



Kathleen Marika Schleinitz (Autor)

**Genetic analysis of stereospecific cleavage of the
chiral herbicide 2-(2,4-dichlorophenoxy) propionate
(dichlorprop) in *Delftia acidovorans* MC1**

Kathleen Schleinitz

Genetic analysis of stereospecific cleavage of the
chiral herbicide 2-(2,4-dichlorophenoxy)propionate
(dichlorprop) in *Delftia acidovorans* MC1



Cuvillier Verlag Göttingen

<https://cuvillier.de/de/shop/publications/2452>

Copyright:

Cuvillier Verlag, Inhaberin Annette Jentzsch-Cuvillier, Nonnenstieg 8, 37075 Göttingen,
Germany

Telefon: +49 (0)551 54724-0, E-Mail: info@cuvillier.de, Website: <https://cuvillier.de>

Chapter 1

Introduction

Chiral agrochemicals currently make up around 25% of all agrochemicals used worldwide and are employed with increasing tendency (Williams, 1996). Their effects on biological systems, such as toxicity for target and non-target organisms, as well as their transport, transformation and degradation are generally recognised to be enantioselective. Although stereospecific processes are of considerable importance for the assessment of the impact and fate of chiral agrochemicals in the environment, they are often not well understood on a molecular level (Hegeman and Laane, 2002). This also holds true for the degradation of the herbicide dichlorprop (*R,S*-2-[2,4-dichlorophenoxy]propionate).

1.1 Use of dichlorprop and its fate in the environment

Dichlorprop and mecoprop (*R,S*-2-[2-methyl-4-chlorophenoxy]propionate) belong to the phenoxyalkanoate group of herbicides, which is one of the most widely used group of herbicides worldwide (Environmental Protection Agency, <http://www.epa.gov/>). In the European Union more than 500 t are produced annually (Hegeman and Laane, 2002). There are currently five herbicides containing dichlorprop and eight herbicides containing mecoprop registered for use in Germany (Biologische Bundesanstalt/Bundesamt für Verbraucherschutz und Lebensmittelsicherheit; <http://www.bvl.bund.de/pflanzenschutz/psmdbstart.htm>).

Phenoxyalkanoate herbicides act as auxin analogues and have been employed as broad leaf herbicides since the 1940s (Environmental Protection Agency, <http://www.epa.gov/>). Formulae of principle members are shown in Figure 1.1. They are characterised by a chlorine substituent at the C-4 position and a chlorine atom or a methyl group at the C-2 position of the aromatic ring. In contrast to the phenoxyacetate and phenoxybutyrate derivatives the phenoxypropionates contain an asymmetric C-atom, render-

ing the herbicides chiral. Only the *R*-enantiomers display significant toxicity. Nevertheless the racemates have been applied as herbicides for decades. Since the 1990s many products are sold containing only the active enantiomer named dichlorprop-P and mecoprop-P (Williams, 1996). This is also the case with all of the products currently available in Germany.

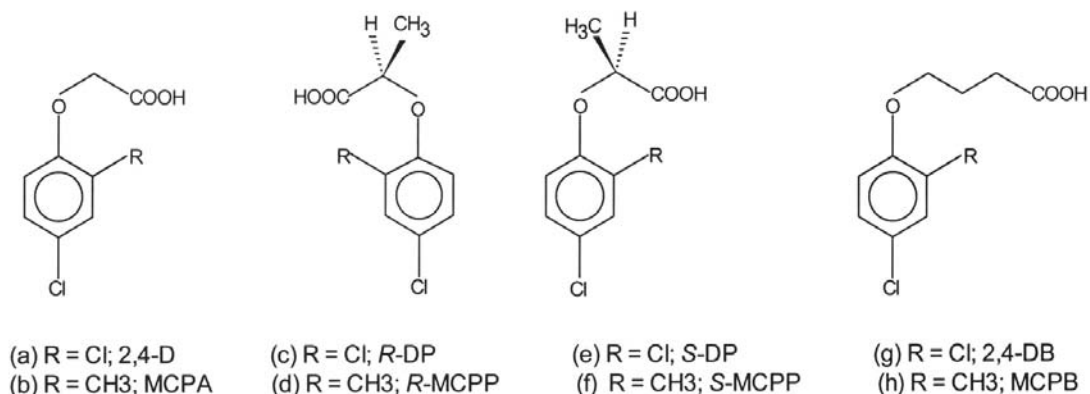


Figure 1.1: **Chemical structures of principle members of the phenoxyalkanoate group of herbicides.** 2,4-D – 2,4-dichlorophenoxyacetate; MCPA – 2-methyl-4-chlorophenoxyacetate; *R*-DP and *S*-DP – *R*- and *S*- enantiomer of 2,4-dichlorophenoxypropionate, respectively; *R*-MCPP and *S*-MCPP – *R*- and *S*-enantiomer 2-methyl-4-chlorophenoxypropionate, respectively; 2,4-DB – 2,4-dichlorophenoxybutyrate; MCPB – 2-methyl-4-chlorophenoxybutyrate

When applied to the soil dissipation of dichlorprop and mecoprop is usually rapid with half lives under aerobic conditions ranging between 3 and 62 days (Hassall, 1990; Garrison et al., 1996; Vink and van der Zee, 1997b; Romero et al., 2001, an literature therein). However, under anaerobic conditions they can be much more stable. Several authors have reported that phenoxypropionate herbicides are not degraded under anaerobic conditions (Harrison et al., 1998; Zipper et al., 1999; Larsen et al., 2000; Baun et al., 2003) or that the rate of disappearance is decreased by one or more orders of magnitude compared to aerobic conditions (Vink and van der Zee, 1997a; Larsen et al., 2001; Larsen and Aamand, 2001). Furthermore, abiotic factors indirectly influence the breakdown of phenoxypropionate herbicides by affecting microbial activity. Suboptimal or extreme levels of moisture content, temperature, pH or macro-/micronutrients lead to a decrease in the rate of degradation, thereby enhancing their recalcitrance (Helweg, 1993; Vink and van der Zee, 1997b; Romero et al., 2001). Likewise high concentrations of phenoxypropionates (> 5 g/kg) delay or prevent degradation due to toxic effects on microbial communities (Reffstrup et al., 1998; Henriksen et al., 2003).

Besides their recalcitrance under certain conditions the ease with which

phenoxypropionates are translocated from the site of application can be a problem. Dichlorprop and mecoprop are highly water soluble, while adsorption onto soil particles is low (Felding, 1995) rendering them highly mobile. Leaching and surface runoff, especially after heavy rainfall, can then lead to the contamination of surface and ground water (Felding, 1995; Bergström, 1995; Matallo et al., 1999; Gerecke et al., 2002; Fletcher et al., 2004). Concentrations in groundwater samples have been reported in the range of micrograms per litre, e.g. 0.3–975 $\mu\text{g}/\text{l}$ in landfill and soil leachate samples (Bergström, 1995; Felding, 1995; Zipper et al., 1998). Besides leaching and surface runoff, atmospheric processes contribute considerably to the translocation and deposition of dichlorprop and mecoprop. Distinct geographical and seasonal patterns with maximum concentrations in air and precipitation during and after local use have been observed (Lode et al., 1995; Rawn et al., 1999; Donald et al., 1999). However, atmospheric transport also occurs to a significant extent on a regional and long range scale (Donald et al., 2001).

Abiotic processes, namely photodegradation, are known to lead to the transformation of mecoprop and dichlorprop (José-Climent and Miranda, 1997; Romero et al., 1998; Meunier and Boule, 2000; Meunier et al., 2002) and complete breakdown of mecoprop has been observed under laboratory conditions (Topalov et al., 2000). However, under environmental conditions these processes play only a minor role and the only significant route of removal of these herbicides from the environment is microbial degradation (Alexander, 1981; Heider and Fuchs, 1997).

1.2 Microbial degradation of phenoxypropionate herbicides

The ability to degrade the achiral phenoxyacetate herbicides is widespread among bacteria (Hägglom, 1992). In contrast, breakdown of the chiral phenoxypropionate derivatives is far less widespread and only few strains capable of mineralising these compounds have been described (Table 1.1).

Degradation proceeds along the same route as that of 2,4-D (Horvath et al., 1990; Müller et al., 2001) (Fig. 1.2), which is well studied in *Wautersia* [formerly *Ralstonia*, then reclassified by Vanechoutte et al., 2004, but see also Vandamme and Coenye, 2004] *eutropha* JMP134 (Hägglom, 1992). It is initiated by the etherolytic cleavage of the side chain yielding 2,4-dichlorophenol (2,4-DCP). 2,4-DCP is then hydroxylated to give 3,5-dichlorocatechol (3,5-DCC). Subsequent conversion of 3,5-DCC to 3-oxoadipate follows the modified *ortho*-pathway. Only the first step is specific for the single herbicide substrates. Side chain cleaving activity is therefore crucial and determines the substrate range that can be utilised by a given microorganism.

To date, two types of 2,4-D-cleaving enzymes have been described. In

Table 1.1: **Bacterial strains able to mineralise phenoxypropionate herbicides**

Strain	Source	Reference
<i>Sphingobium herbicidovorans</i> MH	Soil	Horvath et al., 1990
<i>Alcaligenes denitrificans</i>	Garden soil	Tett et al., 1994
<i>Rhodofera</i> sp. P230, P212	Building rubble	Ehrig et al., 1997
<i>Delftia acidovorans</i> MC1	Building rubble	Müller et al., 1999
<i>Alcaligenes</i> sp. CS1	Agricultural soil	Smejkal et al., 2001b
<i>Ralstonia</i> sp. CS2	Agricultural soil	Smejkal et al., 2001b
<i>Stenotrophomonas maltophilia</i> PM	Groundwater sediment	Mai et al., 2001

Wautersia eutropha JMP134 side chain cleavage is catalysed by TfdA, a well-studied Fe(II)-dependent dioxygenase, which couples the oxidative decarboxylation of α -ketoglutarate (α KG), yielding succinate and carbon dioxide, to the hydroxylation of 2,4-D, yielding 2,4-DCP and glyoxylate (Streber et al., 1987; Fukumori and Hausinger, 1993a; Fukumori and Hausinger, 1993b; Hausinger and Fukumori, 1995). Although affinity and catalytic activity were found to be highest with 2,4-D, TfdA is capable of converting several other mono- and dichlorinated phenoxyacetates and also exhibits some activity towards dichlorprop (Hausinger and Fukumori, 1995). Spectroscopic and mutagenesis studies as well as primary sequence analysis identified the functionally essential motif HX(D)_{23–26}(T/S) X_{114–183}HX_{10–13}R/K, comprising the Fe(II) and α KG binding residues (Whiting et al., 1997; Hogan, 1999; Hogan et al., 2000). The presence of this motif indicates that TfdA is related to several other (referred to as group II) α KG-dependent dioxygenases (Hogan, 1999). Recently, a three-dimensional model of the active site was proposed (Elkins et al., 2002) on the basis of the crystal structures of two homologous enzymes, taurine/ α KG dioxygenase (TauD; Elkins et al., 2002) and clavamate synthase (CAS1; Zhang et al., 2000). The identity of the suggested substrate-binding residues was confirmed by site-directed mutagenesis and inactivation studies (Dunning Hotopp and Hausinger, 2002). The *tfdA* genes are widespread among bacteria (Maltseva et al., 1996; Suwa et al., 1996; Vallaeyts et al., 1996; Vallaeyts et al., 1997; McGowan et al., 1998; Vallaeyts et al., 1998; Saari et al., 1999; Vedler et al., 2000; Hoffmann et al., 2001; Müller et al., 2001; Smejkal et al., 2001), also among such unable to degrade 2,4-D (Hogan et al., 1997). Recently, several α -proteobacterial strains were shown to carry *tfdA*-like genes designated *tfdA α* (Itoh et al., 2002; Itoh et al., 2004). They show between 46 and 60% nucleotide identity to the canonical *tfdA* genes and form a phylogenetic clade distinct from that of *tfdA*. A second type of 2,4-D-cleaving-enzyme, a putative monooxygenase, was found to be encoded by the genes *cadAB*, identified in *Bradyrhizobium* sp. strain HW13 (Itoh et al.,

2002). The *cadA* genes were subsequently detected in several 2,4-D degrading and non-degrading *Bradyrhizobium* sp. and *Sphingomonas* sp. strains, and showed between 46 and 53% nucleotide sequence identity to *tftA*, a 2,4,5-trichlorophenoxyacetate monooxygenase gene of *Burkholderia cepacia* AC1100 (Itoh et al., 2004).

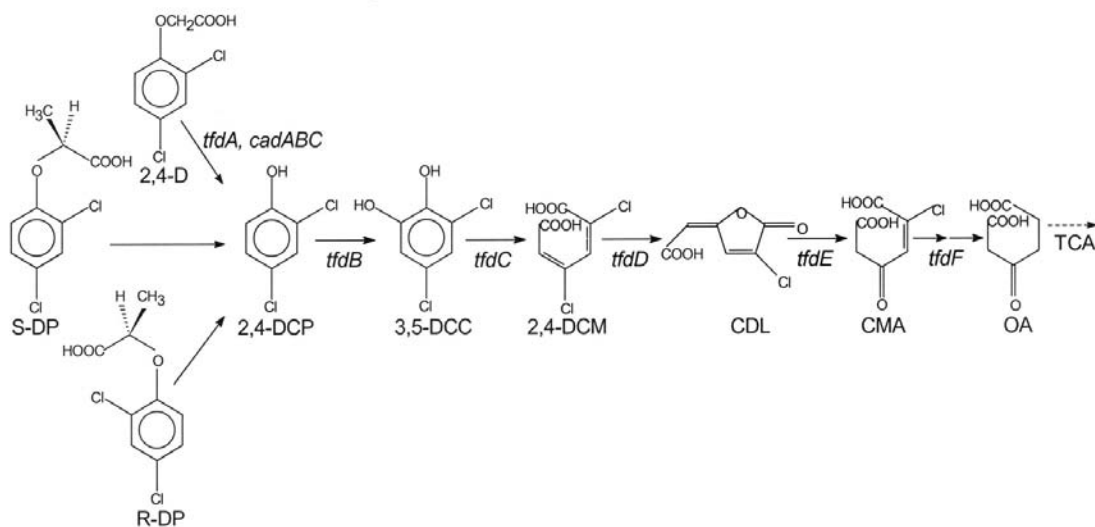


Figure 1.2: **Modified *ortho*-pathway for the degradation of chlorinated phenoxyalkanoate herbicides** 2,4-D, 2,4-dichlorophenoxyacetate; *R*-DP and *S*-DP, *R*- and *S*-enantiomers of 2(2,4-dichlorophenoxy)propionate, respectively; 2,4-DCP, 2,4-dichlorophenol; 3,5-DCC, 3,5-dichlorocatechol; 3,5-DCM, 3,5-dichloromuconate; CDL, *cis*-2-chlorodiene-lactone; CMA, chloromaleylacetate; MA, maleylacetate; OA, 3-oxoadipate; TCA, tricarboxylic acid. Genes given alongside the arrows encode the following enzymes: *tfdA*, 2,4-D/ α KG-dependent dioxygenase; *cadABC*, putative 2,4-D monooxygenase; *tfdB*, 2,4-dichlorophenol hydroxylase; *tfdC*, chlorocatechol 1,2-dioxygenase; *tfdD*, chloromuconate cycloisomerase; *tfdE*, dienelactone hydrolase; *tfdF*, maleylacetate reductase

Studies on *Sphingobium* (formerly *Sphingomonas*, Takeuchi et al. (2001)) *herbicidovorans* MH and *Delftia* (formerly *Comamonas*, Wen et al. (1999)) acidovorans MC1 indicated that enantiospecific dichlorprop and mecoprop cleavage is catalysed by Fe(II) and α KG-dependent dioxygenases (Nickel et al., 1997; Müller et al., 1999). In strain MH one of the dioxygenases was specific for *R*-mecoprop and *R*-dichlorprop, whereas the other showed highest activity towards the *S*-configurations (Zipper et al., 1996; Nickel et al., 1997). The enzyme acting on the *R*-enantiomers was inducible by its substrates, whereas the *S*-specific enzyme was constitutively expressed and repressed during growth on the *R*-enantiomers (Nickel et al., 1997). Three inducible transport systems separately specific for the *R*- and *S*-enantiomers of dichlorprop and for 2,4-D were identified in *S. herbicidovorans* MH (Zipper et al., 1998).

In *D. acidovorans* MC1 the enzyme cleaving the *R*-enantiomers was highly specific for its substrates and did not exhibit significant activity towards the *S*-configuration or the phenoxyacetate derivatives (Müller and Babel, 1999; Westendorf et al., 2003). The enzyme cleaving the *S*-enantiomers showed a broader substrate specificity. 2,4-D was converted at a rate almost equal to that of the *S*-enantiomers of dichlorprop, and mecoprop and even the *R*-configuration was attacked to some extent. Both dioxygenases were found to be constitutively expressed (Müller et al., 2001). Herbicide uptake has not been studied in this strain.

No data regarding the genetic background of dichlorprop cleavage are available for any of the strains. So far, the dioxygenases have been assumed to be *tfdA* gene products (Saari et al., 1999), because some dichlorprop- and mecoprop-degrading strains carry *tfdA* genes (Saari et al., 1999; Müller et al., 2001), and cell extracts of *W. eutropha* JMP134 showed some activity toward cleaving *S*-dichlorprop (Saari et al., 1999). However, no activity for *S*-mecoprop or the *R*-enantiomers was detected in strain JMP134, and the organism was unable to grow on phenoxypropionate herbicides. Recently available N-terminal and internal amino acid sequences of the enantiospecific dioxygenases from *D. acidovorans* MC1 showed only low similarities to TfdA enzymes (Westendorf et al., 2002), and *tfdA* genes were not detected in this strain (Müller et al., 2001). This suggested that genes other than *tfdA* are involved in dichlorprop degradation in strain MC1.

Currently nothing is known about the basis of stereoselectivity of these dioxygenases and the protein structure that enables them to distinguish between the dichlorprop enantiomers. To date, only few pairs of enantioselective enzymes have been structurally solved (Kirsch et al., 1995; Sugio et al., 1995; Stoll et al., 1996; Nakajima et al., 1998). These studies have shown that such pairs of enzymes can vary widely in their degree of structural similarity and that stereoselectivity can develop by divergent as well as convergent evolution. The former is the case with the strictly enantiospecific tropinone reductases TR-I and TR-II (Nakajima et al., 1998), involved in the synthesis of tropane alkaloids in *Datura stramonium* and *Hyoscyanus niger*. These enzymes catalyse the reduction of their common substrate tropane to 3- α -hydroxy-tropane (TR-I) or 3- β -hydroxy-tropane (TR-II). TR-I and TR-II share 64% amino acid identity and the overall folding of the enzymes is almost identical. The binding sites for the cofactor NADPH and positions of the active site residues are conserved. The active sites are mainly composed of hydrophobic residues, but differently charged amino acids create different electrostatic environments resulting in the opposite orientation of the positively charged substrate in TR-I compared to TR-II. Stereoselectivity in L- and D-lactate dehydrogenases (L-LDH, D-LDH) of *Lactobacillus pentosus* on the other hand is the result of convergent evolution (Stoll et al., 1996). L-LDH and D-LDH catalyse the conversion of pyruvate to L- and D-lactate, respectively. Despite sharing around 20% sequence identity their

3D-structures are completely different showing that L-LDH and D-LDH belong to different protein families. Convergent evolution was also proposed for L-amino acid aminotransferases (L-AAT) and D-amino acid aminotransferases (D-AAT) of *Bacillus* sp. that completely lack sequence identity and display different 3D-structures (Sugio et al., 1995). However, they resemble each other in their enzymatic mechanism and their active centres were found to have some striking similarities. These similarities include the interaction between the cofactor pyridoxal phosphate (PLP) and the enzyme as well as the orientation of the α -amino and α -carboxy groups of the amino acid substrate relative to the orientation of PLP. The amino acid side chain is, due to inverse chirality, orientated in the opposite direction.

Both dichlorprop dioxygenases exhibit the same enzymatic mechanism. N-terminal and internal peptide sequences display similarities to TfdA (Westendorf et al., 2002); therefore it may be anticipated that they are homologous to each other.

1.3 Instability of dichlorprop cleavage in *Delftia acidovorans* MC1

Dichlorprop cleaving ability in *Delftia acidovorans* MC1 is highly unstable and easily lost under non-selective conditions (Müller et al., 1999). Loss was observed to be irreversible. The resulting mutants, generally designated MC1-DP⁻, are unable to use *R,S*-dichlorprop, but are still able to grow on 2,4-DCP, indicating that only the first step in degradation is affected.

The spontaneous loss of catabolic functions in bacteria is a well-known phenomenon (Wyndham et al., 1994) and has also been described for four strains degrading phenoxyalkanoate herbicides. In three of them – *Achromobacter xylosoxidans* EST4002 (pEST4011), *Burkholderia cepacia* CSV90 (pMAB1) and *Burkholderia cepacia* 2a (pIJB1) – the genes encoding 2,4-D degradation are located on the plasmids (Mäe et al., 1993; Bhat et al., 1994; Xia et al., 1998). Plasmids pEST4011 and pMAB1 are segregationally unstable and curing of the plasmids gives rise to the 2,4-D⁻ phenotype (Mäe et al., 1993; Bhat et al., 1994). Furthermore, all plasmids were observed to be structurally unstable, undergoing deletion of the region carrying the genes involved in degradation. In plasmid pEST4011 deletion of a 48 kb fragment was the result of homologous recombination between two copies of an IS1071::IS1471 hybrid insertion element flanking the deleted region (Vedler et al., 2000; Vedler et al., 2004). The same was true for plasmid pIJB1, where the *tfd* genes residing on the 41 kb composite transposon Tn5530 were lost by homologous recombination between the IS1071::IS1471 elements terminating the transposon (Xia et al., 1998; Poh et al., 2002). In plasmid pMAB1 the deletion comprised 20 kb, but mechanisms causing it were not studied (Bhat et al., 1994). The fourth strain, *Delftia acidovorans*

P4a, carries the genes encoding 2,4-D degradation on a transposon located on the chromosome (Hoffmann et al., 2003). As in the other strains these genes were deleted during cultivation under non-selective conditions, but the exact extent and mechanism of the deletion were not studied.

In *Delftia acidovorans* MC1 the location of the genes encoding the dichlorprop dioxygenases is not known. However, the strain possesses a 120 kb plasmid which harbours *tfdB*, *tfdC* and *tfdD* and which undergoes the deletion of a 40 kb fragment when the strain is cultivated under non-selective conditions (Müller et al., 2001). It was therefore suggested that the genes encoding the initial step in degradation are located on this fragment and that its deletion causes the loss of dichlorprop cleaving ability (Müller et al., 2001).

1.4 Aims of this study

The aim of this study is to analyse the genetic background of stereospecific dichlorprop cleavage in *Delftia acidovorans* MC1.

At the outset of this work dichlorprop dioxygenases were thought to be *tfdA* gene products known to be responsible for the cleavage of the achiral 2,4-D, but this hypothesis was questioned because of the lack of *tfdA* genes in *Delftia acidovorans* MC1 and other findings. Therefore, the first part of this study focuses on identifying and sequencing the two dichlorprop dioxygenase genes. These genes are analysed with regard to their phylogeny and their distribution in other dichlorprop degraders. Homology modeling of the derived amino acid sequence is carried out in order to study structural implications, particularly the basis of enantioselectivity.

The second part of this study investigates the organisation of the dioxygenase genes and their association with lower pathway genes, genes involved in transport and regulation as well as mobile genetic elements. It describes the localisation of the dioxygenase genes within the genome of strain MC1, and the sequencing and sequence analysis of a 28 kb fragment on which they were located.

The third part reports results regarding the instability of dichlorprop cleavage. Loss of dichlorprop cleavage in the wild type strain is monitored in continuous flow cultivations on nonselective medium. Further continuous flow cultivations are performed with two mutants able to degrade either of the dichlorprop enantiomers and one mutant with a seemingly stabilised dichlorprop cleavage. Loss of the degradative trait in them is compared with that of the wild type. In subsequent Southern hybridisations are also used to characterise some of the mutants with regard to the presence or absence of the dioxygenase genes as well as lower pathway genes.

Chapter 2

Material and Methods

2.1 Microbiological methods

2.1.1 Strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 2.1. *Delftia acidovorans* MC1 and *Rhodofera* sp. P230 isolated from contaminated building rubble (Müller et al., 1999; Ehrig et al., 1997) and *Sphingobium herbicidovorans* MH isolated from soil (Horvath et al., 1990) were all maintained on a mineral salts medium (MM) containing (in mg/l): NH₄Cl, 761; KH₂PO₄, 272; K₂HPO₄, 348; CaCl₂* 6 H₂O, 5.47; MgSO₄* 7 H₂O, 71.2; FeSO₄* 7 H₂O, 4.98; CuSO₄* 5 H₂O, 0.785; MnSO₄* 4 H₂O, 0.812; ZnSO₄* 7 H₂O, 0.44 and Na₂MoO₄* 2 H₂O, 0.25. For strains P230 and MC1 the pH was adjusted to 8.5 with 20% (v/v) KOH, strain MH was cultivated at pH 7. If not stated otherwise, media and reagents were sterilised at 121 ° for 20 to 60 min, depending on the volume. CDP was added as a sole source of carbon and energy at a final concentration of 0.5 mM. Plates were incubated at 30 °C. The wild type strain MC1 was not passaged on agar plates for strain maintenance, but always streaked out from a glycerol stock culture dating from 11/1999. For the maintenance of Tn5::*gfp* insertion mutants the medium was additionally supplemented with 27.8 mM fructose and 100 µg/ml Gm. Growth on complex medium was performed using LB medium which was supplemented with 50 µg/ml Gm when cultivating Tn5::*gfp* insertion mutants. Plates were incubated at 30 °C for 5 (MM) and 2 (LB medium) day, and were stored at 4 °C for one month.

Wautersia eutropha JMP134 (pJP4) and *W. eutropha* JMP222 were grown on LB agar. The plates were incubated at 30 °C for 2 days and stored at 4 °C for up to three weeks.

Escherichia coli S17-1 (pAG408) and *E. coli* XL Blue MR (pScosPA1) were maintained on LB medium containing 50 µg/ml Gm and 50 µg/ml Ap, respectively. *E. coli* XL Blue MRF' was grown on LB medium without antibiotics. The strains carrying reference plasmids for the different in-