Control of polyhydroxyalkanoate synthesis in
Azotobacter vinelandii strain UWD

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INTRODUCTION

In recent years the controlled, large-scale production of polyhydroxyalkanoates (PHAs) by bacteria has become a subject of increasing interest. This material is represented most commonly by poly-β-hydroxybutyrate (PHB), which is stored in an intracellular inclusion by a great variety of bacteria (Anderson & Dawes, 1990). In 1982, ICI discussed the exploitation of this polyester as a natural, biodegradable plastic (Howells, 1982; King, 1982). PHB shares characteristics with polypropylene, but is more brittle (Howells, 1982). However, the random incorporation of 8 to 20% 3-hydroxyvalerate (HV) subunits into the polymer results in P(HB-co-HV), a copolymer

**Abbreviations:** AcCoA, acetyl-coenzyme A; AcAc, acetocetate; AcAcCoA, acetocetyl-coenzyme A; BOHB-DH, d-(-)-β-hydroxybutyrate dehydrogenase; CoA, coenzyme A; HV, hydroxyvaleric acid; 4-PA, 4-pentenoic acid; PHA, poly-β-hydroxyalkanoic acid; PHB, poly-β-hydroxybutyric acid; P(HB-co-HV), poly-(β-hydroxybutyric-co-β-hydroxyvaleric) acid; SNAD(P)*, thio-NAD(P)*; Y<sub>non-PHB</sub> yield of g polymer (P) per g non-PHB residual mass (RM).

Keywords: Azotobacter vinelandii, polyhydroxyalkanoate synthesis, biopolymers, 3-ketothiolase, acetocetyl-CoA reductase, 3-hydroxybutyrate dehydrogenase
with superior mechanical properties (Luzier, 1992). Co-polymer formation is promoted by feeding precursors to the microbial culture during its growth on a primary C source, usually a sugar (Byrom, 1987). Propionic or valeric acid, or their salts, are most frequently used as precursors for HV formation. ICI is currently producing P(HB-co-HV) in a two-stage fermentation using Alcaligenes eutrophus (Byrom, 1987) and has successfully test-marketed bioplastic shampoo bottles in Europe (Emsley, 1991).

Recently, a stable, capsule-negative mutant of Azotobacter vinelandii was described that produced relatively large amounts of PHB during exponential growth (Page & Knosp, 1989). Az. vinelandii UWD was constructed by the transformation of heavily mutagenized DNA from Az. vinelandii strain 113 into the capsule-negative recipient strain UW (Page & Knosp, 1989). Although the mutation leading to PHB accumulation was determined to be a defect in the respiratory oxidation of NADH, it was possible that other mutations affecting PHB synthesis had been inherited as well. Also, an examination of the enzymes involved in PHA synthesis could shed light on substrate specificity for copolymer formation, the apparent rate-limiting step in HV synthesis (Page et al., 1992), and why Azotobacter accumulates such a high molecular mass polymer (usually 1-4 MDA). Although Azotobacter beijerinckii has been used extensively in the past for studies of PHB synthesis, enzymology and control (Dawes & Senior, 1973; Jackson & Dawes, 1976; Ritchie et al., 1971; Senior et al., 1972; Senior & Dawes, 1971, 1973; Stockdale et al., 1968; Ward et al., 1977), interest in substrate specificity has only arisen recently, with the evolution of commercial applications in this field. Az. beijerinckii was a leading contender for commercial PHB production, but was abandoned because of strain instability and capsule formation (Byrom, 1987).

The first step in PHB biosynthesis is catalysed by 3-ketothiolase which condenses two molecules of acetyl-CoA (AcCoA). This step is strongly regulated by free CoA, with a high ratio of AcCoA to CoA being the trigger for PHB biosynthesis (Oeding & Schlegel, 1973). The next steps are the reduction of the acetoacetyl-CoA (AcAcCoA) to 3-(−)-3-hydroxybutyryl-CoA and polymerization. Recently, the control of these enzymes in Al. eutrophus was described (Haywood et al., 1988a, b, 1989). These studies demonstrated two thiolases, enzyme-A involved in PHA biosynthesis and enzyme-B involved in β-oxidation. Also, two reductases were found, one NADPH-specific which produced 3-(−)-3-hydroxyacyl-CoA for PHB synthesis, and one NADH-specific which reacted with dl-3-hydroxyacyl-CoA. It was suggested that the NADH-specific enzyme played a role in removing 3-(−)-3-hydroxyacyl-CoA from the β-oxidation pathway and directing it into PHA biosynthesis, via a 3-ketoacyl-CoA intermediate. Another point of control in PHB accumulation is at the 3-hydroxybutyrate dehydrogenase, which mediates the internal recycling of AcAc from polymer turnover (Senior & Dawes, 1973). This step is negatively regulated by NAD(P)H in Al. eutrophus (Oeding & Schlegel, 1973) and Az. beijerinckii (Senior & Dawes, 1973).

Interest in the commercial production of PHAs is high because the worldwide demand for biodegradable plastics is growing and in many cases the demand is driven by legislation (Leaversuch, 1987). The market demand has been estimated at 1.4 million tonnes per year by the year 2000 (Leaversuch, 1987). With degradable polymers produced by petrochemical companies valued at approximately $6-6 (USA) per kg, this will be a multi-billion dollar per year market in the future (Lindsay, 1992). However, P(HB-co-HV) is not economically priced (Lindsay, 1992), and alternative routes for the production of PHA must be sought. Az. vinelandii strain UWD produces PHB during active (exponential) growth in media containing unrerefined sugars or molasses, which will offer significant savings in the cost of the primary substrate (Page, 1992a, b; Page et al., 1992). This strain forms commercially useful P(HB-co-HV) when fed sodium valerate and β-oxidation has been implicated in generating the intermediates for HV formation (Page et al., 1992). However, nothing has been reported about the substrate specificity or control of the enzymes involved in PHA synthesis in this organism.

METHODS

Bacterial strains and growth conditions. Azotobacter vinelandii UWD (ATCC 53799) and its parent strain UW (ATCC 13705; Page & Knosp, 1989) were grown in a glucose medium composed of 0.81 mM MgSO\(_4\), 0.58 mM CaSO\(_4\), 50 μM ferric citrate and 1 μM Na\(_2\)MoO\(_4\) in 5 mM potassium phosphate buffer, pH 7.2, containing 3% (w/v) glucose, 0.1% (w/v) ammonium acetate, and 0.1% (w/v) fish peptone (product H0100BT from Protan A/S) (Page, 1992b). Azotobacter beijerinckii (DSM 1041) was grown in glucose medium, but without fish peptone addition. Alcaligenes eutrophus (DSM 545) was grown in the medium described by Ramsay et al. (1990). Fish peptone was determined to not have a beneficial effect on PHB yield in Az. beijerinckii or Al. eutrophus. The medium (50 ml) was contained in a 500 ml Erlenmeyer flask and inoculated (4%, v/v) with a culture pregrown in the same medium overnight. Sodium valerate or other α-alkanotes were used as primary carbon sources (30 mM final concentration) or were added as precursors (10 mM final concentration) after 12 h incubation of the cultures in glucose medium (Page et al., 1992). The culture was incubated for 20–24 h at 28–30 °C with rotary shaking at 225 r.p.m. on a New Brunswick Scientific model G-10 shaker.

Chemical analysis of cell polymer content and culture fluids. The cells were collected from the culture fluids by centrifugation and the total dry weight, PHA dry weight, cell protein and copolymer content were determined as described previously (Page et al., 1992).

Cell extract preparation and enzyme purification. Strain UWD cells were washed once in 8 mM Tris/HCl buffer, pH 7.8, then resuspended in the same buffer and ruptured by sonication (five to seven 20 s bursts at medium power at 1 min intervals). The debris was removed by centrifugation at 10000 g for 15 min and the clear cell extract was stored at −20 °C.
(a) 3-Ketothiolase. The enzyme was partially purified as described by Haywood et al. (1988a). The enzyme activity was followed by the thiolysis reaction of Nishimura et al. (1978). One unit of enzyme activity was the amount of enzyme required to catalyze the cleavage of 1 μmol AcAcCoA min⁻¹. The cell extract was precipitated with protamine sulfate and 1-3 ml of the supernatant fluid containing about 100 units of enzyme were applied to a DEAE Sepharose CL-6B (Pharmacia) column (16 cm diameter, 15 cm height). The recovery was about 100 units, with a specific activity of 186 units (mg protein)⁻¹; a 12.5-fold purification over the activity in the original cell extract.

The ability of the 3-ketothiolase to catalyze the condensation of AcCoA and another enoyl-CoA molecule (usually AcCoA) was measured as described by Oeding & Schlegel (1973). One unit of enzyme activity was the amount of enzyme needed to produce 1 μmol AcAcCoA min⁻¹. The assay was started with the addition of AcCoA, rather than enzyme. The condensation reaction of Nishimura et al. (1978), as used by Haywood et al. (1988a), was found to be unacceptable because of a 2-3 min lag before the reaction started.

(b) AcAcCoA reductase. The enzyme was partially purified and assayed as for enzyme-A as described by Haywood et al. (1988b). One unit of activity was the amount of enzyme required to oxidize 1 μmol NAD(P)(H) min⁻¹. The cell extract was made to 1 mM dithiothreitol, precipitated with protamine sulfate and about 100 units of enzyme activity were applied to the DEAE Sepharose CL-6B column used for 3-ketothiolase purification. A single AcAcCoA-reductase was eluted from the column and the recovery was about 39 units with a specific activity of 6'9 units (mg protein)⁻¹, a purification of 1.5-fold over the activity in the original cell extract.

(c) β-Hydroxybutyrate dehydrogenase. This enzyme (BOHB-DH) was confirmed to be unstable and lost activity after freezing and thawing (Jurshuk et al., 1968). Therefore, the cell extract was prepared by sonication in 0.2 M potassium phosphate buffer, pH 8.0, containing 1 mM MgCl₂ and was made to 20% (v/v) glycerol immediately after centrifugation. The enzyme was partially purified as described by Tal et al. (1990). The cell extract was precipitated with 55% ammonium sulfate and the resulting pellet was dissolved in phosphate buffer containing MgCl₂ and glycerol, dialysed against the same buffer containing MgCl₂, then made to 20% glycerol after dialysis. The enzyme was applied to the Sepharose CL-6B column equilibrated with 5 mM potassium phosphate, pH 8.0, containing 1 mM MgCl₂ and 100 mM KCl. Enzyme activity was followed in a 1 ml assay containing 0.1 mM potassium phosphate, pH 8.0, 10 mM β-hydroxybutyrate and 0.6 mM NAD⁺. Assays of crude extracts also contained 2 mM KCN. One unit of activity was the amount of enzyme required to generate 1 μmol NADH min⁻¹. The enzyme eluted from the column was purified 15'8-fold over the crude cell extract and had a specific activity of 6 units (mg protein)⁻¹.

(d) Pyridine nucleotide transhydrogenase. Enzyme activity was measured by following NADPH-thio-NAD⁺ or NADH-thio-NAD⁺ transhydrogenase activities (Chung, 1970). The reduction of thio-NAD(P)⁺ [SNAD(P)⁺] was followed at A₂₆₅ and one unit of activity was the amount of enzyme required to reduce 1 μmol SNAD(P)⁺ min⁻¹. Two buffer systems were used in the assays. The reduction of SNAD⁺ by NADH was inhibited by phosphate ions (Chung, 1970), so it was assayed in 50 mM Tris/HCl, pH 7.0. The reduction of SNAD⁺ by NADPH, however, was greatest in 50 mM potassium phosphate buffer, pH 7.0. These assays also contained 2 mM KCN.

Enzyme molecular mass determination. Molecular masses of the 3-ketothiolase, AcAcCoA reductase, and BOH-DH were determined as described by Haywood et al. (1988a, b) by gel filtration in a Sepharose CL6B column (2.5 cm diameter, 35 cm height) following the procedures described by Andrews (1970). The column was calibrated with β-amylase (M, 200000), alcohol dehydrogenase (M, 150000), bovine serum albumin (M, 66000), carbonic anhydrase (M, 29000) and cytochrome c (M, 12400) as described in the Sigma technical bulletin GF-3.

Substrate specificity of 3-ketothiolase and AcAcCoA reductase. Determination of the substrate specificities of the 3-ketothiolase and AcAcCoA reductase of strain UWD required the synthesis of C₄-, C₅-, and C₆-trans-2-enoyl-CoA substrates by the mixed anhydride method of Schulz (1974). The concentration of trans-2-enoyl-CoA substrates were estimated (Schulz, 1974) using crotonase (enoyl-CoA hydratase; Sigma) which was also used to generate L-β-hydroxyacyl-CoA for the specificity assays. This product was then reacted with NAD⁺ and 1-specific 3-hydroxyacyl-CoA dehydrogenase (from pig heart; Sigma), in a linked assay with lactic dehydrogenase (Haywood et al., 1988a), to generate the corresponding 3-ketoacyl-CoA.

The substrate specificity of the 3-ketothiolase was measured by following the thiolysis of the 3-ketoacyl-CoA substrates crotonyl-CoA (Sigma) and those generated above. Cleavage of the 3-ketoacyl-CoA substrate by 3-ketothiolase was followed as a decrease in A₂₆₅ (Haywood et al., 1988a). The substrate specificity of the UWD AcAcCoA reductase was determined by following the reduction of the ≥ C₄-3-ketoacyl-CoA substrates to 3-hydroxyacyl-CoA (Haywood et al., 1988b).

RESULTS
3-Ketothiolase activity and control in Az. vinelandii UWD

Az. vinelandii UWD formed PHB during exponential growth in glucose medium (Page & Knosp, 1989), a characteristic that could be explained by altered 3-ketothiolase control. Therefore, the 3-ketothiolase of strain UWD was partially purified as described in Methods. The enzyme eluted from a DEAE Sepharose CL-6B column with 75-80 mM KCl as a single, symmetrical peak of activity and had an apparent molecular mass of 150000 Da.

In the condensation reaction, a plot of velocity versus 0–20 mM AcCoA substrate showed typical Michaelis-Menten kinetics, but the substrate saturation curves became increasingly sigmoidal in the presence of increased CoA, the Hill coefficient (n) was 1-0 and in the presence of 0·15 mM CoA this increased to n = 1-4, exactly as described by Oeding & Schlegel (1973).
Thiolysis by the UWD 3-ketothiolase had an apparent $K_m$ for AcAcCoA of 52.6 μM (at 67 μM CoA) and an apparent $K_m$ for CoA of 11.8 μM (at 67 μM AcAcCoA). Thiolysis displayed typical Michaelis–Menten kinetics as the AcAcCoA substrate concentration was increased to 67 μM (Fig. 1a). When CoA was increased from 10 to 67 μM, the $V_{max}$ of the enzyme increased, but very high CoA concentrations (133 to 667 μM) had a negative effect on $V_{max}$ (Fig. 1a). A Lineweaver–Burk plot of these data (Fig. 1b) suggested competitive substrate inhibition by CoA in a Ping-Pong bireactant system (Segal, 1975). This model was confirmed by varying the concentration of AcAcCoA in assays with CoA as the enzyme substrate (Segal, 1975). The Lineweaver–Burk plot of these data (Fig. 1c) demonstrated a family of parallel lines with increasing $V_{max}$ (intercepts) as the concentration of AcAcCoA was increased to the point of saturation. That is to say, the inhibition caused by CoA was overcome by a saturating concentration of the normal first substrate, AcAcCoA.

3-Ketothiolase activity and control in A. vinelandii UW

The regulation of the UWD thiolysis reaction differed significantly from that described for the 3-ketothiolases of Az. beijerinckii (Senior & Dawes, 1973) and Al. eutrophus (Oeding & Schlegel, 1973). These enzymes demonstrated substrate inhibition by 25–68 μM AcAcCoA which was overcome by increasing the concentration of CoA in the assay. Therefore, the properties of the 3-ketothiolase from the parent strain Az. vinelandii UW were examined.

The partially purified UW 3-ketothiolase was eluted from the DEAE Sepharose CL-6B column with 75 mM KCl as a single, uniform peak of activity. In the condensation and thiolysis reactions the UW enzyme demonstrated kinetics identical to those described for the UWD enzyme (data not shown).

3-Ketothiolase activity in strain UWD grown with valerate

Strain UWD was grown in medium containing 30 mM sodium valerate instead of glucose, with the expectation that PHA biosynthesis would involve the β-oxidation of valerate to provide the intermediates for PHB-co-HV synthesis (Page et al., 1992), and this may induce a second 3-ketothiolase. After 24 h incubation, the cells were harvested and determined to contain 22% PHA (dry wt),

<table>
<thead>
<tr>
<th>3-Ketoacyl-CoA substrate</th>
<th>Lag time (min)</th>
<th>$\Delta A_{303}$ (min⁻¹)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_4$</td>
<td>0</td>
<td>0.676</td>
<td>100</td>
</tr>
<tr>
<td>$C_6$</td>
<td>1–2</td>
<td>0.150</td>
<td>22</td>
</tr>
<tr>
<td>$C_8$</td>
<td>1–2</td>
<td>0.140</td>
<td>21</td>
</tr>
<tr>
<td>$C_9$</td>
<td>2–3</td>
<td>0.120</td>
<td>18</td>
</tr>
</tbody>
</table>

* The substrate, derived from 40 μM-trans-2-enoyl-CoA (see Methods), was reacted with partially purified strain UWD 3-ketothiolase and assayed in the direction of thiolysis.
† Lag time before the decline in $A_{303}$ was linear.
‡ Based on $\Delta A_{303}$.
comprising 33 mol % HV. The 3-ketothiolase activity from these cells was eluted from the DEAE Sepharose CL-6B column with 70 mM KCl as a single, uniform peak of activity. This partially purified 3-ketothiolase exhibited enzyme kinetics identical to those described for UWD grown in glucose medium (data not shown). Thus, β-oxidation did not induce an additional 3-ketothiolase.

**Substrate specificity of the UWD 3-ketothiolase**

The UWD 3-ketothiolase was most active with AcAcCoA (C₄-substrate), but also was active with C₅-, C₆- and C₇-substrates (Table 1). Considering the enzyme activity rates and the lag times before thiolysis was observed, the substrate preference appeared to be C₄ ≫ C₅ = C₆ > C₇.

**AcAcCoA reductase activity and control in Azotobacter vinelandii UWD**

In strain UWD, the mutation has been described as a defect in the respiratory oxidation of NADH (Page & Knosp, 1989). PHA biosynthesis then acts as an electron sink to recycle NAD⁺ (Page & Knosp, 1989). However, the AcAcCoA reductase of *Azotobacter beijerinckii* has been shown to be about five times more active with NADPH than NADH (Ritchie et al., 1971). Therefore, it was important to examine cofactor specificity and control of the AcAcCoA reductase of strain UWD.

The partially purified UWD AcAcCoA reductase eluted from a DEAE Sepharose CL-6B column with 130 mM KCl as a single, symmetrical peak of enzyme activity, and used NADPH or NADH as a reductant. The enzyme had a molecular mass of 77000. The pH optima for the reduction of AcAcCoA and the oxidation of DL-β-hydroxybutyryl-CoA were pH 5.5 and pH 8.0, respectively, as observed by Ritchie et al. (1971). However, the oxidative reaction had only one-tenth of the rate of the reductive reaction.
The partially purified strain UWD AcAcCoA reductase had only 15–22 % as much activity with 100 μM NADH as it did with 100 μM NADPH. A Michaelis–Menten plot of the reduction assay showed sigmoid curves which became increasingly sigmoid at higher concentrations of NADPH (Fig. 2a). Substrate inhibition was observed at greater than 10 μM AcAcCoA and there also was evidence of inhibition by 200 μM NADPH. A Lineweaver–Burk plot of these data gave a pattern of curved lines characteristic of cooperative substrate binding to an allosteric enzyme (Fig. 2b). When the reciprocal of the velocity was plotted versus the reciprocal of 3 or 3, the latter plot gave a straight line relationship with an apparent n = 4. Similarly, a Hill plot of these data gave a straight line relationship with an apparent n = 4 and a K_m of 11 μM AcAcCoA (at 100 μM NADPH).

The enzyme kinetics were examined using NADPH as the variable substrate and AcAcCoA as the fixed substrate. The Michaelis–Menten plots were non-linear and indicated product inhibition at > 25 μM NADPH which was most evident at 2.5–20 μM AcAcCoA (Fig. 2c). The apparent K_m for NADPH (at saturating 32 μM AcAcCoA) was 20 μM from Lineweaver–Burk plots. This plot was non-linear except at saturating AcAcCoA concentration (Fig. 2d). A Hill plot of these data indicated an apparent n = 2.

The AcAcCoA reductase was inhibited 22 % by the addition of 100 μM NADP+ to the assay, thus confirming product inhibition. However, no inhibition of the enzyme was caused by up to 200 μM NAD+. When NADH was used as the substrate, the AcAcCoA reductase had an apparent K_m of 385 μM for NADH (at saturating 32 μM AcAcCoA) and n = 1 (from Hill plots), thus confirming a clear preference for NADPH.

**Substrate specificity of AcAcCoA reductase**

The AcAcCoA reductase of strain UWD had a preference for AcAcCoA as substrate, rather than 3-ketovaleryl-CoA. In a control assay, the reduction of authentic or generated (see Methods) 20 μM AcAcCoA resulted in a decrease in ΔA_303 of 0.135 or 0.140 min⁻¹, respectively. Use of 20 μM 3-ketovaleryl-CoA as a substrate resulted in ΔA_303 of 0.022 min⁻¹.

Reaction of the UWD enzyme with 60 μM 3-ketovaleryl-CoA alone, or in combination with 20 μM AcAcCoA, resulted in ΔA_303 of 0.072 and 0.200 min⁻¹, respectively. Thus, the presence of the C₃-substrate did not significantly prevent C₂-substrate reduction (i.e. the total ΔA_303 was almost additive).

**Copolymer formation from n-alkanoate precursors**

Strain UWD was able to form P(HB-co-HV) copolymer when sodium valerate, heptanoate or nonanoate were fed to cells in glucose medium or when these salts were used as the sole C source (Page et al., 1992). The mol % HV in P(HB-co-HV) was increased by adding 0.5–1.0 mM 4-pentenoate (4-PA) to the medium containing valerate. 4-PA is believed to inhibit the 3-ketothiolase in vivo and increase the flow of L-(+)-3-hydroxyvaleryl-CoA or 3-ketovaleryl-CoA intermediates from β-oxidation into copolymer production (Page et al., 1992).

In the present study, the experiment with 4-PA addition was repeated with acetate, valerate, hexanoate or heptanoate as sole C source (Table 2). The results show that 4-PA had little effect on the growth of UWD in acetate, but was increasingly inhibitory to growth on longer chain n-alkanoates where β-oxidation would be required to generate AcCoA. Although the growth of UWD in valerate was inhibited 25 % by 4-PA, more of the C-source was diverted into PHA synthesis, resulting in an increased yield of PHA with increased HV content. When 4-PA was added to cultures containing hexanoate or heptanoate, the generation of intermediates (e.g. 3-hydroxyacyl-CoA or 3-ketoacyl-CoA) for PHB-co-HV synthesis and growth (AcCoA) was efficiently blocked. Also, the PHA extracted from cells grown in the presence of 4-PA did not contain subunits longer than 3-hydroxyvalerate.

**Pyridine nucleotide transhydrogenase activity and nitrogen effects on PHA synthesis**

Pyridine nucleotide transhydrogenase was considered to be an important enzyme to examine because the UWD mutation would generate NADH, but AcAcCoA reductase had a definite preference for NADPH. Also, the best PHA yields were obtained in NH₄⁺-limited, nitrogen-fixing strain UWD cells (Page, 1992), where the preferred

<table>
<thead>
<tr>
<th>Substrate*</th>
<th>4-PA (mM)†</th>
<th>PHA (g l⁻¹)</th>
<th>YP,RM (g g⁻²)‡</th>
<th>HV (mol %)</th>
<th>Growth inhibition (%)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>0</td>
<td>0.42</td>
<td>1.8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.40</td>
<td>1.9</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>Valerate</td>
<td>0</td>
<td>0.34</td>
<td>0.3</td>
<td>31</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.50</td>
<td>0.5</td>
<td>56</td>
<td>25</td>
</tr>
<tr>
<td>Hexanoate</td>
<td>0</td>
<td>0.92</td>
<td>0.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.00</td>
<td>1.0</td>
<td>0</td>
<td>54</td>
</tr>
<tr>
<td>Heptanoate</td>
<td>0</td>
<td>0.90</td>
<td>0.6</td>
<td>35</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.02</td>
<td>0.2</td>
<td>0.8</td>
<td>91</td>
</tr>
</tbody>
</table>

* All substrates (pH 7.0) were added to a final concentration of 30 mM (see Methods) and cultures were incubated for 24 h before harvesting.
† 4-PA was added to the medium before inoculation.
‡ Yield of g polymer (P) per g non-PHA residual biomass (RM).
§ Calculated from non-PHA residual biomass, where uninhibited growth in acetate = 0.24 g l⁻¹, valerate = 1.22 g l⁻¹, hexanoate = 0.97 g l⁻¹ and heptanoate = 1.87 g l⁻¹.
PHA synthesis in *Azotobacter vinelandii* UWD

### Table 3. Effect of ammonium in the growth medium on pyridine nucleotide transhydrogenase and AcAcCoA reductase activity

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth condition*</th>
<th>Transhydrogenase specific activity†</th>
<th>AcAcCoA reductase specific activity‡</th>
<th>g PHB (g protein)⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NADPH-SNAD⁺</td>
<td>NADH-SNAD⁺</td>
<td></td>
</tr>
<tr>
<td>UW</td>
<td>N₂</td>
<td>430</td>
<td>540</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>NH₄⁺-sufficient</td>
<td>340</td>
<td>360</td>
<td>2.7</td>
</tr>
<tr>
<td>UWD</td>
<td>N₂</td>
<td>270</td>
<td>290</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>NH₄⁺-limiting</td>
<td>200</td>
<td>260</td>
<td>1.9</td>
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<tr>
<td></td>
<td>NH₄⁺-sufficient</td>
<td>140</td>
<td>180</td>
<td>2.5</td>
</tr>
</tbody>
</table>

* All cultures were incubated for 24 h as described in the Methods. NH₄⁺-sufficient medium contained 15 mM ammonium acetate (strain UW) or 15 mM ammonium acetate plus 15 mM NH₄HCO₃ added at 12 h (strain UWD). NH₄⁺-limiting UWD culture contained 15 mM ammonium acetate only.

† μmol SNAD⁺ or SNAD⁺ reduced min⁻¹ (mg protein)⁻¹.

‡ μmol NADPH oxidized min⁻¹ (mg protein)⁻¹.

Reductant for nitrogen fixation was NADPH (Benemann *et al.*, 1971; Klugkist *et al.*, 1986).

In both strains UWD and UW, the transhydrogenase specific activity was greatest in nitrogen-fixing cells (Table 3). The transhydrogenase was slightly more active when measured in the direction of SNAD⁺ reduction. In strain UW, AcAcCoA reductase activity was high in NH₄⁺-sufficient cells, where nitrogenase was repressed and where NADPH should be more available for PHA synthesis (Table 3). However, strain UWD AcAcCoA reductase appeared to be equally active with or without NH₄⁺ present. This was consistent with the UWD mutation causing a surplus of reducing power under all growth conditions (Page, 1992). The yield of PHB in strain UWD correlated well with the NADH-SNAD⁺ transhydrogenase activity (Table 3). That is, a 38% decrease in transhydrogenase activity in NH₄⁺-sufficient cells was accompanied by a similar decrease in PHB yield.

### Table 4. Comparison of β-hydroxybutyrate dehydrogenase activity in *Az. vinelandii* UWD and other PHB-producing bacteria

<table>
<thead>
<tr>
<th>Organism</th>
<th>Age (h)</th>
<th>PHB (g l⁻¹)</th>
<th>Y_PFRM (g g⁻¹)</th>
<th>BOHB-DH specific activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Az. vinelandii</em></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>UW</td>
<td>24</td>
<td>3.5</td>
<td>1.7</td>
<td>0.16</td>
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<td></td>
<td>48</td>
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* μmol NADH min⁻¹ (mg protein)⁻¹.

† Grown in glucose medium containing 0.1% (w/v) fish peptone.

‡ Grown in glucose medium without fish peptone.


Mutant bearing a mutation causing a surplus of reducing power under all growth conditions (Page, 1992). The yield of PHB in strain UWD correlated well with the NADH-SNAD⁺ transhydrogenase activity (Table 3). That is, a 38% decrease in transhydrogenase activity in NH₄⁺-sufficient cells was accompanied by a similar decrease in PHB yield.

#### BOHB-DH activity and control

When strain UWD was grown and harvested as described by Senior & Dawes (1973), cell extracts had 100-fold less BOHB-DH specific activity compared to that reported for *A. beijerinckii*. However, these cells were harvested after 18 h incubation and contained very little PHB (0.43 g l⁻¹, Y_PFRM = 0.7 g g⁻¹). Since BOHB-DH is involved in PHB degradation, it was more likely to be active in older cells that were using PHB as an endogenous carbon and energy reserve. This was confirmed, using strain UWD and *A. beijerinckii* DSM 1041 harvested at 24–72 h (Table 4). Both strains had similar BOHB-DH activities after 24 h incubation and enzyme activity increased at later times when the polymer was turned-over.

The partially purified BOHB-DH was eluted from a Sepharose CL6B column as a single, uniform peak of activity and had an apparent molecular mass of 76000 Da. The enzyme had an apparent K_m of 42 μM for NAD⁺ (at saturating 10 mM 3-hydroxybutyrate) and 1.06 mM for 3-hydroxybutyrate (at saturating 1 mM NAD⁺). Only 5 to 6% of the enzyme activity was observed with 0.6 mM NADP⁺ instead of NAD⁺. Kinetic plots suggested a partial ordered Bi Bi reaction. Enzyme activity was unaffected by 0.25 mM NADH or 1.0 mM AcCoA. Activity was inhibited only 13% by 2 mM pyruvate, 7% by 3 mM oxaloacetate, and 5% by 3 mM α-ketoglutarate, while activity was increased 1.5-fold in the presence of 0.25 mM NADP⁺.
**DISCUSSION**

PHA synthesis in *A. vinelandii* UWD shares many similarities with other PHA-producing organisms, but also demonstrates fine differences in the control of the biosynthetic and degradative enzymes. Overall, the PHB biosynthetic pathway (Fig. 3) appears to be a typical *A. eutrophus*-type route (Steinbüchel, 1991), with the exception that HV subunits are not generated by the condensation of propionyl-CoA and AcCoA (Page et al., 1992). Instead, the only demonstrated source of HV subunits is from the \( \beta \)-oxidation of odd-number \( n \)-alkanoates (Page et al., 1992). The type of copolymer formed seems to be limited to P(HB-co-HV) (Page et al., 1992), despite attempts to make longer side-chain precursors available by inhibition of \( \beta \)-oxidation with 4-PA (this study).

Polymer synthesis in strain UWD is triggered by a mutation in the respiratory NADH oxidase (Fig. 3, step 1), which through the action of transhydrogenase (step 2) leads to increased levels of NADPH. This most likely causes feedback inhibition of citrate synthase and isocitrate dehydrogenase by NAD(P)H (step 3), thus slowing the tricarboxylic acid cycle and increasing the ratio of AcCoA to CoA (Dawes & Senior, 1973; Oeding & Schlegel, 1973; Senior & Dawes, 1973). The first enzyme in the production of PHA, the 3-ketothiolase condensation reaction (step 4) has an apparent \( K_m \) of 0.83 mM for AcCoA, similar to the values shown for *A. beijerinckii* (\( \approx 1 \) mM, Senior & Dawes, 1973) and 3-ketothiolase-A of *A. eutrophus* (1.1 mM, Haywood et al., 1988a). In all three organisms, the condensation of AcCoA is inhibited by free CoA. Thus, active sugar catabolism and an active tricarboxylic acid cycle should result in a relatively high ratio of CoA to AcCoA and the inhibition of PHA biosynthesis. The UWD condensation reaction was inhibited by low CoA concentrations when the AcCoA concentration was low, but \( V_{\text{max}} \) was attained at higher concentrations of AcCoA, even in the presence...
of inhibitor. This pattern was essentially identical to the Ping-Pong mechanism described by Oeding & Schlegel (1973).

The second enzyme in PHA biosynthesis is the AcAcCoA reductase (Fig. 3, step 5). The UWD enzyme had at least 100-fold greater affinity for NADPH than NADH, very similar to the \( K_m \) values observed with the \( \text{Al. eutrophus} \) AcAcCoA reductase-B (Haywood et al., 1988b). This coenzyme preference also necessitates an active NADH-NADP\(^+\) transhydrogenase (step 2) in the UWD cells. Furthermore, the UWD AcAcCoA reductase was negatively affected by 100 \( \mu \)M NADP\(^+\), while this enzyme was unaffected by NAD(P)\(^+\) in \( \text{Az. beijerinckii} \) (Senior & Dawes, 1973) or \( \text{Al. eutrophus} \) (Haywood et al., 1988b). Thus the transhydrogenase could play an additional role in keeping the concentration of NADP\(^+\) low in strain UWD.

The AcAcCoA reductases of \( \text{Az. beijerinckii} \), \( \text{Al. eutrophus} \) and strain UWD had apparent \( K_m \) values of 2, 5 and 10 \( \mu \)M AcAcCoA (at saturating NADPH), respectively (Haywood et al., 1988b; Ritchie et al., 1971). However, the \( \text{Az. beijerinckii} \) AcAcCoA reductase demonstrated normal Michaelis-Menten kinetics and straight line Lineweaver-Burk plots with AcAcCoA as substrate (Ritchie et al., 1971). The UWD enzyme apparently does not follow normal Ping-Pong bireactant kinetics because the Lineweaver-Burk plot failed to generate parallel lines. The most likely mechanism is a general hybrid Ping-Pong random mechanism (Segal, 1975). In this model, there are two steps at which NADPH (substrate A) can bind and four steps at which AcAcCoA (substrate B) can bind. The velocity equation will contain \([A]^2\) and \([B]^4\) terms, generating a number of non-hyperbolic plots and sigmoid curves.

Although a complete survey of 3-ketoacyl-CoA substrates was not performed, the UWD AcAcCoA reductase had only 16% as much activity with the \( C_2 \)-substrate compared to the \( C_4 \)-substrate, similar to that reported for \( \text{Al. eutrophus} \) (Haywood et al., 1988b). However, when mixed substrates are present, the \( C_4 \)-substrate does not appear to interfere with use of the \( C_2 \)-substrate. The marked substrate preference of the AcAcCoA reductase must contribute to the fixed rate of HV incorporation into copolymer (\( \approx 2.5 \text{ mol% h}^{-1} \)) which occurs despite variations in the rate or manner of feeding valerate to the culture (Page et al., 1992). The PHA synthase (Fig. 3, step 8) has not been examined in detail in \( \text{Az. vinelandii} \) or \( \text{Az. beijerinckii} \). However, this enzyme in \( \text{Al. eutrophus} \) also has a definite preference for \( C_4 \)-substrate versus \( C_3 \)-substrate (Haywood et al., 1989).

In the direction of polymer turnover, the intracellular depolymerase (Fig. 3, step 9) has not been examined in \( \text{Az. beijerinckii} \) or \( \text{Az. vinelandii} \), but as described by others, yields free \( \beta \)-hydroxybutyrate (Dawes & Senior, 1973). The BOHB-DH specific activity (step 10) was not unlike that observed in \( \text{Az. beijerinckii} \) or \( \text{Al. eutrophus} \) (this study). The enzyme is NAD\(^+\)-specific in UWD, \( \text{Az. beijerinckii} \) (Senior & Dawes, 1973), and \( \text{Azospirillum brasiliense} \) (Tal et al., 1990) and has similar \( K_m \) values for NAD\(^+\) (70 and 104 \( \mu \)M in \( \text{Az. beijerinckii} \) and UWD, respectively) and \( \beta \)-hydroxybutyrate (0.88, 0.94 and 1.0 mM in \( \text{Az. beijerinckii} \), UWD, and \( \text{Azospirillum brasiliense} \), respectively). However, the UWD enzyme was unaffected by NADH, marginally affected by pyruvate, and appeared to be stimulated by 0.25 mM NADPH. Thus there appeared to be little control of BOHB-DH in strain UWD, contrary to the model drawn for \( \text{Az. beijerinckii} \) (Dawes & Senior, 1973; Senior & Dawes, 1973) and \( \text{Al. eutrophus} \) (Oeding & Schlegel, 1973). The control of the formation of the high-molecular-mass polymer in strain UWD (Budwill et al., 1992; unpublished data), therefore, cannot be accounted for by a slower rate of polymer turnover, regulated at the level of BOHB-DH.

The AcAc from the degradative cycle is converted to AcCoA (Fig. 3, step 11) by a succinyl-CoA transferase in \( \text{Az. beijerinckii} \) (Senior & Dawes, 1973). The \( 3 \)-ketothiolase now plays a role in generating AcCoA from AcAcCoA (step 12), the ACaCoA enters the tricarboxylic acid cycle (Hitchins & Sadoff, 1973) and the level of free CoA rises, thus inhibiting the condensation reaction (Oeding & Schlegel, 1977). The 3-ketothiolase of strain UWD shares characteristics with the \( \text{Al. eutrophus} 3 \)-ketothiolase-A, involved primarily in PHB synthesis (Haywood et al., 1988a; Oeding & Schlegel, 1977), and the 3-ketothiolase-B, involved primarily in fatty acid catabolism (Haywood et al., 1988a). The isolation, molecular mass and \( K_m \) values resemble enzyme A, while the inhibition of the condensation reaction resembles enzyme B. In addition, the UWD enzyme used \( C_4 \)-, \( C_5 \)-, \( C_6 \)- and \( C_8 \)-substrates in an order of preference like that of enzyme B. There was no evidence of the induction of a second thiolase in strain UWD during growth of \( \pi \)-alkanoates, so the one enzyme must function in both PHB biosynthesis and fatty acid degradation capacities.

In the thiolysis reaction, AcAcCoA is the first substrate bound to the \( \text{Al. eutrophus} \) enzyme, followed by CoA, in a bireactant Ping-Pong mechanism (Oeding & Schlegel, 1977). In both \( \text{Al. eutrophus} \) and \( \text{Az. beijerinckii} \) the thiolysis is substrate-inhibited by AcAcCoA at low concentrations of CoA (Oeding & Schlegel, 1977; Senior & Dawes, 1973). This combination of events must prevent AcAcCoA degradation when the condensation reaction is favoured. Substrate inhibition is overcome by higher concentrations of free CoA. However, thiolysis was not inhibited by AcAcCoA in strain UWD, but it was inhibited by free CoA. The kinetic plots suggested that CoA was a competitive inhibitor in a bireactant Ping-Pong system (Segal, 1975). In this model, CoA could combine with the enzyme before AcAcCoA to form a dead-end [enzyme:CoA] complex. However, the inhibition caused by CoA was overcome by a saturating concentration of the normal first substrate, AcAcCoA. This control pattern could allow the catabolism of AcAcCoA derived from PHB turnover (Fig. 3, step 11) or \( \beta \)-oxidation (step 7), even in the presence of exogenous sugar (and high CoA). The high levels of AcAcCoA
should inhibit the AcCoA reductase (step 5), while high levels of CoA generated by AcCoA use should inhibit the thiolase condensation reaction (step 4), effectively turning off PHB synthesis and promoting degradation.

Experimentally, the growth of strain UWD on sodium valerate as sole C source results in only 5% (w/w) of the added valerate appearing as HV in the polymer, as β-oxidation is used primarily for catabolism (Page et al., 1992). However, when sodium valerate is fed to UWD cells actively growing in glucose medium, 49–56% (w/w) of the added valerate appears in HV (Page et al., 1992). Furthermore, very little of the added valerate is incorporated into PHA in the parent strain UW growing in glucose medium (unpublished data), which indicates that the UWD mutation must promote copolymer formation under these growth conditions. The increased NADPH levels in the mutant cells could promote AcCoA reductase activity under these conditions, consistent with the observation that the \( V_{\text{max}} \) of the UWD AcCoA reductase was reached at high AcCoA concentration only when the NADPH concentration also was high.

This work lays a foundation for understanding PHA biosynthesis in strain UWD. Although the 3-ketothiolase, AcCoA reductase and BOHB-DH kinetics differ from those described for other organisms, these enzymes appeared to be normal for \( Az. vinelandii \) UW and elevated PHA production in strain UWD could be explained based on a mutation in the respiratory oxidation of NADH (Page & Knosp, 1989; Fig. 3). Of the enzymes that remain to be examined, the intracellular PHA depolymerase (Fig. 3, step 9) may be the most likely regulator of polymer molecular mass in this organism, because there seems to be little impediment to polymer degradation after the generation of β-hydroxybutyrate monomers.

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