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Screening of Soil Bacteria for Poly-β-Hydroxybutyric Acid Production and Its Role in the Survival of Starvation

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ABSTRACT

Seventy-three bacterial strains were isolated from a clay-loam soil and screened for poly-β-hydroxybutyric acid (PHB) production. When grown in a nitrogen-free, carbon-rich medium, 23 strains produced PHB homopolymer. The pseudomonads, which were the majority of the Gram negatives, had a very high frequency (67%) in PHB production, and most of the strains with high productivity of the energy reserve material were placed in this group.

When suspended in Winogradsky salt solution and starved at 21°C, most strains survived very well over the starvation period (150 days and 70 days for two experiments, respectively). The survival ability was strain specific and depended on the growth conditions prior to starvation. The strains capable of producing PHB were not necessarily superior to the others. Accumulation of the energy reserve material had a positive effect on survival of starvation for an individual strain. One strain that did not produce any energy reserve material displayed a prolonged viability under starvation conditions when grown in a nitrogen-free, carbon-rich medium prior to starvation.

Introduction

It is generally believed that the availability of energy substrates remains the main limiting factor for microbial growth in soil. Reacting to occasional influx of organic substance, soil microorganisms grow sporadically. All soil microorganisms, except the rhizospheric microorganisms, are starving most of the year [25], and oligotrophic growth is one way for soil microorganisms to survive a long period of starvation. Another important strategy for soil bacteria is to accumulate energy reserve materials during periods of organic carbon availability. One of the common energy reserve materials is poly-β-hydroxybutyric acid (PHB), found in bacterial cells. PHB has been demonstrated in a wide range of bacterial species [9, 27], and normally accumulates to 2–50% of cell dry weight (DW), varying with bacterial strains and growth

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conditions. Some bacterial species even accumulate PHB to more than 65% of the cell dry weight [29, 30].

PHB is a straight-chain homopolymer of D(−)-β-hydroxybutyric acid [21]. Recent studies have also demonstrated the formation of copolyesters, β-hydroxybutyrate, and β-hydroxyvalerate [10], or 3-hydroxybutyrate and 4-hydroxybutyrate [19], and heteropolymers of short-chain β-hydroxy acids [11, 20]. A new name, poly-β-hydroxyalkanoate (PHA), has been suggested for the heteropolymers [11].

The accumulation of energy reserve materials, such as polysaccharides and PHB, is commonly associated with unbalanced growth of bacterial cells. PHB is produced by bacteria in the presence of excess carbon, when either oxygen, nitrogen, or phosphorus becomes a limiting factor [2, 7, 9]. The conditions for bacterial PHB production can be met in soil, due to its heterogenous nature. For instance, nitrogen availability in soil varies with microsites. It may become a limiting factor for bacterial growth, especially in some nitrogen-poor (carbon-rich) sites [31]. Bacterial growth is also stressed by lack of phosphorus and other mineral nutrients [2, 16, 24]. In a model for simulating relationships between carbon, nitrogen, and microbial biomass during wheat straw decomposition in soil, it was suggested that, when nitrogen was limited, excess carbon apparently was immobilized as polysaccharides [18]. PHB production is also greatly associated with plant roots [12]. Foster and his coworkers [12, 13, 14] demonstrated, with ultrastructural histochemical techniques, that bacteria may produce PHB in rhizosphere and nonrhizosphere soil.

It has long been known that living plant roots represent an important carbon and energy source for soil microorganisms [28], and it was also indicated that the competition between plant roots and microorganisms for nitrogen results in a persistent nitrogen limitation of microbial growth [31]. In the present study we attempted to investigate the fraction of the soil bacteria that may produce PHB under nitrogen deficiency, the potential role of PHB accumulation as an indicator to nitrogen deficiency, and PHB accumulation as an aid in the survival of starving soil bacteria.

Materials and Methods

Soil

The bacteria were isolated from a clay-loam soil, located in the experimental farm of the Department of Soil and Water Sciences, Agricultural University of Norway, Aas-NLH, Norway. The soil has been classified as Typical Haplaquet, containing 26% clay, 42% silt, and 32% sand, with 3% organic carbon, 0.3% organic nitrogen, and pH 5.7.

Isolation and Purification of Soil Bacteria

The soil was mixed 1:10 with the sterilized Winogradsky salt (WS; containing 0.25 g K_2HPO_4, 0.125 g $MgSO_4$, 0.125 g NaCl, 2.5 mg $Fe_2(SO_4)_3$, and 2.5 mg $MnSO_4$ in 1,000 ml distilled water) solution and homogenized for 3 × 1 min with a Warring blender (Warring, Conn), with intermittent cooling in an ice bath. After homogenization, dilutions of 10^{-4}, 10^{-5}, and 10^{-6} g ml^{-1} were prepared from the soil suspension for plate counts with a cold soil extract agar medium (CSEA) and a yeast extract-peptone agar medium (YP). CSEA comprised to a 400 ml autoclaved agar solution, 100 ml of 1:1 cold water soil extract, 1 ml of nutrient stock solution (10 g per 1,000 ml of sodium citrate, sodium succinate, glucose, fructose, xylose, peptone, and yeast extract), and 1 ml of phosphate buffer stock solution (64 g KH_2PO_4 and 34 g Na_2HPO_4 in 1,000 ml distilled water) were added and adjusted to pH 7. YP consisted of 5 g peptone, 1 g yeast extract, 0.01 g $FePO_4 \cdot 2H_2O$, and 20 g agar in 1,000 ml distilled water, pH 7.2 [25].

After incubation at 21°C for 1 week (YP) or 2 weeks (CSEA), all colonies growing on eight of the 10^{-6} dilution plates with YP medium and five of those with CSEA medium were picked up; examined microscopically, and purified by dilution plating on a YPF agar medium (5 g of glucose, yeast extract, and peptone; 1 g KH_2PO_4, 0.5 g $(NH_4)_2SO_4$, 0.25 g $MgSO_4 \cdot 7H_2O$, and 20 g agar, pH 7). After purification, they were preserved both in a glycerol solution at −70°C (Protect Bacterial preserves, Technical Service Consultants Ltd., Lance, UK), and in the test tubes with YPF medium at 4°C for later use.

Screening of Soil Bacteria for PHB Deposition

To test for the production of PHB, each strain was grown to a stationary phase in a nitrogen-free, carbon-rich medium (WSC). The medium consisted of nine parts WS solution and one part carbohydrates stock solution (C), pH 7 and filter sterilized. The stock solution contained 20 g $liter^{-1}$ of each of six sugars (glucose, fructose, galactose, xylose, mannose, and succinate). The soil bacterial isolates from both media, having grown on petri dishes with YP agar medium at 21°C for 5–10 days, depending on strain, were harvested and transferred to WSC medium (20 ml WSC liquid medium in each 50-ml Erlenmeyer flask). The flasks were placed on a reciprocal shaker and incubated at 21°C for one day.

All the cultures were examined by fluorescence microscopy after staining with Nile red. Nile red (9-diethylamino-5*H*-benzo(α)-phenoxazine-5-one) is a hydrophobic stain and intensively fluoresces in all organic solvents, while, in water, fluorescence is quenched. It has been confirmed that Nile red is an excellent vital stain for the detection of intracellular lipids, when applied to cultured tissues and macrophages [15]. The stain is poorly water soluble and crystallizes in water with time, so it was kept as stock solution in acetone at 10 μg ml^{-1} and diluted to 0.10 μg ml^{-1} with water just before use.

The cells were flame-fixed on slide glass, then a drop of 0.1 μg ml^{-1} Nile red solution was added to the smear. After being heated over a flame for 1 second (a test indicated that a slight heating of the smear over a flame for few seconds gave stronger fluorescence), the smear was covered with a cover-glass and examined under the fluorescence microscope (Ploemopak Orthoplan with fluoblock I 2/3, bandpass filter 450–490, Leitz, Germany). Immersion oil must be prevented from entering the smear, since it will absorb Nile red stain and give strong fluorescence.

PHB Analysis

All cultures, regardless of the presence of visible Nile red–fluorescing spots, were subjected to chemical extraction and PHB determination. PHB was extracted from the lyophilized cells with hot chloroform, a modified method of Findlay and White [11] as follows. About 1 mg of cells (DW), with 5 ml chloroform, was boiled with reflux in a 50-ml round-bottom flask on a thermostated heating mantle for 2 h. The extract was applied to a chloroform-saturated silicic acid column (SI, 1 ml, Analytichem International, Calif.) while it was still hot. The flask was washed twice with 2 of hot chloroform, and the washes were also applied to the column. The column was washed with 2 ml chloroform once again, then the PHB was eluted with 10 ml of acetone (glycolipid fraction) [11].

One μl of ethylated sample was subjected to a gas chromatographic (GC) analysis (Carlo Erba 4200, Carlo Erba, Milano, Italy). The column was a 30-m × 0.25-mm capillary column with a film thickness of 0.25 μm (DB-5, J&W Scientific, Calif.). The chromatograph was operated in the split-less mode, with a 30-s venting time. The temperature program was initiated at 45°C for 7 min and then increased to 200°C at 4°C min^{-1}; a 10-min isothermal period was sustained. The helium carrier gas flowed at a rate of 4.5 ml min^{-1}. The injector and detector temperature were 220°C and 250°C, respectively.

The recovery of PHB with present method was tested by extraction and analysis of known amounts of a purchased copolymer (poly(3-hydroxybutyric acid)/-poly(3-hydroxyvaleric acid), Aldrich Chemical Company, Inc., Wis.,). By assuming an equivalent FID (flame ionization detector) response per gram dry weight for ethyl esters of malic acid and the two monomers, > 95% recovery of PHB was obtained.

Taxonomy of the Soil Bacterial Isolates

Bacterial isolates were first characterized, using an API #20B test (API System S.A. Montalieu-Vercieu, France). At first, the soil bacteria were placed within main bacterial groups, based on *Bergey's Manual of Determinative Bacteriology,* 8th edition [3]. Another classification was based on the new edition [4, 5]. The classification of the soil bacterial isolates was only tentative, and the main limitation was the insufficient description of many soil bacteria.

Electron Microscopic Examination

Some of the strains capable of producing PHB were examined by thin-section transmission electron microscopy. The cells were fixed with 2% OsO_4 and stained with uranyl acetate followed by lead citrate.

Protocol for Starvation Experiments

Growth of Bacterial Cells prior to Starvation. A total of 10 out of 73 strains were tested for their ability to survive energy starvation in two separate experiments. The main characteristics and suggested names for the tested soil bacteria are shown in Table 1. In the first experiment, four strains were selected, including two PHB-producing strains and two strains not showing accumulation of any energy reserves. In the second experiment, six PHB-producing strains were tested, and two of them (no. 21 and no. 23) were included in the first experiment.

Prior to starvation, two treatments (treatment A and treatment B) were prepared. In treatment A, exponentially growing cells were obtained by a brief growth period in liquid YP medium. The cells harvested from YP agar plates were transferred to a 500-ml Erlenmeyer flask containing 200 ml YP broth, to an initial cell density of 10^7 cells ml^{-1}, and incubated on a reciprocal shaker at 21°C. Before reaching the stationary phase (12–24 h), cells were harvested and exposed to starvation conditions.

In treatment B, the cells harvested from the same medium plates as in treatment A were grown in a nitrogen-free, carbon-rich medium (WSC medium), with a final cell density of 10^9 ml^{-1}. The suspensions in 200 ml WSC medium in 500-Erlenmeyer flasks were incubated on a reciprocal shaker for 2 days.

Process of Starvation. Prior to starvation, the cells were centrifuged (10,000 *g* for 10 min, Sorvall RC-5B refrigerated superspeed centrifuge, Du Pont, Newtown, Conn.) and washed with WS solution twice. Finally, the cells were resuspended in WS solution to a density of 10^9–10^{10} ml^{-1}. After being shaken for 2 h, the suspension was partitioned into four 500-ml presterilized Erlenmeyer flasks, with 200 ml WS solution, with a final cell concentration of 1–5 × 10^8 ml^{-1}. The remaining cells were stored at −20°C for lipid analysis. The flasks were stoppered with cotton wool and placed on a reciprocal shaker at 80–100 rev min^{-1} at 21°C.

Table 1. The characteristics of the soil isolates tested in two starvation experiments

Isolate number	Tested in experiment[a]	Gram reaction	Energy reserves	Preliminary identification
5a	1	−	No	*Flavobacterium* sp.[b]
21	1,2	+	PHB	*Arthrobacter* sp. C (−)
23	1,2	−	PHB	*Pseudomonas* sp.
39	2	+	PHB	*Arthrobacter* sp.
111a	2	+	PHB	*Arthrobacter* sp.
115	2	−	PHB	*Pseudomonas* sp.
131	1	+	No	*Arthrobacter* sp.
136	2	−	PHB	*Pseudomonas* sp.

[a] 1, Experiment 1; 2, experiment 2
[b] C (−), Catalase negative

Sampling. For the first starvation experiment, the original intention was to sample four times (one flask removed in each sampling) and to finish the experiment within 10 days. After the third sampling (7 days of starvation), we realized that there was only a slight reduction in viability, causing a postponement in the last sampling point. Thus, the last flask of each treatment was maintained for an extended period (5 months) on the shaker. During this period, small subsamples (1 ml) were removed to count numbers of cells and viability, and the remaining cells were harvested for PHB analysis.

For the second starvation experiment, samples were taken after 7, 21, 42, and 70 d of starvation (1 flask taken for each sample).

Counting. Acridine orange direct counts (AODC) and plate counts on YP solid medium were applied to each sample. The water losses due to evaporation (around 20 ml per month, measured by weight loss in the flasks) have been corrected, so that the concentration of cells in the flasks is based on the starting volume.

Microscopic Examination. A combination of fluorescence (Nile red stain) and phase-contrast microscopy was used to check the frequency and the size of granules in the cells.

Statistical Analysis

Standard statistical methods were used to test the difference in survival ability during starvation, and between treatments. Both total (AODC) and viable counts (colony forming units, CFU) were transferred in percentage to the original value, and samplings were treated as blocks.

Results

Identification of Soil Bacterial Isolates

A total of 73 strains were isolated, 37 from YP and 36 from CSEA. Among the strains isolated, 50 strains were Gram positive and 23 strains were Gram negative. The predominant morphological types were rods and the more or less pleomorphic forms. Only one facultative aerobic strain was obtained. The bacteria were preliminarily placed into four main groups: Gram-negative aerobic rods, Gram-positive aerobic irregular rods (coryneform group of bacteria), endospore-forming aerobic rods, and cocci. They were further split into nine smaller groups (genera), namely, *Bacillus, Arthrobacter, Aureobacterium, Corynebacterium, Curtobacterium, Pseudomonas, Flavobacterium, Micrococcus,* and *Acinetobacter.* The profiles of the bacteria isolated from both media show that the frequency of sporulating species was evidently higher on YP than that on CSEA (7 vs. 1), but a little higher frequency of coryneform group of bacteria (22 vs. 17) and *Pseudomonas* (11 vs. 7) was observed on CSEA than on YP.

PHB Production

When stained with Nile red, PHB-containing cells gave bright reddish to orange fluorescence, which was easily distinguished from a weak, dark red fluorescence background from the cell membrane. Chemical analysis confirmed that the strains without bright reddish to orange fluorescing spots did not contain a detectable amount of PHB (<0.01% of cell DW).

A total of 23 soil bacterial isolates were able to accumulate PHB when grown in a nitrogen-free medium, and the PHB production ranged from about 1% to more than 30% of cell DW (Table 2). Most of the isolates contained about 10% PHB of cell DW, with four strains containing more than 20%. For those strains with fluorescing spots seeming to fill the whole cell and no individual granules visible, the symbol "+++" is given; for those strains with small fluorescing spots and/or distinguishable granules, the symbol "+" is indicated (Table 2).

Table 2. Screening of PHB producers by fluorescence microscopy with Nile red stain and chemical analysis

Strains	Fluorescence with Nile red strain		PHB produced (% DW)	Suggested strain name
	Color	Visible granules		
4b	Orange	++	9.6	*Pseudomonas* sp.
8	Orange	+	5.7	*Pseudomonas* sp.
9	Reddish	+++	21.7	*Pseudomonas* sp.
16	Orange	++	8.9	*Curtobacterium* sp.
21	Reddish	++	16.3	*Arthrobacter* sp.
22	Yellow	+	1.5	*Pseudomonas* sp.
23	Orange	+++	31.5	*Pseudomonas* sp.
24	Orange	++	13.2	*Pseudomonas* sp.
30	Orange	+	3.2	*Bacillus* sp.
38	Reddish	+	4.3	*Arthrobacter* sp.
39	Orange	++	10.5	*Arthrobacter* sp.
40	Orange	++	10.6	*Aureobacterium* sp.
42	Reddish	+	4.0	*Bacillus* sp.
49	Reddish	++	19.8	*Bacillus* sp.
105a	Orange	+	3.4	*Pseudomonas* sp.
106	Orange	++	10.3	*Pseudomonas* sp.
110	Orange	+	6.4	*Curtobacterium* sp.
111a	Orange	+	3.3	*Arthrobacter* sp.
115	Orange	+++	22.7	*Pseudomonas* sp.
116	Orange	+++	20.1	*Pseudomonas* sp.
123	Orange	++	12.0	*Pseudomonas* sp.
126	Orange	+	1.5	*Curtobacterium* sp.
136	Orange	+++	24.6	*Pseudomonas* sp.

Table 3. Distribution of PHB-producing strains among the soil bacterial isolate group

	Isolate number	Sporul.	Coryneform group					G-Rods		Cocci	
		Baci.	Total	*Arth.*	*Aur.*	*Cor.*	*Cur.*	*Pseu.*	*Flav.*	*Mc.*	*Ac.*
Sum	73	8	39	15	10	6	8	18	4	3	1
PHB	23	3	8	4	1	0	3	12	0	0	0
PHB/sum (%)	31.5	37.5	20.5	26.7	10.0	0	37.5	66.7	0	0	0

Distribution of Soil Bacterial Isolates with PHB

The distribution of PHB-producing strains among the soil bacterial isolates is shown in Table 3. Based on preliminary classification, the bacillus and pseudomonad strains were commonly PHB producers.

The Examination of PHB with Electron Microscopy

The micrographs of one PHB-producing strain are shown in Fig. 1. The PHB granules were not able to be stained with OsO_4 and left the electron transparent holes in the sections [14]. Several small granules were commonly found in PHB-containing cells.

Survival Ability of Soil Bacteria Containing PHB

The total counts (AODC) and viable counts (CFU) at day 42 and day 150 are shown in Table 4. for 2 PHB producers and 2 other strains that apparently did not produce PHB or other carbon reserve materials, as observed by phase-contrast microscopy and Nile-red fluorescence microscopy. With time, both total bacterial numbers and CFUs in starving cultures decreased. There was a tendency toward the bacterial numbers (AODC and CFU) of exponentially grown cells decreasing faster than cells previously grown in a nitrogen-free medium. At the end of the first experiment, the total bacterial cell numbers dropped to 14–50% of the zero-time values for treatment B, and 9–32% for treatment A (Table 4). However, there was no statistically significant difference ($p \leq 0.05$) in the ability to survive starvation among strains tested in the first experiment, regardless of ability to produce energy reserves.

To further explore the effects of growth state on viability, we compared survival in six PHB-producing strains during the second experiment. Each strain was harvested either during exponential growth or after 2-day exposure to nitrogen-free medium. In each strain, survival was greater in cultures exposed to nitrogen-free medium compared to those harvested during exponential growth (Table 5).

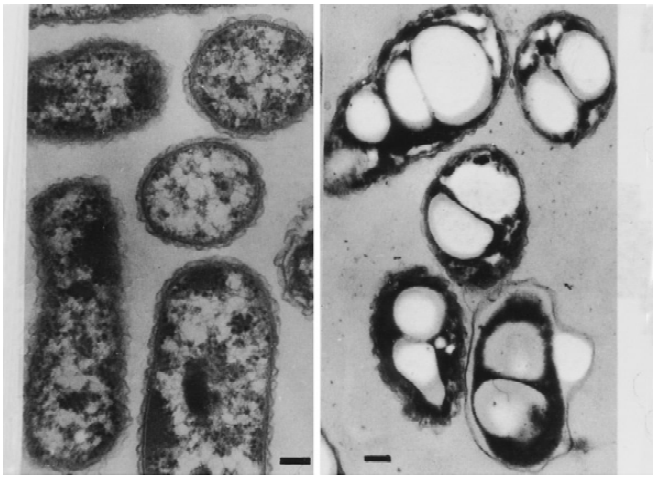

Fig. 1. Electron photomicrographs showing the bacterial cells of strain no. 23 (*Pseudomonas* sp.) with (*right*) and without (*left*) PHB accumulation. *Bar* = 100 nm.

Degradation of PHB and Membrane Lipid During Starvation

The degradation of PHB contained in the starving bacterial cells in both experiments is shown in Fig. 2. The fastest PHB

Table 4. Total counts and viable counts during starvation expressed as % of zero-time total counts, first experiment

Strain	Energy reserves	Treatment	Counts as % of AODC at $t = 0$			
			42 days		150 days	
			AODC	CFU	AODC	CFU
5a	None	B	61.9	67.4	35.3	5.3
		A	70.4	0.8	19.2	1.0
131	None	B	47.3	43.1	28.1	6.2
		A	53.2	47.4	32.1	17.4
21	PHB	B	48.0	43.2	26.1	6.9
		A	43.3	29.2	14.2	3.3
23	PHB	B	64.4	52.3	28.7	17.7
		A	72.4	49.2	27.6	1.1

Table 5. Total counts and viable counts during starvation expressed as % of zero-time total counts, for PHB producers in the second experiment

		Counts as % of AODC at $t = 0$			
		42 days		70 days	
Strain	Treatment	AODC	CFU	AODC	CFU
21	B	81.0	80.3	80.0	46.7
	A	84.0	23.7	76.2	13.9
23	B	83.3	75.9	68.7	66.7
	A	79.8	43.7	63.2	34.3
39	B	95.9	38.3	68.3	19.6
	A	73.6	ND[a]	69.8	4.3
111a	B	76.1	0.7	89.3	0.3
	A	38.8	0.3	29.5	0.1
115	B	87.2	72.3	58.6	22.4
	A	59.8	45.9	47.3	10.8
136	B	71.4	39.3	53.7	34.9
	A	65.3	11.5	49.4	10.0

[a] ND, Not determined

degradation was observed during the beginning of the starvation; 50–90% of initial PHB had been degraded by the end of 1 week, the exception strain was 23, in which cells degraded PHB much slower, but by the end of both experiments, considerable PHB in the cells was still detected.

When observed with Nile-red fluorescence microscopy in the beginning of the starvation, cell membrane of all strains tested fluoresced dark red, making cells without PHB deposition visible. There were some small spots of more intensive and bright fluorescence on the background of dark red fluorescence. Fluorescence of both in the cells grown in nitrogen-free medium was much stronger than in the exponentially grown cells. With time, during starvation, Nile red fluorescence from the membranes weakened, and fluorescing spots in the envelope disappeared.

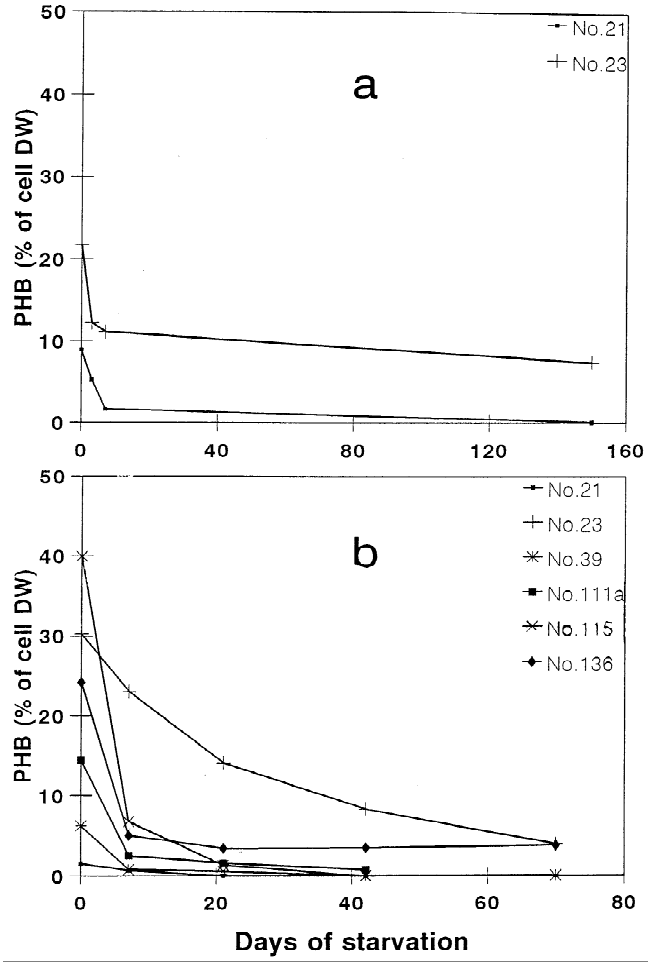

Fig. 2. The degradation of PHB contained in the bacterial cells during starvation for the C-exposed cells in the first (**a**) and the second (**b**) experiments.

Discussion

A large proportion (32%) of the soil bacterial isolates obtained as part of this study were able to produce PHB as energy reserve material. The accumulation of PHB in visible intercellular structure varied with bacterial groups. The highest frequency of PHB-producing bacteria was found within pseudomonads (67%). About 20% of the coryneform bacteria, and 37% of bacilli, were PHB producers. The number of strains in the other genera was too low to judge the frequency of bacteria capable of the energy reserve material accumulation. Dawes and Senior [9] and Preiss [27] claim that PHB has not been observed among coryneform bacteria. This might be because very few bacterial strains in this group have been examined for PHB production [6], considering the high frequency of PHB-producing strains in this group (Table 2). However, the actual amounts of PHB (percent of cell DW) were generally lower for the coryneform bacteria than for the other groups.

PHB produced in the soil bacteria was exclusively of the homopolymer type. The co- or heteropolymer of short-chain hydroxy acids was not been detected. In the marine sediment samples, more than 10 short-chain β-hydroxy acids have been demonstrated [11]. As for industrial purposes, some bacterial strains have been shown to accumulate copolyesters of β-hydroxybutyrate and β-hydroxyvalerate [10], and heteropolymers [17, 20]. The substrates for copolymer and heteropolymer production were short-chain

alkanes and alkenes [20], and short-chain fatty acids [10], or short-chain β-hydroxy fatty acids [17]. When glucose was used as carbon substrate, only homopolymer of β-hydroxybutyrate was produced [10]. In the marine sediments, a variety of organic carbon substrates may be present, whereas, in aerobic soil, the concentrations of more reduced forms of organic carbon are probably very low.

It is well established that PHB, as well as other energy reserve materials including glycogen, is used for endogenous metabolism during starvation [6, 9, 27], especially in the early phase [22, 23]. In the present investigations, a positive effect of PHB utilization on prolonged bacterial viability was observed. The higher initial content of PHB for a certain strain (treatment B vs. A) implies more supply of energy substrates. Subsequently this would result in a better survival. As demonstrated by Matin et al. [23], the resistance to starvation for a freshwater *Spirillum* sp. correlated directly with the initial PHB content in the cells subjected to starvation.

The PHB degradation rate in the present study was much slower (Fig. 2) compared with marine bacteria, in which PHB was degraded to zero (not detectable level) within a few hours of starvation [22]. It seemed that the PHB degradation rate was a more important factor for survival ability than the initial PHB content. The higher demand for maintenance energy by some of the strains resulted in a quicker disappearance of PHB from the cells and, consequently, a lower ability to survive starvation. For instance, two strains, no. 23 and no. 136, when grown in a nitrogen-free medium, retained a considerable amount of PHB throughout the entire starvation period (70 days, Fig. 2) and had exceptionally high survival ability (Table 5). This is clearly in contrast to strains 111a and 115 (Fig. 2 and Table 5).

The ability to produce PHB was apparently not necessary for having an excellent survival ability during starvation. This is supported by the observation that the strains not able to accumulate energy reserve materials (nos. 5a and 131) survived equally as well as the strains capable of accumulating energy reserve materials. The present study agrees well with a general review that suggests that PHB enhances the survival of some, but not all, of the bacteria [1]. As reviewed by Preiss [27], other the presence of energy reserve compounds in bacterial cells, for instance glycogen, does not always imply a better survival rate during starvation.

During starvation, membrane lipid degradation was also visible, as indicated by general weakening of Nile red fluorescence from the membranes and a disappearance of fluorescing spots in the envelope. The indication of a reduction in membrane-bound lipids occurred in the exponentially grown cells of all strains tested, as well as in the cells of strains incapable of producing energy reserve materials, even those grown in a nitrogen-free, carbon-rich medium. Thus, a reduction in membrane-bound lipids during starvation seems to be a common feature in all strains. The utilization of lipids other than PHB by the bacteria during starvation was also demonstrated by others [22, 23, 26]. Endogenous metabolism is essential for the bacterial cells undergoing starvation. This does not necessarily imply that just energy reserves are utilized as substrates. It has been reviewed that microorganisms degrade cellular materials including amino acids, sugar, and some macromolecules during starvation [8]. The utilization of such substances might account for prolonged survival for the other strains (nos. 5a and 131).

In conclusion, as an adaptational mechanism to occasional availability of carbon substances, a considerable proportion of soil bacterial isolates we found to be capable of accumulating PHB as an energy reserve material. For an individual strain, the accumulation of the energy reserve material implies enhanced survival during starvation. However, strains capable of producing PHB were not necessarily superior to others.

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