Metabolism of mono- and dihalogenated C1 and C2 compounds by *Xanthobacter autotrophicus* growing on 1,2-dichloroethane

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Abstract

The conversion of and toxic effects exerted by several mono- and dihalogenated C1 and C2 compounds on cultures of *Xanthobacter autotrophicus* GJ10 growing on 1,2-dichloroethane were investigated. Bromochloromethane, dibromomethane and 1-bromo-2-chloroethane were utilized by strain GJ10 in batch culture as a cosubstrate and sole carbon source. The rate of degradation of dihalomethanes by whole cells was lower than that of 1,2-dichloroethane, but a significant increase of the rate of dihalomethane biodegradation was observed when methanol or ethanol were added as a cosubstrate. Products of the degradation of several tested compounds by haloalkane dehalogenase were analyzed and a new metabolic pathway based on hydrolytic conversion to formaldehyde was proposed for the dihalomethanes. Strain GJ10 growing on 1,2-dichloroethane converted 2-fluoroethanol and 1-chloro-2-fluoroethane to 2-fluoroacetate, which was tolerated up to a concentration of 2.5 mM. On the basis of the results from batch cultures an inert (dichloromethane), a growth-supporting (dibromomethane) and a toxic (1,2-dibromoethane) compound were selected for testing their effects on a continuous culture of strain GJ10 growing on 1,2-dichloroethane. The compounds were added as pulses to a steady-state chemostat and the response of the culture was followed. The effects varied from a temporary decrease in cell density for dibromomethane to severe toxicity and culture washout with 1,2-dibromoethane. Our results extend the spectrum of halogenated C1 and C2 compounds that are known to be degraded by strain GJ10 and provide information on toxic effects and transformation of compounds not serving as a carbon source for this bacterium.

Abbreviations: 2-FE – 2-fluoroethanol; BCE – 1-bromo-2-chloroethane; BCM – bromochloromethane; CFE – 1-chloro-2-fluoroethane; CSTB – continuous stirred tank bioreactor; CT – carbon tetrachloride; DBM – dibromomethane; DCE – 1,2-dichloroethane; DCM – dichloromethane; DCY – 1,2-dichloroethylene; DFA – 2,2-difluoroacetate; DIM – diiodomethane; EDB – 1,2-dibromoethane; MFA – 2-fluoroacetate; MIA – 2-iodoacetate; OD – optical density; TBM – tribromomethane; TCA – 2,2,2-trichloroacetate; TCE – trichloroethane; TCM – trichloromethane; TCY – trichloroethylene

Introduction

The biodegradation of 1,2-dichloroethane (DCE), an abundant chlorinated C2 groundwater pollu- tant, has been the subject of intensive studies. The best-known bacterial strain capable of complete mineralization of DCE, *Xanthobacter autotrophicus* GJ10, can utilize this xenobiotic compound as

a sole carbon and energy source (Janssen et al. 1985) (Figure 1). Removal of DCE by this microorganism was demonstrated to occur in a continuous stirred tank bioreactor (Ferreira Jorge & Livingston 1999; van den Wijngaard et al. 1993), a membrane bioreactor (Freitas dos Santos & Livingston 1995), and at full scale in a rotating disk biological contactor (Stucki & Thüer 1995). In these studies DCE was the only organic substrate fed to the reactor. While such a situation may occasionally exist in polluted groundwater, industrial wastewaters and contaminated groundwater often contain a diverse mixture of halogenated contaminants. The presence of more than one pollutant can affect the microbial activity in a variety of ways. A non-inert compound present in a mixture of pollutants may be either toxic to an organism that is of key importance for reactor performance and hinder its growth, or it may be used as an additional carbon substrate (Hu et al. 2005; Kovárová & Egli 1998).

Whilst microbial interactions during biodegradation of DCE by strain GJ10 in the presence of another pollutant and a strain capable of its degradation have been studied (Ferreira Jorge & Livingston 2000; Hartmans et al. 1992), few data are available on the influence of additional compounds on strain GJ10 itself. Moreover, the spectrum of C1 or C2 halogenated compounds that can be degraded by strain GJ10 (Table 1) is not well characterized. Although the substrate range of both dehalogenases was studied thoroughly (Keuning et al. 1985; Schanstra et al. 1996; van der Ploeg et al. 1991), the data cannot always be used to predict the catabolic potential of whole cells of GJ10. For instance, the soil fumigant 1,2-dibromoethane is rapidly dehalogenated by haloalkane dehalogenase, but it cannot be mineralized by whole cells due to formation of toxic intermediates (van der Ploeg et al. 1996). Similarly, bromoacetic acid is hydrolyzed by haloacid dehalogenase, but it is toxic to the wild-type strain GJ10 (van der Ploeg et al. 1995). On the other hand, we recently demonstrated the biodegradation of monochloroacetate by strain GJ10 (Torz & Beschkov 2005), although earlier reports did not suggest such possibility.

In this paper we present the results of an investigation of the degradation of DCE by strain GJ10 in the presence of other C1 or C2 halogenated compounds of environmental importance. Halogenated methanes and ethanes that were chosen in this study are often produced in large amounts and have genotoxic effects (Ahmed et al. 1980). Several fluorinated analogues of chlorinated compounds degraded by GJ10 were included in the research. Fluorinated compounds are of growing interest in industry (Maximov 1998) but their metabolism and toxicity are still not fully understood (Keller et al. 1996).

The aim of the work was to evaluate the fate of the secondary halogenated substrates and to assess their influence on strain GJ10 growing on DCE. Incubations containing binary mixtures of DCE and other C1 or C2 compounds and cells of strain GJ10 were screened for products and intermediates. The compounds which were converted by strain GJ10 were further tested as a sole carbon and energy source. The products of their dehalogenation by haloalkane dehalogenase were identified and metabolic pathways were proposed. Finally, the effects of three selected compounds on the continuous degradation of DCE were investigated. For this purpose an inert compound, a utilizable substrate, and a toxic compound were separately added as pulses to a chemostat culture growing on DCE and their fate as well as the culture response were followed. The results show
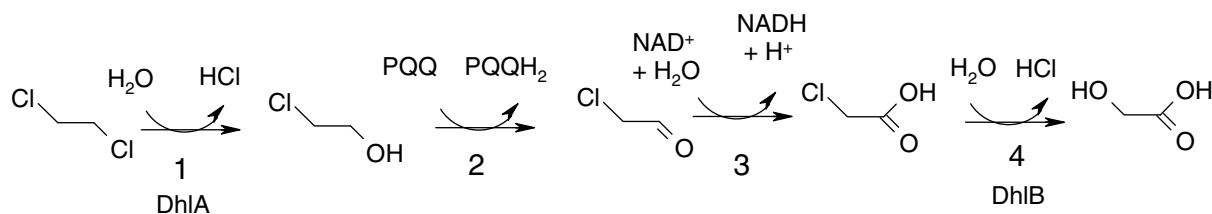

Figure 1. Metabolism of DCE by *Xanthobacter autotrophicus* GJ10 (Janssen et al. 1985). The enzymes are: 1 – Haloalkane dehalogenase (DhlA); 2 – Pyrrolo-quinoline quinone-containing alcohol dehydrogenase; 3 – NAD-dependent aldehyde dehydrogenase; 4 – Haloacetate dehalogenase (DhlB).

Table 1. Spectrum of halogenated methanes and ethanes utilized by *X. autotrophicus* GJ10 as a sole carbon and energy source

Utilized as a sole carbon source	Not utilized as a carbon source
1,2-Dichloroethane ($\leq$ 12 mM) (Ferreira Jorge & Livingston 1999; Van den Wijngaard et al. 1993)	Methyl chloride
2-Chloroethanol (Janssen et al. 1987)	Dichloromethane
Monochloroacetic acid ($\leq$ 48.8 mM) (Torz & Beschkov 2005)	Chloroethane
Dichloroacetic acid ($\leq$ 20 mM) (Meusel & Rehm 1993)	1,1-Dichloroethane
Dibromoacetic acid	1,1,1-Trichloroethane
Bromoethane	1,1,2-Trichloroethane
Bromoacetic acid (mutant, van der Ploeg et al., 1995)	1,1,2,2-Tetrachloroethane
	Trichloroacetic acid
	1,2-Dibromoethane
	2-Bromoethanol

Source: Janssen et al. (1985) and the references indicated.

that the spectrum of halogenated C1 and C2 substrates that are utilized by strain GJ10 can be extended with two halomethanes and a dihaloethane, and that toxic effects may accompany conversion of compounds that do not support growth.

Materials and methods

Batch growth conditions

Xanthobacter autotrophicus GJ10 cells were grown in the darkness at 30 °C under rotary shaking at 200 rpm. Glass flasks of 350–500 ml were filled to one-fifth of their volume and tightly closed with metal caps containing rubber and Teflon septa. Growth medium (MMY) contained (g/l): 5.37 $Na_2HPO_4 \cdot 12H_2O$, 1.36 KH_2PO_4, 0.5 $(NH_4)_2SO_4$ and 0.2 $MgSO_47 \cdot H_2O$. Salts solution (5 ml/l) (Torz & Beschkov 2005) and yeast extract (15 mg/l) were also added. For growth tests on binary substrate mixtures, cultures were inoculated with 1 ml of a late-exponential phase preculture grown on 0.8% nutrient broth (Difco) and for growth tests with single carbon sources, the inoculum was pregrown on 3.5 mM DCE.

Carbon sources were added with a glass syringe or a micropipette without sterilization, except for nutrient broth, which was autoclaved, and MFA, MIA, TCA and sodium acetate, which were prepared as aqueous solutions and filter sterilized (0.2 μm, Schleicher & Schuell). Sterility of all solutions was checked in control experiments carried out in the same way as growth tests but without inoculation. No growth or substrate deg-

radation was ever observed in a non-inoculated flask. All experiments were done at least in duplicate.

All chemicals were of $>$ 98% purity except for sodium fluoroacetate, which was of 90% purity. All compounds were used as received. Acids (MIA, TCA) were added to the flasks together with an appropriate volume of 2.5 mM sodium hydroxide solution in order to avoid a pH change.

Conditions of cultivation in CSTB

The 2.5 l fermentor used in this work was described in detail elsewhere (Torz & Beschkov 2005; van den Wijngaard et al. 1993). The substrate (15–20 mM DCE) was continuously fed to the fermentor together with the growth medium through Viton rubber tubing. The medium contained 0.46 g/l $Na_2HPO_4 \cdot 12H_2O$, 0.16 g/l KH_2PO_4 and 30 mg/l yeast extract. The concentrations of the other components were the same as in batch experiments. DCM, DBM and EDB were added as pulses (each in a separate experiment) directly to the fermentor *via* rubber septa at a steady-state of bioreactor performance. The response of the bioreactor to the pulses was followed by measuring the concentrations of biomass, both substrates (DCE and the compound added) and degradation products. Operational conditions were as follows: dilution rate 0.029 h^{-1}, temperature 30 $\pm$ 0.1 °C, pH 6.90–7.15, stirring rate 400 rpm. The aeration rate was controlled by the actual oxygen concentration in the broth and was within the range of 0–40 ml/min of air.

On-line monitoring of substrate depletion in batch incubations

Degradation rates of DBM, BCM and DCE in batch incubations were determined by on-line monitoring of headspace concentrations. The experimental setup was described in detail previously (van Hylckama Vlieg et al. 1996). Briefly, 25 ml of cells suspended in MMY medium were placed in a tightly closed 120-ml incubation vessel that was thermostated at 30 °C and continuously mixed with a magnetic stirrer. After addition of the volatile substrate a 35-μl sample loop was filled with regular time intervals with gas from the vessel and the samples were injected into a gas chromatograph (model Chrompack 9001, Middelburg, the Netherlands) equipped with a CP-Sil-5CB column (Chrompack, 0.53 mm × 25 m, film thickness 5 μm) and a flame-ionization detector supplied with hydrogen, air and nitrogen. Helium (26 ml/min) was used as the carrier gas. The column temperature was 100 °C and the detector was set at 150 °C.

Cells to be used for these kinetic experiments were grown on 6.8 mM DCE in 750 ml medium. At the late exponential phase the cells were centrifuged, washed with MMY medium, and resuspended in MMY medium to an OD_{500} of 3.5. Substrates were prepared as concentrated aqueous solutions and added with a glass syringe to the incubation vessel *via* Teflon-lined rubber septa. Calibration curves were prepared by measuring the gas chromatography peak areas after introducing known amounts of an appropriate stock solution to the vessel filled with cell-free medium. Concentrations were expressed as the total amount of substrate in the vessel divided by the volume of the liquid phase. After each experiment degradation of the respective compound was confirmed by determination of the halide concentration in the vessel by means of ion chromatography (data not shown).

To estimate the apparent first-order rate constants for substrate degradation, the following equation previously described by van Hylckama Vlieg et al. (1996) in a modified form was fitted with the experimental data:

$$S = S_0 \exp\left(-\frac{V_{max}}{K_m a} t\right)$$

where S is the total amount of substrate in the vessel divided by the volume of the liquid phase (V_w), V_{max} and K_m are the usual Michaelis–Menten constants, and $a = 1 + V_g \cdot H/V_w$. This model assumes that the K_m is much higher than the concentration of each substrate in the aqueous phase, which simplifies Michaelis–Menten kinetics to first-order kinetics. The following values for the Henry's constants ($H = C_g/C_w$, dimensionless) were used: 0.057 for DBM (Moore et al. 1995), 0.075 for BCM (estimated with the HENRYWIN 1.90 program) and 0.058 for DCE (Leighton & Calo 1981).

Expression and purification of haloalkane dehalogenase

Escherichia coli TOP10 (pBADdhlA), which contains the *dhlA* gene (Janssen et al. 1989), was used for haloalkane dehalogenase expression. A preculture was grown at 37 °C to an OD_{600} of 5 in terrific broth (TB) medium containing (g/l): tryptone 12, yeast extract 24, KH_2PO_4 2.31, $K_2HPO_4 \cdot 3H_2O$ 16.45, and 4 ml/l glycerol. The broth was supplemented with 100 μg/ml ampicillin. Cultivation was continued at 17 °C in TB with 100 μg/ml ampicillin and 0.5% arabinose. Cells were harvested after 3 days ($OD_{600} = 18$), washed with TEMAG buffer containing 25 mM Tris · SO_4 (pH 7.9), 1 mM EDTA, 1 mM 2-mercaptoethanol and 10% (v/v) glycerol and disrupted by sonication. The enzyme was purified as described earlier (Schanstra et al. 1993). The final concentration of the protein was measured by its absorbance ($\epsilon_{280} = 4.87 \times 10^4$ M^{-1} cm^{-1}) and was equal to 10 mg/ml. The enzyme was stabilized with TEMAG buffer and kept in at −20 °C until used for assays.

Enzyme assays

Incubations with haloalkane dehalogenase were carried out in 40-ml tightly closed vials fitted with Teflon septa and containing 20 ml of 5 mM substrate (DCE, BCE or CFE) in 50 mM Tris · SO_4 buffer (pH = 8.2). Reactions were started with 5–10 μl of purified haloalkane dehalogenase solution (corresponding to 50–100 μg of enzyme). The flasks were incubated at 30 °C on a rotary shaker at 50 rpm. Samples of 0.5 ml were taken with appropriate time intervals and halide concentrations were measured. Finally, 1–2 ml of the incubation mixture was extracted with 1 ml of diethyl ether containing 0.1 mM 1-chlorohexane as an

internal standard and products were identified by GC analysis.

Transformation of BCM was carried out in 25 mM phosphate buffer (pH = 8.2) since Tris was found to strongly interfere with the formaldehyde determination method that uses chromotropic acid. All other conditions were the same as in the transformation tests with dihaloethanes.

Kinetic studies were carried out in 15 ml 50 mM Tris · SO$_4$ buffer (pH = 8.2) containing 5 mM of substrate and 100 μg of enzyme. The activities of haloalkane dehalogenase are expressed as μmol of substrate degraded (calculated on the basis of halide concentrations) per min per mg of protein added (U/mg).

Analytical methods

Biomass growth was monitored by measuring optical densities at 500 nm. Halide and MFA concentrations were determined by ion chromatography. A Dionex type DX-120 chromatograph equipped with an Alltech column type Allsep A-2 was used. The eluent was prepared by dissolving 2.7 mmol/l sodium carbonate and 0.3 mmol/l sodium bicarbonate in deionized water. Halide concentrations in enzyme incubations were determined by the colorimetric method of Bergmann & Sanik (1957). Formaldehyde was determined by a chromotropic acid method described in detail elsewhere (Georghiou & Ho 1989). Monobromoacetate was determined by means of an HPLC equipped with a Varian ChromSep SS Microspher C18 column and a UV detector working at 210 nm. The eluent was prepared as follows: deionized water and acetonitrile were mixed at a ratio of 7:3 and 680 mg/l KH$_2$PO$_4$ and 340 mg/l of sodium dodecylsulphate were added. The pH was adjusted with H$_3$PO$_4$ to a value of 3. Finally, the solution was mixed 1:1 with deionized water and used as eluent.

Haloalkanes were determined by gas chromatography. Aqueous samples of 1–2 ml were extracted with 1 ml of diethyl ether containing 0.1 mM 1-cyclohexane as an internal standard. Samples of 5 μl were injected manually with a Hamilton syringe in a Chrompack 9001 gas chromatograph as described above. The oven was temperature programmed as follows: 3 min isothermal at 30 °C followed by an increase of 10 °C/min to 150 °C. DCM was analyzed with a model 6890 gas chromatograph (Hewlett–Packard) equipped with a HP-5 column, autosampler and electron capture detector. The temperature program was as follows: 5 min isothermal at 30 °C followed by an increase of 10 °C/min to 120 °C.

Results

Growth on binary substrate mixtures in batch cultures

The effects of the presence of a range of halogenated C1–C2 compounds on the growth of strain GJ10 on DCE in batch culture were investigated. Binary mixtures of DCE and a compound of interest were used at concentrations of 2.5 mM each. At this concentration of DCE the yield of biomass was high enough to observe a potential toxic effect from the test compound. Furthermore, possible intermediates and transformation products of the tested xenobiotics could be detected.

In the absence of another compound the growth on DCE was almost complete after 36 h of cultivation (Figure 2). With three of the test compounds (BCM, DBM, and BCE), a higher growth yield was obtained when they were added together with DCE. This indicated that they served as additional carbon sources, even though the growth rate was lower.

A reduced growth rate was also observed with the two fluorinated compounds (MFA and 2-FE) as well as with several other compounds. Four of the tested compounds were even found to be highly toxic to strain GJ10. The iodinated substrates (DIM and MIA) and TBM completely inhibited growth, whereas CT allowed only very poor growth on DCE. Several other compounds were inert or only slightly toxic, such as the chlorinated ethylenes (Figure 2).

The effect of MFA, which was expected to be a potential inhibitor of the tricarboxylic acid cycle, was also tested in growth experiments with other carbon sources and a 5-fold lower amount of inoculum (initial OD$_{500}$ = 0.01). In this case, the presence of MFA indeed resulted in a delay of the growth of strain GJ10 as well as a lower yield of biomass (Figure 3). The latter was not observed when citrate was the growth substrate although the lag-phase was even longer in this case.
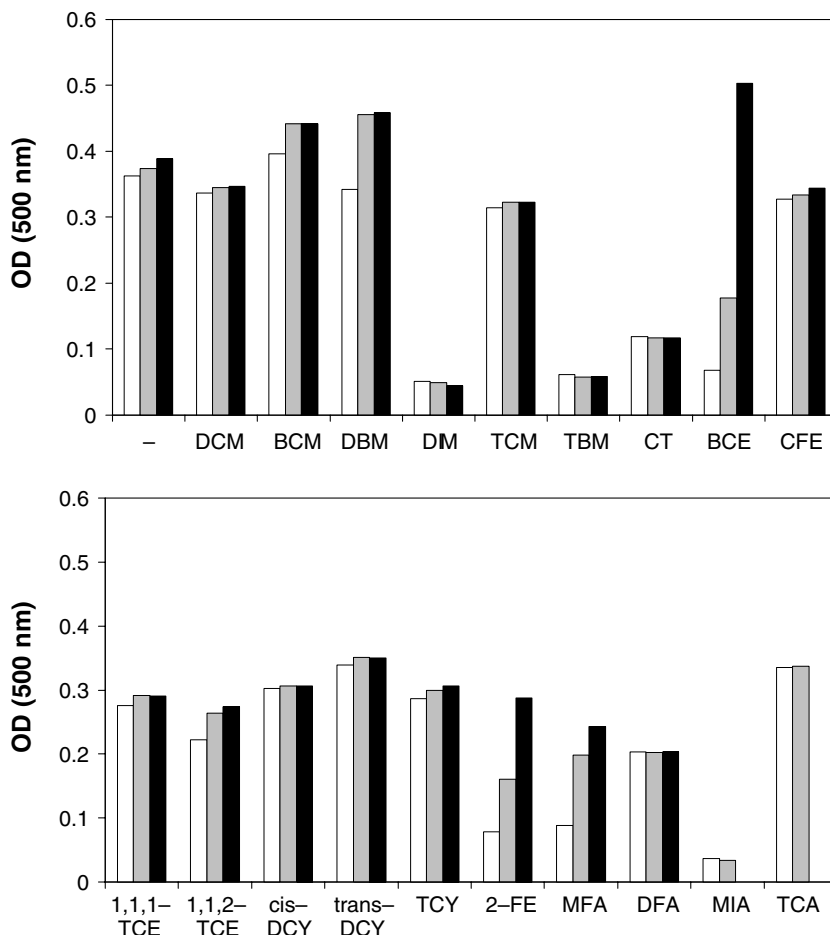
Figure 2. Effect of halogenated C1–C2 compounds on the growth of *Xanthobacter autotrophicus* GJ10 on DCE in batch culture. All compounds were used at 2.5 mM concentration. The OD was measured after 36 h (white bars), 60 h (gray) and 84 h (black). The OD after 84 h was not measured in the MIA- and TCA-containing flask.

The halide concentrations that were detected in the culture media of cells exposed to binary mixtures of substrates were in agreement with results of the growth experiments. Thus, inhibition of growth (Figure 2) was accompanied by a lower rate of chloride release from DCE (Figure 4), for example in cultures containing both DCE and BCE or 2FE. The measured halide levels also indicated that complete mineralization of BCM, DBM and BCE occurred. However, the rate of release of halogens from the dihalomethanes was lower than the rate of chloride production from DCE.

Analysis of products that accumulated in cultures containing CFE and 2-FE revealed that both these compounds were partially converted to MFA, which was detected at levels up to *ca.* 0.9 mM (Figure 4). Apparently, MFA was not

rapidly degraded or used as a carbon source by strain GJ10.

Hydrolytic degradation of BCM, DBM and BCE

The growth tests described in the previous section revealed that the dihalomethanes BCM and DBM could be utilized as a cosubstrate and supported growth of strain GJ10. In order to compare the rate of their degradation with the rate of DCE conversion, substrate depletion in batch cultures was followed by on-line GC analysis of the culture headspace. The depletion curves obtained after the addition of 0.05 mM of DBM, BCM and DCE (each in a separate experiment) could be fitted to first-order substrate depletion curves, indicating that the
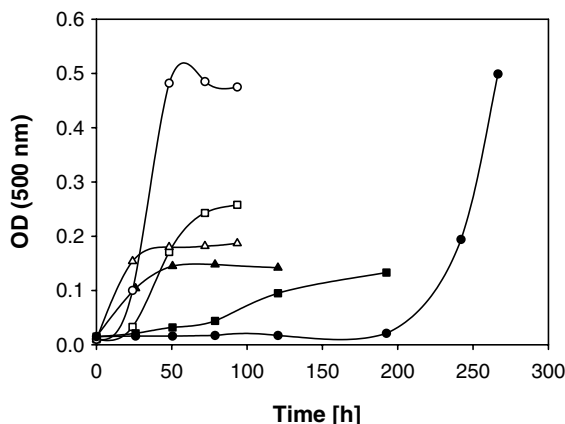
Figure 3. Growth of *Xanthobacter autotrophicus* GJ10 on different carbon sources and the effect of 2.5 mM MFA. Symbols: 2.5 mM DCE (■ with MFA, □ without MFA); 2.5 mM sodium acetate (▲ with MFA, △ without MFA); 2.5 mM trisodium citrate (● with MFA, ○ without MFA).

substrate concentrations were below the K_m values (Figure 5). The values were related to cell dry mass and were equal to 1.01×10^{-1} ml per min per mg of cells for DBM, 0.98×10^{-1} ml min^{-1} mg^{-1} for BCM, and 3.9×10^{-1} ml min^{-1} mg^{-1} for DCE. These numbers indicate that the first-order rate constants for the transformation of dihalomethanes were almost four times lower than the k_{cat}/K_m value for DCE transformation.

Subsequently, it was tested if BCM, DBM and BCE could serve as a sole carbon and energy source for strain GJ10 (Figure 6). Complete biodegradation of 5 mM BCE was observed after about 75 h and resulted in a biomass optical density of 0.370. Under the same conditions, 5 mM DCE was completely degraded within approximately 50 h (data not shown) and the optical density at the end of the exponential phase was as high as 0.650. The growth of GJ10 on the dihalomethanes BCM and DBM was much slower than on BCE and did not result in a visible exponential phase. After 190 h only 45% of the DBM added and 65% of the BCM was degraded. Complete transformation of both compounds was found after 400 h of incubation (not shown in Figure 6).

The poor kinetics of BCM and DBM utilization by strain GJ10 might be a serious limitation in the use of the organism for the removal of dihalomethanes. However, the rate of biodegradation was greatly accelerated when 5 mM ethanol was added as a cosubstrate (Figure 6). After 165 h,

about 90% of 5 mM DBM was mineralized. Since the exponential phase of growth occurred between 25 and 50 h and the fastest rate of halide release occurred between 50 and 75 h, the increased rate of DBM dehalogenation was probably caused by the higher cell concentration that was achieved in the presence of ethanol.

A similar increase of the rate of halocarbon degradation was observed with 5 mM DBM when 15 mM of methanol was used as a cosubstrate. The growth of strain GJ10 on methanol, with the exponential phase occurring between 50 and 75 h, was slower and the period of dibromomethane degradation lasted longer than with ethanol. The highest rate of bromide release was observed between 75 and 105 h and approximately 90% of the compound was degraded after 200 h of cultivation (Figure 6).

Dehalogenation of BCM, BCE and CFE by haloalkane dehalogenase

The conversion of DBM, BCM, BCE and CFE was also tested with purified haloalkane dehalogenase in order to identify dehalogenation products. BCM (5 mM) transformation was quantitatively accompanied by halide release and formation of formaldehyde (Figure 7). Similar results were obtained for DBM (data not shown). Incubation of BCE with haloalkane dehalogenase also led to the production of bromide, but even after 45 min of incubation no chloride could be detected in the flask (Figure 7). Gas chromatographic analysis at that time indicated the presence of remaining substrate and 2-chloroethanol. The dehalogenation of CFE by isolated dehalogenase resulted in the production of chloride. No fluoride was detected after conversion of CFE.

Rate measurements showed that haloalkane dehalogenase had an activity with various C1 and C2 haloalkanes (Table 2) that is slightly lower than the rate found with 1,2-dichloroethane, which was 4.9 μmol of substrate per min per mg of protein, close to the value of 6 μmol min^{-1} mg^{-1} found by Pries et al. (1994). Especially compounds containing a carbon–bromine bond were rapidly converted.

Effect of pulses of DCM, DBM and EDB on the continuous biodegradation of DCE in CSTB

The batch experiments with the four dihalomethanes described above revealed different effects on
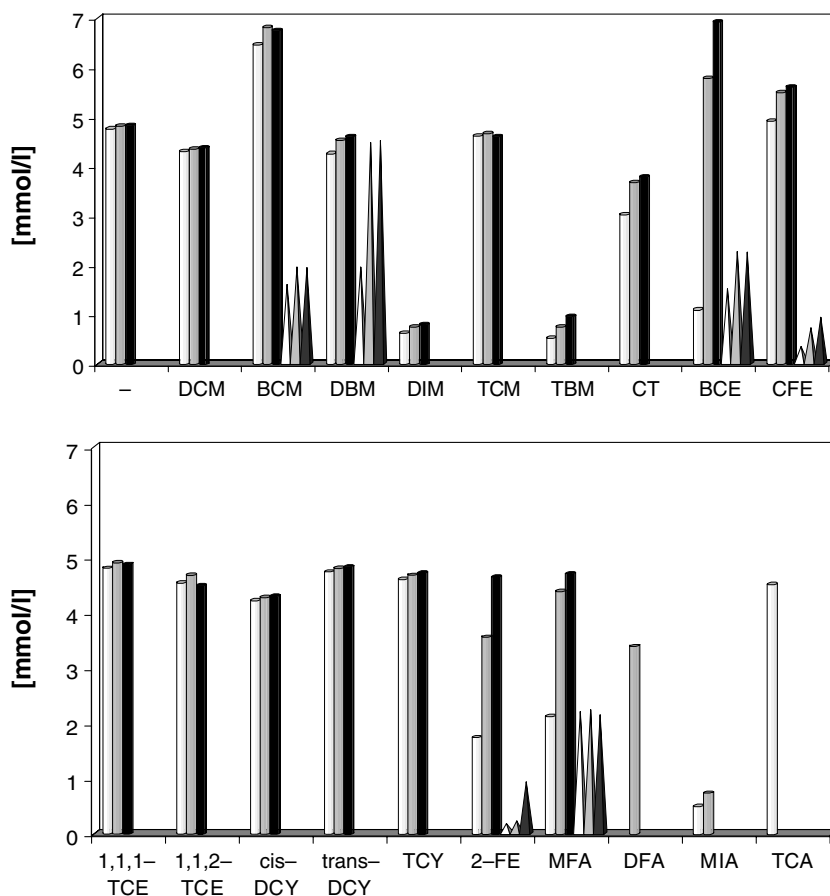
Figure 4. Chloride, bromide and MFA released during growth of *X. autotrophicus* GJ10 on 2.5 mM DCE in the presence of other C1 or C2 halogenated compounds. All compounds were used at 2.5 mM. Chloride (rectangles), bromide (triangles at BCM, DBM and BCE profiles), and MFA (triangles at CFE, 2-FE and MFA profiles) were determined after 36 h (white bars), 60 h (gray) and 84 h (black). One analysis was carried out in case of DFA and TCA and two analyses in case of MIA.

the growth and dehalogenation activity of strain GJ10. The dihalomethanes DBM and BCM enhanced the growth yield, whereas other compounds such as CF were toxic. To test the effects of such compounds on cells of strain GJ10 growing in continuous culture on DCE, pulse experiments were performed. Pulses of 20 mM DCM and DBM and 10 mM EDB were added to the CSTB and the response of the culture was followed. The compound EDB was chosen instead of DIM, since the latter was virtually insoluble in the aqueous phase. According to van der Ploeg et al. (1996), EDB was also highly toxic to strain GJ10.

The profiles of biomass, chlorides, DCE and DCM after addition of a 20 mM pulse of DCM are presented in Figure 8a. In agreement with the data obtained in batch tests, no effect of DCM on

the degradation of DCE by GJ10 was found. DCM was not degraded and it was only physically removed from the bioreactor. It was estimated through integration of the profile of the DCM concentrations in the reactor outlet that 34% of the compound was washed out with the medium; the remaining 56% is attributed to removal *via* the gas phase.

The removal of a pulse of 20 mM DBM (Figure 8b) was accomplished *via* physical removal and biological activity. The bromide and DBM profiles revealed that about 38% of the DBM added was degraded and 25% left the bioreactor dissolved in the medium. The removal *via* the gas phase stands for the remaining 37%, as calculated from a mass balance. The pulse exerted a mildly toxic effect on the cells. Immediately after DBM
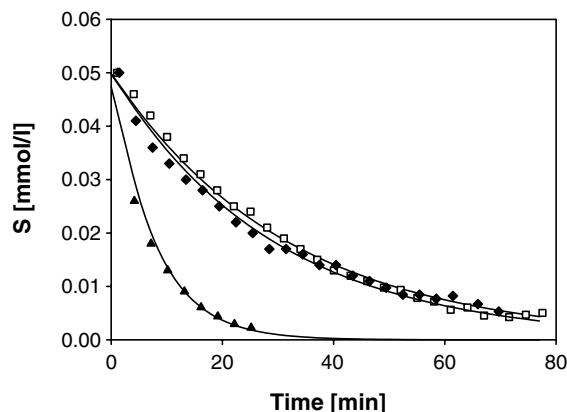
Figure 5. Substrate depletion curves obtained during incubations of cells of strain GJ10 on DCE, BCM and DBM under non-growth conditions. Each substrate was added separately to freshly prepared cells. *S* is the total amount of substrate in the incubation vessel (in mmol) per one unit volume of liquid medium (l). Symbols: ▲ DCE; □ BCM; ♦ DBM; continuous lines are the results of fitting of the model equation (see in the text).
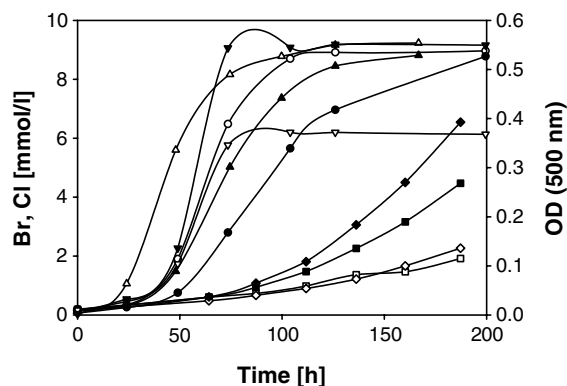

Figure 6. Halide release and growth of *X. autotrophicus* GJ10 on BCE, BCM and DBM and effect of a cosubstrate. Cultures contained 5 mM of halogenated compound with or without ethanol or methanol. Symbols: BCE culture (∇ OD, sum of halide concentrations); BCM culture (◇ OD, ♦ sum of halide concentrations); DBM culture (□ OD, ■ [Br⁻]); culture with DBM+5 mM ethanol (△ OD, ▲ [Br⁻]); culture with DBM+15 mM methanol (○ OD, ● [Br⁻]).

was added to the bioreactor a decrease in biomass optical density occurred. After the OD had decreased to 76% of its initial value, the cells started to restore and the culture density increased, even though DBM was still present.

The effect of a toxic substrate on the continuous culture was investigated by the addition of 10 mM EDB (Figure 8c). It was estimated that
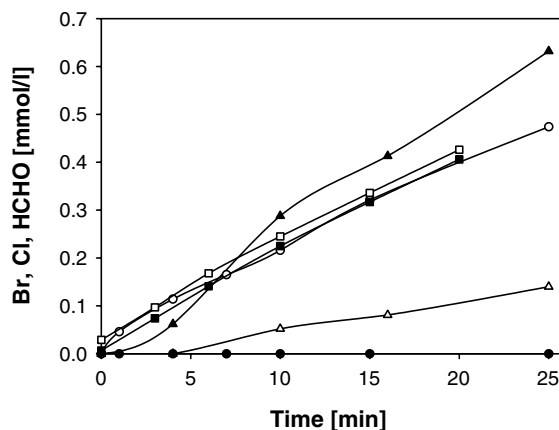

Figure 7. Transformation of BCM, BCE, CFE and DCE by purified haloalkane dehalogenase. All substrates were used at 5 mM. Symbols: BCM (□ total free halide concentration divided by 2, ■ formaldehyde formed); BCE (○ bromide released, ● chloride); CFE (△ chloride released); DCE (▲ chloride released).

Table 2. Dehalogenation of various C1 and C2 haloalkanes by purified haloalkane dehalogenase

Substrate	Activity (%)
1,2-Dichloroethane	100
1,2-Dibromoethane	78
1-Bromo-2-chloroethane	76
1-Chloro-2-fluoroethane	25
Bromochloromethane	99
Dibromomethane	71

Dehalogenation rates are expressed as the percentage of the activity found with 1,2-dichloroethane (4.9 μmol min^{-1} mg^{-1}). Values concern substrate turnover.

31% of the substrate was converted into monobromoacetate while 62% was washed-out with the medium. The toxic effects from the substrate or degradation products resulted in complete decay of the cells. Immediately after EDB had been added the utilization of DCE was stopped and its concentration increased. The system did not recover within 150 h.

Discussion

Growth tests carried out with *X. autotrophicus* strain GJ10 revealed that the presence of a range of halogenated methanes and ethanes did not stop the biodegradation of DCE or its use as a carbon

source. The majority of the C1 and C2 halocompounds, including fluorinated ones, which were not utilized as a carbon source by strain GJ10
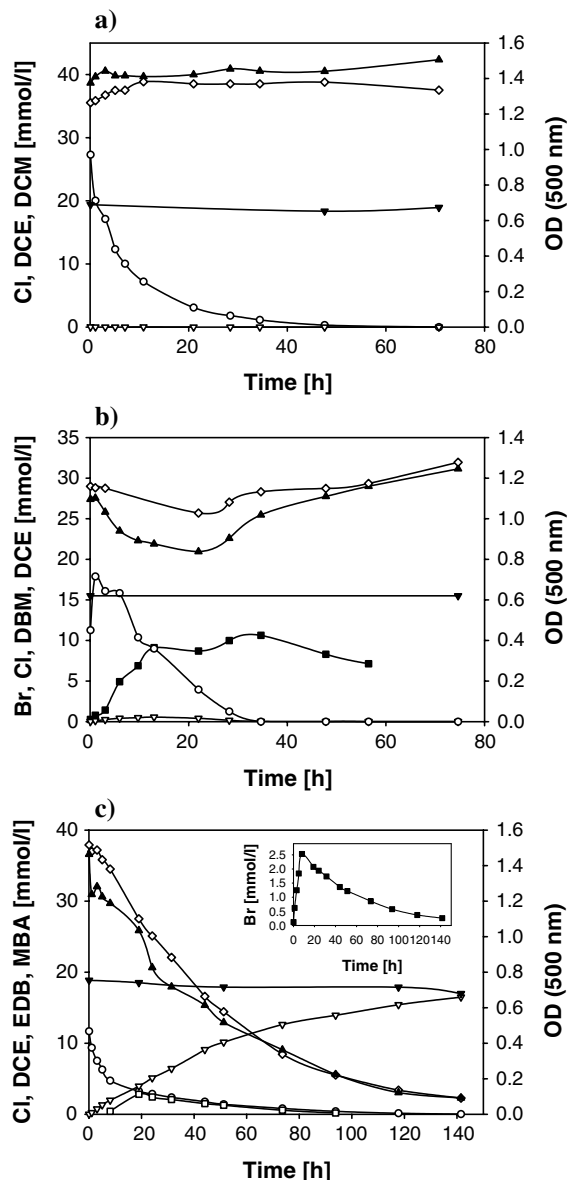

Figure 8. Effect of pulses of DCM (a), DBM (b), and EDB (c) on the biodegradation of DCE by strain GJ10 in a continuous culture. The CSTB was operated under chemostat conditions as described in Materials and methods. A pulse of the halocarbon indicated (20 mM for DCM and DBM, and 10 mM for EDB) was added directly to the reactor at $t = 0$ and substrate, biomass, and products were followed in time. Concentrations: DCE inlet; ▽ DCE outlet; ○ DCM, DBM or EDB outlet, respectively; ◇ chloride outlet; ■ bromide outlet; □ MBA outlet; ▲ biomass outlet.

caused only a mild decrease of the conversion rate of DCE and of the biomass concentration that was reached.

The most toxic compounds were DIM, TBM, CT, and MIA, which caused strong growth inhibition. Although the mechanism of toxicity of these compounds has not been established, it is likely that the iodinated compounds acted as selective or aselective alkylating agents, whereas CT may disrupt membrane integrity. We do not assume that specific (competitive) inhibition of haloalkane dehalogenase activity plays an important role, since dehalogenase assays carried out here and those done in previous studies (Keuning et al. 1985; Schanstra et al. 1996) have always failed to yield a simple haloalkane that acts as a strong competitive inhibitor or irreversible inhibitor. Most substrate analogues have very low affinities for the enzyme active site, and the only known haloalkane inhibitor is 1,1,2-trichloroethane, which has a K_i of around 27 mM. Even in the absence of a mechanistic explanation, it is worth to notice that toxic effects of 2-haloacetates and dihalomethanes followed the order: chlorinated < brominated < iodinated. The brominated 1,2-dihaloethanes and trihalomethanes also inhibited the growth to much stronger degree than their chlorinated analogues, which can be explained for the bromoethanes by the very high toxicity of the intermediate 2-bromoacetaldehyde (Van der Ploeg et al. 1996). It remains to be determined whether the differences in toxicity are mainly related to the variations in the rate of formation and conversion of toxic intermediates or if they are due to differences in the reactivity of such intermediates toward cellular components.

The resistance of strain GJ10 to 2.5 mM MFA, which is a strong inhibitor of energy production in the tricarboxylic acid (Krebs) cycle, is an interesting feature. A possible explanation for this phenomenon lies in the similarity of toxic effects caused by MCA and MFA. It was suggested by Hayes et al. (1973) that MCA acts in a similar way as MFA, i.e. interfering with central metabolism and energy generation by blocking the tricarboxylic acid cycle. Since MCA is an intermediate of DCE metabolism by strain GJ10, the organism may well have evolved a resistance mechanism to it, which may also provide enhanced tolerance to MFA. Since the presence of MFA did not inhibit growth on acetate, the resistance to MFA was

probably not due to blocking of acetate kinase or phosphotransacetylase, a phenomenon observed in some mutants of other strains capable of growth in the presence of MFA (McKenney & Melton 1986).

Five compounds (CFE, 2-FE, BCE, BCM and DBM) were found to be efficiently converted by strain GJ10. The cometabolic conversion of CFE by haloalkane dehalogenase (Figure 7) and the transformation of 2-FE by whole cells found in the growth test (Figure 4) support the following metabolic pathway for CFE (compare with Figure 1): 1-chloro-2-fluoroethane → 2-fluoroethanol → 2-fluoroacetaldehyde → 2-fluoroacetate. Conversion of CFE to MFA was also described to occur in rats *via* a pathway that could involve cytochrome P450 (Keller et al. 1996).

Three haloalkanes (BCM, DBM and BCE) can now be added to the spectrum of C1–C2 halogenated compounds serving as a sole carbon and energy source for strain GJ10. The degradation of BCE by haloalkane dehalogenase led to the formation of 2-chloroethanol, which was probably further converted as in the DCE metabolic pathway (Figure 1). The formation of 2-chloroethanol instead of 2-bromoethanol is in agreement with the kinetic preference of haloalkane dehalogenase for carbon-bromine bonds as compared to carbon chlorine bonds (Schanstra et al. 1996). The mineralization of BCE by the cells was slower than the degradation of DCE and resulted in a lower yield of biomass. The observation supports the conclusion on elevated toxicity of brominated analogues, and may be related to minor amounts of 2-bromoethanol being formed.

A compound that is rapidly converted but very toxic is EDB. When added to the bioreactor, EDB was partially dehalogenated by the cells. Unlike formaldehyde, the intermediate formed after dehalogenation of EDB could not be further converted and resulted in cell wash-out. According to van der Ploeg et al. (1996) the toxic intermediate was 2-bromoacetaldehyde. The observed accumulation of monobromoacetate was thus likely to be caused by oxidation of accumulated 2-bromoacetaldehyde.

The biodegradation of BCM and DBM proceeded *via* formaldehyde. The same metabolite was found in a group of methylotrophic bacteria utilizing DCM, BCM, DBM and DIM (Leisinger & Braus-Stromeyer 1995). These methylotrophs degraded dihalomethanes by a glutathione-dependent DCM dehalogenase. The degradation proceeded through nucleophilic displacement of halide by the thiol group of glutathione followed by a reaction with a water molecule. In contrast, the dehalogenation of BCM and DBM by haloalkane dehalogenase is a cofactor-independent reaction. According to the mechanism of DCE conversion, the dehalogenation of BCM and DBM would result in chloromethanol and bromomethanol, respectively. As such molecules are highly unstable, complete conversion to formaldehyde probably takes place in the enzyme active site, although it cannot be excluded that the halomethanol itself is released from the active site and contributes to toxicity. Possibly, the formaldehyde that is generated from dihalomethanes is degraded by the H_4MPT-dependent formaldehyde oxidation system found in autotrophic *Xanthobacter* strains (Vorholt et al. 1999).

Since haloalkane dehalogenase is a cytoplasmic enzyme, whereas the methanol dehydrogenase that is involved in growth on methanol is located in periplasm (Janssen et al. 1987), this creates the unusual situation that formaldehyde is generated directly in the cytoplasm when cells are exposed to dihalomethanes that are a substrate for haloalkane dehalogenase. It is likely that the cytoplasmic production of formaldehyde, a compound toxic for all organisms due to its non-specific reactivity with proteins and nucleic acids, is the main reason for the low rate of growth with and utilization of dihalomethanes. Also the transient toxic effects that were observed after a DBM pulse was added to the CSTB could be ascribed to excessive formaldehyde production following dehalogenation of DBM. Such an effect was not observed after the pulse of DCM, as the compound was not rapidly converted by haloalkane dehalogenase.

The rate of halomethanes degradation was significantly increased when methanol or ethanol were used as a cosubstrate. These non-halogenated compounds were preferably utilized as a growth substrate by strain GJ10 and degradation of DBM and BCM was facilitated due to higher biomass concentration. Even though the rate of growth of strain GJ10 on dihalomethanes was not very high, the hydrolytic pathway involving haloalkane dehalogenase in the first step provides a new route for bacterial dihalomethane utilisation.

Acknowledgements

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