Metabolic Control Between the Symbiotic *Chlorella* and the Host *Paramecium*

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Contents

Abstract Metabolic control, including the transfer of materials between a host and a symbiont, is important for understanding symbiotic relationships. However, sugars, mainly maltose, are the only confirmed class of material transferred from symbionts to *Paramecium bursaria*. An axenic Japanese *Chlorella* symbiont, which had been thought hard to isolate and maintain, was found to irreversibly adapt to its symbiotic milieu. Analysis of its features, such as the unique availability of nitrogenous compounds (e.g., amino acids) and its uncommon stimulation of carbon fixation by the host extract, revealed that three constitutional amino acid transport systems that can be controlled by Ca^{2+} and sugar are present, and that the carbon fixation

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ability of the symbiont depends on the extracellular cation concentration. These novel features of the Japanese symbiont imply metabolic control between the host and the symbiont.

1 Introduction

Cells of the green paramecium, *Paramecium bursaria*, contain hundreds of endosymbiotic *Chlorella* cells within perialgal vacuoles in the cytoplasm. *P. bursaria* can live under autotrophic conditions, e.g., in a medium for microalgae containing inorganic minerals and some vitamins but lacking carbohydrates and amino acids (Loefer 1936), clearly indicating that the protozoa and the green algae are mutually beneficial to each other; the protozoa can only obtain the organic compounds they need as an energy source from the symbiotic algae. In exchange for the algal photosynthetic products, the host provides the symbiont with several materials including minerals as nutrients. This exchange of substances probably requires the sharing of information to maintain homeostasis in *P. bursaria*.

Although the transfer of materials has been thoroughly investigated, the details of the transfer in host–symbiont relationships have not been elucidated. Different types of photosynthate, mainly maltose, have been reported as being released from cultured symbiotic algae (Brown and Nielsen 1974; Reisser 1976, 1986). Maltose produced by the symbiont is an essential energy supply for the host during symbiosis under autotrophic conditions. However, given the autotrophic growth habit of algae, determining which compounds are essential for their growth is more difficult.

If all classes of materials around the *Paramecium* cell could pass through freely to algal cells, it may seem that there is no requirement for the supply of materials by the host, but that is not the case. After carbon, nitrogen is the material transferred from the host to the symbiont in the largest amounts and thus has received major research interest. Studies focusing on the nitrogen sources of symbionts have revealed the differential availability of nitrate between the algal cells before and after isolation from the host, and the possibility of amino acid transfer, especially of L-glutamine, from the host to the symbiont (Albers et al. 1982). Plausible hypothetical control methods between the host and the symbiont during symbiosis have been proposed (Reisser 1988). However, the diversity of symbiont features found (Reisser and Widowski 1992) indicated that the relationship between the host and the symbiont may vary on a case-by-case basis, making it difficult to discuss in general terms.

Reisser et al. (1988) stated that "a typical symbiotic *Chlorella* strain common to all *P. bursaria* strains does not exist." Symbiotic *Chlorella* strains vary and each possesses characteristic features, such as differing availabilities of amino acids, nitrate, and ammonium. Previous studies on symbiotic *Chlorella* were carried out on strains that could be cultured in standard inorganic media. Even these cultivatable strains showed great diversity. Symbiotic *Chlorella* did not show any specific adaptation to the symbiotic milieu except for the pH-dependent release of photosynthate

(Reisser et al. 1988). Recently, phylogenetic analyses determined the different origins of European and American *P. bursaria* (see Hoshina and Imamura, this volume). Consequently, the differing host-symbiont relationships for the species resulted in a diverse range of symbiont features.

The Japanese symbionts of *P. bursaria* (F36-ZK), by way of their inability to utilize nitrate, were thought to be difficult to isolate and cultivate. The many novel features of F36-ZK suggest that it is an extreme example with adaptations to the symbiotic milieu, implying that its features should reflect the symbiotic surroundings. Thus, analyzing the features of F36-ZK could provide important information about the transfer of material and metabolic control between the host and the symbiont. In this chapter, we will first describe the novel features of the Japanese symbiotic *Chlorella*, which appears greatly dependent on the host, and we will then consider the metabolic control between the Japanese symbiont and its host.

2 Features of Symbiotic *Chlorella* Species

Symbionts are thought to be special *Chlorella* species or strains. For example, symbiont-free *P. bursaria* can distinguish a symbiotic *Chlorella* from a free-living one and can generally make a stable association (Reisser et al. 1982). This ability to be recognized is a novel feature of symbiotic *Chlorella*. In 1983, a virus that came to play an important role in *P. bursaria* studies was reported (Van Etten et al. 1983). This virus, known as chlorovirus, could infect cultured symbiotic *Chlorella* strains isolated from green hydra or *P. bursaria*, but not free-living *Chlorella* strains. In 1991, Reisser et al. reported that the virus could distinguish between *P. bursaria* collected in Europe and that collected in America. Since then, the geographical origin of *P. bursaria* has been important when considering the features of symbiotic *Chlorella* (Reisser and Widowski 1992). Recently, Hoshina et al. (2005) classified the European and American symbiotic *Chlorella* strains in different groups using phylogenetic studies based on ribosomal DNA. Thus, symbiotic *Chlorella* strains in *P. bursaria* around the world should not be considered identical.

2.1 European and American Symbiotic *Chlorella*

Before European and American strains of *Chlorella* were distinguished by chlorovirus, Reisser et al. (1988) compared the morphological and physiological features of symbiotic *Chlorella* strains isolated from European and American *P. bursaria* with those of free-living *Chlorella* strains from an ecological standpoint. They found that carbohydrate release of symbiotic *Chlorella* was higher at acidic pH than at neutral pH, whereas that of free-living *Chlorella* was not significantly dependent on pH. Furthermore, many physiological and cytochemical features were different

in individual strains, regardless of whether they were symbiotic or free-living. Because these symbiotic *Chlorella* strains did not show common properties, no underlying features could be found to account for the differences between symbiotic and free-living *Chlorella*. Most free-living *Chlorella*, including the European and American symbionts tested, can utilize nitrate (Reisser and Widowski 1992). This nitrate-utilization ability was a characteristic common to symbiotic and free-living *Chlorella* because the symbiont was isolated on agar plates containing nitrate as the sole nitrogen source. However, these observations were only applicable to cultivable symbiotic *Chlorella* strains.

2.2 Japanese Symbiotic *Chlorella*

In contrast to many studies using European and American symbionts, few studies have been conducted on the symbiotic algae in Japanese *P. bursaria*. Takeda (1995) studied six symbiotic strains of Japanese *P. bursaria.* On the basis of cell wall composition comparisons, all the Japanese symbionts appeared identical to each other, but were different from German symbionts. The Japanese symbionts were isolated as a homogeneous colony on an agar plate using nitrate as the sole nitrogen source. Nishihara et al. (1998) reported that the Japanese symbionts always coexisted with bacteria; therefore, no bacteria-free Japanese symbionts could be isolated, regardless of the type of culture medium used. However, Nakahara et al. (2003) succeeded in culturing Japanese symbionts under axenic conditions using a medium containing ammonium nitrate (NH_4NO_3) as the sole nitrogen source, although the strains could not be kept for more than a few months.

Symbiotic algae previously associated with *P. bursaria* were isolated and maintained on an inorganic medium using nitrate as the sole nitrogen source (Reisser 1984). The Japanese symbionts were thought to possess characteristic properties that were distinguishable by nitrogen utilization from the cultivatable strains reported. To investigate the Japanese symbionts, axenic strains were established (Kamako et al. 2005). Isolation efforts were first carried out on an agar plate using inorganic nitrogen sources for algal autotrophic growth (C medium: Ichimura 1971). After incubation for several weeks, clones on the initial agar plate were transferred to a flesh agar plate; all colonies were contaminated with bacteria, which varied by colony. This indicated that there was no species-specific relationship between the symbiont and the contaminating bacterial strain; colonies on the agar plate, visible to the naked eye, were not axenic. Agar plates were observed under a microscope after a short period of incubation and small colonies that appeared to be free of bacteria were marked. After further incubation for several weeks, xenic colonies were observed to grow well and became visible to the naked eye, but the marked colonies remained almost the same size (80–100-μm diameter) as when initially measured. After cultures on several media had been attempted, the axenic Japanese symbiotic strain was found to grow on agar containing modified Bold's basal medium (MBBM) (Nichols and Bold 1965). Once the axenicity of the algal strains isolated from three Japanese *P. bursaria* strains had been confirmed,

phylogenetic studies based on 18S ribosomal DNA analyses revealed that all the Japanese symbiotic *Chlorella* strains were identical. The most closely related free-living species was considered to be *C. vulgaris*. The Japanese symbionts were classified into an American symbiotic *Chlorella* group; virus sensitivity also supported this classification.

The characteristic constituents of MBBM, Bacto peptone and sucrose, were examined separately to determine which components were required for algal growth; Bacto peptone was found to be essential, while sucrose was not (Kamako et al. 2005). Axenic symbionts showed some growth on C medium owing to organic nitrogen impurities in the agar, which were detected using the ninhydrin reagent. However, xenic clones growing on the medium were visible with the naked eye, perhaps supported by organic nitrogen compounds produced from nitrate by coexisting bacteria. Therefore, for the Japanese symbiotic strains, the availability of nitrate seemed to depend on whether the strain was axenic or not.

The utilization of saccharides by Japanese symbionts was also examined, because knowledge of saccharide utilization was poor. Results of algal growth in media containing every respective sugar showed that the monosaccharides glucose and fructose seemed to be available, while the disaccharides sucrose and maltose were not (Kamako et al. 2005). However, recent experiments using radioactive tracers revealed that these saccharides could not be imported in symbiotic algal cells (Kato and Imamura 2008b). Thus, the growth-stimulating effect of glucose and fructose was not caused by nutrition, and the effect of glucose will be discussed later.

2.3 Nitrogen Utilization of Symbiotic *Chlorella*

One of the characteristic features of Japanese symbiotic algae is nitrogen utilization. Preliminary nitrogen-utilization experiments were performed using Bacto peptone, casamino acids, ammonium (NH_4^+), nitrate (NO_3^-), and nitrite (NO_2^-) as the respective sole nitrogen sources. The symbiotic algal strains, American NC64A obtained from the American Type Culture Collection (ATCC) and Japanese F36-ZK, showed similar trends in nitrogen utilization. Both grew most rapidly in the medium containing casamino acids, more slowly with ammonium, and poorly with Bacto peptone, but neither grew with nitrate or nitrite. The nitrogenous compounds in Bacto peptone are mainly oligopeptides with small amounts of free amino acids, while those in casamino acids are free amino acids. Because the nitrogen content was adjusted to equivalent amounts in these experiments, these results indicate that amino acids were more effective for F36-ZK growth than ammonium (Kamako et al. 2005). Kessler and Huss (1990) reported that all four European *Chlorella* symbiotic strains tested were able to use nitrate, while only three could utilize nitrite. Reisser and Widowski (1992) also stated that most *Chlorella* symbionts in European and American *P. bursaria* use nitrate as a nitrogen source. The American symbiotic strain NC64A was reported to grow in a medium containing nitrate as a sole nitrogen source (Reisser et al. 1988), although the NC64A strain from the ATCC

could not use nitrate in a recent experiment (Kamako et al. 2005). The growth of the free-living strains *C. kessleri* and *C. vulgaris* was also examined using Bacto peptone, casamino acids, ammonium, nitrate, and nitrite as the sole nitrogen sources. *C. vulgaris* grew well in media containing inorganic nitrogen compounds and casamino acids, but not with Bacto peptone, whereas *C. kessleri* grew well in all media. The utilization of nitrogenous compounds by *Chlorella* strains should depend on the assimilation capacity of inorganic nitrogen and on the transport and metabolism of organic nitrogen. The latter were investigated to better understand the features of nitrogen utilization by F36-ZK.

2.3.1 Nitrate and Nitrite Assimilation

Nitrate reductase (NR) and nitrite reductase (NiR) catalyze the first steps of nitrogen assimilation by reducing nitrate to ammonium. Both enzymes can be activated by inducers such as nitrate and glucose. The activities of these enzymes in NC64A and F36-ZK cells were measured under conditions of enzymatic induction. NC64A exhibited immediate NR and NiR activity with nitrate induction, although the activities of the symbiont remained low over 48 h. The NR activity of NC64A was not sufficient to allow survival. On the other hand, F36-ZK did not produce NR under any experimental conditions, but NiR activity was observed with nitrate induction as in free-living strain cells. The difference in response to nitrate induction between F36-ZK and NC64A indicated important differences related to NR expression. The distinct properties of NR and NiR activities of the two symbionts resembled those of two spinach mutants, an NR regulatory gene mutant and an NR structural gene mutant (Ogawa et al. 1994). The regulatory gene mutant showed low NR and NiR activities with nitrate induction, whereas the structural mutant exhibited undetectable NR and potent NiR activities, because NR messenger RNA was considered to play a role as a signal for NiR gene expression although the messenger RNA could not be correctly translated to NR. These similarities suggest that F36-ZK may have a mutation in the NR gene and that NC64A may be a regulatory gene mutant. Symbiotic algae do not need NR in the host cell because *P. bursaria* cannot import nitrate (Alberts et al. 1982); hence, the lack of NR in F36-ZK seems to be an irreversible adaptation to the symbiotic milieu.

2.3.2 Amino Acid Utilization

The preliminary results of the experiment described in the previous sections clearly indicated that F36-ZK could use amino acids. To confirm which amino acids can be utilized, the growth of the Japanese symbiont F36-ZK was measured in C medium supplemented with 20 individual amino acids (Kato et al. 2006). The six amino acids L-arginine, L-asparagine, L-glutamine, L-serine, L-alanine, and glycine allowed healthy and remarkable growth of F36-ZK, as summarized in Table 1. The total concentration of these amino acids in Bacto™ casamino

Table 1 Amino acid utilization and uptake of *Chlorella* spp. (Modified from Kato et al. 2006)

		Doubling time (days)[a]	Rate of uptake ($\times 10^{-8}$nmol min^{-1}cell^{-1})[b]	
Amino acid		F36-ZK	F36-ZK	C. vulgaris NIES-227
Basic	Arg	1.9	3.30	1.34
	Lys	NG	0.64	ND
	His	NG	2.46	ND
Acidic	Asp	NG	0.14	ND
	Glu	NG	2.08	ND
Polar	Asn	3.0	0.42	ND
	Gln	4.7	6.92	ND
	Ser	2.9	2.28	ND
	Thr	NG	3.16	ND
	Tyr	NG	1.08	ND
Nonpolar	Ala	2.9	2.86	ND
	Val	NG	2.52	ND
	Leu	NG	3.26	ND
	Ile	NG	2.34	ND
	Pro	NG	3.46	ND
	Phe	NG	3.22	ND
	Met	NG	2.32	ND
	Trp	NG	3.16	ND
	Gly	3.4	3.20	ND
	Cys	NG	5.62	ND

NG no growth, ND not detectable (i.e., counts per minute values at 1 and 2 min almost did not change)

[a]Endosymbiotic algae were cultured in C medium added with amino acid (200 μg ml^{-1}). The culture conditions were 25°C, 30 μmol photons^{-1}m^{-2} s^{-1}, light-dark 16 h-8 h. Doubling times were calculated from a period of 3–6 days. Data variations less than 2.0%, $n = 2$.

[b]1×10^8 cells ml^{-1} algae were incubated under the condition of 25°C, 90 μmol photons m^{-2} s^{-1}. Uptake rates were determined by filtering 100-μl samples at 1 and 2 min after the addition of 1 mM [^{14}C]amino acid (specific activity 2–4 (μCi /(μmol(1). Data variations less than < 9.5%, n = 2. n.d.: not detectable, i.e., cpm values at 1 and 2 min didn't almost change.

acids is less than 20% (BD Technical Center 2003). No L-glutamine or L-asparagine was present in the media because they were converted to L-glutamic acid and L-aspartic acid, respectively, by acidic hydrolysis. All six amino acids are candidate nitrogen sources for symbionts from the host. Ammonium is an additional candidate, although its efficiency for the growth of F36-ZK should be quite low considering the available amino acid content of casamino acids. The use of three dipeptides containing the utilizable amino acids glycine and L-arginine, namely, glycylglycine, glycyltyrosine, and arginylarginine, was also examined. As expected from the results of F36-ZK growth with Bacto peptone, no algal growth was observed.

Albers et al. (1982) speculated that L-glutamine and ammonium were the key nitrogen compounds transported from the paramecium host to its symbionts. L-Glutamine was then thought to be the most important candidate (Reisser and Widowski 1992). In many reports, only a few amino acids such as L-glutamine and L-glutamic acid were used for symbiotic algal growth experiments. McAuley (1986) studied the relationship between amino acid utilization and uptake of the American symbionts 3N813A and NC64A.

He used eight amino acids, L-arginine, L-glutamine, L-proline, L-serine, L-alanine, glycine, L-lysine, and L-glutamic acid, and reported that NC64A used L-arginine and L-glutamine and imported L-arginine and L-lysine. L-Glutamine stimulated the growth of NC64A (although the cells were described as pale and shrunken), but it was not imported by the organism. In contrast to NC64A, 3N813A imported all amino acids except L-glutamic acid and used every amino acid except L-glutamic acid and L-lysine. From these results, McAuley (1986) concluded that these algal amino acid utilizations could be correlated with their amino acid uptakeabilities.

3 Amino Acid Transport of Symbiotic *Chlorella* and Its Regulation Factors

Most free-living *Chlorella* are known to show little amino acid uptake under usual growth conditions, but amino acid uptake can be induced in some cases. For example, glucose induced the amino acid uptake systems of *C. kessleri* for basic and neutral amino acids (Cho et al. 1981). Studies on amino acid transport systems of symbiotic *Chlorella* have not been performed. The major characteristic features of the Japanese symbiont are its developed amino acid transport systems and novel regulation with some materials, as described next.

3.1 Amino Acid Uptake by Symbiotic *Chlorella*

Amino acid uptake was examined with radioactive tracers (Kato et al. 2006). The results of amino acid uptake by F36-ZK and free-living *C. vulgaris* NIES-227 are displayed in Table 1. Surprisingly, F36-ZK imported all amino acids at adequate rates without any induction, but *C. vulgaris* imported only L-arginine. Contrary to McAuley's aforementioned conclusion regarding American symbiotic strains, only six amino acids supported the growth of F36-ZK among the 20 amino acids imported. Some amino acids with no growth-stimulating effects, such as L-leucine, L-proline, and L-cysteine, are imported more rapidly than growth-stimulating ones such as L-alanine, for instance. These results clearly indicate that the six growth-stimulating amino acids are used as nitrogen sources via the intracellular metabolic pathway. These six amino acids are thought to generate ammonium ions; L-arginine, L-glutamine, and L-asparagine have a nitrogen-containing group in their side chain, which easily releases nitrogen during metabolism. L-Alanine and L-serine can be converted to glycine, two molecules of which generate each molecule of ammonium, carbon dioxide, and L-serine in photorespiration processes. Compared with amino acids, extracellular ammonium is not a good source of nitrogen, but intracellular ammonium could play an important role as a nitrogen source.

The activities of enzymes responsible for ammonium assimilation in F36-ZK were also measured, but clear results were not obtained. When cells were grown under weak light intensity (50 μmol photons s^{-1} m^{-2}) in media containing ammonium

as the sole nitrogen source, the total activity of glutamine synthetase (GS), a key enzyme in ammonium assimilation, in F36-ZK cells was either not detectable (Kato et al. 2006) or low compared with that of *C. vulgaris* and NC64A. However, GS activity in F36-ZK cells grown under strong light conditions (150 μmol photons s^{-1} m^{-2}) was similar to that observed in *C. vulgaris* and NC64A. Among these algae cultured in media containing ammonium, F36-ZK was the most sensitive organism to the GS inhibitor, methionine sulfoximine (MSX). MSX is a chemical analogue of L-glutamic acid and L-glutamine, and could therefore be imported by F36-ZK but not by other algal strains. Because MSX showed lethal effects against F36-ZK in media containing L-serine or L-arginine as a nitrogen source, it is very likely that GS plays a role in ammonium assimilation from L-serine or L-arginine.

3.2 Amino Acid Transport Systems

F36-ZK transported more amino acids than the American *Paramecium* symbionts (McAuley 1986, 1989), whereas free-living *C. vulgaris* transported only one amino acid, L-arginine (Kato et al. 2006). These differences suggest that the amino acid transport systems in symbiotic *Chlorella* F36-ZK evolved over time.

3.2.1 Amino Acid Transport Systems in F36-ZK

To analyze the amino acid transport systems, the uptake of all amino acids was performed using radioactive tracer methods by kinetic analyses (Kato and Imamura 2009). From the K_m values, L-arginine and L-tyrosine showed higher affinity, whereas L-glutamine, L-histidine, L-asparagine, and acidic amino acids showed lower affinity to their transporters. For all amino acids, except L-glutamine, V_{max} ranged from 1.02 to 31.4×10^{-8} nmol min^{-1} $cell^{-1}$. L-Glutamine showed the highest V_{max}, 250×10^{-8} nmol min^{-1} $cell^{-1}$, although the affinity of L-glutamine for its transporter was low. L-Lysine and L-alanine were transported by at least two systems because the kinetic data for their uptake on a Lineweaver–Burk plot were biphasic in nature. The other 18 amino acids had single K_m values, indicating transport by one system.

To sort amino acids into their respective transport systems, competitive experiments were carried out. In other words, the uptake of a radioactive amino acid was measured in the presence of another unlabeled amino acid. The uptake of L-lysine was strongly inhibited by the addition of L-arginine, indicating that these amino acids were transported via the same basic amino acid transport system. The uptake of most amino acids, including L-lysine, L-histidine, L-aspartate, and L-glutamic acid, was inhibited by the addition of neutral amino acids. Therefore, the existence of a common broad transport system that transports 19 amino acids but not L-arginine was revealed and is referred to as a general amino acid transport system. In addition, a Lineweaver–Burk plot suggested the existence of an alanine transport system. Thus, kinetic analyses and competitive experiments disclosed three amino

acid transport systems in F36-ZK, a basic amino acid transport system for L-arginine and L-lysine, a general amino acid transport system for 19 amino acids, and a separate L-alanine transport system.

3.2.2 Features of Amino Acid Transport Systems

Studies focused on amino acid transport systems of microalgae have been scarce; the uptake of several amino acids by F36-ZK was measured in the pH range of 4.0–8.0 to obtain general information on these amino acid transport systems. Optimal uptake of all neutral amino acids tested, such as L-serine, L-alanine, and L-glutamine, was observed near pH 5.0; L-arginine uptake showed a broad optimal pH range between pH 5.0 and 6.5. A slight positive charge seemed to be required for amino acid transport, because the optimal pH was slightly more acidic than the isoelectric points. These results support an amino acid–proton symport mechanism which has been reported in many organisms (Bush 1993; Cho and Komor 1983; Young et al. 2003). In the case of L-aspartic acid and L-glutamic acid, their uptake was very sensitive to external pH, i.e., uptake increased when the pH became acidic and little uptake was measured above pH 6. Previous studies (Albers et al. 1982; Kato et al. 2006; McAuley 1986) have reported that symbiotic *Chlorella* could not utilize L-glutamic acid; however, since those experiments were carried out in a medium with a pH above 6.3, and L-glutamic acid uptake showed strict pH dependence as mentioned above, the utilization of L-glutamic acid by symbiotic *Chlorella* should be reviewed (Kato and Imamura 2009).

To assess whether the transport system in F36-ZK is active or passive, intracellular and extracellular concentrations of L-arginine (for the basic amino acid transport system), L-serine (for the general amino acid transport system), and L-alanine (for the alanine transport system) were measured. Intracellular concentrations of all amino acids were higher than extracellular ones even at 1 min after the addition of tracers. The differences between the intracellular and extracellular amino acid concentrations increased after 30 min of incubation, indicating that the transport systems were active. All amino acid transport systems in F36-ZK were considered to be active transporters carrying out amino acid–proton symport, on the basis of the following results: (1) a decrease in external proton concentration was observed after the addition of L-serine and L-alanine; (2) a protonophore (carbonyl cyanide *m*-chlorophenyl hydrazone) and an inhibitor of ATP synthesis (sodium azide) strongly inhibited amino acid uptake, while an inhibitor of phosphorylation (vanadate) also inhibited amino acid uptake. Amino acid–proton symport systems were also supported by the above-mentioned pH-dependent amino acid uptake.

In the case of microbes, the uptake of amino acids was reportedly inhibited by ammonium ions (Grenson et al. 1970; Willis et al. 1975). Thus, the uptake of several amino acids by F36-ZK was measured in the presence of nitrate and ammonium. Nitrogen ions from inorganic sources did not affect the uptake of L-arginine, L-serine, or L-glutamine. Furthermore, to examine the effect of cultivation with ammonium, the amino acid uptake of F36-ZK was measured using cells grown with ammonium as the sole nitrogen source. The cells transported all 20 amino acids

and, thus, all of the transport systems were expressed under culturing conditions with ammonium. Overall, the expression of the amino acid transport systems was independent of the nitrogen source, i.e., all transport systems are constitutive systems and represent one of the specific features of F36-ZK.

Many microalgae grown photoautotrophically import a few amino acids but possess interesting inducible amino acid transport systems (Cho and Komor 1985; Kato et al. 2006; Kirk and Kirk 1978). For example, amino acid transport systems in free-living *C. kessleri* can be induced by glucose (Cho et al. 1981). The induced general system of *C. kessleri* exhibited broad specificity for ten amino acids (Sauer 1984). In the case of *C. vulgaris*, sugars or glycine induce an amino acid transport system for four neutral amino acids (Plakunov et al. 1995; Seifullina et al. 1995). Therefore, free-living *Chlorella* can build various amino acid transport systems that are usually repressed under photoautotrophic conditions.

3.3 Factors Affecting Amino Acid Transport

Although environmental conditions under which the symbiont is living are a very important consideration for *P. bursaria* symbiosis, the underlying mechanistic details are unknown. Examples of extracellular conditions affecting the amino acid uptake of fungal and plant cells have been reported (Cameron and Lejohn 1972; Harrington et al. 1981; Rickauer and Tanner 1986; Smith 1978). For instance, the divalent cations Ca^{2+} and Mg^{2+} have been found to activate amino acid uptake. The mechanism of activation by such cations is still unclear, although several hypotheses have been proposed. The first suggests that divalent cations create a proton motive force for amino acid transport in tobacco cells (Smith 1978); another implicates Ca^{2+} as the activator of the p-type ATPase of *Neurospora crassa* (Lew 1989), and lastly Ca^{2+} was shown to affect the permeability of the cell membrane in *Phaseolus vulgaris* (Rickauer and Tanner 1986). Whatever the mode of activation, divalent cations stimulate amino acid uptake. Although no reports deal with cation transport in the symbiosis of *Paramecium*, the host must supply cations to its symbionts because *Chlorella* requires divalent cations for survival. Studies on amino acid uptake have found that the Ca^{2+} concentration in test cell suspensions affects amino acid uptake (Kato and Imamura 2008a). The novel effects of cations and glucose on amino acid uptake of F36-ZK will be described further.

3.3.1 Effects of Divalent Cations on Amino Acid TransportSystems in F36-ZK

Divalent cations such as Ca^{2+} and Mg^{2+} generally activate amino acid uptake as previously mentioned, but there are no specific reports of these effects in *Chlorella*. The effects of divalent cations on amino acid uptake in free-living *C. vulgaris* and in the Japanese symbiont F36-ZK are displayed in Fig. 1. The uptake of L-arginine by *C. vulgaris* was enhanced by Ca^{2+} and Mg^{2+} (Fig. 1a). However, the uptake of L-serine by the symbiont F36-ZK was decreased by the cations, especially by Ca^{2+}

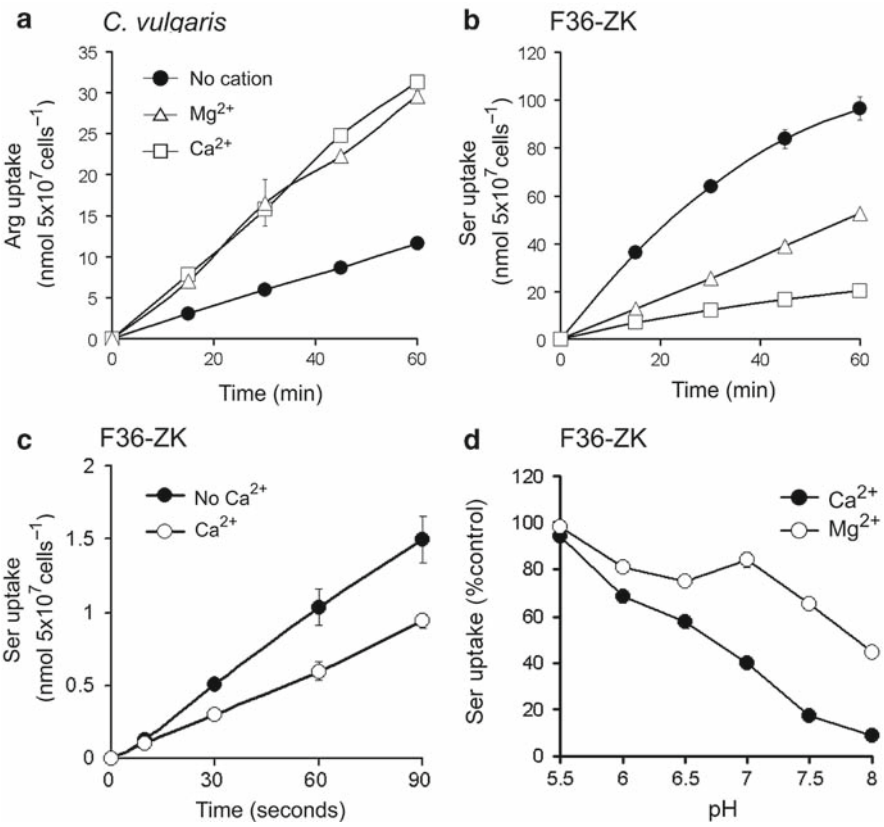

Fig. 1 Uptake of amino acids by *Chlorella* in the presence of divalent cations (Kato and Imamura 2008a). **a** Arginine uptake by free-living *Chlorella vulgaris* and **b** serine uptake by the *Paramecium* symbiont F36-ZK in the presence of 0.64 mM Ca^{2+} (*squares*), 0.64 mM Mg^{2+} (*triangles*), or no cations (*circles*) at amino acid concentrations of 1 mM. Algae were treated with these cations for 15 min prior to the experiments. *Bars* represent the mean ± the standard deviation (SD) of three (F36-ZK) or four (*C. vulgaris*) replicates. **c** Uptake of serine by the *Paramecium* symbiont F36-ZK in the presence (*open circles*) or absence (*filled circles*) of 1 mM Ca^{2+}. Serine was supplied at a concentration of 0.1 mM. *Bars* represent the mean ± SD of three replicates. **d** Uptake of serine by the *Paramecium* symbiont in relation to pH. Cells were pretreated with 1 mM Ca^{2+} (*filled circles*) or 4 mM Mg^{2+} (*open circles*). Serine was supplied at a concentration of 1 mM. Each point is the mean of three experiments

(Fig. 1b). When L-serine and Ca^{2+} were added at the same time, a rapid decrease in L-serine uptake was observed within 30 s (Fig. 1c); the rate of L-serine uptake decreased with a preincubation time lasting up to 30 min. The uptake of L-serine with Ca^{2+} or Mg^{2+} treatment was decreased from pH 5.5 to 8.0 (Fig. 1d).

The phenomenon of divalent cations decreasing amino acid uptake is novel and is one of the features of F36-ZK. To characterize this phenomenon in detail, a dose–response relationship between divalent cation concentrations and decreased

L-serine uptake was measured. The half-maximal effective concentration (EC_{50}) of Ca^{2+} was 0.21 mM and the minimum L-serine uptake, which was less than 10% in nontreated F36-ZK, was observed at a Ca^{2+} concentration greater than 1 mM. In the case of Mg^{2+} the minimum L-serine uptake, which was approximately 40% in nontreated F36-ZK, occurred at a Mg^{2+} concentration greater than 5 mM. The effects of monovalent and polyvalent cations on serine uptake by F36-ZK were also examined. Monovalent cations, such as Li^{+}, Na^{+}, and K^{+}, did not significantly affect serine uptake; divalent cations, such as Ni^{2+}, Mn^{2+}, and La^{3+} (a calcium ion analogue), also decreased serine uptake. Ca^{2+} was the strongest inhibitor among the cations tested.

F36-ZK has three amino acid transport systems. To evaluate the effect of Ca^{2+} on the transporters, the uptake rates of L-arginine, L-serine, L-alanine, and L-glutamine were measured after Ca^{2+} treatment. Little inhibition was observed for L-arginine uptake, but the uptake of L-serine, L-alanine, and L-glutamine was decreased to be less than 20% of that for nontreated F36-ZK. Ca^{2+} inhibited the uptake via the general amino acid transport system, but not via the basic amino acid transport system (Kato and Imamura 2009). The alanine transport system also appeared inhibited by Ca^{2+} because the transport system should function at the concentration of L-alanine used in the experiment. The effects of Ca^{2+} on the uptake of L-serine were also monitored by kinetic analysis. No effect on the K_m value was observed, but the V_{max} value increased about twofold. These results indicate that the noncompetitive inhibition of serine uptake via the general amino acid transport system was Ca^{2+}-dependent.

Calcium ions are essential for survival in living organisms. To analyze the mechanism of the above-mentioned process, Ca^{2+} uptake of F36-ZK was measured using radioactive $^{45}Ca^{2+}$. The symbiont F36-ZK imported Ca^{2+} with no observed change in external pH during uptake, indicating that Ca^{2+} uptake occurred without a proton motive force. Free-living *C. vulgaris* also showed similar results. To identify the major membrane transport protein for Ca^{2+} uptake in F36-ZK, experiments were performed using calcium channel blockers and a Ca^{2+}-ATPase inhibitor. Calcium channel blockers, especially the hydrophobic compounds verapamil and diltiazem, inhibited F36-ZK's Ca^{2+} uptake, but the Ca^{2+}-ATPase inhibitor erythrosine B did not. Although F36-ZK imported Ca^{2+}, it was not clear whether the intracellular Ca^{2+} contributed to the inhibition of L-serine uptake. To determine this, extracellular Ca^{2+} was removed using ethylene glycol bis(2-aminoethyl ether)tetraacetic acid (EGTA), a Ca^{2+} chelator, and the inhibition of amino acid uptake by Ca^{2+} was recorded. The uptake of L-serine that was inhibited by Ca^{2+} was restored by the addition of EGTA. In contrast, the uptake of L-arginine in *C. vulgaris* was accelerated by Ca^{2+} in the presence of EGTA. Therefore, extracellular Ca^{2+} inhibited the uptake of L-serine in F36-ZK and intracellular Ca^{2+} accelerated the uptake of L-arginine in *C. vulgaris*.

The detailed mechanism behind these observations remains unclear. The activity of an H^{+}-ATPase, an energy source provider for amino acid transport in general (Bush 1993; Cho and Komor 1983; Young et al. 2003), and the membrane permeability of amino acids were changed by Ca^{2+} in other organisms. However, there was no effect of Ca^{2+} on the uptake of L-arginine by F36-ZK, indicating that the inhibition of L-serine uptake was not due to a decrease in H^{+}-ATPase activity (Kato and Imamura 2009). The possibility that Ca^{2+}affects membrane permeability should be neglected

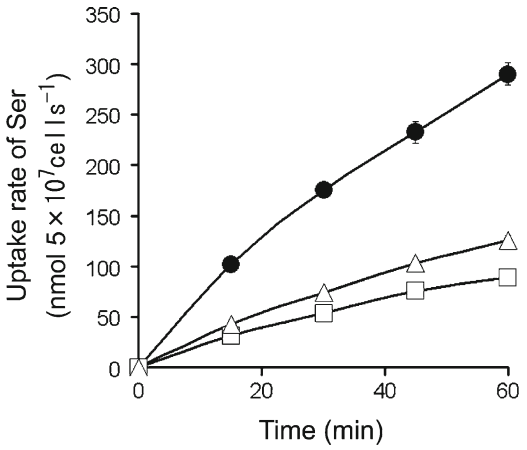

Fig. 2 Uptake of serine by an general amino acid transporter induced with glucose in *C. kessleri* in the presence of Ca^{2+}. Serine uptake by *C. kessleri* in the presence of 0.64 mM Ca^{2+} (*squares*), 0.64 mM Mg^{2+} (*triangles*), and no added cation (*circles*) at a concentration of 1 mM serine. Algae were pretreated with 13 mM glucose for 3 h

because Ca^{2+}exhibited opposing effects on membrane permeability, and indeed, efflux of intracellular L serine by $Ca^{2+}$was not observed.

As stated above, the amino acid transporters of free-living *C. kessleri* could be induced by glucose and the induced amino acid transport system could import L-serine in the manner of general amino acid transport of F36-ZK (Sauer 1984). The general amino acid transport system of F36-ZK is inhibited by Ca^{2+}, and it would be interesting to determine whether the transport system induced in *C. kessleri* can be inhibited by the cation. It should be noted that Ca^{2+} and Mg^{2+}inhibited the induced amino acid transport system in *C. kessleri*, as shown in Fig. 2 (Kato and Imamura 2008a). Thus, free-living *C. kessleri* have an amino acid transport system similar to that of F36-ZK, but it is not expressed under usual conditions.

3.3.2 Effects of Glucose on Amino Acid Transport Systems in F36-ZK

In a symbiotic *P. bursaria* cell, the transfer of sugars and amino acids occurs in opposite directions, just like a barter transaction between the symbiotic alga and the host. Sugars are a common nutrient and can also act as signaling substances in many organisms: sugar sensing and signaling pathways have been found in yeasts, plants, and mammals (Rolland et al. 2001). In free-living *C. kessleri* glucose and its analogues cause induction of a hexose and of the amino acid transport system (Cho et al. 1981; Tanner 1969). This suggests that green algae also have a sugar sensing and signaling system. Symbiotic *Chlorella* cells release sugars (Brown and Nielsen 1974; Pardy et al. 1989; Reisser and Windowski 1992) and each *Chlorella* cell is enclosed in a vacuolar membrane in the host cell (Meier et al. 1984). Therefore, sugars should exist around the algae, implying that they import amino acids in the presence of sugar in

their host. Thus, the effect of sugars on algal amino acid uptake was studied. Glucose is the typical sugar known to play a signal role and is the first metabolite of maltose in *P. bursaria*, which is produced and supplied by the symbiont *Chlorella*. Hence, studies were first performed using glucose (Kato and Imamura 2008b).

The rate of L-serine uptake by F36-ZK increased in the presence of glucose. A pulse treatment with glucose for 1 min also increased the rate to nearly the same level as the treatment for 30 min. L-Serine uptake increased with increasing glucose concentration and reached a maximum value approximately double that observed without glucose. The EC_{50} was 3 μM, assuming that the effect observed with 27.8 mM glucose was maximal, although glucose treatment in the range 0.05–27.8 mM showed almost the same effect. The rate of uptake of cells treated with glucose for 1 h was 3.06×10^{-8} nmol min^{-1} $cell^{-1}$, that of cells treated with glucose for 1 h and then left standing for 1 h without glucose was 2.40×10^{-8}nmol min^{-1} $cell^{-1}$, and that of untreated control cells was 1.40×10^{-8} nmol min^{-1} $cell^{-1}$. Although some reduction in L-serine uptake was observed after the removal of glucose, the higher L-serine uptake rate was sustained for at least 1 h after cells had been treated with glucose. The effect showed pH dependence; L-serine uptake increased sixfold at pH 8.0 and an external acidic pH caused a decrease in the effect. At pH 5.5, no change was observed in L-serine uptake relative to that of cells without glucose treatment. This observation is in contrast to the pH dependence of L-serine uptake, which was optimal near pH 5.0. The transport of L-serine was more affected by $Ca^{2+}$and glucose under alkaline than acid conditions; both were pH-dependent. The uptake of other amino acids was measured using cells treated with glucose. The general amino acid transport system proved more sensitive than the basic amino acid transport system. Kinetic analysis of L-serine uptake with glucose treatment was performed. The V_{max} value of L-serine uptake was doubled by treatment with glucose, but no change occurred in the K_m value, suggesting that glucose treatment produced an increase in the amount of the transporter. However, cycloheximide, an inhibitor of protein synthesis, had no effect on accelerated L-serine uptake by glucose, indicating that post-translational modification and transcript levels were unchanged by glucose. The effect of glucose on the incorporation of L-serine into protein was also evaluated, but no effect was observed under either light or dark conditions. Furthermore, the amount of protein in the cells did not differ with or without glucose treatment.

Symbiotic *Chlorella* release sugars, but little is known about sugar uptake by symbionts. The uptake of glucose and other sugars by *Chlorella* spp. was measured to determine whether sugar is actually imported into cells. Free-living *C. vulgaris* imported glucose, sucrose, and maltose at uptake rates of 8.44, 18.92, and 0.68×10^{-8} nmol $cell^{-1}$, respectively, over 15 min; fructose was not imported. In contrast, F36-ZK did not import any sugars at pH 6.0 or 7.5 under either light or dark conditions. No uptake of glucose confirmed that F36-ZK could not utilize glucose as a nutrient although glucose obviously stimulated its growth, as mentioned above. Because the increase in the rate of L-serine uptake resulted from glucose treatment even at a low concentration (EC_{50} = 3 μM) and with a pulse treatment lasting only 1 min, a glucose sensing and signaling pathway seems to be involved in this response. In a glucose sensing system, some nonmetabolizable analogues of glucose are known to act as agonists (Rolland et al. 2001). To determine the presence of a glucose sensing and signaling pathway,

the response of L-serine uptake to glucose-related compounds was measured. The uptake of L-serine increased following treatment with almost all of the monosaccharides tested. However, treatment with L-glucose and 6-amino-6-deoxyglucose did not increase the uptake of serine; application of D-glucuronic acid inhibited the uptake of L-serine. Cells treated with the disaccharides maltose, cellobiose, trehalose, and gentiobiose, which contain only glucose as a building block, responded to treatment. Nonmetabolizable glucose analogues such as 2-deoxyglucose and 3-*O*-methylglucose also activated L-serine uptake, indicating that the message is transmitted via a glucose sensing and signaling pathway. These sugars were thought to be sensed at the same site on the plasma membrane, because treatment with other sugars, such as maltose, xylose, and 3-*O*-methylglucose, in addition to glucose, produced no additive effect on the rate of L-serine uptake.

Mechanisms by which glucose transduces its effects via the signaling pathway could be (1) the activation of amino acid transporters and (2) the translocation of amino acid transporters. Given that H^+-ATPase creates a H^+ gradient for amino acid transport, activation of H^+-ATPase by sugars is most probable (Camoni et al. 2006). To examine this, the effects of inhibitors on a protein kinase (*N*-6-dimethylaminopurine and chelerythrine), inhibitors of a protein phosphatase (cyclosporine A and okadaic acid), and a calmodulin antagonist (W-7), were measured. Inhibitors of the protein kinase and the phosphatase showed no effect; the calmodulin antagonist exhibited a strong inhibitory effect, supporting the involvement of calmodulin in the signaling pathway, but activation of H^+-ATPase was not clear.

Because the uptake of L-serine by F36-ZK was inhibited by Ca^{2+} (Kato and Imamura 2008a), whether glucose could counter the inhibition caused by Ca^{2+} was investigated next. Glucose restored L-serine uptake in Ca^{2+}-treated cells. Sugars such as fructose and sucrose contributed to the restitution of serine uptake in Ca^{2+}-treated cells, although they had only a small effect on the rate of serine uptake. These findings, regarding the regulatory effects of Ca^{2+} and sugars on the amino acid transport system of symbiotic *Chlorella* cells, imply the presence of a control system using these materials in the perialgal vacuole enclosing the symbiotic algae. Because the symbionts released sugars, calcium ions are likely to be transported from the host to the symbiont. Calcium has indeed been observed using electron microscopy in *Paramecium* cell symbionts (Kato and Imamura 2008a).

4 Photosynthesis of Symbiotic *Chlorella* and Its Regulatory Factors

Many examples of symbiosis between invertebrates and algae have been reported in marine organisms. For example, the dinoflagellate *Symbiodium* spp. has established symbiotic relationships with the anemone (Trench 1971), coral (Schlichter et al. 1983), and the giant clam (Ishikura et al. 1999; Streamer et al. 1988) and supplies glycerol, amino acids, or sugars to the host (Cook 1983; Hinde 1988; Trench 1971, 1979, 1993). Muscatine (1967) first reported that host tissue homogenates of symbiotic coral and clam activated excretion of fixed carbon by its symbiotic algae.

Further studies on anemones (Whitehead and Douglas 2003), corals (Gates et al. 1995), and giant clams (Masuda et al. 1994) also revealed that host homogenates stimulate release of photosynthetic products from symbiotic algae. In the case of the sea anemone, a low molecular mass fraction of host homogenate accelerated glycerol release from algae severalfold (Grant et al. 1997). Thus, it is thought that excretion from symbiotic algae could be stimulated by some specific compound(s) in the host homogenate, referred to as a host release factor. This factor has not been identified owing to its lability and given inherent difficulties in cultivating experimental organisms (Grant et al. 1998). An inhibitory effect on photosynthesis by a symbiotic coral homogenate was also observed (Sutton and Hoegh-Guldberg 1990) and the photosynthesis inhibiting factor was also suggested to be a low molecular mass compound (Grant et al. 2001).

On the basis of these symbiotic relationships in marine invertebrates, it is conceivable that there is a similar relationship, established through a host factor, in the green freshwater paramecium. In cell-free extracts of Japanese *P. bursaria*, the host factor that affects carbon fixation by symbiotic algae will be described.

4.1 Features of Photosynthesis in Symbiotic *Chlorella*

To obtain fundamental knowledge about the features of carbon fixation by the symbiont F36-ZK, the pH dependence of F36-ZK's carbon fixation was compared with that of free-living *C. vulgaris*. Radioactive carbon dioxide was used and the photosynthetic products of F36-ZK were studied. Surprisingly, the pH dependence of gross carbon fixation by these algae showed opposing tendencies, i.e., the gross carbon fixation by F36-ZK was increased at alkaline pH, while that of *C. vulgaris* was decreased, as shown in Fig. 3 (Kamako and Imamura 2006). In *Chlorella* spp., a decrease in carbon fixation at alkaline pH has been previously reported (Beardall 1981; Beardall and Raven 1981; Gehl et al. 1990; Shelp and Canvin 1985). Thus, the enhancement of carbon fixation at alkaline pH seems to be a notable feature of the Japanese symbiont F36-ZK. However, the release of photosynthetic products by F36-ZK was increased at low pH, as observed in an endosymbiotic *Chlorella* isolated from a green hydra (McAuley et al. 1996). The photosynthate of F36-ZK released at low pH was analyzed by silica gel thin-layer chromatography and the major photosynthetic product was identified as maltose. This observation strongly suggests that the Japanese symbiotic algae F36-ZK provides maltose to its host, like the European and American symbionts (Brown and Nielsen 1974; Reisser 1976, 1986).

4.2 Effects of Host Extracts on Carbon Dioxide Fixation by Symbiotic *Chlorella*

The effects of cell-free extracts of *P. bursaria* F36 on carbon fixation and the photosynthate release of symbiotic *Chlorella* F36-ZK were investigated (Kamako

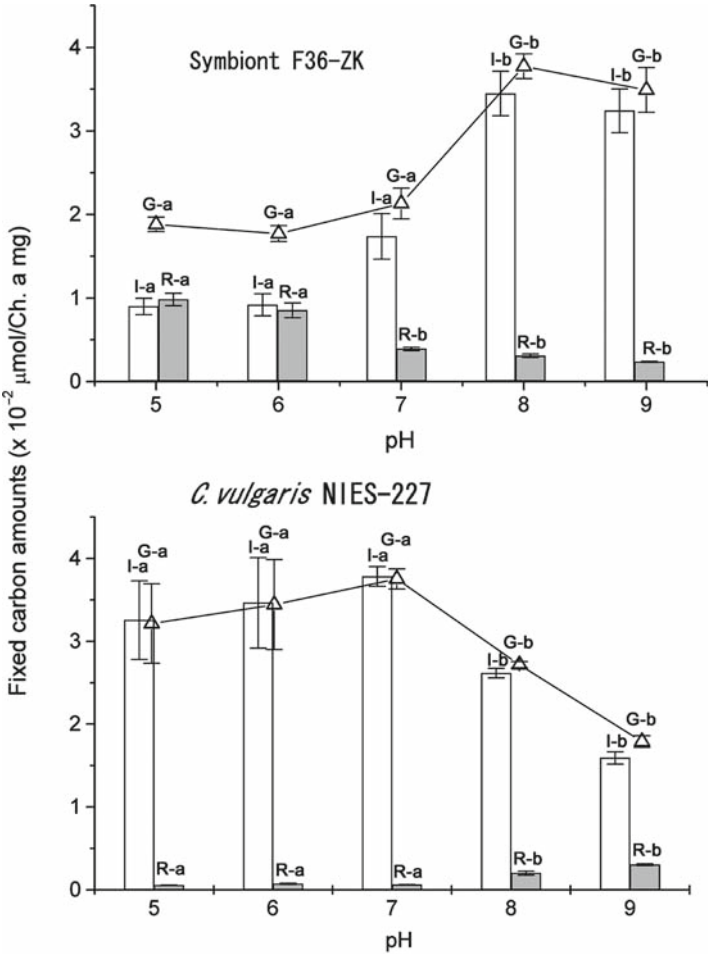

Fig. 3 pH dependence of carbon fixation of F36-ZK and *C. vulgaris*. (Kamako and Imamura 2006). The *white bar* shows intracellular carbon fixation (*I*), the *shaded bar* represents the released photosynthate (*R*), and the *line chart* depicts gross carbon fixation (*G*). Mean values ± the standard error (SE; $n = 3$). Means showing statistical differences, based on one-way analysis of variance (ANOVA) followed by a Tukey test ($p < 0.05$), are indicated

and Imamura 2006). Intracellular carbon fixation by F36-ZK in sodium phosphate buffer (pH 7.0) increased with increasing host extract concentration and reached a maximum value about threefold that without the host extract, but the release of photosynthetic products hardly changed. Well-known factors limiting photosynthesis are light intensity and carbon dioxide concentration (Taiz and Zeiger 2002). Light intensity was kept constant, and carbon dioxide and bicarbonate were excluded as enhancing factors, because carbon fixation increased with host extract from which carbon dioxide was ready eliminated by acid treatment. From

these experiments, proof was obtained for a host factor that stimulates algal carbon fixation.

The pH dependence of the effects of the host factor was studied at a pH of 5.0, 7.0, and 9.0. Intracellular carbon fixation by F36-ZK was enhanced with increasing host extract concentration at pH 5.0 and 7.0, although it remained high at pH 9.0 with or without host extract. Carbon fixation by F36-ZK is optimal at alkaline pH as mentioned above and thus it might be impossible to enhance the carbon fixation at pH 9.0. While the amount of photosynthate released was affected only at pH 5.0, about 45% of the total carbon fixed was released (a relatively high percentage compared with results at pH 7.0 or 9.0), irrespective of host extract concentration (Fig. 4). Therefore, photosynthate release depended on pH but not on the concentration of the host extract at any pH. In *P. bursaria* cells, a perialgal vacuole encloses the symbiotic algae and maintains acidic conditions (Schüßler and Schnept 1992). Inside the vacuole, the host factor could enhance symbiotic algal carbon fixation and the acidic environment could favor release of the photosynthate, maltose, from the symbiont. The enhancement of carbon fixation by an agent is still an unusual phenomenon, but evidence for increased symbiotic algal carbon fixation by a host cell homogenate was reported for coelenterates (Trench 1971) and giant clams (Gates et al. 1995). Dinoflagellates were the symbiotic algae in both cases, and several free amino acids were shown to enhance carbon fixation in the symbionts.

4.3 Factors Affecting Photosynthesis of Symbiotic *Chlorella*

Further studies on the host factor in *P. bursaria* extract were carried out and the host factor in Japanese *P. bursaria* F36 extract was characterized as described below. In many marine organisms, the host factors were known to be of low molecular mass. Experiments using ultrafiltration showed that the host factor in *P. bursaria* F36 extract was also a low molecular mass substance of less than 5 kDa (Kamako and Imamura 2006). Heat stability was measured, because some host factors in marine organisms were reported to be too labile to be isolated (Grant et al. 1998), and it was found that the activity remained even when the extract was autoclaved at 121°C for 20 min. Considering the role of minerals as potential host factors, the activity of the inorganic fraction of the extract was examined. After removal of the organic compound by burning at 700°C, the remaining ash solution and the extract enhanced algal carbon fixation, indicating that the active ingredient is inorganic material.

The major cellular inorganic materials are cations, such as K^+, Ca^{2+} and Mg^{2+}, and the mixture of these cations were considered to be the host factor. Every cation is important for photosynthesis; K^+ is present in the chloroplast at approximately 100 mM (Wu and Berkowitz 1992a), and contributes to the stability of enzymes for carbon fixation and for the regulation of stromal pH (Berkowitz and Wu 1993). Activation of ribulose bisphosphate carboxylase/oxygenase and fructose 1,6-bisphosphatase requires Mg^{2+} (Ishijima and Ohnishi 2002). Furthermore, the activity of a chloroplastic ATPase, which also requires Mg^{2+} for function, drastically increases

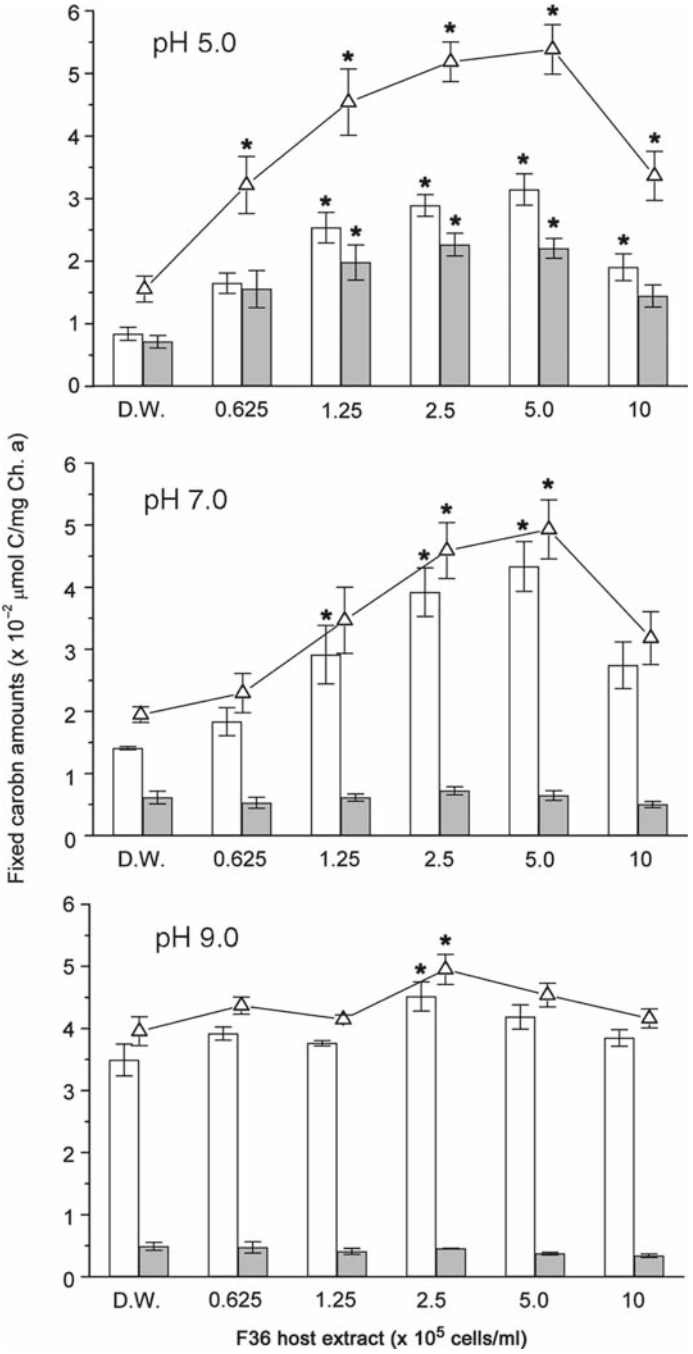

Fig. 4 The pH dependence effect of the cell-free extract on carbon fixation of F36-ZK. (Kamako and Imamura 2006). The *top*, *middle*, and *lower* charts show carbon fixation at a pH of 5.0, 7.0, and 9.0, respectively. The *white bar* shows intracellular carbon fixation, the *shaded bar* shows released photosynthate, and the *line chart* shows gross carbon fixation. Mean values ± SE ($n = 3$). *Asterisks* indicate a statistically significant difference from the control (distilled water, *D.W.*) based on one-way ANOVA followed by a Dunett test ($p < 0.05$). The concentrations of the host extract were expressed as the cell density before homogenization

in the presence of K^+ and Mg^{2+} (Wu and Berkowitz 1992b). Calcium ions also seem to play a role in oxygen evolution (Yocum 1991), although the effect of Ca^{2+} on photosynthesis remains unclear.

In marine organisms, organic molecules have been postulated to be the host factors (Gates et al. 1995; Ritchie et al. 1997). However, the inorganic materials K^+, Ca^{2+}, and Mg^{2+} were seemed to play important roles in the symbiosis of Japanese *P. bursaria* F36.

5 Conclusions

Many novel features of the Japanese symbiont F36-ZK were demonstrated. The lack of NR activity in F36-ZK indicated its irreversible adaptation to symbiotic circumstances. Information gathered about the transfer of materials and metabolic control between the host and the symbiont led to the following conclusions. The ability of F36-ZK to utilize nitrogen provided in the form of ammonium and amino acids implied that F36-ZK lives in environments in which several free amino acids are available but where ammonium is not. The utilizable amino acids seem to easily generate ammonium ions in the algal cells. Extracellular ammonium is not a good growth stimulant; therefore, these amino acids could be carriers of nitrogen in symbiosis. Although it remains unclear which amino acid is used as the nitrogen carrier, multiple amino acids are candidates, because F36-ZK possesses three developed amino acid transport systems. However, the presence of sugars in the symbiotic circumstances complicates this situation. Sugars are known to induce amino acid transport systems in free-living *C. kessleri* and *C. vulgaris* and *P. bursaria* symbionts release maltose in a perialgal vacuole during the entire day (Ziesenisz et al. 1981). Therefore, amino acid transport systems of symbiotic *Chlorella* evolved in a perialgal vacuole, and might become a constitutional system. Because a number of amino acid transport systems can be induced by sugars, it is doubtful that the three amino acid transport systems directly support the theory that the host supplies multiple amino acids. An interesting Ca^{2+}-dependent regulatory phenomenon in the F36-ZK amino acid transport systems was unveiled. The inhibition of L-serine uptake by Ca^{2+} was a common feature with the transport system induced by glucose in *C. kessleri*. The general amino acid transport system of F36-ZK resembles that induced in free-living *C. kessleri*, indicating that these amino acid transport systems are common features of *Chlorella* spp. regardless of their expression. Therefore, the amino acid transport systems of F36-ZK do not appear to acquired characters but seem to be genetic characters of *Chlorella* spp. Another regulatory phenomenon involving glucose was also found; glucose restored the inhibition of L-serine uptake by Ca^{2+}. Ca^{2+} and glucose appear to be an inhibitor-activator pair for the regulation of the amino acid uptake. The transfer of sugars and amino acids between the symbiont and the host occurs in opposite directions in the *P. bursaria* cell. When the Ca^{2+} concentration in the perialgal vacuole is constant, the symbiont releases more sugar, leading to an import of more amino acid from the host, analogous to a barter transaction between the symbiotic alga and the host.

From the results of the study on host factors, the pH and cation concentrations of the extracellular fluid triggered drastic changes in carbon fixation by the Japanese symbiont. Quite unexpectedly, the photosynthetic ability of F36-ZK showed the opposite pH dependence compared with that of free-living *C. vulgaris*, because carbon dioxide is converted to bicarbonate under alkaline conditions and the impermeability of the plasma membrane to bicarbonate causes a general decrease in the rate of photosynthesis. The dependency of F36-ZK's photosynthetic ability on extracellular cation concentration is also unusual. However, the possibility remains that the host controls the cation concentration in the perialgal vacuole for optimal algal photosynthesis. Although further analyses of the host factor are still ongoing, it is assumed that several signals exchanged between the host and its symbiotic cells control each other to maintain the symbiotic relationship.

Finally, a schematic of the above-mentioned metabolic control mechanisms is illustrated in Fig. 5. It is important to remember that the symbionts are wrapped with a lipid bilayer in the host cell and that the perialgal vacuole membrane is thought to be one of the barriers to overcome for the transportation of materials between the host and the symbiont. The permeability of this membrane regulates material transfer between the host and the symbiont and the surroundings of the symbionts in the vacuole. Studies on

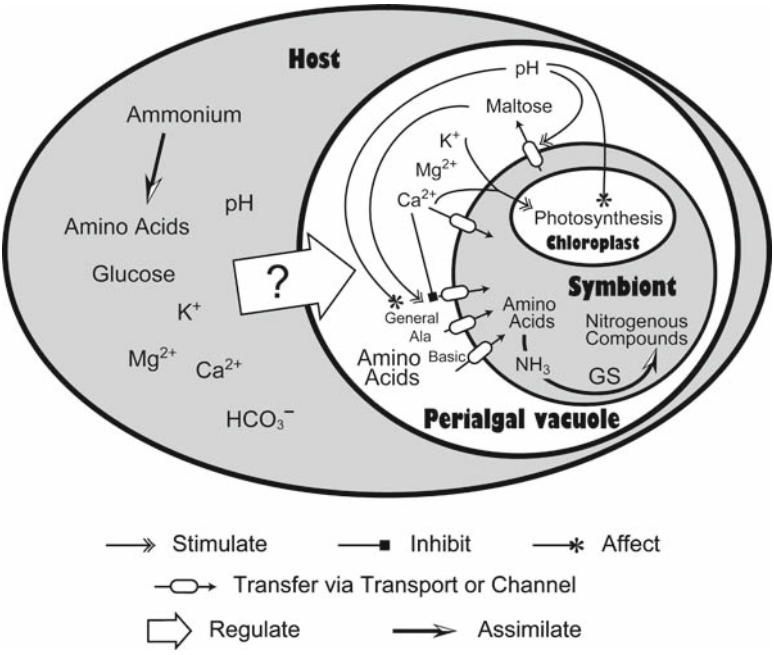

Fig. 5 Material supposedly transported, and metabolic control mechanisms, between the host and the symbiont

the perialgal vacuole membrane are at early stages and little is known about the dynamics of membrane permeability. Recently, Kodama and Fujishima (2008) found a novel effect caused by the protein synthesis inhibitor cycloheximide on the perialgal vacuole. They speculated that a protein produced and released by the symbiont during photosynthesis plays an important role in the transfer of maltose on the perialgal vacuole membrane. Interestingly, proteins permitting the transportation through the perialgal vacuole membrane are thought to be produced both by the host and by the symbiont. Certainly, the surroundings in the perialgal vacuole are also important, e.g., the pH and cation concentrations are important regulatory factors for the physiological features of the symbiont. The Japanese symbiont F36-ZK possesses many unique features that differ from previously reported symbiotic algal characters, and thus seems an interesting symbiotic alga to study further. However, the surroundings of the perialgal vacuole of Japanese *P. bursaria* could be very similar to those of *P. bursaria* worldwide. Regardless of adaptation to the symbiotic milieu, material transport between the host and the symbiont could be almost the same. Details regarding the role of the perialgal vacuole in the symbiotic relationship should be investigated in the future.

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