Identification of an Algal Carbon Fixation-Enhancing Factor Extracted from *Paramecium bursaria*

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Z. Naturforsch. **66 c**, 491–498 (2011); received March 12/May 3, 2011

The green ciliate *Paramecium bursaria* contains several hundred symbiotic *Chlorella* species. We previously reported that symbiotic algal carbon fixation is enhanced by *P. bursaria* extracts and that the enhancing factor is a heat-stable, low-molecular-weight, water-soluble compound. To identify the factor, further experiments were carried out. The enhancing activity remained even when organic compounds in the extract were completely combusted at 700 °C, suggesting that the factor is an inorganic substance. Measurement of the major cations, K^+, Ca^{2+}, and Mg^{2+}, by an electrode and titration of the extract resulted in concentrations of 0.90 mM, 0.55 mM, and 0.21 mM, respectively. To evaluate the effect of these cations, a mixture of the cations at the measured concentrations was prepared, and symbiotic algal carbon fixation was measured in the solution. The results demonstrated that the fixation was enhanced to the same extent as with the *P. bursaria* extract, and thus this mixture of K^+, Ca^{2+}, and Mg^{2+} was concluded to be the carbon fixation-enhancing factor. There was no effect of the cation mixture on free-living *C. vulgaris*. Comparison of the cation concentrations of nonsymbiotic and symbiotic *Paramecium* extracts revealed that the concentrations of K^+ and Mg^{2+} in nonsymbiotic *Paramecium* extracts were too low to enhance symbiotic algal carbon fixation, suggesting that symbiotic *P. bursaria* provide suitable cation conditions for photosynthesis to its symbiotic *Chlorella*.

Key words: Carbon Fixation, Symbiosis, *Paramecium bursaria*

Introduction

Symbiosis between invertebrates and algae often occurs in marine organisms. For example, the dinoflagellate *Symbiodinium* sp. establishes a symbiotic relationship with anemone (Trench, 1971; Sutton and Hoegh-Guldberg, 1990), coral (Schlichter *et al.*, 1983), and giant clam (Streamer *et al.*, 1988; Masuda *et al.*, 1994; Ishikura *et al.*, 1999), supplying them with glycerol, amino acids or sugars (Trench, 1971, 1979; Hinde, 1988). Muscatine (1967) first reported that a homogenate of symbiotic coral and clam activates the excretion of fixed carbon by its symbiotic algae and that this excretion is believed to be stimulated by specific compound(s) in the host homogenate (Grant *et al.*, 1998), referred to as a host release factor (HRF). The HRF has not been identified due to the difficulty in cultivating experimental organisms and the lability of the HRF (Grant *et al.*, 1998). Gates *et al.* (1995) proposed free amino acids and mycosporine-like amino acids (MAAs) as candidates for the HRF, although these amino acids did not completely reproduce the activity of the HRF. Moreover, an inhibitory effect on photosynthesis caused by the symbiotic coral homogenate was also observed (Sutton and Hoegh-Guldberg, 1990; Grant *et al.*, 2001). Grant *et al.* (2001) suggested that the photosynthesis-inhibiting factor is of low molecular weight, but it was not identified. As noted above, the host is thought to affect the release of photosynthate and the photosynthesis using certain compound(s) such as HRF in marine symbiosis.

Amongst symbiotic freshwater organisms, the green ciliate *Paramecium bursaria* has been used in many studies on endosymbiosis because *P. bursaria* can be easily cultured; in addition, host and symbiotic *Chlorella* species can be separated and cultured independently, and their symbiotic relationship can be reconstructed via "reinfection" of cultured symbiotic *Chlorella* with symbiotic

algae-free host cells (Bomford, 1965). Symbiotic *Chlorella* was reported to supply its host mainly with maltose (Brown and Nielsen, 1974; Pardy *et al*., 1989; Reisser and Widowski, 1992). We previously evaluated the effect of a Japanese *P. bursaria* extract on the photosynthesis of its symbiotic *Chlorella.* As a result, carbon fixation by symbiotic *Chlorella* increased in the host extract. This suggested the existence of an algal carbon fixation-enhancing factor, referred to as a host factor, in the *P. bursaria* extract (Kamako and Imamura, 2006). Since cultivation of *P. bursaria* is easier than that of marine symbiotic organisms, and the phenomenon is interesting, further studies on the host factor in the *Paramecium* extract were carried out. In this paper, we describe the identification of the host factor in *P. bursaria* extracts.

Methods and Materials

Strains and culture conditions

Japanese symbiotic *Paramecium bursaria* F36, obtained from Prof. Yanagi, Ishinomaki Sensyu University, Miyagi, Japan, and nonsymbiotic *P. audatum* TA2, obtained from Prof. Fujishima, Yamaguchi University, Yamaguchi, Japan, were cultured in aka-endomame medium (Tsukii *et al.*, 1995) buffered with 1.65 mM Tris (pH 7.0) containing *Klebsiella pneumoniae* NBRC 3512 as food for *Paramecium*. Symbiotic *Chlorella variabilis* F36-ZK (Hoshina *et al.*, 2010) isolated from Japanese *P. bursaria* F36 (Kamako *et al*., 2005) were cultured in C medium (Ichimura, 1971) plus L-serine (200 μg ml^{-1}) without aeration. Free-living *Chlorella vulgaris* NIES-227 was grown in C medium. All cultivations were performed with a 16 h:8 h light (50 μmol photons m^{-2} s^{-1}) and dark cycle at 25 °C.

Estimation of the Paramecium cell volume

To estimate the cell volumes of *P. bursaria* F36 and *P. caudatum* TA2, major and minor axes of these cells were measured from photomicrographs. A *Paramecium* cell is shaped similar to a rugby ball; the cell volume was calculated using the formula: $V = 4/3\ \pi\ r_1^2\ r_2$, where r_1 is the minor axis and r_2 is the major axis.

Preparation of the Paramecium extract

Cultured *P. bursaria* F36 cells were collected on a paper filter (Advantec No. 101; Toyo Roshi, Tokyo, Japan), and the medium was completely replaced with distilled water. A portion of the suspension was taken for measurement of the cell number, and the remaining cells were disrupted by vacuum filtration (approximately 10 hPa) with a glass filter (GF/C; Whatman, Maidstone, Kent, UK). Subsequently, the filtrate was passed through a membrane filter (Advantec, pore size 0.2 μm, cellulose acetate; Toyo Roshi). The fluid was dried under reduced pressure at 40 °C, and the residue was dissolved with distilled water in an amount corresponding to one-tenth of the total estimated volume of *Paramecium* cells in the suspension. This solution was stored as *P. bursaria* F36 extract at –20 °C. Prior to experiments, the extract was completely thawed at room temperature and centrifuged at 12,000 × *g* for 5 min to remove a slight amount of insoluble material. The extract of *P. caudatum* TA2 was prepared in the same manner.

Measurement of the chlorophyll a concentration

Cultured *Chlorella* cells were washed with 50 mM sodium phosphate buffer (pH 7.0) three times by centrifugation at 1,200 × *g* for 5 min at room temperature. Washed cells were resuspended with 90% aqueous acetone and then disrupted using an ultrasonic disruptor (Microson XL2005; Heat Systems, Inc., Farmingdale, NY, USA). The suspension was centrifuged at 12,000 × *g* for 5 min at 4 °C, and then the absorbance of the supernatant at 750, 664, and 647 nm was measured using a photospectrometer (U-1100; Hitachi, Tokyo, Japan). The concentration of chlorophyll *a* (Chl. *a*) was calculated according to the method of Jeffrey and Humphrey (1975).

Measurement of carbon fixation

Chlorella cells at the log phase were washed with 50 mM sodium phosphate buffer (pH 7.0) three times by centrifugation at 1,200 × *g* for 5 min at room temperature, and resuspended with sodium phosphate buffer at approximately 5 μg Chl. *a* ml^{-1}. The algal suspension (170 μl) was transferred into a tube equipped with a cup holding a membrane filter (ultrafree-MC, pore size 0.45 μm; Millipore, Bedford, MA, USA). Subsequently, 5 μl of $NaH^{14}CO_3$ solution (1.85 MBq ml^{-1}; Amersham, Little Chalfont, Buckinghamshire, UK) at the final concentration of 10 mM and 75 μl of sample solution (*e.g.*, diluted

P. bursaria F36 extract) were added to the algal suspension and then incubated at 25 °C under 120 μmol photons m^{-2} s^{-1} for 30 min. After incubation, the algal suspension in the tube was filtered by centrifugation at 10,000 × *g* for 20 s at 4 °C to separate algal cells and filtrate. The algal cells on the membrane filter were resuspended with 200 μl of ice-cold sodium phosphate buffer and centrifuged at 10,000 × *g* for 2 min at 4 °C. Algal cells in the filter cup were put into a scintillation vial, and 5 ml of scintillation cocktail (Clear-sol I; Nacalai Tesuque Inc., Kyoto, Japan) were added to the vial, which was allowed to stand for 1 d in the dark. Radioactivity was measured using a liquid scintillation counter (LS6000TA; Beckman Coulter, Fullerton, CA, USA) to calculate the amount of fixed carbon. The filtrate in the tube was transferred into a glass vial and acidified with 50 μl of 1 M HCl, then heated at 90 °C for 10 min to remove unfixed carbon. After cooling down, 5 ml of scintillation cocktail were added to the vial to measure the amount of photosynthate excreted. Distilled water was used instead of a sample solution as a control. In all carbon fixation experiments, the *P. bursaria* F36 extract was diluted tenfold in the experimental solution to reproduce the cytoplasmic environment. When C medium was used as an experimental buffer, experiments were performed with replacement of sodium phosphate buffer by the medium.

Statistical analysis

All experiments were performed at least three times. Carbon fixation data were statistically analysed using the statistical software programme SPSS 12.0J for Windows (SPSS Inc., Chicago, IL, USA).

Preparation of the inorganic fraction

To obtain inorganic compounds from the *P. bursaria* F36 extract, 2 ml of extract were transferred into an aluminium crucible (As One, Osaka, Japan). The crucible was heated to 700 °C at a rate of 10 °C min^{-1} and kept at 700 °C for 1 h using a programmable furnace (MMF series; As One). The ash in the crucible was dissolved with in 2 ml distilled water and used as the inorganic fraction.

Measurement of cation concentrations

The concentration of K^+ in the tenfold diluted *P. bursaria* F36 extract was measured using a potassium ion-selective electrode (model 93–19; Orion, Fukui, Japan). KCl solutions at concentrations of 0.015 to 12.5 mM were used as standards.

The concentrations of Ca^{2+} and Mg^{2+} were measured by chelate titration. Tenfold diluted *P. bursaria* F36 extract (2 ml) was mixed with 2 ml of 2 M KOH and 5 drops of Dotite NN solution (Dojindo, Kumamoto, Japan), or with 2 ml of 2.6 M ammonium buffer (pH 10) and 2 drops of Dotite BT solution (Dojindo). Each mixture was allowed to stand for 5 min at room temperature and was then titrated with 1 mM Na_2EDTA solution. Concentration of Ca^{2+} and total concentrations of Ca^{2+} and Mg^{2+} were calculated from the volume of 1 mM Na_2EDTA solution required to reach the endpoint of the titration as indicated by the colour change of the NN and BT metals, respectively. The concentration of Mg^{2+} was calculated from the difference between the concentrations. The concentrations of K^+, Ca^{2+}, and Mg^{2+} cations in *P. caudatum* TA2 extracts werewas measured in the same manner.

Effect of cations on carbon fixation by symbiotic C. variabilis F36-ZK

To evaluate the effects of K^+, Ca^{2+}, and Mg^{2+} on carbon fixation by *C. variabilis* F36-ZK, carbon fixation was measured in the presence of 1.32 mM K^+, 0.67 mM Ca^{2+}, 0.38 mM Mg^{2+}, or a mixture of the three species. In experiments that evaluated the effect of two cations, carbon fixation was measured at K^+ concentrations from 0 to 50 mM and 0.38 mM Mg^{2+}; at Mg^{2+} concentrations from 0 to 10 mM and 1.32 mM K^+; and at Ca^{2+} concentrations from 0 to 1 mM and 1.32 mM K^+. When carbon fixation was measured in the presence of the three-cation mixture, concentrations of K^+, Ca^{2+}, or Mg^{2+} were fixed at 1.32 mM, 0.67 mM, and 0.38 mM, respectively, and concentrations of the other cations were altered between 0 and 3 mM for K^+, 0 and 1 mM for Ca^{2+}, and 0 and 10 mM for Mg^{2+}. All cation solutions were used with chloride as the counterion.

Results

Estimation of the Paramecium cell volume

Cell volumes for *P. bursaria* F36 and *P. caudatum* TA2 were estimated to be $1.18 \cdot 10^5$ and $1.63 \cdot 10^5$ μm^3, respectively. To reproduce the cytoplasmic environment in these experiments, *P. bursaria* F36 and *P. caudatum* TA2 extracts

were prepared as concentrates, at a volume that was one-tenth of the total estimated cell volume used for the preparation, and diluted according to experimental objectives.

Characterization of the carbon fixation-enhancing factor in P. bursaria extracts

Carbon fixation by *C. variabilis* F36-ZK was enhanced in the presence of *P. bursaria* F36 extract (Fig. 1) as reported by Kamako and Imamura (2006). Heat stability of the carbon fixation-enhancing factor was examined, and the activity was entirely maintained even when the extract was autoclaved at 121 °C for 20 min (data not shown). Thus, the activity of an inorganic fraction of the *P. bursaria* F36 extract was examined. After the removal of organic compounds by combustion at 700 °C, the ash solution enhanced algal carbon fixation equally as well as the *P. bursaria* F36 extract (inorganic fraction in Fig. 1), indicating that the active principle is the inorganic material.

The concentrations of the major cellular cations, K^+, Ca^{2+}, and Mg^{2+}, were measured in tenfold diluted *P. bursaria* F36 extract to reproduce the cytoplasmic environment in the *Paramecium* cell, and found to be 1.32, 0.67, and 0.38 mM for K^+, Ca^{2+}, and Mg^{2+}, respectively.

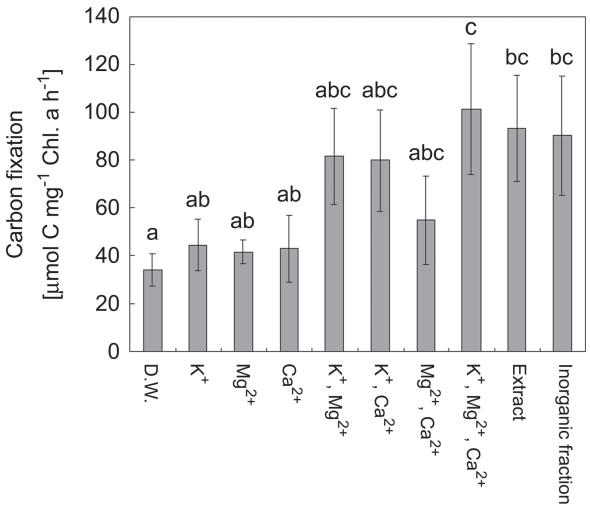

Fig. 1. Effect of inorganic compounds on carbon fixation by symbiotic *C. variabilis* F36-ZK. Carbon fixation was measured in the presence of 1.32 mM K^+, 0.38 mM Mg^{2+}, 0.67 mM Ca^{2+}, their mixture, and inorganic compounds in the *Paramecium* extract for 30 min. Bars indicate ±SD of three replicates. Data were statistically analysed based on one-way ANOVA followed by the Tukey test ($p < 0.05$), resulting in three significantly different groups designated as a, b, and c. Small letters above each bar indicate the data groups. D.W., distilled water.

Effect of cations on carbon fixation by symbiotic Chlorella

The effects of the cations K^+, Ca^{2+}, and Mg^{2+} on algal carbon fixation were evaluated (Fig. 1). In the presence of each cation alone, little enhancement of carbon fixation was observed; however, when a second cation was added, algal carbon fixation increased approximately twofold. Furthermore, carbon fixation was enhanced by the three-cation mixture to the same level as with the *P. bursaria* extract. With respect to excretion of fixed carbon, no effect of the three cations on excretion by *C. variabilis* F36-ZK was observed at pH 7.

The effect of various concentrations of one cation on carbon fixation was measured in the presence of another cation (Fig. 2). In 0.38 mM Mg^{2+}, carbon fixation increased with the concentration of K^+, and reached twice the level of the control at more than 0.78 mM (Fig. 2A). In 1.32 mM K^+ solution, carbon fixation was gradually enhanced along with increasing concentrations of the other divalent cation and reached a maximum at concentrations greater than 0.01 mM Mg^{2+} and 0.5 mM Ca^{2+}, as shown in Figs. 2B and C, respectively.

To clarify the details of the combinatorial effect of the three cations on carbon fixation, carbon fixation was measured under the following conditions: the concentrations of two cations were altered and the third cation was kept at the same level as in the *P. bursaria* F36 cytoplasmic environment (Fig. 3). Carbon fixation increased in the presence of two cations, and it could be further enhanced by the addition of the third cation. Higher amounts of fixed carbon were observed in the presence of more than 0.2 mM K^+, 0.2 mM Mg^{2+}, and 0.3 mM Ca^{2+}.

When C medium, which contains 0.99 mM K^+, 0.64 mM Ca^{2+}, and 0.16 mM Mg^{2+}, was used in the assay instead of sodium phosphate buffer, cells in the medium also showed higher carbon fixation activity [(122 ± 7.6)%, $n = 3$ compared to cells treated with *P. bursaria* extract].

Effect of cations and P. bursaria extract on carbon fixation by free-living Chlorella

To evaluate the effect of the three-cation mixture and *P. bursaria* extract on photosynthesis of free-living *C. vulgaris*, carbon fixation was meas-

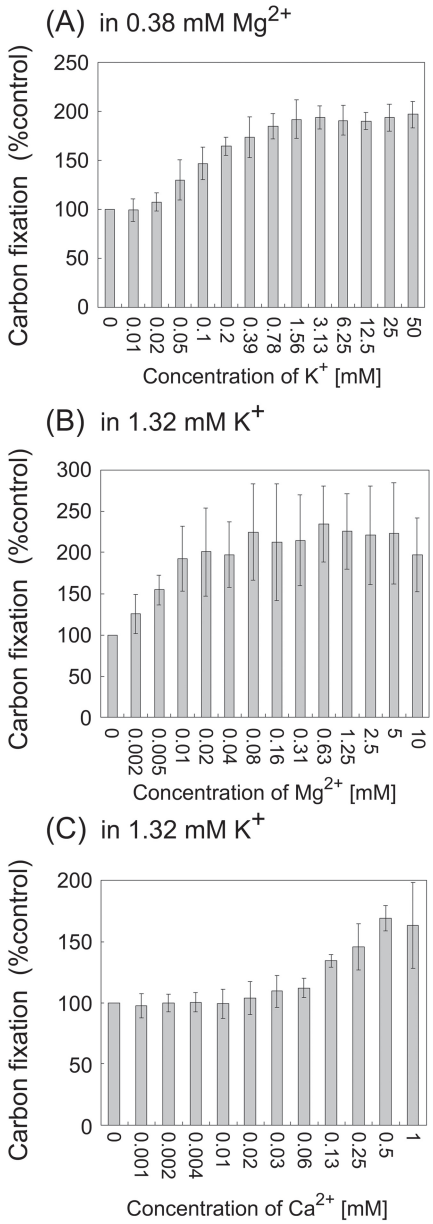

Fig. 2. Effect of monovalent and divalent cation mixtures on carbon fixation by symbiotic *C. variabilis* F36-ZK. Carbon fixation was measured for 30 min in the presence of K^+ and the divalent cations Ca^{2+} and Mg^{2+}. Various concentrations of the divalent cations Ca^{2+} or Mg^{2+} were added to the algae in phosphate buffer containing 1.32 mM K^+ to evaluate their effect (B, C). When the concentration of K^+ was varied, carbon fixation was performed in the presence of 0.38 mM Mg^{2+}. It was confirmed prior to the experiments that no change in the external pH value was caused by the addition of these cations to the mixture. Bars indicate ±SD of three replicates.

ured in the presence of the mixture and the extract (Fig. 4). Although carbon fixation by symbiotic *C. variabilis* F36-ZK was enhanced by the mixture (cations in Fig. 4A) and the extract (extract in Fig. 4A), there was no effect of these additives on carbon fixation by free-living *C. vulgaris* (Fig. 4B).

Concentration of cations in symbiotic and nonsymbiotic Paramecium extracts

Concentrations of the cations K^+, Ca^{2+}, and Mg^{2+} of the two *Paramecium* extracts adjusted to the same concentrations like inside the cells are listed in Table I. Concentrations of Ca^{2+} in these two solutions were similar, but the concentration of K^+ in the *P. caudatum* TA2 extract was lower than that of the symbiotic organism. Little Mg^{2+} was detected in *P. caudatum* TA2 extracts.

Discussion

Kamako and Imamura (2006) reported that the algal carbon fixation-enhancing factor in *P. bursaria* F36 extracts was a heat-stable and low-molecular-weight substance. Our findings revealed that inorganic compounds in the extract enhanced the carbon fixation. A mixture of three cations, K^+, Ca^{2+}, and Mg^{2+}, provided the same level of activity as the extract. Thus, the mixture of these cations was concluded to be the carbon fixation-enhancing factor in the *P. bursaria* F36 extract.

Each cation is known to be important for photosynthesis; for example, K^+ is contained in the chloroplast at approximately 100 mM (Demmig and Gimmler, 1983; Wu and Berkowitz,

Table I. Concentration of cations in symbiotic and nonsymbiotic *Paramecium* extracts.

Extract	Cation concentration [mM]					
	Symbiotic *P. bursaria* F36			Nonsymbiotic *P. caudatum* TA2		
	K^+	Ca^{2+}	Mg^{2+}	K^+	Ca^{2+}	Mg^{2+}
Lot 1	1.32	0.67	0.38	0.26	0.47	0.07
Lot 2	0.60	0.50	0.14	0.17	0.45	n.d.
Lot 3	0.79	0.47	0.10	0.17	0.36	0.03
Average	0.90	0.55	0.21	0.20	0.43	0.03
Ratio	4.3	2.6	1.0	6.0	12.8	1.0

The *Paramecium* extracts were dissolved with distilled water to produce an intracellular milieu of *Paramecium* cells as mentioned in Methods and Materials. n.d., not detected.

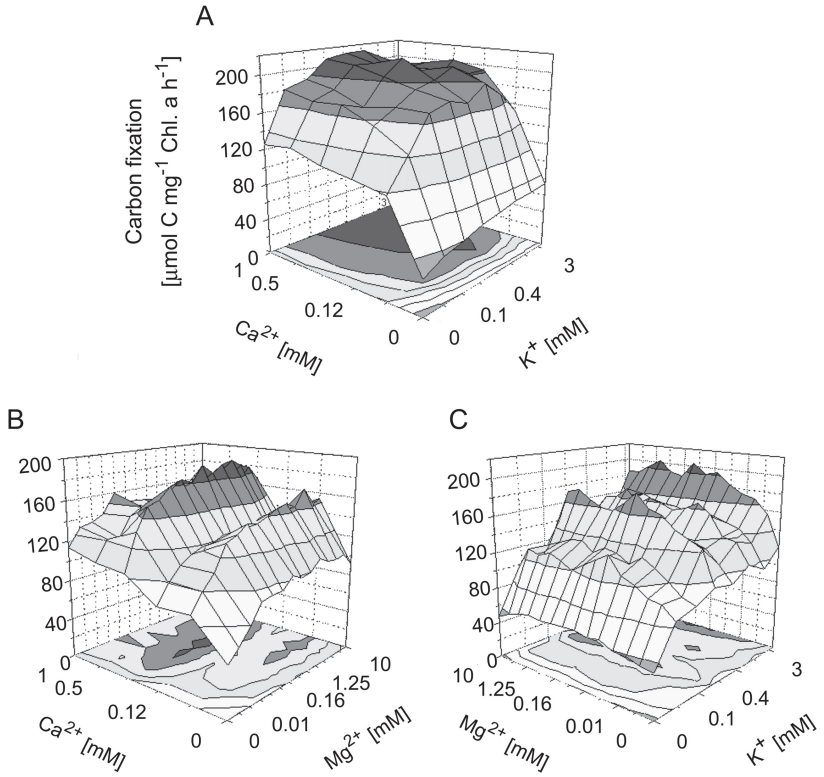

Fig. 3. Carbon fixation by symbiotic *C. variabilis* F36-ZK at various cation concentrations. (A) Modification of Ca^{2+} and K^+ concentrations in 0.38 mM Mg^{2+}. (B) Modification of Ca^{2+} and Mg^{2+} concentrations in 1.32 mM K^+. (C) Modification of K^+ and Mg^{2+} concentrations in 0.67 mM Ca^{2+}. Values are the averages of at least three replicates.

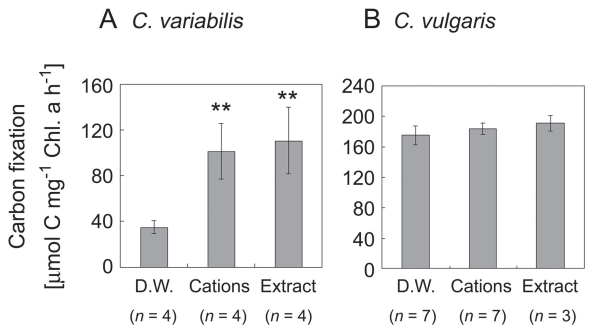

Fig. 4. Effect of the three-cation mixture and the *P. bursaria* extract on carbon fixation by *Chlorella* spp. Carbon fixation by (A) symbiotic *C. variabilis* F36-ZK and (B) free-living *C. vulgaris* NIES-227 was measured in the presence of the three-cation mixture or the *P. bursaria* extract. The mixture gave the final concentrations of 1.32 mM K^+, 0.38 mM Mg^{2+}, 0.67 mM Ca^{2+}, which are the same concentrations like those inside the cells of *P. bursaria* (see Table I). Bars indicate ±SD of multiple replicates. Double asterisks indicate a significant difference from control (D.W.) based on one-way ANOVA followed by Dunnett test ($p < 0.01$).

1992a) and contributes to the stability of the carbon fixation enzymes and regulation of stromal pH (Berkowitz and Wu, 1993). Activation of rubisco and fructose 1,6-bisphosphatase (FBPase) requires Mg^{2+} (Ishijima and Ohnishi, 2002), and the activity of chloroplastidic ATPase, which also requires Mg^{2+} for its function, increases dramatically in the presence of K^+ and Mg^{2+} (Wu and Berkowitz, 1992b). The calcium cation also seems to play a role in oxygen evolution (Yocum, 1991), although the effect of Ca^{2+} on photosynthesis remains unclear.

Carbon fixation by symbiotic *C. variabilis* F36-ZK was not increased by any of the cations separately, but was enhanced by a mixture of two of the three cations, and further enhancement was observed by the addition of the third cation. Therefore, enhancement of carbon fixation was considered to be due to multiple effects of the cations, and the maximum enhancement required all three cations, K^+, Ca^{2+}, and Mg^{2+}. Although

the three-cation mixture at the concentrations found in the *P. bursaria* F36 cytoplasm strongly enhanced carbon fixation, carbon fixation was enhanced more by other ratios of the three-cation mixture, indicating that carbon fixation by symbiotic *C. variabilis* F36-ZK can be controlled by host cellular concentrations of the three cations. Perhaps this property is a unique feature of Japanese symbiotic *C. variabilis* F36-ZK, since there was no effect of the cation mixture on carbon fixation in the case of free-living *C. vulgaris*.

A comparison of the concentrations of the three cations in *P. bursaria* F36 and *P. caudatum* TA2 extracts revealed lower concentrations of K^+ and Mg^{2+} in the *P. caudatum* TA2 extract. These differences could imply that intracellular conditions in *P. bursaria* F36 were better than those in *P. caudatum* TA2 for carbon fixation by symbiotic *C. variabilis* F36-ZK.

In this study, we demonstrated suitable intracellular cation conditions for algal carbon fixation; however, the ideal conditions are not exactly the same as observed in the cytoplasm of the host. In *P. bursaria* cells, each symbiotic *Chlorella* cell is enclosed in a lipid bilayer membrane to form a perialgal vacuole (Meier *et al.*, 1984; Kodama and Fujishima, 2005). Therefore, they are strictly separated from cytoplasm of the host. Taking the membrane into consideration, the most important condition for carbon fixation by the symbiont is the cation concentration in the aqueous milieu between the perialgal vacuolar inner membrane and the algal cell. The enhancing effect of the three cations was not maintained as the higher levels of carbon fixation disappeared once algal cells in culture medium (C medium plus Ser) were washed with sodium phosphate buffer, whilst cells in C medium, which contains K^+, Ca^{2+}, and Mg^{2+} at concentrations similar to the host cell, had a higher carbon fixation activity. Therefore, the possibility exists that the host can maintain the cation concentration, perhaps regulating the cation concentration in the perialgal vacuole for optimal algal photosynthesis, resulting in an efficient sugar supply from its symbiotic *Chlorella*. Future studies will investigate the cation concentration in the perialgal space, which has yet to be determined.

This is the first report concerning the host factor in a freshwater symbiotic organism. In marine organisms, organic molecules have been suggested to be the host factors (Gates *et al.*, 1995; Ritchie *et al.*, 1997). In contrast, we conclude that the inorganic materials, K^+, Ca^{2+}, and Mg^{2+}, act as host factors for Japanese *P. bursaria* F36. We do not know why marine organisms use organic molecules whilst *P. bursaria* uses inorganic ones. We will further investigate the mechanism involved in enhancement of carbon fixation by symbiotic *C. variabilis* F36-ZK in response to cations.

Acknowledgements

This study was supported by the Sumitomo Foundation (053377). We thank Prof. Haruki Shiraishi and Mr. Hiroki Hayashi of Ritsumeikan University, Japan, for their advice on the measurement of inorganic cations; Associate Prof. Kazuyoshi Takagi and his student, Mr. Hiroki Kawade, for their helpful advice on removal of organic compounds and chelate titration; and Dr. Hazel B. Gonzales, a lecturer at Ritsumeikan University, Japan, for checking our English.

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