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Intracellular PHB conversion in a Type II methanotroph studied by ^{13}C NMR

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Poly-β-hydroxybutyrate (PHB) formation under aerobic conditions *via* incorporation of [^{13}C-2]acetate as a cosubstrate and its intracellular degradation under anaerobic conditions in a Type II methanotroph was studied by ^{13}C NMR. During PHB synthesis in the presence of labelled acetate, low levels of β-hydroxybutyrate, butyrate, acetone, isopropanol, 2,3-butanediol and succinate were observed. Subsequent anaerobic PHB breakdown showed enhanced levels of these products at the expense of PHB. Fermentative metabolism occurring during anaerobic PHB degradation was confirmed in experiments with fully ^{13}C-enriched cells, which were grown on ^{13}C-labelled methane. β-hydroxybutyrate, butyrate, acetate, acetone, isopropanol, 2,3-butanediol and succinate were detected as multiple ^{13}C-labelled compounds in the culture medium. Our results suggest that intracellular PHB degradation can be used as a reserve energy source by methanotrophs under anoxic conditions. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 15–21.

Keywords: PHB; obligate methanotroph; ^{13}C NMR

Introduction

Methanotrophic bacteria are highly specialized microorganisms that have the ability to use methane as a sole carbon and energy source. These bacteria are widespread in nature. They have been found in different types of natural environments as well as in bioreactors, gas pipelines and water-treatment systems [6,14,26].

Methanotrophs have been classified into two groups (Types I and II) on the basis of their primary carbon assimilation pathways (the RuMP and the serine pathway, respectively), different membrane arrangements, the ability to form different long-chain unsaturated fatty acids and the ability to fix molecular nitrogen [6]. Types I and II methanotrophs also have different survival strategies. Type I methanotrophs are characterized by rapid growth under favorable conditions and a rapid rate of die-off under stress conditions. Methanotrophs of group II grow slower, but survive better. In addition, Type II methanotrophs outcompete Type I methanotrophs under oxygen- and nitrogen-limiting conditions [12,14]. Both groups of bacteria form poly-β-hydroxybutyrate (PHB); however, very little is known about the way of conversion of this storage material in methanotrophs.

Principally there are two general approaches to investigate intracellular PHB degradation: in living cells under starvation conditions [18,19,24] and in the isolated natural granules of PHB [13,15,21]. Self-degradation of isolated native granules of PHB mostly gives rise to D-β-hydroxybutyrate as the main and sometimes sole product of decomposition. It is most likely carried out by hydrolases entrapped in the biopolymer granules [13,21]. Different kinetics of intracellular PHB degradation were demonstrated for a number of microorganisms in cell suspensions under aerobic and anaerobic conditions [1,9,18]. β-Hydroxybutyrate, acetoacetate and a small amount of acetate were found in cell suspensions of *Bacillus megaterium* and *Bacillus cereus* as a result of PHB conversion under both conditions [18]. However, in the presence of oxygen these products of degradation were rapidly converted into CO_2 and H_2O. Butyrate, lactate and ethanol were demonstrated as minor metabolites next to β-hydroxybutyrate and acetate as major components of anaerobic PHB degradation in cell suspensions of *Alcaligenes eutrophus* [9]. No extracellular metabolites of PHB breakdown were observed in the presence of oxygen.

^{13}C NMR spectroscopy has been successfully used to study the physical properties and biochemistry of PHB [3–5,9,10,20,22]. In this study we applied ^{13}C NMR techniques to investigate reactions related to PHB conversion in a Type II methanotroph. Particularly, we focused on intracellular PHB degradation in intact cells under anaerobic conditions.

Materials and methods

Organism

The obligate methanotrophic bacterium strain MTS was isolated from a denitrifying bioreactor operated with methane gas as electron donor [7]. Phylogenetic analysis revealed that the strain was a Type II methanotroph. About 91% of the 16S rRNA gene of the strain was sequenced and deposited in the GenBank nucleotide sequence database under the accession number AF107461. Strain MTS has been deposited in the National Collection of Industrial and Marine Bacteria, Scotland, Aberdeen, UK under the accession number NCIMB 13654.

PHB determination

PHB was obtained from freeze-dried cells using a hypochlorite treatment followed by extraction in hot chloroform [25]. The

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concentration of PHB was measured according to the assay of Law and Slepecky [17].

In vivo ^{13}C NMR

Cells of the methanotrophic bacterium strain MTS were grown at 30°C in four 1-l shake flasks each containing 300 ml mineral NMS medium [26] and a methane–air gas headspace (1:3, v/v). Cells of fully grown cultures were harvested under aseptic conditions by centrifugation and wet pellets were subsequently resuspended in fresh NMS medium to an end volume of 14 ml. The concentrated cell suspension was aseptically transferred into a sterile 20-mm NMR tube containing three glass balls (4-mm diameter) to assure adequate mixing of the cells with the applied gas phase in the spinning NMR tube. The final concentration of the biomass in the tube was about 18.0 mg dry weight/ml.

Aerobic acetate conversion was carried out by adding 2 mM [^{13}C-2]acetate (99.3%, sodium salt, Isotec, Miamisburg, Ohio, USA) to the cell suspension. During the experiment the concentrations of CH_4 and O_2 were maintained between 30% and 20%, and 20% and 10%, respectively. ^{13}C NMR spectra (duration, 6 h) were obtained at 75.47 MHz on a wide-bore AMX-300 NMR-spectrometer (Bruker, Karlsruhe, Germany) at 30°C; a spinning rate of 3–4 Hz was used. The concentrations of methane and oxygen in the headspace of the NMR tube were controlled by a Packard 417 gas chromatograph equipped with a Molsieve 13× (2 m×0.25 in. OD) packed column connected to a thermal conductivity detector (TCD) with argon as carrier gas (20 ml/min).

When nearly all the cosubstrate [^{13}C-2]acetate was consumed, the aerobic conditions in the NMR tube were changed to anaerobic conditions. The gas phase was replaced by sterile N_2 gas by flushing it through a 0.2-μm pore-size bacterial filter (Rotrand/Red rim, Schleicher and Schuell, Germany). The culture was incubated for 2 weeks under anaerobic conditions. *In vivo* ^{13}C NMR spectra (duration 6 h) were obtained with the same acquisition parameters as for the aerobic experiment.

The purity of the suspensions was checked during all experiments by light microscopy and by examination of growth in complex organic media. No growth of possible heterotrophic contaminants was observed.

^{13}C NMR of supernatants

For these experiments, cells of the methanotrophic bacterium were grown in mineral NMS medium in four 1-l shake flasks containing 15% (v/v) of solely ^{13}C-labeled CH_4 (99% ^{13}C, Isotec) in the air headspace. After growth (3 days) cells were concentrated and resuspended in fresh NMS medium to a volume of 14 ml. The cell suspension was transferred to 60-ml serum bottles and anoxic conditions were established as described above. The suspension

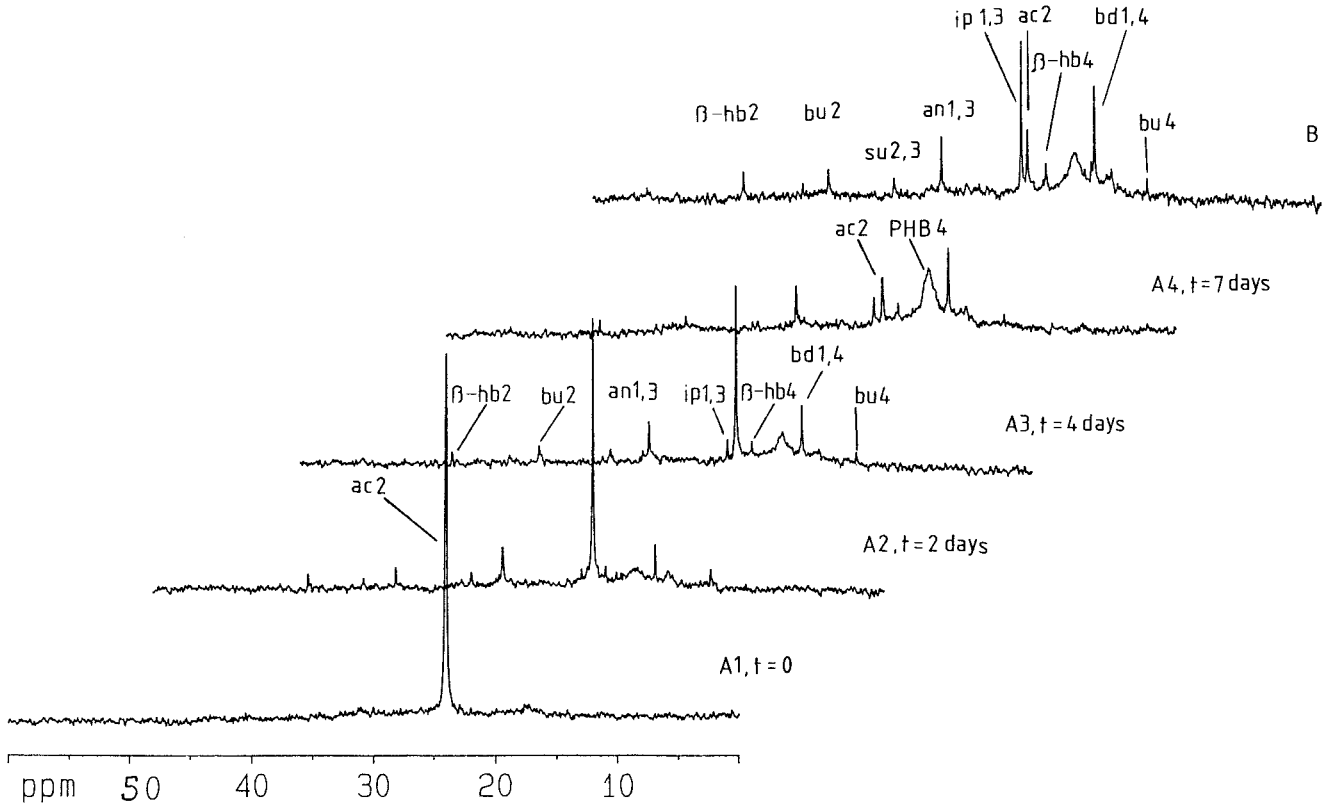

Figure 1 ^{13}C NMR spectra of a cell suspension of the obligate methanotrophic bacterium strain MTS incubated aerobically with natural abundance methane and [^{13}C-2]acetate as a cosubstrate, recorded at 75.47 MHz at the indicated times (A). Subsequent anaerobic PHB breakdown recorded after 14 days incubation under anoxic conditions (B). Abbreviations: β-hb, β-hydroxybutyrate; ac, acetate; an, acetone; bu, butyrate; ip, isopropanol; bd, 2,3-butanediol; su, succinate.

was incubated anaerobically in the dark at 30°C. Samples (0.6 ml) were taken in time, and after centrifugation the supernatants were analyzed at 125.76 MHz in 5-mm NMR tubes using a narrow bore AMX-500 NMR spectrometer (Bruker). To aliquots (400 μl) of supernatant, 50 μl of D_2O (10%) to provide a lock signal and 50 μl of 250 mM potassium fumarate were added as internal standard. All observed resonances were assigned by spiking with small amounts of pure compounds. Spectra were referenced against TMS by setting the methanol resonance at 50.2 ppm according to Barnard and Sanders [3].

Multiple labelling

Because the only carbon source was 99% ^{13}C-labelled CH_4, the carbons of all compounds formed during growth are 99% ^{13}C-labelled. Consequently, the methyl carbon in butyrate (C_4) has only a ^{13}C-labelled methylene carbon (C_3) as neighbour and the $^{13}C_4-^{13}C_3$ fragment is doubly labelled with a probability of 99%×99%. The (proton decoupled) ^{13}C NMR resonance of this methyl carbon (C_4), being one single resonance (singlet) in the case of a $^{12}C_3$ methylene carbon as next neighbour, will be split up into a doublet as result of the $^{13}C_4-^{13}C_3$ coupling (34 Hz). The intensity of each doublet component is half of that of the singlet and the distance between the doublet components (coupling constant) is 34 Hz. The C_3 carbon of butyrate has two ^{13}C-labelled neighbours C_4 and C_2, both with coupling constants $^{13}C_3-^{13}C_4$ and $^{13}C_3-^{13}C_2$ of 34 Hz. The resonance of this C_3 carbon of butyrate in this ^{13}C-labelled $^{13}C_4-^{13}C_3-^{13}C_2$ fragment (probability 99%×99%×99%) is a triplet with a separation of 34 Hz between its components and an intensity ratio of 1:2:1, the total intensity being that of the single C_3 resonance when C_3 would have no ^{13}C-labelled neighbours. The C_2 carbon has C_3 and C_1 as neighbours with coupling constants $^{13}C_2-^{13}C_3$ and $^{13}C_2-^{13}C_1$ of 34 and 52 Hz, respectively. The single resonance of C_2 (without ^{13}C neighbours) is first split up into a doublet with the largest coupling constant of 52 Hz, each component of which being subsequently split up by 34 Hz. The result is a quartet for the resonance of the C_2 carbon of butyrate with an intensity ratio of 1:1:1:1. The same is valid for β-hydroxybutyrate. Acetone and isopropanol have two resonances each, of the $C_{1,3}$ and of the C_2, which are doublets because there is only one coupling constant involved: $^{13}C_1-^{13}C_2$ being equal to $^{13}C_3-^{13}C_2$ due to molecular symmetry; the intensity ratio is 2:1. 2,3-Butanediol also has two doublet resonances in a ^{13}C NMR spectrum, occurring from the $C_{1,4}$ and the $C_{2,3}$. There is only one coupling

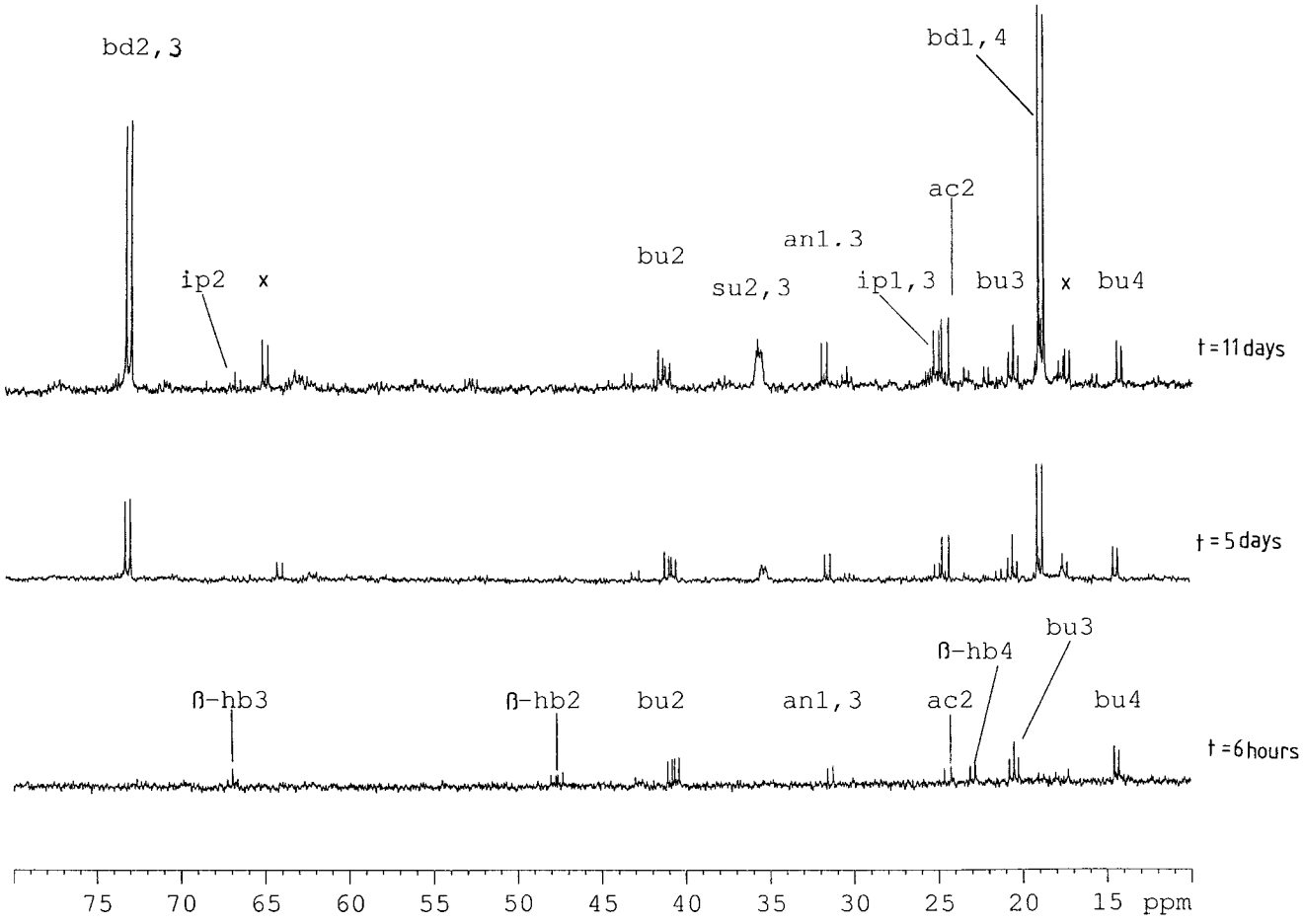

Figure 2 ^{13}C NMR spectra of supernatants of a fully ^{13}C-enriched cell suspension of the obligate methanotrophic bacterium strain MTS incubated under anaerobic conditions. The supernatants were withdrawn from the suspension at the indicated times and the spectra were recorded at 125.76 MHz. Abbreviations as in Figure 1; x, unknown.

constant (34 Hz). The intensity ratio is 2:2 because two carbons are involved in both doublets. Acetate also has two resonances of the C_2 and the C_1 carbons, which are doublets due to the presence of only one coupling constant of 52 Hz. Succinate has two coupling constants $^{13}C_1-^{13}C_2$ (52 Hz) and $^{13}C_2-^{13}C_3$ (34 Hz); as result the spectrum has two resonances,

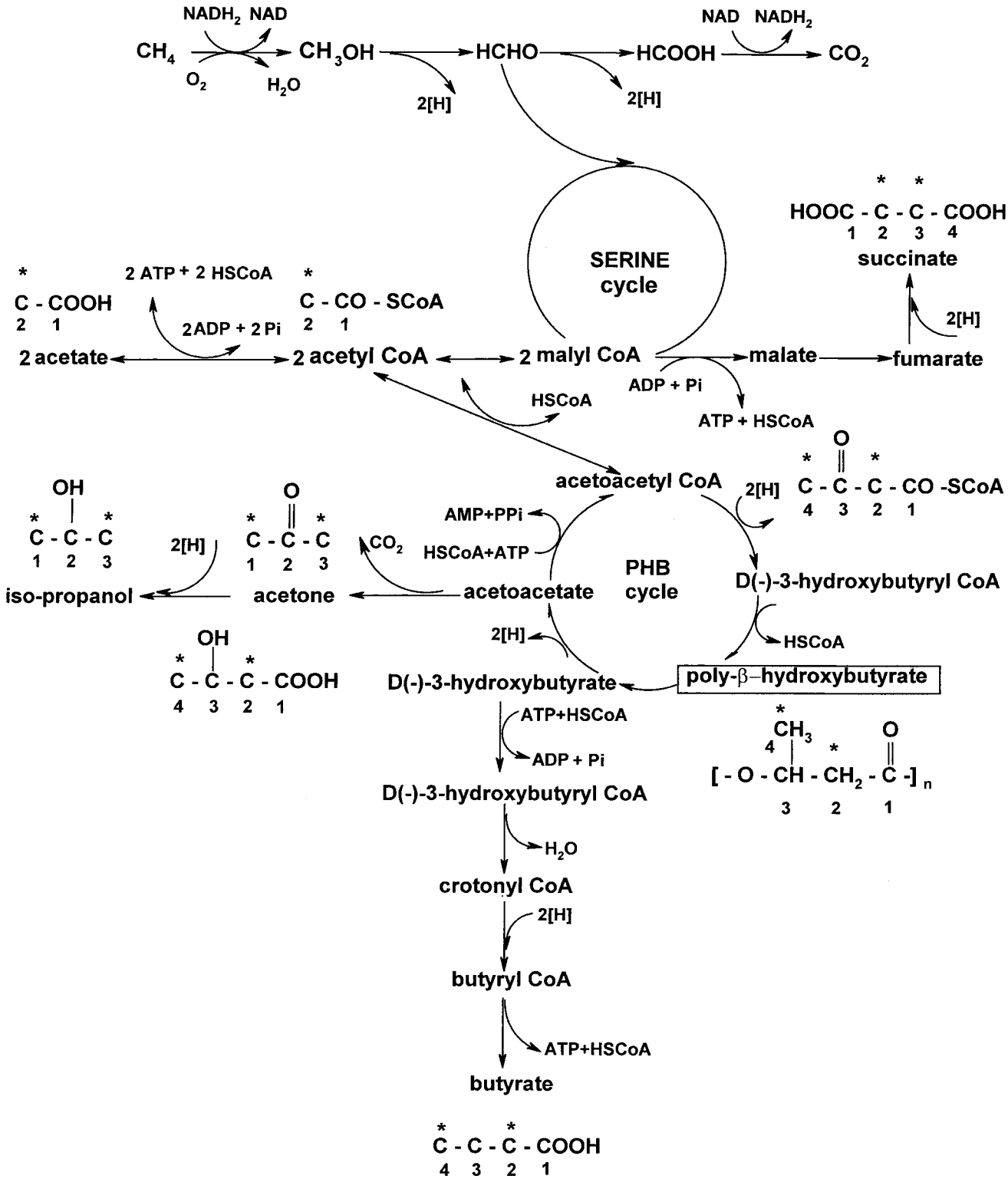

Figure 3 The possible connection of the formation of succinate, acetate, butyrate, acetone and isopropanol with the PHB cycle in a Type II methanotroph strain MTS. Labelling of the various compounds in the case of incorporation of [^{13}C-2]acetate is indicated by asterisks (*). Modified from Ref. [2].

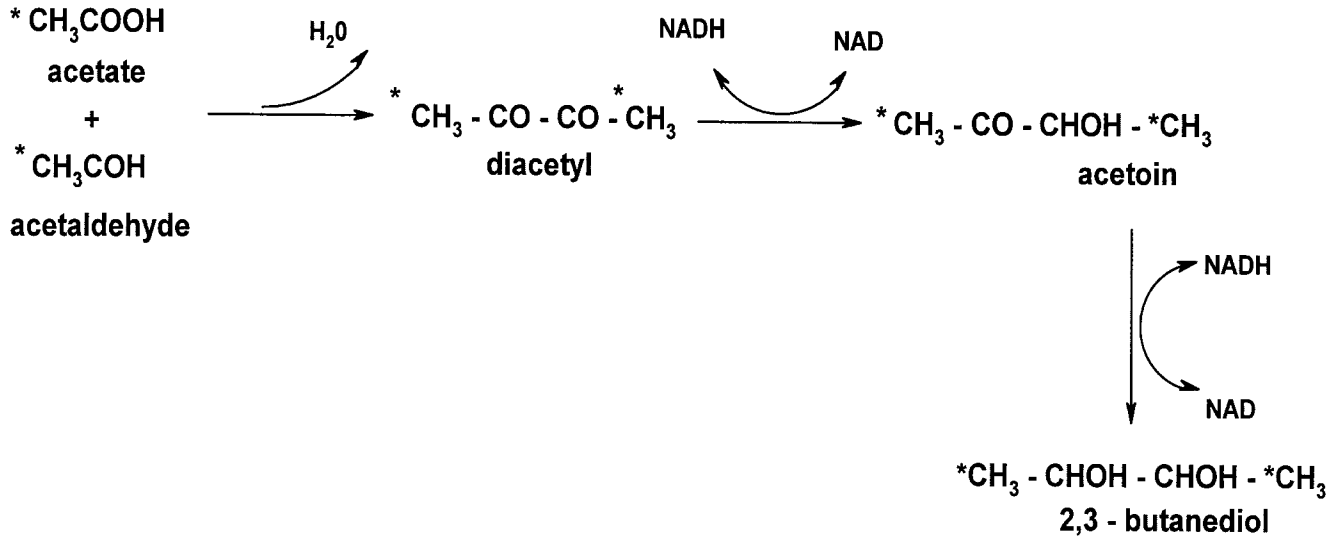

Figure 4 The putative pathway to form 2,3-butanediol during anaerobic fermentation of PHB in a Type II methanotroph strain MTS. Modified from Ref. [23].

being a quartet at the $C_{2,3}$ position, and a doublet at the $C_{1,4}$ position.

Results

PHB

The presence of PHB in the obligate methanotrophic bacterium strain MTS was demonstrated by extraction in chloroform. About 3.0% of the dry weight of methane-grown cells and 7.4% of the dry weight of methanol-grown cells was PHB.

In vivo ^{13}C NMR

The time-course of acetate conversion in a cell suspension of the obligate methanotrophic bacterium strain MTS incubated aerobically together with natural abundance methane and [^{13}C-2]-labelled acetate as a cosubstrate is shown in a series of *in vivo* ^{13}C NMR spectra in Figure 1A. A dynamic incorporation of acetate into PHB was observed. The disappearance of the C_2-carbon resonance of acetate coincided with the appearance of the broad resonance of the $^{13}C_4$ group of PHB at a chemical shift value of 20.4 ppm. During this process of acetate incorporation, a low level of the intermediate β-hydroxybutyrate (visualized by its C_2 and C_4 carbon resonances at 47.64 and 22.96 ppm) was observed. Also, a number of other compounds like butyrate (C_2 and C_4 carbon resonances at 40.41 and 14.40 ppm), acetone ($C_{1,3}$ carbons at 31.42 ppm), isopropanol ($C_{1,3}$ carbons at 24.96 ppm), 2,3-butanediol ($C_{1,4}$ carbons at 18.90 ppm) and succinate ($C_{2,3}$ carbons at 35.11 ppm) were identified.

When $^{13}C_3$-labelled PHB had reached a high value at the expense of acetate, the suspension was transferred to anaerobic conditions. After 2 weeks of anaerobic incubation the ^{13}C NMR spectrum of the intact cells was measured again (Figure 1B). Partial breakdown of PHB was observed whereas the levels of β-hydroxybutyrate, butyrate, acetate, acetone, isopropanol, 2,3-butanediol and succinate had increased.

^{13}C NMR of supernatants

To confirm the results obtained during anaerobic intracellular PHB degradation, a fully ^{13}C-enriched culture (obtained by growth on solely $^{13}CH_4$) was concentrated and incubated directly after harvesting under anaerobic conditions. Three spectra, reflecting the time-course of appearance of the multiple ^{13}C-labelled products in the supernatant, are shown in Figure 2. In the spectrum recorded after 6 h of incubation of cells under anaerobic conditions low levels of excreted β-hydroxybutyrate, butyrate, acetate and acetone were observed. Within 11 days of incubation the signals of β-hydroxybutyrate had disappeared, whereas the resonances of butyrate, acetate, acetone had increased. Succinate and other reduced compounds like isopropanol and a high level of 2,3-butanediol had appeared as well.

Discussion

As shown in previous studies [^{13}C-2]acetate can be selectively incorporated into PHB with labelling in the methyl (C_4) and the methylene (C_2) carbons of the biopolymer [10,20]. In our experiments with [^{13}C-2]acetate, labelled PHB was visualized by the appearance of one broad resonance representing the mobile methyl group when small line broadenings were applied. Our spectra also showed the broader resonance belonging to the immobile methylene group of PHB at 41.1 ppm when larger line broadenings were used. By ^{13}C NMR spectroscopy different physical stages of PHB in various bacteria were demonstrated [3–5,20]. In intact *Rhodopseudomonas sphaeroides* cells labelling with [^{13}C-2]acetate led to similar results as shown by us [20]. On the contrary, all four natural abundance resonances of mobile PHB were resolved in cell suspensions of *A. eutrophus, Methylobacterium* AM1 and *Methylobacterium extorquens* [3,4].

PHB was found in nearly all described methanotrophic bacteria with the RuMP and the serine pathways [6,14]. It is generally believed that in methanotrophic bacteria synthesis and degradation of PHB is similar to that of most heterotrophic microorganisms that

have been investigated in detail [2]. One modification in the PHB cycle for the Type II methanotroph *Methylosinus trichosporium* as well as for *Zooglea ramigera* has been described. PHB is degraded by 3-hydroxybutyrate dehydrogenase and acetoacetyl-CoA synthetase whereas an acetoacetate:succinate CoA transferase is involved in acetoacetate transformation in several heterotrophic bacteria [1] (Figure 3).

The catabolism of intracellular reserve material is intriguing because it serves as carbon and energy source for a wide variety of microorganisms under different stress conditions [1,8]. However, not much is known about intracellular PHB degradation and especially about further metabolism of β-hydroxybutyrate and acetoacetate. The fact that living cells of aerobic methanotrophs can be found in anaerobic zones of many types of environments gave us the idea that PHB degradation might be involved in the survival of these bacteria under anaerobic conditions. Anaerobic fermentation of PHB in cells of the obligate methanotrophs was demonstrated in our experiments. The slow kinetics of degradation, which could be explained by the relatively immobile stage of PHB, allowed us to reveal a number of products like β-hydroxybutyrate, butyrate, acetate and more reduced compounds like acetone, isopropanol and 2,3-butanediol. Figure 3 shows how these products may be derived from the general PHB cycle. The degradation of PHB leads to the formation of acetate whereby ATP can be produced. Acetone, isopropanol and 2,3-butanediol may be sinks of reducing power released during PHB degradation.

Succinate is a compound of the TCA cycle; however, the TCA cycle does not operate under anaerobic conditions. Under anoxic conditions succinate can be formed *via* malyl-CoA, malate and fumarate (Figure 3).

The fact that low levels of reduced end products appeared during aerobic PHB synthesis might indicate that under these conditions an excess of reducing power is present in the cells. However, although the experiment was carried out at relatively high oxygen concentrations (between 10% and 20%) with an excess of methane in the headspace of the NMR tube, a rotation speed of 3–4 Hz of the glass balls may be insufficient to apply nonlimiting oxygen and methane conditions to a dense cell suspension. In this case, microzones with varying gas limitation could exist in the suspension for both gases and the observed compounds might thus reflect simultaneous formation and breakdown of PHB.

Among the products that are formed during PHB degradation, 2,3-butanediol is the most reduced compound. The involvement of this product in PHB-cycle activity was confirmed during anaerobic PHB breakdown following aerobic PHB synthesis (Figure 1B). The resonance of the labelled CH_3 group of 2,3-butanediol increased in intensity with a concomitant decrease in the signal intensity of the $^{13}C_4$ group in PHB. The metabolic pathway of 2,3-butanediol formation is still unclear. In general, 2,3-butanediol is formed by condensation of pyruvate molecules or by the coupling of acetaldehyde and pyruvate. However, pyruvate formation from acetyl-CoA during PHB degradation is unlikely. HS-CoA-dependent pyruvate oxidation to acetyl-CoA in aerobic bacteria is an irreversible reaction [11]. An alternative route for formation of alcohol is related to the 2,3-butanediol cycle [16,23]. We suggest that 2,3-butanediol may be formed by the coupling of acetate and acetaldehyde formed by reduction of acetate. The putative pathway of 2,3-butanediol formation is shown in Figure 4.

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References

1 Anderson AJ and EA Dawes. 1990. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. *Microbiol Rev* 54: 450–472.
2 Asenjo JA and JS Suk. 1986. Microbial conversion of methane into poly-β-hydroxybutyrate (PHB): growth and intracellular product accumulation in a Type II methanotroph. *J Ferment Technol* 64: 271–278.
3 Barnard GN and JKM Sanders. 1988. Observation of mobile poly(β-hydroxybutyrate) in the storage granules of *Methylobacterium* AM1 by *in vivo* ^{13}C-NMR spectroscopy. *FEBS Lett* 231: 16–18.
4 Barnard GN and JKM Sanders. 1989. The poly-β-hydroxybutyrate granule *in vivo*. A new insight based on NMR spectroscopy of whole cells. *J Biol Chem* 264: 3286–3291.
5 Bonthrone KM, J Clauss, DM Horowitz, BK Hunter and JKM Sanders. 1992. The biological and physical chemistry of polyalkanoates as seen by NMR spectroscopy. *FEMS Microbiol Rev* 103: 269–278.
6 Bowman JP, LI Sly, PD Nicols and AC Hayward. 1993. Revised taxonomy of the methanotrophs: description of *Methylobacter* gen. nov., emendation of *Methylococcus*, validation of *Methylosinus* and *Methylocystis* species, and a proposal that the family *Mehtylococcaceae* includes only the Group I methanotrophs. *Int J Syst Bacteriol* 43: 735–753.
7 Costa C, C Dijkema, M Friedrich, P Garcia-Encina, F Fernandes-Polanco and AJM Stams. 2000. Denitrification with methane as electron donor in oxygen limited bioreactors. *Appl Microbiol Biotechnol* 53: 754–762.
8 Dawes EA and PJ Senior. 1973. The role and regulation of energy reserve polymers in micro-organisms. *Adv Microb Physiol* 10: 135–266.
9 Doi Y, Y Kawaguchi, Y Nakamura and M Kunioka. 1989. Nuclear magnetic resonance studies of poly(3-hydroxybutyrate) and polyphosphate metabolism in *Alcaligenes eutrophus*. *Appl Environ Microbiol* 55: 2932–2938.
10 Doi Y, M Kunioka, Y Nakamura and K Soga. 1987. Biosynthesis of copolyesters in *Alcaligenes eutrophus* H16 from ^{13}C-labelled acetate and propionate. *Macromolecules* 20: 2988–2991.
11 Gottschalk G. 1986. Bacterial fermentation. In: Bacterial Metabolism. New York: Springer-Verlag, pp. 210–280.
12 Graham DW, JA Chaudhary, RS Hanson and RG Arnold. 1993. Factors affecting competition between Type I and Type II methanotrophs in two-organism, continuous-flow reactors. *Microb Ecol* 25: 1–17.
13 Griebel R, Z Smith and JM Merrick. 1968. Metabolism of poly-β-hydroxybutyrate: I. Purification, composition and properties of native poly-β-hydroxybutyrate granules from *Bacillus megaterium*. *Biochemistry* 7: 3676–3681.
14 Hanson RS and TE Hanson. 1996. Methanotrophic bacteria. *Microbiol Rev* 60: 439–471.
15 Hippe H and G Schlegel. 1967. Hydrolyse von PHBs durch intracellulare depolymerase von *Hydrogenomonas* H16. *Arch Microbiol* 56: 257–299.
16 Juni E and GA Heym. 1956. A cyclic pathway for the bacterial dissimilation of 2,3-butanediol, acetylmethylcarbinol and diacetyl: I. General aspects of the 2,3-butanediol cycle. *J Bacteriol* 71: 425–432.
17 Law JH and RA Slepecky. 1961. Assay of poly-β-hydroxybutyric acid. *J Bacteriol* 82: 33–36.
18 Macrae RM and JF Wilkinson. 1958. Poly-β-hydroxybutyrate metabolism in washed suspensions of *Bacillus cereus* and *Bacillus megaterium*. *J Gen Microbiol* 19: 210–222.
19 Maimcrona-Friberg K, A Tunlid, P Marden, S Kjellberg and G Odham. 1986. Chemical changes in cell envelope and poly-β-hydroxybutyrate during short-term starvation of a marine bacterial isolate. *Arch Microbiol* 144: 340–345.

20 Nicolay K, KJ Hellingwerf, R Kaptein and WN Konings. 1982. Carbon-13 nuclear magnetic resonance studies of acetate metabolism in intact cells of *Rhodopseudomonas sphaeroides*. *Biochim Biophys Acta* 720: 250–258.
21 Saito T, H Saegusa, Y Miyata and T Fukui. 1992. Intracellular degradation of poly(3-hydroxybutyrate) granules of *Zoogloea ramigera* I-16-M. FEMS. *Microbiol Rev* 103: 333–338.
22 Tavernier P, I Besson, J-Ch Portais, J Courtois, B Courtois and J-N Barbotin. 1998. *In vivo* ^{13}C-NMR studies of polymer synthesis in *Rhizobium meliloti* M5N1 strain. *Biotechnol Bioeng* 58: 250–253.
23 Ui S, T Hosaka, K Watanabe and A Mimura. 1998. Discovery of a new mechanism of 2,3-butanediol stereoisomer formation in *Bacillus cereus* YUF-4. *J Ferment Bioeng* 85: 79–83.
24 Wang JG and LR Bakken. 1998. Screening of soil bacteria for poly-β-hydroxybutyric acid production and its role in the survival of starvation. *Microb Ecol* 35: 94–101.
25 Williamson DH and JF Wilkinson. 1958. The isolation and estimation of the poly-β-hydroxybutyrate inclusions of *Bacillus* species. *J Gen Microbiol* 19: 198–209.
26 Wittenbury R, Philips KC and Wilkinson JF. 1970. Enrichment, isolation and some properties of methane utilizing bacteria. *J Gen Microbiol* 61: 210–218.