Green Paramecia as an Evolutionary Winner of Oxidative Symbiosis: A Hypothesis and Supportive Data

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A single cell of the green paramecia (*Paramecium bursaria*) harbors several hundreds of endo-symbiotic *Chlorella*-like algae in its cytoplasm. Removal of algae from the host organism and re-association of ex-symbiotic host paramecia with ex-symbiotic algae can be experimentally demonstrated in the laboratory. However, the mechanism precisely governing the alga-protozoan association is not fully understood, and the origin of symbiosis in the evolutionary view has not been given. Here, we propose the possible biochemical models (models 1 and 2) explaining the co-evolution between *Paramecium* species and algal symbionts by pointing out that algal photosynthesis in the host paramecia plays a dual role providing the energy source and the risk of oxidative damage to the host. Model 1 lays stress on the correlation between the (re)greening ability of the paramecia and the tolerance to oxidative stress whereas model 2 emphasizes the cause of evolutionary selection leading to the emergence of *Paramecium* species tolerant against reactive oxygen species.

Key words: Green Paramecia, Oxidative Stress, Symbiosis

Introduction

Some algal species grow symbiotically in various invertebrates and protozoa (*e.g.* hydra and paramecia) and represent a type of association that is commonly referred to as symbiosis, resulting from the complex communications between hosts and symbionts (Reisser, 1992). Symbiotic associations are good models for studying the cell-to-cell interactions, mechanism of immunity, and evolution of eukaryotic cells (Gerashchenko *et al.*, 2000).

A single cell of the green paramecia (*Paramecium bursaria*) harbors several hundreds of *Chlorella*-like endo-symbiotic algae (ESA) in its cytoplasm (Reisser, 1980). By receiving the photosynthetically produced sugars from ESA, the host paramecia survives for months without feeding any food organism (Kosaka, 1991). During co-habitation, ESA cells are protected from the severe environmental changes and the algae benefit nitrogenous nutrition from the host organism which feeds various microbes (Kosaka, 1994; Kadono *et al.*, 2004a). A recent study has shown that the algal cell cycle in *P. bursaria* is highly governed by the cell cycle of the host cells (Kadono *et al.*, 2004b). However, the mechanism required for establishment and maintenance of this plant-protozoan symbiosis is not fully understood, and a reasonable explanation in the evolutionary view has not been provided to date. In this article, we like to propose the possible biochemical explanations for the co-evolutionary steps which allow *Paramecium* species to communicate and maintain the symbiosis with *Chlorella*-like algae.

Hypothesis

Among paramecia, only *P. bursaria* possesses ESA. Acquisition of ESA by apo-symbiotic host cells can be experimentally reproducible using the independently cultured artificially prepared apo-symbiotic hosts and algae (Nishihara *et al.*, 1998). Recently it has been shown that such apo-symbiotic white cells of *P. bursaria* seldomly but surely emerge in nature, without lacking the ability of re-association (lasting for some generations) with experimentally added algae (Tonooka and Watanabe, 2002). When mixed up, the host cells take up the algae into cytoplasm very rapidly. Non-symbiotic paramecia (*P. trichium*, *P. caudatum*, and *P. aurelia* complexes) also takes up the algae when experimentally co-incubated with *Chlorella* species or ex-ESA isolated from *P. bursaria*, but the algae fail to retain in the pseudo-host cells since

such non-symbiotic paramecia immediately excretes or digests the algae.

As *P. bursaria* occasionally excretes ESA when exposed to stresses such as heat (Kosaka T., unpublished results), we assume, by analogy, that failure to maintain algae in non-symbiotic paramecia is due to stressful intracellular conditions created by the algae, and thus *P. bursaria* must be tolerant to such alga-oriented stresses. Here, we hypothesize that the reactive oxygen species (ROS) produced by the algae causes such stressful conditions in the paramecia (Fig. 1A). Supporting this view, enhanced excretion of ESA by the *P. bursaria* host cells can be observed when the production of ROS due to the photosynthetic apparatus in ESA is enhanced by addition of a non-lethal level of paraquat (Nishihara *et al.*, 1998; Tanaka *et al.*, 2002).

In green plants, the photosynthetic production of H_2O_2, superoxide and singlet oxygen proceeds through photorespiration, Mehler reaction and photodynamic actions, respectively (Asada, 1999). It is known that the cells and organelles in green plants (Asada, 1999) and green algae such as *Chlorella vulgaris* (Takeda *et al.*, 1998), *Chlamydomonas reinhardtii* (Takeda *et al.*, 1997), and *Euglena gracilis* (Ishikawa *et al.*, 1997) are rich in ROS-detoxifying enzymes and antioxidants, thus protected from photochemically produced ROS. In ESA, the ROS production may proceed via similar paths, but ESA may excrete membrane-permeable ROS (most likely H_2O_2), as many other algae do, so that ROS levels in the algal cells are readily lowered to the safe level (Patterson and Myers, 1973; Collen *et al.*, 1995; Palenik and Morel, 1991; Ishikawa *et al.*, 1993). In turn, the alga-harboring hosts are internally exposed to ROS, possibly resulting in cellular damages. Thus the alga-hosting organisms such as *P. bursaria* must be ROS-tolerant so that they could have survived the evolutionary selection. Therefore it is tempting to speculate that the ROS-tolerancy in *P. bursaria* is much higher than that in non-symbiotic paramecia.

Experimental

Used ciliates were *P. bursaria* green strains [KSK-103 (syngen 1, mating type IV), MB-1 (syngen 1, mating type I), US-2 (syngen 1, mating type I), EZ-22 (syngen 1, mating type IV], *P. bursaria* apo-symbiotic white strain derived from KSK-103, MB-1, and US-2 (KSKw, MBw-1, and USw-2, respectively), *P. bursaria* regreened strain derived from KSK-103 (KSKr), *P. caudatum* (strains KY-1 and TA-2), and *P. trichium* (strain NJ-1). The algae used were ESA clones (SA-1 and SA-2) isolated from *P. bursaria* (Nishihara *et al.*, 1998) and free living *Chlorella kessleri* (C-531; Institute of Applied Microbiology, culture collection, University of Tokyo). Although *Chlorella kessleri* (C-531) is a free-living *Chlorella* species, it has been shown that apo-symbiotic cells of *P. bursaria* take up the cells of C-531 algae and the symbiosis was readily established under artificial conditions (Gerashchenko *et al.*, 2000). The photosynthetic flagellate used in this study was *Euglena gracilis.*

Ciliates and flagellates were statically cultured under white fluorescent light (*ca.* 2000 lux) in lettuce infusion inoculated with *Klebsiella pneumoniae* as a food, as described by Kosaka (1991). *Chlorella* species and ex-ESA were cultured in CA liquid medium with agitation on shakers, under white fluorescent light (*ca.* 2000 lux) as described by Nishihara *et al.* (1998).

In this study, the viability of algae, ciliates and flagellates was tested in the presence of H_2O_2 (12 h-treatment) for obtaining the primary data for discussing the hypothesis. Since H_2O_2 is the only ROS which moves in and out of bio-membranes (Kawano *et al.*, 2000), the exogenously applied H_2O_2 may be delivered into the host cytoplasm and mimics the action of ROS photosynthetically produced and excreted from ESA.

Results

As expected from the reported data for H_2O_2-tolerance in *Chlorella vulgaris* (Takeda *et al.*, 1998), *Chlamydomonas reinhardtii* (Takeda *et al.*, 1997), and *Euglena gracilis* (Ishikawa *et al.*, 1997; Takeda *et al.*, 1992), the photosynthetic organisms used in this study such as free-living *Chlorella*, ex-ESA and *E. gracilis* showed high H_2O_2-tolerance. Similarly, *P. bursaria* was shown to be the most ROS-tolerant among the paramecia tested.

E. gracilis was highly H_2O_2-tolerant (LD_{50} 1.8 mM). The algal viability was assessed by microscopy and flow cytometry as described by Gerashchenko *et al.* (2000, 2001). Both *Chlorella* and *Chlorella*-related ex-ESAs showed high tolerance to H_2O_2 (LD_{50} 0.3–0.6 mM). The viability of paramecia was assessed under microscopes. *P. caudatum* (strains KY-1 and TA-2) and *P. trichium*

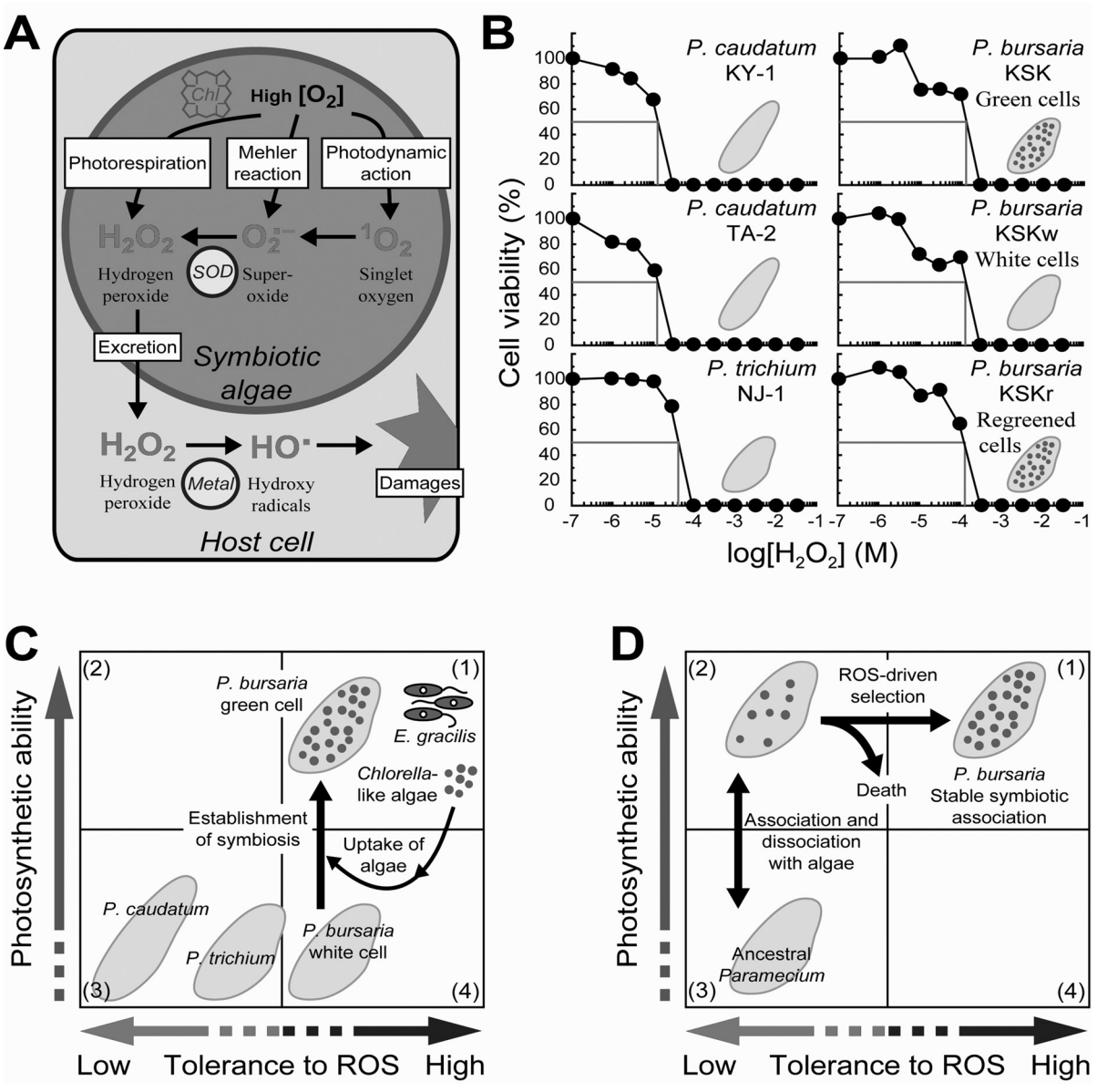

Fig. 1. Possible mechanism of symbiosis in *P. bursaria*. (A) Algal ROS production in *P. bursaria*. Singlet oxygen, superoxide and H_2O_2 are photosynthetically produced. H_2O_2 may be excreted by endo-symbiotic algae and highly reactive hydroxy radicals may be formed via H_2O_2 and damage the host cells. (B) *Paramecium* viability in H_2O_2. Data for *P. caudatum* (KY-1 and TA-2 strains), *P. trichium* (NJ-1 strain), and three cell lines derived from *P. bursaria* KSK-103 strain are shown. (C) Model 1: Emergence of ROS-tolerant paramecia enabled the symbiosis with algae. (D) Model 2: Cohabitation of the ancestral paramecia with algae was followed by the selection of ROS-tolerant species. The quadrants in (C) and (D) are numbered.

(strain NJ-1) were highly sensitive to H_2O_2 so that cells burst out in the presence of H_2O_2, while *P. bursaria* (strain KSK-103) was shown to be more tolerant towards H_2O_2. The LD_{50} values estimated for H_2O_2 in *P. caudatum*, *P. trichium*, and *P. bursaria* were *ca.* 12, 43 and 120 μM, respectively (Fig. 1B). Other strains of *P. bursaria* collected from different lakes or ponds, such as the MB-1

strain (syngen 1, mating type I), apo-symbiotic white cells of the MB-1 strain (MBw-1), US-2 strain (syngen 1, mating type I), apo-symbiotic white cells of the US-2 strain (USw-2), and EZ-22 strain (syngen 1, mating type IV) showed almost identical results with the KSK strain (syngen 1, mating type IV). Compared to *P. bursaria*, other non-symbiotic ciliates such as *Blepharisma japonicum* and *Euplotes* species were shown to be sensitive to H_2O_2 (LD_{50} 10–30 μM; data not shown). Interestingly, alga-free cells of *P. bursaria* (substrain, KSKw derived from KSK-103 green strain) and the cells of *P. bursaria* regained ESA (regreened substrain, KSKr) showed similar viability profiles to the naturally green cells, indicating that the presence of ESA has no contribution to the protection of host cells from the applied H_2O_2.

Discussion

Based on the results, we propose two models explaining the evolution of green paramecia. One model shows that ciliates with higher tolerance to ROS successfully acquire ESA (model 1; Fig. 1C), and the other model claims that the oxidative stress due to the primitive symbiosis with algae, loaded to ancestral green paramecia plays a key role in selection of ROS-tolerant species (model 2; Fig. 1D).

In both models, the freely living algae might have been taken up by the paramecia by chance, and then the majority of algae might have undergone immediate removal processes since the ancestral *Paramecium* species might dislike the presence of ROS-generating objects. The possibility for the algal settlement in *Paramecium* cytoplasm must be higher in the paramecia with higher tolerance to ROS. Therefore the regreening process of the ROS-tolerant cells of apo-symbiotic *P. bursaria* (white cells) in model 1 can be experimentally demonstrated with extremely high reproducibility (Nishihara *et al.*, 1998; Gerashchenko *et al.*, 2000). However, this model does not explain the driving force for emergence of ROS-tolerant *Paramecium* species, in the evolutionary timespan. Any single cell of ancestral paramecia might have successfully acquired the endo-symbiotic partners by chance and its descendants might survive the photosynthetic ROS-driven evolutionary selection for some eras, thus forming the ROS-tolerant host species with stable association with ESA as illustrated in model 2.

Model 1 lays stress on the correlation between the (re)greening ability of the paramecia and the tolerance to ROS whereas model 2 emphasizes the cause of evolutionary selection leading to emergence of ROS-tolerant *Paramecium* species.

There is another possible view that ESA derivatives causing less oxidative damages to the host might be selected in the course of co-evolution. However, this may not be true. It has been experimentally shown that the ex-symbiotic free host cells favor the chlorophyll-rich photosynthetically active free algae (but not the safer algae with less chlorophyll content) as partners for re-association of symbiosis (Gerashchenko *et al.*, 2000). The photosynthetic activity due to chlorophylls and the photo-oxidative stress are tightly related, and thus it is likely that the less photo-oxidatively active algae are less active in photosynthesis thus less attractive as the source of energy. In addition, the algal cell size is another factor favored by the hosts. Among 30 isolates of ex-ESA and other non-symbiotic *Chlorella* species, only three isolates with relatively large cells and extremely high chlorophyll contents were highly active in re-association with the host cells (Gerashchenko *et al.*, 2000). In conclusion, the host cells in *P. bursaria* favors the risky but highly beneficial symbiotic partner.

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