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Auto-/heterotrophic endosymbiosis evolves in a mature stage of ecosystem development in a microcosm composed of an alga, a bacterium and a ciliate

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ABSTRACT

We investigate an ecological mechanism by which endosymbiotic associations evolve, with a particular focus on the relationship between the evolution of endosymbiosis between auto- and heterotrophic organisms, and the stages of ecosystem development. For this purpose we conducted a long-term microcosm culture composed of three species, a green alga (*Chlorella vulgaris*), a bacterium (*Escherichia coli*), and a ciliated protozoan (*Tetrahymena thermophila*) for 3 years. During this culture *T. thermophila* cells harboring *Chlorella* cells emerged by phagocytotic uptake, and increased in frequency, reaching ca. 80–90%. This level was maintained in the late stage of ecosystem dynamics. Analysis of the ecosystem dynamics in the microcosm revealed that a complex causal process through direct/indirect interactions among ecosystem components led to reduction in dissolved O_2 and food (*E. coli*) available to the *T. thermophila*, which gave a selective advantage to the organisms in the endosymbiotic association. This result suggests that the endosymbiosis evolves in a mature stage of ecosystem development, where reproduction and survival of prospective partner organisms is highly resource-limited and density-dependent, favoring efficient matter/energy transfers among participating organisms due to physical proximity. Consequently, a complex web of interactions and pathways of matter/energy flow in ecosystem evolves from an initially simple one.

1. Introduction

Endosymbiosis is widespread in nature, wherein participating organisms acquire new metabolic and ecological capabilities to exploit new niches. Due to its substantial role as a source of innovation in the evolution of life, endosymbiosis is recognized as an essential mechanism of the evolution of a variety of complex biological organizations, and adaptive radiation on earth (Buchner, 1965; Margulis and Fester, 1991; Margulis, 1992; Douglas, 1994; Palmer, 2003; Hackett et al., 2004).

One hypothetical scenario of the evolution of mutualistic symbioses is that they evolve(d) from parasite (or pathogen)–host relationships (Atsatt, 1988; Price, 1984, 1991; Ewald, 1987; Jeon, 1987), because parasites have a pre-adaptation for symbiotic associations, already finely evolved for living intimately with other organisms (Price, 1991). This transition is expected to occur when parasites are transmitted vertically because the parasites share the same host with their offspring, in which they can increase their inclusive fitness by benefiting the host (Ewald, 1987; Herre, 1993; Yamamura, 1993). However, there is another large group of organisms in symbiosis, which seems to have evolved not via parasitism, but via predation by prospective hosts, particularly between photosynthetic and heterotrophic organisms. These include unicellular organisms such as dinoflagellates, flagellates and ciliates, and aquatic invertebrates such as green hydra, sea anemone, sponge, coral, and so on (Stoecker et al., 1987; Douglas, 1994; Paracer and Ahmadjian, 2000; Palmer, 2003; Keeling, 2004; Hackett et al., 2004). Unlike parasites, prospective photosynthetic endosymbionts are nutritionally independent of their prospective hosts because they are autotrophic. In fact, many of the contemporary algal symbionts still have the ability to survive and reproduce outside their hosts. In other words, autotrophic symbionts function as the producer, whereas parasitic symbionts as a consumer in the intracellular metabolic hypercycle of symbiotic cell.

Repetitive ingestion of algae by phagocytosis as food, or incidental ingestion accompanied by food intake, may be an initial step toward the evolution of an endosymbiotic association (Reisser, 1993). Experimental studies of contemporary endosymbiotic organisms have demonstrated that hosts harboring algal

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endosymbionts can have a selective advantage under the conditions of food-limited and/or low dissolved oxygen concentrations. Hosts can increase their rates of survival and reproduction by receiving photosynthetically fixed carbon and O_2 from their endosymbionts (Karakashian, 1963; Douglas and Smith, 1983; Reisser and Kurmeier, 1984; Berninger et al., 1986; Reisser, 1986; Finlay et al., 1988, 1996; Stabell et al., 2002). On the other hand, the algal endosymbionts benefit by receiving nitrogen, other nutrients such as amino acids (Albers et al., 1982), or receiving more light by host's phototaxis (Iwatsuki and Naitoh, 1981). Generally, prior studies suggest that the endosymbiotic association can evolve under strongly resources-limited and density-dependent conditions.

Where does this selective environment come from? The answer may lead us to identify a general ecological law of the evolution of auto-/heterotrophic endosymbiosis, and possibly symbiosis in general. In the study of ecosystem organization, it has been suggested that ecosystems undergo a directional, predictable change in the community structure, energetics, nutrient cycling, and life-history characteristics of component species (e.g. Margalef, 1963; Cooke, 1967; Odum, 1969; Christensen, 1995; Faith et al., 2004; Ludovisi et al., 2005; Jørgensen, 2006). According to the descriptions and predictions, ecosystems develop toward a mature stage, characterized as the equality between gross production and community respiration, low ratio of gross production to standing biomass, high ratio of supported biomass to unit energy flow, detritus-dependent nutrient cycling, high species diversity, web-like food chain, well-organized spatial heterogeneity, etc. This suggests that ecosystem development may have crucial impacts on the evolution of component species. In mature stages of ecosystems, where producers' nutrients are detritus-dependent and consumers' survival/reproduction is highly density-dependent, organisms with higher reproductive and survival efficiency, such as K-strategists, can evolve (Margalef, 1963; MacArthur and Wilson, 1967; Odum, 1969). Symbioses may also prevail due to their efficient growth and survival in this stage. This perspective suggests that those conditions in mature stages may function as a major selective force for symbiotic associations. From this viewpoint, we can hypothesize that auto-/heterotrophic symbiosis evolves in a mature stage of ecosystem development. However, few authors have addressed this issue so far.

In this paper, we investigate the relationship between the stages of ecosystem development and the emergence and maintenance of an endosymbiotic association between auto- and heterotrophic unicellular organisms. For this purpose, we analyze directly the ecological process in which an endosymbiotic association evolves from non-associated organisms, by using a microcosm composed of three species in a medium containing only inorganic salts. It consists of a green alga (*Chlorella vulgaris*) from a single clone, a bacterium (*Escherichia coli*) from a single clone, and a ciliate (*Tetrahymena thermophila*) in the form of a genetically heterogeneous population containing two different mating types (referred to as CET microcosm). The genus *Chlorella* is known as a major group of algal endosymbionts in aquatic symbiotic associations (Muscatine et al., 1967; Reisser, 1986; Douglas, 1994; Dolan, 1992). *T. thermophila* is a bacteriovorous ciliated protozoan, formerly described as *T. pyriformis* syngen 1 (Elliott, 1959; Nanney and McCoy, 1976), which has not been reported to harbor any algae within the cell in laboratory as well as in natural habitat. Starving cells of different mating types conjugate for sexual recombination (Martindale et al., 1982; Karrer, 2000).

In the microcosm, at least initially, *T. thermophila* feeds on *E. coli*, and the latter is nourished by extracellular organic compounds secreted by *C. vulgaris*, where major part of photosynthetic carbon and energy flow from *C. vulgaris* through *E. coli* to *T. thermophila*. We analyze a long-term dynamics of this microcosm over 3 years to test the hypothesis, and discuss how the ecosystem development affects the evolution of endosymbiotic associations in general.

2. Materials and Methods

2.1. Organisms and Media

C. vulgaris Beijerinck: This strain was supplied by Dr. Itayama at National institute for Environmental Studies, which had been maintained in axenic culture under the fluorescent light. One single clone was isolated from this population by purifying it four times on agar, and used as the original ancestral clone in this study (stored at −85 °C). *E. coli* K12 ME7767 was supplied from the Genetic Stock Research Center of the National Institute of Genetics (Mishima, Japan). A single clone was isolated from the population supplied by streaking four times on agar, and used as the original, ancestral strain. *T. thermophila* including two mating types, CU427 (mating type VII) and CU438 (mating type IV), were supplied by Dr. Hori at Yamaguchi University, each of which was maintained axenically in PYG medium (0.2% Peptone, 2% yeast extract and 1% glucose) at 15 °C, and transferred to a fresh medium about every 4–6 months. Medium used for the microcosm, denoted MC medium, contains K_2HPO_4, 1.2; KH_2PO_4, 0.4; $(NH_4)_2SO_4$, 0.5; $MgSO_4{\cdot}7H_2O$, 0.1; NaCl, 0.01; $CaCl_2{\cdot}2H_2O$, 0.02; $MnSO_4{\cdot}5H_2O$, 0.28×10^{-6}; $ZnSO_4{\cdot}7H_2O$, 0.04×10^{-3}; $CuSO_4{\cdot}5H_2O$, 0.02×10^{-3}; H_3BO_3, 0.63×10^{-6}; $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.03×10^{-3}; $CoCl_2{\cdot}6H_2O$, 0.01×10^{-3} (g/l).

2.2. Initiation of Microcosm

The microcosm was initiated by inoculating *C. vulgaris*, *E. coli*, and *T. thermophila*, sequentially to the 200 ml medium in a cylindrical glass bottle with a neck stoppered with an air-permeable silicon foam plug (bottle diameter 7.5 cm, full volume 400 ml). Each population was inoculated as follows: 0.1 ml of *C. vulgaris* suspension grown to the stationary phase in MC medium was inoculated first, and incubated for 13 days. Then, 0.1 ml of *E. coli* grown to the stationary phase in the MC medium with 0.1% glucose, was inoculated. Six days later, i.e. 19 days after the *C. vulgaris* inoculation, *T. thermophila*, grown to the stationary phase in 10% PYG medium and washed twice with MC medium, was inoculated. The microcosm, including 11 replicate lines with a code "No. #", was statically cultured without transfer, while irradiated with lateral fluorescent lights using a 12:12 h LD cycle ($42\ \mu mol\ m^{-2}\ s^{-1}$) at 30 °C.

2.3. Sampling from the Microcosm and Enumeration

After gentle shaking by hand, 1.5 ml of the culture was periodically withdrawn from the microcosm, and used for enumerating populations. *C. vulgaris* cells that radiate fluorescent red (excited at 330–380 nm) were counted as "live" and the cells that did not as "dead" in a hemacytometer under an epifluorescence microscope (Nikon, Eclipse E600). The *E. coli* population was enumerated by two methods, direct and indirect counts: Direct count was done by counting *E. coli* cells in a bacterial counter chamber under the fluorescent microscope (ex. at 330–380 nm) after staining with DAPI (4′-6-diamidino-2-phenylindole, Wako pharmaceutical co.) filtrated through 0.2 μm-pore filter. The viable cell density of *E coli* was determined by the colony count on agar (MC medium supplemented with 0.1% glucose, 0.1% peptone, and 0.1% yeast ext.). The cell density of *T. thermophila* was determined by counting cells under the microscope after fixing with 2% glutaraldehyde. *T. thermophila* cells were enumerated as C-*Tetrahymena* for those containing one or more live *Chlorella* cells, and N-*Tetahymena* for those containing no *Chlorella*. Dissolved oxygen (DO) concentration in microcosm was measured using a DO meter (ABLE, DM-1032). The sensor rod (ABLE, 10AN; diameter 10 mm; length 150 mm) with an air permeable plug sterilized by autoclaving was inserted into the microcosm bottle, in which a sterilized, magnetic stirrer bar (1.5 mm diameter, 8 mm length) was placed beneath the sensor membrane. The microcosm was incubated under the same condition as the long-term culture. TOC was measured using TOC analyzer (Shimadzu, TOC-5000A).

2.4. Observation of the Intracellular Structure of C-Tetrahymena

C-*Tetrahymena* cells were collected by centrifuging 60 ml suspension sampled from a microcosm (No. 11) cultured for 1132 days. These cells were prefixed by 4% glutaraldehyde at 4 °C, and rinsed and washed three times, each 2 h, with 1/15 M phosphoric buffer. Then, the cells were post-fixed in 1% OsO_4 and rinsed and washed four times, each 1 h, with distilled water at room temperature. The fixed samples were dehydrated in an acetone series and embedded in Spurr's resin. Thin sections of the embedded samples were made with a glass knife and stained with toluidine blue. Preparations were observed under the optical microscope. In the control experiment, *T. thermophila* original population (CU 438), which was grown in the PYG medium and washed twice with distilled water, was inoculated into the suspension of *E. coli* in distilled water at the initial density of ca. $10^8\ ml^{-1}$ (without *C. vulgaris*), and grown to the stationary phase.