Abbau von Chlorbenzolen und Chlortoluolen durch natürliche und konstruierte Mikroorganismen

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von:........................................................ Katrin Pollmann
aus:....................................................... Münster/Westf.

1. Referent:............................................. PD Dr. Dietmar H. Pieper
2. Referent:............................................. Prof. Dr. K. N. Timmis
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LEBENSLAUF

DANKSAGUNGEN
Chapter I

GENERAL

INTRODUCTION
1.1 Chloroaromatics in the environment

During the last decades, the production of aromatic compounds and haloaromatics increased significantly due to their widespread use as solvents and for the synthesis of many substances such as dyes and pesticides. This extensive use of chloroaromatics by the chemical industry (e.g., production of 60,000 t chlorotoluenes in 2000 by Bayer (112)) has led to their widespread release into the environment through volatilization, leakage and disposal of industrial wastes (88) (e.g. about 1 t of chlorotoluenes in 1988/89 in West Germany (23)). A prominent example is the industrial waste site near Bitterfeld where significantly increased atmospheric concentrations of chlorobenzenes were detected (97). Since industrial synthesized chloroaromatics are usually not found in nature in such high concentrations, they are often poorly degradable and persist in nature (1). However, a wide variety of naturally occurring organohalogenes has been identified, which may have contributed to the adaptation of microorganisms to xenobiotics and to the evolution of degradation pathways (3).

Chlorotoluenes are produced by chlorination of toluenes. They are widely used by the chemical industry as solvents and intermediates in the synthesis of organic chemicals, dyes, pharmaceuticals and synthetic rubber chemicals and are still produced in high amounts (23, 34, 35). 4-Chlorotoluene is used as a starting material in the synthesis of 2,4- and 3,4-dichlorotoluene and of 4-chlorobenzonitril (35). 2-Chlorotoluene is a starting material in the production of 2-chlorobenzyl chloride, 2-chlorobenzaldehyde, 2-chlorobenzotrichloride, and 2-chlorobenzoic acid, which are in turn precursors for dyes, pharmaceuticals, optical brighteners and fungicides. 2-Chlorotoluene is also used in the production of dichlorotoluenes and o-chlorobenzonitrile (35). Dichlorotoluenes are used as intermediates in the manufacture of fungicides, herbicides, dyes, pharmaceuticals, preservatives, and peroxides (35). Exposure to these compounds cause headache, irritation of the skin, sickness, but also unconsciousness and shortness of breath (23).

Chlorinated benzenes form another group of pollutants, which are used as solvents in pesticide formulations, as solvents for paints and other industrial applications, for degreasing, in the manufacturing of phenol, aniline and for the production of dyes, crop protection agents, pharmaceuticals, rubber chemicals etc. (1, 15, 35). Among others, chlorobenzene has been identified as priority pollutant by the U.S. Environmental protection Agency (53).
**1.2 Aerobic bacterial metabolism of aromatic compounds**

Despite the toxicity of aromatic compounds a large number of microorganisms have been isolated from environment that are capable of degrading and mineralizing these compounds and aerobic as well as anaerobic degradation pathways have been described. In case of aerobic degradation of aromatic compounds, the substrates are generally channeled via a few central metabolites into common pathways (47, 131). In the first stage, the aromatic ring is activated by introduction of two hydroxyl groups catalyzed by specialized mono- or dioxygenases. In the second stage, the resulting dihydroxylated aromatics undergo either intra- or extradiol ring cleavage, catalyzed by intra- or extradiol dioxygenases, respectively. The aliphatic acids formed are metabolized to Krebs cycle intermediates, which are used for biosynthesis or energy production.

**1.2.1 Toluene**

The capability to mineralize toluene is obviously widespread in bacteria. For aerobic degradation, five different metabolic routes have been described (Fig. 1). The TOL plasmid pWW0 of *Pseudomonas putida*, encoding enzymes of the *tol*-pathway, is the most extensively characterized catabolic plasmid (45, 46). In this *tol*-pathway, the methylgroup of toluene is sequentially oxidized to yield benzoate (upper pathway). The carboxylic acid is then oxidized to catechol by the action of toluate dioxygenase and 1,2-dihydroxycyclo-3,5-hexadiene-1-carboxylate dehydrogenase.

In the *tod*-pathway, extensively investigated for *Pseudomonas putida* F1 (132, 133), the aromatic ring is activated by the introduction of two hydroxyl groups, catalyzed by toluene 2,3-dioxygenase. The dioxygenation is followed by rearomatization of cis-toluene-2,3-dihydrodiol by a dehydrogenase to give 3-methylcatechol.

Furthermore, three different types of monooxygenases – toluene-2-, toluene-3- and toluene-4-monooxygenase - have been described, catalyzing the hydroxylation of the aromatic ring of toluene to ortho-, meta- and para-cresol, respectively (58, 106, 114). In case of *o*- and *m*-cresol, the phenolic intermediates are converted to 3-methylcatechol, whereas *p*-cresol is transformed to protocatechuate with *p*-hydroxybenzylalcohol, *p*-hydroxybenzaldehyde and *p*-hydroxybenzoate as intermediates (106) (Fig. 1).
FIG. 1: Bacterial pathways for the degradation of toluene: ortho- and meta-cleavage pathways (45, 46, 58, 106, 114, 132, 133).
Methylcatechols, formed as central intermediates in dioxygenolytic as well as in o- and m-cresol pathways, usually undergo meta-fission to produce hydroxymuconic semialdehydes, which are further transformed into Krebs-cycle products (Fig. 1). Catechol undergoes either intradiol cleavage resulting in the formation of muconate, which is channeled into the Krebs cycle in subsequent reactions or is metabolized via a meta-cleavage pathway. Protocatechuate, the product of p-cresol transformation, like catechol, can be subject to ortho- (3,4-) or to meta- (2,3- or 4,5-) ring-cleavage with the resulting products being channeled into the Krebs cycle.

1.2.2 Chlorinated benzenes

In contrast to toluene degrading organisms, organisms capable to mineralize chlorobenzenes, have been relatively seldom described. Mineralization of chlorobenzene, first described by Reineke and Knackmuss (105), is initialized by a dioxygenolytic attack on the aromatic ring analogues to the toluene 2,3-dioxygenase catalyzed dioxygenation of toluene (Fig 2). Subsequently the resulting cis-dihydrodiol is converted by a dehydrogenase to give 3-chlorocatechol, regaining the aromatic structure. Analogously, degradation of higher chlorinated benzenes such as 1,2-, 1,3-, 1,4-dichloro- and 1,2,4-trichlorobenzene is initialized by dioxygenation followed by dehydrogenation yielding the corresponding chlorocatechols as central intermediates. In case of 1,2,4,5-tetrachlorobenzene, the mineralization of which was described thus far only by two bacterial strains, dioxygenation is accompanied by chloride elimination to give 3,4,6-trichlorocatechol, as described for the strain Ralstonia sp. PS12 (7) (Fig. 2).

The majority of bacteria capable of mineralizing chlorinated benzenes metabolize the resulting chlorocatechols by enzymes of the modified ortho-pathway via the corresponding muconates and dienelactones as intermediates. Elimination of a chloride occurs after intradiol ring cleavage. In contrast, meta-cleavage of chlorocatechols usually leads to substrate misrouting or formation of a suicide product. Nevertheless, in the strain Pseudomonas putida GJ31 the degradation of 3-chlorocatechol via meta-cleavage pathway has been described (76). This strain contains a chlorocatechol 2,3-dioxygenase that can efficiently cleave 3-chlorocatechol at the 2,3-position along with simultaneous dechlorination producing 2-hydroxy-cis,cis-muconate (59, 76).
1.2.3 Chlorinated toluenes

Degradation of aromatic compounds carrying both methyl- and chlorosubstituents was assumed to be difficult. Whereas methylcatechols formed from toluene or xylenes are usually mineralized by a meta-cleavage (extradiol) pathway, intradiol cleavage often results in accumulation of methylsubstituted muconolactones as dead end metabolites (18, 70) and only specialized pathways are capable to further metabolize those lactones (13, 89). On the other hand, chlorosubstituted catechols are usually metabolized via an ortho-cleavage pathway (103), which
involves intradiol cleavage by a chlorocatechol 1,2-dioxygenase. Even though chlorocatechols can be channeled into some meta-cleavage routes, extradiol cleavage usually results in the formation of dead end metabolites or suicide products, e.g. the highly reactive 5-chloroformyl-2-hydroxy-penta-2,4-dionic acid that is assumed to be the product of a distal (1,6-) cleavage of 3-chlorocatechol, which inactivates the catechol 2,3-dioxygenase (4). Additionally, accumulated chlorocatechols can inhibit catechol 2,3-dioxygenases (68). Thus, the simultaneous degradation of methyl- and chloroaromatics was observed to be problematic (99, 25).

However, even though mixtures of methyl- and chlorosubstituted aromatics were observed to be difficult to degrade, mixed substituted chloromethylsubstituted aromatics not necessarily should create problems in degradation via ortho-cleavage routes. As long as a chloride substituent can be eliminated during cycloisomerisation, the formation of methylsubstituted muconolactones will be prevented in favor of the formation of methylsubstituted dienelactones. Several strains have been described to utilize chloro-, and dichlorotoluenes and natural and constructed bacterial strains capable to mineralize 3- or 4-chlorotoluene have been analyzed in detail (11, 40, 109, 123). For metabolism of 4-chlorotoluene, two metabolic routes have been reported. A mutant of strain Pseudomonas JS6 has been described to metabolize 4-chlorotoluene analogous to chlorobenzene via a modified ortho-pathway (40) with 3-chloro-6-methylcatechol as intermediate (Fig. 3). Thus, this mixed substituted catechol seem to create no metabolic problem for the strain. Another route of chlorotoluene degradation has been described by Brinkmann et al. (11). A strain capable of degrading 3-, 4-chlorotoluene and 3,5-dichlorotoluene was constructed by combining the TOL plasmid from Pseudomonas putida strain PaW1, encoding xylene monooxygenase, benzylalcohol dehydrogenase, benzaldehyde dehydrogenase, toluate dioxygenase and toluate dihydrodiol dehydrogenase, with genes encoding the chlorocatechol pathway enzymes of strain Pseudomonas putida B13 (11). Thereby, a derivative strain was obtained capable of transforming 3-, 4-chloro-, and 3,5-dichlorotoluene into 3-chloro-, 4-chloro-, and 3,5-dichlorocatechol via the corresponding benzylalcohols, benzaldehydes and benzoates by the action of enzymes of the tol-pathway and mineralizing the respective catechols by the enzymes of the chlorocatechol pathway (Fig. 3). However, 2-chlorosubstituted toluenes cannot be channeled into such a pathway due to the restricted substrate specificity of xylene monooxygenase.

Only restricted knowledge is available on the degradation of dichlorotoluenes. Vandenbergh (123) described a Pseudomonad able to use several chloroaromatics such as 2,4- and 3,4-dichlorotoluene as growth substrates but no pathways were given. The strain Ralstonia PS12
able to grow on 1,2,4,5-tetrachlorobenzene was described to utilize various dichlorotoluenes as well (109), but no metabolic pathway has been described so far.

FIG. 3: Degradation of 4-chlorotoluene by Pseudomonas sp. strain JS21, a derivative of the strain JS6 (40) and a constructed hybrid strain of Pseudomonas sp., resulting from the mating of the strains B13 and PaW1 (11).
1.3 Aromatic ring-hydroxylating dioxygenases

The initial reaction in bacterial aerobic metabolism of many aromatic compounds is catalyzed by multicomponent aromatic ring hydroxylating dioxygenase systems, which add both atoms of dioxygen to the aromatic ring to produce cis-dihydrodiols (77). These oxygenases are multi-enzyme complexes consisting of three or four proteins. However, since the aromatic hydrocarbon dioxygenase family also comprises monooxygenases and enzymes that do not hydroxylate aromatic rings, Gibson and Parales (32) suggested changing the family name to Rieske non-heme iron oxygenases.

As an example, the recently crystallized and extensively studied naphthalene dioxygenase (NDO) from *Pseudomonas* sp. strain NCIB 9816-4 (17, 25, 26, 38, 39, 72), that catalyzes the conversion of naphthalene into cis-naphthalene dihydrodiol, consists of three functional components that are composed of four proteins (Fig. 4).

Members of the Rieske non-heme iron oxygenases had been classified into three groups as Class I, II and III depending on their structure and the nature of redox centers (5). Recent studies have shown that the oxygenase α-subunits determine the substrate specificities of various dioxygenases, including naphthalene, biphenyl, chlorobenzene and nitrotoluene dioxygenases (8, 28, 33, 84, 85). As discussed by Gibson and Parales the former classification scheme, which does not take into account any information on the oxygenases itself, seems therefore not to be useful anymore (37). As all Rieske non-heme iron oxygenases are related to each other, dioxygenase families can be identified based on amino acid sequences of the catalytic oxygenase α-subunits (129) and four families (naphthalene, toluene/biphenyl, benzoate and phthalate oxygenases) have recently been proposed (37). In general, the thus identified clustering of oxygenases into families correlates with the native substrate oxidized by the members.
FIG. 4: Typical biochemical organization of the toluene/benzene and napthalene
dioxygenase system (5). Red: reductase component; Oxy: oxygenase component; Fd: ferredoxin; the
electrons are transferred from NADH + H⁺ via the reductase containing FAD as prosthetic group (Benzene
dioxygenase) and a plant type Rieske center (only Naphthalene dioxygenase, italic letters) and ferredoxin to
a terminal oxygenase component consisting of α- and β-subunits; both atoms of molecular dioxygen are
incorporated into the aromatic compound at the active site iron (Fe²⁺) resulting in a cis-dihydrodiol.

In naphthalene dioxygenase, electrons are transferred from NAD(P)H through an iron sulfur
flavoprotein reductase and a Rieske iron-sulfur ferredoxin [2Fe-2S] to the terminal catalytic
oxygenase component (25, 26, 38, 39). The oxygenase consists of large α- and small β-subunits in αβ₃-configuration (61). Each α-subunit contains a Rieske [2Fe-2S] center and a mononuclear nonheme iron. The electrons are transferred from the Rieske center in one α-subunit to the
mononuclear iron located in the adjacent α-subunit, that is the site of activation and catalysis (61,
86). Crystallization and determination of the three-dimensional structure of NDO (Fig. 5) and
docking studies with indole, naphthalene and biphenyl suggested the location of substrate and
dioxygen binding and the underlying mechanism of catalysis (17). The substrate pocket is a
flattened and elongated cavity. With the exception of the mononuclear iron and a polar region
located at the bottom, the walls of the active site cavity are mainly hydrophobic, allowing the
accommodation of hydrophobic substrates such as naphthalene and indole (Fig. 5). The crystal
structure allowed the identification of amino acids near the active site iron such as Asp205,
Asn201, and Phe352 (17). The substrate is orientated making a favorable bond to the carbonyl
oxygen atom of Asp205 and close to the carbonyl oxygen of Asn201, such that the distance between the substrate and the iron at the active site is suitable for a dioxygen bridge (17). Recent studies have identified the amino acid Phe352 to determine regioselectivity and enantioselectivity of NDO (85, 87).

**FIG. 5: Structure of naphthalene 1,2-dioxygenase (NDO) with indole bound (17) (Pdb Id: 1EG9).** A: Secondary structure of the a-subunit; only protein backbones with helices (tubes) and sheets are drawn. B: Substrate pocket with binding of indole and active site with iron (Fe); protein backbones are drawn as “fat tubes”, protein side chains are drawn with “wire frames”. Different secondary structures are indicated by different colors; green: helix; orange: strand; blue: coil.

Toluene dioxygenase (TDO) of *Pseudomonas* sp. F1 and tetrachlorobenzene dioxygenase (TecA) of *Ralstonia eutropha* PS12 (7, 8, 33, 120-122, 127, 132, 133) are two other well described oxygenases of broad substrate specificity.

Both enzymes are closely related, having a similar broad substrate spectrum, including various aromatic compounds such as toluene and chlorobenzene. In contrast to TDO, TecA is capable of dechlorinating 1,2,4,5-tetrachlorobenzene, despite the high similarity of the a-subunits of both enzymes (87% identities). Recent site-directed mutagenesis studies have identified the amino acid residue 220 as critical for dehalogenation (8).
1.4 Bacterial aromatic ring-cleavage enzymes

The initial dioxygenation is the first critical step in bacterial degradation of aromatic compounds, which is followed by a dehydrogenation resulting in the respective catechols as key intermediates. These metabolites can undergo either ortho or meta ring cleavage, which is the second key reaction and is catalyzed by ring-cleavage dioxygenases (67, 124). Besides dioxygenases catalyzing the cleavage of catechols or protocatechuates, which can be classified into intradiol and extradiol dioxygenases (44), other types are known such as the gentisate 1,2-dioxygenases which cleaves the aromatic ring of gentisate between the carboxyl and the vicinal hydroxyl group to form maleylpyruvate (73, 130). Catechol 1,2-dioxygenases (intradiol dioxygenases) cleave the aromatic ring between the two hydroxyl groups (as do protocatechuate 3,4-dioxygenases) whereas catechol 2,3-dioxygenases cleave the ring between a hydroxylated and a neighbored non hydroxylated carbon atom (as do protocatechuate 2,3- or 4,5-dioxygenases). Both intradiol and extradiol dioxygenases typically contain a non-heme iron (9, 24, 42, 67, 113). Intradiol enzymes contain ferric ion (Fe$^{3+}$) whereas extradiol enzymes contain ferrous iron (Fe$^{2+}$) in the active site (16, 67). The structures of several extradiol dioxygenases have been solved to date (42, 67, 113), among them the extensively studied catechol 2,3-dioxygenase from Pseudomonas putida mt-2 (67). Several (chloro)catechol 1,2-dioxygenases have been biochemically and genetically characterized and the catechol 1,2-dioxygenases of several sources have been crystallized (9, 12, 22, 31, 98, 124). Apart from the catechol 1,2-dioxygenase from Pseudomonas arvilla, which has two highly homologues monomers that can associate to form hetero- or homodimers (80, 81), all known enzymes of this family are dimers consisting of identical subunits, each containing a ferric iron [(a-Fe$^{3+}$)$_2$] (124). Chlorocatechol 1,2-dioxygenases differ from catechol 1,2-dioxygenases in their high turnover capacity with regard to chlorocatechols (82).

1.5 Muconate and chloromuconate cycloisomerases

Catechol 1,2-dioxygenases incorporate molecular oxygen into catechol and cleave the aromatic ring between its hydroxylated carbons. The formed cis,cis-muconate is then converted to muconolactone by muconate cycloisomerase. The homologous chloromuconate cycloisomerases are involved in the degradation of chlorosubstituted catechols. They catalyze the cycloisomerization of chlorosubstituted cis,cis-muconates to dienelactones with concomitant elimination of chloride. Both classes of enzymes differ from each other with respect to substrate
specificity and product formation. In comparison to muconate cycloisomerases, chloromuconate cycloisomerases usually exhibit high $K_{\text{cat}}$ values for chlorinated cis,cis-muconates but lower $k_{\text{cat}}$ values for cis,cis-muconate (125, 126). Muconate cycloisomerases convert 2-chloro-cis,cis-muconate to a mixture of 2-chloro- and 5-chloromuconate (125), and are thus not capable of dehalogenation during turnover of this substrate. In contrast, chloromuconate cycloisomerases catalyze dominantly a 3,6-cycloisomerization to 5-chloromuconolactone and subsequent dehalogenation of this intermediate to trans-dienelactone (71, 111, 125) (Fig. 6). Further, in contrast to muconate cycloisomerases, chloromuconate cycloisomerases avoid the formation of the bacteriotoxic protoanemonin from 3-chloromuconate (111). However, the biochemistry and genetics of the modified ortho-cleavage pathway have been elucidated dominantly in gram-negative strains. Recently, a muconate and a chloromuconate cycloisomerase have been purified from the gram positive strain *Rhodococcus erythrophilus* ICP (29, 30). Those enzymes differed in their capabilities to transform 3-chloromuconate and both, in contrast to the enzymes isolated from gram-negative organisms converted 2-chloromuconate to 5-chloromuconolactone as the only product. They further differed from the cyclosiomerases of gram negative strains in their inability to convert 2-chloromuconolactone and to catalyze elimination of chloride from 5-chloromuconolactone (117). Also from sequence information, those enzymes were only distantly related to cycloisomerases of gram negative strains (29, 106, 107). The metabolic diversity of (chloro)muconate cycloisomerases was underlined by studies of Plumeier et al. who showed that the chloromuconate cycloisomerase TfdD_{II} of the gram negative strain *Ralstonia eutropha* JMP134 (pJP4) is unable to convert 2-chloro-cis,cis-muconate to trans-dienelactone efficiently (95).

Crystallization and studies of the three-dimensional structure of the muconate cycloisomerase from *Pseudomonas putida* PRS200 and chloromuconate cycloisomerase from the strain JMP134 gave new insights into substrate binding and catalytic mechanism (41, 52). From this information conclusions on the adaptation to the conversion of chloro-substituted compounds could be drawn. (Chloro)Muconate cycloisomerases consist of 8 identical subunits each containing an essential manganese ion at the active site, which does not interact directly with substrate or product (52, 110). Recently, exchanges of specific amino acids of the active site of the muconate cycloisomerases of *Pseudomonas putida* PRS2000 and *Acinetobacter calcoaceticus* ADP1 have shown that the putative evolution from muconate to chloromuconate cycloisomerase was a rather complex process that cannot be explained by simple changes in the active-site cavity, evolving the typical features of chloromuconate cycloisomerases of gram negative strains, these are the acceleration of 2-chloro-cis,cis-muconate turnover, discrimination between the two possible
cycloisomerization directions of this substrate, dehalogenation of (+)-5-chloromuconolactone, acceleration of (+)-5-chloromuconolactone conversion, acceleration of 3-chloromuconate and 2,4-dichloro-cis,cis-muconate conversion and avoidance of protoanemonin formation during 3-chloro-cis,cis-muconate transformation (126). However, recent comparisons of the reaction mechanisms of muconate cycloisomerase and mandelate racemase as well as substitution of the proton donating Lys169 residue of *Pseudomonas putida* muconate cycloisomerase with alanine gave new insights into the mechanism of chloride elimination from 3-chloro- and 2,4-dichloromuconate (36, 60). Thus, an enol/enolate intermediate is proposed to be formed from 3-chloro-cis,cis-muconate and 2,4-dichloro-cis,cis-muconate (60). Whereas the formation of (2-chloro-)protoanemonin from this intermediate as carried out by muconate cycloisomerase requires protonation provided by the lysine residue, enzymatic protonation is not necessary for the formation of (2-chloro-)cis-dienlactone from 3-chloromuconate and 2,4-dichloromuconate as catalyzed by chloromuconate cycloisomerases (60). Structural studies of wild-type muconate cycloisomerase and two other variants gave insights into the mechanism of 2-chloromuconate dehalogenation. It was suggested that chloromuconate cycloisomerases have evolved a new catalytic feature, enabling a rotation of the lactone group of 5-chloromuconolactone relative to each other through binding in a specific orientation, thus enabling a dehalogenation of the substrate to give *trans*-dienelactone (110).

**FIG. 6: Transformation of 2-chloro-cis,cis-muconate by muconate and chloromuconate cycloisomerases.** Most muconate cycloisomerases described thus far catalyze a 1,4-cycloisomerization to form 2-chloromuconolactone (reaction A) and a 1,6-cycloisomerization to form 5-chloromuconolactone (reaction B) (125, 126). Muconate cycloisomerase isolated from *Rhodococcus esterophilus* ICP catalyze reaction B only (117). Chloride elimination from 5-chloromuconolactone to give *trans*-dienelactone is catalyzed exclusively by chloromuconate cycloisomerases (71, 111, 126).
1.6 Problems in degradation of chloroaromatics

1.6.1. Misrouting of substrates into dead end pathways

Recent studies have shown that the tetrachlorobenzene dioxygenase TecA of *Ralstonia* sp. strain PS12 catalyzes not only dioxygenation of the aromatic ring, but also the monooxygenation of methylsubstituents of several chlorosubstituted toluenes (74, 75, 96) (Fig. 7). This strain was the first reported exhibiting reasonable 2-chlorotoluene transforming activities (75). However, the strain failed to grow on 2- and 3-chlorotoluene probably due to insufficient benzylalcohol and bezaldehyde dehydrogenase activities and the absence of a broad-spectrum chlorobenzoate dioxygenase able to transform 2-chlorobenzoate. It was therefore supposed, that 2-chlorotoluene degrading organisms could be designed by assemblage of different catabolic segments (48, 75), i.e. the introduction of genes encoding a) 2-chlorobenzoate dioxygenase and b) chlorobenzylalcohol and chlorobenzylaldehyde dehydrogenases into strain PS12. A derivative strain capable to use 2-chlorobenzoate as growth substrate could thereby be constructed, however, the transconjugant failed to grow on 2-chlorotoluene (74). In the present thesis it is shown, that the monooxygenolytic attack channels chlorinated toluenes into a dead end pathway, thus preventing the degradation of 2-chloro-, 2,3-, 2,6- and 3,5-dichlorotoluene.

![FIG. 7: Metabolism of 2-chlorotoluene by *Ralstonia* sp. str. PS12 as described by Lehning (74,75).](image)

1.6.2 Formation of dead end products by cycloisomerization of substituted muconates

Cycloisomerization of substituted muconates is known to be a critical reaction for channeling substituted aromatics into an appropriate pathway. Muconate cycloisomerase catalyzes the
transformation of 3-chloromuconate predominantly to the bacteriotoxic protoanemonin, which is further transformed only with low activity (10) (Fig. 8). In contrast, chloromuconate cycloisomerase catalyzes dehalogenation with cis-dienelactone as the only product, which is subsequently converted by dienelactone hydrolase (111).

Cycloisomerization of methylsubstituted muconates results in the formation of methylmuconolactones, which are often reported to be dead end products (93). 3-Methyl-cis,cis-muconate, which is the ortho-cleavage product of 4-methylcatechol, is cycloisomerized to give 4-methylmuconolactone. Only strains harboring a specialized 4-methylmuconolactone methylisomerase capable of transforming 4-methylmuconolactone into 3-methylmuconolactone as described for the strain *Ralstonia eutropha* JMP 134 are reported to utilize 4-methylcatechol via an ortho-cleavage pathway (13, 92) (Fig. 9).

In contrast, 4-chloro-2-methylmuconate, the ring-cleavage product formed during 4-chloro-2-methylphenoxyacetate transformation, is mineralized via 2-methyldienelactone as cycloisomerization product (90, 91, 99). Thus, provided a chloride can be eliminated during cycloisomerization resulting in the formation of a substituted dienelactone, bacteria are able to metabolize chloromethylcatechols due to the unspecificity of dienelactone hydrolases.

On the other hand, 2-chloro-4-methylmuconate, which is the product of 2-chloro-4-methylphenoxyacetate transformation by the strain *Ralstonia eutropha* JMP134, is cycloisomerized to give the dead end product 2-chloro-4-methylmuconolactone. Thus, in case of
chloromethylsubstituted aromatics, the cycloisomerization direction determines if a substrate can be used as a growth substrate.

![Diagram of degradation of 4-methylcatechol](image_url)

**FIG. 9: Degradation of 4-methylcatechol.** Enzymes involved are: catechol 1,2-dioxygenase (DO), muconate- or chloromuconate cycloisomerase (CI); 4-methylmuconolactone methylisomerase (MLMI) is harbored by *Ralstonia eutropha* JMP134, enabling the strain to mineralize 4-methylcatechol; in other strains 4-methylmuconolactone is a dead end metabolite (13, 92).

### 1.6.3 Toxicity of solvents

Organic solvents with logP\textsubscript{O/W} values (the partition coefficient of the compound in a mixture of octanol/water) between 1 and 5 are considered to be highly toxic to bacterial cells (56). The main target for their toxicity is the cell membrane. The incorporation of the hydrophobic solvents in the bilayer destabilizes the membrane, causing a leakage of proteins, lipids, and ions and disruption of the membrane potential, leading to cell death (56, 115, 116). Thus, bacteria are usually very sensitive to organic solvents (32, 105).

However, several *Pseudomonas* strains capable of tolerating solvents in high concentrations have been isolated from the environment in the recent years (21, 54, 55, 66, 128). Various mechanisms have been identified that contribute to this solvent tolerance. An important adaptive reaction is a change in the fatty acid composition of membrane lipids, including the isomerization of \textit{cis} to \textit{trans} unsaturated acids as rapid response, the increase of the degree of saturation of membrane lipids (Fig 10 A), an increased phospholipid biosynthesis and the increase of the protein:lipid ratio as longterm responses, resulting in decreased membrane fluidity (45, 49-51, 57, 94, 102). Additionally, the strains *Pseudomonas putida* S12 and DOT-T1 possess energy dependent efflux pumps that catalyze the active efflux of solvents, thus enabling...
the strains to grow in the presence of high concentrations of solvents (Fig. 10 B) (62, 63, 78, 100, 107).

1.7 The strain *Ralstonia* sp. PS12

The strain *Ralstonia* PS12 (formerly *Pseudomonas, Burkholderia*) was isolated in 1991 by P. Sander from the industrial waste deposit-site Hamburg-Georgswerder. It is described to grow on several chlorinated benzenes and toluenes such as all three dichlorobenzenes, 1,2,4-trichlorobenzene, 1,2,4,5-tetrachlorobenzene, 4-chlorotoluene, and several dichlorotoluenes (6, 109). Thus, PS12 is one of the few strains known to utilize tetrachlorobenzene and chlorinated toluenes, but no degradation pathways for chlorotoluenes were given so far. The broad spectrum initial tetrachlorobenzene dioxygenase (TecA) was found to catalyze dioxygenation as well as monooxygenation of the methyl group of 2- and 3-chlorotoluene enabling the strain to transform these substrates efficiently, although it is unable to mineralize them (75).
1.8 The strain *Pseudomonas putida* DOT-T1E

The toluene-degrading strain *Pseudomonas putida* DOT-T1 was isolated in 1995 from a wastewater treatment plant in Granada (101). Due to its high tolerance to organic solvents, and its capability to grow in the presence of high concentrations of solvents with a logP value higher than 2.3, the strain is considered as an interesting candidate for environmental applications (54, 101). Recently, the mechanisms responsible for the solvent tolerance of this strain have been extensively studied. So far, three efflux pumps have been identified being responsible for solvent tolerance (78, 100, 107). Two of them, both possessing a broad substrate spectrum including 1,2,4-trichlorobenzene, are constitutively expressed, whereas one pump is inducible by aromatic compounds. In addition to the active removal of the solvents from the cells, changes of membrane composition as short- and longterm-responses contribute to solvent tolerance (57, 102), whereas toluene metabolism is not involved significantly (79). Nevertheless, the genes encoding the inducible pump of the strain DOT-T1 are linked to the genes for toluene metabolism (78), which show high similarity to the genes of the *tod*-pathway (79).

1.9 Engineering of bacteria for environmental applications

Since organohalogenes occur naturally only in low concentrations (3) while industry has released synthetic chloroaromatics in environment in high amounts during the last decades (23, 88), microorganisms were not able to evolve appropriate metabolic pathways for these compounds in this short time period. For some xenobiotics no degradative pathways have been described, others are transformed incompletely or inefficiently or the mixture of xenobiotics at the contaminated site prevents degradation (2, 69). Other problems microorganisms have to deal with are the low bioavailability of compounds with low solubility in water and the toxicity of the contaminants at high concentrations (32). Different genetic engineering strategies have been used to improve the biodegradation.

1.9.1 Genetic engineering by patchwork assembly

The combination of pathway segments in a suitable host by conjugative transfer has been proven as a powerful means of accelerating the evolution of new biological activities by generating new functioning hybrid pathways (103, 104, 108). For example, Brinkmann et al. constructed a strain degrading 3-, 4-chlorotoluene and 3,5-dichlorotoluene by transferring the TOL plasmid from...
Pseudomonas putida strain PaW1 to the strain Pseudomonas putida B13 carrying the chromosomal encoded enzymes of the chlorocatechol pathway (11). By combining enzymes from five different catabolic pathways into a functional ortho-cleavage route for the degradation of methylphenols and methylbenzoate a transconjugant was obtained, which was able to simultaneously mineralize mixtures of chloro- and methylnaphthalenes that were toxic for bacteria (108) as well as to protect the indigenous microbial community from shock loads of phenol mixtures (27).

1.9.2 Protein engineering

Bottlenecks in the degradative pathways can be removed by altering activities of existing enzymes using site-directed mutagenesis, thus extending substrate spectra and avoiding dead end products. Resolved crystal structures may aid a rational design of active sites and substrate pockets by computer-assisted modeling of the three-dimensional structure of the enzyme (19, 43). For example, Parales et al. changed the product range of naphthalene dioxygenase by substitution of the residue Phe352 (85, 87). Substitutions of Val for Asn260 in 2NTDO resulted in an enzyme that no longer oxidized the aromatic ring but formed the monooxygenation product 2-nitrobenzyl alcohol (85). Beil et al. (7) identified the residue Ala220 to be responsible for dechlorination of 1,2,4,5-tetrachlorobenzene. Substitution of Met220 by Ala in toluene dioxygenase resulted in a gain of dehalogenation activity. However, this approach is only applicable to protein families in which the three-dimensional structure of at least one member protein has been resolved (43).

1.9.3 DNA shuffling and random mutagenesis

In vitro evolution of enzymes using DNA shuffling involves the assembly of DNA segments into a full-length gene by homologous, or site-specific, recombination (20, 43, 83, 118, 119). Before the assembly, the segments are often subjected to intensive mutagenesis, e.g. random mutagenesis. When DNA shuffling is done between a set of related genes utilizing naturally occurring mutations, the method is called family shuffling (20, 43). These techniques are useful techniques to generate diversity by recombination, thus combining useful mutations from individual genes (20) and have been shown to be powerful tools for protein engineering in various cases, e.g. improving of catechol 2,3-dioxygenase by family shuffling (64, 65, 83) and extending the substrate specificity of biphenyl dioxygenase (14).
1.10 Aim of the thesis

Chlorobenzenes and chlorotoluenes are important environmental pollutants since these toxic synthetic chemicals persist in nature. Whereas a couple of strains capable of mineralizing toluene and chlorobenzenes have been isolated and pathways have been studied in detail, only poor information is available about the degradation of aromatic compounds with both methyl- and chlorosubstituents. However, some strains are described to utilize higher chlorinated toluenes, among these the strain Ralstonia PS12, which is described to grow on 4-chlorotoluene and several dichlorotoluenes, but no pathways were given so far. The channeling of dichloromethylecatechols as the putative products of a dioxygenolytic attack on dichlorotoluenes catalyzed by the TecA tetrachlorobenzene dioxygenase of the strain PS12 and subsequent dehydrogenation into an ortho-cleavage pathway might be problematic due to the formation of methylmuconolactones as dead end metabolites as described for the cycloisomerization of 2-chloro-4-methylmuconate. On the other hand, monooxygenation, catalyzed by TecA as well, might channel chlorotoluenes into a dead end pathway as described for the transformation of 2-chlorotoluene.

Another problem microorganisms have to deal with is the toxicity of the contaminants. As the concentrations of xenobiotics are often high at contaminated sites, the isolation and the development of organisms tolerant to high concentrations of these toxic compounds are of special interest for bioremediation.

This thesis aims to characterize the pathways of (di)chlorotoluene degradation, to identify bottlenecks in the pathways and to possibly remove these bottlenecks by enzyme engineering. Another task related to metabolic bottlenecks was the construction of a solvent tolerant strain capable of mineralizing high concentrations of chlorobenzene to obtain a strain suitable for the bioremediation of heavily contaminated sites.

In chapter II the initial reactions involved in the transformation of various di- and trichlorinated toluenes and catalyzed by the tetrachlorobenzene dioxygenase (TecA) and the chlorobenzene dihydrodiol dioxygenase (TecB) of strain Ralstonia sp. PS12 are investigated. Only those compounds which are predominantly dioxygenated by TecA (2,4-, 2,5- and 3,4-dichlorotoluene) served as growth substrate, whereas those compounds, which are predominantly or exclusively subject to monooxygenolytic attack (2,3-, 2,6-, 3,5 di- and 2,4,5-trichlorotoluene) were no growth substrates, suggesting, that monooxygenation channels the substrates into a dead end pathway.
In chapter III the pathways of 2,4-, 2,5- and 3,4-dichlorotoluene degradation, used as growth substrates by the strain PS12, were investigated, bottlenecks in degradation pathways identified and enzymes of the pathways characterized. The intermediate dichloromethylcatechols are further degraded via a modified ortho-cleavage pathway producing the respective substituted muconates, muconolactones and dienelactones as intermediates, and the complex metabolic fate could be followed by in situ $^1$H-NMR. Beside 2,4-, and 2,5-dichlorobenzoate, resulting from the monooxygenolytic pathway, no significant amounts of dead end metabolites were formed, suggesting that inefficient degradation is preferentially caused by the initial monooxygenation.

One strategy to remove bottlenecks in metabolic pathways is the rational design of enzymes based on their crystal structure. In chapter IV, site-directed mutagenesis based on the crystal structure of naphthalene was performed with TecA in order to change the direction of attack, with the primary goal of preventing the monooxygenation of the methyl group. An amino acid critically determining enzyme activity and a residue determining regioselectivity of attack by the enzyme were identified. Based on the structural information given for naphthalene dioxygenase and the biochemical and genetic information given for TecA, the catalytic mechanism of TecA could be modulated.

Mineralization of chlorinated toluenes via a dioxygenolytic pathway by avoiding monooxygenation of the methylgroup as suggested in chapter IV, necessitates the degradability of the respective chloromethylcatechols as central intermediates. In chapter V, the metabolic fate of 4-chloro-3-methyl/3-chloro-4-methylcatechol (from 2-chlorotoluene) and of 5-chloro-3-methyl/3-chloro-5-methylcatechol (from 3-chlorocatechol) was investigated. Degradation of both chloromethylcatechols formed from 2-chlorotoluene was found to be problematic, due to the inability of the chloromuconate cycloisomerase to eliminate the chloride of the two cycloisomerisation products. In contrast, 5-chloro-3-methylcatechol, the major dioxygenation product formed from 3-chlorotoluene, is subject to dehalogenation after transformation by chlorocatechol 1,2-dioxygenase and chloromuconate cycloisomerase resulting in the formation of 2-methyldienelactone, which serves as substrate for the subsequent dienelactone hydrolase.

Chlorobenzenes, which are often present in high concentrations at contaminated sites, are highly toxic to microorganisms. In chapter VI, the construction of a chlorobenzene degrading strain that is tolerant to high concentrations of chlorobenzene is described. Genes encoding enzymes of the chlorocatechol pathway were introduced into the solvent tolerant strain DOT-T1E, thus combining them with genes of the tod-pathway, resulting in a chlorobenzene degrading strain exhibiting new yet undescribed features.
1.11 References


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Chapter II


Transformation of Chlorinated Benzenes and Toluences by TecA Tetrachlorobenzene Dioxygenase and TecB Chlorobenzene Dihydrodiol Dehydrogenase of Ralstonia sp. Strain PS12

Katrin Pollmann, Stefan Beil§, Dietmar H. Pieper*

Division of Microbiology, German Research Centre for Biotechnology (GBF), D-38124 Braunschweig

§ Present address: IMH Industrie Management Holding GmbH, D-30159 Hannover

* Corresponding author. Mailing address: Bereich Mikrobiologie, AG Biodegradation, Gesellschaft für Biotechnologische Forschung mbH, Mascheroder Weg 1, D-38124 Braunschweig, Germany. Phone: 49/(0)531/6181-467. E-mail: dpi@gbf.de
2.1 Abstract

The *tecB* gene located downstream of *tecA* encoding tetrachlorobenzene dioxygenase in *Ralstonia* sp. strain PS12 was cloned into *E. coli* DH5a together with the *tecA* genes. The identity of the *tecB* gene product with a chlorobenzene dihydrodiol dehydrogenase was verified by transformation into the respective catechols of chlorobenzene, the three isomeric dichlorobenzenes as well as 1,2,3- and 1,2,4-trichlorobenzene, all of which are transformed by TecA into the respective dihydrodihydroxy derivatives. Di- and trichlorotoluenes were either subject to TecA mediated dioxygenation (the major or sole reaction observed for the 1,2,4-substituted 2,4-, 2,5- and 3,4-dichlorotoluene) resulting in the formation of the dihydrodihydroxy derivatives, or to monooxygenation of the methylsubstituent (the major or sole reaction observed for 2,3-, 2,6- and 3,5-dichloro- and 2,4,5-trichlorotoluene), resulting in formation of the respective benzylalcohol. All chlorotoluenes subject to dioxygenation by TecA were transformed, without intermediate accumulation of dihydrodihydroxy derivatives, into the respective catechols by TecAB, indicating dehydrogenation to be no bottleneck for chlorobenzene/chlorotoluene degradation. However, only those chlorotoluenes subject to a predominant dioxygenation were growth substrates for PS12, confirming monooxygenation to be an unproductive pathway in PS12.

2.2 Introduction

Chlorotoluenes are important intermediates in many chemical processes and are still produced in high amounts (9, 28). Dichlorotoluenes, known as moderately toxic chemicals (2, 3, 10), are used as precursors for the production of pesticides, dyes and peroxides (9). Their high chemical stability causes their accumulation in environment (10). Thus far, only a few studies reported on the degradation of chlorotoluenes. Whereas organisms capable of mineralising 3- or 4-chlorotoluene have been described in detail (8, 11), degradation routes for dichlorotoluenes have not been analysed yet. Vandenbergh et al. described a Pseudomonad able to utilize several chloraromatics including 2,4-dichlorotoluene and 3,4-dichlorotoluene, but no further indications of the metabolic pathway were given (33). *Ralstonia* sp. PS12 (formerly *Pseudomonas, Burkholderia*), capable of mineralizing various chlorobenzenes, i.a. 1,2,4,5-tetrachlorobenzene was also reported to mineralize various dichlorotoluenes, again without indication of a metabolic sequence (5, 27).
Two distinct metabolic routes were reported for the mineralization of 4-chlorotoluene. Brinkmann et al. (8) constructed bacterial strains mineralizing 4-chlorotoluene (as well as 3-chloro- and 3,5-dichlorotoluene) via the corresponding chlorinated benzoates and catechols by combining the TOL plasmid (12) with genes encoding enzymes of the chlorocatechol pathway (25). However, 2-chlorosubstituted toluenes were no substrates for the TOL plasmid encoded xylene monooxygenase, thus they cannot be degraded by such a catabolic route. In *Pseudomonas* sp. JS6, 4-chlorotoluene is subject to dioxygenation in this strain, and 3-chloro-6-methylcatechol is formed after dehydrogenation of the intermediate dihydrodiol (11). Enzymes of the chlorocatechol degradation pathway are responsible for further metabolism of this compound. *Ralstonia* sp. PS12 (27), was shown to be capable of growing with several chlorinated benzenes and, similar to JS6, to initialize the degradation by dioxygenolytic activation (5). The broad substrate spectrum tetrachlorobenzene dioxygenase TecA of PS12, catalysing this initial step, has been extensively described (5, 6). The most prominent feature of this enzyme system is its capability to transform 1,2,4,5-tetrachlorobenzene and thereby to attack a chlorosubstituted carbon atom. Such an attack results in the formation of an unstable diol intermediate which spontaneously rearomatize with concomitant chloride elimination. Like the lower substituted chlorobenzenes, 4-chlorotoluene, which is used as a growth substrate by PS12, is subject to dioxygenation at two unsubstituted carbon atoms. In contrast, 2- and 3-chlorotoluene were subject to monooxygenation of the methylsubstituents with 2- and 3-chlorobenzylalcohol as the main products (18). Apparently, TecA can catalyze dioxygenating, dechlorinating and monooxygenating reactions. In the present report, we analyzed which of these three types of reaction was performed with higher chlorosubstituted toluenes and which of those substrates by PS12 derived activities can be transformed into chlorocatechols as central intermediates of chloroaromatic degradation.
2.3 Materials and methods

Organisms and culture conditions. *E. coli* DH5α pSTE7 containing the *tecA* tetrachlorobenzene dioxygenase genes (5), and *E. coli* DH5α pSTE44 containing the tetrachlorobenzene dioxygenase and dehydrogenase genes *tecAB* were grown at 37°C in Luria broth medium containing 0.1 mg/ml ampicillin and 1 mM IPTG.

Cloning of the *tecAB* genes. First the 3’-end of the *tecB* gene was PCR amplified from template plasmid pCR12, containing the region located downstream of the *tecA* gene (7), with the primers prSTB86 (5′-ctgctctacaccgcgggc-3′) and prSTB68 (5′-gctttgagagctcatgttgtc-3′), the latter containing an artificial SacI site (indicated in bold). For sequence confirmation the resulting 0.4-kb fragment was cloned into plasmid pCR2.1 (Invitrogen) resulting in pCR17. A 0.3-kb BamHI-SacI fragment of pCR17 was then cloned into the BamHI-SacI site of pSTE7 encoding TecA resulting in pSTE44 carrying the *tecA* and *tecB* genes.

Sequence analysis. Plasmid DNA for sequencing was extracted with the Plasmid Maxi kit (Qiagen). Sequencing reactions on both strands were performed with the Applied Biosystems 373A DNA sequencer according to the protocols of the manufacturer (Perkin-Elmer, Applied Biosystems) for Taq cycle-sequencing with fluorescent-dye labeled dideoxynucleotides, as described previously (14). The GeneWorks software package V2.45 (IntelliGenetics) was used for sequence evaluation. Similarity searches in non-redundant databases were made with the FASTA program. Sequence comparisons and calculations of evolutionary distances were carried out using the PILEUP, DISTANCE and UPGMA programs of the GCG software (Wisconsin Package, Version 8, Genetics Computer Group, Madison, WI, USA).

Resting cell assays. Resting cell assays were performed as described by Beil et al. (5). For kinetic experiments, at each time point 400 µl aliquots were removed and shock frozen in liquid nitrogen. The samples were stored at -20 °C for subsequent analyses.

Extraction of and derivatization of metabolites. Metabolites were extracted as described previously (5). For subsequent GC-MS analysis, dihydrodiol and catechol intermediates were derivatized with butylburonic, whereas benzyl alcohols were derivatized with Me3SOH (5).

Analytical methods. For HPLC-analyses of metabolites, 10 µl of the samples were injected after removing cells by centrifugation (20°C, 10 min, 13000 rpm). Product formation was analyzed with a Shimadzu HPLC system (LC-10AD liquid chromatograph, DGU-3A degasser, SPD-
M10A diode array detector and FCV-10AL solvent mixer) equipped with a SC125/Lichrospher 5-µm (Bischoff, Leonberg, Germany) column. The aqueous solvent system (flow rate 1 ml/min) contained 0.1% (v/v) of H₃PO₄ (87%) and 50% or 58% (v/v) of methanol for the determination of metabolites or 80% (v/v) of methanol for the determination of substrates. The alcohol intermediates were identified and quantified by comparison with authentic standards.

GC-MS-analyses were performed as previously described (5).

For NMR-analyses, the extracted metabolites (about 2 mg) were dissolved in 1 ml D₆-acetone and, in case, spiked with an appropriate aromatic standard. ¹H and ¹³C nuclear magnetic resonance spectra were recorded on a Bruker CXP 300 (Bruker, Rheinstetten Germany) with Aspect 2000 software using tetramethylsilane as internal standard.

**Chemicals.** 3,5-dichlorotoluene and 2,4,5-trichlorotoluene were synthesized and kindly provided by W. Reineke. BuB(OH)₂ was obtained from Acros organics (Geel, Belgium), trimethylsulfonium hydroxide from Machery-Nagel (Düren, Germany), 2,3-dichlorobenzyl alcohol from TCI (Japan) and 3-chloro-, 4-chloro-, 3,4-dichloro- and 3,4,5-trichlorocatechol from Helix Biotech. 3,5-, 3,6- and 4,5-dichlorocatechol were kindly provided by H.-A. Arfmann. All other chemicals were purchased from Aldrich Chemie (Steinheim, Germany), Fluka AG (Buchs, Switzerland), or Merck AG (Darmstadt, Germany).

**Nucleotide sequence accession number.** The nucleotide sequence data of the TecB cis-chlorobenzene dihydrodiol dehydrogenase have been submitted to the GenBank sequence data bank and are available under accession number U78099.
2.4 Results

The *tecB* gene encodes a chlorobenzene dihydrodiol dehydrogenase. The deduced amino acid sequence of the 0.83-kb ORF downstream of the *tecA* genes and designated *tecB* shows the highest similarity (>99%) to the TcbB *cis*-chlorobenzene dihydrodiol dehydrogenase from *Pseudomonas* sp. P51 (34) and shows the typical features of the short-chain alcohol dehydrogenase family (23) including the short-chain alcohol dehydrogenase consensus pattern (23) and the binding site for the ADP moiety of the NAD<sup>+</sup> coenzyme (4, 30). To verify that *tecB* encodes a chlorobenzene dihydrodiol dehydrogenase, the TecA dioxygenase and the *tecB* gene product were simultaneously produced in *E. coli* (pSTE44) cells, and the transformation of various chlorinated benzenes was analysed by high-performance liquid chromatography (HPLC). All chlorobenzenes analyzed and shown to be transformed by TecA into the respective dihydrodiols were converted by TecA + TecB into the corresponding aromatic dihydroxy compounds (Table 1). The identity of the products was confirmed by comparison with authentic standards. A single catechol product was observed in each case, except for chlorobenzene transformation, where 3-chlorocatechol was the major product, but 4-chlorocatechol was formed in minor amounts (Table 1). This indicates that TecB is the second enzyme in the degradative pathway of (chloro)benzenes and encodes a chlorobenzene dihydrodiol dehydrogenase. Accumulation of dihydrodiols was not detected, except for minute amounts in the case of 1,4-dichlorobenzene and 1,2,4-trichlorobenzene transformations. Thus, TecB dehydrogenase constitutes no pathway bottleneck for the transformation of products from TecA catalysis into the corresponding aromatic intermediates.

Transformation of di- and trichlorotoluenes by TecA and TecB. The potential of TecA chlorobenzene dioxygenase to transform 2,3-; 2,4-; 2,5-; 2,6-; 3,4- and 3,5-di- as well as 2,4,5-trichlorotoluene (23DCT, 24DCT, 25DCT, 26DCT, 3,4DCT, 35DCT, 2,4,5TCT) was analysed initially by high-performance liquid chromatography (HPLC) using supernatant fluid of resting *E. coli* cells carrying plasmid pSTE7 incubated with the respective substrates. HPLC analysis revealed the formation of products from all substrates tested (Table 1). Formation of the respective benzyl alcohol from dichlorotoluenes could be directly verified due to comparison of retention volumes and UV absorption spectra with authentic standards.
The corresponding benzyl alcohol was the only product formed from 23DCT and 35DCT, the apparent major product formed from 2,6DCT, the apparent minor product from 24DCT and 25DCT and obviously not produced from 34DCT.

The ultraviolet spectra of the other products formed during dichlorotoluene turnover were indicative for the formation of dihydrodiols ($\lambda_{\text{max}} = 275-280$ nm). The only product formed during 245TCT transformation did not exhibit such an UV spectrum.

Confirmation of the identity of the intermediates formed from dichlorotoluenes as the corresponding dihydrodiols was obtained by GC-MS analysis of the boronated derivatives. Whereas no such products were detected from 23DCT and 35DCT, prominent signals showing the expected molecular ion of $m/z$ 260, 262, and 264 (rel. intensities 100:62:9) were observed from 34DCT, 24DCT, 25DCT and 26DCT. The products showed the fragmentation pattern typical for $n$-butylboronated chlorobenzene or methylbenzene dihydrodiols (5, 16), i.e. loss of $\text{C}_4\text{H}_9 [\text{M-57}]^+$, O-B-$\text{C}_4\text{H}_9 [\text{M-84}]^+$ as well as loss of one or two chlorine atoms [M-35]$^+$ (Fig. 1).

$^1$H- as well as $^{13}$C-NMR-data of the product formed after transformation of 245DCT indicated the formation of 2,4,5-trichlorobenzylalcohol ($^1$H NMR: $\delta = 7.76$ ppm (bs, H-6), 7.62 ppm (bs, H-3), 4.68 ppm (bs, 2 H, CH$_2$); $^{13}$C NMR: $\delta = 141.6$ ppm (s, C-1), 131.6 ppm (s, C-4), 131.4 ppm (2*s, C-2/C-5), 131.1 ppm, 129.9 ppm (2*d, C-3/C-6), 61.0 ppm (t, C, CH$_2$)) due to a monooxygenolytic attack of TecA. GC/MS-analysis of the methylated product confirms the identity of the product with 2,4,5-trichlorobenzylalcohol (Fig. 1). Molecular ion signals at $m/z$ 224, 226, 228, and 230 (rel. intensities 100:98:31:4) showed the expected mass peak of the benzyl alcohol with its three chlorines. Intense signals at $m/z$ 193 or 189 resulted from a loss of O-CH$_3$ [M-31]$^+$ or a chlorine atom [M-35]$^+$. Analysis of the boronated extract gave no indication for the formation of dihydroxylated products. Thus attack on 2,4,5-trichlorotoluene is clearly different from that on 1,2,4,5-tetrachlorobenzene, where dioxygenolytic attack on an unsubstituted and a chlorosubstituted carbon atom leads to an unstable intermediate, which spontaneously rearranges to form 3,5,6-trichlorocatechol.

The HPLC analyses of TecA plus TecB mediated transformation of dichlorotoluenes showed, as expected, no difference in the amounts of benzyl alcohols formed, when compared to TecA mediated transformation. However, in no case, accumulation of dihydrodiols was detected, but new products were observed to be formed from 34DCT (only product), 24DCT and 25DCT (major product) and 26DCT (minor product). GC-MS analysis of the boronated derivatives...
confirmed the hypothesis that those products are identical with dichlorosubstituted methylcatechols (Fig. 1). The recorded intensities of molecular ion signals at $m/z$ 258, 260, and 262 were always in close agreement with the theoretical pattern for dichlorosubstituted catechols. Intense signals at $m/z$ 202, 204, and 206 result from the loss of butene $[\text{M-C}_4\text{H}_8]^+$ as described for the butyl boronates of 3-chlorocatechol, 3,6-dichlorocatechol (16) and 3,4,6-trichlorocatechol (5). To our knowledge, this is the first report about MS spectra of boronated dichloromethyl dihydrodiols, dichlorosubstituted methylcatechols and methylated 2,4,5-trichlorobenzylalcohol.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Proposed product</th>
<th>[MeOH]</th>
<th>RV</th>
<th>( ?_{\text{max}} )</th>
<th>Growth substrate</th>
</tr>
</thead>
</table>
| Chlorobenzene                 | \(^a\) 3-chloro-
 cis-1,2-dihydroxy-1,2-dihydrocyclohexa-3,5-diene | 36     | 3.4 | 272           | +               |
|                               | \(^a\) 4-chloro-
 1,2-dihydroxy-1,2-dihydrocyclohexa-3,5-diene     | 36     | 4.4 | 265           |                 |
|                               | \(^b\) 3-chloro-
 cis-1,2-dihydroxybenzene                           | 58     | 2.6 | 210           |                 |
|                               | \(^b\) 4-chloro-1,2-dihydroxybenzene                  | 58     | 2.9 | 210           |                 |
| 1,2-Dichlorobenzene           | \(^a\) 3,4-dichloro-
 cis-1,2-dihydroxy-1,2-dihydrocyclohexa-3,5-diene   | 36     | 6.8 | 272           | +               |
|                               | \(^b\) 3,4-dichloro-1,2-dihydroxybenzene              | 58     | 4.2 | 210           |                 |
| 1,3-Dichlorobenzene           | \(^a\) n.d.                                          | 58     | 5.3 | 210           | +               |
| 1,4-Dichlorobenzene           | \(^a\) 3,6-dichloro-
 cis-1,2-dihydroxy-1,2-dihydrocyclohexa-3,5-diene   | 36     | 4.1 | 283           | +               |
|                               | \(^b\) 3,6-dichloro-1,2-dihydroxybenzene              | 58     | 4.0 | 210           |                 |
| 1,2,3-Trichlorobenzene        | \(^a\) 3,4,5-trichloro-
 cis-1,2-dihydroxy-1,2-dihydrocyclohexa-3,5-diene   | 72     | 2.3 | 282           | -               |
|                               | \(^b\) 3,4,5-trichloro-1,2-dihydroxybenzene           | 58     | 8.6 | 210           |                 |
| 1,2,4-Trichlorobenzene        | \(^a\) 3,4,6-trichloro-
 cis-1,2-dihydroxy-1,2-dihydrocyclohexa-3,5-diene   | 58     | 3.4 | 288           | +               |
|                               | \(^b\) 3,4,6-trichloro-1,2-dihydroxybenzene           | 58     | 8.3 | 210           |                 |
| 1,2,4,5-Tetrachlorobenzene    | \(^a\) 3,4,6-trichloro-
 1,2-dihydroxybenzene                                  | 58     | 8.3 | 210           | +               |
| 2,3-Dichlorotoluene           | \(^a\) 2,3-dichlorobenzylalcohol                      | 60     | 5.4 | 201           | -               |
|                               | \(^b\) 2,3-dichlorobenzylalcohol                      | 60     | 6.2 | 201           | +               |
| 2,4-Dichlorotoluene           | \(^a\) 2,4-dichlorobenzylalcohol                      | 60     | 6.2 | 201           | +               |
|                               | \(^a\) 4,6-dichloro-
 3-methyl-cis-1,2-dihydrox-1,2-dihydrocyclohexa-3,5-diene | 60     | 3.7 | 278           |                 |
|                               | \(^b\) 4,6-dichloro-3-methyl-cis-1,2-dihydroxybenzene | 60     | 7.5 | 205           |                 |
| 2,5-Dichlorotoluene           | \(^a\) 2,5-dichlorobenzylalcohol                      | 60     | 6.1 | 201           | +               |
|                               | \(^a\) 3,6-dichloro-4-methyl-cis-1,2-dihydrox-1,2-
 dihydrocyclohexa-3,5-diene                           | 60     | 2.9 | 280           |                 |
|                               | \(^b\) 3,6-dichloro-4-methyl-cis-1,2-dihydroxybenzene | 60     | 6.0 | 204           |                 |
| 2,6-Dichlorotoluene           | \(^a\) 2,6-dichlorobenzylalcohol                      | 60     | 3.9 | 203           | -               |
|                               | \(^a\) 3,5-dichloro-4-methyl-cis-1,2-dihydrox-1,2-
 dihydrocyclohexa-3,5-diene                           | 60     | 3.7 | 275           |                 |
|                               | \(^b\) 3,5-dichloro-4-methyl-cis-1,2-dihydroxybenzene | 60     | 6.3 | 203           |                 |
| 3,4-Dichlorotoluene           | \(^a\) 3,5-dichloro-6-methyl-cis-1,2-dihydrox-1,2-
 dihydrocyclohexa-3,5-diene                           | 60     | 3.5 | 276           | +               |
|                               | \(^b\) 3,4-dichloro-6-methyl-cis-1,2-dihydroxybenzene | 60     | 6.6 | 204           |                 |
| 3,5-Dichlorotoluene           | \(^a\) 3,5-dichlorobenzylalcohol                      | 60     | 7.4 | 203           | -               |
|                               | \(^b\) 3,4,5-dichlorobenzylalcohol                    | 60     | 12.1| 205          | -               |

The proposed products derived from transformation of the indicated substrates with \(^a\) E. coli (pSTE7) producing TecA dioxygenase or \(^b\) E. coli (pSTE44) producing simultaneously TecA tetrachlorobenzene dioxygenase and TecB dehydrogenase are shown. [MeOH], percentage of methanol used in the solvent system; RV, retention volume in ml; \( ?_{\text{max}} \), wavelength of the maximal absorption in nm. +/−, used /not used as growth substrate (27).
FIG. 1. Mass spectra of the boronated (A-H) or methylated (I) products formed by *E. coli* (pSTE44) (A-D) and *E. coli* (pSTE7) (E-I) from di- and trichlorotoluenes.

A 4,6-dichloro-3-methylcatechol; B 3,6-dichloro-4-methylcatechol; C 3,5-dichloro-4-methyl-catechol; D 3,4-dichloro-6-methyl catechol; E 4,6-dichloro-3-methyl-1,2-dihydrox-1,2-dihydrocyclohexa-3,5-diene; F 3,6-dichloro-4-methyl-1,2-dihydrox-1,2-dihydrocyclohexa-3,5-diene; G 3,5-dichloro-4-methyl-1,2-dihydrox-1,2-dihydrocyclohexa-3,5-diene; H 3,5-dichloro-6-methyl-1,2-dihydrox-1,2-dihydrocyclohexa-3,5-diene; I 2,4,5-trichlorobenzyl-alcohol.
Quantification of product ratios and product formation rates for TecA and TecB catalyzed turnover of di- and trichlorotoluenes. Dichlorobenzyl alcohols were available commercially and their production rate could thus be quantified. No standards were available for 2,4,5-trichlorobenzyl alcohol, dihydrodiols or dichloromethylcatechols, such that neither product formation rates nor the ratio in which dioxygenation versus monooxygenation occurred could be quantified. However, as the absorption at \(\lambda=210\) nm of all dichlorosubstituted catechols varied only by a factor of 0.05, the absorption of dichloromethylcatechols can be assumed to be similar. Under this assumption, the dichloromethylcatechols can be proposed to constitute 5 +/- 0.25% of the products formed during transformation of 26DCT, 80 +/- 4% during transformation of 25DCT, and 90 +/- 4.5% during transformation of 24DCT (Fig. 2).

To verify above assumption, the ratio of monooxygenation versus dioxygenation was quantified by \(^1\)H-NMR analysis in case of TecA + TecB catalyzed 25DCT transformation.
Analysis of the product mixture showed the expected signals for 2,5-dichlorobenzylalcohol ($^1$H NMR: $\delta = 7.63$ ppm (dt, H-6, $J_{6,6} = 2.6$ Hz, $J_{6,7} = 1.3$ Hz), 7.39 ppm (d, H-3, $J_{3,4} = 8.6$ Hz), 7.31 ppm (ddt, H-4, $J_{4,7} = 0.6$ Hz), 4.70 ppm (bs, H-7)) as verified by a standard and 3,6-dichloro-4-methylcatechol ($^1$H NMR: $\delta = 8.31$ ppm, 8.40 ppm (3-OH, 4-OH), 6.84 ppm (bs, H-6), 2.25 ppm (d, H-7, $J_{7,7} = 0.7$ Hz)). Comparison of the integrals of the signals of the respective H-6 protons showed that these products were present in the mixture in a 1:4 ratio. This confirms the previous result of a 80 % formation of 3,6-dichloro-4-methylcatechol during transformation of 25DCT. For quantification of 2,4,5-trichlorobenzylalcohol, 245TCT was transformed by TecAB. The product prepared for $^1$H-NMR analysis was spiked with a defined concentration of 2,3-dichlorobenzaldehyde as an internal standard, thus the concentration of 2,4,5-trichlorobenzylalcohol in the mixture could be determined by $^1$H-NMR analysis. This sample served as a HPLC standard.

The transformation rates, expressed as the amount of product formed per time unit, were in the same order of magnitude compared to that of 1,2,4,5-tetrachlorobenzene (5) (Table 2). The product formation rates of dichlorotoluenes which were mainly subject to dioxygenation were significantly higher than those of dichlorotoluenes which were subject to monooxygenation (Table 2). The transformation rate of 2,4,5-trichlorotoluene was about threefold the rate observed with 1,2,4,5-tetrachlorobenzene and 2-3fold the rate observed with dichlorotoluenes. Comparisons of the product formation rates as catalyzed by E. coli (pSTE7) and E. coli (pSTE44) showed, that the rates of E. coli (pSTE7) expressing TecA were twofold higher than the rates of E. coli (pSTE44) expressing TecAB (Table 2).
TABLE 2. Absolute and relative rates of transformation of chlorinated toluenes and tetrachlorobenzene catalyzed by the strains E. coli DH5α pSTE7 and pSTE44 expressed as the formation of product per minute.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pSTE7 (µM/min)</th>
<th>Rel. Transformation rate (%)</th>
<th>pSTE44 (µM/min)</th>
<th>Rel. transformation rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4,5-Trichlorotoluene</td>
<td>3.7</td>
<td>100</td>
<td>1.9</td>
<td>100</td>
</tr>
<tr>
<td>1,2,4,5-Tetrachlorobenzene a</td>
<td>1.0</td>
<td>27</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>2,3-Dichlorotoluene</td>
<td>1.3</td>
<td>35</td>
<td>0.6</td>
<td>32</td>
</tr>
<tr>
<td>2,4-Dichlorotoluene</td>
<td>n.d.</td>
<td>n.d.</td>
<td>1.0</td>
<td>53</td>
</tr>
<tr>
<td>2,5-Dichlorotoluene</td>
<td>n.d.</td>
<td>n.d.</td>
<td>1.0</td>
<td>53</td>
</tr>
<tr>
<td>2,6-Dichlorotoluene</td>
<td>1.3</td>
<td>35</td>
<td>0.7</td>
<td>37</td>
</tr>
<tr>
<td>3,4-Dichlorotoluene</td>
<td>n.d.</td>
<td>n.d.</td>
<td>1.3</td>
<td>68</td>
</tr>
<tr>
<td>3,5-Dichlorotoluene</td>
<td>1.4</td>
<td>38</td>
<td>0.6</td>
<td>32</td>
</tr>
</tbody>
</table>

a according to Beil et al. (5); all rates were determined at a cell density of A₆₀₀=1.8.

2.5 Discussion

Dioxygenation as carried out by multicomponent dioxygenases is the initial step in the degradation of various aromatic compounds and commonly results in the formation of the respective cis-dihydrodiols, which are further transformed into dihydroxyaromatics by the action of cis-dihydrodiol dehydrogenases (20, 26, 29). Among these dioxygenases, naphthalene dioxygenase (NDO) from Pseudomonas NCIB 9816-4 and toluene dioxygenase (TOD) from Pseudomonas putida F1 have been extensively studied (17, 31, 35, 36). These enzymes were reported to have relaxed substrate specificities and in addition to a stereospecific cis-dihydroxylation, they catalyse monooxygenation, desaturation, dealkylation and sulfoxidation reactions (21). The TecA tetrachlorobenzene dioxygenase of Ralstonia sp. PS12 as well carries out cis-dihydroxylations and has been shown to transform toluene and
various chlorinated benzenes in a dioxygenolytic manner (5). The enzyme differs from TOD dioxygenase of *Pseudomonas putida* F1 in its failure to attack benzene, but is superior in its capability to transform 1,2,4,5-tetrachlorobenzene. In addition to chlorobenzenes, TecA dioxygenase, like TOD dioxygenase, also transforms 4-chlorotoluene by a dioxygenolytic attack, whereas 2- and 3-chlorotoluene were subject mainly to monooxygenation of the methyl side chain (18). All dichlorotoluenes recently indicated to be growth substrates for *Ralstonia* sp. PS12, i.e. 24DCT, 25DCT, and 34DCT were mainly or exclusively subject to dioxygenation (Fig. 2). It was interesting to note that obviously all 1,2,4-substituted dichlorotoluenes are subject to mainly dioxygenation (like the 1,4-disubstituted 4-chlorotoluene), whereas the chlorinated toluenes substituted in 1,2-, 1,3-, 1,2,3- or 1,3,5-position were subject to mainly monooxygenation (Fig. 2). As already outlined by Sander et al. (27), 1,3,5-trichlorobenzene is not subject to dioxygenolytic attack by PS12, thus a dioxygenolytic attack on 35DCT could be restricted for similar yet unknown reasons. Surprisingly, also 245TCT was exclusively transformed into the corresponding benzyl alcohol, even though a dioxygenation followed by spontaneous chloride elimination, as observed for the structural analogue 1,2,4,5-tetrachlorobenzene (5, 27), was expected. As differences in electronic properties of the different chlorinated toluenes seem to be negligible for the specificity of the attack, it is more likely, that the reaction depends on the position of the substrate to the active and on the active site’s structure. It has been elucidated that even small differences in the amino acid sequences leads to major differences in the enzymatic properties, such as substrate range and regioselectivity (19). The 2-nitrotoluene-dioxygenase from *Pseudomonas* sp. str. JS42 (1) catalyses predominantly a dioxygenation of 2-nitrotoluene, whereas the closely related 2,4-dinitrotoluene dioxygenase from *Burkholderia* sp. DNT (32) catalyses monooxygenation. Toluene dioxygenase of *Pseudomonas putida* F1 catalyses, like dinitrotoluene dioxygenase, a monooxygenation of 2-nitrotoluene, whereas 4-nitrotoluene is subject to dioxygenation. Evidently, the regiospecificity depends in a complex fashion on both the substitution pattern and active site structure. Based on its crystal structure, several amino-acids were identified near the active site of naphthalene dioxygenase (15) and this information has been used to identify amino acids, that control regioselectivity and enantioselectivity of naphthalene dioxygenase. However, only poor information is available on amino acids governing the selectivity of benzyl alcohol versus *cis*-dihydrodiol formation (22).
All cis-dihydrodiols shown to be formed in the present study by TecA dioxygenase are obviously transformed at a high rate by the TecB cis-dihydrodiol dehydrogenase. TecB evidently does not constitute a bottleneck for chlorobenzene/chlorotoluene transformation. The tecB gene product, belonging to the family of short-chain-alcohol-dehydrogenases (23), is closely related to the TcbB cis-chlorobenzene dihydrodiol dehydrogenase from Pseudomonas sp. 51 (34), an enzyme recently described to be of broad substrate specificity (24). However, if those gene products, like the respective tcbA or tecA gene products, are specifically adapted towards the metabolism of chlorosubstituted derivatives still remains to be elucidated.

Dichlorotoluenes subject to monooxygenation of the methylfunction cannot be used by PS12 as growth substrates (27). This is similar to the observation that 2- and 3-chlorotoluene cannot be mineralized by this strain (18). The failure of growth was attributed to further oxidation of the benzyl alcohols produced into the corresponding benzoates at rates too low to support growth and, in the case of 2-chlorobenzoate, to the restricted substrate range of the probably chromosomally encoded benzoate dioxygenase. Like 2-chlorobenzylalcohol, 2,3-, 2,4-, 2,5- and 2,6-dichlorobenzylalcohol are transformed by PS12 into the corresponding benzoates, which cannot be mineralized by PS12 due to, at least, the absence of a broad spectrum 2-halobenzoate dioxygenase (data not shown). Thus, monooxygenation in this strain results in channeling of all (in case of 23DCT), the major part (26DCT) or a minor part of the substrate into an unproductive pathway, explaining the failure of the strain to grow with 23DCT and 26DCT. Similarly, no effective pathway is present for 35DCT degradation. Complementation of a degradative pathway for 2-chlorotoluene by recruitment of different pathway modules from various bacteria has been proposed recently (18) and respective experiments are currently been performed (13). This strategy can be adapted, at least theoretically, for the mineralization of dichlorotoluenes. Alternatively, the search for new chlorobenzene dioxygenases with new substrate specificities and regioselectivities of attack as well as protein engineering strategies will, in case, lead to the isolation of enzymes with capabilities more suited for dichlorotoluene mineralization.
FIG. 2. Proposed transformation of different di- and trichlorotoluenes by tetrachlorobenzene dioxygenase TecA and dehydrogenase TecB. Substrates: A 2,3-dichlorotoluene; B 2,4-dichlorotoluene; C 2,5-dichlorotoluene; D 2,6-dichlorotoluene; E 3,4-dichlorotoluene; F 3,5-dichlorotoluene; G 2,4,5-trichlorotoluene. → major reaction catalyzed by TecA; ← minor reaction catalyzed by TecA; ⇄ reaction catalyzed by TecB.
Chapter II  Transformation of Chlorinated Benzenes and Toluens ...

Acknowledgements

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2.6 References


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Metabolism of Dichloromethylcatechols as Central Intermediates in the Degradation of Dichlorotoluenes by

*Ralstonia eutropha* sp. Strain PS12

Katrin Pollmann¹, Stefan Kaschabek²*, Victor Wray³, Walter Reineke² and Dietmar H. Pieper¹*

Department of Environmental Microbiology¹, and Department of Structure Research³, GBF - German Research Center for Biotechnology, Mascheroder Weg 1, D-38124 Braunschweig, Germany¹ and Chemical Microbiology, Bergische University, Wuppertal

Running Title: *tfd* genes of *Ralstonia eutropha*

*Corresponding author. Mailing address: Bereich Mikrobiologie, AG Biodegradation, Gesellschaft für Biotechnologische Forschung mbH, Mascheroder Weg 1, D-38124 Braunschweig, Germany. Phone: 49/(0)531/6181-467. E-mail: dpi@gbf.de

Present address: TU Bergakademie Freiberg, Interdisziplinäres Ökologisches Zentrum, D-09599 Freiberg, Germany
3.1 Abstract

*Ralstonia* sp. strain PS12 is able to use 2,4-, 2,5- and 3,4-dichlorotoluene as growth substrates. Dichloromethylcatechols are central intermediates formed by TecA tetrachlorobenzene dioxygenase mediated activation at two adjacent unsubstituted carbon atoms followed by TecB chlorobenzene dihydrodiol dehydrogenase catalyzed rearomatisation and channeled into a chlorocatechol ortho-cleavage pathway involving a chlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase, and dienelactone hydrolase. However, completely different metabolic routes were observed for the three dichloromethylcatechols analysed. Whereas 3,4-dichloro-6-methylcatechol is quantitatively transformed into one dienelactone (5-chloro-2-methyl-dienelactone), and thus degraded via a linear pathway, 3,5-dichloro-2-methylmuconate formed from 4,6-dichloro-3-methylcatechol is subject to both 1,4- and 3,6-cycloisomerization and thus degraded via a branched metabolic route. 3,6-dichloro-4-methylcatechol, on the first view, is transformed predominantly into one (2-chloro-3-methyl-trans-) dienelactone. In-situ $^1$H NMR analysis revealed the intermediate formation of 2,5-dichloro-4-methylmuconolactone, showing both 1,4- and 3,6-cycloisomerization to occur with this muconate and indicated a degradation of the muconolactone via a reversible cycloisomerisation reaction and the dienelactone forming branch of the pathway. Diastereomeric mixtures of two dichloromethylmuconolactones were prepared chemically to proof such hypothesis. Chloromuconate cycloisomerase transformed 3,5-dichloro-2-methylmuconolactone into a mixture of 2-chloro-5-methyl-cis- and 3-chloro-2-methyl-dienelactone, affording evidence for a metabolic route of 3,5-dichloro-2-methylmuconolactone via 3,5-dichloro-2-methylmuconate into 2-chloro-5-methyl-cis-dienelactone. 2,5-Dichloro-3-methylmuconolactone was transformed nearly exclusively into 2-chloro-3-methyl-trans-dienelactone.
3.2 Introduction

Chlorotoluenes are important intermediates in many chemical processes such as the production of dyes and pesticides and are still produced in large amounts (21, 59). Their high chemical stability causes their accumulation in the environment (24). Whereas many bacterial strains are able to degrade various chloro- or methylsubstituted aromatics have been studied, only a few strains are known that are able to mineralize chlorinated toluenes. Degradative pathways have been described only for 3- and 4-chlorotoluene (1, 8, 26). Some strains have been reported to mineralize dichlorotoluenes (55, 64), however as yet no pathway has been analyzed in detail.

*Ralstonia* sp. strain PS12 (former *Pseudomonas, Burkholderia*) is known to mineralize a broad spectrum of chlorosubstituted benzenes, as well as 3- and 4-chlorotoluenes and 2,4-, 2,5-, and 3,4-dichlorotoluenes (55). Recently, the initial steps in the metabolism of chlorinated toluenes catalyzed by the broad substrate spectrum tetrachlorobenzene dioxygenase TecA and chlorobenzene dihydrodiol dehydrogenase TecB have been analyzed (34, 49). In the case of 3,4-dichlorotoluene transformation, TecA catalyzes exclusively a dioxygenation of the aromatic nucleus, which is followed by a TecB catalyzed dehydrogenation to give 3,4-dichloro-6-methylcatechol as product. In the case of 2,4- and 2,5-dichlorotoluene transformation, TecA catalyzes mainly a dioxygenation, although a minor but significant portion of the substrates was subject to monooxygenation (49). As only those dichlorotoluenes, which are predominantly subject to dioxygenation were reported as growth substrates for strain PS12, it may be assumed that dichloromethylcatechols are the central intermediates in the degradation of dichlorotoluenes. However, the further degradation routes have not yet been described.

The intermediate di- and trichlorocatechols formed from di-, tri-, and tetrachlorobenzene by strain PS12 are metabolized via a chlorocatechol ortho-cleavage-pathway, involving a chlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase, dienelactone hydrolase and maleylacetate reductase (55). Thus, it may be assumed that dichloromethylcatechols formed from dichlorotoluenes are degraded by a similar sequence of reactions.

Cycloisomerization of intermediary muconates is known to be a critical reaction for routing substituted aromatic substrates into an appropriate pathway. Blasco et al. (5) have shown that muconate cycloisomerase convert 3-chloro-*cis,cis*-muconate predominantly to the antibiotic protoanemonin, whereas chloromuconate cycloisomerase catalyze dehalogenation only with *cis*-dienelactone as a product (Fig.1) (58). Whereas *cis*-dienelactone is transformed by dienelactone hydrolase efficiently, activity of the enzyme with protoanemonin is only 1% of this rate (10).
Both, muconate and chloromuconate cycloisomerases catalyze a 3,6-cycloisomerization of 3-chloromuconate. However, it is assumed that in the reaction of chloromuconate cycloisomerases with 3-chloromuconate, the enol/enolate intermediate (25) loses the negative charge by chloride abstraction, whereas a protonation reaction is involved in the formation of protoanemonin (31). In all cases reported thus far, the product of chloromuconate in a muconate cycloisomerase catalyzed transformation is cis-dienelactone, and not trans-dienelactone. Similarly, 2-chloro-cis-dienelactone is the product of cycloisomerization of 2,4-dichloromuconate (31, 33).

In contrast, trans-dienelactone is the product of cycloisomerization of 2-chloromuconate by chloromuconate cycloisomerases (Fig.1) (58). It has been proposed that the lactone ring of the intermediary 5-chloromuconolactone rotates before dehalogenation to bring the acidic C4 proton close to the general acid/base at the active site (56) and chloride is eliminated to yield trans-dienelactone. Substituted muconates can dock into cycloisomerases in two different orientations, yielding 5-chloromuconolactone (3,6-cycloisomerization) or 2-chloromuconolactone (1,4-cycloisomerization) and binding in a mode leading to 3,6-cycloisomerization was obviously favored in the case of chloromuconate cycloisomerase (67). In contrast, proteobacterial muconate cycloisomerases form a pH-dependent equilibrium mixture of 2- and 5-chloromuconolactone (68).

Cycloisomerization of methylsubstituted muconates results in the formation of methylsubstituted muconolactones, which are often reported to be dead-end products (Fig.1) (12, 32, 46). Their dead-end nature explains the failure of various bacterial strains to mineralize methylcatechols via ortho-cleavage pathways. 4-Methylmuconolactone is reported as the product of cycloisomerization of 3-methylcatechol (32). Only bacteria harboring a special enzyme activity capable of transforming 4-methylmuconolactone into 3-methylmuconolactone (9, 45) were reported to be able to grow on methylcatechols via an ortho-cleavage route. Direct formation of 3-methylmuconolactone from 3-methylmuconate has never been observed in bacteria. In accordance with this observation, Pseudomonas sp. strain B13, which is capable of 3-methylmuconolactone degradation (52) and harbors catechol ortho-cleavage activities but is devoid of catechol meta-cleavage activities, only grows on methylaromatics after introduction of a gene encoding 4-methylmuconolactone methylisomerase. The importance of proper cycloisomerization for degradation has also been shown by Pieper et al. (43) for the metabolism of chloro- and methylsubstituted aromatics. 4-Chloro-2-methylphenoxyacetate is mineralized by Ralstonia eutropha JMP134 and cycloisomerization of intermediary 4-chloro-2-methylmuconate can be assumed, by analogy with the transformation of 3-chloromuconate by this strain, to result
in the formation of 2-methyl-cis-dienelactone, which can then be transformed by dienelactone hydrolase (Fig. 1). 2-Chloro-4-methylphenoxyacetate, however, is not mineralized but converted into 2-chloro-4-methylmuconolactone as dead-end product (Fig. 1) (41, 44, 51). In the case of 2-chloromuconolactone it was shown that this compound can be transformed by chloromuconate cycloisomerasers (68) via 2-chloromuconate and 5-chloromuconolactone into trans-dienelactone. However, such an activity was not observed with 2-chloro-4-methylmuconolactone, indicating that cycloisomerization of 2-chloro-4-methylmuconate results predominantly or exclusively in 2-chloro-4-methylmuconolactone formation. It was therefore astonishing to observe growth of Ralstonia sp. strain PS12 on 2,4-, 2,5-, and 3,4-dichlorotoluene, as according to the accumulated information, it could be speculated that some intermediary dichloromethylmuconates would be problematic for further degradation. Therefore, in the present report we investigated in detail the metabolic fate of the intermediates 4,6-dichloro-3-methyl-, 3,6-dichloro-4-methyl-, and 3,4-dichloro-6-methylcatechols in the complete metabolisms of the respective substituted chlorinated toluene substrates.
3.3 Materials and methods

**Bacterial strains and growth conditions.** *Ralstonia* sp. strain PS12 (DSM 8910) (previously *Burkholderia, Pseudomonas*), *Pseudomonas* sp. strain B13 (18) and *Ralstonia eutropha* JMP134 (pJP4) (17) were routinely grown in mineral salts medium (18) containing 50 mM phosphate buffer (pH 7.4), supplemented with the indicated carbon source (usually 3 - 5 mM) or in Luria broth medium. Flasks were sealed with Teflon coated screw caps and incubated at 30°C on a rotary shaker (150 rpm). 1,2,4,5-Tetrachlorobenzene as sole source of carbon was added as fine
mortal-ground crystals corresponding to a concentration of 5 mM. For growth on toluene or chlorinated toluenes mineral salt medium was supplemented with 1% (v/v) of a 300 mM solution of the respective carbon source in 2,2,4,4,6,8,8-heptamethylnonane (thus corresponding to a final concentration of 3 mM).

*E. coli* DH5α (pSTE44) containing the tetrachlorobenzene dioxygenase and chlorobenzene dihydrodiol dehydrogenase genes *tecAB* (3) was grown at 37°C in Luria broth medium containing 1 mM IPTG and 0.1 mg/ml ampicillin.

**Monitoring growth and preparation of cell extracts.** Growth on di- and trichlorotoluenes was monitored spectrophotometrically (A$_{600}$) and cell free supernatants were stored at −20°C for subsequent analysis of metabolites. Cell extracts were prepared from cells harvested during late exponential growth phase, as described previously (47).

**Enzyme assays and analyses of kinetic data.** (Chloro)catechol 1,2-dioxygenase, catechol 2,3-dioxygenase, chloromuconate cycloisomerase and dienelactone hydrolase were measured as previously described (20, 58, 69). Activity of partially purified chloromuconate cycloisomerase was determined in the presence of an excess of dienelactone hydrolase partially purified by anionic exchange chromatography and free of any interfering enzyme activity. Usually, activity was determined by quantifying the decrease in concentration of the respective muconate. Extinction coefficients ($\varepsilon$) for 2,5-dichloro-3-methyl-, 3,5-dichloro-2-methyl-, and 2,3-dichloro-5-methylmuconate (9200 M$^{-1}$ cm$^{-1}$, 12800 M$^{-1}$ cm$^{-1}$ and 10000 M$^{-1}$ cm$^{-1}$, respectively) were determined spectrophotometrically after complete transformation of 20 – 100 µM solutions of the corresponding catechols by partially purified chlorocatechol 1,2-dioxygenase. Substrate concentrations as well as complete turnover were characterized by HPLC-analysis. In the case of muconate, 2-methyl-, 3-methyl-, 2-chloro-, 3-chloro-, and 2,4-dichloromuconate, substrate transformation was analyzed spectrophotometrically at $\lambda$=299 ($\varepsilon_{cis,cis}$-muconate=457 M$^{-1}$ cm$^{-1}$ (67)), $\lambda$=285 nm ($\varepsilon_{2-methyl-cis,cis}$-muconate=6050 M$^{-1}$ cm$^{-1}$ (67)), $\lambda$=280 nm ($\varepsilon_{3-methyl-cis,cis}$-muconate =5050 M$^{-1}$ cm$^{-1}$) or at $\lambda$=260 nm as described previously (19, 33). In the case of 3-chloromuconate, for concentrations above 0.15 mM, the formation of cis-dienelactone was analyzed at $\lambda$=305 nm ($\varepsilon_{cis}$-dienelactone=5300 M$^{-1}$cm$^{-1}$). The kinetic parameters were calculated by non-linear regression analysis using SigmaPlot (v. 6, SPSS Science). Turnover numbers ($k_{cat}$ values) were calculated assuming a subunit molecular mass of 38 kDa.
Protein concentrations in the cell extracts were determined by the Bradford procedure (6). One enzyme unit is defined as the amount of enzyme that catalyzes the transformation of 1 µmol substrate per min.

**Enzyme purification.** Chlorocatechol 1,2-dioxygenase, and dienelactone hydrolase from strain PS12 and chloromuconate cycloisomerase from strains B13 and JMP134 were partially purified using a Fast Protein Liquid Chromatography system (Amersham Biosciences, Freiburg, Germany), whereas chloromuconate cycloisomerase from strain PS12 was purified to homogeneity. Cell extract of strain PS12 pregrown in 2 l mineral salt medium containing 3 mM 3-chlorobenzoate as carbon source (volume 2 ml, total protein 100 mg, total activity of chlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase and dienelactone hydrolase with 0.1 mM 3-chlorocatechol, 2-chloromuconate or cis-dienelactone of 16 +/- 5 U, 7 +/- 0.5 U or 14 +/- 5 U, respectively) were applied to a MonoQ HR 10/10 column and eluted by a linear gradient of NaCl (0 to 0.5 M over 200 ml) in Tris HCl (50 mM, pH 7.5 supplemented with 2 mM MnCl₂) (flow rate 0.5 ml/min; fraction volume 3 ml). Fractions containing dienelactone hydrolase activities (eluting at 0.18 +/- 0.02 mM NaCl) were stored for their subsequent use as auxiliary enzyme, whereas the fractions containing high chlorocatechol 1,2-dioxygenase (eluting at 0.24 +/-0.03 M NaCl) and chloromuconate cycloisomerase (eluting at 0.26 +/- 0.04 mM NaCl) were pooled and concentrated to a final volume of 0.5 ml using centricons (Millipore, Eschborn, Germany). Chlorocatechol 1,2-dioxygenase and chloromuconate cycloisomerase were further purified by hydrophobic interaction chromatography. (NH₄)₂SO₄ was added to the pooled fractions to give a final concentration of 1M. The solution was applied onto a Phenylsuperose HR 5/5 (Pharmacia) column and eluted with a linear gradient of (NH₄)₂SO₄ (1 M to 0 M over 32 ml) in Tris HCl (50mM pH 7.5 + 2 mM MnCl₂) (flow rate 0.25 ml/min, fraction size 0.5 ml). The fractions containing high chlorocatechol 1,2-dioxygenase (eluting at 0.67 +/- 0.02 M (NH₄)₂SO₄) or chloromuconate cycloisomerase (eluting at 0.80 +/- 0.02 M (NH₄)₂SO₄) activities were pooled and concentrated to a final volume of 0.5 ml using centricons. A chlorocatechol 1,2-dioxygenase preparation (total protein 1 mg, total activity 2.4 U) with a specific activity of 2400 U/g protein, corresponding to a purification factor of 15 and a yield of 15 %, and a chloromuconate preparation (total protein 3 mg, total activity 1.7 U) with a specific activity of 560 U/g protein, corresponding to a purification factor of 8 and a yield of 24 % were obtained. Chloromuconate cycloisomerase was further purified by gelfiltration. The solution was applied onto a HiLoad 16/60 Superdex200 column and eluted with Tris HCl (50 mM pH7.5 + 2mM MnCl₂) over 140 ml (flow rate 1 ml/min, fraction volume 1 ml). The fractions containing high chloromuconate cycloisomerase activity (eluting at 59-63 ml) were pooled and concentrated to a
final volume of 1 ml using centricons. A chloromuconate cycloisomerase preparation (total protein 1.64 mg, total activity 1.5 U) with a specific activity 900 U/g protein, corresponding to a purification factor of 13 and a yield of 21% was obtained. Homogeneity of the preparation of the chloromuconate cycloisomerase of strain PS12 was verified by SDS-gel electrophoresis with subsequent Coomassie brilliant blue staining (47), resulting in one visible band at 38000 Da. For partial purification of chloromuconate cycloisomerase from strains B13 and JMP134, cell extracts of cultures (total volume of 1 l) pregrown on 3-chlorobenzoate (3 mM), were applied to a MonoQ HR10/10 column (volume of cell extract, 2 ml each, total protein 23 and 68 mg, respectively, total activity with 3-chloromuconate 4 U and 6U, respectively) and eluted as described for the purification of chloromuconate cycloisomerase from strain PS12. Fractions with high chloromuconate cycloisomerase activity were pooled, concentrated and chromatographed on a Phenylsuperose column as described for the strain PS12 derived enzyme. After pooling and concentration of fractions containing high cycloisomerase activity, preparations with total activities of 0.8 and 1.5 U, respectively, with 3-chloromuconate as substrate were obtained.

**Measurement of chloride.** To each 200 µl of the samples mixed with 800 µl MilliQ H2O 200 µl of a 0.1 M AgNO3 solution was added. After 10 min the turbidity was determined at $\lambda = 525$ nm. The chloride concentration was calculated by comparison with a calibration curve (22).

**Transformation of substrates including acid transformation of muconates.** The chlorinated methylcatechols were transformed into the corresponding muconates by addition of cell extract of 3-chlorobenzoate grown cells of strain PS12 or partially purified 1,2-dioxygenase (corresponding to a total activity of chlorocatechol 1,2-dioxygenase with 3-chlorocatechol of 15-20 mU) in a total volume of 1 ml 33 mM Tris/HCl buffer (pH 8.0) containing 0.1 mM of substrate. The chloromuconate cycloisomerase present in the cell extract was inactivated by the addition of 1.3 µmol EDTA. The formed muconates were transformed by acidification of the solution to pH 4.0. After neutralisation further cell extract or partially purified chloromuconate cycloisomerase and dienelactone hydrolase of strain PS12 (corresponding to a total activity of chloromuconate cycloisomerase and dienelactone hydrolase with 3-chloromuconate and cis-dienelactone of 15-20 mU, respectively) was added. After 10 min incubation at room temperature, 0.5 mM of NADH + H+ and additional cell extract (corresponding to a total activity of maleylacetate reductase with maleylacetate of 15-20 mU) was added. The single reactions were monitored spectrophotometrically for changes in absorption spectra and by HPLC analyses.
**Transformation of substrates exclusively by enzymes.** The reaction mixture contained in 33 mM Tris/HCl-buffer (pH 8.0) had chlorinated methylcatechol as substrate (0.1 mM) and cell extract corresponding to a final protein content of 0.1 mg/ml. The reaction was monitored for 1 hour for changes in the absorption spectrum. Every 10 min an aliquot was taken for subsequent HPLC analyses.

Stepwise transformation of dichloromethylcatechols was achieved by sequential transformation of dichloromethylcatechols (0.1 mM in 1 ml 33 mM Tris/HCl-buffer, pH 8.0) by partially purified chlorocatechol 1,2-dioxygenase, purified chloromuconate cycloisomerase and partially purified dienelactone hydrolase. All enzymes were added in amounts corresponding to 10 – 20 mU after completion of the previous reaction as judged by HPLC analysis. All reactions were monitored spectrophotometrically for changes in absorption spectra.

**Extraction and derivatization of metabolites.** To 10 ml Tris/HCl buffer (33 mM, pH 8.0) containing 0.1 mM dichloromethylsubstituted catechol cell extract of 3-chlorobenzoate grown cells of PS12 (to give a final protein content of 0.1 mg/ml) was added. Substrate depletion and accumulation of intermediates were monitored by HPLC. After appropriate time periods (see results section), the enzyme reactions were stopped by acidifying the solution to pH 4.0 with HCl. All products were extracted twice with equal volumes of ethyl acetate. The organic phases were dried over MgSO$_4$ and evaporated under vacuum with a rotary evaporator. Prior to GC analysis, the products were esterified by methylation with diazomethane. The products were stored at –80°C.

**HPLC- analyses.** Metabolites were analysed by injection of 10 µl samples. Product formation was analyzed with a Shimadzu HPLC system (LC-10AD liquid chromatograph, DGU-3A degasser, SPD-M10A diode array detector and FCV-10AL solvent mixer) equipped with an SC125/Lichrospher 5-µm (Bischoff, Leonberg, Germany) column. The aqueous solvent system (flow rate 1 ml/min) contained 0.01% (v/v) of H$_3$PO$_4$ (87%) or 0.005 M PicA (Waters) and 35%, 40%, 55% or 50% (v/v) methanol for the determination of metabolites or 50 or 60% (v/v) methanol for the determination of substrates. Where possible, the metabolites were identified by comparison of retention volume and UV absorption spectra with those of authentic standards.

**GC-MS-analyses.** 1 µl of derivatized samples was injected and analyzed with a Shimadzu (Kyoto, Japan) GC-17A gas chromatograph equipped with an XTI-5 column (30 m by 0.25 mm; film thickness 0.5 µm; Resteck, Bellefonte, P.a.) and coupled to a QP-5000 quadropole mass spectrometer as described previously (3).
In-Situ-NMR-analyses of the transformation of substrates. Prior to NMR analyses the Tris/HCl buffer of enzyme preparations was exchanged for borate buffer (0.2 M H$_3$BO$_3$, 0.05 M Na$_2$B$_4$O$_7$ at pH 7.8) using centricons. The substrates were dissolved in aqueous borate buffer (200 mM H$_3$BO$_3$, 50 mM Na$_2$B$_4$O$_7$ at pH 7.8, supplemented with 20% (v/v) D$_2$O) to give a final concentration of 0.7 mM and transferred to the NMR sample tube (0.7 ml). The one-dimensional $^1$H NMR spectra were recorded at 300°K on a Bruker AVANCE DMX 600 NMR spectrometer (Bruker, Rheinstetten Germany) locked to the deuterium resonance of D$_2$O in the solution. Spectra were recorded using the standard Bruker 1D NOESY suppression sequence with 280 scans each with a 1.8 s acquisition time and 1.3 s relaxation delay. The center of the suppressed water signal was used as internal reference (δ 4.80 ppm).

After recording the in-situ $^1$H spectrum of the substrates partially purified chlorocatechol 1,2-dioxygenase, purified chloromuconate cycloisomerase and partially purified dienelactone hydrolase (dissolved in borate buffer) were added sequentially in amounts sufficient to quantitatively transform the respective substrate within 1-3 hours. Spectra were recorded immediately after each addition and then every hour. The subsequent enzyme on the pathway was added after completion of the previous reaction.

$^1$H NMR analyses. For identification of compound Ia and Ib or Ic and Id, 20 ml of phosphate buffer (50 mM, pH 7.4) containing 0.17 mM 4,6-dichloro-3-methylcatechol or 0.25 mM 3,6-dichloro-4-methylcatechol and 0.4 mM EDTA were supplemented with cell extract of 3-chlorobenzoate grown cells (corresponding to 2 mg of protein) and the reaction monitored by HPLC analyses. After complete transformation of the substrate, the solutions containing 3,5-dichloro-2-methylmuconate or 2,5-dichloro-3-methylmuconate were acidified to pH 4 to yield compounds Ia and Ib or Ic and Id, respectively. These products were extracted twice with equal volumes of ethyl acetate, dried over MgSO$_4$ and evaporated under vacuum on a rotary evaporator. The compounds were dissolved in 0.7 ml acetone-d$_6$ (Chemotrade). $^1$H NMR spectra were recorded on a Bruker AVANCE DMX 600 NMR spectrometer (Bruker, Rheinstetten Germany) using tetramethylsilane as internal standard.

Chemicals. Dichloromethylcatechols were prepared as follows: Strain E. coli pSTE44 was grown in 200 ml Luria broth medium containing 1 mM IPTG and 0.1 mg/ml ampicillin. After reaching an A$_{600}$ of 2.7, the cells were harvested by centrifugation (6000 x g, 10 min, 20°C). The pellet was washed twice with assay buffer (35), resuspended in 200 ml of the same buffer and supplemented with 0.5 mM of 2,4-, 2,5- or 3,4-dichlorotoluene (from a 100 mM stock solution in MeOH). The flasks were sealed with Teflon coated screw caps and incubated at 30°C on a rotary
shaker (150 rpm) for 5 hours. After centrifugation (10000 x g rpm, 10 min, 20°C) the cell-free supernatants were acidified to pH 4 and extracted twice with an equal volume of ethyl acetate. The organic phases were dried over MgSO₄ and evaporated under vacuum with a rotary evaporator. The residues were stored at –20°C.

3-Chloro-, 2-methyl-, 3-methyl-, 2,4-dichloro-, and dichloromethylmuconates as substrates for chloromuconate cycloisomerase were prepared in situ from 4-chloro-, 3-methyl-, 4-methyl-, 3,5-dichloro-, and dichloromethylcatechols using partially purified chlorocatechol 1,2-dioxygenase which was free of any (chloro)muconate cycloisomerase activity.

2-chloro-3-methyl-trans-dienelactone and 2-chloro-5-methyl-cis-dienelactone were prepared as follows: The tert. butylester of 2-chloro-3-methyl-trans-dienelactone was obtained as one of several isomers from the Wittig reaction between equimolar amounts of (tert. butoxycarbonylmethylene)triphenylphosphorane (Ph)₃P=CHCO₂tBu and chlorocitraconic anhydride as described in Kaschabek (27). The t-butylester of 2-chloro-5-methyl-cis-dienelactone was prepared in a similar way, using (Ph)₃P=CCH₃CO₂tBu and 3-chloro-3,4,7,7-tetrahydro-4,7-epoxyisobenzofuran-1,3-dione. From the resulting dienelactone-t-butylesters the free acids were obtained by proton-catalyzed thermal decomposition in toluene. 2-Chloro-3-methyl-cis-dienelactone and 2-chloro-5-methyl-trans-dienelactone were produced by the irradiation of aqueous solutions of these substrates (0.2 mM) using an ultraviolet lamp and irradiation with ?=254 nm light at a distance of 6 cm for 2 hours as described for the preparation of 2-chloro-trans-dienelactone from 2-chloro-cis-dienelactone (31). Reactions were monitored by HPLC. The E₂₁₀/E₂₆₀ ratio and the absorption maxima of the products (λ=286 and 299 nm, respectively), which were identical to those of the substrates, confirmed the identity of the expected isomers. The methylated dichlorocatechols were prepared as described above. All other chemicals were purchased from Aldrich Chemie (Steinheim, Germany), Fluka AG (Buchs, Switzerland), or Merck AG (Darmstadt, Germany).

3.4 Results

Growth of Ralstonia sp. PS12 on dichlorotoluenes. Out of the range of di- and trichlorotoluenes analyzed (2,3-, 2,4-, 2,5-, 2,6-, 3,4-, 3,5-dichloro-, and 2,4,5-trichlorotoluene), only 2,4-, 2,5-, and 3,4-dichlorotoluene could be used as growth substrates by Ralstonia sp. PS12. Growth yield, as indicated by the optical density obtained after complete transformation of the substrate (3 mM), was higher with 2,4- and 2,5-dichlorotoluene (A₆₀₀= 0.44 +/- 0.05 and 0.31
+/- 0.05, respectively) compared to 3,4-dichlorotoluene ($A_{\text{600}} = 0.16 +/ - 0.06$). The growth yield with 2,4-dichlorotoluene was similar to that observed with 1,2,4,5-tetrachlorobenzene (3). Whereas 2,4- and 2,5-dichlorotoluene were reasonable growth substrates with generation times of 14 – 15 hours (compared to a generation time of 9.5 hours reported for 1,2,4,5-tetrachlorobenzene (3)), growth with 3,4-dichlorotoluene was poor (generation time of 22 hours). Chloride elimination during growth on 2,4-, 2,5- and 3,4-dichlorotoluene corresponded to 65 +/- 5, 60 +/- 5 and 25 +/- 5 %, respectively, of the concentration expected in the case of complete mineralization, indicating that in all cases dead-end metabolites were formed. As shown previously, both 2,4- and 2,5-dichlorotoluene are subject preferentially to dioxygenation by tetrachlorobenzene dioxygenase TecA and the resulting dihydrodiols are further transformed by chlorobenzene dihydrodiol dehydrogenase TecB into the corresponding catechols (49). However, in addition to dioxygenation these dichlorotoluenes also undergo monooxygenation by TecA resulting in the formation of the corresponding benzyl alcohols (5 and 20%, respectively, of the supplied substrate).

Analyses of the supernatants after growth with 2,4- or 2,5-dichlorotoluene revealed the accumulation of 2,4- and 2,5-dichlorobenzoate respectively. Both compounds were identified by comparison of their retention volume and UV absorption spectra with those of authentic standards (Table 1). Formation of dichlorobenzoates from dichlorobenzylalcohols involves unspecific enzymes as described for the strain PS12 catalyzed oxidation of 2- and 3-chlorobenzylalcohol to 2- and 3-chlorobenzoate (34). The 2,4- and 2,5-dichlorobenzoate concentration amounted to 2 and 15% of substrate transformed and was only slightly lower than expected from experiments using recombinant tetrachlorobenzene dioxygenase. These dichlorobenzoates were not transformed further and are thus dead-end products for strain PS12.

In contrast, during growth on 3,4-dichlorotoluene neither accumulation of 3,4-dichlorobenzylalcohol nor of 3,4-dichlorobenzoate was observed. This is in accordance with the observation that TecA dioxygenates 3,4-dichlorotoluene exclusively. Of note however, during growth on 3,4-dichlorotoluene a yellow coloration of the culture supernatant was observed with an absorption maximum of $\lambda_{\text{max}} = 361$ nm. As products formed by extradiol cleavage of several unsubstituted and substituted catechols are known to exhibit similar absorption maxima (53, 54), it can be assumed that part of the 3,4-dichlorotoluene transformed is misrouted into a catechol meta-cleavage pathway which, with a few exceptions (2, 36), is usually not suited for mineralization of chlorosubstituted catechols.
Metabolites later identified as the respective dichloromethylmuconates and chloromethylidenelactones accumulated only transiently during growth with all three dichlorotoluenes as verified by HPLC analyses.

**Ring-cleavage of catechols.** Cell extracts of toluene, 3-chlorobenzoate or 2,4-dichlorotoluene grown cells exhibited exclusively 1,2-dioxygenase activity with catechol, 3-chlorocatechol and 4,6-dichloro-3-methylcatechol as substrates. The activities with 3-chlorocatechol were 760, 770 and 740 U/g and those with catechol and 4,6-dichloro-3-methylcatechol roughly 60% and 125%, respectively, of the activity determined with 3-chlorocatechol. Catechol 2,3-dioxygenase activity was not observed in these extracts (< 1 U/g). Hence, 2,4-dichlorotoluene is mineralized exclusively via an *ortho*-cleavage pathway. Since the respective chlorocatechol transforming activities were expressed equally during growth on the three substrates tested, cell extracts of 3-chlorobenzoate-grown cells were chosen in further experiments, as those were more easily accessible. Purified enzymes of these extracts were used to follow the fate of dichloromethylcatechols formed from 2,4-, 2,5- and 3,4-dichlorotoluene during their further processing by enzymes of the chlorocatechol *ortho*-cleavage pathway of strain PS12.
TABLE 1: Retention volumes and absorption maxima during HPLC analyses of the products formed from 4,6-dichloro-3-methylcatechol, 3,6-dichloro-4-methylcatechol and 3,4-dichloro-6-methylcatechol by enzymes of the chlorocatechol ortho-cleavage pathway of *Ralstonia* sp. strain PS12

<table>
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<tr>
<th>Substrate</th>
<th>Product</th>
<th>[MeOH] (%)</th>
<th>RV (ml)</th>
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<tr>
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<td>2.7</td>
<td>223</td>
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<td></td>
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Cells of *E. coli* (pSTE44) expressing both tetrachlorobenzene dioxygenase TecA and dihydrodiol dehydrogenase TecB from strain PS12 were incubated with dichlorotoluenes. The formed catechols were extracted and used as substrates for enzymes of the chlorocatechol ortho-cleavage pathway of strain PS12. The proposed products derived from the transformations are shown. [MeOH], percentage of the methanol used in the solvent system, with or without PicA (Waters); RV, retention volume in ml. ?<sub>max</sub>, wavelength of the maximal absorption in nm.

Substituted muconates, products formed after intradiol cleavage, have been reported to exhibit absorption maxima of 260-280 nm (58). Since substituted muconates, specifically those substituted in the 3-position, are known to be very unstable under acidic conditions (58, 60), HPLC analysis using ion-pair chromatography was performed to characterize the products after incubations of 4,6-dichloro-3-methyl-, 3,6-dichloro-4-methyl-, and 3,4-dichloro-6-methylcatechol (0.1 mM each) in the presence of cell extract (total activity of chlorocatechol 1,2-dioxygenase with 3-chlorocatechol 15-20 mU) and EDTA. HPLC analyses revealed the formation of single products with absorption maxima at 270 nm, 273 nm and 289 nm (Table 1) respectively, that are indicative of the formation of 3,5-dichloro-2-methyl-, 2,5-dichloro-3-methyl-, and 2,3-dichloro-5-methylmuconates, respectively (Fig. 2-6).
Transformation of dichloromethylmuconates. Acidification (to a final pH of 4) of the above reaction mixtures containing 3,5-dichloro-2-methyl- or 2,5-dichloro-3-methylmuconate resulted in the formation of two new products in each case as shown by HPLC analyses (Table 1). Compounds Ia and Ib formed from 3,5-dichloro-2-methylmuconate and Ic and Id formed from 2,5-dichloro-3-methylmuconate exhibited absorption maxima at about 222 nm (Table 1). Hence, these products do not contain a conjugated double-bound system as present in the dienelactone structure. Since the acid catalyzed transformation of 2-chloro-4-methylmuconate has been reported to result in the formation of 5-chloro-3-methyl-muconolactones (due to the presence of two asymmetric carbon atoms the formation of 4 stereoisomers was described) (44, 51), we postulated that compounds Ia-d might also be muconolactone isomers.

In contrast, acidification of 2,3-dichloro-5-methylmuconate gave a single product with an absorption maximum of 299 nm, which is in the range expected for substituted dienelactones (28, 46, 58).

After neutralisation (pH 7) of the solutions containing Ia and Ib or Ic and Id and partially purified chloromuconate cycloisomerase (total activity with 2-chloromuconate 2-5 mU) was added. Products with absorption maxima between 278 and 299 nm (Table 1) indicating the formation of dienelactones (58) were monitored. Ia and Ib were mainly converted to 2-chloro-5-methyl-cis-dienelactone (2C5MDL) (Fig. 4), confirmed by comparison of the retention volume and UV-absorption spectrum with an authentic standard (Table 1). A second product formed from Ia and Ib, which was not identical to trans-2C5MDL, could not be identified unambiguously because of the absence of an authentic standard, but its absorption maximum of 278 nm indicates that it is a dienelactone, probably 3-chloro-2-methyl-dienelactone (3C2MDL) (Fig. 4). More than 60% of Ia and Ib were transformed by chloromuconate cycloisomerase. Spontaneous transformation of Ia and Ib was negligible under the given conditions.

Products formed from compounds Ic and Id were 2-chloro-3-methyl-trans-dienelactone (trans-2C3MDL) as the major product as well as cis-2C3MDL as the minor product (Fig. 5) (approximately 2% comparing signal intensities at 270 nm after separation by HPLC). These products were verified by comparison with authentic standards (Table 1). More than 60% of each, Ic and Id were converted into dienelactones after addition of chloromuconate cycloisomerase.

In contrast to the acid catalyzed reaction, addition of purified chloromuconate cycloisomerase to reaction mixtures containing 3,5-dichloro-2-methyl-, 2,5-dichloro-3-methyl-, or 2,3-dichloro-5-
methylmuconate resulted in the formation of compounds, according HPLC retention volumes and UV absorption spectra, identical with those described above for acidic catalysis followed by chloromuconate cycloisomerase catalyzed transformation (Table 1).

Transformation of 3,5-dichloro-2-methylmuconate resulted in the formation of \textit{cis}-2C5MDL and 3C2MDL of unknown stereochemistry (Fig. 4). Assuming similar extinction coefficients at λ=230 nm both compounds were produced in approximately a 1:1 ratio that is different to the ca. 2:1 ratio observed during the subsequent acid and chloromuconate cycloisomerase catalyzed reaction.

2,5-Dichloro-4-methylmuconate was transformed by chloromuconate cycloisomerase into \textit{trans}-2C3MDL as the major and \textit{cis}-2C3MDL (Fig. 5) as the minor product in a ratio similar to the one observed during the subsequent acid and chloromuconate cycloisomerase catalyzed reaction.

Compounds Ia through Id were detected as intermediate products of chloromuconate cycloisomerase catalyzed conversion in amounts not exceeding 5% of those observed during acid catalysis.

Chloromuconate cycloisomerase catalyzed transformation of 2,3-dichloro-5-methylmuconate resulted in the formation of a dienelactone (most probably an isomer of 5-chloro-2-methylidenelactone [5C2MDL]), without any evidence for the accumulation of intermediate products (Fig. 6).

\textbf{Identification of products formed by chloromuconate cycloisomerase by GC/MS.} GC/MS analysis of three reaction mixtures supposedly containing a mixture of \textit{cis}-2C5MDL and 3C2MDL of unknown stereochemistry, \textit{trans}-2C3MDL as the major product or 5C2MDL, confirmed the identity of all these products as chloromethylsubstituted dienelactones. Prominent signals showing the molecular ion of m/z 202 and 204 (3:1) were evident in samples derivatized with diazomethane. All products exhibited intense signals at M⁺- m/z 31 and/or M⁺- m/z 32 typical of methyl esters. Two such products were observed from the mixture supposedly containing \textit{cis}-2C5MDL and 3C2MDL. One of these, exhibiting a retention time of 11.6 min (relative intensities m/z 202/171/170, 30/50/85 % of basepeak), was identified as \textit{cis}-2C5MDL by comparison with an authentic standard. The second product (retention time of 12.4 min) showed a fragmentation pattern (relative intensities m/z 202/171/170, 15/95/3 % of basepeak, see table) very similar to that of authentic \textit{trans}-2C3MDL with a basepeak of m/z 69. Significant
fragments with this mass (at least 50% of basepeak) have been observed for 2-methyl, 3-methyl- as well as 2-chlorodienelactone (26, 41, 50, 60), indicating the fragment is not comprised of C2 and C3, but the C5 carbon atom of the dienelactone structure. As a consequence, the m/z 69 fragment observed thus far for dienelactones is absent in the mass spectrum of cis-2C5MDL. Instead a fragment with m/z 83, not present in the other spectra, is observed and indicates an exocyclic position of the methyl substituent. The basepeak of m/z 69 suggests it is 3C2MDL.

Out of the reaction mixture supposedly containing trans-2C3MDL as the major product, two products with nearly identical fragmentation patterns (relative intensities m/z 202/171/170, 13/70/48% of basepeak) and retention times of 13.8 (major product) and 11.6 min (minor product) were identified as trans-2C3MDL and cis-2C3MDL by comparisons with authentic standards. Both products exhibit a basepeak of m/z 69. Themethylester of the dienelactone tentatively identified as 5C2MDL has a retention time of 12.5 min (relative intensities m/z 202/171/170, 40/27/60% of basepeak). Only a minor fragment of m/z 69 was observed, and fragments of m/z 105/103 (33/12% of baspeak) indicate it is actually 5C2MDL.

Identification of Ia - Id by \(^1\)H NMR-analysis. As compounds Ia–Id were intermediates in the formation of dienelactones from chlorosubstituted muconates, their identity with muconolactones is to be expected. Depending on the direction of cycloisomerization, two differently substituted muconolactones can theoretically be formed in each case, namely 2,4-dichloro-5-methyl- and 3,5-dichloro-2-methylmuconolactone from 3,5-dichloro-2-methylmuconate, and 2,5-dichloro-3-methyl- and 2,5-dichloro-4-methylmuconolactone from 2,5-dichloro-3-methylmuconate. To clarify the identity of these compounds, mixtures of Ia/Ib or Ic/Id, respectively, were extracted from the reaction mixtures after chlorocatechol 1,2-dioxygenase mediated transformation of 4,6-dichloro-3-methyl- or 3,6-dichloro-4-methylcatechol, respectively, followed by acid catalyzed conversion of the intermediate 3,5-dichloro-2-methyl- or 2,5-dichloro-3-methylmuconate. The \(^1\)H NMR spectrum showed the presence of 6 signals originating from Ia plus Ib, with three signals from each compound from their intensities. No signals were observed at chemical shifts higher than 5.8 ppm, which excluded the presence of 2,4-dichloro-5-methylmuconolactone in the compound mixture, as olefinic protons located at the C-3 carbon atom of muconolactones exhibit chemical shifts significantly higher than 7 ppm (44, 58, 65). Both compounds exhibit very similar \(^1\)H NMR spectra. One of the compounds, comprising approximately 60% of the mixture, exhibited signals of a methyl function at 1.89 ppm and signals corresponding to single protons at 5.30 and 5.75
ppm respectively, whereas the second compound had signals at 1.92 (methyl function), 5.25 and 5.80 ppm, respectively. Those data are compatible with the assumption that Ia and Ib are different stereoisomers of 3,5-dichloro-2-methylmuconolactone (Fig. 4). A similar formation of two diasteromers (5-chloro-3-methylmuconolactone), each comprising two different enantiomers has recently been reported after acid catalyzed transformation of 2-chloro-4-methylmuconate (51). The chemical shift values of 5.25 and 5.30 ppm, resemble those observed for the 5-H protons of 5-chloro- (5.14 ppm (65)) or 5-chloro-3-methylmuconolactone (5.20 and 5.24 ppm for the different diastereomers, respectively (44)) and the chemical shift values of 5.75 and 5.80 ppm in turn, resemble those observed for the respective methine (4-H) protons (5.60 – 5.83 ppm). In addition the vicinal couplings 3J(4-H,5-H) of 3.0 and 2.0 Hz are compatible with the postulated structures.

Similarly the mixture of Ic and Id showed no 1H NMR signals at chemical shifts greater than 6 ppm again excluding the formation of a muconolactone with a proton at C-3, and thus excluding the formation of 2,5-dichloro-4-methylmuconolactone. As with Ia/Ib, the 1H NMR spectrum indicated Ic/Id was a mixture of diastereomers of 2,5-dichloro-3-methylmuconolactone (Fig. 5). The chemical shift values of 5.20 and 5.26 ppm, resemble those observed for the 5-H protons of 5-chlorosubstituted muconolactones (5.15 – 5.30 ppm, see above) and chemical shift values of 5.72 and 5.83 ppm in turn, resemble those observed for the respective methine (4-H) protons (5.60 – 5.83 ppm). As with Ia/Ib, vicinal couplings 3J(4-H,5-H) of 3.0 and 2.0 Hz were observed.

**Biological conversion with chloromuconate cycloisomerases of highly substituted muconates.** Cycloisomerization of methylsubstituted muconates often leads to methylsubstituted muconolactones as dead-end products (46) and the formation of 2-chloro-4-methylmuconolactone from 2-chloro-4-methylmuconate has been suggested to be the reason for the failure of strain *Ralstonia eutropha* JMP134 to grow with 2-chloro-4-methylphenoxyacetate (44). As such dead-end products obviously did not occur during transformation of dichloromethylsubstituted muconates by strain PS12, the chloromuconate cycloisomerase of *Ralstonia* sp. strain PS12 was purified in order to compare its biochemical properties with those of other known chloromuconate cycloisomerases, including the JMP134 derived enzyme.

Turnover numbers of PS12 chloromuconate cycloisomerase for the various substrates tested were usually slightly lower, but similar to those of previously described chloromuconate cycloisomerasers (Table 2). Specificity constants varied by a factor of 10, showing this enzyme
Transformation of chloromethylsubstituted dienelactones. After addition of partially purified dienelactone hydrolase (total activity of dienelactone hydrolase with cis-dienelactone 15-20 mU) to reaction mixtures (1 ml) supposedly containing trans-2C3MDL plus cis-2C3MDL, cis-2C5MDL plus 3C2MDL, or 5C2MDL, all these compounds, with the exception of cis-2C3MDL, were transformed within 30 min. The activity of dienelactone hydrolase with cis-2C3MDL was thus lower than 1% of the activity with cis-dienelactone as substrate.

Dienelactone hydrolase catalyzed transformation of trans-2C3MDL resulted in the formation of a new product exhibiting an UV absorption maximum under the acidic conditions used for HPLC separation at $\lambda_{\text{max}} = 225$ nm. (Table 1). The same product was observed upon transformation of trans-2C3MDL using cell extract. Upon addition of NADH to the last reaction mixture, further transformation of this product was observed. This suggests trans-2C3MDL is transformed by dienelactone hydrolase into 2-chloro-3-methylmaleylacetate which is present in its cyclic lactone structure (2-chloro-4-hydroxy-3-methylmuconolactone) under acidic conditions (Fig. 5) (42). Addition of NADH would then result in its reduction by maleylacetate red utcase present in the cell extract to 5-chloro-4-methyl-3-oxoadipate.

Only one new signal exhibiting an absorption maximum at $\lambda_{\text{max}} = 223$ nm (HPLC diode array analysis) was observed after transformation of cis-2C5MDL and 3C2MDL by partially purified dienelactone hydrolase or cell extract. It is clear that the products 2-chloro-5-methylmaleylacetate and 3-chloro-2-methylmaleylacetate (Fig. 4) could not be separated by HPLC under the given conditions. The new HPLC signal disappeared completely after addition of NADH to the reaction mixture containing cell extract.
Addition of partially purified dienelactone hydrolase or cell extract to reaction mixtures supposedly containing 5C2MDL resulted in the formation of a new product exhibiting an absorption maximum at $\lambda_{\text{max}} = 207$ nm, a significantly shorter wavelength compared to the products formed from other dienelactones. This product was not transformed to any significant extent within 30 min after addition of NADH + H$^+$ to reaction mixtures containing cell extracts (total protein 0.2 mg). Thus, its identity with a maleylacetate is rather improbable. Maleylacetate has been reported to be subject to spontaneous decarboxylation, resulting in the formation of cis-acetylacrylate (42, 57), which exhibits an absorption maximum at $\lambda = 195$ nm under acidic conditions (13, 62) and is quite different to maleylacetate at 206 nm (50, 61). Thus, it is proposed that 5-chloro-2-methylmaleylacetate (Fig. 6) formed by hydrolysis of 5C2MDL is subject to a fast decarboxylation.
### TABLE 2: Substrate specificities of chloromuconate cycloisomerases from different strains

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Ralstonia sp. PS12</th>
<th>Pseudomonas sp. B13</th>
<th>Ralstonia sp. JMP134</th>
<th>Pseudomonas sp. P51</th>
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<td>$k_{\text{cat}}^a$ (µM)</td>
<td>$k_{\text{cat}}^a$ (min$^{-1}$)</td>
<td>$k_{\text{cat}}^a/k_m^a$ (µM$^{-1}$ min$^{-1}$)</td>
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<td>9</td>
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$^a$ The data for the strains P. B13, R. JMP134 and P. P51 are from Vollmer et al. (67)

$^b$ The data for the strains P. B13, JMP134 and P. P51 except the values for the dichloromethylmuconates are calculated from Vollmer et al. (67)

$^c$ For cis,cis-muconate: $\varepsilon_{299\text{nm}}=457$ M$^{-1}$ cm$^{-1}$ (67), substrate concentrations: 0.2-0.9 mM

$^d$ For 2-methyl-cis,cis-muconate: $\varepsilon_{285}=6050$ M$^{-1}$ cm$^{-1}$ (67), substrate concentrations: 0.025-0.2 mM

$^e$ For 3-methyl-cis,cis-muconate: $\varepsilon_{280}=5050$ M$^{-1}$ cm$^{-1}$, substrate concentrations: 0.05-0.35 mM

ND, not done.
In-situ NMR-analyses. As various metabolites in the degradation of highly substituted aromatics such as muconates, muconolactones, dienelactones or maleylacetates are often unstable under non-physiological conditions (29, 44, 51, 57, 60), HPLC- and GC-MS-analyses may lead to artefacts (41, 60). One dimensional $^1$H NMR spectroscopy in H$_2$O has recently been shown to be an excellent tool for monitoring enzyme activities and assessing solution structure of substrates and products (7, 15, 16, 23, 48). In order to characterize intermediates formed during dichloromethylcatechol metabolism phosphate buffer, known to severely inhibit muconate cycloisomerizing enzyme activities (33), was replaced by borate buffer. Even though 1,2-dioxygenase activity in borate buffer was only 10% of that observed in Tris/HCl buffer, products accumulating in this system could easily be characterized as no interfering signals originating from organic buffer systems were present.

The three catechol systems behaved as follows:

a) 4,6-Dichloro-3-methylcatechol (0.7 mM) dissolved in borate buffer exhibited signals at $\delta=6.85$ ppm, (aromatic proton) and $\delta=2.19$ ppm (methylfunction). After addition of partially purified 1,2-dioxygenase (total activity with 3-chlorocatechol, 20 mU) to the assay mixtures of 1 ml, the signals originating from the substrate disappeared. Two new signals with chemical shifts at $\delta=2.02$ ppm and $\delta=6.56$ ppm are indicative of the presence of one methylfunction and one olefinic proton in the product and in agreement with the postulated structure 3,5-dichloro-2-methylmuconate (Fig. 4).

These signals decreased after addition of purified chloromuconate cycloisomerase (total activity with 3-chloromuconate, 6 mU) and the formation of two distinct products was obvious from the presence of signals of two olefinic protons evidently due to the formation of cis-2C5MDL ($\delta = 8.05$ ppm) and 3C2MDL ($\delta = 5.93$ ppm) (Fig. 4).

Those values are in close agreement with literature values of chemical shifts of the C3 protons of cis-dienelactone (8.27 ppm) and 2-chloro-cis-dienelactone (8.39 ppm) and of C5 protons of dienelactones (ranging from 6.0 – 6.1 ppm for cis- and from 5.5 – 5.8 ppm for trans-dienelactones). $^1$H NMR analysis of a standard of cis-2C5MDL verified its identity with one component of the mixture. The olefinic proton of 2C5MDL resonates at 8.04 ppm when dissolved in buffer at pH 7.5. Under acidic conditions, a shift of the resonance line to 8.22 ppm was observed.
b) *3,6-Dichloro-4-methylcatechol* solution (0.7 mM in borate buffer) showed two signals with chemical shifts of $\delta=2.26$ ppm (methyl function) and $\delta=6.69$ ppm (aromatic proton) (Fig. 2). Upon addition of chlorocatechol 1,2-dioxygenase these signals disappeared with concomitant appearance of two new signals at $\delta=2.05$ ppm (methyl function) and $\delta=6.75$ ppm (olefinic proton), indicative of the formation of 2,5-dichloro-3-methylmuconate (Fig. 2). After addition of chloromuconate cycloisomerase, these signals disappeared. The spectrum of the product mixture was dominated by two strong signals with chemical shifts of $\delta=5.93$ ppm (olefinic proton) and $\delta=2.21$ ppm (methyl function). These signals originate from the formation of 2-chloro-3-methyl-trans-dienelactone as verified by comparison with authentic material (Fig. 2). However, the transient observation of three additional signals ($\delta=5.80$ ppm, 7.79 ppm and 1.70 ppm) was indicative of the formation of a dichloromethylsubstituted muconolactone intermediate. Two such structures can be postulated from 2,5-dichloro-3-methylmuconate, namely 2,5-dichloro-4-methyl- (after 3,6-cycloisomerization) and 2,5-dichloro-3-methylmuconolactone (after 1,4-cycloisomerization). The signal at $\delta=7.79$ ppm is evidently from an olefinic proton (44), which is present only in 2,5-dichloro-4-methylmuconolactone (Fig. 2). After addition of partly purified dienelactone hydrolase (total activity with *cis*-dienelactone, 60 mU) all signals decreased.
c) 3,4-Dichloro-6-methylcatechol (0.7 mM in borate buffer) showed signals for the methyl function (δ=2.17 ppm) and aromatic proton (δ=6.89 ppm) of the substrate (Fig. 3). Addition of chlorocatechol 1,2-dioxygenase led to their disappearance and the formation of two new signals at δ=1.97 ppm (methyl function) and 6.26 ppm (olefinic proton), indicative of the formation of 2,3-dichloro-5-methylmuconate (Fig. 3). These signals disappeared after the addition of chloromuconate cycloisomerase to give two new signals at δ=2.01 ppm (methyl function) and 8.00 ppm (olefinic proton). As similar chemical shifts were reported for the C3 protons of unsubstituted and substituted dienelactones (e.g. δ= 7.82 for trans-dienelactone, δ= 8.37 ppm for cis-dienelactone and δ= 8.39 for 2-
chloro-cis-dienelactone (60), the identity of the product as 5-chloro-2-methylidienelactone can be assumed (Fig. 3). The signals decreased after addition of dienelactone hydrolase.

**FIG. 3.** Changes in the $^1$H NMR spectra during transformation of 3,4-dichloro-6-methylcatechol. A without enzyme; B after addition of chlorocatechol 1,2-dioxygenase (b); C after addition of chloromuconate cycloisomerase (c). Arrowed signals correspond to the compounds of interest.
3.5 Discussion

Growth on dichlorotoluenes. Recent analyses have shown that the initial tetrachlorobenzene dioxygenase TecA of strain PS12 attacks chlorinated benzenes not only dioxygenolytically, but also catalyzes monooxygenation of the methylgroup (34, 49). Accordingly, during growth on 2,4- or 2,5-dichlorotoluene, which are subject to monooxygenation as a side reaction (49), accumulation of the corresponding benzoates was observed obviously produced by the further oxidation of the dichlorobenzylalcohols (Fig. 4-5). 2-Chloro-, 2,4-, and 2,5-dichlorobenzoate cannot be mineralized by this strain due to the absence of a broad spectrum 2-halobenzoate dioxygenase (34, 49). Thus, monooxygenation results in part in channelling of the substrates into dead-end metabolites. Consequently, only those substrates, which are preferentially attacked in a dioxygenolytic manner were used as growth substrates. Surprisingly 3,4-dichlorotoluene, which is exclusively subject to dioxygenation, was only a poor growth substrate. The yellow color of the culture supernatant during growth indicated that part of the substrate was misrouted into a meta-cleavage pathway. It thus seems, that beside an inactive todF analogue (tlpE* pseudogene) (4) strain PS12 harbors another as yet unidentified active extradiol dioxygenase. However, as such an activity was not observed during growth of strain PS12 on 2,4-dichlorotoluene and thus evidently not necessary for dichlorotoluene mineralization, this activity was not further analyzed here.

Biological fate of dichloromethylmuconates. Intradiol cleavage of dichloromethylcatechols by chlorocatechol 1,2-dioxygenase results in the formation of dichloromethylmuconates. Chloromuconate cycloisomerase catalyzes their cycloisomerization and dehalogenation (Fig. 4-6). Different features of this reaction need special attention. First of all, chloromuconates as symmetric molecules can dock into the active site in two different directions, resulting in a 1,4- or a 3,6-cycloisomerization respectively. For 2-chloromuconate, Vollmer et al. (68) have shown that chloromuconate cycloisomerase catalyze dominantly a 3,6-cycloisomerization to form (4R,5S)-5-chloromuconolactone by syn addition to the double bond followed by chloride elimination to produce trans-dienelactone (14). However, even 2-chloromuconolactone, the product of 1,4-cycloisomerization, can be transformed by chloromuconate cycloisomerase. In this case, chloride elimination does not occur directly from the substrate, but via 2-chloromuconate and 5-chloromuconolactone as intermediates (68). It follows that a “wrong” cycloisomerization can be corrected by the enzyme, if the reaction is reversible and if the “right” cycloisomerization product is taken out of the equilibrium, e.g. by dechlorination.
For 3-chloromuconate, Schmidt and Knackmuss (58) have shown that chloromuconate cycloisomerase catalyzed transformation results in the formation of cis-dienelactone implying 3,6-cycloisomerization (Fig. 1). Kaulmann et al. (25) indicated that during the cycloisomerization of 3-chloromuconate, the corresponding enol/enolate intermediate is not protonated but rather loses the negative charge by chloride abstraction, thus keeping the cis-configuration in the product (31). Similarly, 2-chloro-cis-dienelactone is formed from 2,4-dichloromuconate (31, 41). Like 3-chloro- and 2,4-dichloromuconate, other muconates substituted in β-position relatively to one of the carboxysubstituents were obviously cycloisomerized in a similar fashion. All 3-methyl-, 2-chloro-4-methyl- and 2,4-dimethylmuconate (and probably also 4-chloro-2-methylmuconate), were subject to a cycloisomerization involving the substituted β-carbon atom, with subsequent elimination of the β-chlorosubstituent or addition of a proton resulting in the formation of 4-methylsubstituted muconolactones (Fig. 1). No indications for an alternative mode of cycloisomerization were evident in any of those reports (41, 43-46, 51, 60). As expected, cycloisomerization of 2,3-dichloro-5-methylmuconate resulted in the formation of a 5-chloro-2-methyldienelactone as the only product (Fig. 6). It can be expected that this dienelactone is of a cis-configuration. Similarly, 2-chloro-5-methyl-cis-dienelactone is formed from 3,5-dichloro-2-methylmuconate (Fig. 4). However, the simultaneous formation of a 3-chloro-2-methyldienelactone (probably the trans-isomer) in amounts similar to those of 2-chloro-5-methyl-cis-dienelactone was also monitored. Thus, with this substrate chloromuconate cycloisomerase does not discriminate between the two cycloisomerization reactions.

Cycloisomerization of 2,5-dichloro-3-methylmuconate resulted exclusively in the formation of 2-chloro-3-methyl-dienelactone (Fig. 5). Thus it seems that cycloisomerization is not directed towards the methylsubstituent but involves an attack on the unsubstituted C4-carbon. Then, it should be proposed that 2,5-dichloro-3-methylmuconate is cycloisomerized to form 2,5-dichloro-3-methylmuconolactone, from which the proton is abstracted after rotation of the lactone ring in the active site to give dominantly 2-chloro-3-methyl-trans-dienelactone. However, 1H NMR analysis of this reaction revealed the formation of 2,5-dichloro-4-methylmuconolactone as an intermediate product (Fig. 2 + 5). Thus, cycloisomerization obviously occurs in both possible directions. This indicates again in-situ-1H NMR-analyses to be an appropriate tool to investigate degradation pathways of aromatics without artefacts caused by chemical transformations.

However, the preferred orientation of the substrate in the active site cannot be deduced from these experiments, due to the only intermediate nature of 2,5-dichloro-4-methylmuconolactone, which seems to be transformed by chloromuconate cycloisomerase into 2-chloro-3-methyl-trans-
dienelactone via 2,5-dichloro-3-methylmuconate and 2,5-dichloro-3-methylmuconolactone (Fig. 5). In contrast, there have been no indications that 2-chloro-4-methyl- and 4-methylmuconolactone can be degraded via 5-chloro-3-methyl- and 3-methylmuconolactone involving a cycloisomerase activity. It seemed that in those cases, cycloisomerization is quantitatively directed towards the methylsubstituent (40, 46). However, preliminary experiments indicate that at least chloromuconate cycloisomerase of strain PS12 has some activity with 2-chloro-4-methylmuconolactone, resulting in the formation of 3-methyl-cis-dienelactone as a product, which should occur via 2-chloro-4-methylmuconate and (4R,5S)-5-chloro-3-methylmuconolactone.

As no reports about the metabolism of dichloromethylmuconates were available yet, the chloromuconate cycloisomerase of strain PS12 was supposed to be a specialized enzyme, which catalyzes the cycloisomerization of these compounds. However, comparison with chloromuconate cycloisomerases from other strains with respect to their biochemical and genetic properties showed similarities to the chloromuconate cycloisomerases of the strains Pseudomonas B13 and Pseudomonas P51. All tested cycloisomerases transformed the dichloromethylmuconates to the same products, such that the capability of strain PS12 to grow on dichlorotoluenes seems to depend on the initial tetrachlorobenzene dioxygenase TecA but not on specialized enzymes in the further pathway.

**Biological fate of dichloromethylmuconolactones.** Upon acidification, substituted muconolactones were obtained from 3,5-dichloro-2-methyl- and 2,5-dichloro-3-methylmuconate analogous to the respective formation of 5-chloro-3-methylmuconolactone from 2-chloro-4-methylmuconate and 3-methylmuconolactone from 3-methylmuconate (Fig. 4 + 5) (11, 44, 51). In the acid catalyzed reaction of 2-chloro-4-methylmuconate two diastereomers of 5-chloro-3-methylmuconolactone, each comprising two enantiomers, were produced. Only (+)-diastereomer I (4R, 5R) and (-)-diastereomer II (4R, 5S) were shown to be subject to transformation by muconolactone isomerase (51). According to Vollmer et al, (4R,5S)-5-chloromuconolactone was a substrate for chloromuconate cycloisomerase and dehalogenated to form trans-dienelactone (66). Thus, (4R,5S)-5-chloromuconolactone is a substrate for both chloromuconate cycloisomerase and muconolactone isomerase, resulting however in different products. Whereas muconolactone isomerase catalyzes an anti-elimination, chloromuconate cycloisomerase catalyzes obviously a syn-elimination.
In the acid catalyzed transformation of 3,5-dichloro-2-methyl- as well as 2,5-dichloro-3-methylmuconate also two diastereomers were produced. As the respective diastereomers were not quantitatively transformed by chloromuconoate cycloisomerase, it can be proposed that only one enantiomer each is biologically active. Given the observation of Vollmer et al. (66, 68) that (4R,5S)-5-chloromuconolactone is subject to transformation, it can be speculated, that also the respective higher substituted (4R,5S) 5-chloromuconolactones are transformed. Preliminary experiments indicate, that actually (4R,5S)- and (4R,5R)-5-chloro-3-methylmuconolactone are transformed by chloromuconate cycloisomerase and thus, this enzyme necessitates the same configuration at the C4 atom as muconolactone isomerase. Surprisingly, only 2-chloro-3-methyl-trans-dienelactone is formed from both 2,5-dichloro-3-methylmuconolactone diastereomers by chloromuconate cycloisomerase. However, as suggested by Schell et al. (56) during dehalogenation of 5-chloromuconolactone, the carboxylate remains bound to the Mn$^{2+}$ ion in the catalytic site and the lactone ring rotates to bring the acidic C4 proton next to the general acid/base. Thus, for both (4R,5S)- and (4R,5R)-2,5-chloro-3-methylmuconolactone the formation of 2-chloro-3-methyl-trans-dienelactone seems reasonable. The diastereomeric mixture of 3,5-dichloro-2-methylmuconolactone was transformed in both a 3,5-dichloro-2-methyl- dienelactone isomer and 2-chloro-5-methyl-cis-muconolactone (Fig. 4). The last mentioned compound can only be reasoned to be formed by ring-opening of one of the enantiomers to reproduce the 3,5-dichloro-2-methylmuconate followed by cycloisomerization and dehalogenation. Further studies on pure enantiomers of 5-chlorosubstituted muconolactones are necessary to elucidate the underlying mechanisms of dechlorination.

In conclusion problems in degradation of dichlorinated toluenes are mainly due to the unspecific attack of TecA, misrouting part or all of the substrate in an unproductive pathway resulting in the production of dichlorobenzaldehydes or dichlorobenzoates. For naphthalene dioxygenase as well as for biphenyl dioxygenase, several amino acids were identified, that control regioselectively and enantioselectivity (37-39, 63). Based on this information, exchanges of amino acids in tetrachlorobenzene dioxygenase TecA can be performed which may prevent such a misrouting. However, even in this case, mineralization can be problematic. We had observed the intermediate accumulation of dichloromethylmuconates and chlorodi enelactones during growth of strain PS12 on dichlorotoluenes, showing that at least chloromuconate cycloisomerase activity is rate limiting for transformation. Whereas numerous studies have been performed on substrate specificities of chlorocatechol 1,2-dioxygenases, chloromuconate cycloisomerases and maleylacetate reductases, information on substrate specificities of dienelactone hydrolases is scarce, however those enzymes have not yet been described as constituting a pathway bottleneck.
Out of the dienelactones described in the present report, only 2-chloro-3-methyl-\textit{cis}-dienelactone was transformed at a low rate. Similarly, 3-methyl-\textit{cis}-dienelactone was described as a poor substrate for dienelactone hydrolase of strain JMP134 (50). It seems thus, that 3-methyl-\textit{cis}-dienelactones are generally poor substrates for dienelactone hydrolase. Applying the rule that \textit{cis}-dienelactones are formed after elimination of chloride from the $\beta$-position of chloromuconates, whereas \textit{trans}-dienelactones are formed by elimination of chloride from the $\alpha$-position, it can be deduced, that 3-methyl-\textit{cis}-dienelactones are probably formed during the degradation of 2,3- or 2,6-dichlorotoluenes via the respective chlorocatechols. Thus, studies on the degradation of those compounds would be of special interest.
FIG. 4. Proposed pathway for the degradation of 2,4-dichlorotoluene in *Ralstonia* sp. strain PS12.
FIG. 5. Proposed pathway for the degradation of 2,5-dichlorotoluene in Ralstonia sp. str. PS12.
FIG. 6: Proposed pathway for the degradation of 3,4-dichlorotoluene in *Ralstonia* sp. strain PS12.
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Chapter IV

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Directed Engineering of the Regioselectivity of TecA Tetrachlorobenzene Dioxygenase for the Transformation of Chlorinated Toluences

Katrin Pollmann#, Victor Wray, Hans-Jürgen Hecht and Dietmar H. Pieper*

Departments of Environmental Microbiology and Structural Biology, GBF - German Research Center for Biotechnology, Mascheroder Weg 1, D-38124 Braunschweig, Germany

*Corresponding author. Maili ng address: Bereich Mikrobiologie, AG Biodegradation, Gesellschaft für Biotechnologische Forschung mbH, Mascheroder Weg 1, D-38124 Braunschweig, Germany. Phone: 49/(0)531/6181-467. E-mail: dpi@gbf.de
#Present address: Institut für Radiochemie, Forschungszentrum Rossendorf, Bautzner Landstraße 128, D-01328 Dresden, Germany
4.1 Abstract

The tetrachlorobenzene dioxygenase (TecA) of *Ralstonia* sp. strain PS12 carries out the first step in the aerobic biodegradation of chlorinated toluenes. Besides dioxygenation of the aromatic ring of 4-chloro-, 2,4-, 2,5- and 3,4-dichlorotoluene as main reaction, it also catalyzes monooxygenation of the methyl groups of 2,3-, 2,6-, 3,5-di-, and 2,4,5-trichlorotoluene as the main reactions, channeling these compounds into dead end pathways. Based on the crystal structure of the related naphthalene dioxygenase (NDO) and alignment of the α-subunits of NDO and TecA the substrate pocket of TecA was reconstructed. Recently, for NDO and the related 2-nitrotoluene dioxygenase two amino acids (Phe-352 and Val-260) were identified, which control the regioselectivity of these enzymes. The corresponding amino acids at Phe-366 and Leu-272 of TecA were substituted to change the regioselectivity and to expand the product spectra. Position 352 was shown to control regioselectivity of the enzyme although mutations resulted in decreased or lost activity. Amino acid substitutions at Leu-272 had little or no effect on the regioselectivity of TecA, but had significant effects on the product formation rate. Substitutions at both positions changed slightly the site of oxidation of 2,4,5-trichlorotoluene. As new products, 3,4,6-dichloro-1-methyl-1,2-dihydroxy-1,2-dihydrocyclohexan-3,5-diene, 4,6-dichloro-3-methylcatechol, 3,6-dichloro-4-methylcatechol and 3,4-dichloro-6-methylcatechol were identified.
4.2. Introduction

The aerobic degradation of many natural and xenobiotic aromatic compounds is initialized by dioxygenases, which catalyze the incorporation of dioxygen into the aromatic ring to form arene-cis-dihydrodiols followed by a dehydrogenation reaction catalyzed by a cis-dihydrodiol dioxygenase to give catechols or substituted catechols which serve as substrates for oxygenolytic cleavage of the aromatic ring. The aromatic ring dioxygenases, such as benzoate (20), toluate (11), naphthalene (6, 7, 17), 2-nitrotoluene (22), biphenyl (9), benzene (13), chlorobenzene (37) or tetrachlorobenzene dioxygenase (1) are enzyme complexes consisting of different electron transport proteins (a ferredoxin, a reductase or a combined ferredoxin-NADH-reductase) and the terminal oxygenase (iron sulfur protein), which determines the substrate specificity of the enzyme to carry out the substrate activation (10).

The recently characterized tetrachlorobenzene dioxygenase TecA of the 1,2,4,5-tetrachlorobenzene degrading strain *Ralstonia* sp. PS12 (1) is a broad spectrum enzyme which catalyzes the first step in the degradation of a wide variety of chlorinated benzenes and toluenes (1, 19, 27, 32). Like other dioxygenases acting on hydrophobic substrates, the enzyme is composed of an electron transport chain involving a reductase and a ferredoxin and the terminal dioxygenase consisting of α- and β- subunits. The α-subunit of TecA contains a Rieske (2Fe2S) center and a mononuclear nonheme iron. Recent studies have shown that the α-subunit is responsible for determining the substrate specificity of the enzyme (2). Similarly the α-subunits have been reported to be dominantly responsible for the substrate specificities of biphenyl, naphthalene and nitrotoluene dioxygenases (14, 23, 36). Besides dioxygenation of the aromatic nucleus, TecA catalyzes monooxygenation of the methyl group of various chlorosubstituted toluenes, with the ratio of di- versus monooxygenation dependent on the substitution pattern (19, 27).

Recent analyses have shown, that only those chlorinated toluenes, which were exclusively or predominantly subject to dioxygenation, such as 4-chlorotoluene, 2,4-dichlorotoluene, 2,5-dichlorotoluene and 3,4-dichlorotoluene, can be used as growth substrates by PS12 whereas monooxygenation channels the substrates into dead end pathways (19, 28). Thus, the regioselectivity of attack as determined by TecA controls if and to what extent a substrate can be mineralized by PS12. It is, therefore, important to know which amino acids of the protein control regioselectivity and if the specificity of the enzyme can be changed to prevent or reduce monooxygenation reactions. To date the most thoroughly studied dioxygenase acting on
hydrophobic aromatics is the naphthalene dioxygenase (NDO) from *Pseudomonas* sp. strain NCIB 9816-4 (6, 7, 34), the α-subunit of which, on the amino acid level, is 36% identical to that of TecA. NDO catalyzes the oxidation of naphthalene and a wide variety of aromatic compounds as well as monooxygenation (7, 18, 24), desaturation, sulfoxidation and O- and N-dealkylation reactions with selected substrates. This enzyme has been crystallized and amino acids ligated or close to the active-site iron have been identified (5, 14). Recent studies have identified that the amino acid Phe-352 plays a major role in controlling the regioselectivity of attack on bi- or tricyclic aromatics and the stereochemistry of the products (24, 25). 2-Nitroluene dioxygenase (2NTDO) is a related enzyme, which catalyzes dioxygenation of the aromatic nucleus of 2-nitroluene (22). Substitutions in 2NTDO of Val260 for Asn were claimed to result in an enzyme, that no longer oxidized the aromatic ring but formed the monooxygenation product 2-nitrobenzyl alcohol, whereas substitution of Val for Asn in NDO were claimed not to change the specificity (24) although no detailed data were reported. Based on the sequence alignments of the α-subunits of NDO (34), 2NTDO (22), toluene dioxygenase (38) and TecA (1), the amino acids Phe and Leu in positions 366 and 272 of the TecA α-subunit could be identified as corresponding to the amino acids Phe-352 and Val-260 of NDO, respectively, and thus are promising candidates for the introduction of single mutations to change the direction of the attack.

In this study, five TecA derivatives with amino acid substitutions at Phe-366 and Leu-272 were generated and characterized.
4.3 Materials and methods

Bacterial strains, plasmids and culture conditions. Bacterial strains and plasmids used were *Escherichia coli* DH5α (Clontech) as host for cloning; pBluescript II KS(+) (Stratagene); pSTE44, carrying the genes *tec*AB coding for tetrachlorobenzene dioxygenase TecA and dihydrodiol dehydrogenase TecB (27); pKPM1 was obtained by cloning a 3976 bp *Eco*RI-fragment from a digest of pSTE44 into the *Eco*RI-site of pBluescript II KS(+); pKPM2, pKPM3, pKPM4, pKPM5 and pKPM6 were obtained by cloning the 1164 bp *Rsr*II and *Mlu*I digested 1240 bp PCR-products obtained from SOE-PCR carrying mutations in F366W, F366Y, L272W, L272F and F366L, respectively, into *Rsr*II and *Mlu*I digested pKPM1; pKPM7, pKPM9, pKPM10, pKPM11 and pKPM12 were obtained by cloning a 1922 bp *Eco*47III fragment from pKPM2, pKPM3, pKPM4, pKPM5 and pKPM6 into the *Eco*47III-site of pSTE44.

Strains were routinely grown in Luria Bertani (LB) medium containing 100 µg/ml ampicillin and incubated at 30°C on a rotary shaker operated at 130 rpm.

**DNA manipulations.** Standard procedures were performed as described by Sambroek (31). Plasmid DNA was extracted with the Plasmid Midi Kit (Qiagen, Hilden, Germany). Restriction enzymes were purchased from New England Biolabs (Frankfurt, Germany) and MBI Fermentas (St. Leon-Rot, Germany). Isopropyl-β-D-thiogalactoside (IPTG) and X-Gal were obtained from Carl Roth GmbH (Karlsruhe, Germany). Oligonucleotides were purchased from Invitrogen (Karlsruhe, Germany). Taq polymerase was obtained from Qiagen. Elution of DNA from 1% agarose gels was performed with QIAquick gel extraction kit (Qiagen). PCR products were purified with QIAquick PCR Purification Kit (Qiagen). Sequencing reactions on both strands were performed with the Applied Biosystems 3100 DNA sequencer according to the protocols of the manufacturer (Applied Biosystems, Weiterstadt, Germany) for Taq cycle-sequencing with fluorescent-dye labelled dideoxynucleotides. Site specific mutations were introduced using splicing by overlap extension (SOE)-PCR (12). Sequences of all de novo synthesized DNA molecules and correct insertion of fragments in the plasmids were confirmed by sequencing.
Oligonucleotides and Site-Directed Mutagenesis. The designation, sequence (5'→3') and priming direction of the oligonucleotide primers used for amplification of DNA fragments and SOE-PCR were as follows:

- 1970forw CATCCCCTGCAACTGGAAACT;
- 3187Rev GCTGGAAATCTCGTACTCGCCTTC;
- L272Wforw AGAACCCGATGGCTGCTCGCCAT;
- L272Wrev TGGCGAGCACTCCATCGGGTTCT;
- L260Fforw AGAACCCGATTTCCTGCTCGCCAT;
- L260Frev ATGGCGAGCAGGAATCGGGTTCT;
- F366Yforw TGCGCACCTACTCCGCTGGC;
- F366Yrev GCCAGCGGAGTAGGTGC;
- F366Wforw TGCGCACCTGGTCCGCTGGC;
- F366Wrev GCCAGCGGACCAGGTGCGCA;
- F366Lforw TGCGCACCTCTCCGCTGGC;
- F366Lrev GCCAGCGGAGAGGTGCGCA.

Nucleotides, which were changed using SOE-PCR are indicated in bold type. To obtain PCR fragments carrying the mutations, three PCR reactions for each mutation were performed. In the first reaction, a PCR fragment was amplified from pSTE44 using the 1970forw and a reverse mutagenic primer. In a second reaction, a PCR fragment was amplified from pSTE44 using the 3187rev primer and a mutagenic forward primer corresponding to the first reaction. The PCR products obtained from both reactions were purified, mixed and used as template in a third PCR reaction with 1970forw and 3187rev as primers. Each PCR reaction contained 5 µl 10x PCR buffer containing 15 mM MgCl₂, 200-500 ng template DNA, 1 pmol forward primer, 1 pmol reverse primer, 200 µM of each dNTP and 2.5 U Taq DNA polymerase in a volume of 50 µl. PCR conditions were as follows: 5 min 94 °C; 30 cycles with 1 min 94 °C, 1 min 60 °C, 1 min 72 °C; 10 min 72°C; 4°C.

Resting cell assays. E. coli strains were precultured in Luria broth medium containing 1 mM IPTG and 0.1 mg/ml ampicillin at 30°C on a rotary shaker at 130 rpm. 200 - 1000 ml of the same medium was inoculated with 1 ml of the preculture. The cells were harvested at A₆₀₀=2.7, pelleted (10 min, 20°C, 5900 x g) and washed twice with assay buffer (10 mM Glucose + 0.1 X M9 minimal medium) (31). Prewarmed assay buffer (10 – 100 ml), containing 0.5 mM of 2-chlorotoluene, 3-chlorotoluene, 4-chlorotoluene, 2,3-, 2,4-, 2,5-, 2,6-, 3,4- 3,5-dichlorotoluene or 2,4,5-trichlorotoluene (from a 100 mM stock solution in methanol) was inoculated with the
suspension to give a final $A_{600}$ of 1.8. The flasks were sealed with Teflon coated screw caps and incubated at 30 °C in an Aquatron shaker (130 rpm).

**Quantification of proteins.** To quantify the expression of TecA mutants, *E. coli* producing the wild-type TecA dioxygenase, the mutated TecA dioxygenases and, as control, *E. coli* carrying pBluescript KS(+) were grown in LB supplemented with 1 mM IPTG to an optical density of $A_{600} = 2.7$. Harvested cells were resuspended in assay buffer and disrupted using a French press (Aminco, Silver Spring, MD, USA). Cell debris was removed by centrifugation, at 100,000 x g for 40 min at 4°C. Protein concentrations in the extracts were determined by the method of Bradford using bovine serum albumin as the standard (3). Proteins of the cell extracts (corresponding to 10 µg) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), as described previously (26). For quantification of TecA, gels were stained using the fluorescent dye Sypro® Ruby (MoBiTec GmbH, Göttingen, Germany). Gels were scanned using a Fujifilm LAS-1000 CCD camera. The relative amounts of the soluble TecA $\alpha$-subunit protein band with a size of 50-51 kDa and TecB dihydrodiol dioxygenase band with a size of 31-32 kDa were determined using the AIDA 2.1 software package (Raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany).

**Analysis of transformation products by HPLC.** To monitor product formation, 200 µl aliquots were removed from resting cell assays at regular time intervals between 0-120 min and after 16 h and shock-frozen in liquid nitrogen. The samples were stored at -20°C for subsequent HPLC analyses. Use of the same bacterial host and assay conditions allowed a direct comparison of transformation rates of the different dioxygenase variants. Product formation was analyzed with a Shimadzu HPLC system (LC-10AD liquid chromatograph, DGU-3A degasser, SPD-M10A diode array detector, FCV-10AL solvent mixer) equipped with an autosampler and a sample cooler operating at 4°C on a SC125/Lichrospher 5-µm (Bischoff, Leonberg, Germany) column. The aqueous solvent system (flow rate 1 ml/min) contained 0,1% (v/v) of $\text{H}_3\text{PO}_4$ (87%) and 50% or 60% (v/v) of methanol. 10 µl of the samples were injected after removing cells by centrifugation (20°C, 10 min, 10000 x g). The alcohol products were identified and quantified by comparison with authentic standards. Concentrations of dichloromethylcatechols and 2,4,5-trichlorobenzylalcohol were determined by HPLC analyses as described previously (27). Substrate transformation rates were expressed as the amount of product formed per time as determined by HPLC.
Preparation of metabolites for GC-MS and $^1$H-NMR analysis. Resting cells (100 ml) of *E. coli* pKPM10 (L272W) were incubated with 2,4,5-trichlorotoluene or 4-chlorotoluene (0.5 mM) for 10 hours, and resting cells of *E. coli* (pSTE44) were incubated with 2-chlorotoluene or 3-chlorotoluene (0.5 mM) for 4 hours. After centrifugation (20°C, 10 min, 5900 x g) the acidified supernatants (pH 6) were extracted twice with equal volumes of ethyl acetate. The organic phases were dried over MgSO$_4$ and evaporated under vacuum on a rotary evaporator.

Characterization of transformation products by GC-MS. Metabolites formed from 2,4,5-trichlorotoluene were dissolved in 1 ml dry acetone. 50 µl of the solution was transferred to an autosampler vial and dried under a stream of nitrogen, 20 µl of the borating reagent (50 mg/ml BuB(OH)$_2$ in acetone) was added and incubated for 10 min at 50°C (15). After evaporation under a stream of nitrogen the intermediates were redissolved in 30 µl n-hexane. Products formed from 2,4,5-dichlorotoluene were analyzed by GC-MS. 1 µl of derivatized samples were injected and analyzed with a Shimadzu (Kyoto, Japan) GC-17A gas chromatograph equipped with an XTI-5 column (30 m by 0.25 mm; film thickness 0.5 µm; Resteck, Bellefonte, P.a.) and coupled to a QP-5000 quadrupole mass spectrometer. The mass spectrometer was operated in the electron impact mode at 70 eV with an ion source temperature of 320°C. Helium was used as carrier gas with a flow rate of 1 ml/min. The oven temperature was maintained at 35°C for 5 min and then increased to 100°C at a rate of 5°C/min, followed by an increase to 320°C at a rate of 10°C/min. GC-MS was operated in the splitless mode with an injection temperature of 270°C.

Characterization of chloromethylcatechols by $^1$H-NMR. Extracted metabolites were dissolved in 0.7 ml d$_6$-acetone. To prepare a defined standard of 3-chloro-4-methylcatechol (from 4-chlorotoluene), the metabolite solution was spiked with a defined concentration of 3-chlorobenzylalcohol. $^1$H nuclear magnetic resonance spectra were recorded on a Bruker DPX 300 (Bruker, Rheinstetten, Germany) using tetramethylsilane as internal standard and the concentration of 3-chloro-4-methylcatechol in the mixed sample was quantified by comparison of the resonance lines at $\delta = 4.61$ ppm (protons of the alcohol residue of 3-chlorobenzylalcohol) and $\delta = 6.59$ ppm (H-2 proton of 3-chloro-4-methylcatechol). The composition and identity of the product mixtures formed from 2- and 3-chlorotoluene were analyzed using 1D ($^1$H and nuclear Overhauser enhancement difference spectra with 10 s delays between pulses) and 2D (COSY) NMR spectra recorded on a Bruker ARX 400 NMR spectrometer.

Chemicals. 3,5-dichlorotoluene and 2,4,5-trichlorotoluene were synthesized and kindly provided by W. Reineke and S. Kaschabek. BuB(OH)$_2$ was obtained from Acros organics (Geel, Belgium).
and 2,3-dichlorobenzyl alcohol from TCI (Chemos GmbH, Regenstauf, Germany). All other chemicals were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany), or Merck AG (Darmstadt, Germany).

**Enzyme modelling.** The structure model of TecA tetrachlorobenzene dioxygenase was generated using Modeller (30) based on the naphthalene dioxygenase structures (pdb-ID’s: 1NDO and 1EG9).

### 4.4 Results

**Expression of mutant TecA α-subunits.** After electrophoretic separation and staining of proteins from cell extracts of *E. coli* cells expressing dihydrodiol dehydrogenase TecB and wild-type tetrachlorobenzene dioxygenase TecA or mutant TecA, distinct bands with a size of 50-51 kDa, which can be related to the TecA α-subunit (2), and a size of 31-32 kDa, which can be related to TecB (27), were present in all extracts except those of the control not producing TecAB. The expression of the proteins varied to some extend (rel. intensities wild-type TecA : F366W : F366Y : F366L : L272W : L272F = 100 : 90 : 98 : 66 : 72 : 59). Since all variants showed similar expression of TecA and TecB, the inability of the variants F366W and F366Y to transform chlorinated toluenes (see below) was not caused by the absence of the proteins.

**Transformation of chlorinated toluenes by TecA variants and TecB.** The potential of the TecA variants to transform various chlorinated toluenes was characterized by HPLC analysis of supernatants of resting *E. coli* cells expressing the different enzyme variants and incubated with the respective substrates. Strains producing enzymes carrying the mutations F366L, L272F and L272W were able to transform all tested substrates, that is 2-, 3- and 4-chlorotoluene, 2,3-, 2,4-, 2,5-, 2,6-, 3,4- and 3,5-dichloro- and 2,4,5-trichlorotoluene. However, the TecA variants F366W and F366Y were inactive towards all these compounds. Products formed from chlorotoluenes by the active enzymes could, in most cases, be identified by HPLC analyses through comparison of their retention volumes and UV absorption spectra with those of authentic standards (27). Like wild-type TecAB, all active variants transformed 2-, 3-chloro-, 2,3-, 2,4-, 2,5-, 2,6-, 3,4-dichloro- and 2,4,5-trichlorotoluene (either quantitatively or in part) into the corresponding benzylalcohols, and 2-, 3-, 4-chloro-, 2,4-, 2,5-, 2,6- and 3,4-dichlorotoluene (either quantitatively or in part) into the corresponding catechols. Qualitative differences were observed during 2,4,5-trichlorotoluene turnover. Whereas wild-type TecAB formed exclusively 2,4,5-trichlorobenzylalcohol by monooxygenolytic attack, four additional products were formed by the
variants F366L, L272F and L272W. Three of these products could be identified by HPLC analysis as 4,6-dichloro-3-methylcatechol (net retention volume (RV) = 14.8 ml, $\lambda_{\text{max}}$ = 205 nm, using 50% MeOH as eluent), 3,6-dichloro-4-methylcatechol (RV = 8.2 ml, $\lambda_{\text{max}}$ = 204 nm, 50% MeOH) and 3,4-dichloro-6-methylcatechol (RV = 10.4 ml, $\lambda_{\text{max}}$ = 204 nm, 50% MeOH), respectively (27). The fourth product (RV = 3.7 ml, 50% MeOH) showed an UV spectrum with $\lambda_{\text{max}}$ = 284 nm indicative of the formation of a dihydrodiol (27). Confirmation of the identity of this intermediate with a dihydrodiol was obtained by GC-MS analysis of the boronated derivatives. Besides three signals showing molecular ion masses of $m/z$ 258, 260 and 262 originating from 4,6-dichloro-3-methylcatechol, 3,6-dichloro-4-methylcatechol and 3,4-dichloro-6-methylcatechol (27), one signal showing a molecular ion mass of $m/z$ 294, 296, 298 (relative intensities, 100:94:31), indicative of a trichlorinated compound, was observed. The fragmentation pattern showed the typical characteristics described for boronated dihydrodiols formed from substituted benzenes (1, 27), that is loss of $\text{C}_4\text{H}_9 [M-57]^+$, loss of $\text{Cl-C}_4\text{H}_9 [M-91]^+$ and loss of one or two chlorines (Fig. 1). As dioxygenolytic attack on 1,2,4-trichlorotoluene involving a chlorosubstituted and an unsubstituted carbon atom, as shown above, (and as previously described for 1,2,4,5-tetrachlorobenzene transformation, (2)) in all three possible cases resulted in a spontaneous chloride elimination and concomitant formation of a catechol, the observed dihydrodiol should have been formed by a distinct dioxygenolytic attack. Thus, the product 3,4,6-dichloro-1-methyl-1,2-dihydroxy-1,2-dihydrocyclohexan-3,5-diene results from dioxygenolytic attack on a methyl substituted carbon and unsubstituted carbon atom. Such a product would not be a substrate for TecB.
Identification of 4-chloro-3-methylcatechol and 5-chloro-3-methylcatechol as the dominant dioxygenation products formed from 2- and 3-chlorotoluene, respectively. From 2-chlorotoluene, two different catechols ($\lambda_{\text{max}} = 201$ nm, RV = 4.1 ml and 6.6 ml, 50% MeOH) were formed apart from 2-chlorobenzylalcohol. Previous studies have shown the formation of 3-methyl-5-chloro-cis-1,2-dihydroxy-1,2-dihydrocyclohexa-3,5-diene and 5-methyl-3-chloro-cis-1,2-dihydroxy-1,2-dihydrocyclohexa-3,5-diene by TecA mediated dioxygenation (19). Thus, the formation of 3-chloro-4-methylcatechol and 4-chloro-3-methylcatechol from the dihydrodiols by TecB dehydrogenase or from 2-chlorotoluene by the activity of the TecAB enzyme system may be assumed (Fig. 2). Assuming similar extinction coefficients at 210 nm, the two catechols (RV = 4.1 ml and RV = 6.6 ml) were formed in a 1:4 ratio. Similarly, from 3-chlorotoluene two products ($\lambda_{\text{max}} = 201$ nm, RV = 4.1 ml and 6.2 ml, 50% MeOH) were formed apart from 3-chlorobenzylalcohol which may be assumed to be 3-chloro-5-methylcatechol and 5-chloro-3-methylcatechol (Fig. 2). Those two catechols (RV = 4.1 ml and RV = 6.2 ml) were formed in a 1:10 ratio.

The $^1$H-NMR spectrum of the product mixture formed from 3-chlorotoluene showed the presence, in addition to 3-chlorobenzylalcohol, of one major catechol derivative with two aromatic protons resonating at 6.64 and 6.71 ppm (Fig. 2). The coupling constant of 2.3 Hz indicate these are located in meta-position to one another. The methyl group of the chloromethylcatechol resonates at 2.18 ppm and upon low power irradiation affords a nuclear Overhauser enhancement (nOe) only to the signal at 6.64 ppm. This is unambiguous evidence that only this proton is located in an ortho-position with respect to the methyl substituent and thus the product is 5-chloro-3-methylcatechol.


<table>
<thead>
<tr>
<th>m/z</th>
<th>0</th>
<th>75</th>
<th>111</th>
<th>123</th>
<th>159</th>
<th>168</th>
<th>177</th>
<th>203</th>
<th>237</th>
<th>259</th>
<th>261</th>
<th>294</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rel. Abundance</td>
<td>50-</td>
<td>50-</td>
<td>30-</td>
<td>10-</td>
<td>10-</td>
<td>5-</td>
<td>5-</td>
<td>10-</td>
<td>10-</td>
<td>5-</td>
<td>5-</td>
<td>5-</td>
</tr>
</tbody>
</table>

- Fig. 2: Diagram showing the mass spectrum of the boronated product formed from 2,4,5-trichlorotoluene by TecA L272W, L272F and F366L variants. The spectrum indicates the relative abundances of various m/z values, with 5-chloro-3-methylcatechol and 4-chloro-3-methylcatechol being the dominant products.
The major catechol formed from 2-chlorotoluene exhibited the signals of a methyl group at 2.24 ppm and two ortho aromatic protons at 6.69 and 6.72 ppm with coupling constants of 8.6 Hz (Fig. 2). These signals obviously overlap with those of one signal of a further aromatic system of a second catechol derivative present in the mixture. The second aromatic proton of this compound resonates at 6.64 ppm. Low power irradiation of the methyl signal at 2.24 ppm afford an nOe only to the small signal at 6.64 ppm. Thus, the major catechol derivative formed from 2-chlorotoluene is 4-chloro-3-methylcatechol, and the derivative formed in minor amounts is 3-chloro-4-methylcatechol.

**FIG. 2.** $^1$H-NMR data of chloromethylcatechols formed from 2-chlorotoluene and 3-chlorotoluene. Chemical shifts (in ppm) are indicated as well as coupling constants (in Hz), with the respective coupling protons. Signal assignments were evident from the $^1$H nOe difference data and from the detectable long-range couplings of ortho and para protons to their respective methyl groups.
FIG. 3. Products and relative product distributions (%) formed by wild-type and mutant TecA enzymes with different chlorinated toluenes as substrates. A: 2-chlorotoluene; B: 3-chlorotoluene; C: 2,4-dichlorotoluene; D: 2,5-dichlorotoluene; E: 2,6-dichlorotoluene; F: 2,4,5-trichlorotoluene. The data shown are averages from three independent experiments.
Regioselectivity of chlorotoluene transformation by modified TecA proteins. Products formed from chlorotoluenes, dichlorotoluenes and 2,4,5-trichlorotoluene were quantified by comparison with authentic standards in the case of mono- and dichlorobenzylalcohols or as previously described (27) in the case of dichloromethylcatechols or 2,4,5-trichlorobenzylalcohol. For quantification of chloromethylcatechols formed from 2- and 3-chlorotoluene, a standard of 3-chloro-6-methylcatechol was prepared and the concentrations of the differently substituted chloromethylcatechols was determined, assuming similar absorption at $\lambda = 210$ nm for all the monochlorinated methylcatechols.

Wild-type TecAB oxidized 2,4-, 2,5- and 2,6-dichlorotoluene to 94:6, 81:19 and 11:89 mixtures of the corresponding benzylalcohols and substituted catechols, respectively (Fig. 3). In the case of 2-chlorotoluene, 2-chlorobenzylalcohol was the main product (64.5 %) and in addition 3-chloro-4-methylcatechol and 4-chloro-3-methylcatechol were produced in a 7:29 ratio. The mixture produced from 3-chlorotoluene consisted of 3-chlorobenzylalcohol as main product (80 %) and byproducts of 3-chloro-5-methylcatechol and 5-chloro-3-methylcatechol in a 2:18 ratio. For 2,4,5-trichlorotoluene monooxygenation of the methyl group produced 2,4,5-trichlorobenzylalcohol exclusively (Fig.3).

Whereas two out of the three mutants carrying substitutions in position F366, i.e. F366W and F366Y did not form detectable products from any tested substrate, the F366L mutant was active with all substrates and showed significant changes in regioselectivity. In contrast to wild-type TecA, 2,5-dichlorobenzylalcohol was the major product (54 %) formed from 2,5-dichlorotoluene. Similarly in the case of the other chloro- and dichlorotoluenes tested, the direction of attack usually shifted significantly towards monooxygenation to form the corresponding benzyl alcohols (Fig. 3). In contrast, for 2,4,5-trichlorotoluene significant amounts of dioxygenation products (comprising about 14 % of the products) were formed.

TecAB mutant enzymes with changes at position 272 differed only slightly from the wild-type enzyme with regard to the regioselectivity of attack (Fig. 3). However, similar to the F352L variant, significant amounts of products resulting from a dioxygenolytic attack were formed from 2,4,5-trichlorotoluene.

Relative activities of the mutant TecA enzymes. Whereas the F366W and F366Y variants were not active with any of the substrates, the F366L variant converted the substrates at
significantly reduced rates of 0.2–25% compared to those of wild-type TecA (Table 1). Thus, all enzymes with substitutions at position 366 were severely defective in catalyzing oxygenation of the chlorinated toluenes. Interestingly, 3,4-dichlorotoluene, which was exclusively dioxygenated, was transformed by F366L at slightly reduced rates.

Enzymes with substitutions in position 272 showed high activity with all tested substrates (Table 1). Whereas transformation rates with monochlorinated toluenes were, at a first glance, significantly reduced compared to wild-type TecA (32-64%), transformation rates of dichlorotoluenes were mostly elevated. However, taking into account the lower expression level of the L272F and L272W enzyme variants (60-70% of wild-type TecA, see above), these mutants showed approximately the same activity with chlorotoluenes as the wild-type enzyme. In particular the variant L272W showed increased transformation rates with 2,3-, 2,5-, 2,6, 3,4- and 3,5 dichlorotoluene (200-380% of wild-type TecA).

### TABLE 1. AbsOLUTE and RELATIVE RATES of Transformation of Chlorinated Toluenes Catalyzed by Wild-type and Mutant TecA Enzymes.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product formation (µM/min)</th>
<th>(relative transformation rate [%])</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TecAB&lt;sup&gt;a&lt;/sup&gt;</td>
<td>L272W</td>
</tr>
<tr>
<td>2-CT</td>
<td>5.5 (289)</td>
<td>2.2 (88)</td>
</tr>
<tr>
<td>3-CT</td>
<td>7.4 (389)</td>
<td>4.7 (189)</td>
</tr>
<tr>
<td>4-CT</td>
<td>5.7 (300)</td>
<td>1.8 (74)</td>
</tr>
<tr>
<td>2,3-DCT</td>
<td>0.6 (32)</td>
<td>1.2 (50)</td>
</tr>
<tr>
<td>2,4-DCT</td>
<td>1.0 (53)</td>
<td>1.8 (72)</td>
</tr>
<tr>
<td>2,5-DCT</td>
<td>1.0 (53)</td>
<td>3.8 (155)</td>
</tr>
<tr>
<td>2,6-DCT</td>
<td>0.7 (37)</td>
<td>1.4 (57)</td>
</tr>
<tr>
<td>3,4-DCT</td>
<td>1.3 (68)</td>
<td>2.8 (114)</td>
</tr>
<tr>
<td>3,5-DCT</td>
<td>0.6 (32)</td>
<td>1.4 (55)</td>
</tr>
<tr>
<td>2,4,5-TCT</td>
<td>1.9 (100)</td>
<td>2.5 (100)</td>
</tr>
</tbody>
</table>

<sup>a</sup>The data for the DCTs and 2,4,5-TCT are from Pollmann et al. (24). All rates were determined at a cell density producing an A<sub>600</sub> of 1.8.
4.5 Discussion

**General docking of substrates and mechanism of action.** Based on the crystal structure of the related naphthalene dioxygenase, it was possible to model the substrate pocket and the active site of TecA. This model gave information about the docking of the chlorinated toluenes and the mechanism of action.

As described for the naphthalene dioxygenase (5) the surface of the substrate pocket of TecA is mainly hydrophobic with the exception of the iron center and a polar region located on the bottom of the pocket. On one side of the iron, an elongation of the cavity is formed by the side-chains from several residues, among them Phe-366 (Fig. 4). The distribution of products and product yields points to a preferred orientation of substrates such that at least one chloride points preferentially not towards Phe366 but towards the opposite side of this elongation and might interact with the side-chains there. In this position, the space between substrate and iron is suitable for a dioxygen bridge, initializing the transformation of the substrate. The methyl groups of the substrates 2-, 3-chloro-, 2,3-, 2,6-, 3,5-dichloro- and 2,4,5-trichlorotoluene are orientated preferentially towards the elongation of the cavity, thus having suitable space for interaction with the side chain of Phe-366 (Fig. 4A, C-D). In this position the methyl group is close to the iron of the active site, favoring monooxygenolytic attack. In contrast, 4-chloro-, 2,4-dichloro-, 2,5-dichloro- and 3,4-dichlorotoluene are in a reverse orientation that affords preferential dihydroxylation of the aromatic ring with the methyl group pointing towards the opposite side of the active site (Fig. 4B).
FIG. 4: Binding of chlorinated toluenes into the active site of TecA. The preferential binding of A: 3-chlorotoluene, B: 2,5-dichlorotoluene, C: 2,6-dichlorotoluene D: 2,4,5-trichlorotoluene into the substrate pocket (blue) of different L272 variants (L272W/F) is shown. The orientation of the methyl group towards the iron (the ligation with two His residues and an oxygen is drawn) is preferred in cases A, C and D, forming an oxygen bridge with the iron, whereas 2,5-dichlorotoluene is orientated towards the iron preferentially with its aromatic ring. Blue: positively charged surface; red: negatively charged surface; pink colored atoms: chlorine. (Prepared with Molscript (16), GRASP (21) and rendered with POV-Ray (TM), www.povray.org.)
**Effect of position 366.** The hydrophobic residue Phe366 was substituted by the larger hydrophobic amino acids tryptophane or tyrosine in order to change the size of the pocket close to the active site and thus the orientation of the chlorinated toluenes towards the active site by distancing the methyl group from the iron, thus favoring a dioxygenolytic attack of the aromatic nucleus. However, substitutions at this position resulted in a loss of activity. It may be assumed, that introduction of these larger side chains reduced the pocket’s size to such an extend that productive binding of substrates is prevented.

When Phe366 was exchanged by the smaller hydrophobic amino acid leucine, the enzyme retained its activity, although it was significantly decreased. In contrast to the wild type, the mutant enzyme preferentially catalyzed monooxygenation of 2,5-dichlorotoluene and in the case of 2-, 3-chloro-, 2,4- and 2,6-dichlorotoluene the direction of attack also shifted towards the methyl group. Introduction of a smaller residue expands the pocket near the active site, facilitating an orientation of the methyl group towards the iron of the active site (Fig. 5), though significantly reduced activities point to a suboptimal binding of the substrates. The space might be too large for fixing the substrate into a position suitable for transformation. In contrast to chloro- and dichlorotoluenes, the direction of attack shifted towards dioxygenation of the aromatic ring with 2,4,5-trichlorotoluene as substrate. Obviously, the mutation (as well as the mutations in position 272) facilitates multiple orientations of the substrate in the substrate pocket (Fig. 5). However, the enzymes activity was also significantly reduced for 2,4,5-trichlorotoluene.

Similarly, the variants F352W and F352Y of naphthalene dioxygenase were unable to transform naphthalene, biphenyl and phenanthrene, whereas other variants showed reduced rates, but were still efficient in catalyzing substrate transformation (25). These mutants exhibited significantly different regioselectivity of attack.
FIG. 5: Binding of 2,4,5-trichlorotoluene and 2,5-dichlorotoluene into the active site of the F366L variant. The surface of the substrate pocket of the F366L mutant is shown. A: Binding of 2,4,5-trichlorotoluene; the substrate has multiple orientations towards the iron (the ligation with two His residues and an oxygen is drawn) due to an expansion of the substrate pocket (white). B: Binding of 2,5-dichlorotoluene; the orientation of the methyl group of the substrate molecule is shifted towards the iron due to an expansion of the substrate pocket. Pink colored atoms: chlorine. (Prepared with Molscript (16), GRASP (21) and rendered with POV-Ray (TM), www.povray.org.)

Effect of position 272. Substitutions of valine for Asn-260 in 2-nitrotoluene dioxygenase have indicated that this residue controls regioselectivity of the enzyme. Therefore, it was thought that replacement of the corresponding Leu-272 in TecA by larger hydrophobic amino acids such as phenylalanine and tryptophane would change the orientation of the aromatic ring of the chlorinated toluenes towards the active site, thus favoring dioxygenolytic attack. However, experiments showed only a slight effect on the regioselectivity for some substrates, although the mutants catalyzed transformation of dichlorotoluenes significantly more efficiently. The side chains Phe and Tyr do not project in the hydrophobic cavity as expected but seem to interact with adjacent side-chains such that the methyl groups of the substrates have still enough space to orientate towards the iron of the active site. The increased activity of the mutants, especially that of mutant L272W is indicative of a more stable binding of the substrates or an improved orientation of the dichlorotoluene towards the active site facilitating the formation of a dioxygen bridge.

Relative transformation rates indicate a more efficient transformation of dichlorotoluenes than the monochlorinated compounds. Thus, mutations at position 272 result in enzymes with
increased tendency to oxidize highly substituted toluenes, probably due to a better binding of the substrate into the active site.

**Rational engineering of TecA.** Recently a number of strategies have been used to expand the substrate spectra of a variety of dioxygenases. Mutants of toluene dioxygenase were generated by random and saturation mutagenesis that exhibited higher activity towards toluene and 4-picoline (29). Mutants of biphenyl dioxygenases with enhanced substrate specificity and changed regioselectivity were obtained by random and site directed mutagenesis and gene shuffling (4, 8, 35). In this study, rational design was used in order to change the product spectra of tetrachlorobenzene dioxygenase (TecA). For naphthalene dioxygenase, several amino acids of the active site were identified, out of which F352 and Val260 were shown to control regioselectivity (24). Based on the crystal structure of NDO (5) and alignments, we could reconstruct the substrate pocket of TecA. With this information it was possible to identify amino acids that may contribute to regioselectivity and activity. Among the, the residue Phe-366 was shown to control regioselectivity. However, mutations at Phe-366 resulted in loss or decrease of activity. In contrast, changes at Leu-272 resulted in increased efficiency but only slight changes of regioselectivity. Even though much information is known about an enzyme, it is difficult to predict the effects of substitutions. Additionally the limited range of possible substitutions hinders the rational design of improved enzymes. Thus, many attempts to alter specifically the properties of enzymes by rational design of new enzymes have failed because small changes in amino acid composition had unexpected effects on the structure and function of the target enzyme (33).

Under the assumption that a chloride of the chlorinated toluenes interacts with side-chains of residues on the opposite site of the F366 pocket, mutations in this region to improve this interaction might be more successful. Recently, Beil (1) has identified residue 220, which forms a part of this region, to be responsible for dechlorination of 1,2,4,5-tetrachlorobenzene and thus enabling the strain PS12 to mineralize this substrate. However, further investigations are necessary.

**Acknowledgements**

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Chapter V

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Chloromethylmuconolactones as Critical Metabolites in the Degradation of Chloromethylcatechols: Why 2-Chlorotoluene is so Difficult to Degrade

Katrin Pollmann#, Victor Wray and Dietmar H. Pieper*

Department of Environmental Microbiology, GBF - German Research Center for Biotechnology, Mascheroder Weg 1, D-38124 Braunschweig, Germany

*Corresponding author. Mailing address: Bereich Mikrobiologie, AG Biodegradation, Gesellschaft für Biotechnologische Forschung mbH, Mascheroder Weg 1, D-38124 Braunschweig, Germany. Phone: 49/(0)531/6181-467. E-mail: dpi@gbf.de

#Present address: Institut für Radiochemie, Forschungszentrum Rossendorf, Bautzner Landstraße 128, D-01328 Dresden, Germany
4.1 Abstract

2- and 3-chlorotoluene besides being monooxygenated, are subject to dioxygenation by tetrachlorobenzene dioxygenase with 4-chloro-3-methyl-/3-chloro-4-methylcatechol and 5-chloro-3-methyl-/3-chloro-5-methylcatechol formed as product after dehydrogenation. Out of these catechols, only 5-chloro-3-methylcatechol, the major dioxygenation product formed from 3-chlorotoluene, is subject to quantitative dehalogenation after successive transformation by chlorocatechol 1,2-dioxygenase and chloromuconate cycloisomerase resulting in the formation of 2-methylidenelactone. 3-Chloro-5-methylcatechol is transformed to 2-chloro-4-methylmuconolactone. None of the two cycloisomerization products formed during 3-chloro-4-methylcatechol transformation is subject to dehalogenation, such that this catechol obviously cannot be mineralized by reaction sequences related to catechol ortho-cleavage pathways known thus far. 4-Chloro-3-methylcatechol suffers only poor dehalogenation during enzymatic processing, due to the kinetic properties of chloromuconate cycloisomerases analyzed. Thus, degradation of 2-chlorotoluene via a dioxygenolytic pathway is evidently problematic.
In the recent years an overwhelming amount of microorganisms capable to degrade various chlorinated aromatics have been described (4). As an example bacterial isolates are available for the degradation of all mono- and all dichlorobenzoates, with the exception of 2,6-dichlorobenzoate (7-9, 15). Also for the majority of chlorobenzene congeners (chlorobenzene, all dichlorobenzenes, 1,2,4-trichlorobenzene, 1,2,4,5- and 1,2,3,4-tetrachlorobenzene (25, 28, 29)) or chlorotoluene congeners (3- and 4-chlorotoluene as well as 2,4-2,5-, 3,4-, 3,5-dichlorotoluene (1, 5)) isolates capable to use those compounds as sole carbon and energy source have been isolated. It is thus surprising, that despite the severe attempts to isolate bacteria with new metabolic properties, and the broad capabilities and plasticity of microorganisms, some simple chloroaromatics such as 2-chlorotoluene obviously are highly recalcitrant, and no organisms capable to mineralize them have been isolated thus far.

Two distinct metabolic routes have been described for the metabolism of chlorotoluenes. Degradation can be initiated by dioxygenation, such that the methylsubstituent stays intact and the degradation occurs via the respective chloromethylsubstituted catechols (5). Alternatively, degradation can be initiated via oxidation of the methylsubstituent, such that the substituent is eliminated during further processing reactions resulting in chlorocatechols as intermediates (1). Both such routes were shown to be productive for the mineralization of 4-chlorotoluene.

We had determined previously, that only those chlorotoluenes, which are subject to dioxygenation by tetrachlorobenzene dioxygenase of Ralstonia sp. PS12 are used as growth substrates by this strain (23). Monooxygenation, the dominant reaction catalyzed by tetrachlorobenzene dioxygenase with 2-chloro-, 3-chloro- or 2,3-, 2,6- and 3,5-dichlorotoluene (22) as substrates, results in misrouting of the substrates into a dead-end route. However, pathways via monooxygenation of the side chain of chlorotoluenes have been reported to be functional for 3-chloro- 4-chloro- as well as 3,5-dichlorotoluene in strains harboring the TOL plasmid encoded upper pathway for the transformation of the substrates into the respective benzoates and catechols, and harboring a chlorocatechol pathway for degradation of chlorocatechols (1). Due to the restricted substrate specificity of toluene monooxygenase and of toluate dioxygenase, 2-chlorotoluene cannot be degraded by such strains, and engineering for the purpose of 2-chlorotoluene degradation via a monooxyenolytic pathway failed thus far (6). Recently the optimization of chlorotoluene degradation by rational engineering of the regioselectivity of toluene dioxygenases to avoid monooxygenation, was suggested (24). Such a strategy necessitates, that the supposed central intermediates, chloromethylcatechols are easily degradable. Commonly, the degradation of chlorocatechols requires the elimination of a
chlorosubstituent from chloromuconates (formed after intradiol cleavage) by chloromuconate cycloisomerase and the formation of (substituted) dienelactones. Dehalogenation can occur either directly, as is the case for 3-chloromuconate, resulting in the formation of cis-dienelactone, or via a chloromuconolactone intermediate, as is the case for 2-chloromuconate transformation, with dehalogenation occurring after abstraction of a proton of intermediate 5-chloromuconolactone, with the formation of trans-dienelactone (11, 31). Dehalogenation thus requires either a cycloisomerization towards a chlorosubstituted carbon atom or towards an unsubstituted carbon atom, allowing proton abstraction and dehalogenation. As cycloisomerization of unsymmetrically substituted muconates can occur in two different directions (1,4- as well as 3,6-cycloisomerization), the cycloisomerization direction can critically determine if dehalogenation is possible. The importance of proper cycloisomerization for degradation has been shown by Pieper et al. (18, 19) for the metabolism of chloro- and methylsubstituted phenoxyacetates. Whereas 4-chloro-2-methylphenoxyacetate is mineralized by *Ralstonia eutropha* JMP134 (18) and cycloisomerization of intermediary 4-chloro-2-methylmuconate can be assumed, by analogy with the transformation of 3-chloromuconate by this strain, to result in the formation of 2-methyl-cis-dienelactone, which should be a substrate for dienelactone hydrolase. 2-Chloro-4-methylphenoxyacetate, is not mineralized but converted into 2-chloro-4-methylmuconolactone as dead-end product evidencing that mineralization can only be achieved if cycloisomerization proceeds in a way allowing dehalogenation during cycloisomerization (19). In analogy to problems encountered during the metabolism of chloromethylphenoxyacetates, it can similarly be assumed, that chloromethylcatechols formed during the metabolism of chlorotoluenes are transformed into dead-end products by enzymes of chlorocatechol pathways.
Transformation of chloromethylcatechols by chlorocatechol 1,2-dioxygenases and chloromuconate cycloisomerases. To elucidate the metabolic fate of chloromethylcatechols, a mixture of 3-chloro-4-methyl- and 4-chloro-3-methylcatechol was prepared from 2-chlorotoluene and a mixture of 3-chloro-5-methyl and 5-chloro-3-methylcatechol was prepared from 3-chlorotoluene using *E. coli* (pSTE44) as previously described (23) (Fig.1).

Chlorocatechol 1,2-dioxygenase and chloromuconate cycloisomerase were partially purified from *Ralstonia eutropha* JMP 222 (pBBR1-MI) (21) and PS12 (23) by anion exchange chromatography on a MonoQ HR 5/5 column as described previously. For subsequent HPLC analysis, 0.1 ml aqueous solutions of chloromethylcatechol mixtures (corresponding to concentrations of approx. 0.2 mM) were supplemented with an equal volume of Tris/HCl (pH 7.5, 100 mM) and 2 mM MnCl₂. Samples were then supplemented with chlorocatechol 1,2-dioxygenase (10 – 50 mU) and either acidified (pH 4) or further supplemented with the respective chloromuconate cycloisomerase (10 - 50 mU). Samples were analyzed by HPLC as described previously, (24) using an acidified aqueous solvent system as described containing 50 % or 25 % (v/v) of methanol.
The substrate mixtures were analyzed with a solvent system containing 50 % (v/v) of methanol and consisted, as previously described (24) of 2-chlorobenzylalcohol (net retention volume using 50 % of methanol as eluent, RV\textsubscript{50% MeOH} = 3.8 ml), 3-chloro-4-methylcatechol (RV\textsubscript{50% MeOH} = 3.5 ml) and 4-chloro-3-methylcatechol (RV\textsubscript{50% MeOH} = 5.6 ml) (from 2-chlorotoluene) and of 3-chlorobenzylalcohol (RV\textsubscript{50% MeOH} = 4.3 ml), 3-chloro-5-methylcatechol (RV\textsubscript{50% MeOH} = 3.7 ml) and 5-chloro-3-methylcatechol (RV\textsubscript{50% MeOH} = 5.6 ml) (from 3-chlorotoluene). Upon addition of chlorocatechol 1,2-dioxygenase, all 4 chloromethylcatechols were transformed, whereas 2- and 3-chlorobenzylalcohol remained unchanged. The products formed from 3-chloro-5-methyl-/5-chloro-3-methylcatechol after successive ring-cleavage and cycloisomerization could easily be characterized by comparison with authentic standards. 2-Chloro-4-methylmuconolactone, 5-chloro-4-methylmuconolactones as well as 3-methyl-\textit{cis}- and 3-methyl-\textit{trans}-dienelactone were available from previous preparations (19, 27), whereas 2-methyl-\textit{cis}- as well as 5-methyl-\textit{cis}-dienelactone (synthesized as previously described (10, 23)) were kindly supplied by Walter Reineke and Stefan Kaschabek. No significant difference was observed between experiments performed with JMP222 (pBBR1-MI) derived or PS12 derived enzymes. 2-Methyl-\textit{cis}-dienelactone (\(\lambda_{\text{max}} = 291\) nm, RV\textsubscript{25% MeOH} = 5.6 ml) dominated the product mixture and 2-methyl-\textit{trans}-dienelactone (\(\lambda_{\text{max}} = 291\) nm, RV\textsubscript{25% MeOH} = 2.3) was formed in amounts 10 ± 2 % those of the \textit{cis}-isomer. Both those compounds evidently originated from 3-methyl-5-chlorocatechol and can supposed to be formed by 1,4-cycloisomerization of 2-methyl-4-chloromuconate. 2-Chloro-4-methylmuconolactone (\(\lambda_{\text{max}} = 220\) nm, RV\textsubscript{25% MeOH} = 2.6) was observed to be formed in minor amounts, representing 5 ± 2 % of the product mixture. Two new products M1 (\(\lambda_{\text{max}} = 273\) nm, RV\textsubscript{50% MeOH} = 4.2 ml, RV\textsubscript{25% MeOH} = 27.6 ml) and M2 (\(\lambda_{\text{max}} = 278\) nm, RV\textsubscript{50% MeOH} = 2.1 ml, RV\textsubscript{25% MeOH} = 10.7) were formed by transformation of 3-chloro-4-methyl-/4-chloro-3-methylcatechol and should be identical to chloromethylmuconates. Addition of chloromuconate cycloisomerase resulted in the formation of one obviously dominant product L1 (\(\lambda_{\text{max}} = 223\) nm, RV\textsubscript{25% MeOH} = 3.9 ml), besides three obviously minor products L2 (\(\lambda_{\text{max}} = 291\) nm, RV\textsubscript{25% MeOH} = 6.2 ml), L3 (\(\lambda_{\text{max}} = 223\) nm, RV\textsubscript{25% MeOH} = 2.5 ml), and L4 (\(\lambda_{\text{max}} = 206\) nm, RV\textsubscript{25% MeOH} = 1.7 ml). The absorption spectra indicated L1, 3 and 4 to be identical with muconolactones. L2 could be identified as 5-methyl-\textit{cis}-dienelactone, by comparison with an authentic standard. Acidification of the reaction mixture containing M1 and M2 prevented the formation of 5-methyl-\textit{cis}-dienelactone.
**CHAPTER V**

**METABOLISM OF 2-CHLOROTOLUENE**

Elucidation of the structure of products formed from 3-chloro-4-methyl-/4-chloro-3-methylcatechol. To elucidate the structure of the cycloisomerization products formed from chloromethylcatechols arising from 2-chlorotoluene, 20 ml of a solution containing approx. 0.5 mM of chloromethylcatechols in Tris/HCl (pH 7.5, 50 mM + 2 mM MnCl₂) were supplemented with 3 U of JMP 222 (pBBRMI) derived chlorocatechol 1,2-dioxygenase and 2 U of JMP 222 (pBBRMI) derived chloromuconate cycloisomerase. After complete transformation of chloromethylcatechols and M2 as well as partial transformation of M1, 80% of the reaction mixture was extracted at different pH values to allow differential extraction of reaction products. By extracting twice with an equal volume of ethylacetate at pH 6.5, 2-chlorobenzylalcohol could be eliminated. The extract obtained by extracting at pH 3.8 (extract 1) contained, as measured by HPLC, L1 and L3 in a ratio similar to their presence in reaction mixture, beside a slightly elevated amount of L2 (5-methyl-cis-dienelactone). The residue obtained by extracting at pH 2 (extract 2) contained L1 and L3 in a ratio similar to the reaction mixture, beside a slightly reduced amount of L2. Exclusively this extract contained M1 as well as L4. The rest of the reaction mixture was extracted after complete transformation of M1: the reaction mixture was extracted twice after acidification to pH 6.5 to eliminate residual 2-chlorobenzylalcohol. Then, the reaction products were extracted after acidification of the reaction mixture to a pH of 2. The extract (extract 3) contained L1 through L4 in ratios similar to the reaction mixture.

¹H NMR analysis was performed on all three extracts comprising different amounts of intermediates with d6-acetone as solvent. The composition and identity of the product mixtures were analyzed using 1D (¹H and nuclear Overhauser enhancement difference spectra with 10 s delays between pulses) and 2D (COSY) NMR spectra recorded on a Bruker AVANCE DMX 600 NMR spectrometer (Bruker, Rheinstetten Germany). Signals arising from 5-methyl-cis-dienelactone were identified by comparison with an authentic standard. Two olefinic protons resonate at 6.52 and 8.41 ppm, respectively, and the small coupling constant of 5.6 Hz indicates the presence of the olefinic system in a closed five-membered ring-system (16). Protons of the methylgroup resonate at 2.09 ppm. This compound could be estimated to comprise 3%, 4% and 4% respectively of the products present in the different extracts. The product dominating in all extracts showed an NMR spectrum similar to those previously reported for 2-methyl- (12), 3-methyl- (17) and 2,3-dimethylmuconolactone (33) and evidently contains a CH₂-CH coupling system. Two protons of a methylene group resonate at 2.66 and 3.07 ppm, respectively. The signals were split into doublets of doublets due to a geminal coupling of 16.7 Hz and vicinal couplings of 8.2 or 3.6 Hz, respectively. A proton resonating at 5.34 ppm showed the respective
vicinal coupling constants of 8.2 and 3.6 Hz, in addition to a homoallylic coupling of 1.8 Hz with a methylsubstituent (1.85 ppm). Such a homoallylic coupling can be supposed to occur only in 3-chloro-2-methylmuconolactone. Moreover, the chemical shift is similar to those of 2-methylsubstituted muconolactones (12, 20, 33), but significantly lower than those of 3-methylsubstituted muconolactones (17, 20, 33), evidencing the structure of the dominant cycloisomerization product to be 3-chloro-2-methyl- and not 2-chloro-3-methylmuconolactone (Fig. 2). This postulation was confirmed by low power irradiation of the methyl signal which did not afford any nOe. 3-chloro-2-methylmuconolactone was thus the major product comprising 70–80% of all three product mixtures, and is thus identical to the product previously designated L1.

A third product showed a $^1$H NMR spectrum very similar to that of 3-chloro-2-methylmuconolactone by comprising an ABX system. It differed from the major metabolite by the chemical shift of the methyl protons (2.17 ppm) and the lower coupling of the methyl protons of 0.9 ppm (Fig. 2). Such behavior is in accordance with its structure to be 2-chloro-3-methylmuconolactone. In accordance with the postulated structure, low power irradiation of the methyl signal afforded an nOe at the signal at 5.40 ppm. 3-chloro-2-methylmuconolactone was comprising 15–20% of all three product mixtures, and is thus identical to the product previously designated L3.

A fourth product (L4) was present in the extracts 2 and 3 only. Its NMR characteristics were similar to those previously reported for 5-chloromuconolactone and 5-chloro-3-methylmuconolactones (19, 38) by absence of the typical AB spectrum of two diastereotopic methylene protons. In accordance with a chlorine substituted 4-carboxymethyl side-chain, a single proton with a chemical shift of 4.77 ppm was observed. Two olefinic protons resonate at 6.22 and 7.77 ppm with a small coupling constant of 5.7 Hz indicative for the presence of the olefinic system in a closed five-membered ring-system. In accordance with the postulated structure (5-chloro-4-methylmuconolactone), low power irradiation of the methyl signal afforded nOes at the signals at 4.77 and 7.77 ppm. No further signals with chemical shifts similar to 4.77, 6.22 and 7.77 ppm were observed, excluding the presence of a diastereomeric mixture of 5-chloro-4-methylmuconolactone (Fig. 2). Thus, this compound was obviously produced by enzymatic action. Extract 3 was shown to contain about 5% of this metabolite.

A fifth product (M1) was observed in extract 2, only. Two olefinic protons resonate at 6.36 and 8.29 ppm, respectively, and the large coupling constant of 15 ppm indicate that they are located
in an open chain configuration, as present in muconates (32). The absence of any NOe upon low power irradiation of the methyl signal at 2.25 ppm indicate the structure of M1 to be 3-chloro-2-methylmuconate (Fig. 2).

**Fig. 2.** Metabolic fate of 3-chloro-4-methyl-, 4-chloro-3-methyl-, 3-chloro-5-methyl- and 5-chloro-3-methylcatechol, when subjected to intradiol cleavage by chlorocatechol dioxygenase and cycloisomerization by chloromuconate cycloisomerase.

**Bottlenecks in the degradation of chloromethylcatechols.** We had previously reported that 2-chlorotoluene, by tecA dioxygenase is dominantly monooxygenated to form 2-chlorobenzylalcohol (13), but more than 30% of the substrate is subject to dioxygenation with 3-chloro-4-methylcatechol and 4-chloro-3-methylcatechol being formed in an approximately 1:4 ratio (24) (Fig. 1). Similarly, a minor amount of 3-chlorotoluene (aprox. 20%) is subject to dioxygenation, giving rise to mainly 5-chloro-3-methylcatechol (24) (Fig. 1). As suggested previously for the degradation of 4-chloro-2-methylphenoxyacetate, 4-chloro-2-methylmuconate, the ring-cleavage of 5-chloro-3-methylcatechol is exclusively subject to 1,4-cycloisomerization with 2-methyl-cis-dienelactone as dominant cycloisomerization product (18, 19). 2-Methyl-cis-dienelactone, in turn is a subject for dienelactone hydrolase (data not shown) such that mineralization of 5-chloro-3-methylcatechol, in contrast to 3-chloro-5-methylcatechol should be rather easily achieved. However, obviously, neither 3-chloro-4-methylcatechol nor 4-chloro-3-methylcatechol obviously can be mineralized by enzymes of the chlorocatechol pathway. This is easily evident for 3-chloro-4-methylcatechol metabolism. 3-chloro-4-methylcatechol is
transformed into a mixture of 2-chloro-3-methyl- and 5-chloro-4-methylmuconolactone, and thus dehalogenation does occur neither during 1,4- nor during 3,6-cycloisomerization. However, dehalogenation cannot be expected, as none of the two dehalogenating mechanisms described for chloromuconate dehalogenation (11, 30) can occur. It has recently been observed, that muconolactone isomerase, which has been described to be capable to abstract, like chloromuconate cycloisomerase, the C-4 proton of 5-chlorosubstituted muconolactones (26), can be integrated into a functional pathway for 3-chlorocatechol degradation (14). Transformation of 2-chloromuconolactone by this enzyme into protoanemonin has been described (34). However, even if muconolactone isomerasers can be assumed to exhibit some activities against 2-chloro-3-methylmuconolactone, no pathways for the degradation of protoanemonin derivatives or even protoanemonin itself have been described thus far. Thus, degradation of 2-chloro-3-methylcatechol is not possible by any of the known metabolic pathways for chloroaromatic degradation after intradiol cleavage.

4-Chloro-3-methylcatechol is transformed into a mixture of 3-chloro-2-methylmuconolactone and 5-methyl-cis-dienelactone. Evidently, dehalogenation occurs during 3,6-cycloisomerization, analogous to the dehalogenation of 3-chloromuconate. Previously, Vollmer et al. (38) had shown that muconate cycloisomerase catalyze the formation of an equilibrium between 2-chloromuconate, 2-chloro- as well as 5-chloromuconolactone, and that all three compounds are substrates for the cycloisomerase. Transformation of 2-chloromuconolactone by chloromuconate cycloisomerase was described to proceed via 2-chloromuconate and 5-chloromuconolactone into trans-dienelactone (37). As such, chloromuconate cycloisomerase is capable to correct a “wrong” cycloisomerization reaction in case the cycloisomerization reaction is reversible and the “right” cycloisomerization product is taken out of equilibrium by dechlorination. Such a metabolic route has previously suggested to occur also in the degradation of 3,6-dichloro-4-methylcatechol (23). However, as the ratio of 5-methyl-cis-dienelactone versus 3-chloro-2-methylmuconolactone did not change upon extended incubation with chloromucononate cycloisomerase, it can be suggested, that 3-chloro-2-methylmuconolactone, if at all, is only a poor substrate for chloromuconate cycloisomerases.

Thus it is clear, that dioxygenation of 2-chlorotoluene results in the formation of chloromethylcatechols, which are difficult to degrade. 4-chloro-3-methylcatechol degradation seems to be restricted due to the kinetic properties of chloromuconate cycloisomerases favoring 3-chloro-2-methylmuconolactone formation, and preventing its transformation into 5-methyl-cis-dienelactone, which, in turn, would be a reasonably good substrate for dienelactone hydrolases.
If such kinetic properties are shared among cycloisomerases (36) remains to be established. However, beside the closely related chloromuconate cycloisomerases of protobacterial origin analyzed here, recently, distinct cycloisomerases which differed in their cycloisomerization reaction regarding 2-chloromuconate direction have been observed in Rhodococci (35) (2, 3). If such enzymes are capable of 3-chloro-2-methylmuconate cycloisomerization remains to be analyzed. Nevertheless, it can be stated, that none of the enzymes described thus far and related to aromatic degradation via the ortho-cleavage pathway can be assumed to be capable for degradation of lactones formed from 3-chloro-4-methylcatechol.

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5.2 References


Chapter VI

Construction of a Solvent Tolerant *Pseudomonas putida* Strain Able to Degrade High Concentrations of Chlorobenzene

Katrin Pollmann and Dietmar H. Pieper

Department of Environmental Microbiology, GBF - German Research Center for Biotechnology, Mascheroder Weg 1, D-38124 Braunschweig, Germany

*Corresponding author. Mailing address: Bereich Mikrobiologie, AG Biodegradation, Gesellschaft für Biotechnologische Forschung mbH, Mascheroder Weg 1, D-38124 Braunschweig, Germany. Phone: 49/(0)531/6181-467. E-mail: dpi@gbf.de
6.1 Abstract
Chlorobenzene is an organic solvent highly toxic to microorganisms due to its preferential partition in cytoplasmatic membranes. Even strains capable of utilizing chlorobenzene as energy and carbon source react highly sensitive towards chlorobenzene. In this study, a solvent tolerant chlorobenzene degrading strain was constructed by introduction of genes encoding enzymes of the chlorocatechol pathway into the solvent tolerant toluene degrading strain *Pseudomonas putida* DOT-T1E. This strain was able to grow in medium saturated with chlorobenzene as carbon source and to mineralize cyclodextrin complexed chlorobenzene up to concentrations of 10 mM. This organism might be a promising tool for bioremediation of highly toxic chlorobenzene contaminated environments.

6.2 Introduction
Chlorobenzenes are important building blocks in the chemical industry, and used in the production of dyes and pesticides, and as solvents or additives (2). They are toxic, highly persistant and ubiquitously distributed environmental contaminants that accumulate in the food chain. Despite their toxicity, several bacterial strains capable of using chlorobenzenes as sole carbon source have been isolated (30, 44). Under aerobic conditions the aromatic ring is activated by enzyme catalyzed introduction of two hydroxyl groups resulting in chlorocatechols as central intermediates. These metabolites are usually transformed into Krebs cycle intermediates by enzymes of the chlorocatechol ortho-cleavage pathway (52). An alternative chlorocatechol meta-cleavage pathway for the degradation of 3-chlorocatechol as central intermediate of monochlorobenzene degradation has recently been described (21, 43). However, chlorobenzenes are highly toxic for microorganisms, even for those capable to use these substrates as sole source of carbon and energy.

The toxicity of an organic solvent correlates with its hydrophobicity, which is expressed by the logarithm of its partition coefficient between octanol and water (log $P_{OW}$). Solvents with a log $P_{OW}$ value between 1 and 5 like toluene (log $P_{OW} = 2.8$), benzene (log $P_{OW} = 2.1$) and chlorobenzene (log $P_{OW} = 2.84$) (values are taken from (5, 17) are considered to be highly toxic to microorganisms as those compounds are moderately water soluble and partition well to the cytoplasmic membrane (18). Their accumulation in the cytoplasmic membrane cause an increase in membrane fluidity and thus membrane destabilization, resulting in the leakage of proteins and
low molecular weight molecules and disruption of the cell membrane potential by passive efflux of protons across the membrane (18, 48, 49).

However, recently several *Pseudomonas* strains capable of growth in the presence of high concentrations of solvents have been isolated (6, 16, 17, 25, 53). Different mechanisms were identified that enable bacterial strains to adapt to organic solvents. Isomerization of unsaturated fatty acids from the *cis*- into the *trans*-configuration reduces the membrane fluidity and act against the increase in fluidity caused by organic solvents. This reaction is catalyzed by an energy independent isomerase and allows the bacteria to react very quickly to modifications of their environment (12-14, 20). Long-term responses involve, in addition to changes in fatty acid composition, alteration of the phospholipid head groups and changes of the protein composition, again resulting in a decrease in membrane fluidity (36, 40). Additional, in solvent-tolerant organisms like *Pseudomonas putida* S12, *Pseudomonas putida* GM73, and *Pseudomonas putida DOT-T1* energy dependent efflux systems were observed, that transport solvents from the inner membrane out of the cell (23-25, 29, 38, 45). For DOT-T1, three such pumps were identified that contribute to tolerance to toluene. Two of them, both with a broad substrate range, were constitutively expressed whereas one pump was induced by aromatic compounds (29, 38, 45).

A major problem in applying hydrocarbon-degrading bacteria to industrial and bioremediation processes is their sensitiveness to the substrate used as carbon source. Solvent tolerant bacteria have been considered as potentially good candidates for bioremediation of highly toxic compounds such as toluenes, benzenes etc. (28, 39) and for industrial applications for the production of fine chemicals (54). *P. putida* S12, which is capable of growing on styrene and benzoate (28), has been tested for the production of indigo (31). *P. putida* DOT-T1 is capable of growing on toluene, most probably via a toluene dioxygenase (*tod*) pathway (41), and was shown to be capable of rapidly recovering from solvent shocks not only in liquid culture, but also in soil systems. Even though genes encoding a toluene efflux pump seem to be generally linked to *tod* genes (41), including those of the canonical *tod* pathway of *Pseudomonas putida* F1, the presence of multiple pumps renders DOT-T1 extraordinarily resistant to toluene. Due to their high solvent resistance, both *P. putida* S12 and *P. putida* DOT-T1 were used as recipients for catabolic genes to expand their catabolic functions (54). The catabolic functions of *Pseudomonas putida* S12 were improved by introducing catabolic plasmids for the degradation of aromatic compounds (28) or for the production of 3-methylcatechol (54). Transfer of the TOL plasmid pWWO-Km into strain DOT-T1 enabled the organism to grow on xylene and several related compounds (39).
Biodegradation pathways for chloroaromatic compounds often consist of an “upper” pathway responsible for activation of aromatic compounds with chlorocatechols as intermediate and a “lower” pathway for the transformation of chlorocatechols into Krebs cycle intermediates (51) connected by the central intermediate chlorocatechol (51). The substrate spectrum of a given organism can thus be extended to include chloroaromatics by combining an upper pathway comprising broad substrate specificity enzymes with a chlorocatechol degradation pathway (50), a strategy, which resulted in various recombinant chloroaromatic degraders thus far (42). Chlorobenzene degradation is usually achieved by an upper pathway comprising toluene/(chloro)benzene dioxygenase and toluene/(chloro)benzene dihydrodiol dehydrogenase, in concert with a chlorocatechol pathway. Recombinant chlorobenzene degraders have been reported after transfer of chlorocatechol genes into organisms capable to degrade toluene via a toluene dioxygenase pathway (34, 44, 60). Thus, the solvent tolerant strain *Pseudomonas putida* DOT-T1 which harbors genes encoding enzymes for toluene degradation is a promising candidate for the development of a solvent resistant chlorobenzene degrader, and thus of organisms with a phenotype not reported thus far. In our study, we transferred genes of the chlorocatechol pathway to DOT-T1E to combine the upper dioxygenolytic pathway with the genes for chlorocatechol degradation to enable the strain to grow with chlorobenzene and tested, if the organism is capable of using chlorobenzene as sole carbon source directly applied in high concentrations.
6.3 Materials and Methods

Bacterial strains, plasmids and culture conditions. *Pseudomonas putida* DOT-T1E, which was kindly provided by J. L. Ramos, is a rifampin-resistant derivative of the solvent-tolerant toluene degrading strain *P. putida* DOT-T1 (38, 39) and was chosen as the host for mating experiments. Additionally, *Pseudomonas putida* F1 (55) was used as recipient.

*Pseudomonas* sp. B13 is capable of degrading chlorocatechols by the action of four enzymes of the modified *ortho* cleavage pathway encoded by the *clc*ABDE genes located on the mobile *clc* element and capable of integrating into the chromosome (10, 41). *R. eutropha* JMP222 is a derivative of strain JMP134 (7), cured of plasmid pJP4. *R. eutropha* JMP222 (pBBR1M-I) is a derivative of strain JMP222, containing module I (pBBR1M-I) chlorocatechol genes (*tfd*C,D,E,F1) as described recently (34). pBJ44 is a minitransposon vector (26) containing the *tcb* chlorocatechol gene cluster of *Pseudomonas* sp. strain P51 (52) in a Tn5-based minitransposon (15). pCK05 is a minitransposon vector containing the genes of the upper *tol*-pathway in a Tn5-based minitransposon (33). *Pseudomonas* sp. B13 (9), *Ralstonia eutropha* JMP222 (pBBR1M-I) and *E. coli* CC118(λ*pir*) harboring pBJ44 (22) were used as donor of the chlorocatechol genes. *Escherichia coli* HB101(pRK600) (4, 22) was used as the helper strain in triparental mating. KP26 is a chlorobenzene degrading strain derived from *P. putida* DOT-T1E by conjugation of pBJ44. *P. putida* F1*??CC is a chlorobenzene mineralizing derivative of the solvent sensitive toluene degrader *P. putida* F1 with chromosomal integrations of the genes of the upper *tol*-pathway and the *tcb* chlorocatechol gene cluster of *Pseudomonas* sp. strain P51 (27). This strain was used as control strain in growth experiments.

Bacterial strains were routinely grown in liquid Luria bertani (LB) medium (46) or in mineral salt medium (8). For toluene sensitive strains, toluene and chlorobenzene as carbon source were supplied via the gas phase. For strains able to tolerate high concentrations of toluene, toluene and chlorobenzene as sole carbon source were directly added at a concentration of up to 0.5 % (v/v). To adapt the cells to high concentrations of solvents, the cells were pregrown in 10 ml LB supplemented with 0.1% or 10 % (v/v) toluene. For growth on benzoate and succinate, mineral salt medium was supplemented with 3 mM of the respective carbon source. All flasks were sealed with Teflon coated screw caps and incubated at 30°C at 130 rpm on a rotary shaker.
Matings and isolation of transconjugants. Triparental matings between *P. putida* DOT-T1E and B13, *R. eutropha* JMP222(pBBR1M-1) or *E. coli* CC118(λpir)(pBJ44) were carried out overnight at 30°C on 0.22-µm-pore nitrocellulose filter placed on the surface of an LB plate. Fresh log-phase cultures in a donor-to-helper-to-recipient ratio of 1:1:1 were used. After incubation, cells were resuspended in 1 ml sterile 50 mM MgSO$_4$ and plated on minimal medium with rifampicin (30µg/ml) supplemented with 10 µl chlorobenzene as carbon source in the gas phase. From each mating mixture ten colonies (≈1mm in diameter) that arose within 10 days were selected and used for further experiments.

The presence of chlorocatechol genes in the DOT-T1E background was controlled by PCR. Cells of each conjugant were resuspended in 50 µl sterile MilliQ H$_2$O, boiled for 10 minutes and used as template. To amplify the chloromuconate cycloisomerase gene, degenerated primer pairs TP2 (5’-GTGCASMAGCAGAGCTA) and TP6 (5’-TTGCAMAGCTTCAGCGA) (≈ 745 bp) (37) were used. Conditions for PCR were 95°C for 3 min; 25 cycles of 95°C for 60 s, 50 for 60 s and 72 for 90 s; and then 72°C for 10 min. The identity of the recipient with DOT-T1E was controlled by the amplification of the 16S-23S rDNA interspacer region with primer pairs 16F945 (5’-GGGCCCGACACAAGCGGTGG) and 23R458 (5’-CTTTCCCTACACGTTAC) followed by purification of the PCR-product (Qiagen PCR purification kit) and digestion using *Taq*I (Boehringer Mannheim) (1). The fragments were seperated electrophoretically in a 1% agarose gel (Biozym) and stained by ethidium bromide. The fragmention patterns were compared with those of wild type DOT-T1E and the donor strains.

Growth experiments. Ten transconjugants each from matings between *P. putida* DOT-T1E and *P. putida* B13, *R. eutropha* JMP222(pBBR1M-1) or *E. coli* CC118(?pir)(pBJ44), respectively, were plated on minimal medium containing rifampicin and benzene, chlorobenzene, 1,2-, 1,3- or 1,4-dichlorobenzene, toluene, 2-, 3- or 4-chlorotoluene as carbon source. The substrates were supplied via the gas phase. From each DOT-T1E B13, DOT-T1E(pBBR1M-I) and DOT-T1E(pBJ44) set of tranconjugants one representative transconjugant (colony size >1mm) with benzene, chlorobenzene, toluene and 1,4-dichlorobenzene was selected and used for further experiments.

To test the ability of the three selected transconjugants to grow in the presence of high concentrations of toluene or chlorobenzene, mineral salt medium containing different amounts of solvents directly added as carbon source was inoculated with single transconjugants to give an initial A$_{600}$ of 0.01. The cells were pregrown in LB supplemented with 0.1 % toluene,
harvested during the late log phase by centrifugation (5 min, 6000 x g) and washed twice with mineral salt medium. After 7 days growth the optical density was determined. To test for solvent tolerance, 10 ml LB medium was inoculated with LB grown precultures of the three conjugants, wild-type DOT-T1E and the strain F1*ΔCC as control to give an initial A$_{600}$ of 0.01 and supplemented with 10% (v/v) toluene. In another experiment mineral salt medium containing 1% (v/v) of chlorobenzene was inoculated with a preculture of KP26 grown on 0.1% (v/v) chlorobenzene. This culture was used as inoculum for mineralsalt medium supplemented with 10% (v/v) chlorobenzene. The solvent tolerance of KP26 was further tested by examining growth in 10 ml LB medium supplemented with 10% toluene, 2- or 4-chlorotoluene, 2,4-, or 2,6-dichlorotoluene, benzene or chlorobenzene after inoculation (initial A$_{600}$ = 0.01) with KP26 pregrown on LB + 10% toluene. All experiments were performed in flasks containing 10 ml of liquid phase and 120 ml of additional gas phase.

For batch culture experiments, 1mM or 4 mM chlorobenzene was dissolved in 70 ml mineral salt medium in 70 ml airtight flasks. To achieve higher solvent concentrations, chlorobenzene was complexed with an equimolar amount of α-cyclodextrin (α-CD). α-CD is a cyclic oligomer of six 1,4-α-D-linked glucose units with a cylinder-like shape with an external hydrophilic shell and an apolar internal cavity. Hydrophobic organic molecules can be included in this cavity to form water-soluble complexes. α-, β-, and γ-CD have been used as non-toxic compounds to enhance microbial conversions of toxic or water-insoluble compounds (3, 19).

**Fig. 1:** Custom made double wall flask used in batch culture experiments.
The flask was connected to a circulating waterbath.
The media were transferred to custom-made double wall flasks (total volume 350±15ml) and closed immediately with Teflon coated screw caps. The inner cylinders of the flasks were equipped with two Mininert valves (Alltech) to allow sampling of liquid phase and headspace for subsequent analysis (Fig.1). The outer cylinders were connected to a circulating waterbath tempered at 30°C. The media were inoculated with washed cells of KP26 pregrown in 10 ml LB supplemented with 10µl toluene to give an initial optical intensity of $A_{600} = 0.005$. As control, medium containing 1 mM chlorobenzene or 6 mM chlorobenzene dissolved in a -CD were inoculated with the solvent sensitive strain F1* ΔCC pregrown in 10 ml LB supplemented with 10 µl toluene to give an initial optical density of $A_{600} = 0.005$. The cultures were incubated on a rotary shaker at 130 rpm. Samples from the liquid phase (1 ml) and from the headspace (50 or 100 µl) were taken at appropriate time intervals with sterile single use syringes or gas tight syringes (Hamilton). Growth was monitored by determination of optical density ($A_{600 nm}$).

**Determination of Cl⁻ release.** Samples of the culture fluid were centrifuged (2 min, 13000 rpm) and the supernatants were used for subsequent chloride determination and HPLC-analyses. For determination of the chloride concentration, to each 500 µl of the supernatants mixed with 500 µl MilliQ H$_2$O 200 µl of a 0.1 M AgNO$_3$ acidic solution was added. After 10 min the turbity was measured at $\lambda = 525$ nm (13). The chloride concentration was determined by comparison with a calibration curve.

**HPLC-analyses.** For quantification of metabolite accumulation, 10 µl of the cell free supernatant were analyzed with a Shimadzu HPLC system (LC-10AD liquid chromatograph, DGU-3A degasser, SPD-M10A diode array detector and FCV-10AL solvent mixer) equipped with a SC125/Lichrospher 5-µm (Bischoff, Leonberg, Germany) column. The aqueous solvent system (flow rate 1 ml/min) contained 0.1 % (v/v) of H$_3$PO$_4$ (87%) and 35% (v/v) of methanol. The metabolites accumulating during growth of KP26 with chlorobenzene were identified with authentic standards as 2-chloromuconate (RV = 3.66 ml) and 3-chlorocatechol (RV = 6.11 ml).

**GC-analyses.** To quantify the chlorobenzene content in the gas phase, 50 to 100 µl of sample volume were taken from the headspace with a gastight syringe (Hamilton). For quantification of the chlorobenzene content in the liquid phase, 5 µl of sample volume were taken from the medium. The samples were injected and analyzed immediately with a Shimadzu (Kyoto, Japan) GC-17A gas chromatograph equipped with a XTI-5 column (30 m by 0.25 mm; film thickness 0.5 µm; Resteck, Bellefonte, P.a.) and coupled to a QP-5000 quadropole mass spectrometer.
The mass spectrometer was operated in the electron impact mode at 70 eV with an ion source temperature of 320°C. Helium was used as carrier gas with a flow rate of 1.0 ml/min. The oven temperature was maintained at 39°C for 5 min and then increased to 110°C at a rate of 15°C/min followed by an increase to 200°C at a rate of 40°C/min. GC-MS was operating in the splitless mode with an injection temperature of 270°C.

The ratio between the gas-phase concentration of chlorobenzene and the aqueous phase concentration (partition coefficient) was determined by measuring the distribution of defined amounts of chlorobenzene between the air and the medium by GC analysis as 0.15 which is similar to the values calculated from Henry’s law constants reported in literature ($k_H = 0.22-0.32$ M/atm) (37, 55). Similarly, by measurement of the distribution of defined amounts of chlorobenzene between the headspace and the medium by GC analysis the air-medium distribution ratio for a certain amount of chlorobenzene solved in a -CD could be determined as 0.066.

Thereby, it was possible to determine the chlorobenzene concentration in the aqueous phase by measurement of the chlorobenzene concentration in the headspace as follows:

$$c_m = \frac{n}{V_g \cdot ?_{g/m} + V_m}$$

where $c_m$ is the chlorobenzene concentration in the medium (mol/l), $n$ the total amount of chlorobenzene (mol), $V_m$ is the volume of the medium (l), $V_g$ the volume of the headspace (l) and $?_{g/m}$ is the (partition coefficient) of chlorobenzene. The partition coefficient $?_{g/m}$ is defined as $?_{g/m} = 1/(k_H \times RT)$, where $k_H$ = Henry’s law constant (mol/l atm), $R$ = gas constant (l atm/°K mol) and $T$ = temperature (°K).

Thus, the chlorobenzene concentration in the medium after partition of 1 and 4 mM chlorobenzene in custom made double wall flasks under the experimental conditions was determined as 0.7 +/- 0.1 mM and 2.2 +/- 0.2, respectively, whereas the concentration of 4, 6, 8, 10, 12 and 15 mM a-CD-complexed chlorobenzene was 3.1 +/- 0.1 mM, 4.8 +/- 0.6 mM, 6.3 +/- 0.3 mM, 8.0 +/- 0.2 mM, 9.0 +/- 0.7 mM and 11.5 +/- 0.7 mM, respectively.

**Enzyme assays.** Chlorocatechol 1,2-dioxygenase used for the production of muconate and 2-chloromuconate was purified from *E. coli* overexpressing chlorocatechol 1,2-dioxygenase (TetC) of *Pseudomonas chlororaphis* RW71 as described by Potrawfke (45).
To study the expression of enzymes of the chlorocatechol pathway by strain KP26 during growth on chlorobenzene, two 500 ml flasks with each 70 ml mineral salt medium containing 4 mM chlorobenzene as carbon source were inoculated with washed cells of strain KP26 of a preculture grown in LB supplemented with 0.1 % toluene to give an optical density of $A_{600} = 0.005$ and incubated at 30°C on a rotary shaker (130 rpm). The cells were resuspended in 50 mM Tris/HCl (pH 7.5, supplemented with 2 mM MnCl$_2$), disrupted using a French press and the cell debris were removed by centrifugation (100000 x g, 30 min, 4°C). Protein concentrations in the cell extracts were determined by the Bradford procedure (4). (Chloro)catechol 1,2-dioxygenase, chloromuconate cycloisomerase and dienelactone hydrolase activities were measured as described previously (9, 56, 64). One enzyme unit is defined as the amount of enzyme that catalyzes the transformation of 1 µmol substrate per min.

**Chemicals.** 3-Chlorocatechol and 4-chlorocatechol were obtained from Helix Biotech. Muconate, 2-chloromuconate and 3-chloromuconate were obtained by chlorocatechol 1,2-dioxygenase catalyzed intradiol cleavage of catechol, 3-chlorocatechol and 4-chlorocatechol. All other chemicals were purchased from Aldrich Chemie (Steinheim, Germany), Fluka AG (Buchs, Switzerland), or Merck AG (Darmstadt, Germany).

6.4 Results

**Transfer of chlorocatechol genes to P. putida DOT-T1E and phenotypic characterization of transconjugants.** Ten transconjugants each obtained after mating of $P. putida$ DOT-T1E with $P$. sp. B13 (KP1-10), JMP222(pBBR1M-I) (KP11-20) and $E. coli$ (pBJ44) (KP21-30) and exhibiting a colony size $\geq 1$mm after incubation on agar plates in the presence of chlorobenzene vapors as sole carbon source for 10 days were selected. PCR tests verified the presence of the chlorocatechol genes in the DOT-T1E background in all cases (data not shown).

All 30 transconjugants plated on minimal medium were able to use toluene, benzene, chlorobenzene and 1,4-dichlorobenzene as growth substrate (data not shown) as evidenced by the colony size on agar plates incubated in the presence of solvent vapors compared to those incubated in the absence of carbon source. The transconjugants KP5, KP15, KP26 that showed representative growth with all substrates (colony size $\geq 1$mm) were selected for further experiments. Similar to the wild-type strain, these mutants were able to grow in LB medium in the presence of a toluene concentration of 10 % (v/v) whereas the strain F1*ΔCC, used as
control, failed to grow. In mineral salt medium, directly supplemented with toluene or chlorobenzene as sole carbon sources these derivatives showed growth up to a toluene concentration of 0.2 % (v/v) and a chlorobenzene concentration of 0.05 % (v/v) under the given experimental conditions. KP26 (DOT-T1E(pBJ4/4)), which showed growth even at a chlorobenzene concentration of 0.15 % (v/v) (thus capable to grow in medium saturated with chlorobenzene) was chosen for further experiments. This mutant was able to grow in LB in the presence of chlorobenzene as well as chloro- and dichlorotoluenes and toluene directly added to a concentration of 10 % (v/v), but not in the presence of benzene (Table 1). As expected, this mutant was able to grow with 10 % (v/v) toluene when cells adapted to high solvent concentrations were used as inoculum. However, in contrast to wild-type DOT-T1E cultures, that developed a violet precipitate during incubation with chlorobenzene due to the formation of 3-chlorocatechol (see below), the adapted cells of KP26 showed growth with chlorobenzene up to a concentration of 1% (v/v) (data not shown).

<table>
<thead>
<tr>
<th>Solvent</th>
<th>LogP&lt;sub&gt;O/W&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Growth in LB&lt;sup&gt;a,b&lt;/sup&gt;</th>
<th>DOT-T1E (wild-type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,6-Dichlorotoluene</td>
<td>4.29</td>
<td>++++</td>
<td>n.d.</td>
</tr>
<tr>
<td>2,4-Dichlorotoluene</td>
<td>4.24</td>
<td>++++</td>
<td>n.d.</td>
</tr>
<tr>
<td>2-Chlorotoluene</td>
<td>3.42</td>
<td>+++</td>
<td>n.d.</td>
</tr>
<tr>
<td>4-chlorotoluene</td>
<td>3.33</td>
<td>+++</td>
<td>n.d.</td>
</tr>
<tr>
<td>Chlorobenzene</td>
<td>2.84</td>
<td>+</td>
<td>n.d.</td>
</tr>
<tr>
<td>Toluene</td>
<td>2.73</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Benzene</td>
<td>2.13</td>
<td>-</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

<sup>a</sup> Medium supplemented with 10% solvent was inoculated with cells pregrown in LB medium in the presence of 10% (v/v) toluene to an initial A<sub>600</sub> of 0.002. n.d. not determined

<sup>b</sup> Symbols indicating optical density at 600 nm: ++++, > 5 after 24 h; ++++, >1 after 24 h; ++, >2 after 42 h; + <2, >1 after 42 h; -, < 0.1 after 42 h.

<sup>c</sup> LogP values are taken from: toluene, (24); chlorobenzene, (6); 4-chloro-, 2,6-dichlorotoluene, (17); 2-chloro- and 2,4-dichlorotoluene, (16);
**Growth of KP26 with chlorobenzene.** In contrast to F1*ΔCC, which was not capable to grow on chlorobenzene even in the presence of a concentration as low as 1 mM, the transconjugant KP26 can grow on chlorobenzene if the substrate is supplied directly to the medium at concentrations of 1 - 4 mM (this concentration, present initially in the liquid phase, partition between the liquid and gas phase such that the concentration in the liquid phase corresponds to 62.5 +/- 7.5 % of that value under the given experimental conditions as described in the methods and materials section). Growth occurred after a lag phase of 15 - 24 h and at rates between 0.063 h\(^{-1}\) (in the presence of 4 mM chlorobenzene) and > 0.12 h\(^{-1}\) (in the presence of 1 mM chlorobenzene) (Fig. 2A).

Whereas growth was evident only after extended incubation, chlorobenzene depletion was evident even after 1 day of incubation (Fig. 2B). Concomitant with a low depletion, accumulation of 3-chlorocatechol and 2-chloromuconate was observed. Both metabolites were identified by their HPLC retention volumes and in-situ UV-spectra identical to those of authentic standards. In accordance with those data, chloride release was not observed during this period, but was concomitant with growth. Whereas 3-chlorocatechol accumulated only to low levels (up to 0.04 mM for 1 mM chlorobenzene and 0.1 mM for 4 mM chlorobenzene), high accumulation of 2-chloromuconate, summing up to more than 50% of applied substrate was evident. Thus, chloride release was not quantitative during the growth period.

2-Chloromuconate, excreted during growth on 1 mM chlorobenzene up to a concentration of 0.6 mM, disappeared from the culture medium only at a low rate of approximately 2 µM/h. Thus, even after extended incubation approximately 50% of applied chlorobenzene was not mineralized, which is in agreement with data on chloride release. In contrast, when cells growing on 4 mM chlorobenzene were analyzed, an increase in the external 2-chloromuconate concentration was observed as long as chlorobenzene was present in the culture medium (60 hours after inoculation), but subsequent 2-chloromuconate depletion was significantly faster at a rate of about 180 µM/h, which cannot be explained simply by the higher optical density and thus cell mass, which was only up to 4-fold. Due to the fast 2-chloromuconate depletion, quantitative chloride elimination was observed already after 90 hours of incubation.
FIG. 2. Kinetics of mutant KP26 in batch cultures supplemented with 1 and 4 mM chlorobenzene. Cells were adapted with 0.1 % toluene and transferred into mineralsalt medium with (●) 1 mM and (○) 4 mM chlorobenzene as sole carbon and energy source directly added to the medium. (Q) Strain F1*ΔCC adapted with 0.1 % toluene and transferred into mineralsalts medium containing 1 mM chlorobenzene was used as control. A: Growth kinetics of KP26. B: Depletion of chlorobenzene; total amount of chlorobenzene (n) was determined by GC-analysis as described in the methods and materials section. C: Accumulation of products; filled symbols: accumulation of 2-chloromuconate; opened symbols: accumulation of 3-chlorocatechol. D: Chloride-release.

The strain F1*ΔCC, used as control, showed no growth nor significant chlorobenzene depletion even when exposed to a chlorobenzene concentration of 1 mM. However, a slight 3-chlorocatechol accumulation up to 3µM could be monitored, indicating a low activity with the substrate (data not shown). Nevertheless, activity was too low to detect chlorobenzene depletion or growth.
Activities of enzymes of the chlorocatechol and catechol pathway during growth with chlorobenzene. To test the expression of enzymes of the catechol and chlorocatechol pathway during growth, cells of chlorobenzene (4 mM) grown KP26 were harvested after 47 h ($A_{600} = 0.4$, [2-chloromuconate]$_{medium} = 0.44$ mM) and 53 h ($A_{600} = 0.5$, [2-chloromuconate] = 0.17 mM). The enzyme activities showed features typical for enzymes of the chlorocatechol pathway of Pseudomonas putida P51, e.g. high activities with 3-chlorocatechol, as shown for tcbC chlorocatechol dioxygenase, high activity with 2-chloromuconate (tcbD chloromuconate cycloisomerase) and with cis-dienelactone (tcbE dienelactone hydrolase) (Tab. 3) (62, 64). The low activities with catechol and muconate further indicate that the genes of the chlorocatechol pathway are expressed, but obviously not those of the catechol pathway. The specific activities of the enzymes, especially that of chlorocatechol 1,2-dioxygenase with 3-chlorocatechol, were significantly higher when harvested after 53 h than after 47 h (Tab. 3).

**TABLE 3: Activities of enzymes of the chlorocatechol pathway during growth on chlorobenzene**

Mineralsalts medium containing 4 mM chlorobenzene was inoculated with washed cells of the mutant KP26 pregrown in LB supplemented with 0.1 % toluene. Cells were harvested after 47 h ($A_{600}=0.3$) and 53 h ($A_{600}=0.4$).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$A_{600}=0.3$</th>
<th>$A_{600}=0.4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorocatechol 1,2-dioxygenase</td>
<td>Catechol</td>
<td>133 (123)</td>
</tr>
<tr>
<td></td>
<td>3-Chlorocatechol</td>
<td>108 (100)</td>
</tr>
<tr>
<td></td>
<td>4-Chlorocatechol</td>
<td>329 (305)</td>
</tr>
<tr>
<td>Chloromuconate cycloisomerase</td>
<td>Muconate</td>
<td>6 (17)</td>
</tr>
<tr>
<td></td>
<td>2-Chloromuconate</td>
<td>35 (100)</td>
</tr>
<tr>
<td>Dienelactone hydrolase</td>
<td>cis-Dienelactone</td>
<td>2914</td>
</tr>
</tbody>
</table>
Growth of KP26 with 4-12 mM α-cyclodextrin-complexed chlorobenzene. As chlorobenzene is soluble in water only up to concentrations of 4.4 mM, higher aqueous concentrations were achieved by using α-CD-complexed chlorobenzene. F1*?CC was not capable to grow in mineral salt medium containing 4 mM α-CD-complexed chlorobenzene (α-CD-complexed chlorobenzene partition between the liquid and gas phase such that the concentration in the liquid phase corresponds to 78 +/- 1 % of the given value under the applied experimental conditions as described in the methods and materials section). In contrast, K26 can grow on α-CD-complexed chlorobenzene as shown in Fig. 2. Growth on 4-10 mM α-CD-complexed chlorobenzene was very similar to growth on 4 mM dissolved chlorobenzene as shown above. However, extended lag-phases were observed with increasing chlorobenzene concentration and growth yield as indicated by the final optical density did not increase proportionally with increasing substrate concentration. However, chloride elimination indicated that in all cases more than 80% of the substrate was mineralized.

During growth of KP26 with α-CD-complexed chlorobenzene at concentrations of 10 mM or lower, significant amounts of 2-chloromuconate accumulated temporary, but were, as observed during incubation with 4 mM chlorobenzene, further transformed at significant rates. In contrast, 3-Chlorocatechol accumulated only to low amounts (up to 0.02 mM when incubated with 10 mM chlorobenzene).

Significant different behavior was observed when the strain was confronted with chlorobenzene concentrations higher than 10 mM. When incubated in the presence of 12 mM α-CD-complexed chlorobenzene, a significant accumulation of 3-chlorocatechol up to a concentration of 2.0 mM was observed concomitant with chlorobenzene depletion. Simultaneously, like observed during incubation with lower concentrations of α-CD-complexed chlorobenzene, 2-chloromuconate accumulated. Thus, chlorobenzene depletion during this phase was accompanied by only low chloride elimination. However, in contrast to incubations with lower concentrations of α-CD-complexed chlorobenzene, 2-chloromuconate as well as 3-chlorocatechol disappeared from the culture fluid only at negligible rates. The decline of 3-chlorocatechol at least during later stages of incubation, is obviously caused by the polymerization of 3-chlorocatechol, as indicated by the formation of a dark precipitate during incubation (66). Thus, as after 90 hours of incubation neither further substrate or intermediate transformation nor chloride elimination were observed, it can be stated that the cells during incubation become completely inactive, and 10 % of initially supplied chlorobenzene remained in the medium. However, growth of KP26 with 12 mM α-CD-
complexed chlorobenzene could not be determined accurately due to the extensive dark coloration of the medium.

Neither growth, nor a significant chlorobenzene depletion or chloride release could be monitored during incubation of KP26 with 15 mM α-CD-complexed chlorobenzene. Accumulation of up to 8 µM 2-chloromuconate and up to 2 µM 3-chlorocatechol within 66 h was indicative for a poor activity of the strain under these conditions.
FIG. 3. Kinetics of mutant KP26 in batch cultures supplemented with 6-12 mM chlorobenzene. Cells were adapted with 0.1% toluene and transferred into mineralsalts medium with (A-B, D) \((\odot) 6\) mM, \((\Diamond) 8\) mM, \((\triangle) 10\) mM and \((\square) 12\) mM chlorobenzene complexed with \(\alpha\)-CD. \((\hat{\Sigma})\) Strain F1*ΔCC adapted with 0.1% toluene and transferred into mineralsalts medium containing 6 mM chlorobenzene complexed with \(\alpha\)-CD was used as control. A: Growth kinetics. B: Depletion of chlorobenzene; total amount of chlorobenzene \((n)\) was determined by GC-analysis as described in the methods and materials section. C: Accumulation of products during growth with \((\odot) 6\) mM, \((\Diamond) 8\) mM, \((\triangle) 10\) mM and \((\square) 12\) mM; filled symbols: accumulation of 2-chloromuconate; opened symbols: accumulation of 3-chlorocatechol. D: Chloride-release.
6.5 Discussion

Chlorobenzene is known to be highly toxic for microorganisms even for those strains capable to use chlorobenzene as sole carbon source. As an example strain WR1306, the first chlorobenzene degrading bacterium ever isolated, was reported to be unable to grow with chlorobenzene added directly to the culture medium even at concentrations as low as 0.1 g/l. However, as the actual concentration of chlorobenzene present in the liquid medium depends on the available gas phase, concentrations of chlorobenzene preventing growth can usually not be compared between different reports. However, for WR1306, it can be calculated that the actual liquid phase concentration preventing growth was as low as 0.26 mM. In contrast, Pseudomonas sp. strain RHO1 was obviously less sensitive and could grow on chlorobenzene at liquid phase concentrations as high as 2 mM (13). Zaitsev et al. reported on a benzene and chlorobenzene degrading Rhodococcus strain, and it was claimed that this organism can grow on chlorobenzene present in concentrations as high as 27 mM. However, taking into account the partitioning between liquid and gas phase, it can be calculated, that the strain grows on chlorobenzene only up to liquid phase concentrations of 1.8 – 2.5 mM. Jorge and Livingston carefully examined the growth kinetics of Pseudomonas sp. strain JS150 in a continuous culture system which allowed the adjustment of a predetermined chlorobenzene concentration in the liquid phase (27).

At concentrations above 1.4 mM growth of the strain was strongly inhibited (27) and no growth was obtained at concentrations higher than 1.6 mM.

In accordance with above observations, the chlorobenzene degrading strain F1*ΔCC was found to be highly sensitive to chlorobenzene when added directly to the medium and could not grow on chlorobenzene present in liquid phase concentrations as low as 0.7 mM. In contrast, the solvent tolerant strain DOT-T1E, after introducing of genes encoding enzymes for chlorocatechol metabolism could grow in liquid media saturated with chlorobenzene (approx. 4.4 mM) and on CD complexed chlorobenzene at concentrations up to approx. 9 mM. Thus the derivative KP26 withstands and mineralizes much higher concentrations of chlorobenzene than any other organism reported thus far.

Recently, several mechanisms in DOT-T1E have been characterized which might be responsible for the tolerance of KP26 toward chlorobenzene, including changes in the membrane composition and operation of three energy dependent efflux pumps (28, 48, 50, 54). The TtgABC pump is synthesized at high constitutive levels (9), whereas the TtgDEF pump is synthesized in response to toluene (38). The third pump, designated TtgGHI is formed.
constitutively but the respective genes are expressed at an even higher level in the presence of toluene (54). Based on single, double and triple knock-out mutants, the contribution of the different pumps on resistance to toluene and their substrate specificity regarding different solvents could be assessed. Both the TtgABC and the TtgGHI were observed to be of broad substrate specificity and to be involved in resistance against propylbenzene, ethylbenzene, m-xylene and styrene, whereas the TtgDEF pump, out of the mentioned solvents, was only capable to extrude styrene (54). In an earlier study, Ramos et al. (48) had indicated, that at least two of the mentioned pumps, most probably TtgABC and TtgDEF were capable to extrude 1,2,4-trichlorobenzene (48). However, based on the reported experiments, it cannot be concluded if also TtgGHI can extrude 1,2,4-trichlorobenzene. Even though tolerance against chlorobenzene has never been tested in DOT-T1, nor any of the mutants defective in one or more of the solvent pumps, the broad substrate specificity of TtgABC and TtgGHI could indicate that they are also capable to extrude chlorobenzene, and the capability of TtgDEF to extrude 1,2,4-trichlorobenzene and toluene could indicate that also this pump is involved in chlorobenzene tolerance.

In agreement with previous reports on toluene tolerance of Pseudomonas putida F1 (38), the derivative strain F1*ΔCC was found to tolerate moderate concentrations of toluene (up to 0.1 % v/v). Recently, in strain DOT-T1E the genes *ttg*DEF encoding the inducible efflux pump have been found to be linked to the chromosomal *tod*-genes (38). A similar organization with genes encoding an efflux pump linked to *tod* genes was indicated by results of PCR amplification to occur in P. putida F1 (38). However, as F1*ΔCC showed no significant chlorobenzene tolerance when compared to KP26, it can be suggested that at least the TtgDEF pump of F1 has no major role for chlorobenzene tolerance. However, further investigations are necessary to elucidate the roles of the different solvent pumps of DOT-T1E for chlorobenzene resistance.

Poor performance was observed for growth of KP26 on 12 mM CD complexed chlorobenzene, and the strain failed to grow on 15 mM CD complexed chlorobenzene. The poor performance could, on the first view, be due to insufficient oxygen supply. However, lack of oxygen could not explain the nearly complete absence of growth in the presence of 15 mM chlorobenzene, such that either toxicity chlorobenzene itself, or to metabolites formed thereof should be the reason for absence of growth in this case. Given the fact that no growth disturbance was observed during growth on 10 mM CD-complexed chlorobenzene and quantitative chloride elimination was observed, oxygen, under these conditions was not limiting. It can be calculated that under the experimental conditions used, approx 2.5 mmole of oxygen were available for degradation of 0.7
mmole of chlorobenzene (concentration 10 mM) and thus approximately 3.5 mole of oxygen were sufficient for mineralization of 1 mole of chlorobenzene. Consequently, as, when cells were confronted with 12 mM CD complexed chlorobenzene, high amounts of partially oxidized metabolites (approx 10% of supplied substrate was excreted as 3-chlorocatechol having consumed 1 molecule of oxygen per substrate molecule and more than 20% of applied substrate was excreted as 2-chloromuconate having consumed 2 molecules of oxygen per substrate molecule) appeared in the medium, beside residual chlorobenzene, oxygen depletion at the end of that growth experiment is rather unprobable. In contrast, the performance of the culture ceased as soon as high amounts of 3-chlorocatechol had been accumulated. Chlorocatechols in turn have been reported to be highly toxic to organisms (47). Haigler at al. indicated 3,4-dichlorocatechol to be highly toxic for the 1,2-dichlorobenzene degrading *Pseudomonas* sp. JS100 (11). Thus, the toxicity of 3-chlorocatechol might be crucial for the difficulties of KP26 to mineralize high concentrations of chlorobenzene.

Another metabolite accumulating during growth with chlorobenzene is 2-chloromuconate, reflecting high intracellular concentrations due to inefficient chloromuconate cycloisomerase activity (35). The capacity of KP26 to reutilize previously excreted 2-chloromuconate significantly depended on the incubation conditions and varied between 2 µM/h and 300 µM/min and, taking into account the cell concentration, by a factor of 10-20.

Degradation of 2-chloromuconate can either be due to diffusion of the protonated fraction of 2-chloromuconate followed by intracellular degradation or to the presence of a transport system similar to the muconate transporter (MucK) recently described for *Acinetobacter calcoaceticus* ADP1 (67) allowing active uptake of this compound. However, as DOT-T1 has no intrinsic capability to deal with chlorosubstituted compounds, the presence of a specific 2-chloromuconate transporter seems to be rather unprobable. Moreover, analysis of enzyme activities during growth showed that enzymes of the 3-oxoadipate pathway for catechol degradation like catechol 1,2-dioxygenase or muconate cycloisomerase were evidently not induced during growth on chlorobenzene, making also very unprobable the recruitment of a muconate transporter.

The mutant KP26 faster growth, and decreased adaptation (lag)-phases when confronted with a-CD-complexed chlorobenzene compared to chlorobenzene directly added to the medium. Cyclodextrins are cyclic, non-toxic oligomers consisting of glucose molecules, which interact with hydrophobic organic compounds forming water-soluble or water-insoluble inclusion complexes. They are used as solubilizing agents for hydrophobic molecules in the food and
pharmaceutical industries (59) and to enhance microbial conversion of either toxic or water-insoluble organic substrates. For microbial bio degradation, cyclodextrins were successfully used for improved purification of wastewater (37, 43), aquifers (4) and PCB contaminated soils (10, 11) as well as in liquid pure cultures for enhanced degradation of toluene (56) to diminish the toxic effects and to enhance the bioavailability of the contaminants. Therefore, also for α-cyclodextrin encapsulated chlorobenzene an improved degradation can be assumed due to a diminished toxicity of chlorobenzene and increased bioavailability. Nevertheless, the control strain F1*ΔCC was unable to grow with α-CD-complexed chlorobenzene, suggesting, that these complexes are still toxic enough to prevent growth of solvent sensitive strains.
CHAPTER VI  CONSTRUCTION OF A SOLVENT TOLERANT CHLOROBENZENE DEGRADER

6.6 References


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Chapter VII

GENERAL DISCUSSION
Microorganisms colonizing sites that are heavily polluted by aromatic compounds have to deal with extreme environmental conditions. On the one hand, various of the pollutants in contaminated sites, at least in the amounts present, are new to the bacteria, and organisms had only a very restricted time period to adapt to deal with those pollutants. On the other hand, microorganisms in those sites have to deal with usually very complex mixtures of pollutants. It has been suggested that different metabolic tasks associated with the productive breakdown of pollutants might best be handled by different members of an appropriate indigenous microbial consortium. However, such interactions of community members for pollutant breakdown are usually poorly understood. Moreover, intermediates formed in complex communities may be misrouted into unproductive dead-end pathways, or even transformed into toxic products that may destabilize the community and inhibit biodegradative processes. Examples of last mentioned destabilization are the formation of protoanemonin from chloroaromatics by communities adapted to the degradation of natural aromatic compounds (1) and the degradation of mixtures of chloro- and methyl aromatics resulting in the misrouting of metabolites and formation of suicide substrates (9, 21). The metabolism of xenobiotics containing both chloro- and methyl substituents can be assumed, at least in part, to create metabolic problems similar to the degradation of mixtures of chloro- and methylaromatics. Such compounds, like chlorotoluenes and chloromethylphenols are important environmental pollutants, and their metabolic fate has not been analyzed in detail thus far.

Various of the environmental pollutants can act as organic solvents. Compounds such as chlorobenzenes or chlorotoluenes are therefore highly toxic to microorganisms since they destabilize the cell membrane (24, 25), and are toxic to microorganisms, even if these have the catabolic property to mineralize them. On the other hand, organisms resistant to organic solvents not necessarily contain the potential to mineralize them, and specifically solvent tolerant strains capable of degrading chloroaromatics have not been isolated thus far. Thus, strains capable of mineralizing chlorinated and simultaneously being solvent tolerant like the organism constructed in this thesis are of special interest for biotechnological applications.
7.1 Degradation of chlorinated toluenes

For bioremediation of contaminated sites, complete mineralization of the parent pollutants is essential, as metabolites produced by bacteria and accumulating in environment might be toxic to the indigenous microflora as described for the bacteriotoxic protoanemonin, formed during 4-chlorobiphenyl metabolism as the product of 3-chloromuconate cycloisomerization catalyzed by muconate cycloisomerase (1,10,15,16). Thus, extensive knowledge of degradation pathways, bottlenecks and dead end metabolites in the pathways is essential. The elucidation of dead end metabolites further helps in identifying crucial auxiliary metabolic routes necessary for degradation of environmental pollutants by microbial communities. In this thesis, the degradation pathways of chlorinated toluenes were investigated and bottlenecks were identified.

Degradation pathways of chlorinated toluenes. It was shown, that the degradation pathways of these compounds contain two critical steps. The first critical step is the initial attack of the substrate catalyzed by the TecA tetrachlorobenzene dioxygenase. This enzyme is responsible for the first branching such that the substrate is either misrouted in a monooxygenolytic pathway resulting in the respective benzoates as dead end metabolites for *Ralstonia* sp. PS12, or channeled into a dioxygenolytic pathway, with the corresponding substituted catechol as key intermediate. TecA usually catalyzes a dioxygenation at two neighbored unsubstituted carbon atoms proximal to one of the substituents, and catalyzes dechlorination only in case no two neighbored unsubstituted carbon atoms are available (1,2,4,5-tetrachlorobenzene). Thus, two modes of dioxygenation were catalyzed by TecA dioxygenase on 2- and 3-chlorotoluene leading to a significant branching of the metabolic pathway. The second critical step is carried out by the chloromuconate cycloisomerase, catalyzing the cycloisomerization of substituted muconates formed by intradiol cleavage of substituted catechols. The fact that muconates can be subject to a 1,4- as well as a 1,6-cycloisomerization and the reversibility of the cycloisomerization reaction shows, that different kind of pathways can be followed for a given substituted catechol. Depending on the substitution pattern of the muconates and the mode of cycloisomerization, cycloizomerisation can give rise to muconolactones (in case cycloisomerization is not accompanied by chloride elimination) or to dienelactones (in case cycloisomerization is accompanied by chloride elimination). In the easiest variants, the substrate is (nearly) exclusively subject to one mode of cycloisomerization. If dechlorination occurs during cycloisomerization, like observed during the degradation of 3,4-dichloro-6-methyl- or 5-chloro-3-methyl catechol, the substrate is degraded by a simple linear pathway. If no dechlorination occurs during cycloisomerization, like observed during 3-chloro-5-methylcatechol metabolism,
the respective metabolic precursors would not be used as growth substrate. If the substrate is subject to both 1,4-, and 3,6-cycloisomerization, four modes of metabolic fate were observed. In case both cycloisomerization directions result in dechlorination, the respective catechol, like 4,6-dichloro-3-methylcatechol is degraded via a branched pathway. If none of the modes of cycloisomerization results in dechlorination, like observed for 2-chloro-3-methylcatechol, the catechol cannot be mineralized by known enzymes of the ortho-cleavage pathway variants. In case only one cycloisomerization direction is accompanied by dechlorination, mineralization depends on the kinetics of cycloisomerization. E.g., 3,6-dichloro-4-methylcatechol is transformed into a 4-methylsubstituted muconolactone, which however, is rapidly transformed via the muconate into a dienelactone by chloromuconate cycloisomerase, such that finally, all catechol is routed via the dienelactone branch of the pathway. In contrast, 3-chloro-2-methylmuconolactone formed from 4-chloro-3-methylcatechol is a dead end product, as it is, if at all, only a poor substrate for chloromuconate cycloisomerase.

Unfortunately, despite the availability of crystal structures of both muconate and chloromuconate cycloisomerases (4, 22), site directed engineering of those enzymes was only of limited success, and factors governing the mode of cycloisomerization are still only poorly understood (8, 28).

**In-situ-^1^H NMR-analysis as a new method to investigate degradation pathways.** Since various metabolites in the degradation of highly substituted aromatics such as muconates, muconolactones, dienelactones or maleylacetates are often unstable under non-physiological conditions, conventional methods to investigate the metabolic pathways such as HPLC- and GC/MS-analyses may lead to artefacts. Therefore, in-situ-^1^H NMR-analysis was performed in this thesis as a new tool to study degradation pathways under physiological conditions. The use of this method allowed the characterization of intermediates formed during dichloromethylcatechol metabolism, e.g. the formation of 2,5-dichloro-4-methylmuconolactone during 2,5-dichlorotoluene degradation. Thus, in-situ-^1^H NMR-analysis was proven to be an appropriate tool to investigate degradation pathways of aromatics without artefacts caused by chemical transformations.
7.2 Artificial evolution of enzymes and pathways to create new biocatalysts

Recently, varieties of approaches, involving both ‘rational’ and ‘irrational’ design, have been used successfully to alter protein function (23). The rational design of the catalytic site of a given enzyme requires extensive knowledge of the enzyme structure and the catalytic mechanism (14, 23). However, alignments of homologous protein sequences of proteins of known specificity allow the identification of amino acid residues suitable for engineering to alter the enzyme’s specificity. Rational design has been proven as useful in many efforts. For example, the regioselectivity and stereochemistry of naphthalene dioxygenase was successfully altered using rational site-directed mutagenesis (13).

However, the success of protein engineering by rational design is limited by the structural and mechanistic information available for the enzyme. Additionally, many protein functions are not confined to a small number of amino acids but are influenced by residues far from active sites, making a prediction of reactions more difficult (23).

Rational design of TecA. In this thesis, it was tried to change the regioselectivity of TecA mediated attack on chlorotoluenes by rational design of the enzyme based on the crystal structure of the related naphthalene dioxygenase in order to prevent the monooxygenolytic reaction, thus improving the pathways of chlorotoluene degradation. Position 366 of the α-subunit of TecA was found to control the regioselectivity of the enzyme and position 272 was found to be involved in enzyme activity. However, rational design of TecA was restricted, since mutations at position 366 resulted in decreased or lost activities. Nevertheless, together with the structural information given for the naphthalene dioxygenase, these results allowed to fine-tune the substrate pocket of TecA and the docking of substrates to the active site, thus helping to understand the underlying mechanism of chlorotoluene transformation. These results enable to bring sequence information in relation to functional information, thus allowing a better understanding and assessment of the properties of microorganisms in environment by predicting enzymatic properties from sequence information.

A quite different approach to enzyme redesign, which does not require structural information, is in vitro or directed evolution including random mutagenesis, gene shuffling, family shuffling, or combinations of these. These methods were proven to be useful to create large number of mutants. Thus, directed evolution is considered to have a great potential for the development of proteins with new functions (3, 5, 23, 26, 27). However, good screening methods to screen thousands of mutants are essential.
Another strategy for designing biocatalysts suitable for biotechnological applications is the rational combination of catabolic segments from different organisms (19, 20). This technique has been proven to be useful in the generation of new metabolic routes for xenobiotics and to avoid the formation of dead end products (2, 19). In this thesis, a solvent tolerant chlorobenzene degrading strain was successfully constructed by assemblage of genes of the *tod*-pathway and genes of the chlorocatechol pathway within a solvent tolerant recipient strain.

### 7.3 Construction of a solvent tolerant chlorobenzene degrading strain

A solvent tolerant strain able to grow with high concentrations of chlorobenzene was constructed by introduction of genes of the chlorocatechol pathway into the solvent tolerant strain *Pseudomonas putida* DOT-TE, thus combining them with the genes of the *tod*-pathway. Difficulties to grow at high chlorobenzene degradations were obviously not due to the toxicity of the substrate, but due to the unbalanced activity of the enzymes of the chlorocatechol pathway resulting in the accumulation of the toxic 3-chlorocatechol.

As shown by Huertas et al. (7), in sites heavily contaminated with aromatic hydrocarbons solvent tolerant strains would be expected to establish and colonize the site first and to become predominant in the removal of these compounds. Thus, the constructed solvent-tolerant chlorobenzene degrading strain might be a promising microorganism for bioremediation purposes.
7.4 Outlook

Engineering of strains capable to utilize chlorotoluenes. As shown in this thesis for Ralstonia sp. strain PS12, chlorinated toluenes are misrouted in a dead end pathway by the TecA tetrachlorobenzene dioxygenase catalyzed monooxygenation. Lehning et al. (12) suggested, that introduction of a 2-chlorobenzoate dioxygenase should enable the strain to mineralize 2-chlorotoluene. However, recent experiments to construct such a 2-chlorotoluene degrading strain failed (6, 11). Another strategy to enhance the substrate spectrum of the strain PS12 was used in this thesis. It was tried to change the regiospecificity of TecA by rational protein design in order to prevent the monooxygenolytic reaction. However, rational design could be performed to shift the mode of attack towards monooxygenation, only. Substitutions at the opposite site of the substrate pocket elongation formed by the residue F366 might be more successful, since the residues at this site are supposed to interact with the chlorine of the substrate molecule. Gene shuffling between related dioxygenases might be another promising strategy to enhance the catabolic properties of TecA.

However, even the successful engineering of a dioxygenase to exclusively catalyze dioxygenation does not necessarily result in an optimized pathway. 2-Chlorotoluene mineralization necessitates evidently an attack in 5,6-position, whereas attack in 3,4-position would result in a catechol for which no metabolic sequence has been described thus far. Even the “optimal” 5,6-attack would result after processing in a muconate, which can be, by enzymes characterized thus far, only poorly degraded. Thus, it would be important to characterize new members of the chloromuconate cycloisomerase family for their activity and mechanism to get a more detailed understanding on substrate specificity, including substrate docking into the active site. In addition, a search for new metabolic properties in the environment for degradation of chloromethylmuconolactones would be important to recruit the whole microbial diversity for chlorotoluene degradation. A more promising approach for improved mineralization of mixtures of chlorotoluenes might be the analysis and use of mixed cultures of natural strains, e.g. a mixed culture consisting of the strain PS12 capable of transforming 2-chlorotoluene efficiently and a strain able to utilize 2-chlorobenzoate excreted by the strain PS12. However, essential for both the selection of appropriate strains as well as a rational optimization of metabolic performance of communities in the environment is a good knowledge of the catabolic properties of isolates and the detailed characterization of degradation pathways.

Properties of the transconjugant KP26. In this thesis, a solvent tolerant strain was constructed capable of mineralizing high concentrations of chlorobenzene. Thus, a strain was obtained
having an appropriate catabolic potential for bioremediation purposes. However, since the potential to mineralize chlorobenzene has been studied under lab conditions, further studies of the behavior of the bacteria in microcosms, which are regarded as a suitable approach to evaluate the survival and functioning of genetically engineered microorganisms (GEM), are necessary (17). Recombinant bacteria were found to survive better than the parental strains only when they were introduced in soil under conditions strongly favorable for the GEM (18). Thus, due to its high solvent tolerance, the constructed strain KP26 will have a strong advantage in environments heavily contaminated with organic solvents such as chlorobenzene. Further, studies that are focused on the stability of genetic information introduced in the strain DOT-T1 and on the catabolic properties of the transconjugant in microcosms are necessary (17).

**In conclusion,** the results provided in this thesis expand the knowledge of the catabolic properties of the strain PS12, gave new insights into the structure of the tetrachlorobenzene dioxygenase and contributed to understand the catalytic mechanism. For the first time, the degradation pathways of 2,4-, 2,5- and 3,4-dichlorotoluene have been investigated in detail. Further, a promising biocatalyst capable to mineralize high concentrations of chlorobenzene was engineered that might be suitable for bioremediation applications.
1.5 References


LEBENSLAUF

Katrin Pollmann, geboren am 06.02.1973 in Münster/Westf.
Staatsangehörigkeit: deutsch

Ausbildung:
- 1979-1983 Matthias-Claudius-Grundschule in Münster-Handorf
- 06/1992 Abitur

Beruflicher Werdegang:
- 10/1992-09/1994 Studium Dipl. Biologie an der Westfälischen Wilhelms-Universität Münster
- 09/1994 Vordiplom
- 03/1997-03/1998 Diplomarbeit im Fachbereich Biologie/ AG Spezielle Botanik an der Universität Osnabrück
- 1998-1999 Wissenschaftliche Hilfskraft an der Universität Osnabrück
- 05/1999-04/2002 Wissenschaftliche Hilfskraft der GBF und experimentelle Arbeiten zu der vorliegenden Dissertation
- seit 05/2002 Wissenschaftliche Mitarbeiterin am Forschungszentrum Rossendorf in Dresden
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