Morphological Studies of Synechocystis sp. UNIWG under Polyhydroxyalkanoate Accumulating Conditions

Yew, S.P., Jau, M.H., Yong, K.H., Abed, R.M.M. and Sudesh, K.*

1School of Biological Sciences, Universiti Sains Malaysia, 11800, Minden, Penang.
2Max Planck Institute for Marine Microbiology, Corrensstrasse 1, D-28359 Bremen, Germany.

ABSTRACT

Some cyanobacteria are capable of producing polyhydroxyalkanoate (PHA), among which is the unicellular Synechocystis sp. Here, we report the identification and preliminary characterization of a newly isolated strain of Synechocystis sp. UNIWG that is capable of accumulating unusually high number of PHA granules. This cyanobacterium was isolated from oil-contaminated brackish water sample from Wadi-Geza, Palestine. Surprisingly, Nile Blue A staining of PHA-accumulating cells failed to reveal the accumulated PHA granules. Ultrastructural analysis of Synechocystis sp. UNIWG cells grown under nitrogen limiting conditions revealed the presence of up to 17 electron-transparent granules in the cell cytoplasm. Gas chromatography analysis further revealed that these cells contain up to 14 wt% poly(3-hydroxybutyrate) of the cell dry weight. Ultrastructural analysis also revealed that Synechocystis sp. UNIWG cells from the growth phase were covered with a dense layer of pili like structures. However, these pili like structures were not observed in cells from the PHA-accumulation phase. The possible roles of these pili like structures and PHA accumulation for the survival of this cyanobacterium is discussed here.

Keywords: Polyhydroxyalkanoate (PHA), Synechocystis strain UNIWG, Transmission electron microscopy, Intracellular granules, Pili.

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are accumulated by various bacteria as intracellular carbon and energy storage materials (Dol, 1990; Sudesh et al., 2000). Poly(3-hydroxybutyrate) [P(3HB)] and its copolymers are the most frequently encountered PHAs. Biodegradability and biocompatibility are among the remarkable properties of PHAs synthesized by microorganisms are stored in the form of water insoluble granules in the cell cytoplasm. PHA granules can be visualized readily by light microscopy because of their high refractivity and ability to be stained by dyes such as Nile Blue A. By using electron microscopy technique, the PHA granules appear as discrete electron-transparent granules ranging from 200-500 nm in diameter. The number of PHA granules is usually 2-8 in a bacterial cell (Anderson and Dawes, 1990).

In addition to studies on heterotrophic bacteria as PHA producers, various research groups are exploring PHA production using photosynthetic microorganisms as the production hosts. Studies have shown that some cyanobacteria have the natural ability to synthesize PHA. To date, the occurrence of PHA has been demonstrated for several cyanobacteria such as Chlorella fusca (Carr, 1966), Spirulina sp. (Campbell II et al., 1982; Vincenzini et al., 1990), Aphanothece sp. (Capon et al., 1983), Gloeothecae (Arno et al., 1995), and Synechococcus sp. (Miyake et al., 1966). Like higher plants, cyanobacteria are also oxygen-evolving photoautotrophs with the added advantage that some of them naturally possess the key enzyme in PHA biosynthesis i.e. the PHA synthase (Hein et al., 1998; Taroncher-Oldenburg et al., 2000). The maximum quantity of PHA accumulation that has been reported for cyanobacterium is in Synechococcus sp. MA19 with 27 wt% PHA content. It is thus obvious that production of PHA by cyanobacteria for commercial purposes has attracted a great deal of attention lately, because, in contrast to heterotrophic bacteria, cyanobacteria can obtain the precursors for PHA biosynthesis from CO2 that is assimilated through photosynthesis rather than from more complex organic carbon sources (Asada et al., 1999; Sudesh et al., 2002).

Here, we report the results of our studies in a newly isolated Synechocystis strain UNIWG with respect to its ability to produce PHA. Of particular importance is the accumulation of an unusually large number of PHA granules in the cell cytoplasm. In addition, we have also carried out some preliminary characterization on the pili like structures which formed a dense covering on the cell surface.

MATERIALS AND METHODS

Bacterial strain and cultivation conditions

Synechocystis sp. UNIWG (Abed et al., 2002) was grown in liquid BG-11 medium (Stanier et al., 1971) at 28 ± 2°C. The cells were grown in BG-11 medium under 1020 Lux illumination for nitrogen-sufficient cultivation. In order to obtain nitrogen-starved cells, cultures in the late exponential growth phase were transferred to nitrogen-free BG-11 medium containing different concentration of sodium acetate to promote PHA biosynthesis. All cultures

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were continuously bubbled with filter sterilized air. Cell growth was monitored by measuring the optical density of the culture at 730 nm.

Analytical procedures

In order to determine the PHA content and composition, 25 mg of the freeze-dried cells were subjected to methanolysis with 15% (vol/vol) sulfuric acid and 85% (vol/vol) methanol. The resulting hydroxyacyl methyl esters were then analyzed by gas chromatography (GC) according to standard methods (Brownrigg et al., 1978).

Electron microscopy

Cells were fixed in McDowell-Trump fixative followed by osmium tetroxide. Thin sections of cells were post-stained in methanolic uranyl acetate and lead citrate and then observed under Philips CM 410 microscope at 80 KeV.

Negative staining

Cells from the growth phase and PHA accumulation phase were negatively stained with 1% methylvamine tungstate for 1 min and examined using Philips CM 410 microscope at 80 KeV.

RESULTS

Growth of Synechocystis sp. UNIWG

Synechocystis sp. UNIWG was isolated from Wadi Gaza, Gaza, Palestine. Optical microscopy clearly showed that this strain was coccolid in shape and measured 2-3 μm in diameter. The cells divided by binary division (Fig. 1A). The growth profile of Synechocystis sp. UNIWG cells in balanced BG-11 medium is shown in Fig. 1B. A lag phase of about 4 days was observed for cultures started from colonies on agar plate. The cells reached stationary phase after 12 to 14 days of cultivation. The cells grew photoautotrophically with a doubling time of approximately 17 hours compared to 20 hours for Synechocystis sp. PCC6803 (Nixon et al., 1991). During this time, PHA granules were not detected in the cell cytoplasm (Fig. 2A and B). This was further confirmed by gas chromatography analysis of dry cells from the stationary phase (results not shown).

Ultrastuctural characteristics of Synechocystis sp. UNIWG during growth

Synechocystis sp. UNIWG was subjected to morphological examination using transmission electron microscopy (TEM). After 7 days of growth in balanced BG-11 medium, no PHA accumulation was detected in the cells(Fig. 2A and B).

Figure 2 Transmission electron micrographs of Synechocystis sp. UNIWG. (A) and (B) Cell morphology after 7 days cultivation under balanced growth conditions. (C) and (D) Cell under nitrogen-free culture condition. Cell morphology with PHA granules in the cell cytoplasm. PHA granules appear as electron dense inclusions. Bar represents 500 nm.

In addition, thylakoid membranes were also absent in the cell cytoplasm. TEM also revealed, the presence of spicules on the cell surface during this stage (Fig. 2A and B). The length of spicules was uniform and the spicules were distributed about the entire surface of the cell. They appeared straight and rigid ranging from 150-175 nm in length. During nitrogen starvation after the washed cells had been transferred into BG-11 medium supplemented with 0.5% (w/vol) sodium acetate, PHA was synthesized and the distinct PHA granules can be observed in the cell cytoplasm (Fig. 2C and D). After 21 days of cultivation in the nitrogen-free condition, 10 to 17 electron transparent granules were identified in cells. The PHA granule shape and sizes were very similar to those occurring in other PHA accumulating bacteria (Sudesh et al., 2002).

PHA biosynthesis by Synechocystis sp. UNIWG

Cyanobacteria accumulate PHA under nutrient limiting conditions. Usually nitrogen or phosphate limitations are used. In this study, we used nitrogen limited BG-11 (-N) to induce PHA biosynthesis. In addition to carbon dioxide, we also tested the ability of sodium acetate to promote the biosynthesis of PHA. The addition of sodium acetate was
found to enhance the production of PHA by Synechocystis sp. UNIWG (Table 1). GC analysis revealed that the PHA produced by this new isolate is a P(3HB) homopolymer. In general, P(3HB) concentration increases if acetate was added to the culture medium.

P(3HB) content was relatively low when atmospheric carbon dioxide was used as the sole carbon source. GC analysis revealed the presence of only trace amounts (~1.0 wt% of the dry cell weight) of P(3HB) homopolymer. The concentration of P(3HB) was the highest with the addition of 0.5% (wt/vol) acetate, whereby the P(3HB) content was as high as 14 wt% after 21 days of cultivation. The P(3HB) contents in the cells varied between 1 and 14 wt% of the cellular dry weight depending on the state of the culture and the length of incubation in the nitrogen-deficient BG-11 medium.

Figure 3: Transmission electron micrograph of Synechocystis sp. UNIWG cell stained with 1% methylamine tungstate showing profuse pili. P. plus, between 1 and 14 wt% of the cellular dry weight depending on the state of the culture and the length of incubation in the nitrogen-deficient BG-11 medium.

Negative staining of Synechocystis sp. UNIWG cells

Negative staining revealed that the surfaces of cells contain profuse profusely arranged pili (Fig. 3). The pili were approximately 6 nm thick and profusely distributed all over the cell surface. Bundles of these pili can be seen to originate from specific centers on the cell surface. The presence of pili on the cell surface has been demonstrated for Synechocystis sp. 6803. In addition to the type of pili observed in this study, Synechocystis sp. 6803 was also shown to possess a longer type IV pilus at a much lower concentration. The latter has been proposed to be involved in phototactic motility.

DISCUSSION

Synechocystis sp. UNIWG used in this study is a brackish unicellular cyanobacterium isolated from Wadi Gaza, Gaza Strip, Palestine (Abed et al., 2002). Morphologically, the cells resembled Synechocystis sp. 6803. The latter is a model cyanobacterium that is widely used for molecular studies because the entire genome of this cyanobacterium has been sequenced (Kaneko and Tabata, 1997). However, unlike Synechocystis sp. 6803, Nile Blue A staining of Synechocystis sp. UNIWG could not detect the accumulated PHA granules. This was very surprising because Nile Blue A can be used to stain and visualize PHA granules in bacteria as well as in plant cell (Arai et al., 2001; Pointer, 2001) in a previous study it was shown that PHA granules accumulated by Synechocystis sp. 6803 (Sudesh et al., 2002) can be readily stained and detected by Nile Blue A. In addition, this staining method is also useful to detect PHA granules in yeast cells (Leaf et al., 1996). The inability of the accumulated PHA to be stained by Nile Blue A prompted us to study the ultrastructure of this new cyanobacterial isolate.

Table 1: PHA accumulation by Synechocystis sp. UNIWG

<table>
<thead>
<tr>
<th>Concentration of acetate (wt% vol)</th>
<th>PHA content* (wt % of CDW)</th>
<th>PHA composition 3HB mol %</th>
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<tbody>
<tr>
<td>0</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>0.5</td>
<td>14.0</td>
<td>100</td>
</tr>
<tr>
<td>1.0</td>
<td>3.0</td>
<td>100</td>
</tr>
<tr>
<td>1.5</td>
<td>4.0</td>
<td>100</td>
</tr>
<tr>
<td>2.0</td>
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<td>100</td>
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*Calculated from GC analysis

Cells were cultured in complete BG-11 medium for 12 days then transferred to nitrogen limited BG-11 medium [BG-11/NO] and incubated further for a period of 21 days.

At the ultrastructural level, surprisingly large amount of PHA granules were present. Such large number of granules has not been reported before for cyanobacteria. By comparing the granules shapes and size they were found to be very similar to those found in other PHA-accumulating bacteria (Sudesh et al., 2002). Up to 17 PHA granules were identified in the cytoplasm of some cells with their sizes ranging from 150 nm to 300 nm (Figure 2D). This cyanobacterium revealed particularly high amount of PHA granules when compared to the well-characterized Synechocystis sp. strain PCC6803 (Hein et al., 1998; Sudesh et al., 2002). Specifically, Hein et al. reported the presence of up to five granules in Synechocystis sp. strain PCC6803 (Hein et al., 1998).

Another interesting aspect of this cyanobacterium is the presence of spicules on the cell surface under early balanced growth condition (Figure 2A and B). The length of spicules was relatively constant and the spicules were uniformly distributed about the entire surface of the cell. Surprisingly, under nitrogen starved condition these spicules appeared lesser in number and much longer (Fig. 2C). When PHA accumulation was maximum (Fig. 2D) the cells were devoid of the spicules. This observation indicates that the spicules are either degraded and used as a nitrogen source, or not synthesized under nitrogen-starvation condition. Cyanobacteria are known to degrade cellular components such as phycobilisomes, which serve as a source of nitrogen and carbon during nutrient deficiency (Allen, 1984).

In addition to the spicules observed in thin sections, negative staining of Synechocystis sp. UNIWG from the balanced growth stage revealed the presence of profuse
pili on the cell surface. Such cellular appendages have been reported for Synechocystis sp. PCC6803 (Bhaya et al., 1999). These pili may have important role in the attachment of cells onto solid surface. In addition, they may also be involved in the uptake of heterologous genetic material from the environment. Currently, the involvement of these pili in the motility process of cyanobacteria is being investigated in detail (Bhaya et al., 2000). We have also observed that Synechocystis sp.UNIWG does possess phototactic motility (results not shown).

CONCLUSION

Many electron-transparent granules were observed in nitrogen-starved cultures of Synechocystis sp. UNIWG. This cyanobacterium accumulated unusually large number of PHA granules in the cell cytoplasm. This amounted to a PHA content of about 14 wt% PHA based on GC analysis. Surprisingly, Nile Blue A staining did not detect these PHA granules. TEM analysis also revealed the presence of spicules and pili that were profuse on the cell surface under balanced growth cultivation. These cellular appendages were either degraded or not generated under nitrogen-starved condition. It is suggested that Synechocystis sp. UNIWG cells may have several forms of cellular appendages that have different functions for their survival in various environmental conditions.

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