Effects of Environmental Factors upon Nitrogen Fixation of *Chromatium* sp. Isolated from Lake Kaiike

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Chromatium sp., blooming at the O_2-H_2S interface of Lake Kaiike, could fix N_2. Response of nitrogen fixation of *Chromatium* sp. to environmental factors was established by C_2H_2 reduction method.

Nitrogen fixation of *Chromatium* sp. was light- and H_2S-dependent, and the maximum rate, 32.0 nmol C_2H_4 /hr/(mg dry cell weight), was obtained. Each of optimum environmental conditions was as follows: light intensity, 1000 lux; pH, 8.5; H_2S, 20 mg H_2S-S/*l*; temperature, 30℃.

The bacterium could fix N_2 well at low light intensity and low H_2S concentration under inorganic condition.

It was suggested that *Chromatium* sp., having the ability to fix N_2 photoautotrophically even under low light intensity and low H_2S concentration, might become an important dominant in the ecosystem and a potential contributor to the nitrogen budget.

Key words: *Chromatium* sp., N_2 fixation, C_2H_2 reduction method, Environmental factors.

A large-celled phototrophic bacterium is blooming at the O_2-H_2S interface in Lake Kaiike (Kamikoshiki island) throughout all seasons. The bacterium is probably a new species of Chromatiaceae family.[1] In this paper the bacterium is referred to as *Chromatium* sp..

Since most phototrophic bacteria could grow photoheterotrophically,[2] some species of Rhodospirillaceae are utilized in sewage treatment plant[3),4] or in rice field as soil fertilizer.[5] Similarly, some species of Chromatiaceae play an important role in soil fertilization of lowland rice field where biologically toxic H_2S is found.[6]

The successful nitrogen fixation in phototrophic bacteria may depend upon a number of factors, e. g., light intensity, pH, temperature, or organic compounds. In freshwater and marine environments, most of nitrogen fixation is mediated by cyanobacteria and heterotrophic bacteria.[7]

The energy and reductants required for nitrogen fixation of the bacterium are provided from photosynthesis.[8] In the present study, response of nitrogen fixation of *Chromatium* sp. to environmental factors will be established.

Materials & Methods

The preliminary culture of *Chromatium* sp. isolated from Lake Kaiike had been done at the conditions of 1000 lux, 25℃, pH 7.9~8.4 and 130mg H_2S-S/*l* with the medium of Pfennig.[9] The NaCl and $MgSO_4 \cdot 7H_2O$ contents of the medium were increased 25g and 3.5g/*l*, respectively, for marine habitat of the bacterium.[8] Trace element solution SL 7 was replaced with a solution SL10.[8]

The medium was well buffered by the addition of $NaHCO_3$ (20 mM), Na_2CO_3 (3.3 mM), and H_2CO_3 (by flushing CO_2 into the gas phase of the medium for about an hour) to minimize the pH change during the bacterial growth (ΔpH was usually within 0.5).

Bacterial cells in phase of logarithmic growth were centrifuged (670 x g, 15 min) and resuspended with NH_4^+-free medium (5 times), and which were used for the experiment to measure the nitrogen fixation by C_2H_2 reduction method.[10]

A 50-m*l* bacterial suspension prepared by above procedure was poured into a 100-m*l* syringe. Before the suspension was get into the syringe, N_2 gas had been flowed into the syringe for the removal of other gaseous ingredients. N_2 gas in the syringe was withdrawn by pouring the suspension, and then the volume of it was adjusted to 50 m*l*. After 40 m*l* of N_2 gas was injected into the syringe, C_2H_2 was added to the gas phase (final concentration 20% C_2H_2, v/v).

The ranges of the environmental variables for nitrogen fixation measurement were as follows: light intensity, 0~7000 lux (continuously provided); temperature, 10~37℃; pH, 7.0~8.8 (adjustment by conc. HCl or 1 N NaOH solutions); initial H_2S concentration, 0~100 mg H_2S-S/*l*. But, when there was no specification about experimental conditions, the growth conditions were same with those for the preliminary culture.

The wide range of each environmental variables made the experiment (light intensity and temperature) to be performed several times; e. g., in the experiment for the effect of light intensity, one was performed with the range of 0 to

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2000 lux (including 1400 lux) at pH 8.4, and the other was performed with the range of 1400 to 7000 lux at pH 7.9. Differences of the two data obtained even at same light intensity (1400 lux), generated from discrete environmental conditions (e. g., pH), were quantified. These works were included with making linear curves from those discrete data and obtaining each unit amount of C_2H_2 reduction per time (hr). A ratio of the two unit values was used as a conversion factor for arranging the discrete data on a same basis.

A 0.5 m*l* of sample from the gas phase was collected using a microsyringe, and C_2H_4 formed was measured with a semi-conductor detector-gas chromatograph (Sensortec. Inc. Ltd.). The 1-m, 0.3 cm-diam. column was packed with Porapak N. Flow rate of carrier gas (dry air of pure grade) was 20 m*l*/min. The detector was standardizied with known dilutions of C_2H_4 (Takachiho Kagaku Kogyo Ltd., 99.9%) and quantitated by measuring the peak heights.

100-m*l* syringes having bacterial suspension were gently agitated by hand with 4 to 6 times a day to ameliorate the gas exchange between the gas and the liquid phases. The detection of C_2H_4 formed were done in pairs.

A series of values of C_2H_2 reduction for time (hr) in each experiment were plotted, and by linear regression analysis nitrogen fixation rate (all the data shown, $r \geqq 0.93$), which was expressed as moles of C_2H_4 formed per milligram of dry cell weight, was obtained.

Results

Light

Nitrogen fixation of *Chromatium* sp. at varying light intensities was shown in Fig. 1. There was no nitrogen fixation in the dark. The rate of nitrogen fixation became fast with an increase of light intensity from 50 to 1000 lux, and became lasting to 2000 lux with little difference in its rate, and decreasing over than 2000 lux. The maximum nitrogen fixation rate obtained at 1000 lux was 12.3 nmol C_2H_4/hr/(mg dry cell weight).

Nitrogen fixation of the bacterium was saturated at light intensity of 1000 lux, shown in Figure 1, while many nitrogen fixing phototrophic bacteria excluding some species of *Chlorobium*[11] were saturated at more elevated levels.[12-14] It was of characteristics that a relative nitrogen fixation at 300 lux amounted to 82% of the maximum one (◎). The relatively high nitrogen fixation rates, over than 80% of the maximum one, were observable at light intensities from 300 to 2000 lux (Fig. 1).

pH

Nitrogen fixation of the bacterium was high in alkaline condition (Fig. 2). The optimum pH range for nitrogen fixation of the bacterium was considerably higher than suspension's pH (usually 7.0) of other N_2-growing phototrophic bacteria.[11-13] It was difficult for the bacterium to perform nitrogen fixation at pH of below 7.0.

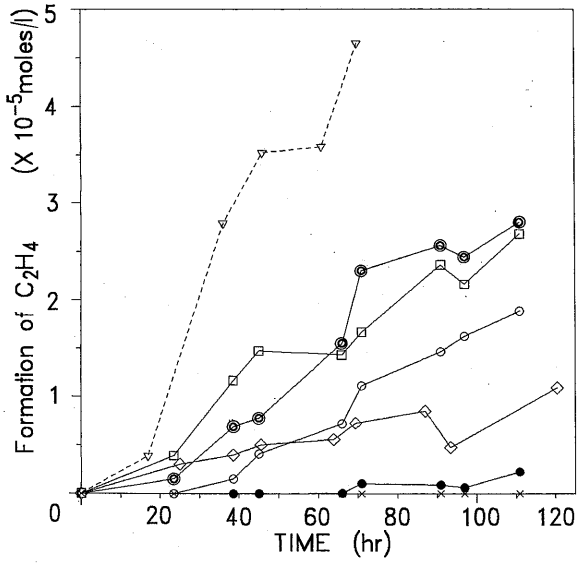

Fig. 1. Nitrogen fixation of *Chromatium* sp. at varying light intensities from 0 to 7000 lux. For experimental conditions (light, H_2S concentration and temperature) see the text, and pH was 8.4 in suspensions illuminated as 0 to 2000 lux, except that pH was 7.9 in case of light intensity of 7000 lux. The maximum rate, 32.0 nmol C_2H_4/hr/(mg dry cell weight), was obtained at the optimum conditions(▽), described below. In the experiment as a function of light intensity, the highest rate was 12.5 nmol C_2H_4/hr/(mg dry cell weight) at 1000 lux(◎).
×, dark; ●, 50 lux; ○, 300 lux; ◎, 1000 lux; □, 2000 lux; ◇, 7000 lux; ▽, optimum conditions of 1000 lux, 30℃, pH 8.4, and 130 mg H_2S-S/*l*.

The optimum pH range for the bacterium grown on NH_4^+-containing medium widely lied between pH 7.5 and 8.5,[1] and that for the bacterium grown on N_2 became narrow around pH 8.5

Hydrogen Sulfide

Nitrogen fixation rate of *Chromatium* sp. at each concentrations of H_2S was shown in Fig. 3. Nitrogen fixation was not perfomed without an addition of H_2S, but became fast from 3 to 20 mg H_2S-S/*l* and steady to 100 mg H_2S-S/*l*, The maximum nitrogen fixation rate was found at 20 to 100 mg H_2S-S/*l*, being 14.2 nmol C_2H_4/hr/(mg dry cell weight). Relatively high nitrogen fixation rate, 9.0 nmol C_2H_4/hr/(mg dry cell weight), was occurred at the concentration of 7 mg H_2S-S/*l*. However, high nitrogen fixation rates by the bacterium were generally occurred at 20 mg H_2S-S/*l* or

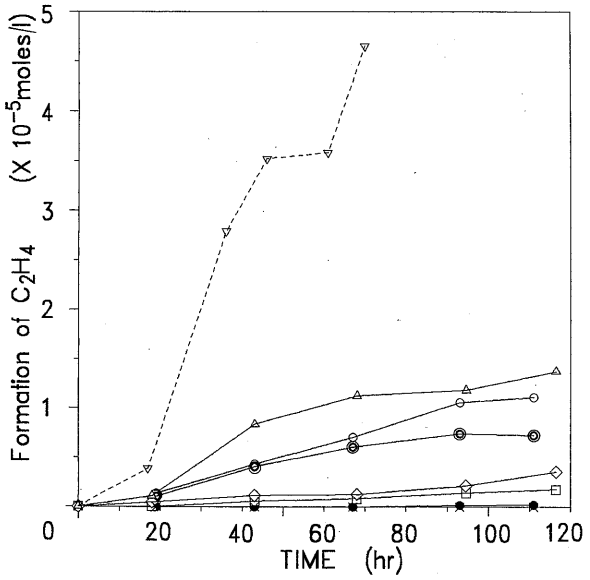

Fig. 2. Effects of pH upon nitrogen fixation of *Chromatium* sp.. The highest rate was 4.8 nmol C_2H_4/hr/(mg dry cell weight) at pH 8.5(△). Generally, high fixation rate was found at alkaline condition.
×, pH 7.0 ; ●, pH 7.4 ; □, pH 7.9 ; ◇, pH 8.1 ; △, pH 8.5 ; ○, pH 8.7 ; ◎, pH 8.9 ; ▽, optimum conditions (as in Fig. 1).

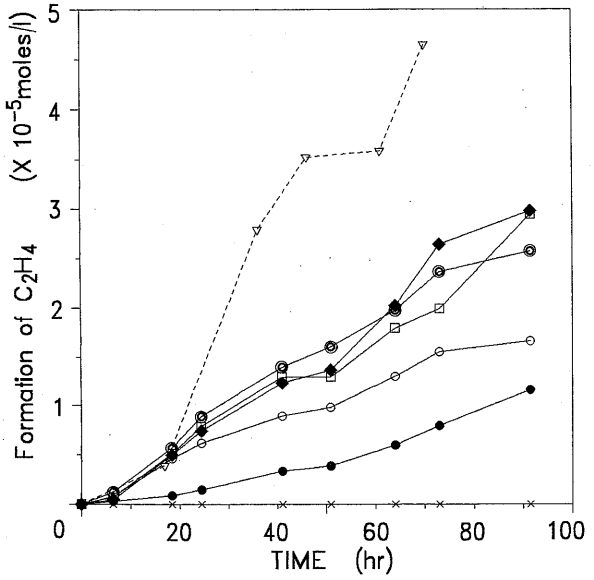

Fig. 3. Response of nitrogen fixation of *Chromatium* sp. to different H_2S concentrations. The highest rate was 14.1 nmol C_2H_4/hr/(mg dry cell weight) at 20 mg H_2S-S/*l*, and at above this concentration little difference between rates was shown. At concentrations from 0 to 20 mg H_2S-S/*l*, pH of suspensions was 8.3, and at more than 20 mg H_2S-S/*l*, pH was 8.4.
×, 0 mg H_2S-S/*l*; ●, 3 mg H_2S-S/*l*; ○, 7 mg H_2S-S/*l*; ◎, 20 mg H_2S-S/*l*; □, 50 mg H_2S-S/*l*; ◆, 100mg H_2S-S/*l*; ▽, optimum conditions (as in Fig. 1)

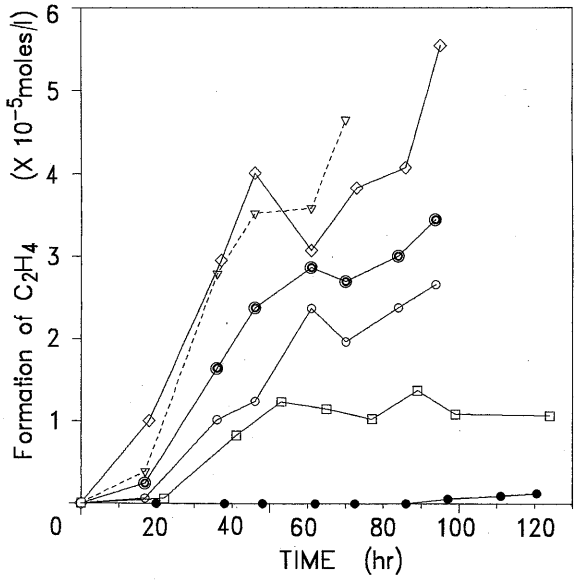

Fig. 4. Nitrogen fixation of *Chromatium* sp. at varying temperatures. The optimum conditions for the bacterial nitrogen fixation were temperature of 30℃, light intensity of 1000 lux, pH of 8.4 and H_2S concentration of 130 mg H_2S-S/*l*(▽), and the maximum rate for that was 32.0 nmol C_2H_4/hr/(mg dry cell weight). pH of the suspension was 8.3 at 10℃, and at the other temperatures pH was 8.4.
●, 10℃ ; ○, 20℃ ; ◎, 25℃ ; ▽, 30℃ ; ◇, 35℃ ; □, 37℃.

more.

Temperature

Nitrogen fixation of *Chromatium* sp. at various temperatures was shown in Fig. 4. High rates of nitrogen fixation were found at 30~35℃, but above which a rapid decrease in fixation rate was appeared.

The optimum temperature for nitrogen fixation in the present study was comparable to that for *C. limicola* forma *thiosulfatophilum's*, however nitrogenase from *C. limicola* was completely inactive at 20℃, and was more active than that of the bacterium at 40℃.[15]

Discussion

The maximum nitrogen fixation rate of *Chromatium* sp., 32.0 nmol C_2H_4/hr/(mg dry cell weight), was quite low compared to those of the other phototrophic bacteria, shown in Table 1. It may be thought that the bacterial differences in capacity to fix N_2 are resulted from an availability of each species to use organic compounds as carbon or nitrogen sources, and from different bacterial environmental requirements for growth.

Light intensity which rendered nitrogen fixation of the

bacterium optimum was comparable to only that of *C. limicola* forma *thiosulfatophilum*,[11] and was comparatively low compared to those of the other bacteria shown in Table 1.

It was shown that the bacterium had not much availability to use carbon compounds, excepted for limited organic compounds such as fructose and lactate.[1] In contrast, many species of Rhodospirillaceae could grow rapidly on organic compounds, and they were able to perform nitrogen fixation in the dark by respiratory energy conversion under microaerobic conditions.[12,21] However, nitrogen fixation by the bacterium was only possible in the presence of light and H_2S(Figs. 1, 3), which indicates that a sufficient ATP and reductants to initiate the nitrogen fixation reaction could be produced by photosynthetic metabolism, not by fermentative energy metabolism.[22]

The utilization of low light intensity by N_2-growing cells of *Chromatium* sp.(Fig. 1) under inorganic condition may become important *in situ*, particularly, in lakes because light intensity of the bacterial habitat has been known to be usually low.[8,23]

The optimum temperature range for nitrogen fixation by the bacterium(Fig. 4) was lower than those for nitrogenase activities of Heliobacteria[13] and some thermophilic bacteria,[15,24] but was comparable to those of some purple nonsulfur and green sulfur phototrophic bacteria.[11,12]

The optimum pH range for the nitrogen fixation comparatively shifted to more alkaline in comparison with that for cells growing on NH_4^+-containing medium(pH 7.5~8.5),[1] which indicates that the effects of pH upon cells growing on N_2 and growing on NH_4^+ seem to be different.

From the results of pH upon nitrogen fixation of the bacterium, nitrogen fixation could not be performed at pH of around 7.0 (Fig. 2), while this range of pH was mostly adopted as growth condition for the nitrogenase activities of the other phototrophic bacteria shown in Table 1.

The ability of a species to cope with environmental restraints, e. g., low light intensity,[25] low concentration of nutrients, or absence of organic compounds possibly becomes important to determine its distribution in nature. In relation to the above, *Chromatium* sp., densely populated in lake Kaiike, has been shown to be an important dominant as a result of minimizing its environmental requirements of light intensity and nutrients concentration for growth.[1,26]

Nitrogen fixation by *Chromatium* sp. may not largely contribute to the recruitment of a deficit in nitrogen budget in the ecosystem because of its inactive state of nitrogen fixation *in situ*.[8] However, results from the present study suggest that *in situ* nitrogen fixation by the bacterium may occur in the water column, if a conceivable restraint, NH_4^+, would be consumed to some critical level.

Table 1. N_2 fixing rates in some diazotrophic bacteria

Species	Maximum N_2 Fixing Rate	Reference
Chlorobium tepidum	6300*	Wahlund & Madigan[15]
Heliobacillus mobilis	4400*	Kimble & Madigan[13]
Rhodopseudomonas capsulata	4320*	Meyer et al.[14]
Chlorobium thiosulfatophilum	8500**	Heda & Madigan[11]
Anabaena variabilis	740*	Prosperi et al.[16]
Nodularia spumigena	310*	Prosperi et al.[16]
Calothrix marchica	525*	Prosperi et al.[16]
Nostoc punctiforme	680*	Prosperi et al.[16]
Actinomycete sp.	20**	Gauthier et al.[17]
Rhizobium sp.	2.5***	Pagan et al.[18]
Rhizobium japonicum	1.89**	Kurz & LaRue[19]
Azotobacter vinelandii	2.5****	Ballesteros et al.[20]
Chromatium sp.	32*	This study

*: nmol C_2H_4/hr/(mg dry cell weight), ***: nmol C_2H_4/hr/segment

: nmol C_2H_4/hr/(mg cell protein), **: μl C_2H_4/hr/10^9 cells

Generally, nitrogen fixers which had the ability to produce high levels of nitrogenase and to grow rapidly on N_2 were suggested to be predominant species in nitrogen-limited environments.[12] The high nitrogenase activities of phototrophic bacteria in Table 1 seem possible when their requirements of each proper organic compounds for growth by nitrogen fixation are met.

It is suggested that *Chromatium* sp. of relatively low nitrogen fixation rate(Table 1) becomes a predominant species because of its ability to fix N_2 photoautotrophically even under low light intensity and low H_2S concentration.

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* : in German.
** : in German with English summary.
*** : in Japanese with English summary.

貝池から分離した *Chromatium* sp. の窒素固定に対する環境要因の影響

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貝池溶存酸素－硫化水素境界で濃密に棲息している *Chromatium* sp. は窒素固定能を持つ。*Chromatium* sp. の窒素固定に対する環境要因の影響をアセチレン還元法によって調べた。

Chromatium sp. の窒素固定は照度及び硫化水素濃度に依存的であり、その最大固定速度、32.0 nmol C_2H_4 /hr/(mg dry cell weight)が得られた。各最適環境条件は、照度1000 lux、pH 8.5、硫化水素濃度20 mg H_2S-S/*l*, 温度30℃であった。

本菌は無機条件下で低い照度及び硫化水素濃度でも速い窒素固定能を示した。

Chromatium sp. は、低い照度及び低い硫化水素濃度の条件下でも光独立栄養的な窒素固定を持つことからその生態系において優点種となり、また窒素固定による窒素収支への寄与が有り得ると考えられた。