the product formation by HPLC as described in chapter 3.

**RESULTS AND DISCUSSION**

Growth of *S. herbicidovorans* MH. *S. herbicidovorans* MH grew exponentially on complex medium to an optical density measured at 546 (OD\(_{546}\)) of about 0.8 (Fig. 1A). Further growth was linear. By adding glucose to the culture, exponential growth could be restored until an OD\(_{546}\) of about 4 (Fig. 1B). Strain MH grew exponentially again, when peptone and yeast extract were added. The maximal OD\(_{546}\) reached with this complex medium and the addition of the components during batch growth was 7 (data not shown). The data indicated that on complex medium, *S. herbicidovorans* MH grows exponentially only in the presence of glucose. A glucose concentration of 0.44 g/l, which was the glucose concentration in the complex medium used, should yield an OD\(_{546}\) of about 0.8
according to rough medium calculations. *S. herbicidovorans* MH grew exponentially to about this turbidity, and at higher densities, exponential growth was only observed, when glucose was added to the medium. Unfortunately, exponential growth was inhibited at too high glucose concentrations as growth studies with glucose concentration of 0.5% (w/v) in the medium showed (data not shown).

![Graph A](image)

![Graph B](image)

**Fig. 1.** (A) Growth of *S. herbicidovorans* MH on complex medium. The triangles represent the measured optical density at 546 nm; the dashed line indicates the trendline for exponential growth. (B) Growth of *S. herbicidovorans* MH on complex medium. The arrows indicate the addition of glucose to the medium.
(S)-dichlorprop dioxygenase activity is ‘dependent’ on growth phase.

Former experiments by Nickel et al. (2) showed that (S)-dichlorprop dioxygenase was expressed when *S. herbicidovorans* MH was grown on complex medium. A closer look at the growth phases revealed, that SdpA was only expressed above an OD$_{546}$ of 0.8 (Fig. 2). Interestingly, highest specific activity in cell-free extract was seen around an OD$_{546}$ of 1.5 whereas above an OD$_{546}$ of 1.7, specific activity decreased significantly. It can be hypothesized that glucose represses the expression of SdpA during the exponential growth phase of *S. herbicidovorans* MH. However, I do not have an explanation for the decreased activity above an OD$_{546}$ of 1.7.

In conclusion, the growth experiments showed that the fastest way to cultivate large quantities of *S. herbicidovorans* MH and to obtain optimal (S)-dioxygenase activity would be to grow the strain in a large volume to an OD$_{546}$ of about 1.5.

![Fig. 2. Specific activity of (S)-dichlorprop dioxygenase at the different growth phases (cell densities indicated as OD$_{546}$). The different markers (triangles, diamonds and circles) represent three independent replicates.](image)

**Partial purification of SdpA.** SdpA was partially purified with different chromatographic methods and procedures. Here, I describe the one, which led to the most purified proteins fractions according to visual inspection on a SDS-PAGE gel. The following procedure was applied: anion-exchange chromatography,
hydroxyapatite chromatography and gel filtration. The most active fractions were loaded on a SDS-PAGE gel (Fig. 3), which was blotted on a PVDF membrane. The N-terminal sequence of the three major protein bands were determined (Table 1) at the Edman-Sequencing facility of ETH Hönggerberg. The N-terminal sequences of band-1 and band-2 showed ambiguous homology whereas the N-terminal sequence of band-3 was similar to an oxidoreductase.

![Figure 3. SDS-PAGE of partially purified (S)-dichlorprop dioxygenase. Lane 1, partially purified (S)-dichlorprop dioxygenase; lane 2, prestained molecular weight marker (Low Range, Bio-Rad) The arrows indicate the three protein bands, which were N-terminally sequenced.](image)

**Reverse genetics.** All obtained N-terminal amino acid sequences were backtranslated into nucleotide sequences (Table 1) and total chromosomal DNA of *S. herbicidovorans MH* was hybridized with the labeled oligonucleotides. The best hybridization result was obtained with oligonucleotide-2, whereas oligonucleotide-1 hybridized weakly to total DNA and oligonucleotide-3 did not hybridize at all. Due to these results and an assumed subunit size of (S)-dichlorprop dioxygenase of 34 kDa, we hybridized the cosmid library of *S. herbicidovorans MH* with oligonucleotide-2. We isolated several positive reacting cosmids, subcloned fragments and sequenced them, but none of the fragments showed any similarity to α-ketoglutarate-dependent dioxygenases genes.
Table 1. The N-terminal sequences of the protein bands and the backtranslated oligonucleotide sequences used for hybridization experiments

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<sup>a</sup> Normal printed amino acid residues could not be identified unambiguously in Edman-sequencing.
<sup>b</sup> R = A or G; Y = c or t; H = a, c, or t; N = a, c, g, or t.
<sup>c</sup> The N-terminal amino acid sequence of SdpA (translated from the nucleotide sequence) is given. Amino acid residues, which do not agree with the residues determined by Edman-sequencing are indicated in normal letters.
<sup>d</sup> Nucleotide sequence of <i>sdpA</i> (accession number AJ628860)
Now, from a distant point of view, having isolated the *sdpA*-gene and being able to compare the nucleotide sequence as well as the deduced amino acid sequences, we suppose that we partially purified the right protein which corresponded to oligonucleotide-2. It is likely, that with further purification steps and a higher amount of purified protein, we would have been able to purify the (S)-dichlorprop dioxygenase, but – luckily – the genetic approach was quicker 😊.
REFERENCES


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