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EFFECT OF GROWTH CONDITIONS ON ENDO- AND EXOPOLYMER BIOSYNTHESIS IN *ANABAENA CYLINDRICA* 10 C

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Abstract—The occurrence and the characterization of a polyhydroxybutyrate (PHB) in *Anabaena cylindrica* 10 C cultivated under different growth conditions were reported. The addition to the culture of *A. cylindrica* of appropriate precursors initiated the formation of a heteropolymer (PHB + PHV). The simultaneous occurrence of an exopolysaccharide and its partial characterization were also reported.

INTRODUCTION

The accumulation of polyhydroxyalkanoate (PHA) is widespread in different taxonomic groups of prokaryotes under conditions of nutrient limitation in the presence of an excess carbon and energy source [1].

Little is known about the lipid reserves of cyanobacteria, such as PHA. However intracellular accumulation of PHA seems to be related to the supplementation in the medium with acetate or to the absence of combined nitrogen [2–4]. It has been reported that cultures of *Oscillatoria limosa* isolated from a marine microbial mat seemed to accumulate 3-hydroxyvalerate (PHV), but there was no clear report about the chemical identification of this polymer and its accumulation by feeding the culture with appropriate precursors [3]. Little is known about the presence of heteropolymers in cyanobacteria. Polyhydroxybutyrate (PHB) and PHV were found in extracts from natural samples of hypersaline microbial mats or freshwater lake, but the ratio between the two monomeric units of the heteropolymers was not reported [3].

Among the nitrogen-fixing cyanobacteria, the most widely studied is *Anabaena cylindrica* which is known as the '*Escherichia coli* of cyanobacteria' [5]. Carr reported the presence of PHB in *A. cylindrica* grown in the presence of acetate and indicated that this polymer was located mainly in the heterocysts providing a reductant store of 2-carbon units [6]. No evidence of the amount of PHB and of its chemical identification was reported, and subsequent studies on members of *Anabaena* did not confirm this report [3]. In fact PHA accumulation in *A. variabilis* or other *Anabaena* spp. could not be found, even in the presence of acetate, consistent with its relative insignificance as a storage material [3]. Some species of cyanobacteria produced a polysaccharide either associated with the cells or as exocellular material [7–9]. From cultures of a strain of *A. cylindrica* a complex polysaccharide bound to the cells was isolated [8], while the presence of an exopolysaccharide in the medium has not been reported until now.

In this paper the occurrence of PHB and of an exopolysaccharide in *A. cylindrica* 10 C has been described. The formation of copolyesters, PHB and PHV, feeding the culture with propionic or pentanoic acid is also described. Modulation of PHB under different autotrophic and mixotrophic conditions was also reported, as well as biosynthetic studies on PHB formation. Comparisons between the parallel occurrence of PHA and of an exopolysaccharide in *A. cylindrica* 10 C under different culture conditions will be reported.

RESULTS AND DISCUSSION

Chemical identification and ^{13}C-acetate incorporation in PHB

A homopolyester of PHB was identified by spectroscopic means (^{1}H and ^{13}C NMR, and IR) in *A. cylindrica* 10 C. The IR spectrum showed a typical absorption peak at 1740 (>C=O) [10], and ^{1}H and ^{13}C spectra fully confirmed that in *A. cylindrica* an isotactic homopolymer with a regular head to tail sequence of 3-hydroxybutyrate units was produced. In fact each peak of PHB in ^{13}C NMR spectrum was very sharp and

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all resonances both in ^{1}H and ^{13}C NMR spectra were typical of PHB [11, 12]. PHB from [1-^{13}C] acetate displayed specific enhancements in the intensity of carbonyl and methine carbon resonances, suggesting that the ^{13}C-labelled carbonyl carbon of acetate was selectively incorporated in the polyester.

PHB accumulation under autotrophic conditions

The intracellular concentration of PHB in *A. cylindrica* remained below the detection limit (0.005% of dry weight) throughout the cultivation period in the presence of combined nitrogen (BG-11 medium) (Fig. 1). On the contrary, when *A. cylindrica* was transferred into a culture medium devoid of combined nitrogen (BG-11$_0$) the intracellular concentration of PHB was 0.2% of dry weight after 21 days of growth. In *A. torulosa* PHB was not found either in the presence or in the absence of nitrogen in the culture medium, within the detection limit of the analytical method used. The presence of PHB only in *A. cylindrica*, although at a very low concentration, among the *Anabaena* species examined so far, could be a constant feature of this photoautotrophically grown species of the genus.

Usually the PHA in many chemo-organotrophic or photoheterotrophic micro-organisms serves as an energy store, and the unbalanced condition has been shown to induce the accumulation of large quantities of PHA [3, 13]. In *A. cylindrica* the unbalanced condition (nitrogen starved) had only a minor effect on PHB accumulation, as was also reported for *Spirulina* [13]. Consequently the results obtained in *A. cylindrica* 10 C strengthen the hypothesis that PHB had only a minor role as an energy storage compound. This distinctive behaviour of cyanobacteria, studied so far, was most plausibly ascribed to the lack of a complete tricarboxylic cycle (TCA) [3, 13], which did not permit dissimilation of acetyl-CoA. Acetyl-CoA, derived from PHB, might be used for biosynthetic purposes. The interrupted TCA cycle in cyanobacteria serves predominantly to provide intermediates in biosynthetic pathways such as synthesis of amino acids, carotenoids, chlorophyll, and in this respect PHB could be regarded as a specific carbon store [3]. Since the PHB is a reduced compound it might act as a sink for an excess of electrons.

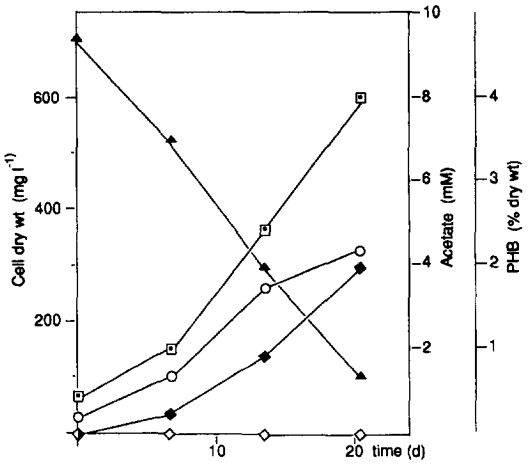

Fig. 1. PHB production from *A. cylindrica* grown in BG11 (○, cell dry wt; ◇, PHB) and in BG11$_0$ plus 8.8 mM initial concentration of acetate (▣, cell dry wt; ◆, PHB; ▲, acetate).

PHB accumulation under mixotrophic conditions

Addition of acetate to the growth medium (BG-11$_0$) of *A. cylindrica* caused an increase both in the cellular yield, (*ca* 40% higher) and in the intracellular PHB concentration up to ten fold (2% of dry weight) (Fig. 1). When the acetate in the medium was exhausted, an immediate and regular decrease in the intracellular PHB content was observed, and a total breakdown of PHB occurred within a few days (data not shown).

When *A. cylindrica* was grown in BG-11$_0$ supplemented with glucose, the intracellular PHB concentration was enhanced *ca* 3-fold with respect to growth in the auxotrophic condition, while citrate supplementation caused a strong decrease of PHB content (*ca* 5-fold). The positive effect of acetate on PHB accumulation could be related to the increase of the intracellular concentrations of acetyl-CoA, which was directly utilized for the synthesis of the polyester by means of the usual pathway operating in prokaryotes. This biosynthesis takes place by the condensation of two moles of acetyl-CoA to acetoacetyl-CoA and the subsequent formation of β-hydroxybutyryl-CoA. The results of the incorporation of [1-^{13}C]acetate in PHB supported the operativity of this pathway in *A. cylindrica* 10 C. As glucose dissimilation in *Anabaena* occurs via the pentose phosphate pathway [3], the positive effect of glucose on PHB content could be due to the production of reduced cofactor.

Copolyesters (PHB + PHV) accumulation

Copolyesters of β-hydroxybutyrate and β-hydroxyvalerate (Fig. 2) were isolated from *A. cylindrica* 10 C grown in nitrogen free culture containing propionate or valerate as carbon source. While the cellular yield was affected by the presence of both fatty acids in the culture medium, causing a decrease of 6-fold for valerate and 20-fold for propionate respectively, the intracellular concentration of PHB remained at the same level found when *A. cylindrica* was grown in BG-11$_0$ medium. The inhibitory effect of fatty acids on

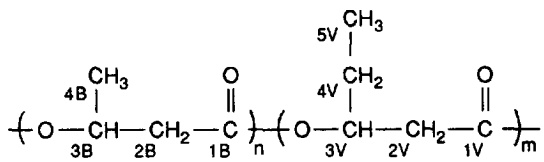

Fig. 2. Copolyesters (PHB/PHV).

growth of *A. cylindrica* was not unusual, in that this phenomenon was reported for other cyanobacteria [14].

^{1}H NMR spectra of copolyesters in $CDCl_3$ indicated that the polymer contained two monomeric units of butyrate (B) and valerate (V). The molar fractions of the two monomeric units (PHB/PHV: 4/6 and 7/3 for copolyesters found feeding valerate or propionate, respectively) were determined from the intensity ratio of the doublet CH_3-proton resonance of B to the triplet CH_3-proton resonance of V. The ^{13}C NMR spectrum of copolyesters (BV) in the presence of valerate in the culture medium showed additional signals with respect to the ^{13}C NMR signals of B, due to the presence of the valerate unit, and all resonances were consistent with the literature data [11, 12]. Moreover, carbonyl, methylene and methine carbon resonances split into several peaks in the expanded ^{13}C NMR spectrum, not always well resolved, which reflected the sensitivity of the carbon nuclei to different sequences of B and V units. The carbonyl resonances in the ^{13}C NMR spectrum of BV were clearly resolved into three peaks arising from different diad sequences of connecting B and V units. The sequence distribution of B and V units were 44%, 37% and 19% for VV, VB + BV and BB respectively, calculated by the integration of the carbonyl resonances in the expanded ^{13}C NMR spectrum [11].

Very often some micro-organisms required more than one substrate to form PHV copolymer in a lower proportion relative to the PHB units [15]. The copolyesters PHV, in *Anabaena* grown in presence of valerate was produced in a large mol%, being up to 50% with respect to PHB. The highest 3-HV content found up until now was about 90% using valeric acid as carbon source in *Alkaligenes eutrophus* [16].

The production of copolyesters with a high 3HV fraction, when the culture of *A. cylindrica* was supplied with valerate, might suggest that most of the valerate was metabolized without decomposition of the carbon skeleton into acetyl-CoA. Valerate was transported into the cells and metabolized to D-3-hydroxyvaleryl-CoA which was then directly incorporated into the heteropolyester as a 3HV unit [14]. When propionate was used as carbon source, the butyrate units of the heteropolymers were formed by two molecules of acetate, which was generated by the elimination of the carbonyl from propionate, while the valerate units were formed by reaction of propionate with the acetate.

Exopolysaccharide production

The presence of an exopolymer was first detected by the observation that the cells in fermentor cultures appeared surrounded by a gelatin matrix. To study the polymer production during growth under different conditions, samples were withdrawn from the fermentor every week. The crude exopolysaccharide (EPS) was isolated as an ethanol ppt. from the culture supernatant solution of *A. cylindrica* 10 C, and the dry weight and the total neutral sugars were determined. The production of EPS was strongly influenced by the growth conditions studied. In fact, from 15 to 2360 mg of EPS was obtained from one liter of supernatant solution (Table 1). As with other EPS, production started early during growth, increasing with increasing cell density and reaching a maximum at the stationary phase of growth.

During autotrophic growth the production of EPS was influenced by the presence of nitrogen in the culture medium, in fact, in BG-11 the EPS content was significantly higher (*ca* 7.5-fold) than in BG-11$_0$ medium. The positive effect of nitrogen on EPS production was also reported for the cyanobacterium *Aphanocapsa halophytia* [17]. Addition of acetate, valerate, glucose or citrate to the growth medium (mixotrophic growth) caused a decrease in exopolymer concentration. The presence of propionate in the medium became limiting both in the cellular yield and in the EPS production.

Table 1. EPS Composition under different growth conditions

Medium	EPS (mg l^{-1})	Fucose	Rhamnose	Xylose	Galactose	Glucose	Mannose
BG11$_0$	325	1.0	1.0	3.1	1.0	2.5	1.5
BG11	2360	1.0^{++}	tr^{++}	3.0^{++}	1.0^{++}	3.6^{++}	1.0^{++}
*BG11$_0$-P	125	1.0	tr	2.0	1.0	2.0	1.0
"+**Acetate	146	1.0	1.0	3.9	1.0	2.6	2.0
"+§Acetate	73	1.0	1.5	3.0	0.5	2.0	1.5
"+Propionate	15	1.3	0.7	2.7	1.5	3.2	1.0
"+Valerate	84	1.0	tr	3.0	tr	2.0	1.5
"+Citrate	103	1.1	0.8	2.0	tr	1.5	1.0
"+Glucose	10	1.2	tr	3.1	1.0	2.8	1.2

Total carbohydrate content of EPS was determined by the method of ref. [22].
Values were reported as molar ratio.
*BG11$_0$ without phosphate.
**Acetate was added in subsequent addition.
§Acetate was added in an early phase of growth.
$^{++}$Values were obtained using purified EPS.
tr = Trace amount.

The component sugars were identified and quantitated by TLC and HPAE-PAD Dionex analysis of hydrolysate-purified EPS (obtained from the growth in BG-11) which showed the glucose, mannose, galactose, xylose, fucose molar ratio to be 3.6, 1.0, 1.0, 3.0, 1.0, respectively, while rhamnose was in a trace amount. Glucuronic acid (17 μg/mg EPS) and an unknown uronic acid were also detected.

The color reaction was positive, with the rhodizonate method indicating the presence of sulfate groups on EPS. These data suggest that the EPS of *A. cylindrica* is an acidic sulfated glucoxylane while polysaccharides isolated from heterocysts and spores were glucomannane as previously reported [8]. The sugar composition of polymer produced under different growth conditions was not strikingly different (Table 1).

EXPERIMENTAL

Organism and culture conditions. *A. cylindrica* 10 C from culture collection of the Centro di Studio dei Microorganismi Autotrofi (Firenze, Italia) and *A. torulosa* B26.79 from Sammlung von Algenkulturen, Gottingen (Germany) were grown in BG-11 [18] at 25° in a 1 l fermentor with an aeration flux of 30 ml min^{-1} using 1 l medium at pH 7; continuous illumination was provided by 32 W cool white fluorescent circular lamp arranged round the fermentor. The culture was started with 2.5% inoculum and collected, by centrifugation at 15 300 *g* for 30 min, after 21 days. Nitrogen- or phosphorus-starved cultures were obtained by adding, under aseptic conditions, centrifuged and washed trichomes, from stock cultures grown in BG-11 medium prepd without addition of $NaNO_3$ (BG-11_0) or K_2HPO_4. Mixotrophic growth conditions were obtained by adding NaOAc (8.8 mM), or Glc (3.3 mM) or citrate (3.1 mM) or valerate (2.5 mM) or propionate (5.2 mM) to the BG-11_0, without changing the other culture parameters. The labelled acetate [1-^{13}C] (99%, Sigma) precursor (0.41 mM) was administered to *A. cylindrica*, grown in BG-11_0 after 9 days of growth. The biomass concn was monitored by determining the cell dry wt on aliquots of the cultures withdrawn daily and washed with 0.05 M Tris-HCl pH 8 containing 16 mM $MgCl_2$. The concn of the residual acetate supplied (both labelled and unlabelled), citrate and glucose in the fermenters was determined enzymatically by using the commercial kits (Boehringer, Mannhein). Large-scale cultures were obtained as previously reported [19].

Isolation and quantitative determination of PHB. Dry cells (1 g) were suspended in 0.05 M Tris pH 8 (30 ml) containing 16 mM $MgCl_2$. Lysozyme (33 mg) and DNasi (0.35 mg) were added to the suspension which was then incubated for 30 min at room temp., chilled to 0° and given a 3 min treatment with ultrasound [20]. The suspension was centrifuged (9 820 *g*) and the pellet was washed 3 times with Et_2O; the polymer was then extracted with $CHCl_3$ overnight. Further purification was effected by precipitating the PHB from $CHCl_3$ soln by the step wise addition of Et_2O, Me_2CO and MeOH, respectively. After evaporation of the solvent, PHB was obtained as a tough, translucent, grey film. Quantitative determination of PHB was effected either by the method of ref. [21] or gravimetrically.

EPS production. Cultural broth (1 l) was centrifuged until the supernatant became clear. Wet cells were washed with 10 mM Na–Pi at pH 7 overnight and completely removed by centrifugation. The two supernatants were pooled and treated, drop by drop, with 2 vol. cold EtOH under magnetic stirring. The EtOH soln, kept at −18° overnight, was centrifuged for 30 min at 15 300 *g* and the ppt. was dissolved in hot H_2O. The polysaccharide soln was dialysed against tap H_2O (72 hr) followed by dist H_2O and dried by lyophilization. Polysaccharide soln was chromatographed on a column, 2.5 cm × 40 cm, of Sephadex G-50 eluted with H_2O–pyridine–HOAc (500:5:2). The EPS fraction was rechromatographed on a column, 1 cm × 40 cm, of DEAE Sepharose CL6B eluted with a linear salt gradient from 0 to 1 M NaCl. EPS, eluted with 0.3 M NaCl, was used for studies on composition. Total carbohydrate content of EPS was detected by the method of ref. [22]. Sodium rhodizonate method was used for sulfate determination [23].

Acid hydrolysis of EPS. 1 ml of 0.5 M (for neutral sugar) or 2 M (for acid sugar) TFA was added to 1 mg of samples (T 80°, 16 hr). Acid was removed under an air stream.

Sugar analysis of EPS. Monomers were identified and quantitated by TLC and by HPAE-PAD Dionex, using standard sugars [24]. The solvent systems for TLC included A: Me_2CO–*n*BuOH–H_2O (8:1:1) for neutral sugars; B: *iso*-PrOH–pyridine–H_2O (3:1:1) for acid sugars. Compounds were visualized with naphthol followed by heating at 150°. Determination of monosaccharides was done by HPAE-PAD Dionex, equipped with Carbo-Pac PA-1 column, using different solvent system: 1) 15 mM NaOH for neutral sugars; 2) H_2O plus 300 mM NaOH in post-column for resolution of mannose and xylose; 3) 100 mM NaOH–150 mM NaOAc buffer for uronic acids.

Instrumental. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Bruker AMX 500 (500.13 MHz for ^{1}H and 125.75 MHz for ^{13}C) spectrometer. Chemical shifts are given in ppm (δ) scale, the $CHCl_3$ signal was used as internal standard (δ 7.26 ^{1}H; δ 77.0 ^{13}C). PHB + PHV δ ^{1}H NMR 1.27 (*d*, J = 6.21; H-4B), 0.89 (*t*, J = 7.34; H-5V); δ ^{13}C NMR 9.32 (C-5V); 19.74 (C-4B); 26.76 (C-4V); 38.65 (C-2V); 40.79 (C-2B); 67.60 (C-3B); 71.91 (C-3V); 169.13 (C-1B); 169.31 (C-1BV + BV); 169.50 (C-1VV). Infrared spectra were recorded at room temp. using TF-IR BIORAD spectrophotometer by using a KBr disc. Ultrasonic treatments were performed using a Sonicator Heat-System (USA) with 5 amp pulses.

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REFERENCES

1. Muller, H. M. and Seebach, D. (1993) *Angew. Chem. Int. Ed. Engl.* **32**, 477.
2. Allen, M. M. (1984) *Annu. Rev. Microbiol.* **38**, 1.
3. Stal, L. J. (1992) *FEMS Microbiol. Rev.* **103**, 169.
4. Vincenzini, M., Sili, C., De Philippis, R., Ena, A. and Materassi, R. (1990) *J. Bacteriol.* **172**, 2791.
5. Sprent, J. I. and Sprent, P. (1990) in *Nitrogen Fixing Organisms*. Chapman and Hall, Cambridge.
6. Carr, N. G. and Bradley, S. (1973) in *Symposia of the Society for General Microbiology Number XXIII, Microbial Differentiation*, pp. 161–188. Printed in Great Britain.
7. Bishop, C. T., Adams, G. A. and Hughes, E. O. (1954) *Can. J. Chem.* **32**, 999.
8. Cardemil, L. and Wolk, C. P. (1981) *J. Phycol.* **17**, 234.
9. Marra, M., Palmeri, A. Ballio, A., Segre, A. and Slodki, M. E. (1990) *Carbohydrate Research* **197**, 338.
10. Carr, N. G. (1966) *Biochim. Biophys. Acta* **120**, 308.
11. Doi, Y., Kunioka, M., Nakamura, Y. and Soga, K. (1986) *Macromolecules* **19**, 2860.
12. Bloembergen, S., Holden, D. A., Bluhm, T. L., Hamer, G. K. and Marchessault, R. H. (1989) *Macromolecules* **22**, 1656.
13. De Philippis, R., Sili, C. and Vincenzini, M. (1992) *J. Gen. Microbiol.* **138**, 1623.
14. Gibson, J. (1981) *Archiv. Microbiol.* **130**, 175.
15. Anderson, A. J., Williams, D. R., Taidi, B., Dawes, E. A. and Ewing, D. F. (1992) *FEMS Microbiol. Rev.* **103**, 93.
16. Doi, Y., Tamaki, A., Kunioka, M. and Soga, K. (1988) *Appl. Microbiol. Biotechnol.* **28**, 330.
17. Sudo, H., Burgess, J. G., Takemasa, H., Nakamura, N. and Matsunaga, T. (1995) *Curr. Microbiol.* **30**, 219.
18. Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M. and Stanier, R. Y. (1979) *J. Gen. Microbiol.* **111**, 1.
19. Soriente, A., Sodano, G., Gambacorta, A. and Trincone, A. (1992) *Tetrahedron* **48**, 5375.
20. Syker, J. (1971) in *Methods in Microbiology* (Norris, J. R. and Ribbons, D. W., eds), Vol. 5B, pp. 189–193. Academic Press, London.
21. Law, J. H. and Slepecky, R. A. (1961) *J. Bacteriol.* **82**, 33.
22. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) *Anal. Chem.* **28**, 350–356.
23. Terho, T. T. and Harthiala, K. (1971) *Anal. Biochem.* **41**, 471–476.
24. Nicolaus, B., Manca, M. C., Romano, I. and Lama, L. (1993) *FEMS Microbiol. Letters* **109**, 203–206.