N₂ Fixation of a Large Phototrophic Bacterium Isolated from the Bacterial Plate of Lake Kaiike with Some Considerations on its *in situ* Growth

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Abstract

N₂ fixation rate of a large phototrophic bacterium, which prevails at the bacterial plate of Lake Kaiike (Kamikoshiki Island), and has been isolated using the medium of PFENNIG (1965), was measured by the C₂H₂ reduction method. The N₂ fixation rate was closely associated with the bacterial growth and required light and H₂S. *In situ* measurement revealed a very low N₂ fixation rate at the bacterial plate on 22 May 1986. But when the bacterial plate sample was exposed at a high light intensity, and particularly when it was supplied with H₂S at the same light intensity, it showed a significantly elevated rate of N₂ fixation. At the bacterial plate the bacterium was suggested to grow at an extremely low rate due to limitation by both factors.

Key words : N₂ fixation rate, phototrophic bacterium, Lake Kaiike.

1. Introduction

The O₂-H₂S interface at the mid-depth of Lake Kaiike (Kamikoshiki Island) is always dominated by two large bacteria, a motile bean-shaped one having 2-3 large pearl-white inclusions, probably *Macromonas bipunctata* (max. number, order of 10⁷ cells·mℓ⁻¹), and a motile ovoid to rod-shaped phototrophic bacterium having several sulfur globules (10⁶ cells·mℓ⁻¹). Their population is so dense that the water at the depth becomes purplish red colored (bacterial plate). A gas-vacuolate bacterium, probably *Lamprocystis roseopersicina*, brown *Chlorobium* species having BChl e, green algae, diatoms, dinoflagellates as well as filamentous blue-green algae are also found, although their abundance is relatively few.

The author has isolated the above large-celled phototrophic bacterium using the medium of PFENNIG (1965) and studied some growth characteristics of the bacterium at different environmental conditions such as pH, light, temperature (MATSUYAMA, 1986).

The bacterium (5 by 7 μm during logarithmic phase, 2 by 4 μm during stationary phase) is close to *Chromatium buderii* with respect to shape, size, motility, plemorphisms, appearance of a single and cell suspension, requirement of NaCl and vitamin B₁₂, which had been designated by TRÜPER and JANNASCH (1968) for the bacterium isolated from

estuarine salt flats on Santa Cruz, Galapagos Islands. But there were marked differences between two bacteria. The bacterium isolated from Lake Kaiike showed an absorption spectrum (maxima at 380, 590, 830 nm and shoulder at 520 nm) very close to the bacterium having okenone (A₅₂₀) as the main carotenoid, while *Chromatium buderii* has warmin-gon (TRÜPER and JANNASCH, 1968). Similarity in the use of sulfur and carbon compounds by these bacteria could not be found (TRÜPER and JANNASCH, 1968 ; MATSUYAMA, 1986). TRÜPER and JANNASCH (1968) stated that *Chromatium buderii* could not fix N₂ and had no hydrogenase. TRÜPER (pers. comm.) suggests that the bacterium isolated from Lake Kaiike may be different from *Chromatium buderii*. The newly isolated bacterium does not match any described species (PFENNIG and TRÜPER, 1983).

One of the characteristics of the bacterium is its relatively low growth rate ; maximum specific growth rate was estimated as 1.0·day⁻¹ (30-32°C, 2,000 lux) and the growth rate during 20-32°C was least among the phototrophic bacteria already isolated from the mid-depth of Lake Kaiike, although all of them had almost the same Q₁₀ in the range (MATSUYAMA, 1986). The bacterium could grow well in the medium without NH₄Cl as a sole nitrogen source suggesting the use of N₂. The bacterium exhibited a vigorous photoevolution of H₂ (MATSUYAMA, unpubl.). The present study aims to test the bacterial N₂ fixation by the C₂H₂ reduction

method in the laboratory and to consider the bacterial growth at the bacterial plate of Lake Kaiike from *in situ* measurement of N₂ fixation.

2. Methods

The bacterial sample was obtained from the bacterial plate at 6 m depth in May 1983, at the point where dissolved O₂ disappeared, and H₂S concentration was 1.5 mg S·ℓ⁻¹. The medium of PFENNIG (1965) was used to obtain an enrichment and pure culture of the bacterium. The medium was modified for the nearly marine condition of the bacterial plate with respect to salinity. NaCl and MgSO₄·7H₂O contents were increased 25 g and 3.5 g·ℓ⁻¹, respectively. Trace element solution SL 7 was replaced with a solution SL 10, recommended by PFENNIG (pers. comm.). The pH of the medium was adjusted to 7.7–8.2. Isolation of the bacterium was achieved by the repeated application of the agar shake culture already mentioned (MATSUYAMA, 1985). More than three successive passages of the agar shake culture yielded a pure colony.

A single colony in the agar shake culture was picked up with a PASTEUR pipette. The colony was suspended in 5–10 ml of the NH₄Cl-deficient medium and mixed well to provide an uniform distribution before being transferred into 1.9 ℓ of the NH₄Cl-deficient medium. It was quickly distributed into a series of 100-ml glass bottles under a N₂ stream and incubated in a water bath at 25°C. Light was provided with incandescent lamps adjusted to about 2,000 lux (saturating) on the bottle surface. Culture was mixed by inverting the bottle twice each day.

Each day following inoculation one bottle was opened to determine the bacterial number and N₂ fixation rate by the C₂H₂ reduction method. The bacterial number was counted in a THOMA hemacytometer. A 100-ml glass gas sampler (12 cm long, 4.0 cm o. d.) with two glass tubings, which were capped with silicon rubber discs, was used for bacterial N₂ fixation measurements in the laboratory as well as field. The sampler, which was previously filled with distilled water, was flushed with argon by introducing the gas through the silicon rubber disc with a hypodermic syringe and venting the water through a second needle. A 10 ml of C₂H₂ (Kanto Acetylene Kogyo Ltd., 99.6%) was injected into the sampler from a syringe, and 50 ml of bacterial suspension (or lake water) was introduced into the sampler using a syringe venting the gas mixture

through the needle to bring the contents to atmospheric pressure. The sampler was incubated in the water bath or at the depth of sampling. A 0.5 ml of the gas phase was successively collected using a microsyringe, and C₂H₄ formed was measured with a semi-conductor detector-gaschromatograph (Sensortec. Inc. Ltd.). The 1-m, 0.3-cm diam. column was packed with Porapak N. Flow of carrier gas (dry air of pure grade) was 20 ml·min⁻¹. The detector was standardized with known dilutions of C₂H₄ (Taka-chiho Kagaku Kogyo Ltd., 99.9%) and quantitated by measuring the peak heights. The limit of detection of C₂H₄ was 3 × 10⁻⁷ moles·ℓ⁻¹.

3. Results

3-1. Bacterial growth and N₂ fixation rate

Figure 1 summarizes the changes of the bacterial number, relative abundance of sulfur globule-containing cells and integration of daily rate of bacterial N₂ fixation expressed as C₂H₄ formed per liter of bacterial suspension during the course of culture growth. Immediately after inoculation the bacterium began to increase, and the culture entered the stationary phase at the sixth day after inoculation. Most of the cells contained sulfur globules until the third day after inoculation. Sulfur-containing cells became fewer, and they were less than 10% at the seventh day indicating complete depletion of H₂S from the medium (MATSUYAMA, 1986).

Figure 2 shows the bacterial N₂ fixation rate during the course of culture growth. The rate of C₂H₄ formation just after inoculation was less than 1 × 10⁻⁶ moles·ℓ⁻¹·day⁻¹. It began to increase rapidly and attained the maximum rate of 6.6 × 10⁻⁵ moles·ℓ⁻¹·day⁻¹ at the third day after inoculation. But the rate began to decrease again to actually zero at the seventh day (not shown in the figure) probably due to complete depletion of H₂S. Integration of the daily rate of N₂ fixation rate (Fig. 1) showed a pattern similar to the increase of bacterial number. This indicates the close association between the bacterial growth and N₂ fixation rate.

The N₂ fixation rate became very poor when the culture entered the stationary phase. Upper panel of Figure 3 shows the N₂ fixation rate of the bacterial suspension at the stationary phase, into which different amounts of neutralized Na₂S·9H₂O (pH=7.0) were added. The rates of C₂H₄ formed for first 4–6 hr (lower panel) were directly proportional to Na₂S·9H₂O added up to the H₂S concentration of about 20

Fig. 1. Changes of the bacterial number, relative abundance of sulfur-containing cells and integration of daily rate of N₂ fixation rate expressed as C₂H₄ formed per liter of bacterial suspension. Daily rate of N₂ fixation was estimated from extrapolation of C₂H₄ formation shown in Figure 2 for 24 hr.

Fig. 2. Bacterial N₂ fixation rate at each day of culture growth. Numeral indicates day after inoculation. N₂ fixation rate at the seventh day was consistently below the detectable level (3×10^{-7} moles · l⁻¹).

Fig. 3. Upper panel: N₂ fixation rate of bacterial suspension at the stationary phase (bacterial number = 1.5×10^7 cells · ml⁻¹), into which different amounts of neutralized Na₂S 9H₂O were added (numeral indicates the initial concentration of H₂S as mg S · l⁻¹).

Lower panel: relation between the initial H₂S concentration and N₂ fixation rate for first 4–6 hr.

mg S · l⁻¹ (Most sulfide was in the forms of H₂S and HS⁻, but here they have been expressed as H₂S.) This indicates that the bacterial N₂ fixation rate was closely dependent upon the H₂S concentration. A maximum concentration of H₂S in Lake Kaiike was up to 35 mg S · l⁻¹ (very changeable depending on season), but the concentration at the bacterial plate was always less than 2 mg S · l⁻¹. So, the concentration of H₂S at the bacterial plate seems to be one of the important factors limiting the bacterial N₂ fixation rate.

Figure 4 shows the relations between light intensity and the N₂ fixation rate of the bacterial suspension just after inoculation and at the fourth day.

Light intensity was changed by enclosing each sampler with different pore sizes of black nylon net. The bacterial suspension was previously darkened for 24 hr. (If it was not darkened, low but significant levels of C₂H₄ were formed even in the subsequent darkness.) In the darkened sampler the rate of C₂H₄ formed was always below the detectable level. The rate increased linearly proportionally to light intensity up to about 100 lux. But above the light level the increase of the rate became gradual. These patterns corresponded well with that for the growth rate of the bacterium in the logarithmic phase grown at different light intensities (MATSUYAMA, 1986).

Fig. 4. Relation between light intensity and the N_2 fixation rate of the bacterial suspension just after inoculation (bacterial number $= 5.8 \times 10^6$ cells $\cdot$ ml $^{-1}$) and at the fourth day (3.4×10^6 cells $\cdot$ ml $^{-1}$). Numeral indicates day after inoculation. Broken line shows the specific growth rate of the bacterium at the logarithmic phase at different light intensities (MATSUYAMA, 1986).

3-2. N_2 fixation rate of bacterial plate sample in Lake Kaiike

In situ measurement of N_2 fixation rate was performed on 22 May 1986. At this period the bacterial plate was observed at 6 m depth, where dissolved O_2 disappeared and H_2S concentration was 0.4 mg S $\cdot$ ℓ^{-1} . Temperature was 25.6°C , and the sky was clear. The number of *Macromonas bipunctata*-like bacterium and the bacterium reported here were 1.4×10^7 cells and 4.3×10^6 cells $\cdot$ ml $^{-1}$, respectively. A 50 ml of samples collected from different depths from surface to 7 m depth was injected into each sampler from a syringe, and the samplers were incubated for 4 hr (10-14) at the depth of sampling. All samples, however, showed a very low rate of C_2H_4 , being less than the detectable level.

The bacterial plate sample filled in a sterile glass bottle was brought back to laboratory keeping it dark and cool and soon delivered into the samplers for measurement of N_2 fixation rate in a water bath at 25°C under the light intensity of 2,000 lux. A neutralized Na_2S $9H_2O$ was added for the H_2S concentration of 11 mg S $\cdot$ ℓ^{-1} (Fig. 5). Exposure of the sample at 2,000 lux led to a formation of C_2H_4 , but addition of Na_2S $9H_2O$ resulted in a more pronounced formation of C_2H_4 .

Fig. 5. N_2 fixation rate of the bacterial plate sample of Lake Kaiike exposed to light intensity of 2,000 lux (Light), and exposed at the same light intensity after supply with neutralized Na_2S $9H_2O$ (initial H_2S , 11 mg S $\cdot$ ℓ^{-1}) (Light, H_2S .)

4. Discussion

From the laboratory experiment it is apparent that the phototrophic bacterium isolated from Lake

Kaiike could use N₂ for a nitrogen source, and that the N₂ fixation rate is closely associated with the growth (Fig. 1), and light and H₂S are both necessary (Figs. 3, 4). The N₂ fixation is thus predominantly controlled by the master reaction, photosynthesis.

Despite the active N₂ fixation of the bacterium under laboratory conditions, *in situ* formation of C₂H₄ was very poor even at the bacterial plate on 22 May 1986. This may be partly responsible for the technical problems; incubation seemed short (4 hr), or the gas phase of sampler (50 mℓ) seemed too large to measure *in situ* N₂ fixation. However, the incubation of the sample at 2,000 lux and supply of H₂S resulted in a significantly elevated formation of C₂H₄ (Fig. 5). This fact suggests that light and H₂S conditions at the bacterial plate may be far from the optimum levels. The bacterium seems to grow at an extremely low rate primarily due to limitation by both factors.

GUERRERO *et al.* (1985), who studied on the dense population of phototrophic bacteria (mainly *Chromatium* species) in Lake Ciso (NE Spain), found that the bacterial plate could be divided into a top part of maximum specific activity (1.75 m), a peak of a maximum abundance (2.0 m), and a bottom part of inactive cells. Phototrophic bacteria at 2 m depth were light-limited and the organisms at 1.75 m depth were H₂S-limited.

The maximum specific growth rate of the bacterium reported here could be estimated as low as 1.0•day⁻¹ (30–32°C, light and H₂S were not limiting). As far as the light condition at the bacterial plate was concerned, the growth rate was reduced less than 0.3•day⁻¹ (doubling time, longer than 2.3 days), because light available at the bacterial plate was 400–600 lux in a maximum, and it was rapidly reduced to actually zero below the plate by selfshading features. The actual growth rate would be far lower than 0.3•day⁻¹, because the H₂S concentration at the bacterial plate was always less than 2 mg S•ℓ⁻¹, and the concentration of H₂S below 20 mg S•ℓ⁻¹ seems to limit severely the bacterial growth suggested from Figure 3. Therefore, it is highly probable that the bacterium grows at an extremely low rate at the bacterial plate primarily due to limitation by light and H₂S. Hence, the observation that no significant C₂H₄ formation was found at the bacterial plate on 22 May 1986 may not be far from the reality. But it is noteworthy that despite possible limitation of H₂S, most of the cells contained sulfur globules.

It has been often observed that the turnover rate (P/B ratio) of phototrophic bacteria at the bacterial plates is lower than expected from the biomass. CLOERN *et al.* (1983) found in Big Soda Lake (Nevada) that the daily rate of oxygenic photosynthesis was a more important source of organic matter than anoxygenic photosynthesis by dominant Chromatiaceae species (*Ectothiorhodospira vacuolata*), although phototrophic bacterial biomass exceeded that of phytoplankton. A similar finding was also reported for the dense population of Chlorobiaceae species. PARKIN and BROCK (1981a, b) found in Knaack Lake (Wisconsin) that species of the *Pelodictyon* and *Clathrochloris* comprised from 41–92% of total phytoplanktonic standing crop on a chlorophyll basis, or 8–58% on a carbon basis, primary production of these species measured by the ¹⁴C method were responsible for 3–6% of total primary production in the lake. Comparable data was reported by MIRACLE and VICENTE (1985). These facts may be due, in part, to a slow turnover rate of phototrophic bacteria in natural waters, which could thrive only at the depth under a reduced light and probably H₂S-limited conditions, although they can grow better *in vitro*. This may be applicable to Lake Kaiike, although experimental data on phytoplanktonic and phototrophic bacterial P/B ratios are lacking.

Why such a slow growing bacterium could sustain the dense population at the O₂–H₂S interface of Lake Kaiike? If the growth rate was considered as a major factor controlling the competition among the species, the bacterium would be soon displaced by other fast growing phototrophic bacteria and ultimately by planktonic algae.

As far as PO₄³⁻ uptake was concerned, the bacterium could attain the order of 10⁶ cells•mℓ⁻¹ (maximum level found in the bacterial plate of Lake Kaiike) in only 3–4 μg atoms P•ℓ⁻¹ consumed (slightly over the level found in the upper water above the plate) (MATSUYAMA, 1986). The bacterial ability to fix N₂ seems to have a lower requirement for environmental NH₄⁺, although both nutrients were greatly accumulated below the bacterial plate. It is plausible that the species having a lower requirement for a nutrient source could effectively displace other competitors, because the species reduced the nutrients to the level at which concentration another species could not sustain a stable population (HSU *et al.*, 1977). Thus, once the dense population of the phototrophic bacterium with a

lower requirement for nutrient sources is formed at the mid-depth, it will act as an effective barrier between the waters above and below, although most of the cells are suggested to exist under starving condition or grow at an extremely low rate (dense population could be a bacterial tactic).

The above discussions are only tentative approaches to understanding how the phototrophic bacterium survive at the mid-depth of Lake Kaiike. More detailed observations of the bacterium at mid-depth are necessary as well as laboratory experiments on the bacterial growth under different environmental conditions including biotic factors such as competition with other species.

摘 要

貝池バクテリアプレートから分離した大型の光合成細菌の窒素固定能と現場での菌の生長についての若干の考察

貝池（上甕島）のバクテリアプレートに優占して出現し、PFENNIG (1965) の培地により分離した大型の光合成細菌の窒素固定能をアセチレン還元法によって確かめた。その窒素固定能は菌の生長と密接に関連し、光と H_2S を必要とした。1986 年 5 月 22 日、バクテリアプレート現場で測定した窒素固定量は著しく低かった。しかし、バクテリアプレート試料を高照度に曝したり、とくに、 H_2S を供給し高照度におくと著しい窒素固定速度を示した。本菌はバクテリアプレートでこの 2 つの要因の制限によって緩慢な速度で生長していると推察された。

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