Symbiosis, 33 (2002) 175–190
Balaban, Philadelphia/Rehovot

Experimentally-Induced Association Between Two Protists: *Physarum polycephalum* (Mycetozoa) and *Chlorella pyrenoidosa* (Chlorophyceae)

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Received June 8, 2002; Accepted July 11, 2002

Abstract

This study reports one of the first detailed analysis of an experimentally-induced association (not occurring naturally) between two unicellular protistans, a slime mold, *Physarum polycephalum* (Mycetozoa), and a chlorophyte alga, *Chorella pyrenoidosa* (Chlorophyceae), in laboratory cultures. The host, *Physarum*, was inoculated with *Chlorella* resulting in the uptake of algae and "greening" of the plasmodia. Ultrastructural analysis showed that the algae were initially engulfed by *Physarum*, subsequently retained in perialgal vacuoles, distributed within the plasmodia and formed a stable association lasting for weeks as long as the plasmodia were active. Some of the *Chlorella* were cyclically exocytized and re-engulfed. The inoculated *Physarum-Chlorella* system had significantly increased survival, some living up to 31 days, as compared to uninoculated hosts. Fluorometric analysis of chlorophyll a and phaeopigments of the *Physarum-Chlorella* cultures and exocytized *Chlorella* resulted in higher acid ratios and chlorophyll a concentrations as compared to unialgal cultures indicating viability of the *Chlorella* in the host cell. At the end of the association, the *Physarum* host, which was transformed from a plasmodium phase containing algae to the sclerotia phase, exocytized viable *Chorella* onto the top of the sclerotia. While not a natural or evolutionary established relationship, the *Physarum-Chlorella* model system

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0334-5114/2002/$05.50

provided notable findings of the initial events during *Chlorella* engulfment and physiological significance of the experimentally induced association between the two protists. This research provides evidence that new host-algal associations may become established rather rapidly under favorable environmental conditions and yields further evidence of the plasticity of host-symbiotic associations that may have promoted endosymbiotic associations during eukaryotic evolution.

Keywords: Symbiosis, *Physarum, Chlorella*, alga, slime mold, experimentally-induced association

1. Introduction

Intracellular symbiosis represents a well-regulated and close association between two genomes of divergent evolutionary origins, which coexist in a shared cytoplasmic environment and represents a functional system of advancing integration which can be studied in terms of the development of the cellular relationships between the two biota. As a microcosmic constituency, algal symbiosis, which occurs broadly across phyla, provides examples of co-existence and relatedness of cells and tissues of widely different genetic origins. In particular, zoochlorellae (green algae) and/or *Chlorella* sp. form associations with hosts in freshwater natural environments in several phyla including 1) Protista, *Paramecium bursaria* (Karakashian, 1975; Karakashian and Karakashian, 1965, 1973), *Vorticella* sp. (Graham and Graham, 1978), *Stentor polymorphous, Ophrydium* sp. (Pardy, 1983) and more recently, by Tsukii et al. (1995), in the heliozoan sarcodine, *Acanthocystis turfacea*, the rhizopodial sarcodine, *Mayorella viridis*, and the ciliates, *Climacostomum virens, Prorodon viridis, Frontonia vernalis, Vorticella monilata*, and *Colpoda* sp. (Esteve et al., 1988); 2) Porifera (sponges), *Spongilla lacustris* (Pardy, 1983; Smith and Douglas, 1987) and *Ephydatia fluviatilis* (Smith and Douglas, 1987); 3) Cnidaria, the hydrozoan coral, *Millipora* sp. (Yonge and Nicholas, 1931a,b), the marine anemone, *Anthopleura* sp. (Smith and Douglas, 1987), *Chlorohydra* and/or *Hydra viridis* (Goestch, 1924; Graham and Graham, 1978; Muscatine et al., 1975); 4) Platyhelminthes (freshwater flatworms), the turbellarian flatworms *Dalyellia viridis* (Smith and Douglas, 1987) and *Phaenocora typhlops* (Smith and Douglas, 1987; Eaton and Young, 1975) and convolutan worms (Keeble and Gamble, 1905, 1907); 5) Mollusca, *Anodonta cygnea* (freshwater clams) (Pardy, 1983; Smith and Douglas, 1987); and 6) Ascomycetes, the lichen-arrangement of the sac fungi (Tiffany, 1958; Smith and Douglas, 1987). Using protists, it is possible to experimentally examine the

details of the initial events during establishment of host-symbiont associations and the factors that promote a stable union.

This report is based on unpublished results of a doctoral thesis which examined the initial endocytic response and subsequent association of two protists, a host slime mold, *Physarum polycephalum*, and a green alga, *Chlorella pyrenoidosa* (Gastrich, 1982). While many scientific studies cited above, including those recently conducted in our laboratory, documented naturally-occurring algal-radiolarian symbioses (Anderson et al., 1983, 1985; Anderson and Matsuoka, 1992) and algal-foraminiferal (planktonic) symbiosis (Gastrich, 1986, 1987; Gastrich and Bartha, 1988), there have been no reports of a *de novo* experimentally-induced algal symbiosis within a protistan host in the last twenty years.

While a preliminary laboratory study reported in the early 1960s that *Chlorella* species were able to enter into full associations (e.g., transfer of radioisotopically labeled phosphorus between host and endosymbiont) with two species of bacteria-free Myxomycetes cultures, including the acellular slime mold *Physarum didermoides* and the cellular slime mold, *Fuligo cinerea*, negative results were reported on trials with other slime mold species, especially *P. polycephalum* (Lazo, 1961; Zabka and Lazo, 1962). Therefore, this study focused on a more extensive ultrastructural and physiological assessment of factors associated with the experimentally induced association of a free-living green alga, *C. pyrenoidosa*, and a host, *P. polycephalum*. The objectives were to document the entry of foreign cells into a recipient cell during the endocyctic process and to study the persistence of the relationship.

Because of its documented fine structure characteristics and phagotrophic activity (Daniel and Jarlfors, 1973; Schuster, 1965; Wohlfarth-Bottermann, 1974), *P. polycephalum* was chosen as a suitable host for endocytic uptake of an algal species known to form associations in nature with other species. More recent studies of *P. polycephalum* ultrastructure and phagotrophic activity confirm its suitability as an experimental host (Anderson, 1992, 1993). Fine structural studies of food particle phagocytosis (Anderson, 1993) provide evidence that food is engulfed within canal-like invaginations on the ventral surface of the plasmodium and that substantial digestion takes place within deeper recesses of these canals where enzymes are secreted. This knowledge provides a context for evaluation of how potential symbiotic cells are endocytized in contrast to how food particles are processed.

In this study, we report morphological and fine structural evidence for laboratory induced uptake of *C. pyrenoidosa* by a white strain of *P. polycephalum* and experimental evidence of a close functional integration leading to sustained association with survival benefits for the host.

2. Materials and Methods

Chlorella cultures

Pure cultures of *Chlorella pyrenoidosa* were obtained from Carolina Biological Supply Company and cultured axenically in sterilized test tubes containing a solution of 20 ml Alga-Gro per liter of distilled water adjusted to a pH 7.4 using 0.5 N KOH and 0.5 N HCL and supplemented with 0.25 ml/l vitamin stock, maintained at 21°C with a 12 h L/D cycle using fluorescent illumination of 1.98×10^2 μW cm^2 in the red range (600–700 nm) and at 4.62×10^2 μW cm^{-2} as measured with an International Light Meter. The cultures were transferred under sterile conditions every two weeks.

Physarum cultures

Pure strains of the rare white plasmodia of *Physarum polycephalum* were obtained from Dr. Charles Holt at Massachusetts Institute of Technology and maintained in sterile Petri dishes containing sterile solidified 2% agar. White strains were used to avoid possible light absorptive interference with photosynthesis of the *Chlorella* after engulfment by the host that otherwise may occur with pigmented *Physarum* sp. Plasmodial transfers were completed weekly under sterile conditions. The cultures were fed (several oat flakes) and incubated in the dark at 22°C.

Inoculation of Physarum with Chlorella

Physarum cultures which had been serially transferred in laboratory culture 22 to 40 times were used for the inoculation of the *Chlorella* into the *Physarum* plasmodia. Healthy dark green cultures of *Chlorella* in logarithmic growth phase were placed in continuous fluorescent illumination for 24 hrs prior to inoculation. Algal cultures were centrifuged and sedimented for 3 min in an EIC clinical centrifuge at 810 rpm. The pellet was drawn up through a pipette with no more than 2 ml of distilled water and placed upon the agar at several inoculation sites or the algal pellet was placed directly upon the advancing plasmodia. In other cases, *Chlorella*, which had previously been taken up by the plasmodia and exocytized on the slime tracks, were used to re-inoculate the plasmodia. All oats were removed prior to the inoculation in order to stimulate the plasmodia to take up the *Chlorella*. Inoculated cultures were placed in fluorescent 12 h L/D alternating cycles or in continuous light at 22°C and some *Physarum-Chorella* cultures were fed oats while others were not fed to determine the possible photosynthate contribution of symbiont to host survival as further explained in the Results section.

Microscopy

Inoculated cultures of *Physarum-Chlorella* were fixed in situ for transmission electron microscopy (TEM) with a 4% glutaraldehyde solution prepared in a 0.1 M phosphate buffer (pH 7.2) at intervals of 1.5 to 3 hrs after the initial inoculation. Fixed samples were kept at 3°C for two hrs and then rinsed three times in a cold Sorensen's (pH 7.2) buffer. The agar containing the *Physarum* was excised and post fixed in a 1% osmium tetroxide solution buffered with 0.1 M phosphate buffer for 2 hrs at 3°C. Following three rinses with distilled water, the specimens were deydrated in an acetone series and embedded in Epon LX-112 resin according to standard TEM techniques. Ultrathin sections were obtained using a diamond knife in a Sorvall MT-2 ultramicrotome and sections were stained with Reynolds lead citrate, and viewed with a JEOL JEM-100S or Philips 200 electron microscope. Inoculated cultures of *Physarum-Chlorella* (*P-C*) were also examined using a Nikon light microscope. A Zeiss Ultraphot using bright field illumination, phase contrast, or Nomarski optics and a Nikon epifluorescence microscope were used to better visualize the chloroplast-containing algae.

Fluorometric determination of chlorophyll a and phaeo-pigments of pure cultures of Chlorella and inoculated Physarum-Chlorella (P-C)

Fluorometric determination of chlorophyll a and phaeo-pigments were used to compare the chlorophyll a and acid ratios of pure *Chlorella* cultures with the inoculated *P-C* cultures to determine the viability of *Chlorella* cells within the host. Pure stocks of *C. pyrenoidosa*, used in the inoculation experiments above, were used to obtain base-line data for chlorophyll a and phaeo-pigments. Data obtained on cell counts, using a Speirs-Levy cell chamber, acid ratios and fluorescence units per sample were used to calculate the picograms of chlorophyll a per *Chlorella* cell using standard procedures (Strickland and Parsons, 1972). A Turner fluorometer, Design #3, was used with a Tau (constant) factor equal to 1.93 and calibrated to samples analyzed with an absorbence spectrophotometer including 10-045 blue lamp, an excitation filter of 10-050 color specification 5-60 filter, a reference filter of 10-052 specification 3-66 filter, emission filter of 10-055 color specification 70 filter nearest the photomultiplier with a 10-053 color specification 16 filter over it, and a R446 infrared sensitive photomultiplier (Strickland and Parsons, 1972). Inoculated experimental samples of *P-C* were weighed on a glass filter using a MicroMettler scale immediately prior to chlorophyll a analysis, because the cytoplasm dehydrates after excision of the agar. Inoculated *P-C* specimens were prepared for fluorometric analysis using the same procedures for

Chlorella above. The longevity of *Physarum* monocultures, *Chlorella* monocultures, and inoculated *P-C* cultures were recorded.

3. Results

The pattern of uptake of *Chlorella* by the *Physarum* was predictable and reproducible for every specimen. Ultrastructural analysis, using light and epifluorescence microscopy and TEM included several phases: 1) the initial endocytic uptake of *Chlorella*, by the advancing *Physarum* plasmodia edge, resulting in the rapid "greening" of the plasmodial fan-shaped edge; 2) subsequent enclosure of *Chlorella* into a single membrane endosomal or "perialgal" vacuole: 3) translocation of the green *Chlorella* cells into the venation network of the *Physarum*, as evidenced by movement of the *Chlorella* within distinctly green veins of the host; and 3) exocytosis and exhabitation of some of the *Chlorella* on the slime tracks of the plasmodia or deposited on top of the *Physarum* sclerotia.

During initial stages of engulfment, the pseudopodia-like strands of the advancing edge of the *Physarum* plasmodia surrounded the *Chlorella* cell and encapsulated it within a single membrane endosomal vacuole and then subsequently transported the *Chlorella* within the plasmodia cytoplasm. Fig. 1A shows the initial stages of engulfment of the *Chlorella* by plasmalemma invaginations. Peripheral membranous capsules, produced by elaboration of the host plasma membrane, enclose algal cells outside the main cytoplasmic body. Subsequently, the *Chlorella* cell is enclosed in a single membrane endosomal or "perialgal" vacuole (Fig. 1B). Typically, the *Chlorella* chloroplastic thylakoids lacked grana and contained numerous starch grains. The *Chlorella* (containing typical cellular organelles of the alga and chloroplasts without grana) encapsulated within a single membrane perialgal vacuole had an electron dense cell wall and cell membrane as compared to free-living algae. Fig. 2 shows that "greening" time for the entire plasmodium (resulting from the uptake of *Chlorella* by *Physarum*) occurred on the average of 5 days after inoculation. However, it was repeatedly observed that the "greening" time of the advancing edge of the plasmodia, which spread over the algal inoculation, was much shorter and accomplished within 2–4 hours after the algal cells were placed in the dish.

There was a significant difference ($p<0.05$) between the mean survival time of *Physarum* monocultures (5.96 days) and inoculated *Physarum-Chlorella* (*P-C*) cultures (17.3 days). Fig. 3 illustrates the cumulative percent survival of *Physarum* monocultures (N=75) and inoculated *P-C* cultures (N=53). In contrast to the inoculated *P-C* cultures, *Chlorella* monocultures survived only 2 weeks without nutrient enrichment. Additionally, there was no significant

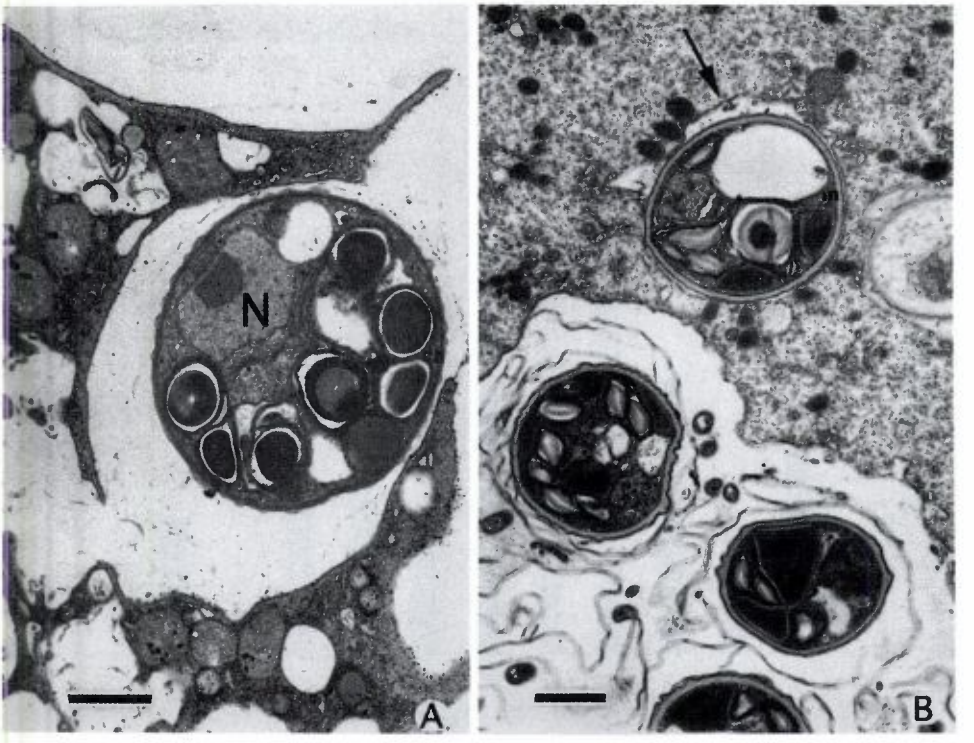

Figure 1. Engulfment of *Chlorella* by white plasmodial *P. polycephalum*. **A**. Early stage of engulfment of *Chlorella* by *Physarum* plasmalemma invaginations. The cytoplasm of *Physarum* contains mitochondria, lipids and vacuoles and the *Chlorella* cell shows a nucleus, nucleolus, starch grains, and chloroplast. Scale bar = 1 μm. **B**. Membraneous system at the periphery of *Physarum* cytoplasm showing algae encapsulated by membranous compartments outside the host cytoplasm, produced by the elaboration of the *Physarum* plasma membrane, and *Chlorella*, showing a thickened cell wall, which has been engulfed and encapsulated into a perialgal vacuole within the host cytoplasm. Scale bar = 1 μm.

difference, using a t-test ($p<0.05$), between the mean length of survival time of the *P-C* cultures that were fed (oats) or those unfed and no significant difference ($p<0.05$) between inoculated *P-C* cultures retained in a culture room with 12 h L/D alternating cycle or in continuous light. The fluorometric analysis included the calculation of a mean acid ratio (indicating the extent of chlorophyll that is intact versus degraded and an estimate of the amount of chlorophyll a which is initially present compared to degraded phaeopigments) that was based upon a ratio of a fluorometric reading before and after acid addition. A mean acid ratio of 1.71 for the *P-C* system ($N=12$) was significantly higher ($p<0.05$) than

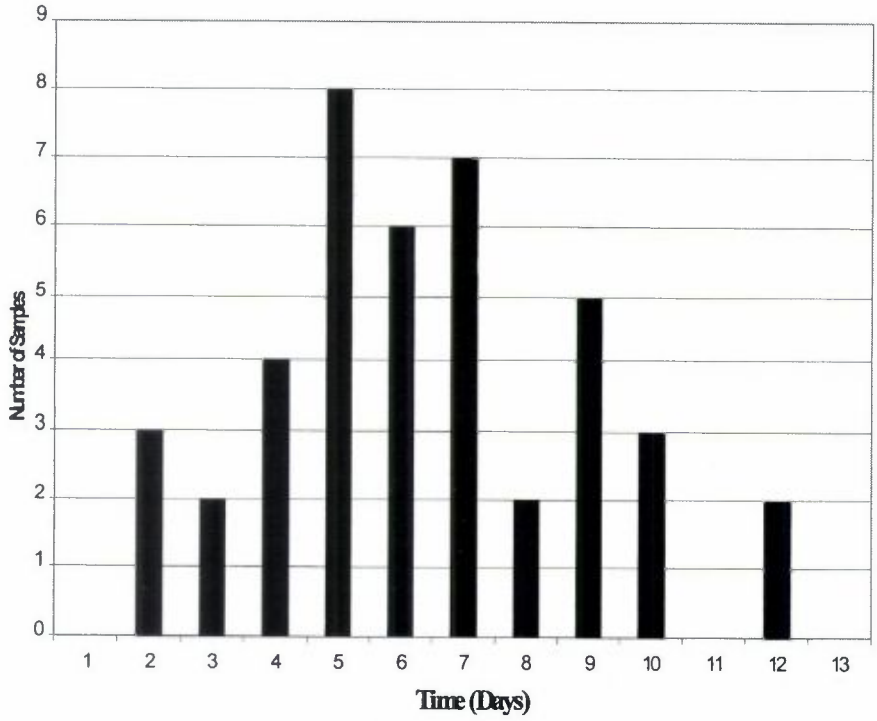

Figure 2. Greening times of *Physarum-Chlorella*.

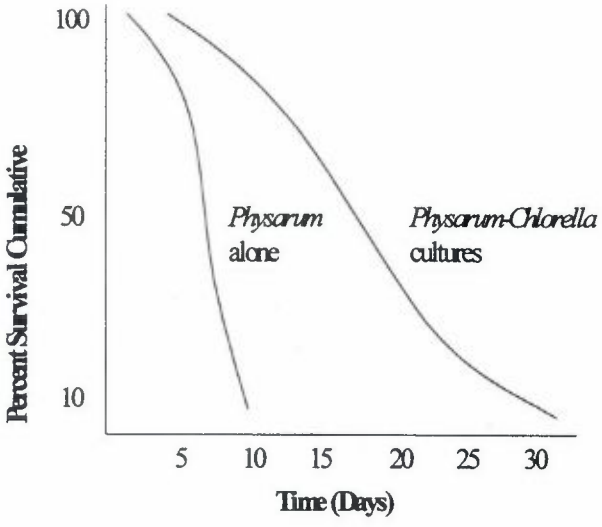

Figure 3. Percent survival of *Physarum* monocultures (N=75) and inoculated *Physarum-Chlorella* cultures (N=53).

the mean acid ratio of 1.57 for the *Chlorella* monocultures (N=12). A high acid ratio for the *P-C* system provided added evidence that the *Chlorella* population of cells were viable and intact inside the host as opposed to severely senescent or digested by the host.

Light microscopy revealed that the green venation of the plasmodia, due to the presence of green algae, connected dense intracellular green areas (of algae) within the main cytoplasmic body of the plasmodium and that the sizes of the green *Chlorella* areas within the host plasmodia differed. For example, five-day inoculated *P-C* showed a size range of green plasmodial areas from 5 mm to 10 mm in diameter. Seven-day inoculated cultures averaged from 6 mm to 11 mm in diameter and the 15-day inoculated cultures had many distinct green veins traversing the Petri dish with dimensions up to 35 mm in length by 25 mm in width. *Chlorella* cells were exocytized by the plasmodia on the agar in the Petri dish in a dense patch. In some cases, it appeared that not all *Chlorella* cells were exocytized as evidenced by the green plasmodia. However, all the *Chlorella* cells were exocytized in the event of the transformation of the plasmodia to sclerotia. The plasmodia were observed to migrate both away from the exocytized *Chlorella* cells and back onto the same algae often engulfing them several times. In some fed *P-C* cultures, the plasmodia appeared to spend equal time between the food patch and the exocytized *Chlorella* deposits. Transfers of green *P-C* cultures survived a week or more without the addition of oats. At the end of 10 days, no *Physarum* monocultures survived whereas at least 89% of the inoculated *P-C* cultures survived.

4. Discussion

The results show a clear association of *Chlorella* with *Physarum* for substantial periods of time even if temporary, and includes evidence of the 1) fairly rapid cycle of uptake of *Chlorella* by *Physarum* plasmodia, sequestration of algae within endosomal vacuoles, the movement of green *Chlorella* cells into the venation network of the plasmodia, and the eventual exocytosis of some of the green algal cells; 2) the stability (only living organisms or cells exhibit viability) of chlorophyll a in the intracellular and exocytized *Chlorella*; and 3) the increased longevity of the *P-C* system, either in fed or unfed cultures, as compared to either protist monoculture. *Physarum* monocultures did not survive individually over 7 days unless they were transferred, while inoculated *P-C* cultures survived an average of 2–3 weeks longer, even if unfed. Because there was no significant difference in survival time between fed and unfed *P-C* cultures, this may indicate that inoculated cultures survive equally well whether or not a constant food source is provided.

These results appear contrary to early experiments (Lazo, 1961) that had

unsuccessful trials in the inoculation of *Chlorella* with several species of slime molds, including *P. polycephalum*. While we used white *Physarum* plasmodium, and the white plasmodia of *P. didermoides* and *Fuligo cinera* similarly exhibited algal uptake (Lazo, 1961), it may be that the unsuccessful trials with *P. polycephalum* in the Lazo study were because yellow plasmodia may have caused some chemical interference (e.g., ptyridine pigments) with the light-capturing ability of the algae. However, our results were similar to Lazo's study (1961) in that 1) new transfers of a green section of older plasmodia maintained their green color; 2) the *P-C* cultures in this study and those of Lazo (1961), using *P. didermoides* and *F. cinera* took approximately 3–5 days to turn entirely green; and 3) the association of plasmodia-algae appears to grow better than plasmodia and/or algae alone. Similar to Lazo's conclusion, it is premature to say that a permanent "symbiosis" occurred, but the results demonstrate how rapidly host-algal associations can be established under favorable conditions.

The fluorometric analysis showed that the engulfed *Chlorella* cells appear to be largely intact and functional. If the *Physarum* had been fed oats within 24 hrs of introduction, movement towards the algae appeared slower. The *Chlorella* cells appeared to be in close association with the *Physarum* host subsequent to enclosure in perialgal vacuoles. However, this relationship is temporary because 1) the host may consume some of the cells; 2) the host exocytizes algae; and 3) the host may uptake some of the exocytized cells and begin the cycle again.

DeBary's use of the term "symbiosis" first connoted a "living together" of living organisms regardless of mutual benefit (Lewin, 1982). However, the term now implies an evolutionary association, which may vary in diversity (e.g., parasitism, commensalism, mutualism) (Ahmadjian, 1983; Goff, 1982) and specifically, a "...physical association (the holobiont) of members of different species of organisms (the bionts) for significant portions of the life history..." (Margulis, 1980; 1993). Margulis (1993) indicates that in the case of "loose symbioses", where environmental conditions may stress the growth of one of the bionts, the association is not likely to continue. For example, lichens, comprised of fungi and algae bionts, may not be "obligate symbionts" (requiring physical contact with the other throughout the life history) (Margulis, 1993). As discussed by Margulis (1993), researchers Ahmadjian and Paracer (1986) showed that changes in specific environmental "macro-or microclimates" and factors (e.g., nutrients, moisture, temperature) surrounding the lichen-forming fungi may lead to establishment and/or dissolution of these relationships. In this study, the induced association between the algae and slime mold is very different from the naturally occurring symbioses in the lichen, which represents a well-established evolutionary symbiosis. However, the transient and

unstable *P-C* association may elucidate some of complexities of natural symbiotic processes that occurred over evolutionary history.

While some researchers consider transient associations as symbioses and scale the process in terms of a continuum of necessity or benefit to the organisms (Starr, 1975), others emphasize the duration (a significant length of time) and stability (regulation) of the relationship in defining symbiosis (Smith and Douglas, 1987). In considering criteria developed for symbiotic associations (Starr, 1975), an associant does not have to remain alive for the relationship to be symbiotic. But Margulis (1993) stresses the duration of the relationship and states that the symbiotic association must occur throughout a major portion of the life history of the bionts. In applying these diverse definitions of symbiosis to the *P-C* system, the *P-C* relationship was maintained for a significant portion of both holobionts life cycle (after Margulis) and *Chlorella* can survive after exocytosis from the *Physarum* plasmodia and even after the host transforms from plasmodium to sclerotia (Starr, 1975).

In terms of other criteria, relative spatial and temporal relationships (Starr, 1975) of the association can be defined by 1) the persistent and cyclic movement of the unfed plasmodia back and forth upon the exocytized algae, including re-engulfment (inhabitational) and exocytosis of algae (exhabitational) (after Schmitz and Kremer, 1977); and 2) the transitory stages including the uptake, endocytosis, transport, and eventual exocytosis of the *Chlorella* by the *Physarum* host. Margulis (1993) also defines symbiosis in terms of similar spatial and temporal aspects. The spatial relationship of the *P-C* system could be considered "facultative" (both partners can complete their life histories in the absence of the other) and the temporal relationship could be considered "cyclical" because the physical association between the bionts is "...periodically established and disestablished..." (Margulis, 1993). Margulis (1993) further states that "...symbioses can form and later dissolve ... stability [of the relationship] is, in part, a function of time and the intensity of environmental selective pressure...". In addition, if the larger biont (e.g., *Physarum*) is threatened, it may digest the symbiont (usually the smaller biont). Potential environmental stressors to the relationship and metabolic relationships, especially metabolites (e.g., carbohydrate), need to be further explored in the *P-C* system.

Because symbiotic relationships may involve benefit (Starr, 1975), the longer survivorship of the *P-C* system as opposed to the survival of the *Chlorella* or *Physarum* monocultures may indicate a benefit of increased survivorship of the relationship as opposed to the free-living organisms. The increased surviorship of the *P-C* relationship, even though the host may predecease the exocytized algae, is consistent with Starr's (1975) expanded definition of symbiosis where both associants are living in the initial stage but may be followed by the death of one of the associants. This finding is also consistent with the Margulis (1993)

definition of the cyclical temporal symbiosis. The *P-C* association is also consistent with Margulis's (1980) definition of symbiosis that includes a consistent association existing for different stages of the life cycle of each species. The fluorometric analysis provides an interesting comparison of a potentially facultative-dependent and/or commensalistic association between these two protists because the chlorophyll acid ratios are higher in the *P-C* system, with lower degraded products, than in pure cultures of *Chlorella*. While other benefits of the *P-C* association may be that the nitrogenous exudates of the *Physarum* may benefit the algae and that the algal production of carbohydrates through photosynthesis may benefit the host, this physiological and/or potentially "commensalistic" relationship needs to be further investigated. Studies have shown that the benefit of the *Chlorella* symbiont to hosts is primarily nutritional and the host organisms receive significant amounts of maltose from the algal cells (e.g., mannitol is the metabolite provided by *Chlorella* to *Hydra viridis*, Margulis, 1993) which may increase the survival of hosts during periods of limited food supply (Smith and Douglas, 1987). The benefit to the *Chlorella* symbiont in well-established symbioses appears to be that a colonized host may provide habitat and possibly, because the transfer of photosynthate from algae to fungi has been well studied in lichen symbiosis (Smith and Douglas, 1987), the relationship may provide a better supply of nutrients than available to free-living algae. Because the experimental synthesis of a lichen from holobionts has been difficult (Smith and Douglas, 1987), the *P-C* experimentally-induced association may be important in terms of understanding the establishment of more evolutionarily advanced symbioses.

Regardless of potential initial host predation and digestion, the *P-C* association is not contraindicative of a potential symbiosis (Goff, 1982) because 1) the emphasis is on survival potential of the population (e.g., longevity of the *P-C* system) involved rather than the individual cells; and 2) the outcome of the *P-C* association clearly indicated a significant benefit, in terms of longevity, to both populations. The significant results include: 1) ultrastructural evidence of endocytosis and sequestration of algal cells; 2) viability of *Chlorella* cells located inside the host and; 3) significant increases in longevity of the *P-C* system as compared to their free-living counterparts. The *P-C* association is marked by a specificity by the host's organization of *Chlorella* into clustered membranous arrays at the periphery of the main cytoplasmic plasmodia and subsequent sequestration in perialgal vacuoles originating from the host plasma membrane. Perhaps this compartmentalization provides a location for the algae and prevents overpopulation within the host cytoplasm.

Survival of *Chlorella* within the plasmodia also needs to be assessed. Altering the fusion of the host phagosome (containing lysosomes) membrane

and/or developing of a thicker or more resilient cell wall are well known strategies of algal symbionts (Karakashian, 1975; Dyson 1979) and may be employed by *Chlorella* sp. It was observed that some cell walls of fully endocytized *Chlorella* appeared more electron dense (or thicker) than *Chlorella* cells undergoing initial engulfment. The cell wall of *Chlorella* sp. is very resistant because it has three layers consisting of cellular microfibrils and a 14 nm trilaminar layer, consisting of a polymerized carotenoid substance similar to sporopollenin, outside the cell wall which may prevent digestion by the host (Dodge, 1973). The dense cell wall in the endocytic *Chlorella* may represent an initial modification of the *Chlorella* to avoid host digestion.

In addition, evidence of algal reproduction (symbiont reproduction during the host's synchronous mitosis) after sequestration in endosomal vacuoles needs to be assessed in order to determine whether the association could be potentially genetically dependent. Further studies should also investigate the capability of *Physarum* to take up other algal species.

5. Conclusions

Over the last 20 yrs since the initial experimental work with *Chlorella* and slime mold species (Lazo, 1961), this is the first study to provide a detailed analysis of an experimentally induced association between two protists that does not occur in nature. The *P-C* system provides a model to study fundamental factors related to the initial stages of the establishment of a symbiotic association. TEM evidence confirms the endocytic uptake of *Chlorella* by *Physarum* and the subsequent intracellular organization of the algae by the host into perialgal vacuoles. This engulfment of *Chlorella* directly into perialgal vacuoles appears to be fundamentally different from the engulfment of food particles that are initially, at least, taken up in canal-like invaginations on the undersurface of the plasmodium (Anderson, 1993). Fluorometric determination of chl a showed that the *Chlorella* within the *P-C* system were viable and had higher acid ratios than uninoculated *Chlorella* which further corroborates that the *Chlorella* are intact and the chlorophyll were not degraded. The increased longevity of the *P-C* system is clearly a benefit to both protists and shows a close and complex association.

In conclusion, the *P-C* system provides a unique and convenient laboratory system, which can be used to examine many aspects of symbiosis and cell biology. Further studies are needed to determine if there is translocation of photosynthates to the host as suggested by our survival data for unfed cultures harboring the *Chlorella*, and to identify the chemical composition of the photosynthates translocated from algae to host. Additional studies should include an evaluation of the extent of algal cell division once encapsulated, a

biochemical assay conducted for sporopollenin, which may be used as a criterion factor in the classification of potential symbionts, and an analysis of reinfection of aposymbiotic hosts with different species of algae.

Acknowledgements

This is Lamont Doherty Earth Observatory of Columbia University Contribution Number 6358. We are grateful for the advice and assistance of Dr. Elizabeth Cosper and Dave Boardman of Lamont Doherty Earth Observatory, Dr. Isobel Contento of the Department of Nutrition at Columbia University Teachers College, Ramapo College of New Jersey for the use of the TEM microscope, especially Dr. Charles Holt of MIT for providing the rare white plasmodia species of *Physarum*, and to Catherine Gastrich who assisted with cultures.

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