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Accumulation of poly-β-hydroxybutyrate in a methane-enriched, halogenated hydrocarbon-degrading soil column: implications for microbial community structure and nutritional status

Peter D. Nichols[1] & D. C. White[2]
[1] *CSIRO Division of Ocanography, GPO Box 1538, Hobart, Tasmania 7001, Australia;* [2] *Institute for Applied Microbiology, University of Tennessee, 10515 Research Drive, Bldg. 1, Knoxville, Tennessee 37932-2567, USA*

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Abstract

The prokarotic, endogenous storage polymer poly-β-hydroxybutyrate (PHB) accumulated in soil from a methane-enriched, halogenated hydrocarbon-degrading soil column. Based on phospholipid ester-linked fatty acid (PLFA) profiles, this mocrocosm has been previously reported to be significantly enhanced in type II methanotrophs. Two strains analysed of the type II methanotroph *Methylobacterium organophilum* were found to contain PHB, with PHB/PLFA ratios similar to those determined for the methane-enriched soil column, suggesting that methanotrophic bacteria enriched in the methane-amended column produced PHB. Control soil and sodium azide-inhibited material, in which methanotroph markers were below detection, did not contain PHB. Biochemical assays, based on the differences observed, can be used to monitor shifts in microbial biomass, community structure and nutritional status of systems used to model microbial biotransformation processes. Further manipulative experiments with pure methanotrophic bacteria will increase our understanding of the mechanism by which PHB is produced. This study illustrates, however, that biochemical procedures have the potential to monitor the stimulated populations of a native soil microbial community capable of degrading pollutants. Such data may ultimately provide information to assist in the selection and optimization of favorable conditions for the adaption of pollutant biotransformation processes to aquifers.

Introduction

Groundwater contains a wide range of organic substances that are of a natural or anthropogenic origin. Many of the pollutants are resistant to microbial degradation in soil and may thus reach groundwater supplies that are used for drinking purposes (Bitton & Gerba, 1984). Carcinogens such as chloroform, carbon tetrachloride, and trichloroethylene (TCE) have, for example, been detected in well water from various regions of the United States (Bitton & Gerba, 1984). The increasing problem of groundwater contamination has prompted research into the biotransformation of contaminants (Bouwer & McCarty, 1983a & b, 1985; Castro & Belser, 1968; Parsons

et al., 1984; Wilson & Wilson, 1985) as an alternative to physical removal procedures (Love & Eilers, 1982; USEPA, 1984).

A number of model systems have been developed to study the biotransformation of pollutant compound. McCarty and coworkers (Bouwer & McCarty, 1983a & b, 1985) have shown that TCE and other substances can be degraded under anaerobic methanogenic conditions. These processes result in the formation of intermediates such as vinyl chloride, which is itself a known human carcinogen. An alternate approach by Wilson and colleagues has employed aerobic processes. Aerobic degradation of TCE and other short-chain chlorinated hydrocarbons has been achieved for a soil column exposed to a mixture of natural gas (0.6 per cent) in air (Wilson *et al.*, 1983; Wilson & Wilson, 1985). Similarly, Fogel *et al.*, (1986) recently reported biodegradation of chlorinated ethenes by a methane-utilizing mixed culture. The next steps will be to categorise the biochemical processes and subsequently to adapt them to aquifers rather than artificial laboratory systems.

In previous studies we have used phospholipid ester-linked fatty acid (PLFA) profiles to assess the microbial biomass and community structure of methane-enriched, halogenated hydrocarbon-degrading (HHD) aerobic soil column (Nichols & White, 1985; Nichols *et al.*, 1987). Relative to control samples and untreated soil, the HHD column showed a significant increase in PLFA markers specific to type II methanotrophic bacteria (Makula, 1978; Nichols *et al.*, 1985). The same column also exhibited the highest biomass.

PLFA profiles can provide information on the microbial biomass and community structure; however, additional analyses are needed to better understand the nutritional status of the sedimentary microbiota. Previous studies have indicated that accumulation of the uniquely prokaryotic endogenous storage polymer poly-β-hydroxybutyrate (PHB) can serve as a measure of unbalanced growth conditions in a range of environments (Herron *et al.*, 1978; Nickels *et al.*, 1979; Findlay & White, 1983); unbalanced growth occurs when a suitable carbon source is present, but one or more essential nutrients are absent (Dawes & Senior, 1973; Nickels *et al.*, 1979). For example, it has been determined that unbalanced growth induced to a small extent by high salinity or incomplete nutrient supplementation, or more extensively by dark upland runoff water, results in rapid accumulation of PHB with little change in PLFA lipid phosphate (Nickels *et al.*, 1979). The result is high PHB/PLFA ratios in the detrital microbiota.

In this study; the PHB content of (a) soil from an HHD column and related controls samples, and (b) methylotrophic bacteria were assayed. The PHB/PLFA ratios were determined to yield further insight into the microbial biota and processes involved in the biotransformation of pollutant compounds.

Materials and methods

Sample preparation

Lyophilized cells of *Methylococcus capsulatus* Bath and *Methylobacterium organophilum* (Strains RG and XX) were obtained from Dr L. Jahnke, NASA-Ames Research Center, Moffett Field, California, and Dr R. S. Hanson, Gray Freshwater Biological Institute, University of Minnesota, Navarre, Minnesota. Culture conditions and isolation of cellular material were as reported elsewhere (Nichols *et al.*, 1985; Jahnke & Nichols, 1986). *Methylococcus capsulatus* was grown in a feed-batch culture with methanol or methane as the sole carbon and energy source (Jahnke & Nichols, 1986). The facultative type II methanotrophs, *Methylobacterium organophilum* strains XX and RG, were grown in AMS basal salts medium (Patt *et al.*, 1974) with an atmosphere of methane and air (1 : 4, v : v) or with methanol (0.1 per cent, w : v) in the absence of methane (Nichols *et al.*, 1985). The methane-enriched HHD soil column samples, the control sodium azide-inhibited soil column samples, and the untreated surface samples were supplied by Drs J. M. Henson and J. T. Wilson, Kerr Environmental Research Laboratory, USEPA, Ada, Oklahoma.

The soil used for column preparation was Lincoln fine surface sand sampled near Ada, Oklahoma. The original vertical profile of the soil was maintained in column preparation. Soil description and column preparation and operating conditions have been described in detail previously (Wilson *et al.*, 1981; Wilson & Wilson, 1985; Henson *et al.*, 1985). Briefly, column A had a headspace containing 0.6 per cent natural gas in air to stimulate bacteria capable of growing using gaseous hydrocarbons. The natural gas was composed of 85 per cent methane, 10 per cent ethane and 3 per cent propane. Column B was inhibited by the addition of 0.1 per cent sodium azide to the water and was not exposed to natural gas. Column A was exposed to natural gas for 3 weeks before an aqueous solution of the halogenated hydrocarbons was applied; a similar solution was also supplied to column B (Henson *et al.*, 1985). After 3 months of operation at 22° to 25 °C, the columns were unpacked, and increments from 0 to 10 cm and 148 to 150 cm were lyophilized prior to lipid extraction.

Cell and soil column lipid extraction by the modified Bligh and Dyer procedure, open column chromatographic fractionation of lipid material into neutral lipids, glycolipids, and phospholipids, and methylation of the phospholipid ester-linked fatty acids (PLFA) were performed according to routine procedures described previously (Guckert *et al.*, 1985; Nichols *et al.*, 1985–1987).

Isolation and derivatization of PHB

As PHB can be quantitatively recovered by a single chloroform-methanol extraction and subsequent column fractionation, PLFA and PHB from environmental samples can be measured simultaneously (Findlay & White, 1986). Thus, PHB was recovered from the acetone fraction rather than by extracting it with boiling chloroform as performed in previous studies (Findlay & White, 1983).

The acetone fraction containing glycolipid and PHB was reduced to dryness under a stream of nitrogen in a teflon-lined, screw-cap test tube. Following the addition of absolute ethanol (1.5 ml) and concentrated HCl (0.5 ml), the test tubes were heated at 100 °C for 4 hours, resulting in cleavage of the PHB polymer and formation of the monomeric ethyl esters. Samples were cooled and partioned with 6 ml distilled water : chloroform (2 : 1, v : v). The chloroform layer containing the β-hydroxy-ethylbutyrate was transferred to a second test tube and the aqueous layer reextracted with 2 ml chloroform. The combined organic layers were dried to approximately one drop under a stream of nitrogen at room temperature. Care was taken to avoid taking the samples to dryness so as to prevent loss of the volatile short-chain ethyl ester. Samples were diluted in a known volume of chloroform for gas chromatographic analysis.

Gas chromatography of β-hydroxy-ethylbuturate

Samples were analyzed with a Hewlett Packard 5880A gas chromatograph (GC) equipped with a flame ionization detector. Samples were injected at 50 °C in the splitless mode with a Hewlett Packard 7672 automatic sampler onto a non-polar, cross-linked, methyl silicone capillary column (50 m × 0.2 mm i.d., Hewlett Packard). After an isothermal period of 10 min, the oven was programmed from 50 to 250 °C per min. Hydrogen was used as the carrier gas (1 ml min^{-1}), and the injector and detector were maintained at 250 °C. Peak areas were quantified with a Hewlett Packard 3350 series programmable laboratory data system and quantitated by calibrated GC response to β-hydroxybutyrate (β-OH 4 : 0) derived from a pure PHB external standard (Dr R. Lafferty, Institut für Biotechnologie, Technische Universität, Graz, Austria). Replicate analyses of all samples were performed, and standard deviations were in the range 5–30 per cent, typically < 15 per cent.

Fatty acid analyses

The sum of the phopholipid ester-linked fatty acids for each sample was obtained based on

calibrated GC response using methylnonadecanoate as the internal injection standard. The same GC described above was used for all analyses; GC conditions have been reported in detail elsewhere (Nichols *et al.*, 1985). Fatty acid profiles for the methane-enriched soil column and *Methylobacterium organophilum* strains have been reported elsewhere (Nichols *et al.*, 1985 & 1987).

Gas chromatography-mass spectrometry (GC-MS)

GC-MS analyses were performed on a Hewlett-Packard 5996A system fitted with a direct capillary inlet. The same column type described above was used for analyses. Samples were injected in the splitless mode at 50 °C with a 0.5-minute venting time. The oven was maintained at 50 °C for 10 min, after which the oven temperature was programmed to 250 °C at either 3° or 4 °C per minute. Helium was used as the carrier gas. MS operating parameters were: electron multiplier between 1300 and 1400 volts; transfer line 250 °C; injector, source and analyzer 250 °C; autotune file DFTPP normalized, optics tuned at m/z 502; electron impact energy 70 eV. Mass spectral data were acquired and processed with a Hewlett-Packard RTE-6/VM data system.

Results

Soil samples

PHB and PLFA content for a methane-enriched, halogenated hydrocarbon-degrading soil column, related control samples and selected methanotropic bacteria are shown in Tables 1 and 2, respectively. Capillary GC and GC-MS showed that β-hydroxybutyrate was the sole monomer produced by hydrolysis of the soil acetone-containing PHB fraction obtained from the extracted soil. Longer chain homologues were below the detection limits of the instruments used in this study.

The concentration of PHB in the upper 10 cm of the methane-enriched HHD soil column ($\bar{x}$ = 125 nmoles g^{-1}) was significantly greater than that observed for the sodium-azide inhibited control column (PHB not detected) or untreated surface soil (1.9 nmoles g^{-1}) (Table 1). A subsurface maximum of the PHB content was observed at 2–4 cm, and both samples from the upper 4 cm contained at least 3 times morePHB than the deeper samples (4–10 cm). The PHB/PLFA ratios obtained for the upper 10 cm of the methane-enriched HHD soil column are illustrated in Fig. 1. The PLFA content for the top 10 cm of the HHD column was relatively constant (Table 1, range 42–74 nmoles g^{-1} dry weight). The PHB/PLFA ratio (Fig. 1) thus generally followed the same trend observed for the sedimentary concentration of PHB.

Table 1. Poly-β-hydroxybutyrate (PHB) and phospholipid ester-linked fatty acid (PLFA) content of soil from a methane-enriched, halogenated hydrocarbon-degrading (HHD) column and related samples.

Soil	PHB nmole g^{-1} [a]	PLFA nmole g^{-1} [a]
Methane-enriched HHD column		
0– 2[b]	170	42
2– 4	336	61
4– 6	60	50
6– 8	26	74
8– 10	31	42
140–150	ND[c]	0.6
Azide-inhibited column		
0– 10	ND	8.9
148–150	ND	0.04
Untreated surface soil		
0– 10	1.9	23

Data are the mean of two analyses.
[a] Dry weight basis.
[b] Depth in cm from top of column.
[c] Not detected.

Methylotrophs

Both strains of *Methylobacterium organophilum* analyzed contained appreciable amounts of PHB (Table 2). In contrast, the type I methylotroph,

Table 2. Poly-β-hydroxybutyrate (PHB) and phospholipid ester-linked fatty acid (PLFA) content of methanotrophic monocultures.

Bacterium (substrate)	PHB μmole g^{-1}[a]	PLFA μmole g^{-1}
Type I		
Methylococcus capsulatus (CH_3OH)	ND[b]	272
Methylococcus capsulatus (CH_4)	0.055	354
Type II		
Methylobacterium organophilum XX (CH_3OH)	26	66
Methylobacterium organophilum RG (CH_4)	104	57

Data are the mean of duplicate analyses.
[a] Dry weight basis.
[b] Not detected.

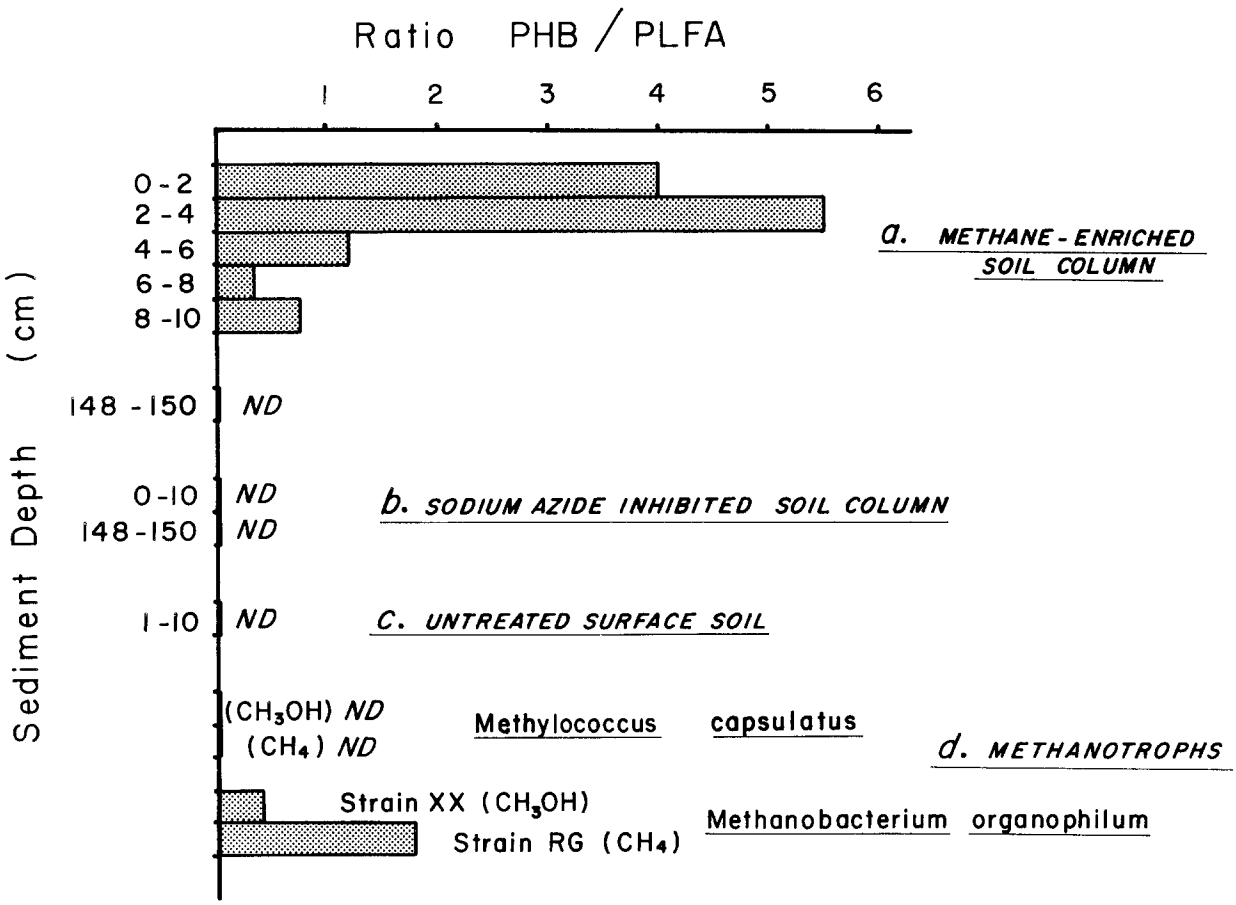

Fig. 1. Ratio PHB/PLFA for (a) samples from a methane-enriched halogenated hydrocarbon-degrading soil column, (b) samples from a sodium azide-inhibited soil column, (c) untreated surface soil, and (d) methanotrophs analyzed. ND: not detected.

Methylococcus capsulatus, contained only trace amounts of PHB when grown with methane, and PHB was absent from this bacterium growth with methanol. The PHB/PLFA ratio for *M. capsulatus* in both cases was < 0.001 (Fig. 1). Values of 0.43 and 1.8 were obtained for *Methylobacterium organophilum* grown on methanol and methane (Fig. 1). The PHB/PLFA ratios obtained for *M. organophilum* were in the range observed for the methane-enriched HHD soil column (Fig. 1).

Discussion

Monomer composition of polyhydroxyalkanoate (PHA) polymer

In this study it is of interest to note than only β-OH 4 : 0 was detected in both the soil material and methanotroph isolates analysed. Previous studies of the monomer composition of PHA recovered from marine sediments and activated sludge have reported conflicting results: Wallen & Rochwedder (1974) found that sludge was composed almost entirely of β-OH 4 : 0 and β-OH 5 : 0 with traces of C_6 and C_7 homologues; Findlay & White (1983) showed the presence of at least 11 short-chain β-hydroxy acids in polymers extracted from marine sediments; Odham *et al.* (1986) have reported β-OH 6 : 0 and β-OH 8 : 0 in addition to β-OH 4 : 0 in several sewage sludge samples, with β-OH 4 : 0 and β-OH 8 : 0 dominating. Odham *et al.* (1986) proposed that these discrepancies may be due to differences in samples and extraction procedures. Findlay & White (unpublished data) in a recent survey of 15 strains of bacteria found that a number of bacteria produced a mixed polymer, with β-OH 5 : 0 present in addition to β-OH 4 : 0. The composition and amount of polymer produced in several bacteria varied with the carbon source used and the metabolic status of the culture. Their data suggest that sample differences, rather than extraction techniques, may be the major cause of the difference in monomer composition noted by Odham *et al.* (1986). Further manipulative experiments need to be performed with methylotrophic bacteria, but it is evident, even at this stage, that under the culture and column operating conditions used in this study only β-OH 4 : 0 was present, with longer chain homologues below detection limits.

Soil microbial community structure

Knowledge of the microbial community structure present in the methane-enriched HHD soil column is necessary for interpretation of the cause and/or role of PHB accumulation in these samples. A brief discussion of the community structure, based on PLFA and other related data, is therefore presented below.

PLFA profiles obtained for the soil samples showed that type II methanotrophic bacteria formed the dominant class of organisms present in the methane-enriched HHD soil column (Nichols *et al.*, 1987); it was estimated that one group alone of the type II methanotrophs (those organisms which contain the PLFA 18 : 1Δ10c as a major fatty acid) contributed between 32 per cent to 56 per cent of the PLFA in the upper 10 cm of the methane-enriched soil column, and a significant input from other type II methanotrophs was also recognized. The enormous increase in markers for the type II methanotrophs was the most significant feature noted comparing the methane-enriched column studies support these findings – both methylotroph enrichment cultures and isolates of pure type II methanotrophs have also been obtained from soil analysed in this study.

Preliminary PLFA analyses of the culture material also confirm the presence of components previously determined to be characteristic for type II methanotrophs. Analysis of the PHB content and monomer composition of the isolates has not been performed due to insufficient sample material being available. Analyses of other methanotrophic bacteria in our laboratory have, however, indicated that two strains of *Methylobacterium organophilum* contain appreciable quantities of PHB, and in fact show PHB/PLFA ratios similar (Table 2) to those observed in the methane-enriched soil column.

Soil PHB content

The PHB/PLFA ratios of the aerobic methane-enriched HHD soil samples were significantly greater than those for material from the control column and untreated soil (Fig. 1). *Methylobacterium organophilum*, a facultative type II methanotroph, has been previously reported to contain PHB (Anthony, 1982), whereas the obligate methanotroph *Methylococcus capsulatus* and other type I bacteria have not been shown to form the polymer (Anthony, 1982; and this study). Based on PLFA profiles, type II rather than type I methanotrophs formed the major group of bacteria present in the methane-enriched soil.

The similarity between the ratios of PHB/PLFA observed in the soil samples and type II methanotrophs is further support for the hypothesis that type II methanotrophs produced the PHB present in the HHD soil column. To our knowledge, although it is recognized that type II methanotrophs such as *Methylobacterium organophilum* do possess PHB, no data exist on the effect of environmental parameters on the synthesis of PHB by these organisms. Data are available, however, which document causes of PHB accumulation in a range of other microbial cultures and environmental samples. It has been shown, for example, that accumulation of PHB in bacterial monocultures can result from oxygen, nitrogen, sulphur, or potassium limitation with adequate carbon and energy sources, or from changes in environment that lead to cyst or spore formation (Dawes & Senior, 1973; Herron *et al.*, 1978). Similarly, studies with estuarine sediments have shown that the detrital microbiota supplemented with carbohydrates also accumulate PHB in the absence of changes in the microbial biomass (based on PLFA lipid phosphate) (Nickels *et al.*, 1979). Indications are that the PHB synthesis was stimulated under conditions of unbalanced growth. In contrast, a freshwater *Spirillum* species, which apparently occupies a niche of low nutritional status (Matin & Veldkamp, 1978), accumulated PHB during lactate-limited growth in continuous culture (Matin *et al.*, 1979). The study did not show how PHB enhanced resistance to starvation, although a correlation between the amount of PHB and viability of starved bacteria has been demonstrated (Jones & Rhodes-Roberts, 1981; Tal & Okon, 1985). A more complete understanding of the conditions under which methanotrophs produce PHB and of the influence of various growth conditions will enable the data presented in this report to be better understood. Taken together, however, the PLFA, PHB and culturing data strongly indicate that type II methanotrophs form the major bacterial group and support the hypothesis that, based on the existing literature for other bacteria (see above), conditions of unbalanced growth exist within the upper layers of the methane-enriched HHD soil column.

PHB/PLFA depth profile

Changes in the PHB/PLFA ratio in the upper 10 cm of the methane-enriched HHD column (Fig. 1) may be the result of shifts in the microbial community structure and/or differences in the nutritional status within the soil column. Interpretation of the PLFA profiles revealed that *Methylobacterium organophilum* and/or related organisms were more abundant in the upper layers (0–2, 2–4 cm), whereas *Methylosinus trichosporium* or similar bacteria were more important in the lower portion (4–6, 6–8, 8–10 cm) of this upper 10 cm region of the column (Nichols *et al.*, 1987). *M. organophilum* contains appreciable proportions of PHB (Anthony, 1982; and this study), and has also recently been shown to contain significantly more PHB than *M. trichosporium* (Findlay & White, unpublished data). Therefore, from the information on microbial community structure obtained from PLFA profiles, it appears that the higher PHB content in the upper layers may well be the result of shifts within the microbial community of the composition of methanotroph species.

The data presented in this paper suggest that methanotrophic bacteria enriched in a methane-amended HHD soil column produce the storage polymer PHB. Biochemical assays, based on the

differences observed in this study, can be used to monitor shifts in microbial biomass and community structure (by means of PLFA profiles) and nutritional status (PHB/PHFA ratios). Interpretation of these data will assist in the selection and optimization of favorable conditions for pollutant biotransformations.

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