Anaerobic degradation of chlorophenol by an enrichment culture

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Summary. An anaerobic mixed culture from sewage sludge was enriched in a yeast extract and peptone-containing medium; it was able to degrade 2-chlorophenol completely to methane and CO₂. Degradation rates of 2-chlorophenol of up to 0.18 g/l per day were observed in suspended cultures without biomass retention and of 0.375 g/l per day in cultures immobilized on Liapor clay beads. Attempts to isolate the dechlorinating organism failed. The mixed culture was reduced to three morphologically distinctive microorganisms using a medium with limited amounts of yeast extract and peptone and n-butyrate as a co-substrate. Under these conditions the phenol-degrading bacterium was lost and phenol accumulated in the medium. No growth and no dehalogenation of 2-chlorophenol was obtained when yeast extract and peptone were omitted completely. Besides serving as a source of supplementary components, yeast extract and peptone were apparently required as the main source of carbon, whereas reducing equivalents for reductive dehalogenation were obtained by oxidation of n-butyrate. A spirochaete-like organism was presumably the dechlorinating bacterium. The mixed culture lost its dehalogenation capability if this organism was lost. n-Butyrate could be replaced by n-valerate, hexanoate, heptanoate, octanoate, pelargonic acid, n-decanoic acid or palmitate as co-substrates for dehalogenation of either 2-chlorophenol, 2-bromophenol or complete dechlorination of 2,6-dichlorophenol, whereas from 2,4-dichlorophenol only the substituent in the ortho-position could be eliminated.

Introduction

In contrast to domestic waste-water, industrial waste-water may contain high concentrations of halogenated compounds. The waste-water from the chlorine bleaching process of cellulose, for instance, can contain up to 500 mg chlorinated compounds/l (Vogel and Winter 1988; Winter et al. 1989).

Degradation of chlorinated chemicals by aerobic or anaerobic microbial consortia or by pure cultures of aerobes has been investigated in some detail (e.g. Knackmuss 1982; Reineke 1984; Sahm et al. 1986; Mikesell and Boyd 1986; Boyd and Shelton 1984; Gibson and Sulfita 1986; Tiedje et al. 1987). Anaerobes that were able to eliminate non-halogen substituents from aromatic compounds could, however, not catalyse dehalogenation (DeWeerd et al. 1986), indicating the specificity of the enzymes responsible. No data on dehalogenation by defined consortia are available and only one strictly anaerobic dehalogenating bacterium, strain DCB-1, has been isolated (Shelton and Tiedje 1984) as yet. In this contribution we describe a selectively enriched, strictly anaerobic, dehalogenating mixed culture, its metabolic capabilities, co-substrate requirements and protein yield.

Materials and methods

Organisms. A 2-chlorophenol-degrading mixed culture was enriched from anaerobically treated sewage sludge by repeated feeding of 0.1% (v/v) portions of 2-chlorophenol and monitoring dechlorination by release of chloride.

Media and culture conditions. Initially 20 ml clarified sewage sludge in serum bottles of 120 ml total volume were supplemented with 0.1% 2-chlorophenol, gassed with nitrogen at a gas station (Balch et al. 1979) to obtain anaerobic conditions and incubated at 37 °C.

After the onset of dechlorination, this enrichment culture was used as an inoculum for continuous cultures growing at 37 °C in a fixed-bed, 7 × 40 cm cylindrical reactor, filled with Liapor clay beads, 1 cm in diameter (Liapor, Pautzfeld, FRG). The working volume of the reactor was 800 ml. The culture liquid was recirculated once per hour and the pH was kept constant at 7.3 with a titrator that supplied fresh medium. The medium for the reactor (medium 1) contained 3.3 mM phosphate buffer, pH 7.3, 2 g/l peptone (Merck No. 7213), 0.4 mM Na₂S·2·9H₂O and 1.6 ml/l (= 2 g/l) 2-chlorophenol. Volatile fatty acids, produced from components of yeast extract and peptone, and HCl, produced from reductive dehalogenation of 2-chlorophenol, lowered the pH and initiated the feed pump.

* Dedicated to Professor O. Kandler on the occassion of his 70th birthday

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To select the essentially required organisms for 2-chlorophenol degradation the medium was varied to contain 0.1 g/l yeast extract, 0.1 g/l peptone and 2 mM n-butyrate as a co-substrate for dechlorination and 2-chlorophenol as indicated (medium 2). The utilization of fatty acids other than n-butyrate as co-substrates for dehalogenation was tested by adding the respective fatty acids from tenfold concentrated, anaerobic stock solutions instead of n-butyrate. The specificity of dehalogenation was tested by replacing 2-chlorophenol with the mentioned haloaromatic substances (Table 1). If utilization of other than the halogenated carbon sources was tested, n-butyrate and the halogenated substrate were replaced by 0.3% (w/v) of either glucose, galactose, fructose, maltose, saccharose, lactose, cellobiose, cellulose, mannose, ribose, mannitol, glycerogen or starch, 0.05% (v/v) of either methanol, ethanol, propanol or 2-butanol, 1 mM of either formate, acetate, propionate, n-valerate, hexanoate, heptanoate, octanoate, palmitic acid, n-decanoic acid, palmitic acid, glutamic acid, glycine or alanine and 0.1% (w/v) of either oxalate, malate, fumarate, succinate, citrate, crotonate, lactate, glycerol or benzoate. Growth on or utilization of these substances was tested in the absence or presence of sodium sulphate (5 mM) or sodium nitrate (5 mM) as electron acceptors. For growth of batch cultures, 20 or 200-800 ml portions of the respective medium were suspended into serum bottles of 120 ml total volume or Schott flasks of 11 volume in a glove box (Araki and Freter 1972). If not otherwise stated, 300 kPa N₂ was introduced at a gas station (Balch et al. 1979). All cultures were incubated at 37°C without shaking. For preparation of petri dishes media were solidified with 2% (w/v) agar-agar.

**Microscopy.** Photomicrographs of tenfold concentrated cell suspensions were taken with a Standard 14 fluorescence phase contrast microscope, equipped with a filter combination G 436, FT 510, LP 520 (Zeiss, Oberkochen, FRG) and a Minolta camera.

Scanning electron photomicrographs were taken with a model DSM 950 scanning electron microscope (Zeiss) at 10 kV accelerating voltage. Cell suspension were fixed as described by Sembiring and Winter (1989).

**Analyses.** Cell numbers in culture suspensions were determined by microscopic counting, using a calibrated Neubauer counting chamber. Volatile fatty acids were determined gas chromatographically and aromatic substances by HPLC reversed phase chromatography, as reported previously (Knoll and Winter 1987). The halogenated compounds 2-, 3- and 4-chlorophenol, 2,4- and 2,6-dichlorophenol, 2-bromophenol, 2- and 4-fluorophenol were separated by gas chromatography (Model 437A, Chrompack, Frankfurt, FRG) on a Silicone OV 351 column (1.5 mm x 2 m, Serva, Heidelberg, FRG) at 170 or 200°C, detected with a flame ionisation detector (210°C) and quantified with a CR3A integrator (Shimadzu, Munich, FRG). Utilization of other halogenated substances, e.g. of 2-chlorobenzoate, 3-methyl-4-chlorophenol, 4-chlororesorcinol, 4,6-dichlororesorcinol, 3,5-dichlorosalicylic acid, chlorohydrochinone, 3-, 4- and 5-chloro-2-methylaniline, 2-, 3- and 4-chloronitrobenzene were determined by quantification of the released chloride with an AOX analyser (Haberkorn and Braun, Munich, FRG).

Cells were harvested and extracts prepared as described elsewhere (Zellner et al. 1989). The protein content was quantified according to Bradford (1976).

**Chemicals.** All chemicals were of analytical grade and were purchased from Merck (Darmstadt, FRG), Serva or Fluka (Neu-Ulm, FRG).

**Results**

**Enrichment of a 2-chlorophenol-degrading consortium**

When sewage sludge was supplemented with 125 mg/l (0.975 mmol/l) 2-chlorophenol and incubated at 37°C, dechlorinating organisms were enriched that degraded the 2-chlorophenol quantitatively within 4 weeks. Upon repeated addition of the same amount of 2-chlorophenol, degradation was improved and was finally completed within 10 days. This enrichment culture served as an inoculum for a pH-auxostatically operated fixed-bed reactor (pH 7.3) containing porous clay beads for immobilization of microorganisms. In the presence of high amounts of yeast extract and peptone, maximum steady-state degradation rates for 2-chlorophenol of approximately 375 mg/l per day (2.925 mmol/l per day) were obtained at a dilution rate of 0.2 day⁻¹ after 140-160 days by pH-guided self-improvement of the system (Fig. 1a). If the dilution rate was further increased manually, phenol accumulated (not shown).

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**Table 1.** Maximum dechlorination rates obtained with the selected consortium in batch (a) and in continuously grown cultures in a fixed-bed reactor with clay beads (b)

<table>
<thead>
<tr>
<th>Cultivation mode</th>
<th>Substrates&lt;sup&gt;a&lt;/sup&gt; (3 mM)</th>
<th>Products</th>
<th>Dehalogenation rates (mmol/1/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Batch cultures</td>
<td>2-Chlorophenol</td>
<td>Phenol, HCl</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>2,6-Dichlorophenol</td>
<td>Phenol, 2 HCl</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>2,4-Dichlorophenol&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4-Chlorophenol, HCl</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>2-Bromophenol</td>
<td>Phenol, HBr</td>
<td>0.1</td>
</tr>
<tr>
<td>(b) Continuous culture</td>
<td>2-Chlorophenol</td>
<td>CH₄, CO₂, HCl</td>
<td>&gt;2.0</td>
</tr>
</tbody>
</table>

Cultures were grown in 20-ml portions of medium 2 (see Materials and methods) with 3 mM of either one of the halogenated substrates in the presence of 2 mM n-butyrate at 37°C

<sup>a</sup>No dehalogenation was observed in the absence of n-butyrate or in the presence of 2 mM n-butyrate and 3 mM of the following substrates: 3- and 4-chlorophenol, 2- and 4-fluorophenol, 2-chlorobenzoic acid, 2-chlorobenzaldehyde, 2-chlorobenzylalcohol, 3-methyl-2 mM n-butyrate and 3 mM of the following substrates: 3- and 4-chlorophenol, 2- and 4-fluorophenol, 2-chlorobenzaldehyde, 2-chlorobenzylalcohol, 3-methyl-

<sup>b</sup>Only dehalogenation of the chlorine-substituent in the ortho-position, but no growth of petri dishes media were solidified with 2% (w/v) agar-agar.