CaCO₃-Inclusions of *Macromonas* sp. Blooming at an Upper Boundary of the H₂S Layer of Lake Kaiike

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Abstract

Two large-celled bacterial species, *Macromonas* sp. and *Chromatium* sp., bloom at an upper boundary of the H₂S layer in Lake Kaiike throughout the year (bacterial plate). Large pearl white refractile inclusions of *Macromonas* sp., which were composed of CaCO₃, disappeared from the cells when the bacterial plate sample was acidified below a pH 6.3. But, as soon as the pH was returned to an initial level (8.5), these cells rapidly deposited them again, indicating that CaCO₃ deposition is one of the essential activities in maintaining cell integrity. The inclusions also disappeared with exposure of live bacteria to a H₂S solution. It was suggested that *Macromonas* sp. inhabits the H₂S boundary using CaCO₃-inclusions as protection from environmental H₂S.

Key words: *Macromonas* sp., CaCO₃-inclusions, H₂S boundary, Lake Kaiike

1. Introduction

Two large-celled bacterial species, *Macromonas* sp. and *Chromatium* sp., bloom so densely at an upper boundary of the H₂S layer in Lake Kaiike that the boundary is easily recognized even with the naked eye due to the coloration of the water (bacterial plate). Maximum numbers of the two species are on the order of magnitude of 10⁶-10⁷ cells·ml⁻¹.

The two species are characterized by intracellular bodies: *Macromonas* sp. by 2 or 3 large pearl white refractile inclusions, and *Chromatium* sp. by numerous sulfur globules. These bodies significantly increased the cellular buoyant densities of both species above 1.26 g·cm⁻³ (MATSUYAMA, 1991a). The ecological role of sulfur globules in *Chromatium* sp. has already been discussed (MATSUYAMA, 1990): sulfur deposition was understood as a cellular device to store environmental H₂S.

Refractile inclusions of *Macromonas* species, which are also found in *Achromonas* species, were thought to be composed of CaCO₃. These inclusions were, without proof, suggested to

exert a buffering effect on H₂SO₄ formed by sulfur oxidation (DE BOER *et al.*, 1971; LA RIVIÈRE and SCHMIDT, 1981).

DUBININA and GRABOVICH (1984) isolated three strains of *Macromonas bipunctata* from the precipitates of sewage aeration tanks. They found that all strains were strictly aerobic heterotrophs rather than chemolithotrophic, sulfur-oxidizing bacteria as was generally believed. They also found that reduced sulfur compounds such as H₂S were not used by the strains as electron donors; rather, their oxidation was due to interaction with H₂O₂, which was a main product of O₂ reduction in respiration.

An aerophilic and heterotrophic growth of *Macromonas* sp. from Lake Kaiike, which closely resembles *M. bipunctata* (WATSON, 1974), was suggested from the enrichment culture, and the strain seemed to grow on some organic matter from coexisting *Chromatium* sp. H₂S was inhibitory for the strain (MATSUYAMA, 1991b). The present study aims to examine the deposition of large refractile inclusions in *Macromonas* sp. with some references to inhabitation at the H₂S

boundary in Lake Kaiike.

2. Methods

Sampling was done at the center of Lake Kaiike using a 3-1 Van Dorn bottle on 4 April 1991. Glass bottles filled with water samples from different depths were brought to the laboratory while keeping them cool and dark. Immediately, the cell numbers of two bacterial species were counted in a Thoma hemacytometer. For measurement of dissolved Ca^{2+} the samples were filtered through a glass-fiber filter (Whatman GF/C). Then, the sample (50 ml) was alkalized to pH 12 with 4 ml of 8 N KOH. After additions of 1 ml of 10% $\text{NH}_2\text{OH}\cdot\text{HCl}$ and 0.5 ml of 10% KCN, the sample was titrated with 0.01 N EDTA using 2-hydroxyl-1-(2-hydroxy-4-sulfo-naphthylazo)-3-naphthonic acid as an indicator (MIYAKE and KITANO, 1976). *In situ* measurements of salinity and temperature were made with a CTD (Alec Electronics, model AST-1000S). Methods for measurements of dissolved O_2 and H_2S in lake water have already been described (MATSUYAMA, 1978). The Ca^{2+} and CO_2 contents in the refractile inclusions of *Macromonas* sp. were determined using a Conway diffusion unit. The bacterium was previously enriched from the bacterial plate sample in the filter-sterilized seawater by daily addition of a medium of DUBININA and GRABOVICH (1984), which contained CH_3COONa , casamino acids and yeast extract. (For details on enriching *Macromonas* sp. in the medium see MATSUYAMA (1991b).) Due to the high cellular buoyant density of *Macromonas* sp., the bacterium formed a solid chalky red sediment by centrifugation (15 min, $1,000\times g$), and above it other microorganisms were gently deposited. The latter were easily separated from the *Macromonas* sediment by gentle swirling. Repeated washing of the bacterial sediment with an NaCl solution ($35\text{ g}\cdot\text{l}^{-1}$) and centrifugation yielded an almost pure suspension of *Macromonas* sp. Then, different amounts of the suspension (0–2.0 ml) were pipetted into side cells of Conway diffusion units. Immediately after addition of 0.2 ml of 1 N H_2SO_4 to the suspension, the units

were tightly covered. Absorption of CO_2 (evolved from the acidified bacterial suspension to a 1.0 ml of 0.1 N $\text{Ba}(\text{OH})_2$ in a central cell) was allowed to proceed for 1 hr. The BaCO_3 formed was determined by the back-titration of excess $\text{Ba}(\text{OH})_2$ with 0.1 N HCl. Acidified bacterial suspension was also used to measure Ca^{2+} by the EDTA titration method. Time change of H_2S concentration during the incubation of *Macromonas* sp. was measured by the method of CLINE (1969).

3. Results and discussion

Figure 1 shows the vertical distribution of the two bacterial species and some environmental factors, as well as dissolved Ca^{2+} in Lake Kaiike. Bacterial distributions were within seasonal variations (MATSUYAMA, 1991b): cell numbers of both species were maximum at the H_2S boundary, and rapidly decreased below the boundary. In the layer above 3 m depth, a concentration of dissolved Ca^{2+} was quite consistent with that of Ca^{2+} , which was calculated from Ca^{2+} /salinity ratio in seawater ($422\text{ mg}\cdot\text{l}^{-1}$ for $S=35\text{‰}$) (RILEY and CHESTER, 1971). Below that depth, however, concentration of Ca^{2+} was somewhat lower than the one calculated, indicating that some part of Ca^{2+} was removed from the water, although the difference between them diminished around 5 m depth. Removal of Ca^{2+} from the lower water may be partly due to Ca^{2+} uptake by *Macromonas* sp. as will be mentioned.

Figure 2 are microphotographs of the bacterial plate samples before and after addition of a few drops of HCl to decrease the pH from 8.5 to 5.0. With the addition of HCl the refractile inclusions rapidly disappeared from the cells of *Macromonas* sp.

Figure 3-A shows the relative abundance of the cells of *Macromonas* sp., which kept the refractile inclusions after environmental pH (8.5) was shifted to different levels with additions of HCl and NaOH solutions. At pH values below 7.3 the inclusion-containing cells began to decrease. Below pH 6.2 the refractile inclusions completely disappeared from the cells.

The bacterial plate sample was acidified with

Fig. 1. Vertical distributions of *Macromonas* sp. (A), *Chromatium* sp. (B) and some environmental factors as well as dissolved Ca²⁺ in Lake Kaike on 4 April 1991. Ca²⁺ observed indicates Ca²⁺ concentration observed by EDTA titration of lake water. Ca²⁺ calculated indicates Ca²⁺ concentration calculated from Ca²⁺: salinity ratio in seawater. S: salinity. T: temperature.

HCl to pH 5.1 to eliminate most refractile inclusions from the cells of *Macromonas* sp. Then, the pH was converted to an initial value (8.5) with the addition of NaOH solution. Figure 3-B shows the time change of the relative abundance of cells which began to deposit the inclusions again. Immediately after the return of pH to 8.5, some bacterial cells began to exhibit small glittering globules, which grew to a size sufficient to fill the cells within a short period (2–3 hr). Along with an increase in size, the inclusion-deposited cells increased in number and also exhibited motility. For *Macromonas* sp., deposition of the refractile inclusions seems to be one of the essential activities in maintaining cell integrity, since the bacterium continued

to grow as long as it contained the inclusions (MATSUYAMA, 1988).

Figure 4 shows the quantities of Ca²⁺ and CO₂ released from different cell numbers of *Macromonas* sp. Comparison of the Ca²⁺ and CO₂ gradients to the cell number showed that these elements existed at a molar ratio of 0.9 : 1.0, indicating that they were present in the bacterial cells as an approximate form of CaCO₃. Figure 4 also shows that the mean content of CaCO₃ in a single cell was 8.8×10^{-12} g. If the density of the bacterial CaCO₃ is assumed to be close to that of calcite ($2.7 \text{ g} \cdot \text{cm}^{-3}$), the volume of the CaCO₃ in a single cell may be estimated as $3.2 \times 10^{-12} \text{ cm}^3$. If we further assume that the CaCO₃ is present as two spheres in a single

Fig. 2. Microphotographs of bacterial plate sample from upper boundary of H_2S layer before (A) and after addition of HCl solution (B). Large refractile inclusions disappeared from cells of *Macromonas* sp. with addition of HCl.

cell, the mean radius of a sphere may be calculated as $1.2\ \mu\text{m}$, which is quite consistent with that of the microscopic observation of the refractile inclusions (Fig. 2-A). (A mean radius of the refractile inclusions of *Macromonas* sp. shown in Fig. 2-A was $1.4\ \mu\text{m}$.) It is safe to say that the refractile inclusions of *Macromonas* sp. are mainly composed of CaCO_3 .

The refractile inclusions of *Macromonas* sp. also disappeared when the bacterial cells were exposed to a H_2S solution as long as the cells were living. In the case of the bacterial cells

killed with formalin, such an elimination of refractile inclusions never occurred, even after prolonged exposure to a H_2S solution. This is markedly different from the disappearance of inclusions treated with acid (purely chemical process).

H_2S was introduced into the living cell suspension of *Macromonas* sp. to give a final concentration of $90\ \text{mgS}\cdot\text{l}^{-1}$. The suspension was then poured into a series of 65-ml glass bottles which were tightly capped for incubation at 25°C . Figure 5 shows the time changes of pH,

Fig. 3. Relative abundance of inclusion-containing cells of *Macromonas* sp. in bacterial plate sample. A: 2 hr after pH of bacterial plate sample (8.5) was shifted to different values with additions of HCl and NaOH solutions. B: time change of inclusion-containing cells after pH of sample was returned from 5.1 to 8.5. Most refractile inclusions previously disappeared from cells with addition of HCl. Vertical dotted line in A indicates pH above which CaCO₃ is more stable form than Ca²⁺ in a water (25°C) containing 400 mgCa²⁺•l⁻¹ and equilibrated with atmosphere having 10^{-3.5} pressure of CO₂ (STUMM and MORGAN, 1970).

Fig. 4. Ca²⁺ and CO₂ released from different cell numbers of *Macromonas* sp. Regression lines estimated by the method of least squares.

Fig. 5. Time changes of pH, H₂S, relative abundance of inclusion-containing cells of *Macromonas* sp. and dissolved Ca²⁺ after H₂S addition to bacterial suspension (90 mg S · l⁻¹). Bacterial cell number was 4.9×10^6 cells · ml⁻¹.

H₂S, the relative abundance of the inclusion-containing cells, and dissolved Ca²⁺. While pH was relatively constant around 8.0 throughout the incubation, disappearance of the inclusions and simultaneous release of Ca²⁺ from the bacterial cells continued. The mechanism underlying the disappearance of the inclusions due to bacterial exposure to H₂S was unknown. However, the author hypothesised that when H₂S infiltrated the bacterial cells, it was oxidized by H₂O₂ as DUBININA and GRABOVICH (1984) suggested, probably due to protective detoxicifying H₂S oxidation, and the H₂SO₄ thus formed was neutralized by the CaCO₃-inclusions. When the rate of H₂S infiltration into the bacterial cells was rapid, the CaCO₃-inclusions would completely disappear from the

cells.

A previous study (MATSUYAMA, 1988) showed that when the cells deprived of inclusions from excess H₂S were exposed to air, they began to deposit inclusions again within 1 hr. Such bacterial ability was found to be valid even after as much as 2 weeks exposure to H₂S and even at an ambient H₂S concentration as high as 40 mg S · l⁻¹.

From the above discussion it may be surmised that large refractile CaCO₃-inclusions of *Macromonas* sp. (or, more strictly speaking, the ability to deposit them inside the cells) may be a bacterial adaptation to survival in the H₂S boundary. These inclusions may serve as a shield from environmental H₂S, although the bacterium is essentially aerophilic.

摘 要

貝池 H₂S 層上端に密集する *Macromonas* sp. の CaCO₃ 顆粒

2 種類の大型の細菌, *Macromonas* sp. と *Chromatium* sp. が貝池 H₂S 層上端に年間を通して密集分布している (バクテリアプレート)。 *Macromonas* sp. の CaCO₃ よりなる大きくて真珠のように白く光沢ある顆粒はバクテリアプレート試料を pH 6.3 以下に酸性化すると急速に細胞から消失した。しかし, pH を初期の値 (8.5) に復帰させると, これら細胞は再び顆粒を急速に貯留し, CaCO₃ の蓄積が細胞機能を維持する上で基本的に重要な活動の一つであることを示した。これら顆粒の消失は細菌が生きている限り, H₂S 溶液に曝しても観察された。 *Macromonas* sp. は CaCO₃ 顆粒を環境中の H₂S に対し遮蔽物として利用することにより H₂S 層上端に生息すると推察された。

References

- CLINE, J. D. (1969): Spectrophotometric determination of hydrogen sulfide in natural waters. *Limnol. Oceanogr.*, 14: 454-458.
- DE BOER, W. E., J. W. M. LA RIVIÈRE and K. SCHMIDT (1971): Some properties of *Achromatium oxaliferum*. *Antonie V. Leeuwenhoek*, 37: 553-563.
- DUBININA, G. A. and M. YU. GRABOVICH (1984): Isolation, cultivation, and characteristics of *Macromonas bipunctata*. *Mikrobiologiya*, 53: 748-755.
- LA RIVIÈRE, J. W. M. (1965): Enrichment of colorless sulfur bacteria, p. 17-27. In H. G. SCHLEGEL (ed.), *Anreicherungskulturen und Mutantenauslese*. Gustav Fischer, Stuttgart.
- LA RIVIÈRE, J. W. M. and K. SCHMIDT (1981): Morphologically conspicuous sulfur oxidizing eubacteria, p. 1037-1043. In P. STARR, H. STOLP, H. G. TRÜPER, A. BALLOWS and H. G. SCHLEGEL (eds.), *The prokaryotes, vol. 1*. Springer-Verlag, Berlin.
- MATSUYAMA, M. (1978): Stratified condition of Lake Kaiike, a small coastal lake on Kamikoshiki Island, Kagoshima Prefecture. *Jpn. J. Limnol.*, 39: 97-102.
- MATSUYAMA, M. (1988): Responses of two bacteria dominating the bacterial plate at the dissolved O₂-H₂S interface of Lake Kaiike to different levels of light and H₂S supply. *Jpn. J. Limnol.* 49: 135-141.
- MATSUYAMA, M. (1990): Intracellular deposition of sulfur globules in a large-celled phototrophic bacterium densely populating an upper boundary of H₂S layer of Lake Kaiike. *Jpn. J. Limnol.*, 51: 1-8.
- MATSUYAMA, M. (1991a): Buoyant density of *Chromatium* sp.: Its effect on the blooming at an upper boundary of H₂S layer in Lake Kaiike. *Jpn. J. Limnol.*, 52: 57-63.
- MATSUYAMA, M. (1991b): Enrichment of *Macromonas* sp. densely populating an upper boundary of the H₂S layer of Lake Kaiike. *Jpn. J. Limnol.*, 52: 215-222.
- MIYAKE, Y. and Y. KITANO (1976): *Methods for Chemical Analysis of Waters*. Chijinshokan, Tokyo (in Japanese).
- RILEY, J. P. and R. CHESTER (1971): *Introduction to Marine Chemistry*. Academic Press, London and New York.
- STUMM, W. and J. J. MORGAN (1970): *Aquatic Chemistry. An Introduction Emphasizing Chemical Equilibria in Natural Waters*. Wiley Interscience, New York.
- WATSON, S. W. (1974): Organisms metabolizing sulfur and sulfur compounds, p. 456. In R. E. BUCHANAN and N. E. GIBBONS (eds.), *Bergey's Manual of Determinative Bacteriology, 8th ed.* The Williams & Wilkins Co., Baltimore.
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