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Metabolism of Dichloromethylcatechols as Central Intermediates in the Degradation of Dichlorotoluenes by *Ralstonia* sp. Strain PS12

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***Ralstonia* sp. strain PS12 is able to use 2,4-, 2,5-, and 3,4-dichlorotoluene as growth substrates. Dichloromethylcatechols are central intermediates that are formed by TecA tetrachlorobenzene dioxygenase-mediated activation at two adjacent unsubstituted carbon atoms followed by TecB chlorobenzene dihydrodiol dehydrogenase-catalyzed rearomatization and then are 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 analyzed. Whereas 3,4-dichloro-6-methylcatechol is quantitatively transformed into one dienelactone (5-chloro-2-methyldienelactone) and thus is 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 is 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 nuclear magnetic resonance analysis revealed the intermediate formation of 2,5-dichloro-4-methylmuconolactone, showing that both 1,4- and 3,6-cycloisomerization occur with this muconate and indicating a degradation of the muconolactone via a reversible cycloisomerization reaction and the dienelactone-forming branch of the pathway. Diastereomeric mixtures of two dichloromethylmuconolactones were prepared chemically to proof such a hypothesis. Chloromuconate cycloisomerase transformed 3,5-dichloro-2-methylmuconolactone into a mixture of 2-chloro-5-methyl-*cis*- and 3-chloro-2-methyldienelactone, 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.**

Chlorotoluenes are important intermediates in many chemical processes such as the production of dyes and pesticides and are still produced in large amounts (22, 59). Their high chemical stability causes their accumulation in the environment (3). Whereas many bacterial strains that are able to degrade various chloro- or methyl-substituted aromatics have been studied, only a few strains that are able to mineralize chlorinated toluenes are known. Degradative pathways have been described only for 3- and 4-chlorotoluene (1, 9, 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 (formerly *Pseudomonas* and *Burkholderia*) is known to mineralize a broad spectrum of chloro-substituted benzenes, as well as 3- and 4-chlorotoluene and 2,4-, 2,5-, and 3,4-dichlorotoluene (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, 48). 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 (48). As only those dichlorotoluenes, which are subject predominantly 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 trichlorocatechol formed from di-, tri-, and tetrachlorobenzenes 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. (6) have shown that muconate cycloisomerases convert 3-chloro-*cis*,*cis*-muconate predominantly to the antibiotic protoanemonin, whereas chloromuconate cycloisomerases catalyze dehalogenation only with *cis*-dienelactone as a product (Fig. 1) (58). Whereas *cis*-dienelactone is transformed by dienelactone hydrolase efficiently, the activity of the enzyme with protoanemonin is only 1% of this rate (11). Both muconate and chlo-

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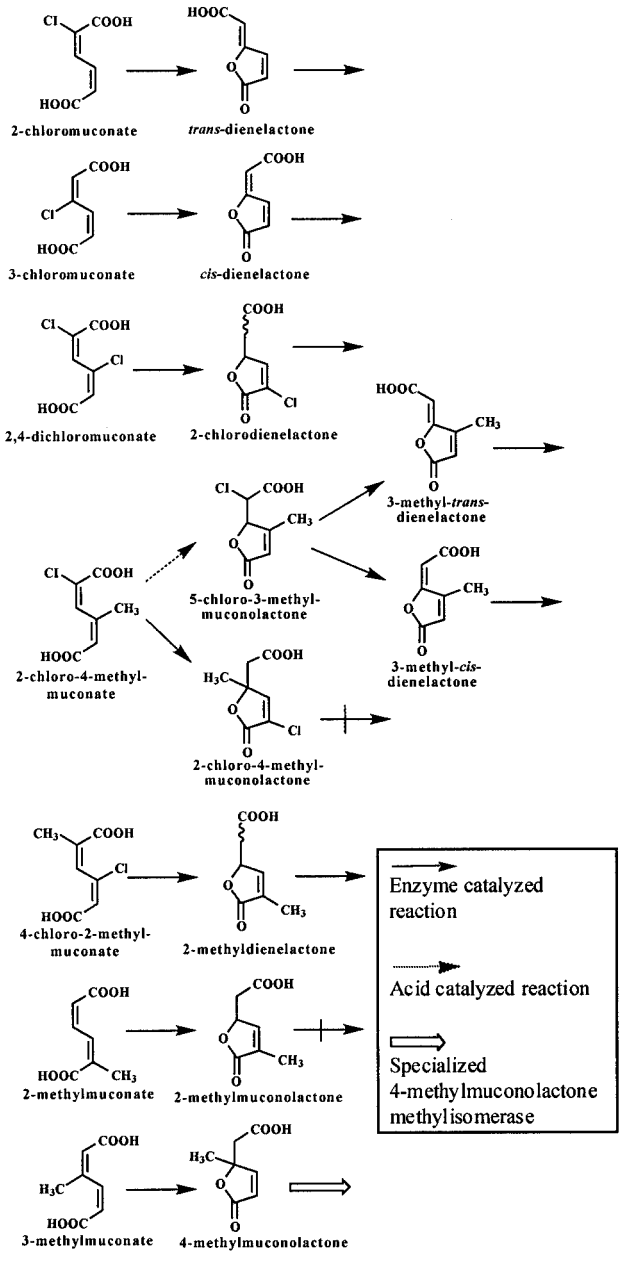

FIG. 1. Metabolism of chloro- and methyl-substituted muconic acids catalyzed by chloromuconate cycloisomerase, as described by Schmidt and Knackmuss (58), Pieper et al. (40, 42–44), Kaschabek and Reineke (30), Vollmer et al. (65), and Prucha et al. (49).

romuconate 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 3-chloromuconate in a chloromuconate 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 C-4 proton close to the general acid-base at the active site (56) and that 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 methyl-substituted muconates results in the formation of methyl-substituted muconolactones, which are often reported to be dead-end products (Fig. 1) (13, 32, 45). 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 (10, 44) 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 (51) and harbors catechol *ortho* cleavage activities but is devoid of catechol *meta*-cleavage activities, grows on methylaromatics only after introduction of a gene encoding 4-methylmuconolactone methylisomerase. The importance of proper cycloisomerization for degradation has also been shown by Pieper et al. (42) for the metabolism of chloro- and methyl-substituted 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 is converted into 2-chloro-4-methylmuconolactone as a dead-end product (Fig. 1) (40, 43, 50). In the case of 2-chloromuconolactone it was shown that this compound can be transformed by chloromuconate cycloisomerases (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 study we investigated in detail the metabolic fates of the intermediates 4,6-dichloro-3-methyl-, 3,6-dichloro-4-methyl-, and 3,4-dichloro-6-methylcatechol in the complete

metabolisms of the respective substituted chlorinated toluene substrates.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Ralstonia* sp. strain PS12 (DSM 8910) (previously *Burkholderia* and *Pseudomonas*), *Pseudomonas* sp. strain B13 (19), and *Ralstonia eutropha* JMP134(pJP4) (18) were routinely grown in mineral salts medium (19) containing 50 mM phosphate buffer (pH 7.4), supplemented with the indicated carbon source (usually 3 to 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 the sole source of carbon was added as fine mortar-ground crystals corresponding to a concentration of 5 mM. For growth on toluene or chlorinated toluenes, mineral salts medium was supplemented with 1% (vol/vol) 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).

Escherichia coli DH5α(pSTE44) containing the tetrachlorobenzene dioxygenase and chlorobenzene dihydrodiol dehydrogenase genes *tecAB* (4) was grown at 37°C in Luria broth medium containing 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and 0.1 mg of ampicillin per ml.

Monitoring of 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 (46).

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 (21, 58, 69). The activity of partially purified chloromuconate cycloisomerase was determined in the presence of an excess of dienelactone hydrolase partially purified by anion-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 at 260 nm (ε_{260}) for 2,5-dichloro-3-methyl-, 3,5-dichloro-2-methyl-, and 2,3-dichloro-5-methylmuconate (9,200, 12,800, and 10,000 M^{-1} cm^{-1}, respectively) were determined spectrophotometrically after complete transformation of 20 to 100 μM solutions of the corresponding catechols by partially purified chlorocatechol 1,2-dioxygenase. Substrate concentrations as well as complete turnover were characterized by high-pressure liquid chromatography (HPLC) analysis. In the case of muconate, 2-methylmuconate, 3-methylmuconate, 2-chloromuconate, 3-chloromuconate, and 2,4-dichloromuconate, substrate transformation was analyzed spectrophotometrically at λ values of 299 nm ($\varepsilon_{cis,cis\text{-muconate}}$ = 457 M^{-1} cm^{-1} [67]), 285 nm ($\varepsilon_{2\text{-methyl-cis,cis-muconate}}$ = 6,050 M^{-1} cm^{-1} [67]), 280 nm ($\varepsilon_{3\text{-methyl-cis,cis-muconate}}$ = 5,050 M^{-1} cm^{-1}), or 260 nm as described previously (20, 33). In the case of 3-chloromuconate, for concentrations above 0.15 mM, the formation of *cis*-dienelactone was analyzed at a λ 305 nm ($\varepsilon_{cis\text{-dienelactone}}$ = 5,300 $M^{-1}cm^{-1}$). The kinetic parameters were calculated by nonlinear regression analysis with SigmaPlot (version 6; SPSS Science). Turnover numbers (k_{cat} values) were calculated by assuming a subunit molecular mass of 38 kDa.

Protein concentrations in the cell extracts were determined by the Bradford procedure (7). One enzyme unit is defined as the amount of enzyme that catalyzes the transformation of 1 μmol of 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 with 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 liters of mineral salts medium containing 3 mM 3-chlorobenzoate as a carbon source (volume, 2 ml; total protein, 100 mg; total activities of chlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase, and dienelactone hydrolase with 0.1 mM 3-chlorocatechol, 2-chloromuconate, or *cis*-dienelactone, 16 ± 5, 7 ± 0.5, or 14 ± 5 U, respectively) was applied to a MonoQ HR 10/10 column and eluted with a linear gradient of NaCl (0 to 0.5 M over 200 ml) in Tris HCl (50 mM and pH 7.5, supplemented with 2 mM $MnCl_2$) (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 subsequent use as auxiliary enzyme, whereas 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) activities were pooled and concentrated to a final volume of 0.5 ml with Centricons (Millipore, Eschborn, Germany). Chlorocatechol 1,2-dioxygenase and chloromuconate cycloisomerase were further purified by hydrophobic interaction chromatography. $(NH_4)_2SO_4$ was added to the pooled fractions to give a final concentration of 1 M. The solution was applied to a phenyl-Superose HR 5/5 (Pharmacia) column and eluted with a linear gradient of $(NH_4)_2SO_4$ (1 to 0 M over 32 ml) in Tris HCl (50 mM and pH 7.5, with 2 mM $MnCl_2$) (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 $(NH_4)_2SO_4$] or chloromuconate cycloisomerase [eluting at 0.80 ± 0.02 M $(NH_4)_2SO_4$] activities were pooled and concentrated to a final volume of 0.5 ml by using Centricons. A chlorocatechol 1,2-dioxygenase preparation (total protein, 1 mg; total activity, 2.4 U) with a specific activity of 2,400 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 gel filtration. The solution was applied to a HiLoad 16/60 Superdex 200 column and eluted with Tris HCl (50 mM and pH 7.5, with 2 mM $MnCl_2$) over 140 ml (flow rate, 1 ml/min; fraction volume, 1 ml). The fractions containing high chloromuconate cycloisomerase activity (eluting at 59 to 63 ml) were pooled and concentrated to a final volume of 1 ml with Centricons. A chloromuconate cycloisomerase preparation (total protein, 1.64 mg; total activity, 1.5 U) with a specific activity of 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 sodium dodecyl sulfate gel electrophoresis with subsequent Coomassie brilliant blue staining (46), resulting in one visible band at 38,000 Da. For partial purification of chloromuconate cycloisomerase from strains B13 and JMP134, cell extracts of cultures (total volume of 1 liter) 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 and 6 U, 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 phenyl-Superose 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 the substrate were obtained.

Measurement of chloride. To each 200 μl of the samples mixed with 800 μl of MilliQ water, 200 μl of a 0.1 M $AgNO_3$ solution was added. After 10 min, the turbidity was determined at a λ of 525 nm. The chloride concentration was calculated by comparison with a calibration curve (23).

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 to 20 mU) in a total volume of 1 ml 33 mM Tris HCl buffer (pH 8.0) containing 0.1 mM substrate. The chloromuconate cycloisomerase present in the cell extract was inactivated by the addition of 1.3 μmol of EDTA. The formed muconates were transformed by acidification of the solution to pH 4.0. After neutralization, additional cell extract or partially purified chloromuconate cycloisomerase and dienelactone hydrolase of strain PS12 (corresponding to total activities of chloromuconate cycloisomerase and dienelactone hydrolase with 3-chloromuconate and *cis*-dienelactone of 15 to 20 mU, respectively) were added. After 10 min of incubation at room temperature, 0.5 mM NADH-H^+ and additional cell extract (corresponding to a total activity of maleylacetate reductase with maleylacetate of 15 to 20 mU) were 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), chlorinated methylcatechol as the substrate (0.1 mM) and cell extract corresponding to a final protein content of 0.1 mg/ml. The reaction was monitored for 1 h 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 of 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 to 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 of Tris HCl buffer (33 mM, pH 8.0) containing 0.1 mM dichloromethyl-substituted 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), 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 gas chromatography (GC) analysis, the products were esterified by methylation with diazomethane. The products were stored at −80°C.

HPLC analyses. Metabolites were analyzed 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 column (Bischoff, Leonberg, Germany). The aqueous solvent system (flow rate, 1 ml/min) contained 0.01% (vol/vol) H_3PO_4 (87%) or 0.005 M PicA (Millipore-Waters, Bedford, Mass.) and 35, 40, 55, or 50% (vol/vol) methanol for the determination of metabolites or 50 or 60% (vol/vol) 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-mass spectrometry (GC-MS) analyses. One microliter 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, Bad Homburg, Germany) coupled to a QP-5000 quadropole mass spectrometer as described previously (4).

In situ NMR analyses of the transformation of substrates. Prior to nuclear magnetic resonance (NMR) analyses, the Tris HCl buffer of enzyme preparations was exchanged for borate buffer (0.2 M H_3BO_3, 0.05 M $Na_2B_4O_7$ [pH 7.8]) with Centricons. The substrates were dissolved in aqueous borate buffer (200 mM H_3BO_3 and 50 mM $Na_2B_4O_7$ [pH 7.8] supplemented with 20% [vol/vol] D_2O) to give a final concentration of 0.7 mM and transferred to the NMR sample tube (0.7 ml). The one-dimensional ^1H NMR spectra were recorded at 300 K on an AVANCE DMX 600 NMR spectrometer (Bruker, Rheinstetten, Germany) locked to the deuterium resonance of D_2O in the solution. Spectra were recorded by 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 an internal reference (δ 4.80 ppm).

After recording the in situ ^1H spectra 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 to 3 h. 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.

^1H NMR analyses. For identification of compounds Ia and Ib or compounds 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 was supplemented with cell extract of 3-chlorobenzoate-grown cells (corresponding to 2 mg of protein) and the reaction was 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 compounds 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 of acetone-d_6 (Chemotrade, Düsseldorf, Germany). ^1H NMR spectra were recorded on an AVANCE DMX 600 NMR spectrometer (Bruker) with tetramethylsilane as an internal standard.

Chemicals. Dichloromethylcatechols were prepared as follows. *E. coli* DH5α(pSTE44) was grown in 200 ml of Luria broth medium containing 1 mM IPTG and 0.1 mg of ampicillin per ml. After reaching an A_{600} of 2.7, the cells were harvested by centrifugation (6,000 × *g*, 10 min, 20°C). The pellet was washed twice with assay buffer (54), resuspended in 200 ml of the same buffer, and supplemented with 0.5 mM 2,4-, 2,5-, or 3,4-dichlorotoluene (from a 100 mM stock solution in methanol). The flasks were sealed with Teflon-coated screw caps and incubated at 30°C on a rotary shaker (150 rpm) for 5 h. After centrifugation (10,000 × *g*, 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 $MgSO_4$ 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 dichloromethylmuconate as substrates for chloromuconate cycloisomerase were prepared in situ from 4-chloro-, 3-methyl-, 4-methyl-, 3,5-dichloro-, and dichloromethylcatechol, respectively, using partially purified chlorocatechol 1,2-dioxygenase which was free of any (chloro)muconate cycloisomerase activity.

2-Chloro-3-methyl-*trans*-dienelactone (*trans*-2C3MDL) and 2-chloro-5-methyl-*cis*-dienelactone (*cis*-2C5MDL) were prepared as follows. The *tert*-butylester of *trans*-2C3MDL was obtained as one of several isomers from the Wittig reaction between equimolar amounts of (*tert*-butoxycarbonylmethylene)triphenylphosphorane [$(Ph)_3P{=}CHCO_2tBu$] and chlorocitraconic anhydride as described by Kaschabek (27). The *t*-butylester of 2C5MDL was prepared in a similar way, using $(Ph)_3P{=}CCH_3CO_2tBu$ 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. *cis*-2C3MDL and *trans*-2C5MDL were produced by the irradiation of aqueous solutions of these substrates (0.2 mM) with a UV lamp and irradiation with 254-nm light at a distance of 6 cm for 2 h as described for the preparation of 2-chloro-*trans*-dienelactone from 2-chloro-*cis*-dienelactone (31). Reactions were monitored by HPLC. The E_{210}/E_{260} ratio and the absorption maxima of the products (λ_{max} = 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).

RESULTS

Growth of *Ralstonia* sp. strain PS12 on dichlorotoluenes. Out of the range of di- and trichlorotoluenes analyzed (2,3-, 2,4-, 2,5-, 2,6-, 3,4-, and 3,5-dichlorotoluene and 2,4,5-trichlorotoluene), only 2,4-, 2,5-, and 3,4-dichlorotoluene could be used as growth substrates by *Ralstonia* sp. strain PS12. The 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 (A_{600} = 0.44 ± 0.05 and 0.31 ± 0.05, respectively) than with 3,4-dichlorotoluene (A_{600} = 0.16 ± 0.06). The growth yield with 2,4-dichlorotoluene was similar to that observed with 1,2,4,5-tetrachlorobenzene (4). Whereas 2,4- and 2,5-dichlorotoluene were reasonable growth substrates, with generation times of 14 to 15 h (compared to a generation time of 9.5 h reported for 1,2,4,5-tetrachlorobenzene [4]), growth with 3,4-dichlorotoluene was poor (generation time of 22 h). 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 (48). 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 volumes 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 concentrations amounted to 2 and 15% of the substrate transformed and were 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, accumulation of neither 3,4-dichlorobenzylalcohol nor 3,4-dichloro-

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[a]

Substrate	Product	[MeOH] (%)[b]	Retention vol (ml)	λ_{max} (nm)
4,6-Dichloro-3-methylcatechol	3,5-Dichloro-2-methylmuconate	35 (+0.005MPicA)	3.3	270
	3,5-Dichloro-2-methylmucono-lactone (compound Ia)	50	4.0	221
	3,5-Dichloro-2-methylmuconolactone (compound Ib)	50	5.0	221
	3C2MDL (*cis* or *trans*)	42	5.5	278
	cis-2C5MDL	42	4.0	299
	2-Chloro-5-methylmaleylacetate or 3-chloro-2-methylmaleylacetate	42	2.7	223
3,6-Dichloro-4-methylcatechol	2,5-Dichloro-3-methylmuconate	35 (+0.005MPicA)	3.9	273
	2,5-Dichloro-3-methylmuconolactone (compound Ic)	42	3.0	223
	2,5-Dichloro-3-methylmuconolactone (compound Id)	42	3.2	223
	cis-2C3MDL	42	6.6	286
	trans-2C3MDL	42	4.3	286
	2-Chloro-3-methylmaleylacetate	42	2.5	225
3,4-Dichloro-6-methylcatechol	2,3-Dichloro-5-methylmuconate	35 (+0.005MPicA)	3.9	289
	5C2MDL (*cis* or *trans*)	50	4.2	299
	5-Chloro-2-methylmaleylacetate	50	2.6	207

[a] Cells of *E. coli* DH5α(pSTE44) expressing both tetrachlorobenzene dioxygenase TecA and dihydrodiol dehydrogenase TecB from strain PS12 were incubated with dichlorotoluenes. The catechols formed were extracted and used as a substrates for enzymes of the chlorocatechol *ortho*-cleavage pathway of strain PS12. The proposed products derived from the transformations are shown.

[b] Percentage of the methanol used in the solvent system, with or without PicA reagent.

benzoate was observed. This is in accordance with the observation that TecA dioxygenates 3,4-dichlorotoluene exclusively. Of note, however, is that during growth on 3,4-dichlorotoluene a yellow coloration of the culture supernatant was observed, with a λ_{max} of 361 nm. As products formed by extradiol cleavage of several unsubstituted and substituted catechols are known to exhibit similar absorption maxima (52, 53), 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, 35), is usually not suited for mineralization of chloro-substituted catechols.

Metabolites later identified as the respective dichloromethylmuconates and chloromethyldienelactones 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, respectively, 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 used in further experiments, as those were more easily accessible. Purified enzymes of these extracts were used to monitor 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.

Substituted muconates, products formed after intradiol cleavage, have been reported to exhibit absorption maxima of 260 to 280 nm (58). Since substituted muconates, specifically those substituted in position 3, 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 to 20 mU) and EDTA. HPLC analyses revealed the formation of single products with absorption maxima at 270, 273, and 289 nm (Table 1), respectively, which are indicative of the formation of 3,5-dichloro-2-methyl-, 2,5-dichloro-3-methyl-, and 2,3-dichloro-5-methylmuconate, respectively (Table 1).

Transformation of dichloromethylmuconates. Acidification (to a final pH of 4) of the above-described 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-bond 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-methylmuconolactones (due to the presence of two asymmetric carbon atoms, the formation of four stereoisomers was described) (43, 50), we

postulated that compounds Ia to Id 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, 45, 58).

After neutralization (pH 7) of the solutions containing compounds Ia and Ib or compounds Ic and Id, partially purified chloromuconate cycloisomerase (total activity with 2-chloromuconate, 2 to 5 mU) was added. Products with absorption maxima of between 278 and 299 nm (Table 1), indicating the formation of dienelactones (58), were monitored. Ia and Ib were converted mainly to *cis*-2C5MDL, as confirmed by comparison of the retention volume and UV absorption spectrum with those of 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-methyldienelactone (3C2MDL). 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 *trans*-2C3MDL as the major product and *cis*-2C3MDL as the minor product (approximately 2% as determined by comparing signal intensities at 270 nm after separation by HPLC). These products were verified by comparison with authentic standards (Table 1). More than 60% each of 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 to 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 *cis*-2C5MDL and of 3C2MDL with unknown stereochemistry. Assuming similar extinction coefficients at a λ of 230 nm, both compounds were produced in approximately a 1:1 ratio, which is different from 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 *trans*-2C3MDL as the major product and *cis*-2C3MDL 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-methyldienelactone [5C2MDL]), without any evidence for the accumulation of intermediate products.

Identification of products formed by chloromuconate cycloisomerase by GC-MS. GC-MS analysis of three reaction mixtures thought to contain a mixture of *cis*-2C5MDL, 3C2MDL of unknown stereochemistry, and either *trans*-2C3MDL as the major product or 5C2MDL confirmed the identity of all these products as chloromethyl-substituted dienelactones. Prominent signals showing molecular ions 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 thought to contain *cis*-2C5MDL and 3C2MDL. One of these, exhibiting a retention time of 11.6 min (relative intensities, *m*/*z* 202, 171, and 170 [30, 50, and 85%, respectively, of base peak]), was identified as *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, and 170 [15, 95, and 3%, respectively, of base peak]) very similar to that of authentic *trans*-2C3MDL, with a base peak of *m*/*z* 69. Significant fragments with this mass (at least 50% of base peak) have been observed for 2-methyl, 3-methyl-, and 2-chlorodienelactone (26, 40, 49, 60), indicating that the fragment is comprised not of C-2 and C-3 but of the C-5 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 base peak of *m*/*z* 69 suggests that it is 3C2MDL.

In the reaction mixture thought to contain *trans*-2C3MDL as the major product, two products with nearly identical fragmentation patterns (relative intensities, *m*/*z* 202, 171, and 170 [13, 70, and 48%, respectively, of base peak]) 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 base peak of *m*/*z* 69. The methylester of the dienelactone tentatively identified as 5C2MDL has a retention time of 12.5 min (relative intensities, *m*/*z* 202, 171, and 170 [40, 27, and 60%, respectively, of base peak]). Only a minor fragment of *m*/*z* 69 was observed, and fragments of *m*/*z* 105 and 103 (33 and 12% of base peak) indicate that it is actually 5C2MDL.

Identification of Ia to Id by ^{1}H NMR analysis. As compounds Ia to Id were intermediates in the formation of dienelactones from chloro-substituted 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 and Ib or of Ic and 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 six signals originating from Ia plus Ib, with three signals from each compound as

judged by 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 (43, 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, whereas the second compound had signals at 1.92 (methyl function), 5.25, and 5.80 ppm. These data are compatible with the assumption that Ia and Ib are different stereoisomers of 3,5-dichloro-2-methylmuconolactone. 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 (50). The chemical shift values of 5.25 and 5.30 ppm resemble those observed for the 5-H protons of 5-chloro-3-methylmuconolactone (5.14 ppm [65]) or 5-chloro-3-methylmuconolactone (5.20 and 5.24 ppm for the different diastereomers [43]), 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 to 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 ^{1}H NMR signals at chemical shifts of 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 ^{1}H NMR spectrum indicated that Ic-Id was a mixture of diastereomers of 2,5-dichloro-3-methylmuconolactone. The chemical shift values of 5.20 and 5.26 ppm resemble those observed for the 5-H protons of 5-chloro-substituted muconolactones (5.15 to 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 to 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 methyl-substituted muconates often leads to methyl-substituted muconolactones as dead-end products (45), and the formation of 2-chloro-4-methylmuconolactone from 2-chloro-4-methylmuconate has been suggested to be the reason for the failure of *R. eutropha* strain JMP134 to grow with 2-chloro-4-methylphenoxyacetate (43). Since such dead-end products obviously did not occur during transformation of dichloromethyl-substituted 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.

The turnover numbers of PS12 chloromuconate cycloisomerase for the various substrates tested were usually slightly lower than, but similar to, those of previously described chloromuconate cycloisomerases (Table 2). Specificity constants varied by a factor of 10, showing that this enzyme was as unspecific as the strain B13- or P51-derived enzyme (67). All chloromuconate cycloisomerases tested showed significant activity with all three available dichloromethyl-substituted muconates. The respective products and the product compositions formed from the different dichloromuconates, as verified by HPLC analyses, were independent of the source of the enzymes. *trans*-2C3MDL, as well as minor amounts of *cis*-2C3MDL, was formed from 2,5-dichloro-3-methylmuconate, 3,5-dichloro-2-methylmuconate was transformed into a 1:1 mixture of *cis*-2C5MDL and 3C2MDL, and 2,3-dichloro-2-methylmuconate was transformed into 5C2MDL. When supplied at concentrations of 0.1 mM, 2,5-dichloro-3-methylmuconate was always transformed significantly faster than the other two dichloromethylmuconates.

Transformation of chloromethyl-substituted dienelactones. After addition of partially purified dienelactone hydrolase (total activity of dienelactone hydrolase with *cis*-dienelactone, 15 to 20 mU) to reaction mixtures (1 ml) containing *trans*-2C3MDL plus *cis*-2C3MDL, *cis*-2C5MDL plus 3C2MDL, or 5C2MDL, all of 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 a substrate.

Dienelactone hydrolase-catalyzed transformation of *trans*-2C3MDL resulted in the formation of a new product exhibiting a UV absorption maximum at 225 nm under the acidic conditions used for HPLC separation (Table 1). The same product was observed upon transformation of *trans*-2C3MDL with cell extract. Upon addition of NADH to the last reaction mixture, further transformation of this product was observed. This suggests that *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 (41). Addition of NADH would then result in its reduction by maleylacetate reductase present in the cell extract to 5-chloro-4-methyl-3-oxoadipate.

Only one new signal, exhibiting an absorption maximum at 223 nm (by 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 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 containing 5C2MDL resulted in the formation of a new product exhibiting an absorption maximum at 207 nm, a significantly shorter wavelength than for the products formed from other dienelactones. This product was not transformed to any significant extent within 30 min after addition of NADH plus 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 (41, 57), which exhibits an absorption maximum at a λ of 195 nm under acidic conditions (14, 62), quite different from that of maleylacetate at 206 nm (49, 61). Thus, it is proposed that 5-chloro-2-methylmaleylacetate formed by hydrolysis of 5C2MDL is subject to a fast decarboxylation.

In situ NMR analyses. As various metabolites in the degradation of highly substituted aromatics such as muconates, mu-

TABLE 2. Substrate specifities of chloromuconate cycloisomerases from different strains[a]

Strain and substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})	Relative specifity constant (%)	Relative activity at 0.1 mM substrate (%)
Ralstonia sp. strain PS12					
cis,cis-Muconate[b]	239	78	0.33	9	35
2-Chloro-*cis,cis*-muconate	146	122	0.84	24	70
3-Chloro-*cis,cis*-muconate	189	171	0.90	26	100
2,4-Dichloro-*cis,cis*-muconate	91	161	1.1	32	165
2-Methyl-*cis,cis*-muconate[c]	100	231	2.31	66	186
3-Methyl-*cis,cis*-muconate[d]	258	898	3.48	100	448
2,5-Dichloro-3-methyl-*cis,cis*-muconate	ND[e]	ND	ND	ND	151
2,4-Dichloro-5-methyl-*cis,cis*-muconate	ND	ND	ND	ND	46
2,3-Dichloro-5-methyl-*cis,cis*-muconate	ND	ND	ND	ND	43
Pseudomonas sp. strain B13					
cis,cis-Muconate	271	481	1.8	12	15
2-Chloro-*cis,cis*-muconate	42	481	11	74	39
3-Chloro-*cis,cis*-muconate	159	2,240	14	92	100
2,4-Dichloro-*cis,cis*-muconate	26	394	15	100	36
2-Methyl-*cis,cis*-muconate	91	744	8.2	54	45
3-Methyl-*cis,cis*-muconate	32	481	15	100	42
2,5-Dichloro-3-methyl-*cis,cis*-muconate	ND	ND	ND	ND	167
2,4-Dichloro-5-methyl-*cis,cis*-muconate	ND	ND	ND	ND	31
2,3-Dichloro-5-methyl-*cis,cis*-muconate	ND	ND	ND	ND	82
Ralstonia sp. strain JMP134					
cis,cis-Muconate	996	704	0.71	0.58	3
2-Chloro-*cis,cis*-muconate	79	215	2.7	2.2	6
3-Chloro-*cis,cis*-muconate	98	3,770	38.0	32	100
2,4-Dichloro-*cis,cis*-muconate	23	2,810	120.0	100	120
2-Methyl-*cis,cis*-muconate	312	5,440	17.0	14	69
3-Methyl-*cis,cis*-muconate	52	3,460	66.0	55	120
2,5-Dichloro-3-methyl-*cis,cis*-muconate	ND	ND	ND	ND	76
2,4-Dichloro-5-methyl-*cis,cis*-muconate	ND	ND	ND	ND	25
2,3-Dichloro-5-methyl-*cis,cis*-muconate	ND	ND	ND	ND	20
Pseudomonas sp. strain P51					
cis,cis-Muconate	177	383	2.2	1	31
2-Chloro-*cis,cis*-muconate	58	407	7.1	13	58
3-Chloro-*cis,cis*-muconate	184	1,260	6.9	13	100
2,4-Dichloro-*cis,cis*-muconate	19	978	53.0	100	185
2-Methyl-*cis,cis*-muconate	92	1,500	16.0	31	176
3-Methyl-*cis,cis*-muconate	145	1,070	7.4	14	98

[a] The data for strains B13, JMP134, and P51 are from reference 67, except for the relative activity values for the dichloromethylmuconates.
[b] For *cis,cis*-muconate, $\varepsilon_{299} = 457\ M^{-1}\ cm^{-1}$ (67). Substrate concentrations were 0.2 to 0.9 mM.
[c] For 2-methyl-*cis,cis*-muconate, $\varepsilon_{285} = 6{,}050\ M^{-1}\ cm^{-1}$ (67). Substrate concentrations were 0.025 to 0.2 mM.
[d] For 3-methyl-*cis,cis*-muconate, $\varepsilon_{280} = 5{,}050\ M^{-1}\ cm^{-1}$. Substrate concentrations were 0.05 to 0.35 mM.
[e] ND, not done.

conolactones, dienelactones, or maleylacetates are often unstable under nonphysiological conditions (29, 43, 50, 57, 60), HPLC and GC-MS analyses may lead to artifacts (40, 60). One-dimensional ^1H NMR spectroscopy in H_2O has recently been shown to be an excellent tool for monitoring enzyme activities and assessing solution structures of substrates and products (8, 16, 17, 24, 47). In order to characterize intermediates formed during dichloromethylcatechol metabolism, phosphate buffer, which is 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.

(i) 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 (methyl function). 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 $\delta = 2.02$ and 6.56 ppm are indicative of the presence of one methyl function and one olefinic proton in the product and are in agreement with the postulated structure 3,5-dichloro-2-methylmuconate. 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). These values are in close agreement with literature values of chemical shifts of the C-3 protons of *cis*-dienelactone (8.27 ppm) and 2-chloro-*cis*-dienelactone (8.39 ppm) and of C-5 protons of dienelactones (ranging from 6.0 to 6.1 ppm for *cis*- and from

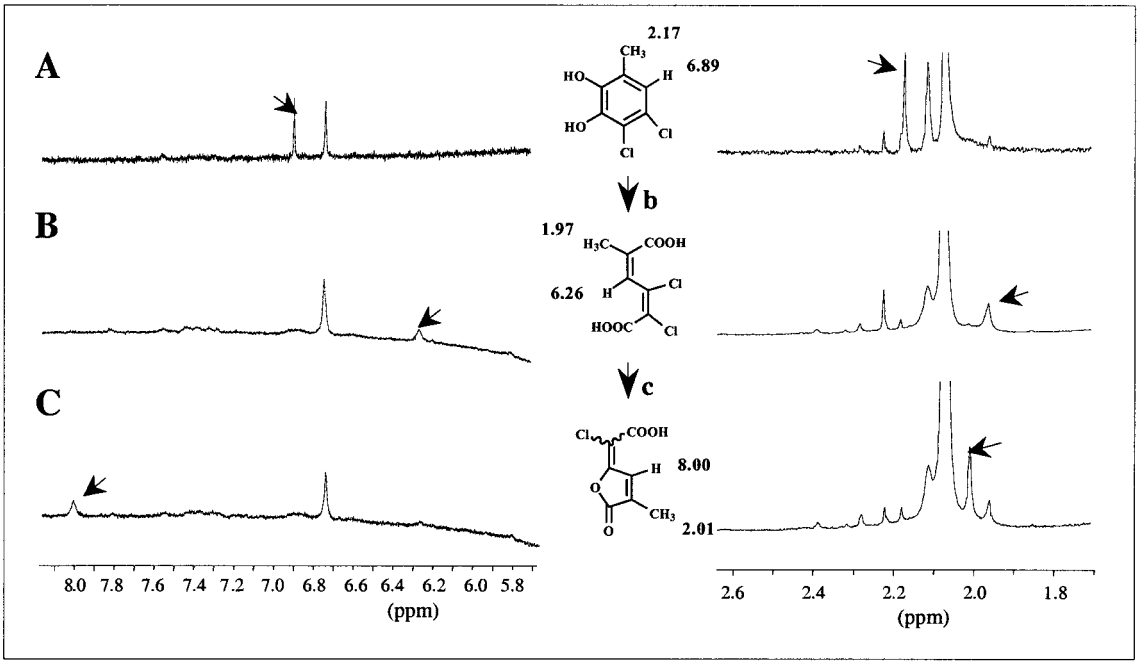

FIG. 2. ^{1}H NMR spectra of 3,6-dichloro-4-methylcatechol and transformation products. (A) before enzyme addition; (B) after addition of chlorocatechol 1,2-dioxygenase (b); (C) after addition of chloromuconate cycloisomerase (c). The peaks originating from relevant substrates and products are marked with arrows.

5.5 to 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.

(ii) 3,6-Dichloro-4-methylcatechol (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 *trans*-2C3MDL as verified by comparison with authentic material (Fig. 2). However, the transient observation of three additional signals ($\delta = 5.80$, 7.79, and 1.70 ppm) was indicative of the formation of a dichloromethyl-substituted muconolactone intermediate. Two such structures can be postulated from 2,5-dichloro-3-methylmuconate, namely, 2,5-dichloro-4-methylmuconolactone (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 (43), 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.

(iii) 3,4-Dichloro-6-methylcatechol (0.7 mM in borate buffer) showed signals for the methyl function ($\delta = 2.17$ ppm) and aromatic proton ($\delta = 6.89$ ppm) of the substrate (Fig. 3). Addition of chlorocatechol 1,2-dioxygenase led to their disappearance and the formation of two new signals at $\delta = 1.97$ 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 $\delta = 2.01$ ppm (methyl function) and 8.00 ppm (olefinic proton). As similar chemical shifts were reported for the C-3 protons of unsubstituted and substituted dienelactones (e.g., $\delta = 7.82$ for *trans*-dienelactone, $\delta = 8.37$ ppm for *cis*-dienelactone, and $\delta = 8.39$ for 2-chloro-*cis*-dienelactone (60), the identity of the product as 5-chloro-2-methyldienelactone can be assumed (Fig. 3). The signals decreased after addition of dienelactone hydrolase.

DISCUSSION

Growth on dichlorotoluenes. Recent analyses have shown that the initial tetrachlorobenzene dioxygenase TecA of strain PS12 not only attacks chlorinated benzenes dioxygenolytically but also catalyzes monooxygenation of the methyl group (34, 48). Accordingly, during growth on 2,4- or 2,5-dichlorotoluene, which are subject to monooxygenation as a side reaction (48), accumulation of the corresponding benzoates, obviously produced by the further oxidation of the dichlorobenzylalcohols, was observed (Fig. 4 and 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, 48). Thus, monooxygenation results in part in channeling of the substrates into dead-end metabolites. Consequently, only those substrates which are preferentially attacked in a dioxy-

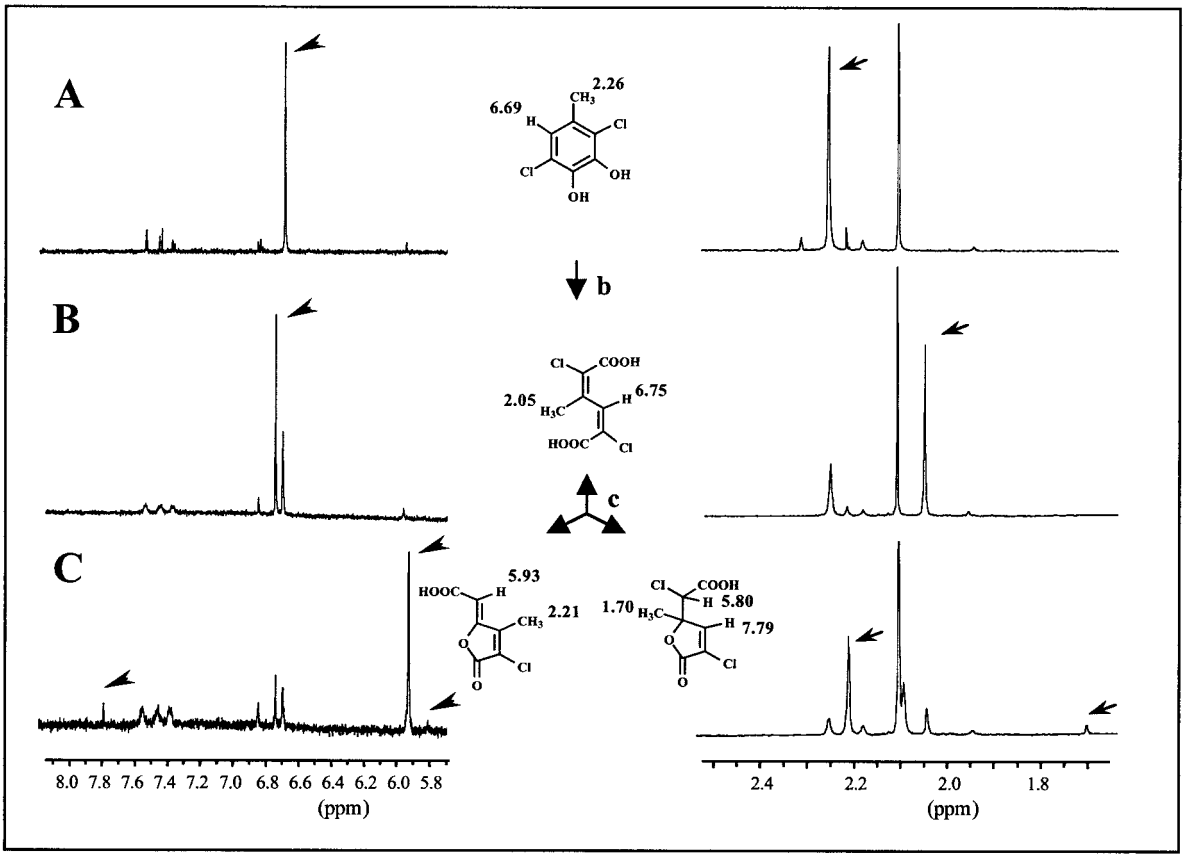

FIG. 3. ^{1}H NMR spectra of 3,4-dichloro-6-methylcatechol and transformation products. (A) Before enzyme addition; (B) after addition of chlorocatechol 1,2-dioxygenase (b); (C) after addition of chloromuconate cycloisomerase (c). The peaks originating from relevant substrates and products are marked with arrows.

genolytic manner were used as growth substrates. Surprisingly, 3,4-dichlorotoluene, which is subject exclusively 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 besides an inactive *todF* analogue (*tlpE** pseudogene) (5), 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 is 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 to 6). Different features of this reaction need special attention. First, chloromuconates as symmetric molecules can dock into the active site in two different directions, resulting in a 1,4- or a 3,6-cycloisomerization. For 2-chloromuconate, Vollmer and Schlömann (68) have shown that chloromuconate cycloisomerase catalyzes dominantly a 3,6-cycloisomerization to form (4*R*,5*S*)-5-chloromuconolactone by *syn* addition to the double bond followed by chloride elimination to produce *trans*-dienelactone (15). 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 occurs 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. 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, 40). Like 3-chloro- and 2,4-dichloromuconate, other muconates substituted in the β-position relative to one of the carboxy substituents 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-chloro substituent or addition of a proton resulting in the

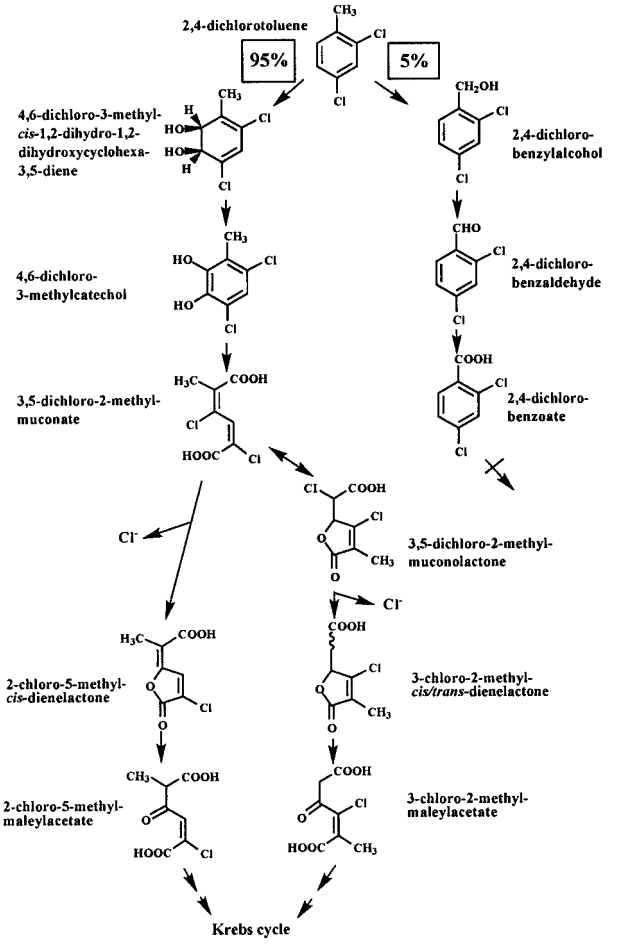

FIG. 4. Proposed pathway for the degradation of 2,4-dichlorotoluene by *Ralstonia* sp. strain PS12.

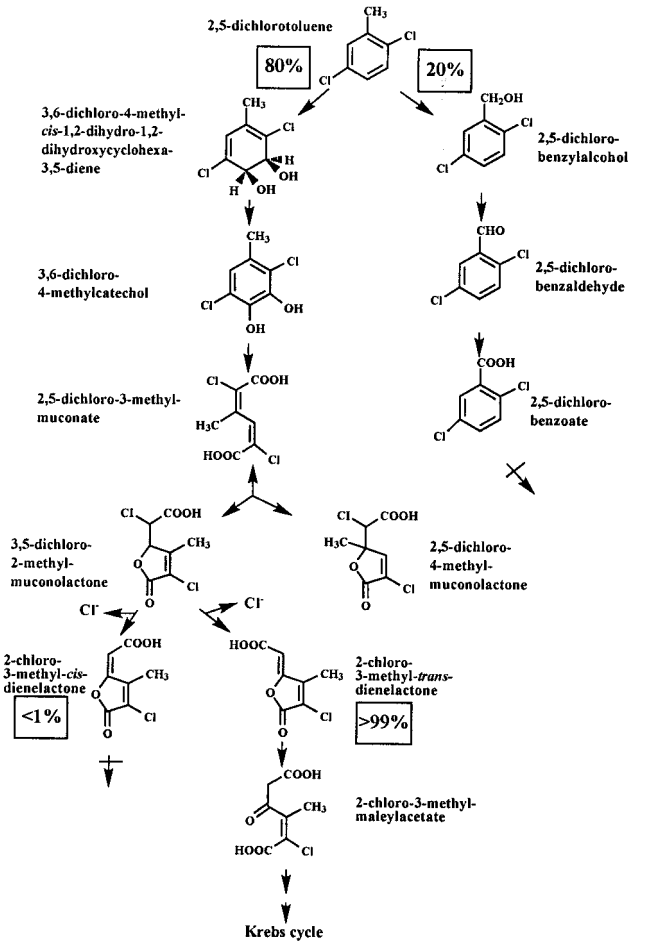

FIG. 5. Proposed pathway for the degradation of 2,5-dichlorotoluene by *Ralstonia* sp. strain PS12.

formation of 4-methyl-substituted muconolactones (Fig. 1). No indications for an alternative mode of cycloisomerization were evident in any of those reports (40, 42–45, 50, 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 2C3MDL (Fig. 5). Thus, it seems that cycloisomerization is not directed towards the methyl substituent but involves an attack on the unsubstituted C-4 carbon. Therefore, 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 *trans*-2C3MDL. However, ^{1}H NMR analysis of this reaction revealed the formation of 2,5-dichloro-4-methylmuconolactone as an intermediate product (Fig. 2). Thus, cycloisomerization obviously occurs in both possible directions. This indicates again that in situ ^{1}H NMR analyses are an appropriate tool to investigate degradation pathways of aromatics without artifacts 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 *trans*-2C3MDL via 2,5-dichloro-3-methylmuconate and 2,5-dichloro-3-methyldienelactone (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 in a reaction involving a cycloisomerase activity. It seemed that in those cases, cycloisomerization is quantitatively directed towards the methyl substituent (39, 45). 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 (4*R*,5*S*)-5-chloro-3-methylmuconolactone.

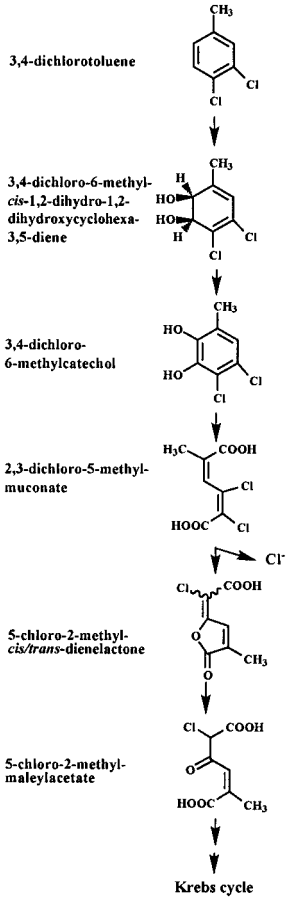

FIG. 6. Proposed pathway for the degradation of 3,4-dichlorotoluene by *Ralstonia* sp. strain PS12.

Since no reports about the metabolism of dichloromethylmuconates were available, the chloromuconate cycloisomerase of strain PS12 was thought 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 *Pseudomonas* sp. strains B13 and P51. All tested cycloisomerases transformed the dichloromethylmuconates to the same products, so that the ability 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 formation of 5-chloro-3-methylmuconolactone from 2-chloro-4-methylmuconate and 3-methylmuconolactone from 3-methylmuconate (Fig. 4 and 5) (12, 43, 50). 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 (4*R*,5*R*) and (−)-diastereomer II (4*R*,5*S*) were shown to be subject to transformation by muconolactone isomerase (50). According to Vollmer et al. (66), (4*R*,5*S*)-5-chloromuconolactone was a substrate for chloromuconate cycloisomerase and was dehalogenated to form *trans*-dienelactone. Thus, (4*R*,5*S*)-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 obviously catalyzes a *syn* elimination.

In the acid-catalyzed transformation of 3,5-dichloro-2-methyl- as well as 2,5-dichloro-3-methylmuconate, two diastereomers also 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 (4*R*,5*S*)-5-chloromuconolactone is subject to transformation, it can be speculated that the respective more-substituted (4*R*,5*S*) 5-chloromuconolactones also are transformed. Preliminary experiments indicate that actually (4*R*,5*S*)- and (4*R*,5*R*)-5-chloro-3-methylmuconolactone are transformed by chloromuconate cycloisomerase and thus that this enzyme necessitates the same configuration at the C-4 atom as muconolactone isomerase. Surprisingly, only *trans*-2C3MDL 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 C-4 proton next to the general acid-base. Thus, for both (4*R*,5*S*)- and (4*R*,5*R*)-2,5-chloro-3-methylmuconolactone the formation of *trans*-2C3MDL seems to be reasonable. The diastereomeric mixture of 3,5-dichloro-2-methylmuconolactone was transformed in both a 3,5-dichloro-2-methyldienelactone 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-chloro-substituted 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 that control regioselectivity and enantioselectivity were identified (36–38, 63). Based on this information, exchanges of amino acids in tetrachlorobenzene dioxygenase TecA which may prevent such a misrouting can be performed. However, even in this case, mineralization can be problematic. We had observed the intermediate accumulation of dichloromethylmuconates and chlorodienelactones 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, these enzymes have not yet been described as constituting a pathway bottleneck. Out of the dienelactones described in the present report,

only *cis*-2C3MDL was transformed at a low rate. Similarly, 3-methyl-*cis*-dienelactone was described as a poor substrate for dienelactone hydrolase of strain JMP134 (49). It thus seems that 3-methyl-*cis*-dienelactones are generally poor substrates for dienelactone hydrolase. Applying the rule that *cis*-dienelactones are formed after elimination of chloride from the β-position of chloromuconates, whereas *trans*-dienelactones are formed by elimination of chloride from the α-position, it can be deduced that 3-methyl-*cis*-dienelactones are probably formed during the degradation of 2,3- or 2,6-dichlorotoluene via the respective chlorocatechols. Thus, studies on the degradation of those compounds would be of special interest.

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