Occurrence of Poly-β-Hydroxybutyrate in the 
Azotobacteriaceae

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Poly-β-hydroxybutyrate (PHB) from various representative strains of the genera Azotobacter, Beijerinckia, and Derxia was isolated and characterized. During growth in shake culture, with glucose as a carbon and energy source, and molecular nitrogen as a nitrogen source, increase in dry weight appeared linear, and PHB formed a constant percentage of the dry weight. In a medium containing 1% (w/v) glucose, PHB declined with the onset of the stationary phase of growth; with 2% (w/v) glucose, an increase in PHB content during stationary phase was noted in the case of some strains, before a subsequent decline. The decrease in PHB as a percentage of dry cellular weight (not of total amount present in the culture) during growth of some strains with 2% as opposed to 1% (w/v) glucose may be ascribed to a greater production of capsular polysaccharide. PHB content could not be used as a taxonomic criterion. Strain differences were as great as or greater than species differences. The only strain of Beijerinckia fluminensis obtained contained PHB, but it could not be grown on the nitrogen-free medium used. Two species of the genus Azotomonas, reported to be aerobic, nonsymbiotic nitrogen-fixers, did not grow on the nitrogen-free medium used and did not produce PHB during growth with a combined nitrogen source.

The presence of poly-β-hydroxybutyrate (PHB) in bacteria has been recognized since 1926 (12). The accumulation and degradation of PHB in Azotobacter chroococcum were observed by Lemoigne and Girard (13), and Schlegel (16) has described a strain of this organism with up to 80% of its dry weight as PHB. A. vinelandii and A. agilis (6) have also been shown to contain PHB.

Interest in the role of PHB as a carbon and energy reserve and in its possible significance for taxonomy prompted us to make a survey of the occurrence of this polymer in those species comprising the family Azotobacteriaceae. A preliminary account has been presented (18).

Materials and Methods

Strains used. Organisms from four genera were used.

In the case of organisms not classified in Bergey's Manual of Determinative Bacteriology, the names given are those proposed by their discoverers. From the genus Azotobacter, the strains used were A. chroococcum NCIB 9125, NCIB 8002, and NCIB 8003; A. beijerincki NCIB 8948 and NCIB 9067; A. vinelandii NCIB 8660 and NCIB 8789; A. agilis NCIB 8637 and NCIB 9473; A. insigne (4) NCIB 9127, VJ5 (isolated by Vagn Jensen, Royal Veterinary and Agricultural College, Copenhagen, Denmark); and A. macrocytogenes (7) NCIB 8700, NCIB 9128. The Beijerinckia strains used were B. indicus NCIB 8712 and NCIB 8997; B. lactofermentum (9) NCIB 8846; B. mobile (3) (unspecified); and B. fluminensis (5) (unspecified). D. gummosa (8) NCIB 9604 represented the genus Derxia, and Azotomonas insolita NCIB 8627A and NCIB 9747 and A. fluorescens ATCC 13544 represented the genus Azotomonas.

Growth media. A modification of Norris' nitrogen-free agar (14) was used routinely as a liquid medium and had the following composition per liter of distilled water. Solution A: glucose, 20 or 40 g; MgSO₄.7H₂O, 0.400 g; CaCl₂, 0.110 g; FeSO₄·7H₂O, 0.012 g; Na₂MoO₄·2H₂O, 0.010 g. Solution B: KH₂PO₄, 2.000 g; NaCl, 0.410 g. Solutions A and B were autoclaved at 15 psi for 10 min, and equal volumes were mixed after cooling; the pH of the medium was 7.7. For solid medium, 2% agar (Kobé powder; Hopkin & Williams, Chadwell Heath, Essex, U.K.) was added to the combined medium. In certain cases, this medium was supplemented with a combined nitrogen source in the form of 1.0 g of yeast extract (Difco) or 2.4 g of NH₄NO₃ per liter or with 5 ml per liter of a trace elements solution having the following constituents per liter of 0.155 M HCl: CaCO₃, 2.000 g; ZnO, 0.406
VOL.

FeCl₃·6H₂O, 5.400 g; MnCl₂·4H₂O, 0.990 g; CuCl₂·2H₂O, 0.170 g; CoCl₂·6H₂O, 0.238 g; and H₂BO₃, 0.062 g.

Growth of organisms. Liquid cultures were grown in conical flasks on a gyratory shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 27 C. Medium was adjusted to occupy 20% of the flask volume, unless otherwise stated. Inocula (1%) from cultures 24 hr old were used to start growth.

Estimation of growth. The optical density (OD) of all cultures was measured with a Unicam SP 600 spectrophotometer at 500 m, with blanks of the appropriate growth media. With strains producing flocculent cultures, heavy clumps of cells were broken up to produce a suspension, stable over 20 min, by agitating a sample (5 ml) in a boiling tube with a Vortex Junior mixer. Suspensions with an OD above 1.0 were diluted with growth medium. Curves relating OD to dry weight were constructed by harvesting cultures at room temperature, washing with distilled water, and resuspending the cells in distilled water to about 10 mg protein per ml. Portions (5 ml) were dried at 100 C and weighed, while further samples were suspended in medium (minus carbon source) to give a series of optical densities up to 1.0.

A complicating factor arose from the fact that A. vinelandii, A. agilis, B. indicus, B. lactoxygenes, and B. mobile produced an extracellular slime which gave high OD readings. In such cases the optical density was read against a culture supernatant blank, diluting the blank in the same ratio as the culture.

Isolation and purification of PHB. Polymer granules were isolated by the method of Williamson and Wilkinson (19). The residue of hypochlorite digestion was washed twice with distilled water to remove soluble salts and with acetone and diethyl ether to remove non-PHB lipid. The residue was dissolved in boiling chloroform and filtered through Whatman no. 1, followed by Whatman no. 42 paper to remove insoluble materials. Chloroform solutions were boiled with activated charcoal and filtered through the same paper sequence. Polymer was precipitated from chloroform by the addition of three volumes of diethyl ether at -15 C and was collected on a Büchner filter. Polymer samples were washed with cold ether and stored as an ether-containing gel.

Characterization. Dried samples of polymer were analyzed for carbon, hydrogen, nitrogen, and phosphorus. Assays were carried out by Drs. Weiler and Strauss (Oxford, U.K.). For qualitative spectrophotometric examination of the material, thin polymer films were prepared by evaporation of chloroform solutions in glass petri dishes at room temperature. Films were separated from the glass by floating them on distilled water, mounted on cardboard frames, and dried at 37 C for several hours. Their absorption spectra, between wave number 5,000 and 650 cm⁻¹, were recorded with a Unicam SP 200 infrared spectrophotometer.

Assay of PHB during growth. Polymer was assayed by the method of Law and Slepecky (11), with slight modifications. Liquid culture (1 ml), containing up to 2 mg of cellular material, was placed in a 10-ml glass centrifuge cone, and 9 ml of alkaline hypochlorite solution (19) was added. The mixture was incubated for about 24 hr; the resulting suspension was centrifuged and the supernatant liquid was decanted. The residue was washed twice with 10-ml portions of distilled water, acetone, and diethyl ether, before it was dissolved in concentrated sulfuric acid. OD was read at 235 m, against a similarly treated medium blank, with a Unicam SP 300 spectrophotometer. Purified polymer samples and sodium pellets were used as standards.

Infrared spectra of whole cells. Lyophilized specimens of water-washed organisms, grown in liquid culture, were pressed into KCl discs, and the spectra between 4,000 and 650 cm⁻¹ were recorded with a Unicam SP 100 spectrophotometer.

RESULTS

Maximal levels of PHB found in organisms grown in nitrogen-free medium with 1 and 2% glucose as carbon source are recorded (Table 1). A wide variation in PHB content was observed with both species and strain. Possession of the polymer or its concentration in the cell, could not, therefore, be used as a taxonomic criterion within the group.

Although some strains, e.g. A. agilis NCIB 9473 and A. beijerinckii NCIB 9067, showed lower percentages of PHB when grown on 2% compared with 1% glucose, the total level of PHB in the cultures did not decrease. Higher glucose concentrations can lead to greater production of capsular polysaccharide, which is not easily removed by washing and centrifugation and which contributes to the dry weight of the organisms.

The final columns of Table 1 give the results of elemental analyses of PHB. Only in the case of A. insigne VJ5 were nitrogen and phosphorus detected as impurities. Theoretical values for "totally polymerized" PHB and free β-hydroxybutyric acid are as follows. β-Hydroxybutyric acid (CH₃CHOHCH₂COOH): C, 46.15%; H, 7.69%; O, 46.15%. Poly-β-hydroxybutyrate [(C₆H₇O₂)₃]: C, 55.81%; H, 6.98%; O, 37.21.

Most values fell within these limits and tended toward those given by the theoretical polymer. Four samples (Table 1) gave a carbon assay higher than 55.81%, namely, those from A. vinelandii NCIB 8660, A. agilis NCIB 8637, A. chroococcum NCIB 9125, and B. indicus NCIB 8712. The sample from A. agilis NCIB 8637 showed also an abnormally high hydrogen content.

Infrared spectra of isolated PHB samples were qualitatively identical, and they correspond to the spectrum for PHB obtained by Norris and Greenstreet (15).

The method of Law and Slepecky (11) was used as a qualitative test for PHB in Azotobacter

POLY-,B-HYDROXYBUTYRATE

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of cells monas appeared carbon sulfuric analyses resistant, ether-insoluble included were tamate, medium with by serves after and strains. Shake cultures, containing 20 ml of Norris' liquid medium supplemented with ammonium nitrate or with trace elements and glutamate, were inoculated. No growth was observed with Norris' medium alone. Cultures were harvested after 36 hr; the cells were resuspended in alkaline hypochlorite, and PHB was estimated. The results obtained with glutamate-grown cells were essentially the same as those for ammonium nitrate-grown cells (Fig. 1). The spectra obtained for A. insigne VJS (1 ml of nitrogen-free medium) and with an authentic sample of crotonic acid were included for comparison. The hypochlorite-resistant, ether-insoluble fractions of Azotomonas appeared devoid of PHB, which would produce an absorption peak at 235 μm after sulfuric acid treatment. The spectra were somewhat similar to those recorded for PHB-deficient cells of Bacillus megaterium by Law and Slepecky (11). PHB was not detected in Azotomonas by infrared analyses of whole cells. Infrared spectra of three strains of Azotomonas were observed immediately after growth with combined nitrogen and after subsequent incubation with excess carbon source (Fig. 2). The two strains of A. insolita showed spectra similar to those of Azotobacteriae strains depleted of their PHB reserves by 10 days of growth in nitrogen-free medium with 0.25% glucose as carbon source. Additional peaks did not appear in the spectra of

- **Table 1. Poly-β-hydroxybutyrate of the Azotobacteriaceae**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Strain</th>
<th>PHB content</th>
<th>Elementary analysis</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1% Glucose</td>
<td>2% Glucose</td>
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<td>22.3</td>
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<tr>
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<td>NCIB 9128</td>
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<td>NCIB 8789</td>
<td>34.4</td>
<td>30.3</td>
</tr>
<tr>
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<td>NCIB 9473</td>
<td>10.3</td>
<td>6.4</td>
</tr>
<tr>
<td>A. glutaectes</td>
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<td>48.6</td>
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<td>NCIB 9127</td>
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</tr>
<tr>
<td>A. beijerinckii</td>
<td>VJS</td>
<td>10.5</td>
<td>14.6</td>
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<td>NCIB 8712</td>
<td>15.9</td>
<td>21.5</td>
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<td>B. mobile</td>
<td>VJS</td>
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</tr>
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<tr>
<td>Derrxia gummosa</td>
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<td>19.7</td>
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</table>

**FIG. 1. Comparison of the ultraviolet spectra of sulfuric acid digests of the ether-insoluble residue of hypochlorite digestion in the Azotomonas and Azotobacter insigne VJS.** (1) Curve A, A. insolita NCIB 9749; curve B, A. insolita NCIB 8627A; curve C, A. fluorescens ATCC 13544. (2) curve A, A. insigne VJS; curve B, crotonic acid.

A. insolita after incubation with glucose, indicating that PHB was not produced. The spectrum of A. fluorescens revealed additional peaks under both conditions. The peak at 1,735 cm⁻¹ can be assigned to ester carbonyl stretching. The peaks at 1,180 and 1,300 cm⁻¹ were more difficult to assign. The former band
could be assigned to C—O stretching, C—N stretching in alkyl amines, or various skeletal carbon stretchings, and the latter band to P—O stretching, O—H bending, or CH₂ scissor vibrations. A spectrum of A. beijerinckii after 48 hr of growth on nitrogen-free medium was included for comparison (Fig. 3).

The production and degradation of PHB was followed throughout the growth cycles of members of the genera Azotobacter, Beijerinckia, and Derxia. Cultures were grown on Norris’ nitrogen-free medium containing 1% and 2% glucose as carbon source. B. fluminensis was exceptional in that growth could only be obtained by supplementation of the medium with trace metals and yeast extract. After the onset of stationary phase, no further increase in turbidity was observed with cultures grown with 1% glucose. Growth with 2% glucose, however, in all cases with the exception of B. fluminensis, resulted in a higher yield of dry weight of cellular material during growth with 2% as opposed to 1% glucose. A further small increase in turbidity during stationary phase occurred in the case of A. macrocystogenes NCIB 8700, A. vinelandii NCIB 8660, A. agilis NCIB 8637, A. insigne NCIB 9127, and B. indicus NCIB 8712. This increase may have been caused by further production of PHB or capsular polysaccharide or of both. The aforementioned organism, along with A. insigne VJ5 and A. beijerinckii NCIB 9067, maintained a steady turbidity during stationary phase when grown with 1% glucose. The total concentration of PHB in the cultures was, in all cases, higher when organisms were grown on 2%, as opposed to 1%, glucose. PHB

![Fig. 2. Infrared spectra of whole cells of the genus Azotomonas. (A) Cells grown for 36 hr with 10 g of glucose and 2.4 g of ammonium nitrate per liter. (B) Cells suspended in nitrogen-free medium with 30 g of glucose per liter for 16 hr.](image1)

![Fig. 3. Infrared spectra of Azotobacter beijerinckii NCIB 9067. (1) After 2 days of growth with 3% glucose; (2) after 10 days of growth with 0.25% glucose; *, absorption peaks not attributable to PHB.](image2)
formed a roughly constant percentage of cell material until the onset of stationary phase, when it declined, rapidly at first, and later more slowly. The turbidities of cultures of *A. chroococcum* NCIB 9125, *B. lactoidegenes* NCIB 8846, *B. mobile*, and *D. gummosa* NCIB 9604 decreased concurrently with PHB content.

**DISCUSSION**

Organisms that accumulate PHB, usually in the presence of excess carbon, are known generally to degrade the polymer on exhaustion of the carbon source (2). PHB production cannot, therefore, simply be considered as a means for the removal of an injurious metabolite or as the end product of a metabolic shunt, although such factors may be contributory to its formation. PHB provides an excellent potential carbon and energy storage compound in that it is neutral and highly reduced, and it exerts negligible osmotic pressure. It is not known whether PHB has a common function in all organisms in which it is found. It may fulfill different roles in different organisms and possibly with various environmental conditions.

The presence of a more or less constant percentage of PHB in *Azotobacter* during growth indicated an apparent departure from the normal pattern of reserve material production. It would appear possible that PHB accumulation during growth was the result of a metabolic “bottleneck” in respiration at the level of pyruvic oxidase or beyond, or it might have been caused by nitrogen limitation preventing the incorporation of acetyl residues into protein or by oxygen limitation preventing the removal of acetyl fragments via terminal respiration. Although all three factors might have been involved, the finding that oxygen tension as measured by an oxygen electrode fell to zero during growth of *A. insigne* VJ5 with free nitrogen in sparged cultures (1.5 liters of air per min per 1,200 ml of medium), and that in shake cultures of the same organism 2,6-dichlorophenol-indophenol was reduced, suggests that oxygen limitation may contribute to PHB accumulation. In the presence of ammonium nitrate, growth of *A. insigne* VJ5 was more rapid than that with free nitrogen, but nevertheless linear, and PHB accumulated to similar levels under both conditions.

The behavior of *A. insolita* and *A. fluorescens* would appear to be characteristic of oligonitrophiles rather than nitrogen-fixers. Their microscopic appearance (10, 17) and lack of PHB would also seem to preclude any close relationship with the *Azotobacteriaceae*.

The ability of the one strain of *B. fluminensis* studied to fix atmospheric nitrogen could not be decided. The description given by Dobereiner and Ruschel (5) suggests that it is a typical *Beijerinckia* species. Becking (1) observed that it showed very slow growth on nitrogen-free media. The fact that cultures achieved almost identical turbidities when grown with 1 and 2% glucose suggests that here the yeast extract provides the nitrogen source. Because the property of aerobic, nonsymbiotic nitrogen fixation is the main criterion for characterizing the *Azotobacteriaceae*, a non-nitrogen-fixing variant of this organism may have been obtained. It may be informative to examine other strains of *B. fluminensis* for nitrogen-fixing ability. Serological tests with other members of the *Beijerinckia* may provide confirmatory evidence as to whether or not the organism is a member of the *Azotobacteriaceae*.

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**LITERATURE CITED**


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