Microbial Degradation of Chlorinated Acetophenones

JÜRGEN HAVEL* AND WALTER REINEKE

Chemische Mikrobiologie, Bergische Universität—Gesamthochschule Wuppertal, Fachbereich 9, Gaußstrasse 20, 42097 Wuppertal, Germany

Received 20 November 1992/Accepted 22 March 1993

A defined mixed culture, consisting of an Arthrobacter sp. and a Micrococcus sp. and able to grow with 4-chloroacetophenone as a sole source of carbon and energy, was isolated. 4-Chlorophenyl acetate, 4-chlorophenol, and 4-chlorocatechol were identified as metabolites through comparison of retention times and UV spectra with those of standard substances. The proposed pathway was further confirmed by investigation of enzymes. The roles of the two collaborating strains were studied by growth experiments and on the level of enzymes. If transient accumulation of 4-chlorophenol was avoided either by the use of phenol-absorbing substances or by careful supplement of 4-chloroacetophenone, the Arthrobacter sp. was able to grow as a pure culture with 4-chloroacetophenone as a sole source of carbon and energy. Several mono-, di-, and trichlorinated acetophenones were mineralized by the Arthrobacter sp.

Chlorinated acetophenones can originate from different sources. After treatment with the insecticides Chlordrin, phos or Gardona, chlorinated acetophenones are found in the soil (6, 7). Chlorinated 2-hydroxyacetophenones are obtained as by-products of the degradation of chlorosubstituted xanthones by an Arthrobacter sp. (27). Formation of mono-, di-, and trichlorinated acetophenones from polychlorinated biphenyls (PCBs) has also been reported, depending on their substitution pattern (3–5). Furthermore, acetophenones occurred as by-products of degradation of ethylbenzene and styrene by a Pseudomonas strain (28).

The microbial degradation of acetophenone has been investigated by Cripps (9) and Cripps et al. (10). The first step in the degradation is a biological type of Baeyer-Villiger-oxidation (Fig. 1). This reaction is observed in the degradation of various compounds such as alkanes and alk-1-enes (1, 2, 17), cyclohexan-1,2-diol (12), cyclohexanone (14), and fluoranthene (29). Phenyl acetate, the product of the Baeyer-Villiger-oxidation, is further degraded via phenol and catechol. Analogous reactions were reported to occur in the conversion of chloroacetophenones in which chlorophenyl acetates and chlorophenols as metabolites were found. Higson and Focht (20) reported some release of chloride during the conversion of chlorinated acetophenones. However, information pertaining to organisms using these substances as growth substrates has not been published.

In contrast, p-hydroxyacetophenone is degraded either via protocatechuateg (11, 21) or via hydroquinone as ring fission substrates. A summary of the pathways for p-hydroxyacetophenone is given by Darby et al. (11).

The present paper describes the isolation and characterization of a mixed culture which mineralizes 4-chloroacetophenone via the Baeyer-Villiger-oxidation mentioned above. One member of the mixed culture, an Arthrobacter sp., was shown to grow with 4-chloroacetophenone and was able to mineralize a broad spectrum of chlorinated acetophenones.

MATERIALS AND METHODS

Media and culture conditions. For all growth experiments a mineral medium was used as described earlier (15). All growth and resting-cell incubations were run at 30°C on a rotary shaker in sealed, fluted 100-ml Erlenmeyer flasks containing 25 ml of medium. Substrates for the growth experiments were added via the gaseous phase. For resting-cell experiments, strains were grown on nutrient broth in the presence of vapor of 4-chloroacetophenone and 4-chlorophenol. Cells were washed twice with and then resuspended in phosphate buffer to give an optical density at 546 nm of 8 to 9. Then, 25 ml of the cell suspensions was incubated in sealed, fluted 100-ml Erlenmeyer flasks. In resting-cell experiments, substrates were directly added to the medium. In the case of 2,4-dichloro- and 2,3,4-trichloroacetophenone, 0.1% (vol/vol) dimethyl sulfoxide was added in order to enhance solubility.

Enrichment procedure. Soil samples of different origins (forest, field, and compost) as well as river sediment samples (Rhine and Lippe) were mixed for enrichment cultures containing 4-chloroacetophenone as the sole source of carbon and energy. A theoretical concentration of the substrate equivalent to 1 mM was added either directly to the medium or via the gaseous phase. Samples of the cultures were transferred into new medium each week. After visible growth occurred a defined mixed culture was created by isolation of eight participating strains and subsequent combination of the pure strains until growth on 4-chloroacetophenone was achieved.

Analytical methods. Acetophenones and metabolites were analyzed by high-performance liquid chromatography on a LiChrosorb RP18 5-μm column (Merck, Darmstadt, Germany). Acetonitrile (30 to 45% [vol/vol]) containing phosphoric acid (2 g/liter) was used as the eluent. Metabolites were identified by means of comparison of the retention times as well as the UV spectra measured with a diode array detector (Merck Hitachi L-3000) with authentic compounds. Chloride and acetate were determined via ion chromatography (Dionex QIC ANALYSER).

Enzyme assays. The preparation of cell extracts has been described previously (9). Oxidation of 4-chloroacetophenone and activity of 4-chlorophenyl acetate esterase were measured as described by Cripps (9) for the nonchlorinated
Degradation of chlorinated acetophenones.

Chlorinated analogs (R=Cl) of acetophenone were obtained from Lancaster (Mühlheim, Germany); 2-chloro-, 2,3-dichloro-, and 2,5-dichlorophenol were obtained from Merck (Darmstadt, Germany); and 3-chloro-, 4-chloro-, 2,4-dichloro-, 2,6-dichloro-, and 2,3,4-trichlorophenol were obtained from Fluka (Neu-Ulm, Germany). The chlorophenyl acetates of 2-chloro-, 3-chloro-, and 4-chlorophenol were synthesized by means of acetylation of the corresponding phenols.

**RESULTS**

Isolation and characterization of a 4-chloroacetophenone-degrading culture. No growth was observed over a period of 15 weeks when the substrate was directly added to the enrichment culture. In a second run, in which 4-chloroacetophenone was supplied via the vapor phase, a growing culture, which could subsequently be transferred, was obtained after 9 weeks. After seven transfers into fresh medium, eight morphologically different strains were isolated on nutrient broth agar. Since none of the isolated pure cultures was able to grow with 4-chloroacetophenone, the eight strains were systematically combined until growth was achieved. In this way a defined mixed culture consisting of two strains was created. The two essential strains were tentatively identified as an *Arthrobacter* sp. (strain M5) and a *Micrococcus* sp. (strain 1B) by the following criteria. Strain M5 was a gram-positive, nonmotile bacterium which showed the typical pleomorphism described by Keddie and Jones (22) for *Arthrobacter* sp. It produced no acidic compounds from glucose whether under fermentative or oxidative conditions. No spores could be detected, and the catalase test was positive. Strain 1B was a coccosid, gram-positive, nonmotile bacterium. The catalase test was positive. Spores could not be detected, and no acidic products were formed from glucose under fermentative or oxidative conditions. When inoculated to an optical density of 0.01, the mixed culture of strains M5 and 1B reached an optical density at 546 nm of 0.55 with 4-chloroacetophenone as the growth substrate after 3 days. The substrate was supplied through the vapor phase. The amount of substrate was equivalent to 2 mM. Sometimes 4-chlorophenol accumulated, which hindered growth on 4-chloroacetophenone. The mixed culture was unable to grow with 2-chloro- or 3-chloroacetophenone as the sole source of carbon and energy.

Resting-cell experiments. To analyze the potential of each organism, both strains were individually incubated with the growth substrate and probable metabolites. Incubation of *Arthrobacter* sp. strain M5 (4 × 10^8 cells per ml) with 4-chloroacetophenone in the presence of paraoxon (0.5 mM) resulted in the accumulation of several intermediates. Although paraoxon has been reported to inhibit ester-hydrolyzing enzymes (13), 4-chlorophenol and 4-chlorocatechol accumulated in the culture medium in addition to 4-chloro-1-phenylethanol and 4-chlorophenyl acetate. When strain M5 was incubated in the absence of paraoxon, nearly stoichiometric amounts of chloride were released from 4-chloroacetophenone (Fig. 2A). 4-Chloro-1-phenylethanol, 4-chlorophenyl acetate, and 4-chlorophenol were totally degraded, yielding stoichiometric amounts of chloride (data not shown). Incubation of strain M5 with 3-chloro- and 2-chloroacetophenone also led to a release of chloride (Fig. 2B and 2C).

The incubations of strain 1B (2.5 × 10^8 cells per ml) with 4-chloroacetophenone, 4-chloro-1-phenylethanol, 4-chlorophenyl acetate, and 4-chlorophenol showed that the strain was lacking the Baeyer-Villiger oxidase which catalyzes the degradation of acetophenone (R=H) and chlorinated analogs (R=Cl) via the corresponding phenyl acetates, phenols, and catechols based on the model of Cripps (9) and Cripps et al. (10). Degradation of 1-phenylethanol can follow the same pathway or occur by ring fission with retention of the side chain.

**Chemicals.** The monochlorinated acetophenones, 4-chlorophenylethanol, and chlorohydroquinone were obtained from Aldrich (Steinheim, Germany); 2,3-dichloro-, 2,4-dichloro-, 2,5-dichloro-, 2,6-dichloro-, 3,4-dichloro-, and 2,3,4-trichloroacetophenone and 2-chloro- and 3-chloro-1-phenoxyethanol were obtained from Lancaster (Mühlheim, Germany); 2-chloro-, 2,3-dichloro-, and 2,5-dichlorophenol were obtained from Merck (Darmstadt, Germany); and 3-chloro-, 4-chloro-, 2,4-dichloro-, 2,6-dichloro-, and 2,3,4-trichlorophenol were obtained from Fluka (Neu-Ulm, Germany). The chlorophenyl acetates of 2-chloro-, 3-chloro-, and 4-chlorophenol were synthesized by means of acetylation of the corresponding phenols (23).

![Chemical Structures](image-url)

**3-Oxoadipate pathway**

FIG. 1. Pathway for the degradation of acetophenone (R=H) and chlorinated analogs (R=Cl) via the corresponding phenyl acetates, phenols, and catechols based on the model of Cripps (9) and Cripps et al. (10). Degradation of 1-phenylethanol can follow the same pathway or occur by ring fission with retention of the side chain.

Protein was determined by the Bradford procedure (8).
insertion of an oxygen atom between the aromatic ring and the side chain forming the phenyl ester. However, strain 1B was capable of catalyzing all other reactions observed in strain M5, i.e., the adjustment of an equilibrium between 4-chloroacetophenone and 4-chloro-1-phenylethylation, the hydrolysis of 4-chlorophenyl acetate (data not shown), and the degradation of 4-chlorophenol with the release of chloride. As shown in Fig. 3, the chlorophenol-degrading enzyme set of strain 1B was specific for 4-chlorophenol. 3-Chlorophenol led to accumulation of chlorohydroquinone and to coloration of the medium accompanied by a decrease in viable count. 2-Chlorophenol was converted very slowly.

**Enzyme activities.** Enzyme activities in cell extracts are listed in Table 1. High activities for 4-chloroacetophenone or 4-chlorophenyl acetate were present only in strain M5. In both strains, catechol 1,2-dioxygenase activity could be detected, while catechol 2,3-dioxygenase was absent. The presence of the ortho pathway was further confirmed by the detection of maleylacetate reductase activity.

Phenol hydroxylase activity could not be detected in cell extracts. Therefore, oxidation of phenol and monochlorophenols was determined with whole cells. As shown in Table 2, oxidation of chlorophenols by strain 1B was achieved by noninduced as well as by induced cells. Resting-cell experiments had indicated that chlorophenols lacking a para-substituent were not suitable substrates for the phenol-hydroxylase of strain 1B but were slowly converted to chlorohydroquinone by para-hydroxylation. This was confirmed by the oxygen consumption assays. The highest oxidation rate was measured with 4-chlorophenol. Oxidation rates of 2-chlorophenol and phenol were below the detection limits of this method. In the resting-cell experiments, strain M5 was shown to be able to mineralize monochlorophenols. However, this activity was too low to show significant oxygen consumption rates in the whole-cell studies.

**Incubation of strain M5 in the presence of a 4-chlorophenol-absorbing substance.** Strain M5 failed to grow on 4-chloroacetophenone but accumulated 4-chlorophenol. From the results described above, it was supposed that strain 1B is important in the mixed culture because of its potential to rapidly remove the 4-chlorophenol produced by strain M5. To prove the assumption that a low concentration of 4-chlorophenol has to be maintained in the mixed culture system to allow the degradation of 4-chloroacetophenone, gelatin was added to mineral medium in order to remove the toxic 4-chlorophenol. Gelatin at 2.5 g/liter was shown to be
TABLE 1. Specific activities of catabolic enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>1B noninduced</th>
<th>1B induced</th>
<th>M5 noninduced</th>
<th>M5 induced</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Chloroacetophenone oxygenase</td>
<td>4-Chloroacetophenone</td>
<td>&lt;1</td>
<td>1</td>
<td>92</td>
<td>58</td>
</tr>
<tr>
<td>4-Chlorophenyl acetate esterase</td>
<td>4-Chlorophenyl acetate</td>
<td>148</td>
<td>170</td>
<td>3,816</td>
<td>4,780</td>
</tr>
<tr>
<td>4-Chlorophenol hydroxylase</td>
<td>4-Chlorophenol</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>238</td>
<td></td>
</tr>
<tr>
<td>Catecho1,2-dioxigenase</td>
<td>Catechol</td>
<td>20</td>
<td>70</td>
<td>254</td>
<td>8</td>
</tr>
<tr>
<td>4-Chlorocatechol</td>
<td></td>
<td>43</td>
<td>12</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Catecho2,3-dioxigenase</td>
<td>Catechol</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Maleylacetate reductase</td>
<td>Maleylacetate</td>
<td>ND</td>
<td>65</td>
<td>ND</td>
<td>250</td>
</tr>
</tbody>
</table>

* The strains were grown overnight on nutrient broth in the presence of a mixture of 4-chloroacetophenone and 4-chlorophenol supplied through the vapor phase. Two hours before the cells were harvested, the inducing compounds (4-chloroacetophenone and 4-chlorophenol) were added directly to the medium at a concentration of 0.1 mM. The cells were washed twice with phosphate buffer (pH 7.2; 50 mM), and crude extracts were prepared immediately.

b ND, not determined.

capable of absorbing 30 μM 4-chlorophenol from the medium. Strain M5 was not able to grow with gelatin as a sole carbon and energy source. The presence of gelatin allowed strain M5 to grow with 4-chloroacetophenone as the sole source of carbon and energy by overcoming the toxic effects of the transient accumulation of 4-chlorophenol at the beginning of the growth experiment (data not shown).

Strain M5 growing alone with 4-chloroacetophenone. To achieve growth of a pure culture of strain M5 with 4-chloroacetophenone without gelatin and without the 4-chlorophenol-degrading strain 1B, the critical phase at the beginning of growth, i.e., low density of strain M5, with increasing concentrations of 4-chlorophenol was overcome by initially adding acetate (4 mM) to reach a higher optical density without accumulation of 4-chlorophenol. Since acetate is a product of the degradation of 4-chloroacetophenone, no negative effect on the induction state of strain M5 was expected. The liquid substrate was added as a drop into a glass inset with an aperture and was placed in the vapor phase in the sealed Erlenmeyer flask. As shown in Fig. 4, slow growth with 4-chloroacetophenone as the sole source of carbon and energy started when acetate was consumed. Since no metabolite (most notably no 4-chlorophenol) was detected after 4 days and chloride was released continuously, the supply of substrate was enhanced after 4 days in order to increase the growth rate. Therefore, from that time the substrate was supplied on a filter paper, which increased the rate of evaporation because of the greater surface in comparison to a drop. However, immediate stoppage of growth and release of chloride occurred, together with an accumulation of high amounts of 4-chlorophenol.

Incubation of strain M5 with more highly chlorinated acetophenones. Strain M5 was tested to convert di- and trichlorinated acetophenones in resting-cell experiments. The results are shown in Fig. 5. The difference in the stoichiometric balances in the cases of 2,4-dichloro-, 2,5-dichloro-, and 2,3,4-trichloroacetophenone after 192 h was mainly due to the fact that the corresponding 1-phenylethanol remained unaffected in the medium. Since dichlorinated 1-phenylethanol was not available, no quantitative measurements could be taken. Depending on the substitution pattern of the compounds, different bottlenecks in the pathway were observed. No conversion of 2,6-dichloroacetophenone took place. Even the reduction to the corresponding 2,6-dichloro-1-phenylethanol, which occurred immediately with all other congeners tested, did not occur. Generally,

TABLE 2. Oxygen uptake responses of whole cells

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Response of strainsa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1B noninduced</td>
</tr>
<tr>
<td>2-Chloroacetophenone</td>
<td>ND</td>
</tr>
<tr>
<td>3-Chloroacetophenone</td>
<td>ND</td>
</tr>
<tr>
<td>4-Chloroacetophenone</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Phenol</td>
<td>&lt;5</td>
</tr>
<tr>
<td>2-Chlorophenol</td>
<td>&lt;5</td>
</tr>
<tr>
<td>3-Chlorophenol</td>
<td>25</td>
</tr>
<tr>
<td>4-Chlorophenol</td>
<td>35</td>
</tr>
</tbody>
</table>

a For culture conditions, see Table 1, footnote a. Rates are expressed in nanomoles of oxygen per minute per milligram of protein.
b ND, not determined.

FIG. 4. Growth of strain M5 with 4-chloroacetophenone as the sole source of carbon and energy after initiation with acetate. The following parameters were determined: 4-chloroacetophenone (○), 4-chlorophenol (□), 4-chlorocatechol (△), optical density at 546 nm (×), acetate (▲), and chloride (○). The arrows indicate the time when substrate supply was raised by enlargement of the surface of vaporizing substrate.
chlorine substitution in the neighborhood of the side chain hindered all reactions in the degradation of chloroacetophenones. The Baeyer-Villiger-oxidation took place if there was at least one nonsubstituted carbon atom next to the side chain. The phenol hydroxylase, however, needed two nonsubstituted carbon atoms next to the side chain. Therefore, degradation of 2,5-dichloroacetophenone ended at the step of the corresponding phenol. During conversion of 2,3-dichloroacetophenone, the transient metabolite 3,4-dichlorocatechol occurred in relatively high amounts (170 μM).

**DISCUSSION**

Decontamination of PCB-polluted sites cannot be evaluated merely by disappearance of the chlorinated biphenyls. It has long been known that degradation often stops on the level of the chlorinated benzoates. However, chlorinated acetophenones have recently been found as final products of PCB degradation (3–5). Accumulation of chlorobenzoates can be avoided by application of hybrid strains, which contain the biphenyl-degrading set of enzymes as well as those necessary for effective degradation of chlorobenzoates (19, 24). The accumulation of chlorinated acetophenones, however, results in other problems for the degrading organisms, since these—in contrast to chlorobenzoates—are toxic. In addition, the chlorophenols, which may occur during degradation of chloroacetophenones, represent another group of critical substances. These facts might explain why strains that are able to grow on chlorinated acetophenones as a sole source of carbon and energy cannot be easily obtained. Enrichment cultures isolated for growth with acetophenone or 4-hydroxyacetophenone failed to grow with chloroacetophenones (9, 20), since these substances select for pathways not suitable for halogenated analogs. 4-Hydroxyacetophenone favors the protocatechuate pathway, which cannot work with 4-chloroacetophenone. Strains enriched with acetophenone do not contain the modified ortho pathway, which effectively degrades chlorocatechols. For these reasons it is necessary to enrich directly for growth with chlorinated acetophenones while paying attention to their toxicity and that of the resulting chlorophenols.

A mixed culture which totally degrades 4-chloroacetophenone could be established, in which one member, strain 1B, was able to immediately remove 4-chlorophenol formed by the other member, strain M5. The participation of both strains in the degradation is summarized in Fig. 6. The results of the enzyme assays clearly indicated that chlorophenols represent the critical step in the degradation of chloroacetophenones by strain M5. The activities of the chloroacetophenone-oxidizing and the chlorophenol acetate-hydrolyzing enzymes are very high compared with those of the phenol hydroxylase. Therefore, under conditions of unlimited supply of substrate, toxic effects of chloroacetophenones might be overcome by rapid conversion of these compounds. As a consequence, however, large amounts of

**FIG. 5.** Incubation of strain M5 (1.1 × 10⁹ cells per ml) with di- and trichlorinated acetophenones. Acetophenones and the corresponding metabolites are marked as follows: (A) 2,3-dichloroacetophenone (○), 2,3-dichlorophenol (□), 3,4-dichlorocatechol (△); (B) 2,5-dichloroacetophenone (○), 2,5-dichlorophenol (□), 3,6-dichlorocatechol (Δ); (C) 3,4-dichloroacetophenone (○), 3,4-dichlorophenol (□), 4,5-dichlorocatechol (△); (D) 2,3,4-trichloroacetophenone (○), 2,3,4-trichlorophenol (□), 3,4,5-trichlorocatechol (Δ); (E) 2,4-dichloroacetophenone (○), 2,4-dichlorophenol (□), 3,5-dichlorocatechol (Δ); (F) 2,6-dichloroacetophenone (○), 2,6-dichlorophenol (□). ×, chloride in all panels.
The results shown are thought to be further steps in the metabolism of PCB-related organic compounds. Chloroacetophenones represent a problematic group of PCB metabolites because of their high toxicity and their tendency to form chlorophenols. Under certain conditions, the organisms described here are able to mineralize chloroacetophenones, and toxic effects can therefore be avoided.

Studies in which chloroacetophenone-degrading organisms are incubated together with PCB-transforming strains to achieve the mineralization of PCBs are in progress.

REFERENCES


