APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1993, p. 1602–1606 Vol. 59, No. 5
0099-2240/93/051602-05$02.00/0
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Influence of the Endogenous Storage Lipid Poly-β-Hydroxybutyrate on the Reducing Power Availability during Cometabolism of Trichloroethylene and Naphthalene by Resting Methanotrophic Mixed Cultures

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Received 28 September 1992/Accepted 9 March 1993

The role of the storage lipid poly-β-hydroxybutyrate (PHB) in trichloroethylene transformation by methanotrophic mixed cultures was investigated. Naphthalene oxidation rates were used to assay for soluble methane monooxygenase activity. The PHB content of methanotrophic cells grown in reactors varied diurnally as well as from day to day. A positive correlation between the amount of PHB in the cells and the naphthalene oxidation rate as well as between PHB and the trichloroethylene transformation rate and capacity was found. Addition of β-hydroxybutyrate increased the naphthalene oxidation rates significantly. PHB content in cells could be manipulated by incubation at different methane-to-nitrogen ratios. A positive correlation between the naphthalene oxidation rate and the PHB content after these incubations could be seen. Both the PHB content and the naphthalene oxidation rates decreased with time in resting methanotrophic cells exposed to oxygen. However, this decrease in the naphthalene oxidation rate cannot be explained by the decrease in the PHB content alone. Probably a deactivation of the methane monooxygenase itself is also involved.

Many groundwater aquifers are contaminated with toxic and/or carcinogenic compounds (28). Therefore, the need for reliable processes for their control is urgent. Trichloroethylene (TCE) is one of the most frequently detected contaminants in groundwater supplies. It is toxic to humans and is a suspected carcinogen (17). Wilson and Wilson (30) first found that TCE can be degraded under aerobic conditions in soil columns exposed to natural gas (77% methane). Subsequently, it was shown that TCE can be degraded cometabolically by a number of different aerobic organisms, including methanotrophs (10, 14, 20). The potential for in situ biodegradation of TCE through biological enhancement of the methanotrophic population in the soil has been demonstrated (23), and many studies have been directed towards on site treatment in bioreactors (2, 16).

TCE degradation by methanotrophs is a cometabolic process in which methane, but not TCE, is used as the primary substrate for energy and cell carbon. Since both TCE and methane are initially oxidized by methane monooxygenase (MMO) (11), methane can act as a competitive inhibitor of TCE oxidation, reducing its rate of transformation (5, 19). As one possible solution to this problem, a two-stage reactor system has been proposed (2, 16). In the first-stage or growth reactor, methane and oxygen are supplied to foster the growth of methanotrophic bacteria. Cells are then moved to the second-stage or treatment reactor, where they are mixed with a TCE-contaminated stream. Here, the resting (nongrowing) cells degrade TCE without competition from methane.

Resting cells have been shown to have a limited capacity to transform TCE (1). This transformation capacity is the result of either limitation in the amount of reducing power available to reactivate MMO or toxicity of metabolic intermediates from TCE degradation (1, 12, 19). By supplying an external source of reducing power such as formate, a limitation in reducing power can be overcome. The finite transformation capacity that then results is likely to be due solely to TCE transformation product toxicity.

Henry and Grbić-Galić (12) demonstrated that resting methanotrophic strains containing sudanophilic storage granules retained the ability to transform TCE longer than strains without storage granules, leading to the suggestion that the storage granules can provide at least some of the reducing power required. Earlier studies by Kallio and Harrington (13) and Whittenbury et al. (29) indicated that such storage granules in methanotrophs contain poly-β-hydroxybutyrate (PHB). Dawes and Senior (8) proposed that PHB can be used as an electron donor in microorganisms. Matin et al. (15) and Tal and Okon (24) demonstrated that PHB accumulation could be an advantage for bacterial cells during starvation, and experiments by Schlegel et al. (21) indicated that PHB can be used for protein synthesis. These results suggest that PHB may act as an endogenous electron donor or source of required reducing power.

The metabolic pathways for PHB are known for several organisms (22, 25). PHB accumulates during unbalanced growth in many organisms (8, 9, 18, 26). By growing methanotrophic cells at different methane-to-nitrogen ratios, different PHB levels can be achieved (4).

One objective of this study was to determine what relationship might exist between the PHB content in methanotrophic cells and their naphthalene oxidation rates. In addition, the relationship between TCE transformation rate and capacity and PHB content was also examined. The possibilities for manipulation of the PHB content were also examined, as was the effect of PHB on the observed decrease in MMO activity with resting aerobic cells.

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MATERIALS AND METHODS

Methanotrophic mixed cultures. A methanotrophic mixed culture, designated LMA, was established as described previously (1, 3). This culture was grown in a 7.5-liter reactor and operated at a residence time of 9 days with continuous methane and air feed but with once-daily liquid medium withdrawal and feed. A second mixed culture, designated LHS, was originally seeded from the LMA culture and was grown in a 2.5-liter reactor with continuous methane and air feed as well as liquid medium withdrawal and feed at a residence time of 4 days.

Analytical procedures. The dry mass of cells was calculated from the A_{600} (1). PHB was determined essentially according to the method of Ward and Dawes (27). Culture samples (three 200-μl replicates) were applied to glass fiber disks (Whatman GF/C; 2.1 cm) and dried at 105°C for 10 min. The cells were digested in 150 μl of sodium hypochlorite solution for 1 h and dried again. Warm chloroform was applied three times, and the disks were transferred to test tubes, successively washed in water, ethanol, and acetone, and then dried. Concentrated H_2SO_4 (2 ml) was added to each test tube, which was then heated for 15 min at 100°C, and the A_{235} was measured. An extinction coefficient of 15,500 M^{-1} cm^{-1} (27) was used to calculate the PHB content of the cells. Pure PHB (Sigma, St. Louis, Mo.) was measured with the same method to check the validity of the extinction coefficient used.

Soluble MMO activity was measured as naphthalene oxidation essentially according to the method of Brusseau et al. (6). Samples (three 1-ml replicates, 0.25 mg [dry mass]/ml) with and without 20 mM sodium formate were incubated at 30°C for 30 min. The A_{530} was measured with identical samples without added naphthalene as blanks (one blank for each replicate). An extinction coefficient of 38,000 M^{-1} cm^{-1} (6) was used to calculate the rate of naphthalene oxidation. Measured naphthalene oxidation rates were normalized to maximum rates for each sample when 20 mM sodium formate was present. Formate acts as a readily available source of reducing power, permitting maximum rates of naphthalene oxidation, with which the effectiveness of PHB as an internal source could be compared. In some cases, β-hydroxybutyrate was added as an alternative electron donor for comparison with both PHB and formate.

TCE transformation was measured as described by Alvarez-Cohen and McCarty (1). Cell suspensions (50 ml each) at a concentration of 0.2 mg of cells per ml in 250-ml bottles were spiked with 1.6 mg of TCE. When TCE transformation was complete (approximately 55 h), the ratio of TCE decrease to the amount of cells initially present (both in milligrams) was used to report the TCE transformation capacity.

Experimental procedures. To manipulate the PHB content in cells, incubation with different methane-to-nitrogen ratios was performed with 10-ml cell suspensions from the LMA reactor (approximately 2.5 mg [dry weight]/ml) in 250-ml bottles. Sodium nitrate (50 μl, 100 g/liter) or water was added to the bottles. Methane corresponding to 15% of the headspace was injected, and the bottles were shaken at 20°C overnight.

In an experiment to determine the effect of aeration on cell activity, 50 ml of resting cells at a concentration of 2.6 mg of cells per ml were shaken for 8 h in 250-ml flasks on a wrist shaker, with samples periodically being taken for analysis.

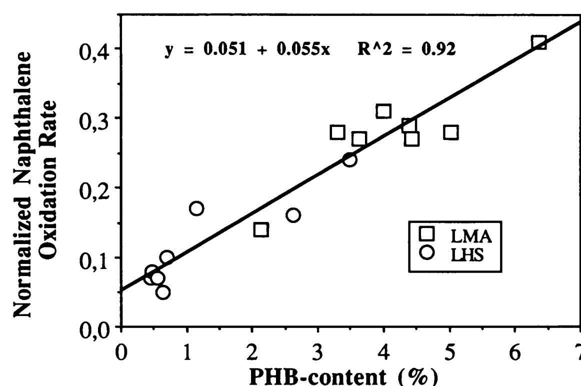

FIG. 1. Correlation between the PHB content and the normalized naphthalene oxidation rate in samples from the LMA and the LHS reactors during a 14-day period. The naphthalene oxidation rate without formate was normalized to the rate with 20 mM formate for each sample.

RESULTS

The methanotrophic mixed culture designated LMA showed a diurnal variation of PHB and cell density. The variation in cell density could be explained by the dilution after each feed, but the variation in PHB content was larger than could be explained by this effect. This variation in the PHB content may have been caused by the higher nitrate concentration just after the once-a-day feeding and withdrawal of liquid medium to and from the reactor. When analyzed at the same time of day over a 40-day period, the PHB content varied between 2 and 8% of the dry mass of cells. The cell density over the same period of time varied between 2.5 and 3.1 mg (dry mass)/ml. The reason for this wide variation in PHB content is not known. There was no significant correlation between the variation in the PHB content and the variation in cell density.

The effect of cellular PHB content on naphthalene oxidation rate and TCE transformation was evaluated with samples from the LMA and LHS reactors. Naphthalene oxidation rates in samples both with and without 20 mM sodium formate addition were measured. This formate concentration was found to give the highest rate of naphthalene oxidation (data not shown). Naphthalene oxidation rate per unit of mass of cells, when formate was present, varied little with time, with cell PHB content, or with reactor operation, giving a coefficient of variation for all 16 samples over the 2-week period of only 15%. Assuming that naphthalene can be oxidized only by soluble MMO and that soluble MMO concentration is rate limiting (6), these results suggest that the soluble MMO content of the cells from each of the two reactors over this period was relatively constant. These results also suggest that as long as 20 mM formate was added, the naphthalene oxidation rate was not limited by reducing power availability.

However, when formate was absent, the naphthalene oxidation rate varied significantly with variation in PHB content. A comparison between the normalized naphthalene oxidation rate (ratio of rates without and with addition of 20 mM formate) and cell PHB content is illustrated in Fig. 1. A positive correlation between the two was indeed found. Results for all samples analyzed during the 2-week period are shown. The coefficients of variation for the PHB assay and the MMO assay were 4 to 16 and 3 to 19%, respectively. These results collectively suggest that the reason for the

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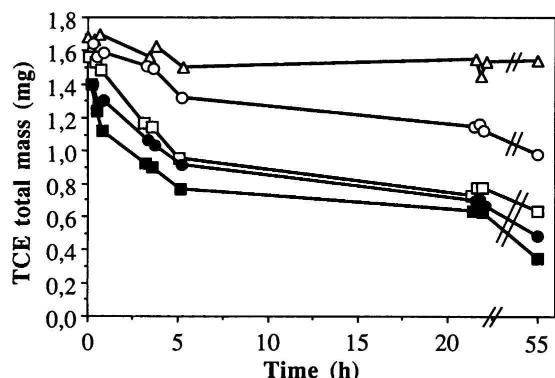

FIG. 2. TCE degradation in samples from the LMA and the LHS reactors measured with and without 20 mM formate. The datum points at 55 h represent final values beyond which no additional degradation occurred and were used to estimate TCE transformation capacity. △, control; ○ and ●, LHS without and with formate, respectively; □ and ■, LMA without and with formate, respectively.

correlation between the PHB content and the naphthalene oxidation rates is not variation in the soluble MMO content but rather a variation in reducing power availability.

TCE transformation and cell PHB content were determined with samples from the two reactors taken during the same 2-week period as above. The TCE transformation was measured both without and with 20 mM sodium formate. The results for one set of samples are shown in Fig. 2. TCE transformation in samples from the LMA reactor was faster than TCE transformation in samples from the LHS reactor. Measured TCE transformation capacities for the two cultures and their PHB contents are summarized in Table 1. The TCE transformation capacities of the two cultures were the same in the presence of formate, which served to overcome a reducing power limitation. The capacity in this case probably resulted primarily from toxicity of TCE transformation products. The significantly lower TCE transformation in both cultures observed when formate was absent suggests that, under these circumstances, the transformation capacity was limited by an inadequate source of reducing power. However, the transformation capacity was much higher in the LMA culture, which had a higher PHB content, which presumably helped to offset the lack of external reducing power. These results again suggest a correlation between the PHB content and the internal reducing power availability in the cells.

The positive correlation between the PHB content and both naphthalene oxidation rate and the TCE transformation capacity might be due to supplemental reducing power availability derived from PHB. However, a second explanation could be that both the high PHB content and the high rates or capacities are simultaneously correlated with some other factor and not directly related. To evaluate PHB as a possible direct source of reducing power, the effect of its monomer, β-hydroxybutyrate, was examined. Addition of β-hydroxybutyrate enhanced the naphthalene oxidation rate (Table 2). These results imply that a higher content of the PHB polymer does have the potential to create a higher reducing power availability. For a more direct comparison, 1 mM hydroxybutyrate in Table 2 corresponds to a PHB content of 8%, and the cells had a PHB content of 3.5% before the hydroxybutyrate addition.

To further investigate the relationship between PHB content and the naphthalene oxidation rate, cells were incubated under different conditions designed to produce samples with differing PHB contents. It is well established that a high methane-to-nitrogen ratio will lead to a higher content of PHB in cells (4). Table 3 contains a summary of the PHB content and naphthalene oxidation rates after batch incubation of cells at different methane-to-nitrate ratios. Again, the naphthalene oxidation rates correlate with the PHB content. Thus, it is possible to manipulate the internal available reducing power in cells. In Fig. 3, the rate of PHB metabolism upon nitrate addition can be seen. The PHB content initially decreased, from 5.6 to 1.3%, within 28 h. Subsequently, the PHB level increased and arrived at the original level after an additional 19 h of incubation. This production probably occurred after nitrate was exhausted, as was expected because the amount of nitrate added corresponded to the normal daily feed for the continuous-feed reactors.

Alvarez-Cohen and McCarty (1) and Henry and Grbić-Galić (12) found that the TCE-transforming ability of resting cells decreased rapidly under aerobic conditions. To investigate a possible explanation for this decreased activity, both the PHB content and the naphthalene degradation rate in a sample that was shaken in air were monitored (Fig. 4). The decrease in the naphthalene oxidation rate was much faster than the decrease in PHB content. After 8 h, the PHB content was still 50% of the original level while the naphthalene oxidation rates, with and without formate, were below

TABLE 2. Normalized naphthalene oxidation rates in samples amended with different concentrations of β-hydroxybutyrate[a]

β-Hydroxybutyrate concn (mM)	Naphthalene oxidation rate (normalized)
0.00	0.22
0.01	0.22
0.10	0.23
1.00	0.32
10.0	0.38

[a] 1 mM β-hydroxybutyrate is equivalent to about 8% PHB in cells.

TABLE 1. TCE transformation capacities[a] and PHB contents for the LMA and the LHS reactors

Reactor	PHB content (%)	TCE transformation capacity (mg/mg)	
		Without formate	With formate
LHS	0.7	0.056	0.11
LMA	4.7	0.091	0.12

[a] Calculated from the same data as in Fig. 2.

TABLE 3. Naphthalene oxidation rates and PHB contents after cell incubation in batch reactors at different methane-to-nitrogen ratios

Addition	PHB content (%)	Naphthalene oxidation rate (μmol/mg/day)		
		Without formate	With formate	Normalized
Nitrate	0.9	0.38	3.12	0.12
None	7.4	1.36	2.14	0.64

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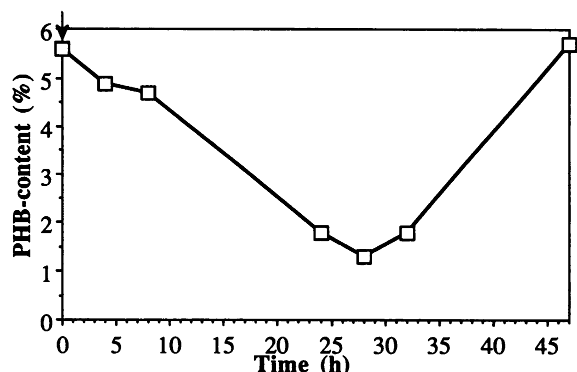

FIG. 3. Changes in the PHB content in a sample from the LMA reactor after addition of sodium nitrate (↓) corresponding to 1-day normal reactor feed.

8% of the initial values. For the unshaken control, no significant decrease in either of the measured parameters was found (data not shown), presumably because of oxygen deficiency (1).

DISCUSSION

PHB occurs in many different bacterial strains and can be used advantageously during cell starvation, one suggestion being that it can be used as a source of reducing power (8). In this study, a direct correlation was found between the PHB content in methanotrophic cells and the naphthalene oxidation rate, as well as between PHB and the TCE transformation rate and capacity. These rates and capacities also appear limited by reducing power availability, as 20 mM formate addition increased all values significantly. There are, thus, at least two possible explanations for these results. The first is that the PHB in resting cells can be used as a direct or indirect source of reducing power. The second is that PHB is a marker for a higher reducing power state in the cells; i.e., when internal reducing power is in excess of growth needs, the cells produce PHB. Such a higher reducing power state could then result in the higher rates and capacities in the cells. The higher reducing power state could

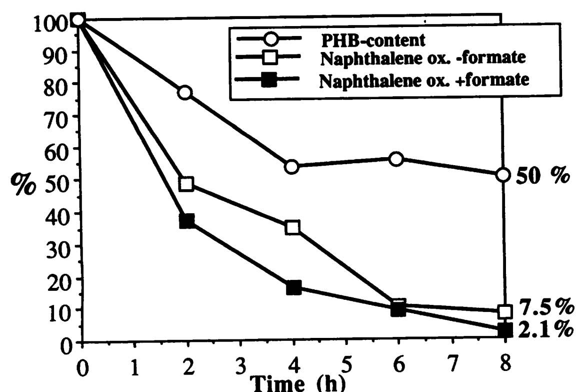

FIG. 4. Decrease in PHB content and naphthalene oxidation (ox.) rates in a shaken sample of resting cells from the LMA reactor. One hundred percent at 0 h corresponds to 2.6% PHB and 1.26 (without formate) and 5.67 (with formate) μmol of naphthalene per mg of cells per day.

be represented by a high NADH-to-NAD^+ ratio or, indirectly, by other metabolites with capacity to produce NADH. The fact that the monomer of PHB, β-hydroxybutyrate, enhanced the naphthalene oxidation rate is at least indicative that PHB has the potential to be used as a reducing power source. Since the correlations between PHB content and the naphthalene and TCE degradation rates was seen only in the absence of formate, this rules out the possibility that the different degradation rates could be explained as resulting from changes in the soluble MMO content.

The decrease in the PHB content of shaken resting cells (50%) was much smaller than the decrease in naphthalene oxidation rates (>90%) (Fig. 4). Such a large decrease in the naphthalene oxidation rate would not be expected on the basis of a 50% change in the PHB content if the latter were the only factor controlling the former. Furthermore, since the naphthalene oxidation rate with 20 mM formate also decreased, it appears that the cause was not a limitation in reducing power availability. These results suggest that under these circumstances soluble MMO itself became rate limiting. The regulation of soluble MMO has been shown to be a fast process, on the order of less than an hour (7). It is possible that such a regulation process also took place in our experiment when the cells were starved for methane, leading to a rapid loss of soluble MMO.

ACKNOWLEDGMENTS

This research was supported in part by a postdoctoral fellowship to T. Henrysson from the Swedish Forest and Agricultural Research Council and, in part, by the Gas Research Institute- and the U.S. Environmental Protection Agency-sponsored Western Region Hazardous Substance Research Center under agreement R-815738.

We also thank L. Smith for permitting use of the culture from the LHS reactor, which he designed and established, and for reviewing the manuscript.

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