A Phototrophic Bacterium of the Chromatiaceae Family
Isolated from the Mid-Depth of Lake Kaiike

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Abstract

A bacterium of the Chromatiaceae family was isolated from a dense layer of phototrophic bacteria at the mid-depth of Lake Kaiike. The bacterium could be purely cultured in the medium given by PFENNIG and LIPPERT (1966). When sulfide was amply present in the medium, a very elongated form of the bacterium, which contained sulfur globules inside the cell, predominated. With decreasing sulfide concentration, it changed to a sulfur-containing and finally to a motile sulfur-free rod-shaped bacterium measuring 1-2 μm in length to achieve the stationary phase of the culture. Some environmental factors, pH, light, temperature, NaCl, vitamin B₁₂ and sulfur compounds, on the bacterial growth were examined.

1. Introduction

The author has made a series of limnological studies of Lake Kaiike on Kamikoshiki Island (surface, 0.15 km²; max. depth, 11.6 m), which is cut off from the sea by a narrow gravel bar (MATSUYAMA, 1977, 1978, 1981a, b; MATSUYAMA and SHIROUZU, 1978).

One of the prominent features of the lake is an obvious development of phototrophic bacteria at mid-depth, at which there is an interface between the upper O₂ and the deeper H₂S-bearing waters. The bacterial population is so dense that the water turns purplish red. These bacteria play a very important role in the organic matter production in the lake.

In the previous reports (MATSUYAMA, 1979, 1980), the author examined some growth conditions of a small coccoid immotile phototrophic bacterium of the Chromatiaceae family isolated from the bacterial dense layer at the mid-depth of Lake Kaiike. The present report aims to characterize another phototrophic bacterium of the same family isolated from the same layer in November, 1981, and to study some environmental factors on the bacterial growth in the medium given by PFENNIG and LIPPERT (1966).

2. Materials and Methods

On 13 November, 1981, the purplish red colored water at the mid-depth of Lake Kaiike (4.5 m) was collected with a HYROHT sampler and it was poured into a 1-liter bottle, to which a small amount of modified FULLER's medium (MATSUYAMA, 1979) was previously added to prevent possible bacterial consumption of the sulfur source. The bottle was tightly stoppered and stored on ice for microscopic observation in the laboratory.

Two kinds of bacteria dominated in the sample. One was ovoid-shaped, about 3 μm long and 1.5 μm wide. The bacterium contained two or three large, colorless hyaline inclusions. The inclusions withered away gradually. The bacterium moved back and forth. By shrinking the inclusions, the motion was increased. This bacterium resembled Macromonas bipunctata, bacterium belonging to Thiobacillaceae (BAVENDAMM, 1924; SKUJA, 1956). The number of this bacterium was about 21×10⁵ cells·ml⁻¹ in the sample. Another bacterium was rod-shaped, about 3 μm long and 1.5 μm wide. It frequently came in pairs or was shortly chained. Sulfur globules were accumulated inside the cell. The bacterium moved with an irregular turning of the body. The bacterial population was about 18×10⁴ cells·ml⁻¹. In this study the latter bacterium was enriched and isolated by the following procedures. Other microorganisms, includ-
ing some Chlorophyceae, were also found in the sample.

**Enrichment and isolation**

It was not easy to culture the above phototrophic bacterium in modified FULLER'S medium. The medium given by PFENNIG and LIPPERT (1966) was applied to the selective enrichment and isolation of the bacterium. This medium has the following composition: NH₄Cl, 60 mg; K₂HPO₄ 3H₂O, 15 mg; CaCl₂ 2H₂O, 75 mg; KCl, 100 mg; MgCl₂ 6H₂O, 100 mg; NaCl, 25 g; Na₂CO₃, 2 g; Na₂S 9H₂O, 200 mg; trace element solution (PFENNIG and LIPPERT, 1966), 10 ml. Distilled water was added up to 1000 ml, and the pH was adjusted to 7.4 with HCl. The medium was sterilized by gentle filtration through a precombusted glass fiber filter (Whatman GF/C) and was good for bacterial growth, although a number of other bacteria as well as some Chlorophyceae were still present in it.

The isolation of pure culture from the enrichment was achieved by the repeated application of the agar shake dilution described by TRUPER (1970). One of the test tubes, which contained 40 ml of the medium of PFENNIG and LIPPERT (1966), was inoculated with a few drops of an enrichment culture and the contents were mixed well. Of this medium about 2 ml was transferred to a second test tube and mixed thoroughly. This dilution series was continued to ten steps. Autoclaved and liquidized agar was added into the test tubes to a final concentration of about 1% and the test tubes were closed with rubber stoppers. Then, the contents were mixed by shaking the test tube upside down. After content solidifying, the test tubes were incubated at room temperature in light provided continually by incandescent lamps. Within 3-4 days, the agar shake culture of the lower dilution rate below 5-6 steps turned evenly pinkish red due to a large number of small colonies, which were so close together that a single colony could not be picked up. In the cultures of the higher dilution rate above 6-7 steps, scattered colonies developed. The test tube was cut and a single colony was picked up with a PASTEUR pipette and it was transferred to a newly prepared agar culture.

In the early series of this agar shake culture, a mass of very small bacterium less than 1 μm in diameter was present within the colony. But three or four applications of the agar shake dilution made it possible to remove this small bacterium from the culture. Purity was checked microscopically.

**Culture experiment**

A single colony of the bacterium in the agar shake culture was picked up and transferred into the test tube containing a small amount of the medium of PFENNIG and LIPPERT (1966). It was mixed vigou-

![Graph](image-url)

**Fig. 1.** Bacterial number cultured in the medium of PFENNIG and LIPPERT (1966). A: sonicated with ultra sonic disruptor, B: unsonicated.
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...ously for uniform distribution of the bacterium and poured into the same medium of 1-2 liters. The inoculated medium was poured into a series of 100-ml glass bottles. These bottles were tightly closed and incubated at 25°C under continual illumination with a 300-W incandescent lamp. Light intensity was adjusted to about 3000 lux on the bottle surface. The culture was mixed by turning the bottle upside down twice a day. Within 2-3 days after inoculation, growth of the bacterium was perceptible. For microscopic observation, 1 ml of sample was taken from the culture. The bottles were immediately filled with the stock medium, which was previously put into several small bottles and stored in a refrigerator. When some compound was added to the culture for the evaluation of its effect on bacterial growth, the compound was previously omitted from the stock medium.

Measurement of bacterial number

In the early period of the culture, the bacterium was mainly elongated and so highly agglomerated that direct microscopic counting was impossible. Hence, the samples were always sonicated briefly (about 3 seconds) with an ultrasonic disruptor (Tomyseiko, Model UR-200P) to break up the long chains. Therefore, in the early period of the culture the estimation of the bacterial number was apt to be overestimated. A few drops of formalin were added to the sample to stop the bacterial motion. The bacteria were counted in a THOMA hemacytometer.

Other measurements

Sulfide concentration of the culture was determined by the iodometric titration of CdS precipitate, which was formed by addition of CdCO₃ suspension into the sample (American Public Health Association, 1965). An absorption spectrum of...
the bacterium was measured spectrophotometrically; the bacterial suspension was passed through a glass fiber filter (Whatman GF/C) and the cells were extracted in 92% acetone at room temperature. Absorption was measured with a spectrophotometer (Beckman-Toshiba, Model Specta-10). The chemicals employed were of special reagent grade of Wako Pure Chemicals.

3. Results

3-1. Growth Characteristics of the Bacterium

Figure 1 shows the change in the number of bacterium in the medium given by PFENNIG and LIPPERT (1966). In the early period of the culture, the bacterium was sedimented as a gray-brown to reddish-brown aggregation. After 5 days of inoculation the bacterium began to spread throughout the bottle to exhibit a homogeneous reddish-brown suspension, or “Wolken,” as described by SCHLEGEL and PFENNIG (1961). In the early period of culture, a great difference in bacterial number was found between the sonicated and unsonicated samples, due to the elongated, highly aggregated formation. But this difference decreased with advance of the culture. The bacterium grew rapidly within 5 days after inoculation and then the culture entered the stationary phase. Final yield of the bacterium was about $2 \times 10^7$ cells ml$^{-1}$. 

Fig. 3. Concentration of sulfide in the medium and relative abundance of sulfur-containing cell.
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The scanning electron and optical micrographs of the bacterium at 4 and 6 days after inoculation are shown in Fig. 2. In the early culture, most of the bacterium was considerably elongated and curved. The cells were somewhat thick and sulfur globules were evenly distributed within. This elongated form was readily broken down so as to form short chains, when slightly pressed. In the middle of the culture, the bacterium was composed of single rod-shaped and short-chained types containing sulfur globules, and they rapidly changed to a sulfur-free form, 1-2 μm long and about 1 μm wide. This sulfur-free form was very motile probably due to flagella, which could not be identified in the micrographs.

Changes in the sulfide concentration in the culture, relative abundance of sulfur-containing cell and size distribution of the bacterium examined at random are shown in Figs. 3 and 4. Sulfide was entirely consumed within 5 days after inoculation. With decreasing sulfide concentration, the relative abundance of sulfur-containing cell also diminished. In the final period of culture, sulfur-containing cells accounted for only 1% of the total bacterial mass. As soon as the sulfide disappeared, the bacterium changed into a small form.

Absorption spectra of the sulfur-containing elongated and sulfur-free rod-shaped ones of the bacterium (Fig. 5) showed a maximum at about 770 mμ, probably due to bacteriochlorophyll-a (CLAYTON, 1963). The maximum was somewhat dropped in the sulfur-free rod-shaped one.

The above growth characteristics of the bacterium shows that it is close to Chromatium vinosum (PFENNIG and TRUPER, 1974). For its specific identification,
further study is necessary.

3-2. Environmental Factors in Bacterial Growth

**pH**

Growth of the bacterium incubated at different pH (Fig. 6) showed that the optimal pH range was limited, between 7.2-7.6. This was slightly more acidic than the optimal pH for the coccoid immotile phototrophic bacterium previously reported (MATSUYAMA, 1979). No marked bacterial growth at pH below 6.4 as well as in the later mentioned medium to which a large amount of Na₂S 9H₂O was added may be partially attributed to an increase of an undissociated form of sulfide, H₂S, which VAN NIEL (1931) suggested to be toxic.

**Light intensity**

Growth of the bacterium incubated at different light intensities is shown in Fig. 7; light intensity was changed by enveloping the bottle with different sheets of black nylon net. The bacterium grew even at a low light intensity of 100 lux, and showed more rapid growth with increasing light intensity. Light saturation appeared to be in the range of 2000-3000 lux. A similar light-growth curve was observed in the previously reported phototrophic bacterium, although the growth rate was much slower than with the present bacterium (MATSUYAMA, 1979).

**Temperature**

Growth of the bacterium incubated at different temperatures between 10-40°C is shown in Fig. 8. Growth did not occur below 15°C. The bacterium grew rapidly by increasing the temperature up to 30°C, but growth was poor above that level. Maximum growth of the previously reported phototrophic bacterium was between 30-34°C, and significant growth was at 36°C (MATSUYAMA, 1980). The present bacterium was somewhat different from
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NaCl

Growth of the bacterium in the media added with different amounts of NaCl is shown in Fig. 9. It was very strange that minimum growth occurred between 1.0-1.5 g·100 ml⁻¹ concentration. Some growth occurred below this level, but most of the bacterium remained in an elongated form. A maximum bacterial growth occurred in the range of 2-4 g·100 ml⁻¹, which accounted for the salinity of water around the dense population of phototrophic bacteria at the mid-depth of Lake Kaiike (25-30%) (MATSUYAMA, 1978).

Vitamin B₁₂

Growth of the bacterium in the media added with different amounts of vitamin B₁₂ (Fig. 10) showed that any different growth was found in the medium depleted of vitamin B₁₂ from those added with vitamin B₁₂. Thus, it was suggested that vitamin B₁₂ was not necessary for the present bacterium. This is consistent with PFENNIG and LIPPERT (1966), who showed that within the Chromatiaceae family a vitamin B₁₂ requirement has been found so far only in a large species such as Chromatium okenii.

Sulfur compounds

Growth of the bacterium in the media...
added with different amounts of Na₂S·9H₂O (Fig. 11) showed that growth did not occur in the absence of Na₂S·9H₂O. In the early period of culture, the bacterium grew rapidly in inverse relation to the initial concentration of Na₂S·9H₂O, but growth continued proportionally with the initial concentration. Therefore, the final yield of the bacterium became proportional to the initial concentration of Na₂S·9H₂O up to 8 mg·100 ml⁻¹, where the population reached about 85 × 10⁶ cells·ml⁻¹. Above this level, however, the growth was greatly depressed, in keeping with the finding of van Gemerden (1974), who reported that the maximum growth rate of Chromatium vinosum was at a sulfide concentration of about 0.1 mmol·l⁻¹; above and below this level, the bacterial growth rate decreased. The concentration of Na₂S·9H₂O above a specific level seems to give rise to an inhibitory effect on the bacterial growth. A similar Na₂S·9H₂O-growth curve was found in the previously reported phototrophic bacterium (Matsuyama, 1979). The sulfide concentration of water around the dense population of phototrophic bacteria in Lake Kaiike was less than 4 mg S·l⁻¹ (Matsuyama, 1980), so the in situ sulfide concentration does not seem to have

Fig. 7. Growth of the bacterium at different light intensities.
an inhibitory effect on the bacterium under study.

Growth of this bacterium in the media added with different amounts of Na$_2$S$_2$O$_3$ is shown in Fig. 12. In the cultures indicated in the right side of the figure, Na$_2$S 9H$_2$O were simultaneously added to a concentration of 20 mg·100 ml$^{-1}$. In the media without Na$_2$S 9H$_2$O, no significant growth occurred within 7 days after inoculation, but then the bacterium began to grow significantly. Meanwhile, in the media added with Na$_2$S 9H$_2$O, the bacterium grew well from the beginning of culture, although some depression was found when the initial Na$_2$S$_2$O$_3$ concentration was increased. This fact demonstrated that in the early period of culture, the bacterium could not use Na$_2$S$_2$O$_3$, but with advance of the culture the bacterium began to use this compound.

Growth of the bacterium in the media added with other sulfur compounds, S$^0$, NaHSO$_3$, NaSCN and HSCH$_2$COONa, is shown in Fig. 13. In the media without simultaneous addition of Na$_2$S 9H$_2$O, the bacterium did not grow at all. Therefore, the bacterium presumably could not use

![Fig. 8. Growth of the bacterium at different temperatures. Suffix: first and second countings at respective time.](image-url)
these compounds in the absence of Na$_2$S 9H$_2$O. Final yields of the bacterium in the media added with S$^0$ and NaHSO$_3$ seem to be somewhat related to the initial concentrations of these compounds. This suggested that these compounds were utilized by the bacterium as sulfur sources to some extent. Meanwhile, NaSCN did not seem to influence the final bacterial yield, and HSCH$_2$COONa showed a strong inhibitory effect on the growth.

4. Discussion

As shown in Figs. 1, 2 and 3, in early culture, when sulfide was ample, the bacterium was mostly an elongated form containing sulfur globules inside the cell. With a lowered sulfide concentration, it rapidly changed to sulfur-containing and further to sulfur-free rod-shaped configuration to enter the stationary phase of the culture. This fact suggests that sulfide is a growth-limiting substance in the culture. A phototrophic bacterium earlier isolated from the coastal sediments of Omura Bay, which was probably the same species as the present bacterium, showed that a terminal part of an elongated form spun very quickly and finally left the elongated body. A similar cell division seems to have taken place in the present bacterium. The rod-shaped bacterium did not develop an
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The bacterium in the sample of Lake Kaiike observed in the laboratory was wholly single rod-shaped or arranged in pairs containing sulfur globules inside the cell. Elongated forms were hardly ever encountered. The bacterium of this form has such limited motility that it would be

Fig. 10. Growth of the bacterium added with different amounts of vitamin B₁₂.

elongated form again; the change from a sulfur-containing elongated form to a sulfur-free rod-shaped one seems irreversible with the culture employed. However, even in the later period of culture a sulfur-containing or sulfur-free elongated form was rarely observed.

The bacterial growth was rapid in early culture. As seen in the right side of Fig. 8, the number of the bacterium incubated at 30°C was $4 \times 10^4$ cells·ml$^{-1}$ at 15 of 1 day after inoculation. It increased to $186 \times 10^4$ cells·ml$^{-1}$ at 11 of the next day. The mean generation time of the bacterium calculated from this increased bacterial population was about 3.6 hr. This rate was much faster than that of the phototrophic bacterium previously reported (Matsuyama, 1980) and roughly comparable to the maximum rate of Chromatium vinosum reported by van Gemerden (1974). Although this estimation was only tentative, the bacterium most likely would exhibit a very fast growth rate in optimal conditions.

The bacterium in the sample of Lake Kaiike observed in the laboratory was wholly single rod-shaped or arranged in pairs containing sulfur globules inside the cell. Elongated forms were hardly ever encountered. The bacterium of this form has such limited motility that it would be
difficult for the cell to remain at the mid-depth of Lake Kaiike due to the limited water motion there caused by the large vertical density gradient. In the culture, the bacterium of this form was highly agglomerated and sedimented on the bottom of the bottle. A homogeneous suspension of the bacterium due to the motility was first found after the change from the elongated to the rod shape. The sulfur-free rod-shaped bacterium was also limited in the sample.

If this observation is valid for a natural population of this kind of bacterium at the mid-depth of Lake Kaiike, the bacterium may well be so amply supplied with sulfide that the intracellular sulfur globules are not consumed; therefore, the natural population of the present bacterium is affected not only by sulfide, but also by other
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Factors. In this study, the culture was always performed at a saturation light intensity of about 3000 lux. As mentioned already (Matsuyama, 1979), the actual light intensity reaching the upper part of the dense population of phototrophic bacteria at the mid-depth of Lake Kaiike was reduced to only a 2-3% light level just below the surface (i.e., 400-600 lux at maximum), and it was rapidly attenuated below due to the self shading feature of the densely populated bacteria. The in situ light intensity, at which the bacterium grows, is thus far lower than this level, and light may indeed be one of the important growth-limiting factors. More detailed observations of a natural population of the present bacterium are necessary, along with growth experiments of the bacterium at a reduced light intensity and in an intermittent light-dark regimen in the laboratory.

Fig. 12. Growth of the bacterium added with different amounts of Na$_2$S$_2$O$_3$. Left: without Na$_2$S 9H$_2$O, right: simultaneously added with Na$_2$S 9H$_2$O (20 mg·100 ml$^{-1}$).
Acknowledgment

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**Fig. 13.** Growth of the bacterium in the media added with different amounts of S°, NaHSO₃, NaSCN and HSCH₂COONa. All media were simultaneously added with Na₂S 9H₂O (20 mg • 100 ml⁻¹).

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References


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