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Differentiation of a free-living alga into forms with ecto- and endosymbiotic associations with heterotrophic organisms in a 5-year microcosm culture

Toshiyuki Nakajima *, Yoshiyuki Fujikawa, Toshiyuki Matsubara, Mimi Karita, Akiko Sano

Department of Biology, Ehime University, Bunkyo-cho 2-5, Matsuyama, Ehime 790-8577, Japan

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The ecological mechanisms underlying the diversification of autotrophic species into endosymbiotic lifestyles and the ways in which the evolution of endosymbiotic species is ecologically and evolutionarily affected by sister lineages/lines that are adapted to extra-host environments remain unclear. In this paper, we investigated a differentiation process of algal species in which an endosymbiotic type was differentiated phenotypically from a free-living ancestral clone, by using an experimental model called the CET microcosm, which contains a green alga (*Micractinium* sp.), a bacterium (*Escherichia coli*), and a ciliate (*Tetrahymena thermophila*) cultured together without an external resource supply for over 5 years. We then analyzed the algal diversification process by comparing algal phenotypic properties regarding cell-aggregate formation and their effects on the survival of *Tetrahymena* (using a clone isolated on day 2668) in the absence of bacteria. We examined 13 *Micractinium* clones, including both ancestral and derived clones isolated from long-term (day 1819–1847) CET microcosm cultures. The results revealed that the free-living ancestral algal strain diversified in sympatry into an aggregate-forming type that associates with *E. coli*, and a non-aggregate-forming type that associates with *Tetrahymena*. Furthermore, a competition experiment revealed that the endosymbiotic (non-aggregate-forming) type was less adapted to the extracellular environment than the aggregate-forming type. This result suggests that severe competition with a nonendosymbiotic sister line in the extra-host environment favors the host-benefiting phenotype in an endosymbiotic line, because such symbionts can enhance the longevity of the host, thereby enabling the survival and reproduction of the symbiont within the host.

1. Introduction

Endosymbiosis between autotrophic microalgae and heterotrophic organisms such as protists and invertebrates is an important biological process in that it unifies auto- (photo-) and heterotrophic metabolic functions into a single cell (Howe et al., 2008; Raven et al., 2009; Nowack and Melkonian, 2010), which includes various stages of associations ranging from tentative, to stable, to genetically unified coupling between auto- and heterotrophic cells. Auto-/heterotrophic endosymbiosis is not a historically rare event; instead, it can occur repeatedly when particular ecological conditions are met with regard to the costs and benefits of symbiosis to participating species (Saldarriaga et al., 2001; Keeling, 2010; Maruyama et al., 2011).

It has been shown that endosymbiotic algae usually have nonendosymbiotic sister lineages. *Chlorella*-like algae have attracted much attention due to their ability to be endosymbiotic with ciliates such as *Paramecium*,*Stentor*, *Climacostomum*, *Coleps*, and *Euplotes*, all of which live either tentatively or stably within cells of heterotrophic species (e.g., Karakashian, 1963; Reisser, 1986; Summerer et al., 2007, 2008; Hoshina et al., 2013), and invertebrates such as green hydra and sponges (Douglas, 1994; Habetha et al., 2003; Venn et al., 2008; Kovacevic, 2012). This group also includes free-living species/strains occurring in the liquid phase of aquatic ecosystems. The term free-living does not necessarily imply that these species have no symbiotic associations with other species, as some may be able to associate with bacteria by forming aggregates, glued with extracellular polymers, to which bacteria are attached as symbiotic partners, although the ecology of these species in natural habitats remains unclear

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* Corresponding author. Tel.: +81 89 927 9639; fax: +81 89 927 9639.
E-mail addresses: nakajima@sci.ehime-u.ac, cognizers@yahoo.co.jp (T. Nakajima).

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(Mouget et al., 1995; Watanabe et al., 2005; Vieira et al., 2006; Imase et al., 2008). Recent studies revisited the phylogeny of *Chlorella*-like algae by using DNA sequencing to examine the phylogenetic relationship among various physiological and ecological forms of algae adapted to free-living (planktonic), edaphic, and endosymbiotic niches. These studies reconstructed phylogenetic relationships between species/strains in this group and revealed that endosymbiotic species/strains isolated from *Paramecium bursaria* (green *Paramecium*) are polyphyletic (Hoshina and Imamura, 2008; Luo et al., 2010; Pröschold et al., 2011). Similarly, it was shown that endosymbiotic algae isolated from green hydra (*Hydra viridissima*) also have a polyphyletic origin and have many related species that are free-living (Huss et al., 1993–1994; Kovačević et al., 2010; Kovacevic, 2012).

Moreover, a similar divergence concerning free-living and endosymbiotic lifestyles has been suggested for dinoflagellates in the genus *Symbiodinium*. This group is known for their symbiotic associations with protists and invertebrates such as sponges, cnidarians, and mollusks. Recently, free-living *Symbiodinium* occurring separately from host animals have received much interest as a potential source of symbionts for many host animals. Notably, recent studies suggest that, in addition to this transient free-living life form, there are *Symbiodinium* species that can be considered “exclusively” free-living, since they have never been reported in invertebrate and protist hosts (LaJeunesse, 2002; Gou et al., 2003; Santos, 2004; Porto et al., 2008; Yamashita and Koike, 2013). The exclusively free-living *Symbiodinium* were usually found in non-host habitats such as sands, tide pools, water columns, stone surfaces, or macroalgal surfaces, but not usually within animal hosts (Yamashita and Koike, 2013).

These phylogenetic studies of endosymbiotic algae suggest that auto-/heterotrophic endosymbioses have evolved through algal differentiation from free-living species and that the endosymbiotic species are polyphyletic and coexist with nonendosymbiotic free-living species in the same locality. Divergent selection in sympatric populations can occur by various mechanisms, including environmental differences, sexual selection, and ecological interactions (Schluter, 2000; Rundle and Nosil, 2005). However, the ecological and evolutionary mechanisms underlying the diversification of autotrophic species into endosymbiotic lifestyles, and the ways in which these species are affected by their free-living sister lineages/lines, remain unclear.

In this paper, we focus on the ecological process underlying the diversifying evolution of algal species and specifically, the way in which an endosymbiotic lineage associating with heterotrophic species can be differentiated from a free-living ancestral lineage, with particular interest in how the evolution of endosymbiotic lineage is causally affected by sister lineages of different species (or sister lines of the same species) that adapt to extracellular environments (In this paper, the term “lineages” is used for different species while lines for different subpopulations of genotypes in the same species). Virulent theory may provide us with a useful perspective for this issue. This theory predicts that the vertical transmission of symbionts favors cooperative traits in the symbiont, because in that way the symbiont can increase its own fitness by benefiting the host; in contrast, horizontal transmission favors exploitative (i.e., parasitic) traits (Ewald, 1983; Herre, 1993; Bull, 1994; Sachs et al., 2004; Sachs and Wilcox, 2006). In extracellular environments, there must be severe competition with sister lineages/lines that adapt to these environments, so that endosymbiotic algal types cannot survive and reproduce better than their sister lineages if adaptation to the host cell reduces adaptation to the extracellular environment. In such a situation, success in the survival and reproduction of these endosymbiotic types may strongly depend on mutualistic associations with hosts, so that the algae benefit from these hosts by spending longer periods of time within them. Therefore, it can be hypothesized that severe competition with sister lineages/lines that are adapted to extra-host environments can promote the evolution of host-benefiting traits in algae. However, this hypothesis has not been addressed effectively, and therefore we focus on this issue in this paper.

In order to resolve this issue, we developed an experimental ecosystem model called the CET microcosm, which contains a green alga (*Micractinium* sp. Ehime [single clone], formerly described as *Chlorella vulgaris*; Germond et al., 2013a), a heterotrophic bacterium (*Escherichia coli* [single clone]), and a ciliate (*Tetrahymena thermophila* [a mixture of the two different mating types CU427 and CU438]; Nakajima et al., 2009). This system was incubated statically without transfer on a 12L:12D cycle at 30 °C using a medium (MC medium) that is without organic compounds and contains only mineral salts and metals. *Micractinium* grows in the light, using the minerals in the medium, and excretes photosynthetic organic compounds into the medium; *E. coli* grows by using the organic compounds; and *T. thermophila* grows by eating *E. coli* but cannot grow on *Micractinium* (Nakajima et al., 2009). These component species can coexist for over three to eight years without transfer, with the length of stable coexistence depending on the frequency of sampling of the culture (unpublished data). Such a self-sustaining ecosystem model is useful for investigating the evolutionary mechanisms underlying the generation of symbioses among organisms of component species under stoichiometric restriction in a semiclosed ecosystem.

The previous studies (summarized in Appendix A) hypothetically suggested that, in the CET microcosm, the ancestral algal population had diversified into different ecological forms that associated with different heterotrophic species; specifically, into an aggregate-forming type that developed a symbiotic ability to associate with *E. coli*, and an non-aggregate-forming algal type that developed a symbiotic ability to associate with *T. thermophila*. However, the previous results were limited in supporting this diversification, because they were based on the results from using only a few isolates and qualitative description of algal characteristics such as aggregation ability. Furthermore, it remains unclear how this algal diversification from the same ancestral clone, if any, can be promoted in terms of intraspecific competition between different algal types mediated through heterotrophic species, such as *E. coli and T. thermophila*, under resource-limited conditions. In this paper, we further investigated algal phenotypic differentiation more clearly using additional algal isolates and employing quantitative measurements of algal characteristics such as aggregating ability and host (ciliate)-benefiting properties. We then investigated the role of intraspecific competition in this algal diversification by testing the hypothesis that severe competition with sister lines that are adapted to the extra-host environment can promote the evolution of host-benefiting traits in algae. For this test, we conducted competition between host-benefiting (non-aggregate-forming) and non-host-benefiting (aggregate-forming) types of algal isolates in the extra-host environment with or without bacteria, in order to examine whether the non-host-benefiting type can competitively exclude the benefitting type.

2. Materials and methods

2.1. Overview of experimental design

Thirteen *Micractinium* clones, including both ancestral and derived clones isolated from three replicate lines of the CET microcosm after they had been cultured for 1819–1847 days were examined for their ability to form cell aggregates and their effects