Degradation of Halogenated Aliphatic Compounds by Xanthobacter autotrophicus GJ10
Janssen, Dick; Scheper, Alex; Dijkhuizen, Lubbert; Witholt, Bernard

Published in:
Applied and Environmental Microbiology

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1985

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 24-03-2018
Degradation of Halogenated Aliphatic Compounds by Xanthobacter autotrophicus GJ10

DICK B. JANSSSEN,1* ALEX SCHEPER,1 LUBBERT DIJKHUizen,2 AND BERNARD WITHOLT1

Department of Biochemistry, Groningen Biotechnology Center, University of Groningen, Nijenborgh 16, 9747 AG Groningen,1 and Department of Microbiology, Groningen Biotechnology Center, University of Groningen, Kerklaan 30, 9751 NN Haren,2 The Netherlands

Received 2 October 1984/Accepted 11 December 1984

A bacterium that is able to utilize a number of halogenated short-chain hydrocarbons and halogenated carboxylic acids as sole carbon source for growth was identified as a strain of Xanthobacter autotrophicus. The organism constitutively produces two different dehalogenases. One enzyme is specific for halogenated alkanes, whereas the other, which is more heat stable and has a higher pH optimum, is specific for halogenated carboxylic acids. Haloalkanes were hydrolyzed in cell extracts to produce alcohols and halide ions, and a route for the metabolism of 1,2-dichloroethane is proposed. Both dehalogenases show a broad substrate specificity, allowing the degradation of bromine- and chlorine-substituted organic compounds. The results show that X. autotrophicus may play a role in the degradation of organochlorine compounds and that hydroytic dehalogenases may be involved in the microbial metabolism of short-chain halogenated hydrocarbons in microorganisms.

Chemical industries produce large amounts of short-chain halogenated aliphatic hydrocarbons, which are used as organic solvents, degrading agents, pesticides, and intermediates for the synthesis of various other organic compounds. As is the case with so many industrial chemicals, haloalkanes have caused numerous cases of environmental pollution due to improper disposal of wastes, accidental spillage, or deliberate release. Contamination of soils, underground waters, and surface waters is frequently observed (12, 17).

Biodegradation is the main process by which xenobiotics disappear from the environment. However, information about the susceptibility of short-chain haloalkanes to biodegradation is scarce. Aerobic transformation has only been described for dichloromethane (6, 13, 20–22) and for 1,2-dichloroethane (11, 23). Anaerobic transformation of halogenated methane in denitrifying cultures has been previously found (4). In methanogenic cultures, transformation was observed for halogenated methanes, 1,2-dichloroethane, 1,1,2,2-tetrachloroethane, and tetrachloroethylene (3, 5). In soil, chemical and biological conversions of haloalkanes that are used as biocides have been reported, and a reductive mechanism for the microbial removal of halide from 1,2-dibromoethane, 1,2-dibromo-3-chloropropane, and 2,3-dibromobutane was proposed (7).

Although aerobic degradation of haloalkanes seems possible and chloride release from 1,2-dichloroethane in crude cell extracts has been observed (11), the precise mechanism of the degradation of haloalkanes remains unclear (13, 19, 23). Dichloromethane degradation by a Hyphomicrobiurn strain involves nucleophilic substitution by a transferase that requires the presence of reduced glutathione for activity in crude extracts and produces S-chloromethyl glutathione. This compound was proposed to be converted nonenzymatically to formaldehyde and glutathione (22). Apart from chloride, the products of the dehalogenation of other haloalkanes have not been identified. The mechanism for haloalkane degradation could also be hydroytic or oxidative (23); the latter is expected to be oxygen dependent and to produce an aldehyde, whereas hydroytic dehalogenation would yield alcohols. Hydrolytic dehalogenation is known to occur during the metabolism of halogenated acetates and propionates (for a review, see reference 18) but has not been reported for haloalkanes (14).

We recently described the isolation of a bacterium that is able to grow aerobically with 1,2-dichloroethane as sole carbon and energy source (11). The organism constitutively produced enzymes that release chloride from 1,2-dichloroethane and chloroacetic acid, but the products and mechanism of these reactions are not known. The results described in this paper demonstrate that the organism belongs to the genus Xanthobacter and degrades haloalkanes to their corresponding alcohols by the action of a hydroytic haloalkane dehalogenase with broad substrate specificity.

MATERIALS AND METHODS

Growth conditions. Cells of strain GJ10 (11) were grown aerobically at 30°C under rotary shaking. To prevent evaporation of substrates, cultivation was carried out in closed flasks, filled to one-fifth of their volume with medium. Growth medium contained per liter: 5.37 g of Na2HPO4 · 12H2O, 1.36 g of KH2PO4, 0.5 g of (NH4)2SO4, 0.2 g of MgSO4 · 7H2O, 5 ml of a salts solution, and 1 ml of a vitamin solution, as described previously (11). Carbon sources were added at 5 mM.

Halide levels in the culture fluids were determined by the colorimetric method of Bergrmann and Sanik (2).

Preparation of crude extracts and enzyme assays. Cells were harvested by centrifugation (10 min at 10,000 × g), washed once with 10 mM Tris · SO4 buffer (pH 7.5), and suspended in this buffer (1 ml/g of wet cells). After ultrasonic disruption of the cells, a crude extract was obtained by centrifugation (30 min at 45,000 × g).

Haloalkane dehalogenase activities were assayed at 30°C in 50 mM Tris sulfate buffer (pH 7.5) containing 5 mM substrate and enzyme in a final volume of 3 ml. At different time intervals, 0.5-ml samples were removed and assayed for halide levels.
Halocarboxylic acid dehalogenase activities were assayed similarly but with 50 mM glycine \( \cdot \) NaOH buffer (pH 9.0) instead of Tris buffer. All dehalogenase activities are expressed as micromoles of halide produced per minute per milligram of protein (milliunits per milligram).

Methanol and 2-chloroethanol dehydrogenase activities were assayed at 30°C by using the oxygen electrode. Phenazine methosulfate was used as an artificial electron acceptor, and the assay was carried out as described by Dunstan et al. (8). Dehydrogenase levels are expressed in micromoles of oxygen consumed per minute per milligram of protein (milliunits per milligram).

Protein concentrations were estimated by the method of Lowry et al. (15), with bovine serum albumin as the standard.

**Gas chromatography.** Degradation of halogenated alkanes in crude extracts was followed by gas chromatography after extraction of incubation mixtures with diethylether containing dodecane as an internal standard. Incubations were carried out as above in volumes up to 10 ml, using closed bottles. Samples of 2 ml were withdrawn and added to 2 ml of 1 mM dodecane in diethylether and 0.2 ml of 10% \( H_3PO_4 \). Etherial extracts were analyzed with a Varian 1400 gas chromatograph equipped with a flame ionization detector. A stainless steel column (4 m by 3 mm) packed with 10% Carbowax 20M on Chromosorb W was used at 110°C for determination of 1,2-dichloroethane and 2-chloroethanol. The same column was temperature programmed from 40 to 120°C at 1°C/min for analysis of 1-propanol and 1-butanol. A stainless steel column (2 m by 3 mm) packed with Chromosorb 102 was used at 150°C for separation of ethanol. In all cases, good separation of alcohols from their corresponding aldehydes, the solvent, and the substrate was obtained, as determined by the analysis of authentic standards.

**Differential heat denaturation of dehalogenases.** Heat treatment was carried out by incubation of crude extracts in a water bath. The thermostat control was manually adjusted from 30 to 70°C at 1°C/min. This gave rise to the rate of temperature increase indicated in Fig. 3. At different time intervals, samples were removed, chilled on ice, and assayed for residual dehalogenase activity.

**Materials.** Halogenated organic compounds were obtained from E. Merck AG, Darmstadt, Federal Republic of Germany, and from Janssen, Beersum, Belgium. The purity of haloalkanes was checked by gas chromatography and exceeded 99% for 1,2-dichloroethane, bromoethane, and 1-chlorobutane, and 1-chloropropane contained 7% of the 2-isomer. Non-halogenated chemicals were of analytical grade.

**RESULTS**

**Characterization of strain GJ10.** The initial characterization of the 1,2-dichloroethane-degrading organism (strain GJ10) described previously revealed that it is a gram-negative (somewhat resistant to decolorization), nonmotile bacterium that is able to grow on methanol, citrate, fructose, and sucrose but not on glucose, galactose, and lactose. Its yellow cellular pigmentation and pleomorphic appearance and the presence of refractile bodies (Fig. 1) suggest that the organism might belong to the genus *Xanthobacter*, which represents the nitrogen-fixing hydrogen bacteria (26). Strain GJ10 is capable of autotrophic growth with a mixture of \( H_2 \) and \( O_2 \) as energy source but not with \( NH_4^+ \cdot O_2 \) (Table 1).

The organism can fix molecular nitrogen, both under autotrophic and heterotrophic growth conditions.

These results show that the organism should be classified as a strain of *Xanthobacter autotrophicus* (26). A comparison of the properties of strain GJ10 with the hypothetical median organism of 35 previously described strains of this species (26) shows a complete fit except for the oxidation reaction, which we scored negative (11) and was described positive for all tested strains (26) but was negative in a preceding report (25). Unlike *Xanthobacter flavus* (16), strain GJ10 does not utilize glucose, histidine, or phenylalanine as a carbon source.

Apart from halogenated compounds, a number of organic chemicals could also support growth: methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, ethylene glycol, acetone, and toluene. No growth was observed with 2-butanol, ethanolamine, pentane, hexane, 1-octanol, phenol, and benzene.

**Degradation and utilization of halogenated compounds.** It was determined whether strain GJ10 could utilize halogenated compounds that are structurally related to 1,2-dichloroethane and its possible degradation products. Since slight growth occurred on plain agar, growth tests were performed in liquid media supplemented with different halogenated compounds. The results (Table 2) show that besides 1,2-dichloroethane, a few other haloalkanes support growth: bromoethane, 1-chloropropane, 1,3-dichloropropane, and 1-chlorobutane. Three different halocarboxylic acids were not detected.

**TABLE 1. Autotrophic growth and nitrogen fixation by strain GJ10**

<table>
<thead>
<tr>
<th>Growth medium*</th>
<th>Atmosphere</th>
<th>Growth*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMV</td>
<td>70% ( H_2 ) + 10% ( CO_2 ) + 20% ( O_2 )</td>
<td>+</td>
</tr>
<tr>
<td>MMZV</td>
<td>75% ( N_2 ) + 10% ( H_2 ) + 10% ( CO_2 ) + 5% ( O_2 )</td>
<td>+</td>
</tr>
<tr>
<td>MMV + NH₄Cl</td>
<td>76% ( N_2 ) + 19% ( O_2 ) + 5% ( CO_2 )</td>
<td>-</td>
</tr>
<tr>
<td>MMZV + citrate</td>
<td>95% ( N_2 ) + 3% ( O_2 )</td>
<td>+</td>
</tr>
<tr>
<td>MMZV + methanol</td>
<td>95% ( N_2 ) + 3% ( O_2 )</td>
<td>+</td>
</tr>
<tr>
<td>MMZV + 2-chloroethanol</td>
<td>95% ( N_2 ) + 3% ( O_2 )</td>
<td>+</td>
</tr>
<tr>
<td>MMZV + 1,2-dichloroethane</td>
<td>95% ( N_2 ) + 3% ( O_2 )</td>
<td>+</td>
</tr>
</tbody>
</table>

* *MMV is the minimal medium described in the text; MMZV is the same medium with \( NH_4\)SO₄ omitted. The compounds indicated were added at 5 \( \mu \)M.
* Growth was scored after 10 days of cultivation at 30°C. +, Increase of the optical density of the culture to at least 0.5 at 450 nm; -, no growth detectable.
found to support growth. In all cases, growth was accompanied by halide release which strongly exceeded uninoculated controls.

The ability of strain GJ10 to utilize halogenated compounds was a very stable property in that 1,2-dichloroethane-negative variants were not detected after 24 serial transfers of the organism on nonselective medium.

 Degradation of halogenated compounds in crude extracts. Since _X. autotrophicus_ GJ10 utilized a number of different halogenated compounds for growth, the degradation of these compounds was tested in crude extracts. Halide production was observed with 1,2-dichloroethane (11), bromoethane, 1-chloropropane, 1,3-dichloropropane, 3-chloropropene, and 1-chlorobutane (Table 3). A number of chlorinated and brominated alkane acids were also dehalogenated in crude extracts, including bromoacetate and chloroacetate, which did not support growth.

### TABLE 3. Dehalogenating activities of crude extracts prepared from 1,2-dichloroethane-grown cells toward various substrates and the effect of heat treatment

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Sp act (mU/mg of protein)</th>
<th>% Activity remaining after heat treatment&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 7.5</td>
<td>pH 9.0</td>
</tr>
<tr>
<td>1,2-Dichloroethane</td>
<td>310</td>
<td>210</td>
</tr>
<tr>
<td>Bromoethane</td>
<td>45</td>
<td>35</td>
</tr>
<tr>
<td>1-Chloropropane</td>
<td>68</td>
<td>57</td>
</tr>
<tr>
<td>1,3-Dichloropropane</td>
<td>167</td>
<td>110</td>
</tr>
<tr>
<td>Chlorobutane</td>
<td>91</td>
<td>54</td>
</tr>
<tr>
<td>3-Chloropropene</td>
<td>97</td>
<td>95</td>
</tr>
<tr>
<td>Chloroacetate</td>
<td>89</td>
<td>367</td>
</tr>
<tr>
<td>Dichloroacetate</td>
<td>167</td>
<td>471</td>
</tr>
<tr>
<td>Bromoacetate</td>
<td>94</td>
<td>520</td>
</tr>
<tr>
<td>2-Chloropropionate</td>
<td>225</td>
<td>670</td>
</tr>
</tbody>
</table>

<sup>a</sup> Halide production at 30°C with different substrates (5 mM) was determined at pH 7.5 and 9.0.

<sup>b</sup> Crude extract of 1,2-dichloroethane-grown cells was heated up to 50°C as shown in Fig. 3. Remaining dehalogenase activities toward the substrates indicated (determined at pH 7.5) are given as percentages of the activity of untreated controls.

The products formed from bromoethane, 1-chloropropane, and 1-chlorobutane were analyzed by gas chromatography, and retention time comparison showed that these compounds were converted to ethanol, 1-propanol, and 1-butanol, respectively. Production of aldehydes was not observed. This suggests a hydrolytic mechanism for halide release.

**Metabolism of 1,2-dichloroethane.** To investigate the route of 1,2-dichloroethane metabolism, the compound was incubated with crude extract, and the products formed were analyzed by gas chromatography. It appeared that 1,2-dichloroethane was converted to 2-chloroethanol with no indication of chloroacetaldehyde formation. Chloride production from 1,2-dichloroethane was accompanied by the formation and disappearance of equimolar amounts of 2-chloroethanol and 1,2-dichloroethane, respectively (Fig. 2A). Furthermore, chloride production in crude extracts was not accompanied by oxygen consumption (Fig. 2B). Both results again indicate that dehalogenation is a hydrolytic reaction in this organism.

Further conversion of 2-chloroethanol is likely to proceed via chloroacetaldehyde and chloroacetate to glycolate. Crude extracts prepared from 1,2-dichloroethane-grown cells contained an ammonia-stimulated 2-chloroethanol dehydrogenase with optimal activity at pH 9.0. The enzyme was active with phenazine methosulfate as an artificial electron acceptor, whereas NAD and NADP were not active. The formation of this activity was induced together with methanol dehydrogenase during growth with 1,2-dichloroethane or methanol (Table 4) but not during growth with citrate.

### TABLE 4. Methanol dehydrogenase and 2-chloroethanol dehydrogenase in _X. autotrophicus_ GJ10

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Sp act* (mU/mg of protein)</th>
<th>Chloroethanol dehydrogenase</th>
<th>Methanol dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2-Dichloroethane</td>
<td>518</td>
<td>535</td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td>490</td>
<td>522</td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td></td>
</tr>
</tbody>
</table>

* Specific activities of methanol dehydrogenase and 2-chloroethanol dehydrogenase were determined in crude extracts of cells grown on the carbon source indicated.
were chloroethane dehalogenase (△) and chloroacetate dehalogenase (○) followed during heat treatment of crude extract prepared from 1,2-dichloroethane-grown cells. The temperature (●) was recorded.

Chloroacetaldehyde could possibly be converted to chloroacetate, followed by dehalogenation of this compound by the chloroacetate dehalogenase described previously (11; see below).

Identification of two dehalogenases. The dehalogenating activities toward various substrates were determined at two pH values, since 1,2-dichloroethane dehalogenase and chloroacetate dehalogenase show different pH optima (11). It appeared that the rate of halide liberation from halogenated carboxylic acids was three- to sixfold higher at pH 9 than at pH 7.5, and haloalkane-dehalogenating activities were lower at the higher pH for the substrates tested (Table 3). This suggests the existence of different dehalogenases for haloalkane and haloalkanoic acid degradation.

Heat denaturation studies confirmed the existence of at least two dehalogenases. The activity involved in chloride liberation from 1,2-dichloroethane was much more sensitive to heat treatment than chloroacetate dehalogenase, which was stable up to 50°C (Fig. 3). Treatment of crude extract up to 50°C also completely and selectively inactivated dehalogenase activity toward the other halogenated alkanes (Table 3).

Regulation of haloalkane dehalogenase formation. It was tested whether the formation of haloalkane dehalogenase activities was increased during growth with any of the respective substrates as carbon source. The results (Table 5) show that there is no significant induction of haloalkane dehalogenase synthesis by any of the compounds tested. The highest dehalogenase levels were observed in extracts prepared from autotrophically grown cells.

**DISCUSSION**

Our results demonstrate that the organism isolated on the basis of its ability to utilize 1,2-dichloroethane also utilizes a number of other haloalkanes for growth and is a nitrogen-fixing hydrogen bacterium that should be classified as a strain of *X. autotrophicus* (26). Carbohydrate utilization pattern, gram reaction, and colony and cell morphology (11; this paper) are in agreement with previously published data for *Xanthobacter* species (26). Up to now, the capability to degrade halogenated aliphatic compounds is mainly observed among members of the pseudomonads (10, 18) and has not been described for chemolithoautotrophic bacteria.

The degradation of haloalkanes in crude extracts was due to a haloalkane dehalogenase with broad substrate specificity. The enzyme is different from the haloalkanoate dehalogenase, as shown by differential heat inactivation. The haloalkane dehalogenase catalyzes the hydrolytic release of halide from haloalkanes, resulting in the replacement of the halogen substituent by a hydroxyl group. This was shown for the degradation of 1,2-dichloroethane, 1-chlorobutane, 1-chloropropane, and bromoethane. In this respect, the enzyme is similar to the dehalogenases that are involved in the metabolism of halogenated carboxylic acids (9, 18). However, microbial dehalogenases that catalyze the hydrolytic conversion of haloalkanes to alcohols have not been described previously (14).

Further conversion of the compounds produced may proceed via oxidation to carboxylic acids. Ammonia-stimulated 2-chloroethanol dehydrogenase activity could be detected in 1,2-dichloroethane- and methanol-grown cells, suggesting the route for 1,2-dichloroethane degradation given in Fig. 4. Oxidation of 2-chloroethanol has also been found in 2-chloroethanol with pseudomonads (11, 24). The possibility

**FIG. 3.** Heat inactivation of dehalogenases. The levels of 1,2-dichloroethane-grown cells. The tested activity was stable up to 50°C. 676 was recorded.

**FIG. 4.** Proposed route for the metabolism of 1,2-dichloroethane in *X. autotrophicus.*

<table>
<thead>
<tr>
<th>Enzyme substrate</th>
<th>Specific dehalogenase activities* (mU/mg of protein)</th>
<th>Substratea</th>
<th>1,2-DCEb</th>
<th>Citrate</th>
<th>CO2c</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2-Dichloroethane</td>
<td>232</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bromoethane</td>
<td>48</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-Chloropropane</td>
<td>76</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,3-Dichloropropane</td>
<td>256</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-Chlorobutane</td>
<td>84</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloroacetic acid</td>
<td>416</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Specific activities with the substrates indicated were determined with extracts from cells grown on the carbon sources indicated.
* Extract was prepared from cells grown on the substrate that was used for the enzyme assay.
* 1,2-DCE, 1,2-Dichloroethane.
* Autotrophic growth was with H2O2 as the energy source (see Table 1 for details).
that 2-chloroethanol is oxidized by a non-specific methanol dehydrogenase is suggested by the observation that the two activities are simultaneously induced during growth with methanol or 1,2-dichloroethane, but this needs further study. The methanol dehydrogenase of a methylothrophic \textit{Pseudomonas} species oxidizes 2-chloroethanol at 80% of the rate observed with methanol (1).

In a second \textit{Xanthobacter} strain, isolated on the basis of its ability to utilize methanol, we could not detect dehalogenase activity (data not shown). This organism did not utilize halogenated compounds for growth. However, 2-chloroethanol dehydrogenase activity was present in extracts of methanol-grown cells of this strain. These results indicate that the production of dehalogenases is not a general characteristic of \textit{Xanthobacter} species and that especially the receipt of the ability to produce these enzymes has enabled strain GJ10 to degrade halogenics.

Both dehalogenases in strain GJ10 are synthesized constitutively. The dehalogenases were also present in autotrophically grown cells, and no induction by haloalkanes that served as carbon source was found. An intriguing question is how such enzymes involved in the degradation of xenobiotic haloalkanes have evolved. The haloalkane dehalogenase is clearly distinct from the haloalkanoate dehalogenase in the same organism. There is no overlap in substrate range, and the enzymes differ with respect to heat stability and do not copurify upon ammonium sulfate fractionation or ion-exchange chromatography (D. B. Janssen and S. Keuning, unpublished data). A further characterization of the haloalkane dehalogenase and a study toward the genetic basis of the ability to utilize haloalkanes should give more insight into this problem.

ACKNOWLEDGMENTS

We thank N. Panman for drawing the figures and K. Gilissen for photography.

This work was supported by the Program Commission for Biotechnology of The Netherlands.

LITERATURE CITED