APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1986, p. 214–216
0099-2240/86/010214-03$02.00/0

Vol. 51, No. 1

Accumulation of Poly(β-Hydroxybutyrate) by Halobacteria

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Received 6 June 1985/Accepted 26 September 1985

Some species of extremely halophilic archaebacteria, *Halobacteriaceae*, have been shown to accumulate large amounts of poly(β-hydroxybutyrate) under conditions of nitrogen limitation and abundant carbon source. The production of poly(β-hydroxybutyrate), at least in large quantities, was restricted to two carbohydrate-utilizing species, *Halobacterium mediterranei* and *H. volcanii*. In addition to the nutrients in the media, the salt concentration also influenced poly(β-hydroxybutyrate) accumulation, which was greater at lower salt concentrations. The possible application of these microorganisms for the production of biodegradable plastics is discussed.

The members of the genus *Halobacterium* belong to the recently described urkingdom of the archaebacteria, a separate branch of cell evolution (8). Halobacteria require high concentrations of NaCl in the medium to grow and survive (4), and their cells lyse if exposed to distilled water (1). Although some years ago it was thought that halobacteria only utilized amino acids as carbon and energy sources, many new groups have recently been described that are able to utilize a wide range of substrates, including carbohydrates (5–7). In the course of our studies of one such new isolate, *Halobacterium mediterranei*, we observed that, when this organism was grown in glucose and afterwards suspended in distilled water to lyse, a whitish precipitate was produced which was easily collected by low-speed centrifugation in considerable amounts (R. F. Castillo, unpublished data). In the present work we have analyzed this substance, which has proved to be poly(β-hydroxybutyrate) (PHB), a common reserve material of eubacteria, which is accumulated in large quantities by *H. mediterranei*. The effects of some environmental factors on PHB production have also been studied.

For the chemical analysis, *H. mediterranei* R-4 ATCC 33500 was grown in 15 liters of a medium containing 10 g of glucose and 1 g of yeast extract (Difco Laboratories) per liter and 25% marine salts, as previously described (5); the pH was adjusted to 7.2 with 1 N KOH. The cells were grown in 10-liter glass containers with 7.5 liters of medium, aerated by a soft humidified air flow, magnetically stirred, and incubated at 37°C. On reaching the beginning of the stationary phase the cells were harvested by centrifugation at 10,000 rpm in a Beckman J2-21 refrigerated centrifuge for 20 min. The pellet was suspended in 1 liter of distilled water and maintained for 24 h at 5°C, conditions which ensure the lysis of the cells. This lysed suspension was centrifuged at 2,000 rpm for 30 min. The pellet was washed 5 to 10 times with the same volume of distilled water, showing finally a pure white color, an indication that there were almost no cells or cell membranes left since these have a pinkish color. The final pellet was dried in an oven at 80°C until constant weight was attained. The white dust resulting from this treatment was dissolved in distilled chloroform. Most of the material dissolved readily, and the undissolved remains were discarded by decantation. Finally, the chloroform was evaporated at room temperature under vacuum and a film was obtained. This film was then characterized by spectroscopic analysis. The infrared spectra were registered in a Perkin-Elmer 457 spectrometer, using film samples. The ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were recorded in a Bruker SP80SY spectrometer at 80 and 20.1 MHz, respectively, using deuterated chloroform as solvent and tetramethylsilane as internal reference.

To study production under different conditions, 250 ml of the different media were grown in 1-liter Erlenmeyer flasks with orbital shaking (200 rpm) and at 37°C. All media were supplemented with 0.1% (wt/vol) yeast extract (Difco) and 1% (wt/vol) D-glucose or the substrate indicated. The various total salt concentrations used (15, 20, 25, or 30%) contained a mixture of marine salts at constant ratios as described elsewhere (5). When the cultures reached the stationary phase, 200 ml was extracted as described above and the film obtained was weighed. The remaining 50 ml was utilized to determine the salt-free dry weight, the cells were harvested by centrifugation and washed with a 25% salt solution, the pellet was dried at 105°C to constant weight, and the ash remaining after heating at 650°C for 4 h was subtracted.

The infrared spectrum of the film showed the presence of methyl (1,385 cm^{-1}) and carbonyl (1,720 cm^{-1}) groups, probably as ester groups (Fig. 1). From the ^{1}H- and ^{13}C-NMR spectra a very simple structure is to be expected for the product obtained. The appearance of only three peaks in both cases, the splitting of these in the proton spectrum, and the chemical shifts lead us to the conclusion that the compound was PHB. This identification was confirmed by the agreement of our results with ^{13}C-NMR and those given in the literature for the same compound (2). The amounts of PHB produced by *H. mediterranei* under different conditions are shown in Table 1. With yeast extract or glucose separately no PHB was detected by this method. However, when glucose and yeast extract were present in the medium, provided that the concentration of the first was much higher, considerable amounts of PHB were produced. The other substrates tested also stimulated the production, although to a lower extent. With amino acids no detectable amount of PHB was found (data not shown). With glucose plus yeast

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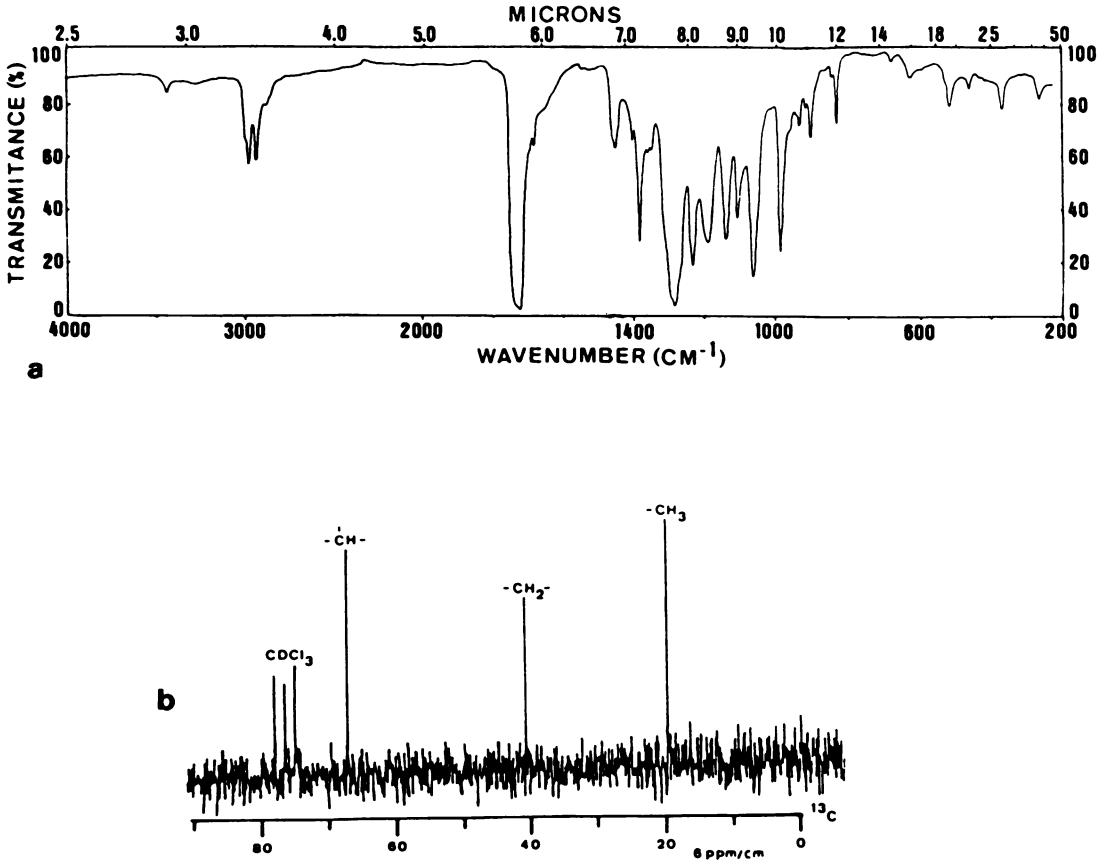

FIG. 1. Infrared (a) and ^{13}C-NMR (b) spectra of the film left after chloroform extraction of cell debris collected by low-speed centrifugation of *H. mediterranei* lysed cells. The cells were grown in a medium containing 1% (wt/vol) glucose and 0.1% (wt/vol) yeast extract and were lysed by exposure to distilled water.

TABLE 1. Amounts of PHB produced by halobacteria

Parameter	PHB (%, dry wt)
Substrate[a]	
α-D-(+)-Glucose	17
Glycerol	7
Sodium citrate	4
D-(+)-Cellobiose	2.5
α-Lactose	
Salts (%)[b]	
15	38
20	35
25	17
30	19
Species[c]	
H. mediterranei	17
H. volcanii	7
H. halobium	
H. gibbonsii	1.2
H. hispanicum	2.4

[a] Amounts produced by *H. mediterranei* grown with different carbon sources; the medium contained 25% (wt/vol) marine salts and 0.1% (wt/vol) yeast extract in addition to 1% (wt/vol) of the substrate assayed.

[b] Effect of salt concentration of the medium (percentage of marine salts) on PHB production in media with 1% glucose and 0.1% yeast extract.

[c] Production of different halobacteria species in a medium with 25% salts, 1% glucose, and 0.1% yeast extract.

extract the PHB accumulation increased at lower salt concentrations, reaching 45% of the cell dry weight. Of the five species of halobacteria assayed, none produced as much as *H. mediterranei*. *H. volcanii* produced about half the amount of the former under identical conditions. *H. hispanicum* and *H. gibbonsii*, which have recently been described in our laboratory (Juez et al., submitted for publication), produced relatively small amounts, and in *H. halobium* none was detected. There is a previous reference in the literature to the presence of PHB in halobacteria, specifically, *H. marismortui* (3). In this work PHB granules were detected by electron microscopy and X-ray diffraction.

Our results confirm this preliminary finding and also show that in *H. mediterranei* PHB is accumulated to a very large extent. PHB has lately become a subject of interest as a potentially useful biodegradable plastic. Considering the large quantities produced by these bacteria, which can be grown relatively rapidly with simple requirements and almost no sterile precautions, these organisms could be interesting candidates to be considered for the production of biodegradable plastics. From a phylogenetic point of view, the presence of PHB in representatives of the archaebacteria establishes a similarity with eubacteria and a difference from eucaryotes, which never accumulate this kind of reserve material.

This investigation was supported by a grant from the Comisión Asesora para el Desarrollo de la Investigación Cientifica y Técnica.

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