Hydroxylation and Dechlorination of Chlorinated Guaiacols and Syringols by Rhodococcus chlorophenolicus

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We show that Rhodococcus chlorophenolicus PCP-I, a polychlorophenol degrader, also degrades various chlorine-substituted guaiacols (2-methoxyphenols) and syringols (2,6-dimethoxyphenols). The substrates investigated were tetrachloroguaiacol, 3,4,6- and 3,5,6-trichloroguaiacol, 3,5- and 3,6-dichloroguaiacol, trichlorosyringol, and 3,5-dichlorosyringol. The first step was a hydroxylation, probably in a position para to the preexisting hydroxy. Tetrachloroguaiacol and trichlorosyringol, with a chlorine substituent in the para position, were both hydroxylated and dechlorinated. The optimum temperature for degradation of polychlorinated guaiacols and syringols was 37 to 41°C. Degradation of polychlorinated phenols, guaiacols, and syringols by R. chlorophenolicus was inducible, and induction was controlled coordinately.

Contamination of the environment with chlorinated phenolic compounds is global. Chlorine bleaching of pulp produces chlorinated guaiacols (2-methoxyphenols) and syringols (2,6-dimethoxyphenols), which are discharged with the spent bleach liquor into recipient waters (12–14, 18, 21, 27). Chlorinated guaiacols also arise from combustion of organic matter (18). Since chlorinated phenolic compounds are toxic to all forms of life, it is important to know their fate in the environment and their routes of biodegradation.

O-methylation of chlorinated guaiacols into chlorinated veratrols (1,2-dimethoxybenzenes) and O-demethylation into chlorinated catechols has been shown to be catalyzed by mixed and pure cultures of bacteria and to occur in aquatic sediments (1, 8, 15–17, 20). O-demethylation of trichlorosyringol has also been reported (17). Anaerobic bacteria were recently shown to reductively dechlorinate 3,4,5-trichloroguaiacol and tetrachloroguaiacol into 3,5-dichlorocatechol and 3,4,6-trichlorocatechol, respectively, which were stable to further microbial attack (16). Mineralization or reactions leading to nonchlorinated products were not described. We have shown that Rhodococcus chlorophenolicus PCP-I degrades polychlorinated phenols and guaiacols (4, 5, 9). In this report we show that R. chlorophenolicus also degrades polychlorinated syringols. The initial reaction in the degradation of both chloroguaiacols and chlorosyringols is shown to be a hydroxylation.

MATERIALS AND METHODS

Abbreviations. The following abbreviations are used in this report: TeCG, tetrachloroguaiacol (3,4,5,6-tetrachloro-2-methoxyphenol); 346-TCG and 356-TCG, 3,4,6-trichloroguaiacol and 3,5,6-trichloroguaiacol, respectively; 36-DCG and 35-DCG, 3,6-dichloroguaiacol and 3,5-dichloroguaiacol respectively; TCS, 3,4,5-trichlorosyringol (3,4,5-trichloro-2,6-dimethoxyphenol); 35-DCS, 3,5-dichlorosyringol; 3-MCS, 3-chlorosyringol; PCP, pentachlorophenol; GLC, gas-liquid chromatography.

The organism and culture conditions. R. chlorophenolicus PCP-I (DSM 43 826), which was used in all experiments, originated from a PCP-degrading mixed culture (3, 22, 26). The organism has been described in detail elsewhere (2) and has been shown to attack 16 different chlorophenols and chloroguaiacols (4, 5, 9). The cells were grown in liquid culture on rhamnose or sorbitol as described previously (5). In some experiments cells were induced by adding 10 μM PCP at 20-h intervals or 2 or 3 times. Cultures were incubated in a gyratory shaker in the dark at 28°C, unless otherwise stated. Bacterial density was 2 × 10⁸ to 5 × 10⁹ ml⁻¹. Toxicity of chlorinated phenolic compounds to R. chlorophenolicus was determined as described previously (9).

Substrates and reference compounds. The chlorinated guaiacols (2-methoxyphenols) and syringols (2,6-dimethoxyphenols) that were used as substrates were synthesized by J. Knutinen (Department of Chemistry, University of Jyväskylä, Jyväskylä, Finland). Chlorinated catechols were synthesized from chlorinated salicylaldehydes by application of the method described by Dakin (7) and converted into guaiacols by limited methylation (11). The compounds 346- and 356-TCG were obtained as a 1:1 (wt/wt) mixture, designated 346/356-TCG, and another 346-TCG preparation contained 10% (wt/wt) TeCG. The other chloroguaiacol preparations were of minimum purity (by GLC). Chlorinated syringols were synthesized by chlorination of syringol with Cl₂ in CS₂ and structures were verified by infrared, nuclear magnetic resonance, and mass spectroscopy and purified by high-performance liquid chromatography. PCP was obtained from E. Merck AG (Darmstadt, Federal Republic of Germany), Trichlorohydroquinone and 2,3-dichlorohydroquinone, which were used as reference compounds, were synthesized by J. Knutinen from chlorinated 4-hydroxybenzaldehyde (10), as described previously for chlorocatechols (7), 2,4,6-Tribromophenol, which was used as an internal standard in the analysis, was obtained from Fluka AG (Buchs, Switzerland).

Analysis. The chlorinated phenolic compounds were analyzed as acetylated derivatives by GLC by using a gas chromatograph (Fractovap 2300; Carlo Erba Strumentazione) equipped with a capillary column (CP Sil 5; Chrompack, Middelburg, The Netherlands) and an electron capture 63Ni detector. Acetylation, with 2,4,6-tribromophenol used as an internal standard, was performed in buffer solution as described previously (4, 9). Metabolites were identified and quantified by GLC-mass spectrometry as follows. A total of 50 to 500 ml of culture was acetylated by adding a 1/10

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TABLE 1. Mobility of acetylated chloroguaiacols, chlorosyringols, and metabolites by GLC

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4,6-Tribromophenol</td>
<td>12.61</td>
</tr>
<tr>
<td>PCP</td>
<td>13.70</td>
</tr>
<tr>
<td>TeCG</td>
<td>13.99</td>
</tr>
<tr>
<td>346-TCG</td>
<td>11.66</td>
</tr>
<tr>
<td>356-TCG</td>
<td>11.67</td>
</tr>
<tr>
<td>35-DCG</td>
<td>9.90</td>
</tr>
<tr>
<td>36-DCG</td>
<td>9.64</td>
</tr>
<tr>
<td>TCS</td>
<td>14.13</td>
</tr>
<tr>
<td>35-DCS</td>
<td>11.73</td>
</tr>
<tr>
<td>3-MCS</td>
<td>10.66</td>
</tr>
<tr>
<td>Metabolite from TeCG</td>
<td>14.87</td>
</tr>
<tr>
<td>Metabolite from 35-DCS</td>
<td>14.88</td>
</tr>
</tbody>
</table>

* A capillary column (HP-1) and a temperature program of 1 min at 100°C, increasing by 10°C min⁻¹ to 300°C, was used.

volume of 1 M K₂CO₃ and a 1/50 volume of acetic anhydride. The acetyl derivatives were extracted 2 times with 50 ml of pentane; the extract was concentrated to 50 µl and analyzed by GLC on a gas chromatograph (HP 5880; Hewlett-Packard Co., Palo Alto, Calif.) by using an Ultra 2 (Hewlett-Packard) or a HP-1 (Hewlett-Packard) capillary column and a mass selective detector (HP 5970 A; Hewlett-Packard). A temperature program of 100°C (1 min), increasing from 100 to 300°C (10°C/min), was used. The retention times of acetylated compounds are given in Table 1. Reference compounds were acetylated as described previously (4, 9). The metabolites were quantified by using the response factor in the total ion chromatogram of trichloro-para-hydroquinone and 2,3-dichloro-para-hydroquinone for the trichlorinated and dichlorinated metabolites, respectively, since authentic compounds were not available.

RESULTS

Degradation of chlorinated syringols by R. chlorophenolicus. The ability of PCP-induced cells of R. chlorophenolicus to degrade chlorosyringols was tested. At a concentration of 10 µM, 100% of TCS and 35-DCS were consumed by R. chlorophenolicus in 200 h. 3-MCS was not significantly consumed by R. chlorophenolicus. In sterile medium the chlorosyringols were stable, with less than a 20% loss in 200 h.

Temperature dependence of the metabolism of chloroguaiacols and chlorosyringols. We determined the rate of degradation of TeCG, 346-TCG, 346/356-TCG, 36-DCG, 35-DCG, TCS, and 35-DCS by PCP-induced cells of R. chlorophenolicus. The amount of substrate remaining in the medium was determined at 1- to 2-h intervals, and the consumption rate was calculated (Fig. 1). The rates of degradation of chloroguaiacols and chlorosyringols increased with temperature, reaching a maximum at 41°C. At 41°C, the rate of degradation varied between 1.1 and 3.7 nmol h⁻¹ ml of culture⁻¹ (2.5 × 10⁶ cells ml⁻¹). The rate of degradation of PCP increased similarly (data not shown), to 9.5 nmol h⁻¹ ml of culture⁻¹ at 41°C, and was more than two times that of TeCG. The tri- and tetrachlorinated guaiacols and syringols were degraded faster than the corresponding dichlorinated compounds. The rate of degradation of all chloroguaiacols and syringols dropped sharply at 45°C.

Intermediate of chloroguaiacol and chlorosyringol degradation. Cells of R. chlorophenolicus, which were induced with PCP, metabolized 10 µM TeCG in 10 h, and there was a concomitant accumulation of a small amount of a metabolite. Metabolites were also found from TCS and 35-DCS. The mass spectra of the acetylated metabolites from TeCG and TCS are shown in Fig. 2. It can be deduced from the fragmentation pattern that the metabolite from TeCG was a trichlorinated methoxyphenyl benzene. It contained one hydroxyl substituent more and one chlorine substituent less than TeCG. TeCG was thus both hydroxylated and dechloro-
The cultures TeCG+++ were of only degradations. Chloramphenicol blocked concentrations 346/356-TCG, as the cases of PCP, 346/356-TCG, 346-TCG, 35-DCG, TCS, 35-DCS were about 5 nM.

**Induction of degradation of chlorinated guaiacols and syringols.** *R. chlorophenolicus* was preincubated for 24 h with 2 µM of one of the following substrates: PCP, TeCG, 346/356-TCG, 346-TCG, 35-DCG, 35-DCS, and 35-DCS. Chloramphenicol (60 µg ml⁻¹) was then added to block further enzyme synthesis; and we tested for the degradation of chloroguaiacols, chlorosyringols, and PCP. The cultures were incubated with 5 µM substrates, and the amount of substrate removed from the medium was measured after 50 h. The results (Table 2) indicate that in the presence of chloramphenicol the compounds were degraded only if there was previous contact with one of the chlorophenol substrates. Results in Table 2 also indicate that PCP and the chloroguaiacols induced the synthesis of the enzymes for the degradation of all the other compounds. The same was true for the chlorosyringols, with three exceptions. 35-DCS was poorly metabolized after induction with either TCS or 35-DCS; 36-DCG and the other chloroguaiacols were poorly metabolized after induction with 35-DCS.

**Effect of chloroguaiacols and chlorosyringols on PCP degradation.** Since the degradation of chloroguaiacols and chlorosyringols was induced by PCP, it could be that these were metabolized by the same enzyme(s) that catalyze para-hydroxylation of chlorophenols (5). We therefore tested for the possible competition of chloroguaiacols and chlorosyringols with PCP—PCP (10 µM) and 5, 10, or 15 µM of one of the following compounds—TeCG, 346/356-TCG, 346-TCG, 36-DCG, 35-DCG, TCS, 35-DCS, or PCP—were added to PCP-induced cells of *R. chlorophenolicus*. The rate of PCP consumption was measured from the linear part of the degradation curve, and the results are shown in Table 3. The results indicate that TeCG, 346-TCG, and 346/356-TCG retarded the degradation of PCP somewhat; and at equimolar concentrations PCP degradation was slowed down by 37, 28, and 21%, respectively. The other compounds had little or no effect on the rate of PCP degradation. The rate of degradation of 20 µM PCP was slightly lower than that of 10 µM PCP, indicating substrate toxicity. The concentration that caused 50% inhibition on the growth of *R. chlorophenolicus* was <5 µM for both PCP.

**TABLE 2. Inducers in the metabolism of chlorinated guaiacols and syringols by *R. chlorophenolicus***

<table>
<thead>
<tr>
<th>Inducer</th>
<th>PCP</th>
<th>TeCG</th>
<th>346/356-TCG</th>
<th>346-TCG</th>
<th>36-DCG</th>
<th>35-DCG</th>
<th>345-TCS</th>
<th>35-DCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (no chloramphenolic)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
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<tr>
<td>None</td>
<td>-</td>
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<td>-</td>
<td>-</td>
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<td>-</td>
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</tr>
<tr>
<td>PCP</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>TeCG</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>346/356-TCG</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>346-TCG</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>35-DCG</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TCS</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+++</td>
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<td>+</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>-</td>
</tr>
</tbody>
</table>

* Cells were first exposed to 2 µM inducers and then incubated with 5 µM substrates. Amounts of substrates removed after 50 h are indicated as follows: +++, more than 90%; ++, more than 70%; +, more than 40%; −, less than 40%.

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**FIG. 3. Degradation of TeCG (■) and formation of trichloromethoxydi hydroxybenzene (○) by *R. chlorophenolicus*.**
and TeCG, 7 μM for 346-TCG, and 17 μM for 346/356-TCG. Substrate toxicity therefore did not account for the inhibition of PCP degradation by tri- and tetrachloroguaiacols.

**DISCUSSION**

*R. chlorophenolicus* was previously shown (5) to initiate the metabolism of polychlorinated phenols through hydrolytic *para*-hydroxylation. In this report we have presented evidence indicating that the bacterium also degrades polychlorinated guaiacols and syringols via hydroxylation.

We showed earlier (9) that *R. chlorophenolicus* degraded five different chlorinated guaiacols, namely, TeCG, 346-TCG, 356-TCG, 35-DCG, and 36-DCG. In this study we have shown that *R. chlorophenolicus* also degrades two chlorinated syringols, TCS and 35-DCS. The substitution pattern of these degradable monomethoxy- and dimethoxychlorophenols differs from that of the degradable chlorophenols (4, 5), in that positions 2 (guaiacol) or 2 and 6 (syringol) carry a methoxyl group instead of a chlorine. TeCG, the chloroguaiacol with the highest rate of degradation, was degraded at only half the rate of PCP. TCS, with two methoxyl substituents, was degraded even more slowly (Fig. 1). It thus seems that replacement of the 2- or 2- and 6-chlorines of the chlorophenol with a methoxyl substituent decreases the rate of degradation. The degree of chlorination also affected the rate of degradation, with the less chlorinated compounds being degraded more slowly than the more chlorinated ones. *R. chlorophenolicus* has a preference for polychlorinated phenols.

The readily degradable methoxylchlorophenols TeCG, 346-TCG, and 346/356-TCG retarded the degradation of PCP, while the poorly degradable ones 35-DCG, 36-DCG, TCS, and 35-TCS had little or no effect on the rate of PCP degradation (Table 3). This suggests that the chlorinated guaiacols may compete as substrates with PCP. Also, the temperature dependence of the rates of degradation of chloroguaiacols and chlorosyringols (Fig. 1) was similar to that of the chlorophenols (5), with an optimum at 37 to 41°C.

Synthesis of the enzyme(s) for the degradation of chlorophenols, chloroguaiacols, and chlorosyringols was inducible and under joint control (Table 2). The inducer is more likely the substrate than some later intermediate, because it was shown previously (5) that the first intermediate, chlorohydroquinone, does not induce the degradation of its parent chlorophenol.

Polychlorinated *para*-hydroquinones seem to have a central role in the degradation of polychlorinated phenolic compounds. Tetrachlorohydroquinone has been reported as a degradation intermediate in PCP metabolism of several bacteria (5, 19, 23–25). Hydrolytic *para*-hydroxylation initiates the degradation of PCP in both *R. chlorophenolicus* (5) and *Flavobacterium* sp. (23). Tetrachlorohydroquinone is further degraded by *R. chlorophenolicus* through hydrolytic and reductive dechlorinations (6).

During the degradation of chloroguaiacols and chlorosyringols, hydroxylated metabolites accumulated transiently (Table 1 and Fig. 2 and 3). The metabolite produced from TeCG was identified as a trichlorinated methoxylhydroxybenzene, and the metabolite produced from TCS was identified as a dichlorinated dimethoxylhydroxybenzene. The metabolites from TeCG and TCS are dechlorination products, and both have an additional hydroxyl group compared with the substrate. We previously detected (9) a dichlorinated methoxylhydroxybenzene as a metabolite in the degradation of 36-DCG by *R. chlorophenolicus*. The isomer configuration could not be determined due to the unavailability of authentic compounds. The degradation of both TCS and 35-DCS resulted into a dichlorinated dimethoxyhydroxybenzene with identical retention times and mass spectra. *para*-Hydroxylation of the substrates into 3,5-dichloro-2,6-dimethoxy-1,4-hydroquinone, with no positional shift of the remaining chlorine atoms, would provide an explanation for the fact that identical metabolites were formed from TCS and 35-DCS. The hydrolytic and dechlorinative hydroxylation of PCP (5) and the proposed initial attack on TeCG and TCS by *R. chlorophenolicus*, resulting in the formation of chlorinated *para*-hydroquinones, is shown in Fig. 4. This is the first report, to our knowledge, which has presented evidence for the degradation of chlorinated guaiacols and syringols via dechlorinative hydroxylation, possibly in the *para* position.

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We thank Juha Knuutinen, Satu Kivelä, and Paul Klein for the synthesis of model compounds and Riitta Boeck for technical assistance. We also thank Veikko Kitunen for advice in GLC-mass spectrometry.

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**LITERATURE CITED**


FIG. 4. Hydrolytic and dechlorinative hydroxylation of PCP into tetrachlorohydroquinone (5) and proposed dechlorinative hydroxylation of TeCG and TCS by *R. chlorophenolicus*. 

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The text describes the metabolism of polychlorinated phenols by *R. chlorophenolicus* and the degradation patterns of chlorinated guaiacols and syringols. It discusses the role of hydroxylated metabolites in the degradation process and the synthesis of enzymes for the degradation of chlorophenols, chloroguaiacols, and chlorosyringols. The study also includes the synthesis of model compounds and technical assistance from Veikko Kitunen. The research was supported by the Academy of Finland and the Maj and Tor Nessling Foundation. The literature cited includes several studies on the metabolism of chlorinated compounds by *Rhodococcus* species.