Biodegradation **6:** 229–235, 1995.

Dichloromethane as the sole carbon source for *Hyphomicrobium* sp. strain DM2 under denitrification conditions

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Key words: denitrification, *Hyphomicrobium*, dichloromethane, dichloromethane dehalogenase, methylotrophic bacteria

Abstract

Hyphomicrobium sp. strain DM2 was found to grow anaerobically in the presence of nitrate with methanol, formaldehyde, formate or dichloromethane. The estimated growth rate constants with methanol and dichloromethane under denitrification conditions were 0.04 h^{-1} and 0.015 h^{-1}, respectively, which is twofold and fourfold lower than the rates of aerobic growth with these substrates. Slight accumulation of nitrite was observed in all cultures grown anaerobically with nitrate. Dichloromethane dehalogenase, the key enzyme in the utilization of this carbon source, was induced under denitrification conditions to the same specific activity level as under aerobic conditions. In a fed batch culture under denitrification conditions *Hyphomicrobium* sp. DM2 cumulatively degraded 35 mM dichloromethane within 24 days. This corresponds to a volumetric degradation rate of 5 mg dichloromethane/l·h and demonstrates that denitrificative degradation offers an attractive possibility for the development of anaerobic treatment systems to remove dichloromethane from contaminated groundwater.

Introduction

Dichloromethane (DCM) is a widely used industrial solvent, recognized to be an environmental pollutant with adverse effects on man. It serves as a growth substrate for aerobic, facultatively methylotrophic bacteria that are readily enriched from soil and groundwater contaminated with the compound (Leisinger et al. 1994). Enrichments from municipal wastewater and from activated sewage sludge have also been successful (LaPat-Polasko et al. 1984; Klecka 1982). All DCM-utilizing facultative methylotrophs studied so far possess DCM dehalogenase. This enzyme catalyzes a glutathione-dependent reaction by which DCM is converted to formaldehyde and inorganic chloride (Leisinger et al. 1994).

In contrast to the information available on biochemistry (Kohler-Staub & Leisinger 1985), genetics (La Roche & Leisinger 1990) and application (Gälli & Leisinger 1985; Stucki 1990) of aerobic DCM degradation, little is known about the utilization of the compound as sole carbon and energy source under anaerobic conditions. Fermentative utilization has recently been observed with strictly anaerobic mixed cultures that either yield acetate (Stromeyer et al. 1991) or methane and carbon dioxide (Freedman & Gossett 1991) as final products. Anaerobic metabolism of DCM by pure cultures of sulfate-reducing or denitrifying bacteria has not been reported so far.

Among anaerobic biotreatment processes, denitrification is most advantageous with respect to substrate turnover, sensitivity to trace amounts of oxygen and overall flexibility. Microbial degradation of pollutants under denitrifying conditions is therefore expected to become increasingly attractive in the development of in situ bioremediation technologies (Sanford & Tiedje 1992). Examples of halogenated aromatics which have been shown to be degraded under denitrifying conditions include p-fluorobenzoate and o-fluorobenzoate (Taylor et al. 1979; Schennen et al. 1985) as well as 2,4-dichlorophenoxyacetic acid and pentachlorophenol (Sanford & Tiedje 1992). Degradation of halogenated aliphatic compounds, under denitrification conditions has been reported for DCM, tetrachloroethene

(Meyer et al. 1992) and tetrachloromethane (Bouwer & McCarty 1983). Most of these studies were conducted with mixed cultures or soil columns, and degradation of the xenobiotics studied was incomplete. In the present contribution we report on the complete degradation of DCM by a pure culture of a denitrifying methylotrophic bacterium.

Materials and methods

Organism, media and growth conditions

Hyphomicrobium sp. strain DM2 (Stucki et al. 1981) is deposited in the American Type Culture Collection as ATCC 43129. The minimal medium used to grow this organism contained in g per liter of distilled water: KH_2PO_4, 2.04; K_2HPO_4, 4.35; $(NH_4)_2SO_4$, 0.4; $MgSO_47H_2O$, 0.024; $Ca(NO_3)_2$, 0.0025 plus 1 ml of the trace element solution described by Scholtz et al. (1988). The final pH was 7.3.

Aerobic cultures were grown in screw-cap Erlenmeyer flasks closed with Mininert valves (Precision Sampling, Baton Rouge, LA). The volume of air above the culture was fourfold larger than the culture volume. After inoculation, undiluted DCM or methanol was added to a maximum concentration of 8 mM DCM or 30 mM methanol, respectively. For denitrifying cultures the minimal medium was supplemented with 10 or 30 mM $NaNO_3$, boiled for 10 min while being vigorously purged with N_2/CO_2 (80% : 20%) and dispensed to 80% of their void volume in serum flasks that had been flushed with N_2/CO_2. Upon sealing the flasks with Viton septa (Maag Technic AG, Dübendorf, Switzerland), an N_2/CO_2 atmosphere was established and the pressure adjusted to 1 bar. After autoclaving, the sterile substrates and the inoculum were injected by a syringe through the septum, and the bottles were incubated on a shaking platform at 30° C.

Large quantities of aerobic or nitrate respiring cells were grown in 1000 or 800 ml of medium respectively with 20 mM methanol and 24 mM (aerobically) or 15 mM (nitrate respiring) DCM. DCM was added in three portions to the medium, each after the pH in the growing culture had been adjusted with 1 mM NaOH to 7.2. Cells were harvested aerobically, washed in 20 mM phosphate pH 7.0 and stored frozen at - 20° C until use.

Analytical methods

DCM in the head space and methanol in the aqueous phase were measured by gas chromatography with a flame ionization detector. A 180 cm stainless steel Porapak P column was used at 120° C (150° C for methanol) and at a flow rate of 30 ml N_2/min. N_2O was determined by gas chromatography with a thermal conductivity detector after separation on a Porapak Q glass column at 30° C and a helium flow of 20 ml/min.

Chloride and nitrate were measured by suppressor-mediated ion chromatography (Dionex Corp., Sunnyvale, CA) and conductivity detection. An Ion Pac AS10 column (Dionex) was used for chloride determination, and a AS9 column (Dionex) for the determination of nitrate.

Nitrite was assayed colorimetrically by the method of Drews (1983), protein in whole cells by a Lowry-type method (Kennedy & Fewson 1968) and protein in cell extracts according to Bradford (1976).

Dichloromethane dehalogenase assay

The preparation of cell-free extracts and the assay of DCM dehalogenase activity, which is based on measuring the rate of formaldehyde formation, have been described previously (Leisinger & Kohler-Staub 1990).

Chemicals

Pure gases and gas mixtures were obtained from Carbagas, Rümlang, Switzerland. Chemicals were of analytical grade and were purchased from Fluka, Buchs, Switzerland.

Results and discussion

Growth of Hyphomicrobium *sp. strain DM2 under denitrification conditions*

Bacteria belonging to the genus *Hyphomicrobium* have long since been known to grow on one-carbon compounds such as methanol (Sperl & Hoare 1971; Attwood & Harder 1972) or methylamine (Harder & Attwood 1978) with nitrate as the terminal electron acceptor. Representatives of this genus are also known to utilize DCM as the sole carbon source under aerobic conditions (Leisinger et al. 1994). The combination of these two metabolic features in one strain thus

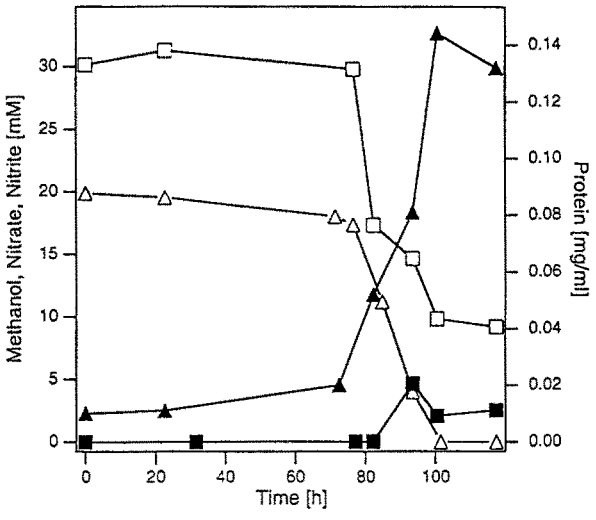

Fig. 1. Growth of *Hyphomicrobium* sp. DM2 with 20 mM methanol under denitrification conditions. The culture was inoculated with 1% (v/v) of a culture grown under the same conditions. (△) Methanol, (□) nitrate, (■) nitrite, (▲) protein.

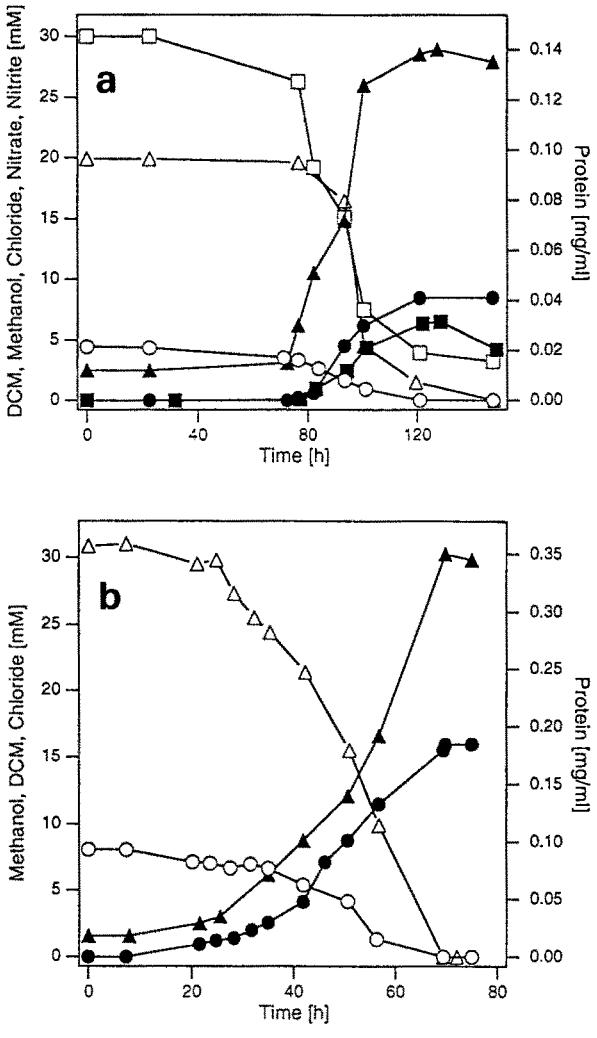

Fig. 2. Growth of *Hyphomicrobium* sp. DM2 with methanol plus dichloromethane a) under denitrification conditions (20 mM methanol plus 4.5 mM DCM), inoculated with 5% (v/v) of an aerobic culture grown with methanol, b) under aerobic conditions (30 mM methanol plus 8 mM DCM), inoculated with 2% (v/v) of an aerobic culture grown on methanol. (△) Methanol, (○) DCM, (●) chloride, (□) nitrate, (■) nitrite, (▲) protein.

should enable growth on DCM under denitrification conditions. We have tested the previously characterized *Hyphomicrobium* sp. DM2 for its ability to anaerobically grow on 20 mM methanol, 10 mM formaldehyde (released from 1.67 mM hexamethylenetetramine) or 20 mM formate in the presence of nitrate. Growth was observed with all three substrates. Figure 1 shows batch growth on methanol. After a lag phase of 3 days strain DM2 consumed 20 mM methanol, and a concomitant decrease of nitrate in the medium was observed. Growth under denitrification conditions on the same substrates was also observed with *Hyphomicrobium* sp. strain GE7, an organism newly isolated from soil according to the protocol described by Sperl & Hoare (1971) (D. Kohler-Staub unpublished).

First attempts to cultivate strain DM2 under denitrification conditions with DCM as the sole carbon source were not successful. The organism however grew and consumed DCM in a medium containing both 20 mM methanol and 4.5 mM DCM. Figure 2a shows that both substrates were utilized simultaneously. The same holds true for growth on a mixture of 30 mM methanol and 8 mM DCM under aerobic conditions (Fig. 2b). DCM consumption in media with mixed substrates was not delayed, even when the cultures were inoculated with methanol-grown cells.

Cultivation of strain DM2 on 5 mM DCM as the sole carbon source under denitrification conditions was achieved by increasing the amount of inoculum from 1% to 5% (v/v). Anaerobically or aerobically grown methanol cultures served equally well as inoculum. The inoculum (5%) was transferred to deoxygenated medium to give a concentration of approximately 10^7 colony forming units per ml. As shown in Fig. 3, after a lag phase of 80 h chloride and biomass were produced and nitrate and DCM were consumed at corresponding rates. In all cultures containing DCM, chloride was produced in stoichiometric amounts. Attempts to grow the DCM-utilizing methylotrophs *Methylobacterium*

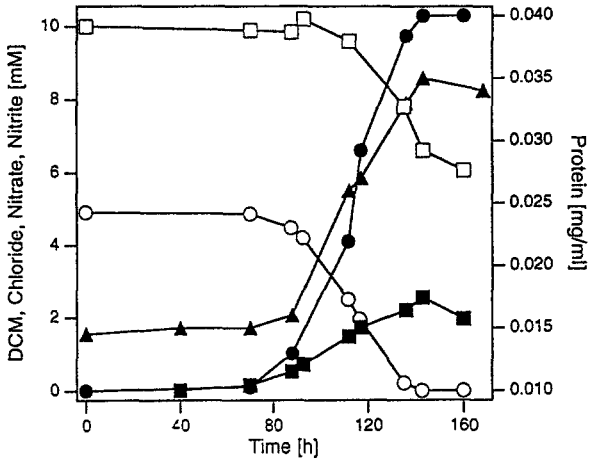

Fig. 3. Growth of *Hyphomicrobium* sp. DM2 with 5 mM DCM under denitrification conditions. Inoculation was with 5% (v/v) of an aerobically grown methanol culture. (○) DCM, (●) chloride, (□) nitrate, (■) nitrite, (▲) protein.

sp. DM4 (Gälli & Leisinger 1988) and *Methylophilus* sp. DM11 (Scholtz et al. 1988) with DCM under denitrification conditions were unsuccessful, and *Hyphomicrobium* sp. GE7 was unable to grow with DCM, both aerobically and anaerobically with nitrate.

Denitrifying batch growth of strain DM2 was always preceded by an extended lag phase of about 80 h. The nature of the inoculum or the substrate concentration had little effect on the length of this period. We presume that this lag phase is due to traces of oxygen which are either introduced by inoculation with aerobically grown precultures or are still present in the medium. The sensitivity of denitrifying bacteria towards oxygen varies, and oxygen controls denitrification by interfering with the induction as well as with the activity of denitrifying enzymes (Tiedje 1988). In the present case, the traces of oxygen in freshly inoculated medium did not support aerobic growth, but may have prevented denitrifying growth and thereby extended the lag phase.

Intermediates of nitrate reduction

To confirm the coupling of DCM degradation to denitrification, inhibition studies were performed. 20 ml of gaseous acetylene, an inhibitor of nitrous oxide reductase (Balderston et al. 1976), was added at the time of inoculation to the oxygen-free gas phase (20 ml) of cultures growing on 5 mM DCM with nitrate as the terminal electron acceptor. Concomitantly with growth, increasing amounts of N_2O accumulated in the gas phase and finally represented approximately 1% (v/v) of the gas phase in the cultures. No N_2O was detected in the gas phase of control cultures that lacked acetylene (data not shown). We conclude from these observations that dissimilative nitrate reduction in *Hyphomicrobium* sp. DM2 proceeds to nitrogen gas as the final product.

In most batch cultures nitrate reduction was accompanied by the accumulation of nitrite. The final concentration of nitrite in the cultures varied between 0.1 and 7.0 mM, with no conceivable correlation to the growth conditions employed. The effect of nitrite on anaerobic growth with nitrate of strain DM2 was evaluated with medium containing 20 mM methanol, 20 mM nitrate plus nitrite at concentrations between 0.1 and 10 mM. The presence of 5 mM nitrite completely inhibited growth, while nitrite concentrations between 0.1 and 4 mM permitted growth at increasingly lower rates.

The slight accumulation of nitrite we observed in cultures of *Hyphomicrobium* sp. DM2 indicates that, under the experimental conditions employed, nitrite reduction occurred at a lower rate than nitrate reduction. This is in contrast to the findings of Timmermans & Van Haute (1983) who observed that nitrate to nitrite reduction was the rate-limiting step in denitrification with methanol by a *Hyphomicrobium* sp.

Specific growth rates and yield coefficients under denitrification conditions

The characteristics of growth with methanol and DCM under aerobic and denitrification conditions are summarized in Table 1. The maximum growth rate constant observed for aerobic growth of *Hyphomicrobium* sp. strain DM2 was close to the value of 0.1 measured by Stucki et al. (1981). The growth rate constant with DCM of 0.07 h^{-1} was identical to the value determined earlier (Stucki et al. 1981). The constant for growth with methanol under denitrification conditions was 0.039 h^{-1}. This is half the rate observed for aerobic growth and corresponds well with the value of 0.035 h^{-1} found by Timmermans & Van Haute (1983) for another *Hyphomicrobium* strain grown under denitrification conditions with methanol. The growth rate with DCM as a substrate under denitrification conditions was four times lower than that observed under aerobic conditions.

The yields of protein per mol of carbon utilized on aerobic growth were in the same range as the values

Table 1. Specific growth rates, yield coefficients and substrate/nitrate ratio of *Hyphomicrobium* sp. DM2 cultures.

Characteristic	Growth conditions			
	Methanol		Dichloromethane	
	Aerobic	Denitrification[1]	Aerobic	Denitrification[1]
$\mu(h^{-1})$	0.08	0.04 ± 0.006	0.07	0.015 ± 0.002
$Y_{P/C}$ (g protein/mol C)	7.9	6.2 ± 1.1	6.6	4.2 ± 0.6
$R_{C/N}$ (mol C/mol NO_3^-)	–	0.85 ± 0.19	–	2.17 ± 0.37

[1] Values for growth under denitrification conditions are means ± standard deviation calculated from three independent experiments.

Table 2. Dichloromethane dehalogenase specific activity in crude extracts of *Hyphomicrobium* sp. DM2 grown under different conditions.

Growth conditions	Dichloromethane dehalogenase specific activity (mkat/kg protein)
Dichloromethane, O_2	5.2
Dichloromethane, NO_3^-	5.85
Methanol, O_2	0.07
Methanol, NO_3^-	0.14

determined in earlier experiments (Gälli 1986; Stucki 1982) and in accordance with yields observed for other aerobic bacteria (Atkinson & Mavituna 1983). Protein yields under denitrification conditions were significantly lower, and this is consistent with the smaller amount of energy released in nitrate respiration as compared to aerobic respiration.

Nitrate respiring cultures can also be characterized by the substrate/nitrate ratio which we define here as mol substrate utilized per mol nitrate reduced (compare Timmermans & Van Haute 1983). As shown in Table 1, this value amounted for growth with methanol to 0.85 which is comparable to the theoretical value of 1.09 determined by Nurse (1980). When estimating the substrate/nitrate ratio for denitrificative growth with DCM, it has to be considered that DCM is hydrolyzed by DCM dehalogenase to formaldehyde which then serves as the actual substrate for growth. The differences in the oxidation states of formaldehyde (0) and methanol (- 2) leads to a 33% reduction in the amount of nitrate required for the anaerobic oxidation of DCM and to a correspondingly higher substrate/nitrate ratio. This was indeed observed (Table 1), but the increase in the ratio on DCM was higher than expected on theoretical grounds.

Induction of dichloromethane dehalogenase

DCM dehalogenase, the key enzyme of DCM degradation, has been shown to be strongly induced in strain DM2 grown aerobically on DCM (Kohler-Staub et al. 1986). To exclude the possibility that DCM dehalogenase specific activity was growth-limiting in denitrifying cells, we have verified its presence and its inducibility under denitrification conditions. As shown in Table 2, similar levels of DCM dehalogenase and similar induction ratios were observed in aerobically grown cells and in cells grown anaerobically with nitrate. Electrophoretic separation of the proteins in crude extracts and immunoblotting using DCM dehalogenase-specific antiserum (Kohler-Staub et al. 1986), confirmed the presence of the same dehalogenase protein in cells grown under the four conditions listed in Table 1 (data not shown). We conclude that the extremely slow growth under denitrification conditions with DCM is not due to insufficient levels of dehalogenase.

Dichloromethane degradation in a denitrifying fed batch culture

The experiment shown in Fig. 4 demonstrates that DCM degradation was maintained in a fed batch culture for 27 days. 400 ml medium containing 6.5 mM DCM and 30 mM nitrate were inoculated. When DCM had disappeared, and thereafter in intervals of 3 to 4 days, the compound was added to bring the concentration in the culture to 4 mM, and the pH of the medium was adjusted to 6.8 by addition of 5 mM NaOH. At day 19 two mmol of nitrate were added to the culture. DCM

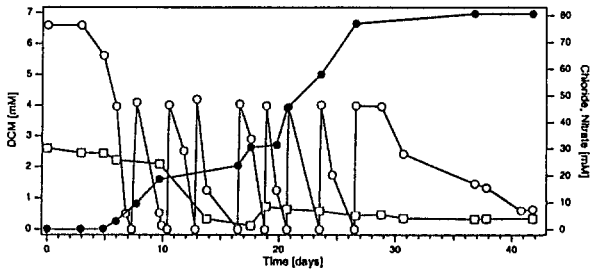

Fig. 4. DCM degradation by *Hyphomicrobium* sp. DM2 under denitrification conditions in fed batch culture. The culture was inoculated with 5% of an aerobic culture grown with methanol and in intervals the concentration of DCM was brought to 4 mM. At day 19 2 mmol nitrate were added to the 400 ml of culture volume. (o) DCM, (•) chloride, (□) nitrate.

degradation and stoichiometric release of chloride proceeded until day 27 when a total of 34.6 mM DCM had been degraded. At this point the cells started to clump and DCM degradation came to a halt. Nitrate consumption was primarily observed in the first part and severely slowed down in the second part of the incubation period. This, together with the low overall protein yield of the fed batch culture (2.2 g protein/mol carbon), indicates that DCM dehalogenation was divorced from growth towards the end of the culture.

The volumetric rate of DCM degradation in the fed batch experiment amounted to 5 mg DCM/h·l, a value similar to the degradation rate of 3.7 mg DCM/h·l observed for the fermentative elimination of DCM by an anaerobic mixed culture (Stromeyer et al. 1991). The rates of these anaerobic systems are about 300 times below the rate of the aerobic fluidized bed system described by Gälli & Leisinger (1985). The anaerobic systems, however, have the advantage that they can be operated with minimal input of mechanical energy in simple fixed bed systems which avoid volatilization of DCM. Fermentative degradation of DCM occurs in mixed cultures by as yet unknown mechanisms. In contrast, the system presented here employs a previously characterized pure culture whose DCM degradation pathway is understood. DCM degradation by *Hyphomicrobium* sp. DM2 under denitrification conditions thus offers an attractive alternative for the development of anaerobic treatment systems to remove DCM from contaminated groundwater.

Acknowledgement

This study was supported by a grant from the Swiss Federal Institute of Technology, Zürich.

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