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Morphological Studies of *Synechocystis* sp. UNIWG under Polyhydroxyalkanoate Accumulating Conditions

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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 Gaza, 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 pilus like structures. However, these pilus-like structures were not observed in cells from the PHA-accumulation phase. The possible roles of these pilus-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; Pilus.

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are accumulated by various bacteria as intracellular carbon and energy storage materials (Doi, 1990; Sudesh *et al.*, 2000). Poly(3-hydroxybutyrate) [P(3HB)] and its copolyesters 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 *Chlorogloea fritschii* (Carr, 1966), *Spirulina* sp. (Campbell III *et al.*,1982; Vincenzini *et al.*, 1990), *Aphanothece* sp. (Capon *et al.*, 1983), *Gloeothece* sp. (Arino *et al.*, 1995), and *Synechococcus* sp. (Miyake *et al.*, 1996). 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 CO_2 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 on 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 pilus 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 (Braunegg *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% methylamine tungstate for 1 min and examined using Philips CM 410 microscope at 80 KeV.

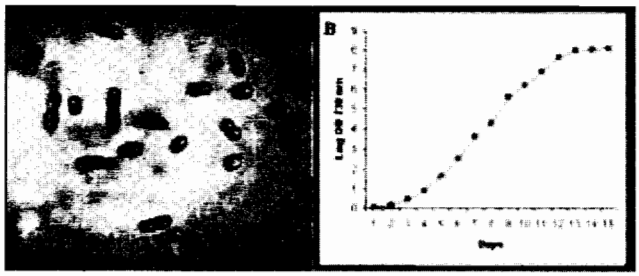

Figure: 1(A) Cell morphology under light microscopy observation. (B) Growth of *Synechocystis* sp. UNIWG in BG-11 medium after 14 days cultivation.

RESULTS

Growth of *Synechocystis* sp. UNIWG

Synechocystis sp. UNIWG was isolated from Wadi Gaza, Gaza, Palestine. Optical microscopy clearly showed that this strain was coccoid 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).

Ultrastructural 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).

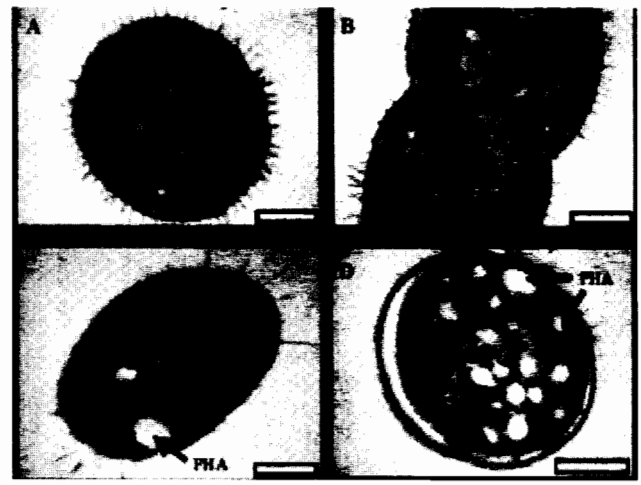

Figure: 2 Transmission electron micrographs of *Synechocystis* sp. UNIWG. (A) and (B) Cell morphology after 7 days cultivation under balanced growth conditions. (C) and (D) Cell under nitrogen-free culture condition. Cell morphology with PHA granules in the cell cytoplasm. PHA granules appear as electron 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% (wt/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 shapes 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.

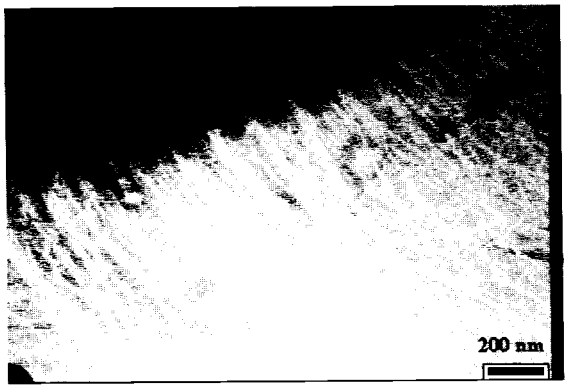

Figure 3: Transmission electron micrograph of *Synechocystis* sp. UNIWG cell stained with 1% methylamine tungstate showing profuse pili. P, pilus. between 1 and 14 wt% of the cellular dry weight depending on the state of the culture and the length of incubation in the nitrogen-deficient BG- 11 medium.

Negative staining of *Synechocystis* sp.UNIWG cells

Negative staining revealed that the surfaces of cells contain profuse peritrichously 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 posses a longer type IV pili 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; Poirier, 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

Concentration of acetate (% wt/vol)	PHA content[a] (wt % of CDW)	PHA composition 3HB mol %
0	1.0	100
0.5	14.0	100
1.0	3.0	100
1.5	4.0	100
2.0	0	100

[a]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(-N)] 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 PHAaccumulating 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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