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Factors Affecting Polyhydroxybutyrate Biosynthesis in the Marine Photosynthetic Bacterium *Rhodopseudomonas* sp. Strain W-1S

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ABSTRACT

A marine photosynthetic bacterium, *Rhodopseudomonas* sp. strain W-1S, accumulated polyhydroxybutyrate (PHB) to 56% of the dry cell weight under microaerobic and photoheterotrophic conditions. The addition of NaCl and phosphate was essential for the accumulation. Acetate was the best carbon source for PHB accumulation, whereas adding malate, succinate, or sugars that did not support the growth in this strain resulted in no accumulation. The strain accumulated PHB to about 50% of the dry cell weight at the logarithmic phase of growth. These results suggested that the accumulation would be growth associated under microaerobic and photoheterotrophic conditions. Vitamins, especially biotin, exerted an inhibitory effect on PHB accumulation and a stimulatory effect on pigment production. There might be regulation of carbon flux to PHB or pigments as storage materials.

Index Entries: Marine; polyhydroxybutyrate; photosynthetic bacteria; hydrogen; *Rhodopseudomonas*.

INTRODUCTION

Many bacteria accumulate polyhydroxybutyrate (PHB) as a carbon and energy storage compound, or use it as a sink for reducing equivalents *(1)*. PHB and probably most other polyhydroxyalkanoates (PHA) are completely biodegradable to CO_2 and H_2O. Since PHA are thermoplastic and can be manufactured on a large scale, they have attracted much attention as possible substitutes for plastics produced from petrochemicals *(2,3)*. Recent studies have shown that the intracellular polymers are produced also by some halobacteria *(4)*. Members of the genus *Halobacterium* belong to archaebacteria, a separate branch of cell evolution *(5)*. Halobacteria are known to require a high concentration of NaCl in the medium in order to survive and grow *(6)*, and their cells lyse if exposed to distilled water *(7)*.

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However, there is little information on the regulation of storage material accumulation in halophilic photosynthetic bacteria. The marine photosynthetic bacterium *Rhodopseudomonas* sp. strain W-1S, which was isolated from marine samples in our laboratory *(8)*, was found to be able to evolve hydrogen under light from fermentative products of microalgae, such as acetic acid and ethanol. The efficiency of energy conversion in the total system with the alga and bacterium was also evaluated, and a high molar yield of hydrogen was achieved by this alga–bacterial combination in an alternating light–dark cycle *(8)*. In recent studies, we found that this strain can also accumulate a considerable amount of PHB as intracellular granules. Under anaerobic conditions, there might be competition between PHB accumulation and hydrogen evolution for reducing equivalents. This article describes the effects of some environmental factors on PHB accumulation in *Rhodopseudomonas* sp. strain W-1S.

MATERIALS AND METHODS

Microorganism

The marine photosynthetic bacterium *Rhodopseudomonas* sp. strain W-1S was used for all the experiments. This strain was newly isolated from marine samples collected near the coast in the Kinki region of Japan *(8)*.

Cultivation

Cells were inoculated into a 2-L culture flask containing 1 L of L- broth consisting of 0.5% Bacto-peptone, 0.3% yeast extract, and 2.5% NaCl. The cells were cultivated aerobically at 30°C with vigorous shaking.

PHB Accumulation

After cultivation for 24 h, cells were harvested by centrifugation, washed aseptically with sterilized distilled water, and resuspended into 1-L culture bottles containing 500 mL of modified Okamoto medium (MOM, pH 8.0), consisting of 30 g NaCl, 0.2 g $CaCl_2 \cdot 2H_2O$, 0.25 g $MgSO_4 \cdot 7H_2O$, 20 mg $FeSO_4 \cdot 7H_2O$, 40.8 mg KH_2PO_4, 495 mg K_2HPO_4, 0.5 mg thiamine, 0.5 mg nicotinic acid, 0.3 mg *p*-aminobenzoic acid (PABA), 0.05 mg biotin, 2.86 mg H_3BO_3, 1.81 mg $MnCl_2 \cdot 4H_2O$, 0.22 mg $ZnSO_4 \cdot 7H_2O$, 0.08 mg $CuSO_4 \cdot 5H_2O$, 0.021 mg Na_2MoO_4, 0.01 mg $CoCl_2 \cdot 6H_2O$, and 50 mg EDTA disodium salt in 1 L, with the addition of 5 m*M* NH_4Cl as a nitrogen source, and sodium acetate, sodium succinate, sodium malate, and sodium pyruvate each at a final concentration of 0.1% as carbon sources. Cells were then incubated microaerobically with a gentle shaking by a magnetic stirrer at 30°C, and illuminated under fluorescent and incandescent lamps at 125 μmol photon/m^2 s for 48 h. After incubation for 48 h, cells were harvested by centrifugation, washed twice with sterilized distilled water, and lyophilized with a freeze dryer (Model VR16, Hitachi, Japan) overnight. After lyophilization, the total cell dry weight was measured.

Analysis of PHB Content

Pigments were extracted from lyophilized cells by acetone extraction with reflux for 5 h at 70°C. After careful removal of the extracts from the cell debris, the remaining cell debris was again suspended with chloroform, and PHB was

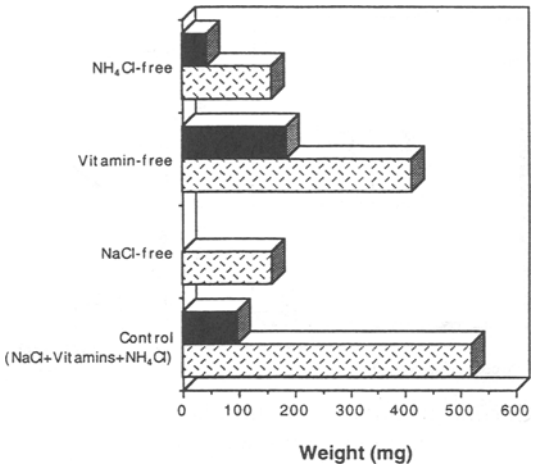

Fig. 1. Effects of NaCl, vitamins, and NH_4Cl on accumulation of PHB in *Rhodopseudomonas* sp. strain W-1S. The control medium contained 3% NaCl, biotin, nicotinic acid, PABA, thiamine, and 5 m*M* NH_4Cl. NaCl, vitamins, or NH4Cl was omitted from the medium, and cells were incubated as described in Materials and Methods. Total cell dry weight (▨) and polymer weight (■) were measured after incubation for 48 h.

extracted for 6 h at 70°C. The cell debris was then removed by passing the suspension through a cellulose filter, and the chloroform solution was concentrated using a rotary vacuum evaporator (Tokyo Rikakikai Co., Ltd., Japan). Finally, the precipitated polymer was separated and the weight of the total PHB content was measured.

RESULTS

The effects of NaCl, vitamins, and NH_4Cl on PHB accumulation in *Rhodopseudomonas* sp. strain W-1S were examined using 0.1% each of acetate, pyruvate, succinate, and malate as substrates. Figure 1 shows the results. In the absence of NaCl, the dry weight of cells after incubation for 48 h was much lower than that of the control, and no PHB accumulation occurred. Cells incubated in the absence of vitamins accumulated twice as much PHB as those incubated in the presence of four vitamins: biotin, nicotinic acid, PABA, and thiamine. The content of PHB under the vitamin-free conditions was 45% (PHB weight/total cell dry weight). Since removal of NH_4Cl decreased both PHB and the total cell dry weight, 5 m*M* NH_4Cl were added in the subsequent experiments.

Figure 2 shows the effect of the NaCl concentration on PHB accumulation. Cells were incubated for 48 h under different NaCl concentrations without adding vitamins. The amount of PHB increased with the NaCl concentration up to 5%, but no PHB accumulation was observed at 10%. The optimum NaCl concentration for growth was 1%. Addition of NaCl at 5% resulted in the best PHB accumulation, but this amount had an inhibitory effect on cell growth. NaCl was therefore added at 3% in the subsequent experiments.

Table 1 shows the effects of vitamins on the PHB content. Each vitamin was omitted in turn to ascertain the inhibitory effect of individual vitamins on PHB accumulation. Removal of thiamine did not affect the PHB content, whereas remov-

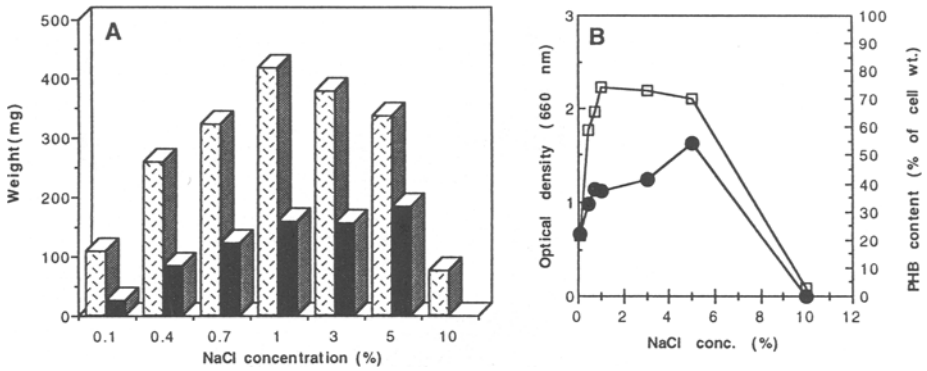

Fig. 2. Effects of NaCl concentration on accumulation of PHB in *Rhodopseudomonas* sp. strain W-1S. Cells were incubated in the presence of various concentrations of NaCl. The medium contained 5 m*M* NH_4Cl and no vitamin. **(A)** Total cell dry weight (⁘) and polymer weight (■). **(B)** Percentage of PHB in total cell dry weight (●) and OD660 (□).

Table 1
Effects of Vitamins on PHB Accumulation in *Rhodopseudomonas* sp. Strain W-1S[a]

Condition	Intitial cell conc., $OK_{660\,nm}$	Final cell conc., $OD_{660\,nm}$	Total cell dry wt, mg	PHB content, mg	PHB ratio, %, PHB/cell dry wt
Control all vitimins	0.3	2.57	521	99	18.9
Vitamin-free	0.3	2.50	411	186	45.2
Biotin-free	0.3	2.32	451	132	29.4
Nicotinic acid-free	0.3	2.09	418	30	7.1
PABA-free	0.3	2.66	561	69	12.4
Thiamine-free	0.3	2.52	504	104	20.7

[a]Cells were grown at 30°C under constant illumination for 48 h in MOM containing 5 m*M* NH_4Cl, and sodium succinate, sodium acetate, sodium pyruvate, and sodium malate each at a final concentration of 0.1%.

ing nicotinic acid and PABA decreased it. However, biotin removal resulted in significantly more PHB accumulation than that obtained under the control conditions in which all four vitamins were added. These results indicate that only biotin had an inhibitory effect on the PHB accumulation in this strain.

Thus far, four carbon sources were used as substrates for PHB accumulation. Next, the effect of each carbon source on the accumulation was examined individually (Table 2). Acetate was shown to be the best substrate, with the PHB content reaching 56% of the cell dry weight. Pyruvate was less effective, giving a PHB content of 12.6%. The addition of malate and succinate, which are good substrates for photohydrogen evolution in this strain, resulted in no PHB accumulation. Cells incubated with glucose, fructose, sucrose, and gluconate also did not accumulate PHB (data not shown). Figure 3 shows the effects of phosphate on PHB accumulation. Cells were incubated for 48 h with 0.5% acetate in the presence and absence of

Table 2
Effects of Carbon Source on PHB Accumulation in *Rhodopseudomonas* sp. Strain W-1S[a]

Carbon source	Initial cell conc., $OD_{660\,nm}$	Final cell conc., $OD_{660\,nm}$	Total cell dry wt, mg	Total PHB content, mg	PHB % of cell dry wt
Acetate	0.24	1.58	211	118	56.1
Malate	0.24	0.26	50	None	—
Succinate	0.24	0.31	53	None	—
Pyruvate	0.24	0.84	135	17	12.6

[a]Cells were grown at 30°C under constant illumination with each carbon source (final concentration, 0.5%) for 48 h in MOM containing 5 m*M* NH_4Cl and without vitamins.

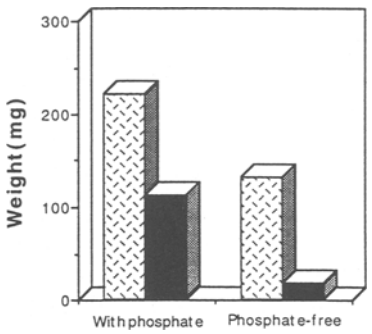

Fig. 3. Effects of phosphate on accumulation of PHB in *Rhodopseudomonas* sp. strain W-1S. Cells were incubated in the presence or absence of phosphate, and the total cell dry weight (▨) and polymer weight (■) were measured after incubation for 48 h. The medium contained 5 m*M* NH_4Cl, 3% NaCl, and no vitamin.

phosphate. Since the removal of phosphate significantly decreased both PHB and the total cell dry weights as well as NaCl, phosphate was shown to be essential for PHB accumulation in this strain.

The relationship between growth phase and PHB accumulation was examined under microaerobic and photoheterotrophic conditions in MOM. The strain accumulated PHB to about 50% of the dry cell weight at both logarithmic and stationary phases of growth.

DISCUSSION

These results demonstrate that the newly isolated marine photosynthetic bacterium *Rhodopseudomonas* sp. strain W-1S is able to accumulate a considerable amount of PHB under a polymer-enhancing medium in which NaCl is provided. No PHB accumulation was observed in the absence of NaCl in the MOM. We also found that under NaCl-free conditions, the cell growth decreased significantly as compared to that in the presence of NaCl. NaCl thus seems to be an essential component for polymer accumulation as well as for growth in this strain. Phosphate was also

shown to be necessary for growth and polymer accumulation, although it has been reported that removal of phosphate greatly enhanced the polymer accumulation in other bacteria *(9,10)*. Usually, cells accumulate more polymer under conditions in which the growth is suppressed by the removal of substrate other than carbon source. On the contrary, the strain W-1S accumulated much polymer in a growth-associated manner under photoheterotrophic conditions. Acetate was shown to be the best carbon source for PHB accumulation. Cells could not accumulate polymer using substrates that did not support photoheterotrophic growth, such as succinate, malate, and sugars. Sugars are, however, good substrates for PHB accumulation in other halobacteria *(9)*.

Removal of vitamins greatly stimulated PHB accumulation. The presence of four vitamins—thiamine, PABA, nicotinic acid, and biotin—might stimulate the production of pigments, such as carotenoid, because the cells displayed a very deep pink color in the presence of vitamins, but were very light pink in their absence. It appears that the carbon flux to PHB or pigments is regulated by certain vitamins under microaerobic and photoheterotrophic conditions in this strain. Under light anaerobic conditions, this bacterium exhibited nitrogenase-dependent hydrogen evolution from organic compounds. Both hydrogen evolution and PHB accumulation could be considered to be electron sinks in the microorganism. Inhibition of nitrogenase greatly enhanced the PHB accumulation under light anaerobic conditions (unpublished data). Therefore, reducing equivalent competition would appear to exist between PHB accumulation and hydrogen evolution.

We have previously proposed a solar energy conversion system based on the microalgal fixation of carbon dioxide *(8)*. The system consists of photosynthetic starch accumulation in microalga, algal fermentation to produce organic compounds, and conversion of the organic compounds to hydrogen by photosynthetic bacteria.

In this study, we have shown that it is possible to control the metabolism in a photosynthetic bacterium. Depending on the purpose, organic compounds from microalga could be used for the production of biodegradable polymer, as well as a source of clean energy—hydrogen.

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