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Pathway of anaerobic poly-β-hydroxybutyrate degradation by *Ilyobacter delafieldii*

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Abstract

Ilyobacter delafieldii produced an extracellular poly-β-hydroxybutyrate (PHB) depolymerase when grown on PHB; activity was not detected in cultures grown on 3-hydroxybutyrate, crotonate, pyruvate or lactate. PHB depolymerase activity was largely associated with the PHB granules (supplied as growth substrate), and only 16% was detected free in the culture supernatant. Monomeric 3-hydroxybutyrate was detectable as a product of depolymerase activity. The monomer was fermented to acetate, butyrate and H_2. After activation by coenzyme A transfer from acetyl-CoA or butyryl-CoA, the resultant 3-hydroxybutyryl-CoA was oxidized to acetoacetyl-CoA (producing NADH), followed by thiolytic cleavage to yield acetyl-CoA which was further metabolized to acetyl-phosphate, then to acetate with concomitant ATP production. The reducing equivalents (NADH) could be disposed of by the evolution of H_2, or by a reductive pathway in which 3-hydroxybutyryl-CoA was dehydrated to crotonyl-CoA and reduced to butyryl-CoA. In cocultures of *I. delafieldii* with *Desulfovibrio vulgaris* on PHB, the H_2 partial pressure was much lower than in the pure cultures, and sulfide was produced. Thus interspecies hydrogen transfer caused a shift to increased acetate and H_2 production at the expense of butyrate.

Introduction

Poly-β-hydroxybutyrate (PHB) has been suggested as a versatile thermoplastic which is biodegradable and obtained from renewable raw materials (Holmes 1985; Steinbüchel 1992; Brandl & Püchner 1992). It is produced by a wide variety of bacteria, both aerobic and anaerobic (Anderson & Dawes 1990). Its degradation has been shown to be mediated by aerobic bacteria (Delafield et al. 1965; Anderson & Dawes 1990) and to occur in a variety of environments, both oxic and anoxic (Holmes 1985; Budwill et al. 1992). Recently, PHB has been shown to support growth of an obligately anaerobic bacterium, *Ilyobacter delafieldii* (Janssen & Harfoot 1990). This organism was enriched on crotonate, and fermented PHB as well as the related organic acids 3-hydroxybutyrate and crotonate to acetate, butyrate and H_2. The pathway of PHB degradation by *I. delafieldii* is the subject of the investigation presented in this paper.

Materials and methods

Enrichment cultures

Enrichment cultures were set up from 3 estuarine

sediments, sediment from a polluted pond, sediment from a meat works settling pond, and sludge from an effluent pond receiving water from an anaerobic solids digester. One ml sediment samples were inoculated into 25 ml medium (Janssen & Harfoot 1990) containing 2.5 g granular PHB and 1 g yeast extract per litre, and incubated at 34° C for one month.

Organisms and culture conditions

Ilyobacter delafieldii strain 10 crl (DSM 5704) was from the authors' collection, and grown as previously described (Janssen & Harfoot 1990). Granular PHB for measuring PHB depolymerase activity was purchased from Sigma Chemical Co. (St. Louis, USA), while fibrous PHB for growth of cultures for measurement of intracellular enzymes was a gift from Dr A Steinbüchel (Göttingen, FRG).

Desulfovibrio vulgaris strain Marburg (DSM 2119) was from the collection of the authors, and grown on a sulfide-reduced bicarbonate-buffered freshwater medium supplemented with 20 mM Na_2SO_4 and a 7 vitamin mixture (Dörner & Schink 1990) with 10 mM Na DL-lactate as the carbon and energy source under a headspace of N_2/CO_2 (4:1). Coculture experiments were carried out in this medium with 20 mM Na_2SO_4 and 1 g/l fibrous PHB.

All cultivations in liquid culture were carried out in 60 ml or 200 ml volumes in closed vessels of 120 ml or 250 ml total volume. Inoculum was usually added to 1% (v/v). Anoxically washed cells of 50 ml late-log/early stationary phase cultures of *Desulfovibrio vulgaris* were used to inoculate the coculture experiments. Thus a ratio of approximately 100:1 cells of *D. vulgaris : I. delafieldii* was obtained at the start of these experiments.

Chemical analyses were carried out as described by Dörner & Schink (1990). All inculations were at 30° C unless noted otherwise. Calculations of free energy changes and dissolved hydrogen concentrations were from Thauer et al. (1977) and Wilhelm et al. (1977), respectively. Sulfide was measured after Trüper & Schlegel (1964).

Depolymerase activity measurements

The formation of 3-hydroxybutyrate was followed as an indication of enzyme activity in essentially cell-free systems. To determine the localization of enzyme activity, three combinations were prepared: (1, supernatant) 10 mg sterile granular PHB was added to 2.5 ml of filtered culture supernatant plus 2.5 ml of uninoculated medium; (2, PHB-bound) the PHB remaining in the culture was washed twice in uninoculated medium, then 10 mg of this PHB was resuspended in 5.0 ml of uninoculated medium; (3, total) 10 mg of washed PHB was added to 2.5 ml of uninoculated medium plus 2.5 ml filtered culture supernatant. These three preparations were incubated aerobically. The accumulation of 3-hydroxybutyrate was followed by HPLC (Patel et al. 1987).

Intracellular enzyme activities

Cultures were harvested anoxically by centrifugation in glass bottles at 3000 × g for 30 min under N_2 at 4° C, washed in N_2-gassed anoxic 100 mM HEPES·KOH, pH 7.6, and centrifuged again. Cells were resuspended in a minimal amount of buffer and disrupted by 3 passages through a French press (Aminco, Silver Spring, Maryland) at 136 MPa under N_2. Unbroken cells and cell debris were removed by centrifugation at 3000 × g for 30 min under anoxic conditions at 4° C. The supernatant was stored on ice or frozen until measurement of enzyme activities. Assays were carried out as described by Brune & Schink (1990) using a PM4 (Carl Zeiss, Oberkochen, FRG) or 100–40 (Hitachi, Tokyo, Japan) photometer at 30° C. Biochemicals and enzymes were obtained from Sigma, München; Serva, Heidelberg; and Boehringer, Mannheim, FRG. Protein was quantified by the micro-assay of Bradford (1976) using bovine serum albumin as a standard.

Acetate kinase (EC 2.7.2.1) was assayed as described by Bergmeyer et al. (1974). Acetyl-CoA acetyltransferase (3-ketoacyl-CoA thiolase, EC 2.3.1.9), enoyl-CoA hydratase (EC 4.2.1.17/55), acyl-CoA dehydrogenase with butyryl-CoA (EC 1.3.99.3) and butyryl-CoA:acetate CoA transferase

(EC 2.8.2.8), were assayed as described by Wofford et al. (1986). Hydrogenase (EC 1.18.99.1) was assayed with methylviologen by the method of Badziong & Thauer (1980).

Phosphate acetyltransferase (EC 2.3.1.8) was assayed as described by Wofford et al. (1986), or in a reaction mixture containing: 100 mM Tris·HCl, pH 8.0, 100 mM KCl, 0.1 mM acetyl-CoA, and cell extract. After monitoring the change in absorbance at 232 nm (ε_{232} = 4.44 mM^{-1} cm^{-1}) due to deacylase activity, the reaction was started by the addition of 20 mM KH_2PO_4. Phosphate butyryltransferase (EC 2.3.1.19) was assayed by the second method, using butyryl-CoA instead of acetyl-CoA.

Acetyl-CoA: 3-hydroxybutyrate CoA transferase (EC 2.8.3.-) and butyryl-CoA: 3-hydroxybutyrate CoA transferase (EC 2.8.3.-) were measured in the following reaction mixture: 100 mM Tricine·KOH, pH 8.0, 10 mM DTE, 2 mM NAD^+, 10 mM 3-hydroxybutyrate, 5 U 3-hydroxybutyryl-CoA dehydrogenase, and cell extract. The reaction was started by the addition of 0.5 mM acetyl-CoA or butyryl-CoA.

3-Hydroxybutyrate: CoA ligase (EC 6.2.1.-) and acetoacetate: CoA ligase (EC 6.2.1.16) were measured in the following reaction mixture: 100 mM Tris·HCl, pH 8.1, 10 mM Li acetoacetate plus 0.2 mM NADH or 10 mM Na 3-hydroxybutyrate plus 2 mM NAD^+, 5 mM $MgCl_2$, 1 mM CoASH, and cell extract. The reaction was started by the addition of 2 mM ATP or GTP.

3-Hydroxybutyrate dehydrogenase (EC 1.1.1.30) and 3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.35/36/157) were determined using the following reaction mixture: 100 mM HEPES·KOH, pH 7.2, 1 mM DTE, 2 mM NAD^+ or $NADP^+$, and cell extract. The reaction was started by the addition of 5 mM 3-hydroxybutyrate or 0.5 mM 3-hydroxybutyryl-CoA.

The oxidation or reduction of electron donors and acceptors was followed as described by Möller-Zinkhan & Thauer (1988). Enzyme activities are expressed as µmol substrate transformed per min per mg cell-free crude extract protein. The reduction of 2 µmol methylviologen was defined as the oxidation of 1 µmol substrate.

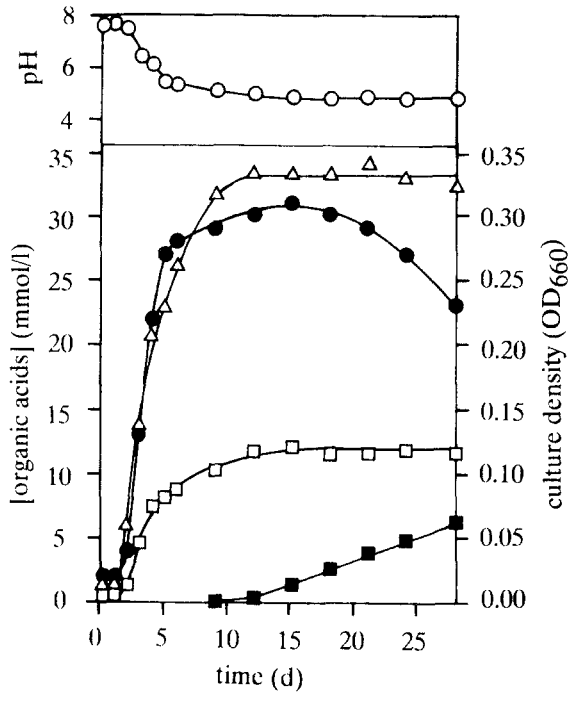

Fig. 1. Growth curve of *Ilyobacter delafieldii* on 2 g/l granular PHB and 1 g/l yeast extract. At the end of growth not all the PHB had been utilized. Symbols: (△) acetate, (□) butyrate, (■) 3-hydroxybutyrate, (●) culture density at 660 nm, (○) pH.

Results and discussion

PHB degradation in anaerobic sediments

Enrichments on PHB showed that granular PHB (approx. 0.1 mm granules) was able to be degraded under strictly anoxic conditions within one month. Acetate and butyrate production occurred in all the enrichments except for that from the effluent pond sludge which produced methane as the main end product. Typically 35 mM acetate plus 11 mM butyrate were formed.

PHB degrading activity

When *Ilyobacter delafieldii* was grown on PHB in liquid culture, 3-hydroxybutyrate began to accumulate once acetate and butyrate production and culture growth halted (Fig. 1). The rate of monomer production was obviously the rate-limiting step in the total degradation. The specific growth rate of *I.*

Table I. Localization of PHB depolymerase activity in a nine day old culture of *Ilyobacter delafieldii* grown on PHB.

Preparation	Activity ($\mu mol \cdot h^{-1} \cdot l^{-1}$)	
Culture filtrate	7.3	45.8 (total)
Washed PHB granules	38.5	
Culture filtrate + washed PHB granules		51.0

delafieldii on 3-hydroxybutyrate was 0.13 h^{-1} (t_d = 5.3 h), whereas on PHB the specific growth rate was 0.034 h^{-1} (t_d = 20 h). Sterile PHB granules autoclaved in medium did not show any degradation.

Observations of a PHB-grown culture by phase contrast microscopy revealed that the cells were not physically attached to the PHB granules. Agar-shake cultures of *I. delafieldii* with PHB suspended in the agar developed clearing zones of up to 5 mm radius around the lens-shaped colonies, corresponding to the disappearance of PHB. This suggests the presence of an extracellular PHB depolymerase.

Depolymerase activity

The location of the PHB depolymerase activity was determined in a nine day old culture of *I. delafieldii* grown on PHB. PHB and cell-free (centrifuged) supernatant were assayed for PHB depolymerase activity. The PHB depolymerase activity (calculated from 3-hydroxybutyrate accumulation) was largely associated with the PHB granules from the nine day old culture of *I. delafieldii* (Table 1). Absorption of the depolymerase to the PHB granules has also been observed in cultures of PHB-degrading *Pseudomonas* spp. (Dawes & Senior 1973).

When *I. delafieldii* was cultured on other growth-supporting carbon sources (crotonate, 3-hydroxybutyrate, lactate or pyruvate, all at 20 mM), no PHB depolymerase activity was detected.

The data obtained suggested the production of monomeric 3-hydroxybutyrate which was then fermented to acetate and butyrate in the same way as the monomer is. Production of dimers or higher polymers could not be excluded since the methods of analysis could not detect these. The end products of PHB and 3-hydroxybutyrate degradation were the same (Janssen & Harfoot 1990). The immediate products of PHB degradation by aerobes have been reported to be monomeric 3-hydroxybutyrate (Chowdhury 1963), dimeric 3-hydroxybutyrate (Merrick et al. 1962, Tanio et al. 1982) and mixtures of monomer, dimer and trimer (Delafield et al. 1965). The characteristics of the depolymerase reaction(s) of *I. delafieldii* remain to be investigated.

Table 2. Enzyme activities measured in crude cell-free extracts of *I. delafieldii* grown on PHB.

Enzyme (acceptor if appropriate)		EC number	Specific activity[a] ($\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$)
1[b]	Acetyl-CoA 3-hydroxybutyrate CoA-transferase	2.8.3.-	1.07
2	Butyryl-CoA 3-hydroxybutyrate CoA-transferase	2.8.3.-	1.18
3	3-Hydroxybutyryl-CoA dehydrogenase (NAD)	1.1.1.35	0.25
4	Acetyl-CoA acetyltransferase [thiolase]	2.3.1.9	0.25
5	3-Enoyl-CoA hydratase [crotonase]	4.2.1.17/55	0.52
6	Butyryl-CoA dehydrogenase (DCPIP)	1.3.99.2/3	0.21
7	Ferredoxin:NAD oxidoreductase	1.18.1.3	1.16
8	Hydrogenase (MV)	1.18.99.1	0.16
9	Phosphate acetyltransferase	2.3.1.8	2.42[c]
10	Acetate kinase	2.7.2.1	0.20
11	Butyryl-CoA acetate CoA transferase	2.8.2.8	0.92

[a] The following enzymes were not detected (< 0.001): 3-hydroxybutyrate CoA ligase (EC 6.2.1.-), acetoacetate CoA ligase (EC 6.2.1.16), 3-hydroxybutyrate dehydrogenase with NAD (EC 1.1.1.30) or NADP (EC 1.1.1.-), 3-hydroxybutyryl-CoA dehydrogenase with NADP (EC 1.1.1.36/157), phosphate butyryltransferase (EC 2.3.1.19), ferredoxin:NADP oxidoreductase (EC 1.18.1.2).

[b] Numbers refer to the reactions in Fig. 2.

[c] Using method 1 (see Materials and methods).

Pathway of 3-hydroxybutyrate fermentation

Measurements of enzyme activities in crude cell-free extracts of PHB-grown cells of *I. delafieldii* (Table 2) revealed that 3-hydroxybutyrate was activated by CoA transfer from acetyl-CoA or butyryl-CoA. The resultant 3-hydroxybutyryl-CoA was then oxidized to acetoacetyl-CoA by an NAD-linked dehydrogenase. Thiolytic cleavage of acetoacetyl-CoA resulted in two acetyl-CoA residues which were then metabolized to acetate with concomitant ATP production. The resultant NADH was reoxidized through reduction of crotonyl-CoA formed by an enoyl-CoA hydratase from 3-hydroxybutyryl-CoA, or alternatively by evolution of reducing equivalents as hydrogen gas. Butyryl-CoA could apparently be used to activate acetate to allow ATP formation. However, since two acyl-CoA esters have to be used to activate two 3-hydroxybutyrate molecules which eventually form three acyl-CoA esters, only one ATP will be formed per two substrate molecules fermented. This is in agreement with the growth yields obtained (Janssen & Harfoot 1990) of 5.1 g cell dry weight per mol 3-hydroxybutyrate. From the enzyme activities measured, a metabolic pathway leading to the products detected could be postulated (Fig. 2).

Kinase activity could also be measured with butyrate (0.18 µmol per min and mg protein), but the absence of phosphate butyryltransferase activity suggests that this was due to enzyme nonspecificity when measuring in the reverse direction with high substrate concentrations, rather than a physiologically important reaction. The measured enzyme activities indicate that butyrate formation is due to CoA transfer to either 3-hydroxybutyrate or acetate. The activation of 3-hydroxybutyrate with either acetyl-CoA or butyryl-CoA and the CoA transferase activity between acetate and butyrate, together with the alternative electron sinks of butyrate formation and H_2 formation, shows that *I. delafieldii* can balance the oxidative and reductive branches of 3-hydroxybutyrate catabolism using various interconnecting mechanisms (Fig. 2).

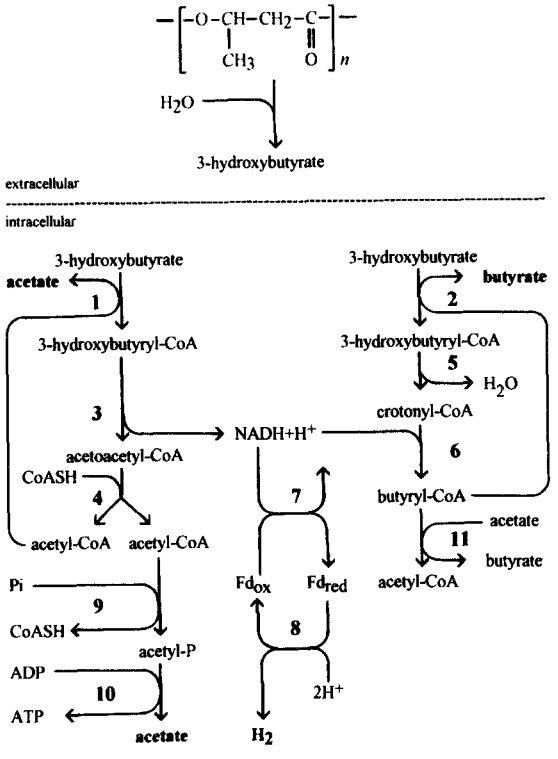

Fig. 2. Pathway of PHB and 3-hydroxybutyrate fermentation to acetate, butyrate, and H_2 by *I. delafieldii*. The numbers refer to the enzyme activities listed in Table 2. Reduced NAD can reduce ferredoxin (Fd) at low pH_2 (reaction 7), or crotonyl-CoA to butyryl-CoA (reaction 6). The possibility of oligomer production during PHB hydrolysis remains to be investigated.

Table 3. Fermentation of 3-hydroxybutyrate (600 µmol) and PHB (577 µmol 3-hydroxybutyrate) by pure cultures of *Ilyobacter delafieldii*, and cocultures of *I. delafieldii* and *Desulfovibrio vulgaris*.

Culture	Substrate	Products (µmol)				Acetate: Butyrate Ratio	Electron Balance (%)[a]
		Acetate	Butyrate	H_2	HS^-		
pure	3-hydroxybutyrate	590	260	9.75	–	2.27	92.1
pure	PHB	552	238	8.58	–	2.32	88.5
coculture	PHB	704	175	0.15	33	4.02	90.5

[a] Electron balance excludes assimilatory metabolism.

PHB degradation in co-culture with a hydrogenotroph

Co-cultures of *I. delafieldii* and the hydrogenotrophic sulfate-reducing bacterium *Desulfovibrio vulgaris* grown on PHB favoured acetate production at the expense of butyrate production (Table 3). In these cultures, the acetate:butyrate ratio was 4.02, in contrast to ratios of about 2.3 found for pure culture fermentation of PHB or 3-hydroxybutyrate.

The dissolved H_2 concentrations in the cocultures were around 37 µM (at 30° C), while in the pure cultures on 3-hydroxybutyrate and PHB the dissolved H_2 concentrations were around 2400 and 2100 µM, respectively. There was also sulfide production in the coculture; the total 33 µmol sulfide produced accounted for 132 µmol H_2. Thus the actual H_2 production in the coculture (132 µmol) was 13 to 15 times that of the pure culture on 3-hydroxybutyrate (9.8 µmol) or PHB (8.6 µmol). This indicated an incompletely-coupled hydrogen removal by the sulfate-reducing bacterium resulting in an increased acetate:butyrate product ratio. From the end-products measured and the pathway of substrate fermentation (Fig. 2), ATP yields of 0.53 and 0.54 per mol 3-hydroxybutyrate for the pure cultures on 3-hydroxybutyrate and PHB, respectively, and a yield of 0.67 ATP per 3-hydroxybutyrate for the culture on PHB can be calculated.

PHB was transformed to methane and CO_2 by an enrichment culture obtained from sewage sludge (Budwill et al. 1992). These authors suggested a fermentation to acetate and H_2, and discounted butyrate production. PHB was fermented to acetate and butyrate by our enrichment cultures from estuarine, freshwater lake, and polluted pond sediments with acetate: butyrate ratios of 3.0 to 3.9. *I. delafieldii*-like organisms were enriched from these culturres (unpublished data). Significant methane production was found only in cultures inoculated with sewage sludge which presumably contained a much higher population of methanogenic and fatty acid-oxidizing syntrophic bacteria. The pathway of 3-hydroxybutyrate fermentation determined in the present study allows a theoretical complete shift to acetate and H_2 production, providing the rate of H_2 removal is not limiting, It can thus be expected that butyrate production only occurs when large amounts of polymer are suddenly made available and the hydrogenotrophic population cannot remove H_2 at a sufficient rate.

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