# $N_2$ Fixation and Growth of *Chromatium* sp. with and without $NH_4^+$ Addition

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$N_2$ fixation and growth of *Chromatium* sp., isolated from Lake Kaiike, were examined with and without $NH_4^+$ addition. The bacterial $N_2$ fixation was inhibited by exogenous $NH_4^+$. Added $NH_4^+$ was rapidly assimilated by the bacterium. The resumption of $N_2$ fixation was found when $NH_4^+$ was reduced to a level of 100 $\mu$M.

Without the addition of $NH_4^+$, the bacterium could grow. However, growth yield added with $NH_4^+$ was larger than that without $NH_4^+$ addition. Different growth yields between the two nitrogen sources seemed to be strongly related to light and $H_2S$. A low light limited the bacterial growth on $N_2$ resulting in the lowest of all the yields. Intracellular sulfur was shown to be promotable to the bacterial growth on $NH_4^+$ rather than that on $N_2$.

Since the bacterial growth on $N_2$ was expected to require more light and $H_2S$ than that on $NH_4^+$, the bacterial $N_2$ fixation at the site of original habitat in Lake Kaiike was suggested to barely maintain the bacterial number.

Key words: *Chromatium* sp., $N_2$ fixation, $NH_4^+$ addition, Growth yield.

*Chromatium* sp. is densely populated at an upper boundary of the $H_2S$ layer in a stratified lake, Kaiike, throughout all seasons. The dense population of *Chromatium* sp. at mid-depth of the lake is called the bacterial plate.[1] The bacterium is shown to be able to fix $N_2$ at an expense of energy provided by photosynthesis,[2] while, for nonphototrophic $N_2$-fixing bacteria exogenous energy supply for reducing $N_2$ is necessary.[3-6] $N_2$ fixation of *Chromatium* sp. and its growth seem to be severely effected by the presence of $NH_4^+$.

Inhibitory effect of $NH_4^+$ for $N_2$ fixation of the bacterium and the resumption of $N_2$ fixation were examined in terms of molar ratio of $H_2S$ and $NH_4^+$.[7] Because $H_2S$ is concurrently consumed by the bacterium during $NH_4^+$ assimilation, $H_2S$ available to the bacterium after $NH_4^+$ consumption is determinative for subsequent $N_2$ fixation.[7]

Growth by $N_2$ fixation requires an extra energy for reducing $N_2$ to $NH_4^+$.[8] The bacterial growth on $N_2$ is expected to show different requirement of light and $H_2S$ compared to the growth on $NH_4^+$ under photolithotrophic growth condition.

The purpose of the present study is to establish an inhibitory effect of $NH_4^+$ for the bacterial $N_2$ fixation, and to make clear the difference of the bacterial growth in the media where $NH_4^+$ or $N_2$ was added as a sole nitrogen source.

## Materials & Methods

*Chromatium* sp. isolated from the bacterial plate of Lake Kaiike had been cultured at the conditions of 1000 lux, 25℃, pH8.2－8.4 and 4.1 mM of $H_2S$ with the medium of Pfennig.[9] The NaCl and $MgSO_4 \cdot 7H_2O$ in the medium were increased 25 g and 3.5g$\cdot l^{-1}$, respectively, for marine habitat of the bacterium, and trace element solution SL 7 was replaced with a solution SL 10.[2]

$NH_4^+$-grown bacterial cells in the exponential growth phase were harvested by centrifugation (670× g, 15 min), and the pellets were resuspended with $NH_4^+$-free medium (3 times), and which were used for the measurement of $N_2$ fixation ($C_2H_2$ reduction method). Cells washed by $H_2S$- and $NH_4^+$-free medium were used for the growths on $N_2$ or $NH_4^+$.

For the $NH_4^+$-growth, $NH_4^+$ stock solution (pH 8.0) was added to the bacteial suspension, while, for the $N_2$-growth a 40 ml of $N_2$ gas was injected as a nitrogen source. A 100-ml syringe was utilized as the culture vessel.

The syringes were placed in a 25℃ water bath at 1000 lux. Illumination was provided by 100-W incandescent lamps, perpendicularly positioned over the water bath. A black nylon net was used for obtaining different light levels by rolling around syringes. Culture vessels in water bath were gently agitated and rotated manually at intervals.

In the culture for the $NH_4^+$-growth, added $NH_4^+$ was utilized and became traceless, and which was possibly found in a stationary growth phase. As a result, dissolved $N_2$ in bacterial suspension was likely to be substitued for $NH_4^+$ as a nitrogen source,because the medium was prepared under $N_2$ stream. However, additions of $H_2S$ and $NH_4^+$ in molar ratio of less than 3 excluded a possibility of the bacterial utilization of $N_2$ for the growth[7].

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The bacterial numbers and its relative one containing intracellular sulfur globules were quantified microscopically using aThoma hemacytometer. In counting work, few drops of 10 % formalin solution were used and enough for stopping the bacterial movement and the de novo deposition of intracellular sulfur globules.[10]

Measurement of the bacterial $N_2$ fixation ($C_2H_2$ reduction method) was performed as previously described.[11]

Measurement of $NH_4^+$ concentration was done by the Indophenol method.[12]

Known volume of neutralized $Na_2S \cdot 9H_2O$ solution was added to the bacterial suspension with a microsyringe. Time-serial changes of $H_2S$ concentration in the bacterial suspension were determined by Cline method.[13]

## Results & Discussion

$N_2$ fixation rate of *Chromatium* sp. in the different amounts of $NH_4^+$ (0~700 $\mu$M) is shown in Fig. 1. The rate was decreasing with increasing $NH_4^+$ concentration, and completely inhibited by 700 $\mu$M. Fig. 2 shows time-serial changes of $NH_4^+$ and $H_2S$ concentration in bacterial suspension, and formation of $C_2H_4$. The bacterium was shown to resume fixing $N_2$ when $NH_4^+$ was reduced to a level of 100 $\mu$M.

Fig. 3 shows the bacterial growth with and without $NH_4^+$ addition at light levels of 250 and 1000 lux, and concurrent bacterial consumption of $H_2S$. Without the addition of $NH_4^+$, the bacterium could grow, but growth yield added with $NH_4^+$ was larger than that without $NH_4^+$ addition. Significantly different growth rates were not observed, while apparently different growth yields at the same light intensity were found, showing those differences became larger in low light intensity. Different growth yields seemed to be strongly related to light and $H_2S$, in connection with kinds of nitrogen sources used for the growth.

At an high light intensity, difference of the bacterial number between both growths began to be large when $H_2S$ in suspension became depleted likely found in a stationary growth phase, shown in Fig. 3. In $NH_4^+$-growth at that period, a significnant increase in bacterial number was observed, ascribed to the bacterial utilization of intracellular sulfur. However, those increase in $N_2$-growth was a slight one, which implied intracellular sulfur did not contribute much to a net increase in bacterial number.

At a low light intensity, slow but, continuous growth was observed in $NH_4^+$-growth, resulting in a high yield. Even after exhaustion of suspension's $H_2S$ the growth could be continued for another 3 days at an expense of intracellular sulfur. In $N_2$-growth, a decrease in growth rate even in the moderate presence of $H_2S$ concentration was found from the 4th day of the incubation (Fig. 3). From that time, only a

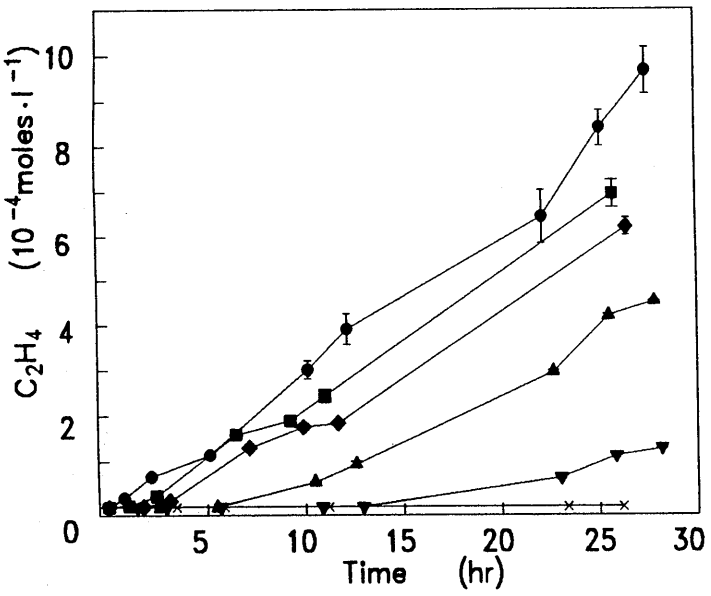


Fig. 1. Effect of $NH_4^+$ upon $N_2$ fixation of *Chromatium* sp. The bacterium grown on $NH_4^+$ at 1000 lux, were harvested at exponential phase and washed by $NH_4^+$-free medium (for details, see the text). Initially, $H_2S$ (1.5 mM) and each $NH_4^+$ concentration were added to each bacterial suspension, and the experiment was started with turning on the light (1000 lux). Each $NH_4^+$ concentration of the bacterial suspension was as follows. ●, Control ($NH_4^+$ was not added); ■, 50 $\mu$M; ◆, 100$\mu$M; ▲, 200 $\mu$M; ▼, 400 $\mu$M; ×, 700 $\mu$M. Vertical bars denote standard deviation of two replicate samples.

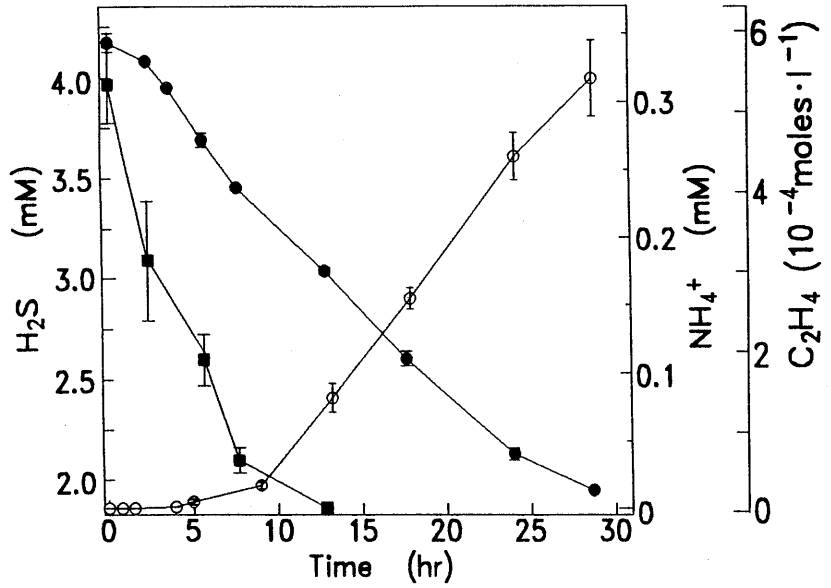


Fig. 2. Effect of $NH_4^+$ upon $N_2$ fixation of *Chromatium* sp. associated with change of $H_2S$ concentration at 1000 lux. Initial concentrations of added $H_2S$ and $NH_4^+$ at zero time were 4.6 mM and 313 $\mu$M, respectively.
●, $H_2S$ concentrations; ■, $NH_4^+$ concentrations; ○, $C_2H_4$ formed.
Vertical bars denote standard deviation of two replicate samples.

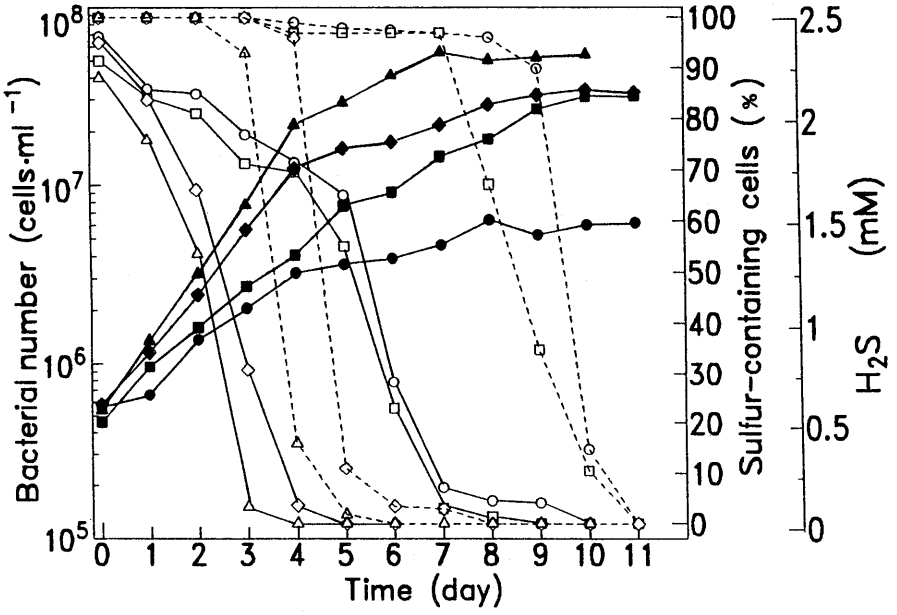


Fig. 3. Changes of the bacterial number, relative abundance of sulfur-containg cells and $H_2S$ consumption in culture of *Chromatium* sp. illuminated with 250 and 1000 lux. Initial $H_2S$ concentrations were in the range of 2.2 to 2.4 mM. In $NH_4^+$-growth, $NH_4^+$ concentration of 2.3 mM was equally added to the bacterial suspensions.
(●) Bacterial number grown at $N_2$ and 250 lux. (■) $NH_4^+$, 250 lux. (◆) $N_2$, 1000 lux. (▲) $NH_4^+$, 1000 lux.
(○——○) Change of $H_2S$ concentration at $N_2$ and 250 lux. (□——□) $NH_4^+$, 250 lux. (◇——◇) $N_2$, 1000 lux. (△——△) $NH_4^+$, 1000 lux.
(○——○) Change of relative abundance of sulfur-containing cells grown at $N_2$, 250 lux. (□——□) $NH_4^+$, 250 lux. (◇——◇) $N_2$, 1000 lux. (△——△) $NH_4^+$, 1000 lux.

rapid $H_2S$ consumption with a little increase in bacterial number, which resulted in the lowest of all the growth yields, was occurred.

In a $N_2$-fixing cyanobacterium, *Aphanizomenon flos-aquae*, of which population was maintained in the surface layer of Wintergreen Lake, it could grow on $NH_4^+$ or $NO_3^-$ at a low light intensity, but not grow on $N_2$ at the same light intensity.[14] At an high light intensity the bacterial growth, regardless of nitrogen sources, was dependent on $H_2S$ (Fig. 3), i.e., its growth would respond to an environmentally available $H_2S$. However, a low light limited the utilization of $N_2$ for the bacterial growth compared to the $NH_4^+$-growth. In another aspect, a low light might limit nitrogenase function or its synthesis.[2),15)]

In conclusion, the bacterium could grow on $N_2$, however, the $N_2$-growth required more light and $H_2S$ than the $NH_4^+$-growth. In considering in situ light and $H_2S$ conditions in Lake Kaiike,[2)] the bacterial $N_2$ fixation is not thought to contribute largely to an increase of the bacterial population in number because of more requirement of light and $H_2S$ for the growth.

In Lake Kaiike vertically sharp change in $NH_4^+$ concentration within the bacterial plate nicely met with that change in $H_2S$ concentration by their molar ratio of 3.[7)] At an upper part of the bacterial plate $H_2S$ and $NH_4^+$ are always in a deficient state.[16)] However, nearly all the bacterium at that place has its intracellular sulfur globules.[2)] Intracellular sulfur in the absence of $NH_4^+$ did not largely promote the bacterial growth compared to that in the presence of $NH_4^+$ (Fig. 3). At an upperpart of the bacterial plate a rapid bacterial growth is not expectable. However, the bacterial $N_2$ fixation that leads a growth with little increase in bacterial number is likely to be occurred.

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*: in German.
**: in Japanese with English summary.
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# $NH_4^+$の添加有無による *Chromatium* sp. の窒素固定および生長

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貝池から分離した *Chromatium* sp. の窒素固定および生長を $NH_4^+$ の添加、無添加によって調べた。本菌の窒素固定は添加した $NH_4^+$ によって阻害された。添加された $NH_4^+$ は本菌によって迅速に同化された。窒素固定の再開は 100$\mu$M 以下の濃度で進行した。

$NH_4^+$ の添加がなくても本菌は生長したが、一定期間培養した後の生長量は常に $NH_4^+$ を添加した方が高かった。それぞれ異なる窒素源による生長量の差は光及び $H_2S$ と強く関わっていると考えられた。低照度は本菌の窒素固定による生長を制限し、最も低い生長量をもたらした。細胞内いおう粒子は $N_2$ 生長より $NH_4^+$ 生長に対してより促進効果を示した。

本菌の窒素固定による生長は $NH_4^+$ による生長に比較し、より多くの光および $H_2S$ を要求することから、貝池の棲息地における本菌の窒素固定は辛うじて菌の個体数を維持するものと推察された。