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Degradation of polychlorinated phenols by *Rhodococcus chlorophenolicus*

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Summary. An actinomycete, *Rhodococcus chlorophenolicus*, isolated from a pentachlorophenol-degrading mixed bacterial culture is a polychlorophenol degrader. It was shown to oxidize pentachlorophenol into carbon dioxide and to metabolize also 2,3,4,5-, 2,3,4,6-, and 2,3,5,6-tetrachlorophenol, 2,3,4-, 2,3,5-, 2,3,6-, 2,4,6-, and 2,4,5-trichlorophenol, 2,5-, and 2,6-dichlorophenol and tetrachloro-p-hydroquinone in an inducible manner. Pentachlorophenol set on the synthesis of enzymes required for the metabolism of all these chlorophenols and of tetrachloro-p-hydroquinone. 2,4,5-, and 2,4,6-trichlorophenol and 2,5-, and 2,6-dichlorophenol were degraded by *R. chlorophenolicus* cells only if these had previous contact to pentachlorophenol. Other chlorophenols mentioned were able to set on the synthesis of enzymes for their own degradation. 2,3,4,5-, and 2,3,4,6-tetrachlorophenol, and 2,3,5-, 2,4,5-, and 3,4,5-trichlorophenol were more toxic to *R. chlorophenolicus* than the other chlorophenols, but nevertheless 2,3,4,5-, and 2,3,4,6-tetrachlorophenol and 2,3,5-trichlorophenol were readily degraded by the bacteria.

Introduction

Chlorophenol contamination of the environment is extensive (Arsenault 1976; Kozak et al. 1979; Valo et al. 1984; Kitunen et al. 1985; Salkinoja-Salonen et al. 1981; 1983; 1984). The bulk of commercially produced chlorophenols is utilized by the wood preserving industry. Soil in the vicinity of wood preserving facilities has been found to contain up to a thousand milligrams of chlorophenols per kilogram (Valo et al. 1984; Kitunen et al. 1985). The chlorophenols had penetrated the soil to a depth of several meters, sometimes to the groundwater level (Valo et al. 1984). Chlorophenols are toxic to all forms of life (Huang and Gloyna 1967; Kozak et al. 1979). Toxicity increases with the degree of chlorination and with the chlorophenol lipophilicity (Beltrame et al. 1984; Liu et al. 1982; Salkinoja-Salonen et al. 1981). The Gram-negative bacteria appear to tolerate 10 to 100 times more chlorophenol than the Gram-positive (Izaki et al. 1981).

The chlorophenol isomers for industrial production were chosen on a basis of biocidal activity and production technology; possible differences in biodegradability were not considered. The distribution of chlorophenol congeners in contaminated soil was found to differ from the chlorophenol composition of the contaminating wood preservative (Kitunen et al. 1985; Valo et al. 1984), possibly due to differences in chlorophenol biodegradability in soil.

Reports on microbial degradation of tri- and tetrachlorophenols are scarce (Chu and Kirsch 1973; Karns et al. 1983). We earlier reported on the mineralization of PCP by a mixed bacterial culture (Apajalahti and Salkinoja-Salonen 1984; Salkinoja-Salonen and Apajalahti 1982; Valo et al. 1985). From this culture we isolated an actinomycete *Rhodococcus chlorophenolicus* sp. nov. (Apajalahti et al. 1986), which, as we show in this paper, not only degrades PCP, but also TeCH and 10 other chlorophenols with 2 to 4 chlorine substituents.

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Abbreviations: DCP, dichlorophenol; TCP, trichlorophenol; TeCP, tetrachlorophenol; PCP, pentachlorophenol; TeCH, tetrachloro-p-hydroquinone

An example of numeration: 2345-TeCP, 2,3,4,5-tetrachlorophenol

Materials and methods

The organism. Rhodococcus chlorophenolicus PCP-I isolated from a PCP-mineralizing bacterial mixed culture (Apajalahti and Salkinoja-Salonen 1984; Salkinoja-Salonen and Apajalahti 1982; Valo et al. 1985), was used in all experiments. The taxonomic description of *R. chlorophenolicus* is reported elsewhere (Apajalahti et al. 1986). The type strain of the species is PCP-I (DSM 43826).

Media and substrates. A mineral salts medium (Sundman 1964) supplemented with 1.5 ml of vitamin solution (Sundman 1964) and 1.5 ml of trace element solution (Bauchop and Elsden 1960) per litre was used in all experiments. For toxicity assays 2 g of yeast extract and for enzyme induction studies 1 g of rhamnose per litre of medium was added. The chlorophenolics used as substrates and reference compounds were produced by Merck (pentachlorophenol), by Fluka, by Egachemie (di-, tri-, and tetrachlorophenols) and by Eastman Kodak Co (tetrachloro-p-hydroquinone). Uniformly ^{14}C-labeled PCP (25.6 mCi/g) was a gift of Dr. M. Fischer (Institut für Wasser-, Boden- und Lufthygiene des Bundesgesundheitsamtes, Berlin, FRG). It was 99,9% pure as determined by gas liquid chromatography and contained only traces of 2346- and 2356-TeCP as impurity (Salkinoja-Salonen and Apajalahti 1982).

Experimental. The degradation experiment with ^{14}C-PCP was performed by trapping evolved $^{14}CO_2$ in 1 M NaOH for radioactivity measurements as described earlier (Apajalahti and Salkinoja-Salonen 1984), and by following the concentration of PCP in the culture by gas chromatography. For enzyme induction studies all the cultures were first grown on rhamnose for two days. Starting from day 3, PCP (10 µM) was introduced three times at 16 h intervals in some of the cultures (PCP-induced), whereas the other cultures were incubated with no amendments for two further days. The cultures were subsequently portioned and chlorophenols were added in subcultures obtained. Bacterial numbers were determined by microscopic enumeration of at least 600 cells counted per sample. In toxicity study the bacteria were grown on yeast extract in the presence of chlorophenol. After 3 days incubation the density of the cultures was measured as $A_{600\,nm}$. All cultures were incubated in a gyratory shaker (150 rpm) at 28° C in the dark.

Gas chromatography. Samples from cultures were acetylated for gas chromatography by mixing an internal reference compound (236-TCP or 246-TCP), 2 volumes of buffer, pH 9.9 (50 ml 0.05 M $NaHCO_3$ +9.1 ml 0.1 M NaOH), and 0.05 volumes of freshly distilled acetanhydride with 1 volume of culture fluid. Acetates were extracted into hexane and analysed by a gas chromatograph (Hewlett Packard 5790 A) using an electron capture ^{63}Ni detector and a SE-30 fused silica capillary column (Orion Analytica). A temperature programme of 1 min at 50° C and 20° C/min to 250° C was used. The carrier gas was H_2 and the make-up gas argon/methane (95/5%).

Results

Mineralization of PCP by Rhodococcus chlorophenolicus

Mineralization of PCP by *R. chlorophenolicus* pregrown on yeast extract is illustrated in Fig. 1. The cells were suspended in a mineral salts medium containing ^{14}C-labeled PCP as a sole source of carbon. The figure shows that PCP disappeared totally from the culture, and about 70% of added ^{14}C-PCP became oxidized into $^{14}CO_2$ in 250 h (Fig. 1).

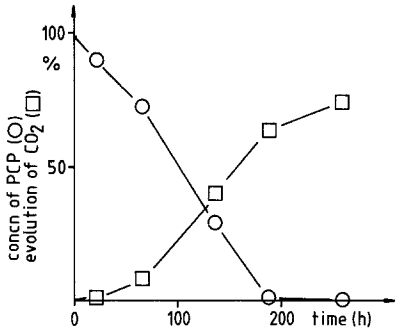

Fig. 1. Mineralization of pentachlorophenol by *Rhodococcus chlorophenolicus*. Bacteria were incubated in a mineral salts medium containing 40 µM ^{14}C-labeled PCP as a sole source of carbon. Concentration of PCP in the medium and cumulative evolution of $^{14}CO_2$ were followed. ○, PCP concentration in the medium (% of initial concentration); □, evolution of $^{14}CO_2$ (% of added ^{14}C). Bacterial density 1.7×10^8/ml

Inducibility of PCP-degradation by *R. chlorophenolicus* was tested. The degradation of PCP was followed in the presence and absence of chloramphenicol by the bacteria pregrown with or without PCP. The results show that the bacteria with no previous contact to PCP did not metabolize PCP in the presence of protein synthesis inhibitor, chloramphenicol, indicating that the enzymes needed for the degradation of PCP were not constitutively expressed. In contrast, PCP was degraded in 6 h by the bacteria pregrown with PCP, even though chloramphenicol was present (Fig. 2).

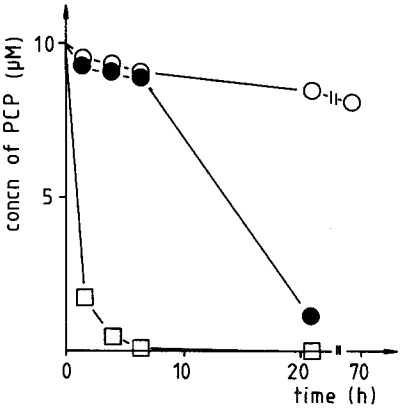

Fig. 2. Time course of PCP degradation by *Rhodococcus chlorophenolicus*. Concentration of PCP was followed in cultures pregrown on rhamnose (●○) and in cultures pregrown on rhamnose+PCP (□). ○□, chloramphenicol (60 µg/ml) added; ●, no chloramphenicol added. Bacterial density 7×10^8/ml

Degradation of tetra-, tri-, and dichlorophenols by Rhodococcus chlorophenolicus

Table 1 shows how 16 different chlorophenols were degraded by *R. chlorophenolicus* cells with chlorophenol as a sole carbon source. All PCP, 2346-TeCP, 2356-TeCP, 235-TCP and 236-TCP was removed from cultures at 2 and 10 μM concentrations in 14 days (Table 1); 234-TCP was degraded to almost the same degree (60 to 83%). From 70% to 100% of 2345-TeCP, 245-TCP, 246-TCP and 26-DCP was removed at a 2 μM, but only 21% to 33% at a 10 μM concentration. 345-TCP, 23-, 24-, 25-, 34-, and 35-DCP were not degraded to any significant degree (Table 1). The disappearance of chlorophenols from sterile medium was less than 10% of starting concentration in 2 weeks.

Inducibility of the degradation of 16 chlorophenols in *R. chlorophenolicus* was tested. In 24 h no chlorophenol disappeared from cultures of rhamnose pregrown cells, when chloramphenicol was present (Table 2). PCP, 2345-TeCP, 2346-TeCP, 2356-TeCP, 235-TCP, and 236-TCP were readily metabolized in parallel cultures containing no chloramphenicol. 234-TCP was also removed to some extent, but 245-TCP, 246-TCP, 345-TCP and dichlorophenols were not attacked (Table 2). The degradation of TeCH by *R. chlorophenolicus* was also tested. It became completely metabolized by the bacteria in 24 h (Table 2). In the presence of chloramphenicol only 26% disappeared, probably due to spontaneous oxidation.

To determine whether the poor attack on 245-TCP, 246-TCP, 345-TCP, and dichlorophenols by *R. chlorophenolicus* resulted from lack of enzyme induction, their degradation by the cells induced with PCP was tested. The results show that the preincubation of the bacteria in the presence of PCP enhanced the degradation of 245-TCP, 246-TCP, 25-DCP and 26-DCP (Table 2). This may indicate that PCP-degrading enzymes had some activity towards these chlorophenols. The degradation level of other chlorophenols was not significantly affected by the induction with PCP.

The following conclusions can be drawn from the results presented in Table 2: a) the degradation of 2345-TeCP, 2346-TeCP, 2356-TeCP, 234-TCP, 235-TCP, 236-TCP and TeCH was inducible; b) each of these chlorophenols set on the synthesis of enzymes for its own degradation; c) the enzymes produced by cells grown with PCP were able to metabolize TeCH, 2345-TeCP, 2346-TeCP, 2356-TeCP, 234-TCP, 235-TCP, 236-TCP,

Table 1. Removal of chlorophenols by the resting cells of *Rhodococcus chlorophenolicus*

Chlorophenol	Degradation[a] (%) of chlorophenols at: 2 μM	10 μM
PCP	100	100
2345-TeCP	100	23
2346-TeCP	100	97
2356-TeCP	100	100
234-TCP	83	60
235-TCP	100	100
236-TCP	100	100
245-TCP	70	21
246-TCP	94	33
345-TCP	25	11
23-DCP	52	40
24-DCP	15	8
25-DCP	42	41
26-DCP	81	30
34-DCP	21	13
35-DCP	28	26

[a] Figures are percentages of chlorophenols removed by the bacteria in 14 days; 100·(1-concn in an inoculated culture/concn in a sterile medium)
Bacterial density 1.7×10^8/ml

Table 2. Inducibility of chlorophenol degradation in *Rhodococcus chlorophenolicus*

Compound 10 μM	Degradation[a] (%) of chlorophenols by the cells[d] pregrown on: Rhamnose −Cm[b]	Rhamnose +Cm[c]	Rhamnose + PCP +Cm[c]
PCP	97	4	100
TeCH	100	26	100
2345-TeCP[e]	98	1	100
2346-TeCP	92	0	99
2356-TeCP	100	3	100
234-TCP	31	0	59
235-TCP	98	5	99
236-TCP	99	8	97
245-TCP	4	0	59
246-TCP	6	1	63
345-TCP	4	2	12
23-DCP	0	10	41
24-DCP	4	11	16
25-DCP	10	12	70
26-DCP	14	3	82
34-DCP	22	13	8
35-DCP	4	1	23

[a] Figures indicate the removal of chlorophenols (% of initial concn) in 24 h
[b] −Cm, no chloramphenicol
[c] +Cm, chloramphenicol (60 μg/ml)
[d] Bacterial density 7×10^8 cells/ml
[e] Concentration of 2345-TeCP was 5 μM

245-TCP, 246-TCP, 25-DCP, and 26-DCP, and d) the degradation was sensitive to chloramphenicol, which indicates that the removal of chlorophenols was true metabolism rather than artefactal disappearance, such as absorption, volatilization, precipitation or oxidation.

Toxicity of chlorophenols to Rhodococcus chlorophenolicus

The growth inhibitory effect of chlorophenols to *R. chlorophenolicus* was tested by growing the cells in yeast extract medium in the presence of chlorophenol at the concentrations of 0, 5, 10 and 20 μM. The concentration of chlorophenol causing 50% inhibition of growth (IC_{50}) was interpolated. The results listed in Table 3 show that 2345-TeCP, 2346-TeCP, 235-TCP, 245-TCP, and 345-TCP were more toxic to *R. chlorophenolicus* than PCP ($IC_{50} < 20$ μM), and 2356-TeCP, 234-TCP, 236-TCP, 246-TCP, and dichlorophenols were less toxic than PCP ($IC_{50} > 20$ μM; Table 3).

The PCP-degrading ability of the cells preincubated with different di- and trichlorophenols was tested to search for possible inhibition by these chlorophenols or their metabolic products on the degradation of PCP. The results presented in Table 3 show that dichlorophenols, excluding 26-DCP, inhibited the degradation of PCP. Trichlorophenols did not affect PCP degradation.

Table 3. Effect of various chlorophenols on the growth and PCP-degrading ability of *Rhodococcus chlorophenolicus*

Chlorophenol	IC_{50}[a]	Inhibition of PCP degradation[b]	log P_{ow}[c]
PCP	20	−	5.01
2345-TeCP	4	ND	4.21
2346-TeCP	8	ND	4.12
2356-TeCP	>20	ND	3.88
234-TCP	>20	−	3.80
235-TCP	17	ND	3.84
236-TCP	>20	ND	3.77
245-TCP	12	−	3.72
246-TCP	>20	−	3.37
345-TCP	6	−	3.83
23-DCP	>20	+	3.42
24-DCP	>20	++	3.41
25-DCP	>20	+	3.42
26-DCP	>20	−	3.36
34-DCP	>20	++	3.47
35-DCP	>20	+	3.47

[a] Chlorophenol concn (μM) causing 50% inhibition on the growth of *R. chlorophenolicus*; interpolated from the experimental values

[b] PCP was added in cultures after 48 h of incubation with other chlorophenols (10 μM). After 30 h of further incubation the concn of PCP was determined. −, 95 to 100%; +, 40 to 60%; ++, 20 to 30% of PCP was metabolized. ND, not determined

[c] Octanol/water partition coefficients as reported by Beltrame et al. (1984)

Discussion

The *Rhodococcus chlorophenolicus* PCP-I used in this study was isolated from a PCP enrichment culture (Apajalahti and Salkinoja-Salonen 1984; Salkinoja-Salonen and Apajalahti 1982; Valo et al. 1985). In this paper we show that this pure strain readily degraded PCP, 2356-TeCP, 2346-TeCP, 234-TCP, 235-TCP, and 236-TCP. 2345-TeCP was completely degraded by *R. chlorophenolicus* at a concentration of 2 μM, but, probably due to its toxicity, not at all at 10 μM concentration (Table 1). We also showed that the degradation of the chlorophenols mentioned was not constitutive but was set on by the presence of each of them. 245-TCP, 246-TCP, 25-DCP, and 26-DCP were degraded to a significant degree by the bacteria pregrown in the presence of PCP, but not at all in 24 h by bacteria with no previous contact to PCP (Table 2). This may indicate a lack of onset of enzyme synthesis. The reason for the failure of *R. chlorophenolicus* to degrade 345-TCP, 23-DCP, 24-DCP, 34-DCP, and 35-DCP remained unclear, since induction with PCP did not turn on their degradation (Table 2).

We found that 2345-TeCP, 345-TCP, 2346-TeCP, 245-TCP and 235-TCP were the most inhibitive to the growth of *R. chlorophenolicus*, the IC_{50} values being 4, 6, 8, 12, and 17 μM, respectively (Table 3). The IC_{50} values obtained for different isomers are similar to those reported by Liu et al. (1982) for dehydrogenase activity, by Beltrame et al. (1984) for phenol degradation, and by Chu and Kirsch (1973) for bacterial growth. There was no clear correlation between the lipophilicity and toxicity of the chlorophenols towards *R. chlorophenolicus* (Table 3).

In the present study most dichlorophenols (or their metabolic products) inhibited PCP metabolism. This inhibition seemed specific for PCP metabolism, since dichlorophenols did not markedly inhibit growth of *R. chlorophenolicus*. On the other hand, 345-TCP and 245-TCP were efficient growth inhibitors, but they did not inhibit PCP degradation (Table 3).

R. chlorophenolicus PCP-I degraded more efficiently polychlorinated than dichlorinated phe-

nols. PCP, all tetrachlorophenols and all but one (345-TCP) trichlorophenols were degraded, whereas only two of dichlorophenols were attacked. Chu and Kirsch (1972; 1973) described a saprophytic coryneform KC-3 with similar properties. Chlorophenol-degrading *Pseudomonas cepacia* AC1100 showed no such preference for polychlorinated phenols (Karns et al. 1983).

Reiner et al. (1978) showed that KC-3 turned PCP initially to TeCH; a mutant strain accumulated this compound. We found that *R. chlorophenolicus* PCP-I metabolized efficiently TeCH and moreover, the metabolism of TeCH was induced with PCP (Table 2). This may indicate that *R. chlorophenolicus* also degrades PCP via TeCH.

Of bacterial strains degrading polychlorinated phenols *P. cepacia* AC1100 and *R. chlorophenolicus* PCP-I are the only ones identified to a species level (Kilbane et al. 1982; Apajalahti et al. 1986). Data on taxonomically important properties, such as type of cell wall sugars and diamino acids, structure of isoprenoid quinones and fatty acid composition, of KC-3 and of the other PCP-degrading coryneforms have not been reported. The strains were suggested to belong to the genus *Arthrobacter*, but since the cell walls of these PCP-degrading bacteria were reported to contain diaminopimelic acid (unknown isomer) and not arabinose (Stanlake and Finn 1982; Edgehill and Finn 1982), they should rather be considered as strains of *Brevibacterium sp.* or *Nocardioides sp.*, or as non-legitimate arthrobacteria. True arthrobacters have lysine as the cell wall diamino acid (Goodfellow et al. 1984). *Flavobacterium* strains degrading PCP were also recently isolated (Saber and Crawford 1985). *R. chlorophenolicus* is a novel, well characterized chlorophenol-degrading species. It has a cell wall typical of mycolic acid containing genera, which none of the previously known PCP-degrading coryneforms has; arabinose and galactose as major cell wall sugars, and *meso*-diaminopimelic acid as the peptidoglycan diamino acid (Apajalahti et al. 1986).

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