Chapter 16
Paramecium as a Model Organism for Studies on Primary and Secondary Endosymbioses

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Abstract Endosymbiosis is a driving force in eukaryotic cell evolution. This phenomenon has occurred several times and has yielded a wide diversity of eukaryotic cells. Despite the importance of endosymbiosis, however, molecular mechanisms for its induction between different microorganisms are not so well known. To elucidate these mechanisms, experiments for synchronous induction of the endosymbiosis by symbionts isolated from the symbiont-bearing host cells and the symbiont-free host cells are indispensable. Also, the infection process needs to be easily observable under a microscope. In many endosymbiotic communities, however, both the endosymbionts and the symbiont-free host cells have already lost the ability to survive and grow independently. Consequently, re-induction of the endosymbiosis was difficult. We have developed optimum experimental conditions for the induction of primary and secondary endosymbiosis using the ciliate *Paramecium* and their endosymbionts.

16.1 Introduction

The ciliate *Paramecium* species are valuable cells to study mechanisms for re-establishment of endosymbiosis, in that they frequently bear prokaryotic or eukaryotic (or both) endosymbionts. Most endosymbiotic bacteria of *Paramecium* species cannot grow outside the host cell because of their reduced genome size. Although the endonuclear symbiotic bacteria species *Holospora* are also unable to grow outside the host cell, they can maintain their infectivity to new host cells for a few days at room temperature even after isolation from the host cells (Fujishima

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et al. 1991). Although the host can acquire various stress resistances by infection of *Holospora*, this symbiont is not necessary for the host's survival. Consequently, re-establishment of endosymbiosis between the *Holospora*-free paramecia and *Holospora* cells isolated from the *Holospora*-bearing paramecia can be induced easily through the host active phagocytosis by mixing them. *P. caudatum* and *Holospora* species are model organisms for researches on the induction of primary symbiosis, because the endosymbiosis can be induced synchronously and whole processes of the re-establishment of endosymbiosis are observable under an ordinal light microscope. Furthermore, macronuclear genomes of *P. tetraurelia* (Aury et al. 2006), *P. caudatum* (McGrath et al. 2014) and draft genomes of three *Holospora* species (Dohra et al. 2013, 2014) were sequenced.

On the other hand, *P. bursaria* and *P. chlorelligerum* (Kreutz et al. 2012) has the ability to keep symbiotic *Chlorella* species in the cytoplasm among *Paramecium* species. Irrespective of the mutual relationship between *P. bursaria* and the symbiotic algae, both cells are still keeping the ability to grow independently, and the endosymbiosis can be re-established synchronously by mixing them. Kodama and Fujishima (2005) found four important cytological events needed for establishing endosymbiosis and their timings in the infection process by pulse-labeling of the alga-free paramecia for 1.5 min with the symbiotic algae isolated from the alga-bearing paramecia and then chasing at known times. *P. bursaria* and the symbiotic *Chlorella* cells also became model organisms for studying the induction of secondary symbiosis. The nuclear genome of the symbiotic *Chlorella variabilis* was sequenced (Blanc et al. 2010), and RNAseq analysis between *P. bursaria* with and without the algae has been done (Kodama et al. 2014). Thus, interactions between *Paramecium* and *Holospora* species and between *P. bursaria* and its symbiotic *C. variabilis* cells provide excellent opportunities to study control mechanisms for establishment of the primary and the secondary symbioses leading to eukaryotic cell evolution. In this chapter, we introduce recent studies on (1) how the symbiont invades the host cytoplasm, (2) how the symbiont can avoid digestion by the host's lysosomal enzymes, (3) how the symbiont can grow synchronously with the host cell, (4) how the host gene expressions are affected by the symbiont, and (5) what benefit the host cell receives which enables it to expand its ecological niche.

16.2 Induction of Re-establishment of Primary Endosymbiosis Between *Paramecium* and *Holospora*

The Gram-negative bacterium *Holospora* species are endonuclear symbionts of the ciliate *Paramecium* species (Fokin and Sabaneyeva 1997; Fokin and Görtz 2009; Fujishima 2009; Gibson et al. 1986; Preer 1969; Ossipov 1973; Ossipov et al. 1975, 1980; Skoblo and Lebedeva 1986) and belong to alfa-proteobacteria (Amann et al. 1991). Phylogenetically most related bacteria with *Holospora* is Rickettsia (Amann et al. 1991; Lang et al. 2005). *Holospora* species are usually found in paramecia

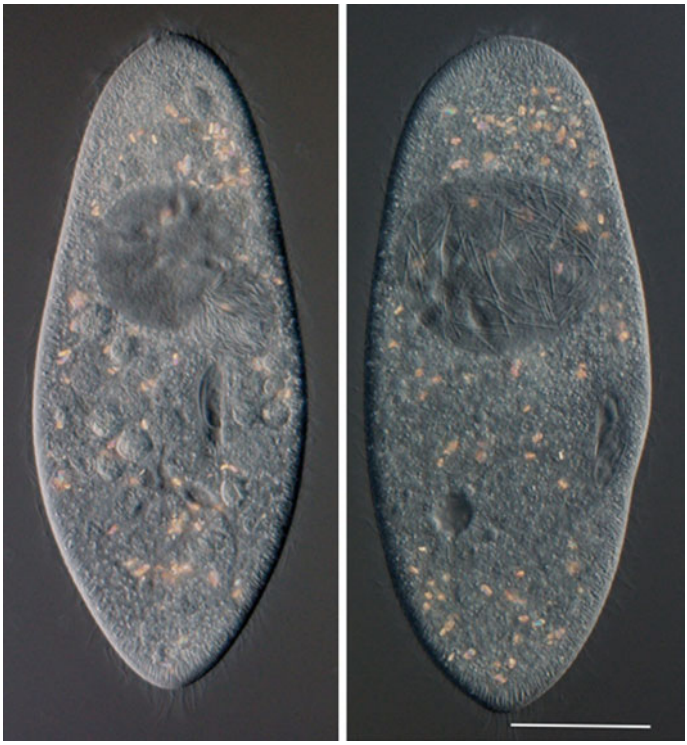

Fig. 16.1 Photomicrographs of *Holospora* in *Paramecium caudatum. Left H. undulata* in a micronucleus. *Right H. obtusa* in a macronucleus. *Bar* 50 μm

living in cold areas, such as northern Europe and the Kamchatka Peninsula (Fokin et al. 1996). To date, nine *Holospora* species have been described (Fokin et al. 1996). All show species-specificity and nucleus-specificity in their habitats (Fig. 16.1). They cannot grow outside the host cell with ordinary culture media because of their reduced genome (Dohra et al. 2013, 2014). *Holospora* species show two different forms in their life cycle: a reproductive short form (RF, 1.5–2 μm long) and an infectious long form (IF, 10–15 μm long) (Fokin et al. 1996; Fujishima et al. 1990b; Görtz 1980; Görtz et al. 1989; Gromov and Ossipov 1981). The bacterium exists as a short RF cell and divides by binary fission in the host nucleus when the host is growing. The RF stops dividing and differentiates into a longer IF cell through intermediate forms when the host cell starves (Fujishima et al. 1990a; Görtz 1983), or the host protein synthesis is inhibited (Fujishima, unpublished data). During this differentiation, the bacterium forms a distinctive structure, one-half of which contains the cytoplasm; the other half is a periplasmic lumen with an electron-translucent tip called as invasion tip (Dohra and Fujishima 1999; Fujishima and Hoshide 1988; Görtz 1980; Görtz and Wiemann 1989; Görtz et al. 1989; Iwatani et al. 2005). The IF cells engulfed into the host digestive vacuoles (DVs) escape with

the invasion tip ahead and penetrate the target nuclear envelope with this special tip (Fujishima and Fujita 1985; Fujishima and Kawai 2004; Görtz and Wiemann 1989). Under a phase-contrast microscope, the cytoplasmic region looks dark, but the periplasmic region looks as a refractile (Dohra and Fujishima 1999). In the macronucleus-specific *H. obtusa* of *P. caudatum*, the IF cells show clear two nucleoids (Fujishima et al. 1990a; Dohra and Fujishima 1999) stained with 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI). This bacterium changes the buoyant density, protein composition (Fujishima et al. 1990a), and surface morphology of the outer membrane (Fujishima et al. 1990b) during differentiation. When the host divides again, the IF cells of *H. obtusa* are collected in a connecting piece of the dividing nucleus and they are freed from the nucleus by wrapping with the nuclear membrane. They are eventually expelled from the host cytoproct (Wiemann 1989). On the other hand, the outer membrane of the RF has a stronger affinity to bind the host chromatin than the IF cells, so that the RF cells can remain in the daughter nuclei when the host divides (Ehrsam and Görtz 1999; Fokin et al. 1996; Görtz et al. 1992; Wiemann 1989). When the macronucleus is filled with so many infectious forms, the host cells cannot grow even in sufficient foods in the culture medium and eventually killed by the bacteria; the infectious forms are freed from the cells (Fujishima, unpublished observation). Consequently, the infectious forms appear outside the host cell by these two means and can then infect new host cells. A *Paramecium* cell has a limited life span. Therefore, *Holospora* species need to escape from the host to infect more young cells. For this reason, a different nature of the outer membranes of these two forms is indispensable for *Holospora*'s survival strategy.

The phenomenon of bacterial invasion into a target nucleus is designated as "infection", and stable growth of the infected bacteria in the nucleus is designated as "maintenance" (Fujishima and Fujita 1985). The infection is controlled by (1) engulfment of the IFs into the host DVs (Fujishima and Görtz 1983), (2) escape from the DV before the host's lysosomal fusion to appear in the host cytoplasm (Iwatani et al. 2005), (3) migration to the target nucleus by a help of the host actins (Fujishima 2009; Fujishima et al. 2007; Sabaneyeva et al. 2009), (4) recognition of a target nuclear envelope by a specific binding between *Holospora*'s outer membrane substance and their target nuclear envelope (Fujishima and Kawai 2004) and by a penetration of the host nuclear envelope with the invasion tip (Iwatani et al. 2005). On the other hand, the maintenance is controlled by the host genotypes (Fujishima and Mizobe 1988). Namely, infection and maintenance are independently controlled phenomena. The whole infection process occurs within 10 min (Fujishima and Görtz 1983). To date, the only organism having an ability to distinguish a somatic macronucleus from a germinal micronucleus of the host *Paramecium* species is *Holospora* species. Thus, these bacteria can recognize some differences between the two kinds of the host nuclei originated from a common fertilization nucleus and timing of the nuclear differentiation (Fujishima and Görtz

1983). After infection, *Holospora* alters the host gene expressions (Hori and Fujishima 2003; Hori et al. 2008; Nakamura et al. 2004), and the host acquires various stress resistances (Fujishima et al. 2005; Hori and Fujishima 2003; Hori et al. 2008; Smurov and Fokin 1998).

16.2.1 Genome of Holospora

Draft genome sequences have been determined in three *Holospora* species of *P. caudatum*; a macronucleus-specific *H. obtusa* and micronucleus specific *H. undulate* and *H. elegans* (Dohra et al. 2013, 2014). Among these three *Holospora* genomes, assembly lengths and GC% varied from 1.27 to 1.40 Mbp and 35.2–36.1 %, respectively (Dohra et al. 2014). The FASTORTHO program (http://enews.patricbrc.org/fastortho/) grouped a total of 3553 protein-coding sequences from the three *Holospora* species genomes into 1610 ortholog clusters, of which 572 were identified as single-copy core orthologous genes shared by the three genomes. Of the 572 *Holospora* core genes, 488 (85.3 %) were assigned to at least one of the cluster of orthologous groups (COGs). The 46 genes were assigned to multiple functional categories; for example, type II secretory pathway proteins were assigned to the COG category of cell motility and of intracellular trafficking, secretion, and vesicular transport.

The cytoplasmic endosymbiotic bacterium *Polynucleobacter necessarius* of the ciliate *Euplotes aediculatus* possesses glycolysis/gluconeogenesis, citrate cycle (TCA cycle), and pyruvate metabolism pathways for energy production (Boscaro et al. 2013). However, The *Holospora* genomes lacked many proteins involved in these pathways, indicating that *Holospora* species strongly depend on the host for energy production (Dohra et al. 2014).

16.2.2 How Does Holospora *Invade the Host Cytoplasm and Migrates to the Target Nucleus?*

Life cycle of *Holospora* is shown in Fig. 16.2. When IF long form of *H. obtusa* cells isolated from the symbiotic host *P. caudatum* cells by Percoll density gradient centrifugation are mixed with the aposymbiotic hosts, the bacteria are soon ingested into the host DVs. The DVs of *P. caudatum* can be classified into four different stages according to Fok and Allen (1988) in *P. multimicronucleatum*. The IF cells ingested in DV-I vacuole escape there by destruction of the DV membrane while the DV-I vacuole is acidified by acidosomal fusion and becomes a condensed DV-II vacuole (Fujishima 2009). In the presence of vacuolar-type ATPase (V-ATPase) inhibitors, concanamycin A, both the acidification of the DV and the bacterial escape from the DV are inhibited completely (Fujishima and Kawai 1997). These

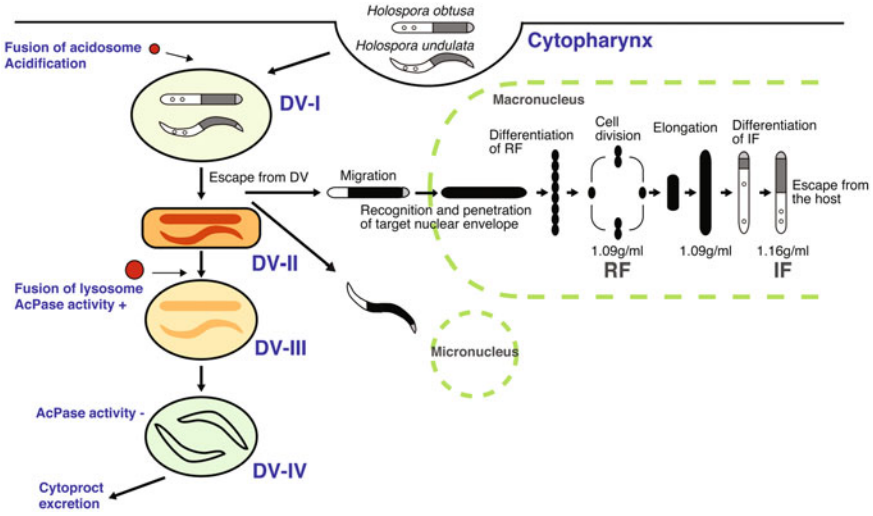

Fig. 16.2 The infection route and life cycle of *Holospora* species. Spherical DV-I vacuole differentiates to condensed and acidified DV-II vacuole by fusion of acidosomes and evagination of the DV membrane to cytoplasm. Then the vacuole differentiates to swollen DV-III vacuole by fusion of lysosomes. Undigested materials remain in the DV-IV vacuole. The DV-IV vacuole fuses to a cytoproct of the host cell and the contents are discharged. Some IF cells of *Holospora* escape from the acidified DV without wrapping with the DV membrane. By acidification of the DV, the bacteria differentiate to the activated forms, and migrate toward the target nucleus with a help of the host actin polymerization. The bacteria distinguish their target nucleus by specific binding between lipopolysaccharides of the outer membrane and the unknown nuclear envelope substance. Then, the bacterium penetrates the target nuclear envelope with an invasion tip leading. After the invasion, bacterial cytoplasmic region increases and large periplasmic region decreases to form constrictions for differentiation to the RFs. During this infection process, the bacterium decreases it buoyant density from 1.16 to 1.09 g/ml. The RF continues to divide by binary fission when the host cell is growing, but the RF halts the binary fission, elongates itself and differentiates to the IF when the host cell starves or the host's protein synthesis is inhibited. During this differentiation, the bacterium increases the buoyant density, forms a large periplasmic region, an invasion tip, and two nucleoids. The infectious forms are freed from the cells (see text). From Fujishima (2009)

results depicted that the acidification of the host DV is an indispensable phenomenon for the bacterial escape from the host DV. Bacteria in the host DVs just before the escape there and those appearing in the host cytoplasm are designated as an activated form (AF) cell. The AF cell looks darker than the IF cell under a phase-contrast microscope (Görtz and Wiemann 1989). The IF cell always escapes from the DV with the invasion tip ahead (Görtz and Dieckmann 1980; Iwatani et al. 2005), and penetrates the target nucleus with the invasion tip leading. We harvested the tips of the IF cells of *H. obtusa* from 3438 bacteria using a laser capture microdissection system (LM 100; Olympus), and loaded to SDS-PAGE. Then, three bands of 89, 76, and 63 kDa were detected by silver staining. Using proteins of 60–90 kDa extracted from the gel as antigens, we developed monoclonal antibodies (mAbs) against the 89 kDa protein in the invasion tip (Iwatani et al. 2005).

Indirect immunofluorescent microscopy and immunoblotting showed that this protein is specific for the invasion tip of *H. obtusa* but not with the RF cells. Subsequently, using partial amino acid sequence of the purified 89-kDa protein, a novel gene encoding the 89 kDa protein was cloned from genomic DNA. The open reading frame of the gene was 2253 nt long with a 32.5 % G + C content. The predicted amino acid sequence of the 89 kDa protein showed two transmembrane signal peptides at N-terminal and two actin-binding motifs near the N-terminal (Iwatani et al. 2005).

Indirect immunofluorescence microscopy with mAbs specific for the 89 kDa protein and the host actin 1–1 showed that the epitopes of the five kinds of mAbs against the 89 kDa protein were present in a lumen of the invasion tip of the IF cell. However, some epitopes translocate outside the bacterial outer membrane of the invasion tip when the IFs were engulfed into the host DVs. Bacterium appeared in the host cytoplasm kept the 89 kDa proteins outside the tip, and the host actins accumulated around the 89 kDa proteins immediately after the bacterial escape from the host DV. When the bacterium penetrated the host macronuclear envelope, a complex of the 89 kDa proteins and the host actins were left behind at the entry point on the nuclear envelope as a cylindrical structure (Iwatani et al. 2005; Fujishima 2009; Fujishima et al. 2007; Fujishima and Kodama 2012). Sabaneyeva et al. (2009) also observed similar actin-based *H. obtusa* motility. These results suggest that the 89 kDa protein and the host actin are responsible for the infection of *Holospora*. However, how the bacteria destruct the host DV membrane by the invasion tip, how the bacterium appeared in the cytoplasm knows a direction to the target nucleus, how the bacterium penetrates the target nuclear envelope, and what is the moving force to push the bacteria from nuclear envelope to the inside the nucleus are unknown.

16.2.3 How Can Holospora *Distinguish Their Target Nucleus in Infection Process?*

To know how the *Holospora* recognizes two kinds of the host nuclei, a macronucleus and a micronucleus, mAbs specific for outer membranes of IF cells of *H. obtusa* and *H. undulata* were developed respectively. When the antigens extracted from the SDS-PAGE gels were mixed with freshly isolated nuclei of *P. caudatum,* indirect immunofluorescence with the mAbs showed that the antigens bound with nuclear envelopes of their target nuclei (Fujishima and Kawai 2004). Namely, outer membrane substances of *H. obtusa* bound only to the macronuclear envelope of *P. caudatum*, and those of *H. undulate* bound with the micronuclear envelope. These antigens are resistant against proteinase K and can be stained neither with Coomassie Brilliant Blue R-250 nor by an ordinary silver stain. However, the bands of the antigens were stained with silver for bacterial lipopolysaccharide (LPS). This indicates that their outer membrane substances are

LPSs (Fujishima and Kawai 2004). These results show that the bacterial recognition of their target nuclei is controlled by a specific binding between the LPS of the IF cell and an unknown receptor substance exposed on the target nuclear envelope.

When the IF cells of *H. obtusa* and exconjugants of *P. caudatum* at various stages were mixed, the infectability against *H. obtusa* was acquired by four of the eight post-zygotic nuclei as soon as the four nuclei differentiated morphologically into macronuclear anlagen. Old macronuclear fragments were also infected. These results indicate that the nuclear envelope of the macronuclear anlagen exposes the receptor substance against *H. obtusa* LPS at almost the same time as the first recognizable change in the macronuclear anlagen, and that the receptor substance has been kept on the old macronuclear fragments (Fujishima and Görtz 1983).

The property of the macronucleus, necessary for it to be recognized and infected by *H. obtusa,* is commonly provided by *P. caudatum*, *P. multimicronucleatum,* and *P. aurelia* species but not by *P. bursaria, P. trichium* (=*P. putrinum*), *P. duboscqui* and *P. woodruffi*, although the bacteria can appear in the cytoplasm through the DVs (Fujishima and Fujita 1985; Fujishima 1986). *P. calkinsi, P. polycaryum* and *P. nephridiatum* also could not be infected by *H. obtusa* (Fujishima, unpublished data). Infectivity of *P. jenningsi* by *H. obtusa* was strain-specific (Fujishima, unpublished data). All strains of *P. caudatum*, *P. multimicronucleatum,* and *P. aurelia* species examined were infected by *H. obtusa*. However, stable maintenance of the infected *H. obtusa* in the host nucleus was achieved only in specific strains of *P. caudatum.* Species-specific infectivity of *H. obtusa* (Fujishima and Fujita 1985; Fujishima 1986) and phylogenetic tree of *Paramecium* species (Fokin et al. 2004; Hori et al. 2006; Kreutz et al. 2012) show that *H. obtusa* recognizes and invades the macronucleus of closely related species with *P. caudatum* (Fujishima 2009).

16.2.4 How Can Holospora *Avoid Digestion by the Host's Lysosomal Enzymes?*

It is known that intracellular symbionts or parasites use one of three strategies to survive against the host lysosomal digestion; (1) escape from the DVs before lysosomal fusion, (2) prevent fusion of the DVs with lysosomes, and (3) survive in the DVs even after the lysosomal fusion. In case of *Holospora*, they escape from the host DVs after acidosomal fusion but before the host lysosomal fusion (see Fig. 16.2). The IFs hole in the acidified DV membrane by their invasion tip and escape there without wrapping with the DV membrane (Fujishima, unpublished observation). Lysosomal fusion occurs 5–10 min after the DV formation in DV-III. The IFs could not escape from the DV-III to appear in the host cytoplasm. Therefore, only few IFs can escape from the DVs, and most of the IFs in the DVs are partially digested in the DV-III and discharged from a host cytoproct. On the other hand, RFs cannot escape from the host DVs and digested.

16.2.5 *How Does* Holospora *Grow in Well Accordance with the Host Growth?*

RF cells of *Holospora* continue binary fission in the host nucleus when the host is growing. However, the RF halts the binary fission and differentiates into an IF cell through intermediate forms when the host cell starves or host's protein synthesis is inhibited (Görtz 1983; Fujishima et al. 1990a). This suggests a possibility that RF cell is importing the host nuclear proteins for their growth and for keeping functions and morphology of the RF cell. Actually, 2D-SDS-PAGE showed more than 60 % of proteins of the RF and the IF cells of *H. obtusa* were different (Fujishima et al. 1990a). During this differentiation, *Holospora* changes nature of their outer membrane. The outer membrane of the RF cells has a stronger affinity to bind the host chromatin than the IF cells, so that the RF cells remain in each daughter nucleus when the host cell divides (Ehrsam and Görtz 1999; Fokin et al. 1996; Görtz et al. 1992; Wiemann 1989). On the other hand, the IF cells are collected in a connecting piece of the dividing nucleus. Then, they are freed from the dividing nucleus by wrapping with the nuclear membrane, and eventually expelled from the host cytoproct (Wiemann 1989). Furthermore, when the macronucleus is filled with many IF cells, the host cannot grow and killed by the bacteria. Eventually, the IF cells appeared outside the host cell by these two means, and can infect new host cells. Because the host *Paramecium* cell has a limited life span, *Holospora* must escape from the host to infect young cells. For this reason, different natures of the outer membranes of the RF and the IF cells are indispensable for *Holospora*'s survival strategy.

16.2.6 *How Does* Holospora *Alter Host Gene Expressions by Infection?*

Differential display and reverse transcribed PCR analysis showed that *H. obtusa* alters multiple gene expression of the host after establishing endosymbiosis (Nakamura et al. 2004) including *hsp60* and *hsp70* gene of the host (Hori and Fujishima 2003). We found that a periplasmic 63 kDa protein of *H. obtusa* might be one of the causes for induction of the host's gene alteration (Abamo et al. 2008). The 63 kDa protein is an IF specific protein and presence in the periplasmic lumen except an invasion tip. Indirect immunofluorescence microscopy showed that not only the pre-existing but also a newly synthesized 63 kDa protein was secreted into the host macronucleus in early infection process (Abamo et al. 2008). A gene encoding the 63 kDa protein was cloned from genomic DNA of *H. obtusa*. This novel gene included 1644 nucleotides encoding a 547-amino acid sequence. Comparison between the deduced amino acid sequence and the N-terminal amino acid sequence of the 63 kDa protein purified from 2D-SDS-PAGE gels revealed that the protein was preceded by a putative signal peptide consisted of 24 amino

acids. Therefore, the mature protein comprises 523 amino acids with a predicted molecular mass of 62.151 kDa and a predicted pI of 8.92 (Abamo et al. 2008). Considering the amount of the 63 kDa protein secreted into the macronucleus and the fact that the fluorescence of the 63 kDa protein cannot be observed in the host cytoplasm, we can speculate that this protein might bind to the host DNA or chromatin and changes the host gene expression to the advantage of the bacteria as shown in pathogenic bacterium *Listeria monocytogenes* (Lebreton et al. 2011).

Alteration of the host's gene expression is a general phenomenon for endosymbiosis. Therefore, *Paramecium* and *Holospora* might serve as a good model system to elucidate the mechanism of the pathogen-induced alteration of the host's gene expression.

16.2.7 *What Kind of Benefit Does the Host Cell Receive by Infection of* Holospora*?*

Three types of *P. caudatum* cells (*H. obtusa*-free cells, reproductive form of *H. obtusa*-bearing cells and predominantly infectious form of *H. obtusa*-bearing cells) cultured at 25 °C were transferred to 4, 10, 25, 35 and 40 °C and their swimming velocities were measured by taking photomicrographs with two-second exposures. The *H. obtusa*-free cells almost ceased swimming at 4 and died soon at 40 °C, while the reproductive form-bearing cells still swam even at these temperatures. Predominantly infectious form of *H. obtusa*-bearing cells also swam though their swimming velocity was statistically slower than that of the reproductive form-bearing *P. caudatum* cells. Thus, *Holospora*-bearing *Paramecium* cells can acquire heat-shock resistance if the host bears RF cells (Fujishima 2009; Fujishima et al. 2005; Hori et al. 2008; Hori and Fujishima 2003). Furthermore, the *Holospora*-bearing paramecia become osmotic shock resistance (Smurov and Fokin 1998). Therefore, *Paramecium* cells become adapted to unsuitable environments for their growth by endosymbiosis with *Holospora* species. Actually, *Holospora*-bearing paramecia can be collected in brackish water.

16.3 Induction of Re-establishment of Secondary Endosymbiosis Between *Chlorella* Species and *P. bursaria*

P. bursaria can maintain several hundred endosymbiotic algae in their cytoplasm (Figs. 16.3a and 16.4a). Each symbiotic alga wrapped with a perialgal vacuole (PV) membrane (Fig. 16.3b), and attaches near the host cell cortex (Fig. 16.3a). The PV membrane has an ability for avoiding host lysosomal fusion (Gu et al. 2002; Kodama and Fujishima 2009b). The association of *P. bursaria* with the

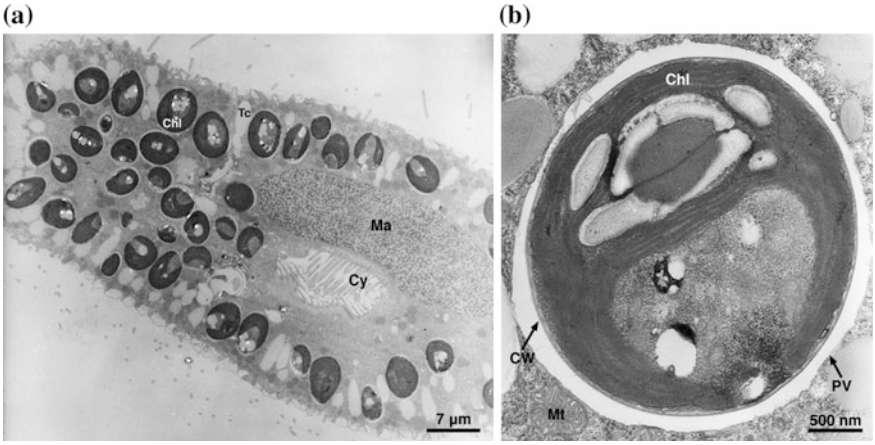

Fig. 16.3 Transmission electron micrographs of *P. bursaria.* **a** Algae-bearing *P. bursaria.* **b** Symbiotic alga near the host cell cortex. Each symbiotic alga wrapped with a PV membrane. *Chl* Chloroplast; *Cy* Cytopharynx; *CW* Cell wall; *PV* Perialgal vacuole; *Ma* Macronucleus; *Mt* Mitochondrion; *Tc* Trichocyst. From Kodama and Fujishima (2010a)

symbiotic *Chlorella* sp. is a mutualism. The host supplies the algae with nitrogen components and CO_2 (Reisser 1976, 1980; Albers and Wiessner 1985), and the host protects algae in the PVs from infection by the *Chlorella* virus (Kawakami and Kawakami 1978; Van Etten et al. 1985; Reisser et al. 1988; Yamada et al. 2006). Also, algal carbon fixation is enhanced in the host (Kamako and Imamura 2006; Kato and Imamura 2009). On the other hand, the symbiotic algae can supply the host with photosynthetic products, mainly maltose (Reisser 1976; Brown and Nielsen 1974; Reisser 1986). The algae in the host show a higher rate of photosynthetic oxygen production than in their isolated state, thereby guaranteeing an oxygen supply for the host respiration (Reisser 1980). Algae-bearing *P. bursaria* can grow better than the algae-free cells (Görtz 1982; Karakashian 1963, 1975); the algae have UV-protective role for the host (Hörtnagl and Sommaruga 2007; Summerer et al. 2009). Because timing of cell divisions of both the algae and the host cells is well coordinated, the symbiotic algae are transferred to the both daughter cells (Kadono et al. 2004; Takahashi et al. 2007).

Irrespective of the mutually beneficial relationships between *P. bursaria* and symbiotic algae, their relationship is facultative mutualism. Algae-free *P. bursaria* as shown in Fig. 16.4b can be easily produced from algae-bearing cells by rapid fission (Jennings 1938), cultivation in darkness (Karakashian 1963; Pado 1965; Weis 1969), X-ray irradiation (Wichterman 1948), treatment with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which is an inhibitor of photosynthesis (Reisser 1976), by treatment with the herbicide paraquat (Hosoya et al. 1995; Tanaka et al. 2002) or by treatment with cycloheximide (Kodama et al. 2007; Weis 1984; Kodama and Fujishima 2008). Furthermore, endosymbiosis between the algae-free *P. bursaria* cells and the symbiotic algae isolated from the algae-bearing

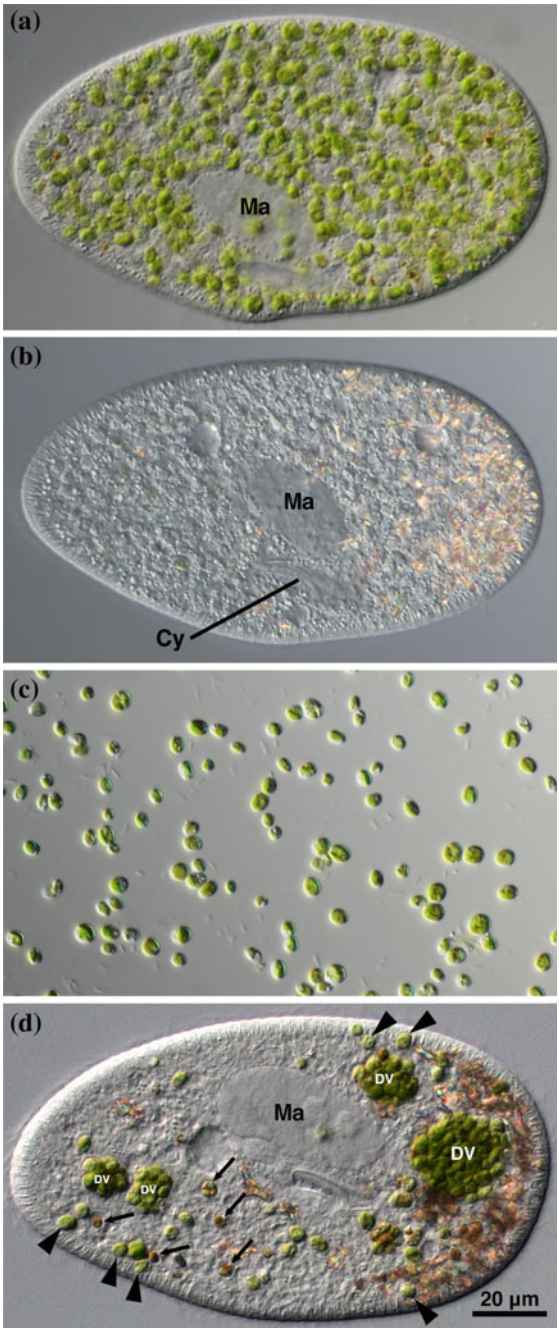

Fig. 16.4 Photomicrographs of *P. bursaria* and isolated symbiotic *Chlorella* sp. **a** Algae-bearing *P. bursaria* cell. **b** Algae-free *P. bursaria* cell. **c** Isolated symbiotic *C. variabilis* cells. **d** Algae-free *P. bursaria* cell, which was mixed with isolated symbiotic algae for 1.5 min, was washed and incubated for 3 h. Arrowhead shows green alga, which establishes endosymbiosis with algae-free cell. The alga localized immediately beneath the paramecium cell cortex. Arrows indicate digested brown alga. *DV* Digestive vacuole. Both green and digested algae appear in the cytoplasm as a result of the budding from the DV. *Ma* Macronucleus; *Cy* Cytopharynx. From Kodama and Fujishima (2012b). (Color figure online)

P. bursaria cells (Fig. 16.4c) is artificially re-established by just mixing them together (Siegel and Karakashian 1959; Karakashian 1975) (Fig. 16.4d). Therefore, the symbiotic associations between these eukaryotic cells are excellent models for studying cell-to-cell interaction and the evolution of eukaryotic cells through secondary endosymbiosis.

16.3.1 Classification of the Host DVs Appearing in Re-Establishment of Endosymbiosis with Chlorella

To understand the re-establishment route of symbiotic *Chlorella* cells, stages of DVs that appear during re-establishment of endosymbiosis were classified and the timing of the appearance of each stage was determined by mixing algae-free paramecia with the isolated symbiotic algae. The cells were mixed at a density of 5×10^3 paramecia per ml with isolated *Chlorella* sp. at 5×10^7 algae per ml in a centrifuge tube (volume, 10 ml) under a fluorescent light (20–30 μ mol photons m^{-2} s^{-1}) for 1.5 min at 25 ± 1 °C. The ciliate-algae mixture was transferred to a centrifuge tube equipped with a 15 μm pore size nylon mesh and filtered. By pouring 30 ml of fresh modified Dryl's solution (MDS, KH_2PO_4 was used instead of $NaH_2PO_4 \cdot 2H_2O$) (Dryl 1959) into this tube, the paramecia were washed and algal cells outside the paramecia were simultaneously removed through the mesh. The paramecia retained on the mesh were harvested and transferred to a centrifuge tube and resuspended in 1 ml of MDS, and then chased for various times under a fluorescent light at 25 ± 1 °C. Aliquot of the cell suspension was fixed by 4 % paraformaldehyde (PFA) at various time points, and the cells were observed under a differential-interference-contrast (DIC) microscope (Kodama and Fujishima 2005).

The DVs observed during the algal infection process were classified into eight different stages on the basis of their morphologies and on changes in algal color and pH in the DVs (Fig. 16.5). The DV-I vacuole has a rounded vacuole membrane containing only green algae. Its membrane is clearly visible under a DIC microscope. DV-II has a reduced size and the vacuole membrane barely visible; the algae are green. In DV-III, the vacuole has increased in size, making its membrane visible; the algae are discolored–either faint yellow or green, or both. The DV-III stage is further classified into three substages: DV-IIIa contains green algae only; DV-IIIb contains both faint yellow and green algae, and DV-IIIc contains faint yellow algae only. In the final stage, DV-IV, the vacuolar size is again reduced, as in DV-II, rendering the vacuole membrane barely visible under a DIC microscope; the algae are green or brown, or both. This vacuole was observed in cells after 20–30 min. DV-IV was also further classified into three sub-stages: DV-IVa contains green algae only; DV-IVb contains both green and brown algae, and DV-IVc contains brown algae only. DVs containing single green *Chlorella* (SGC) were observed in cells fixed 30 min after mixing, but all SGCs present in cells before 30 min after mixing were digested for 30 min (Kodama and Fujishima 2005).

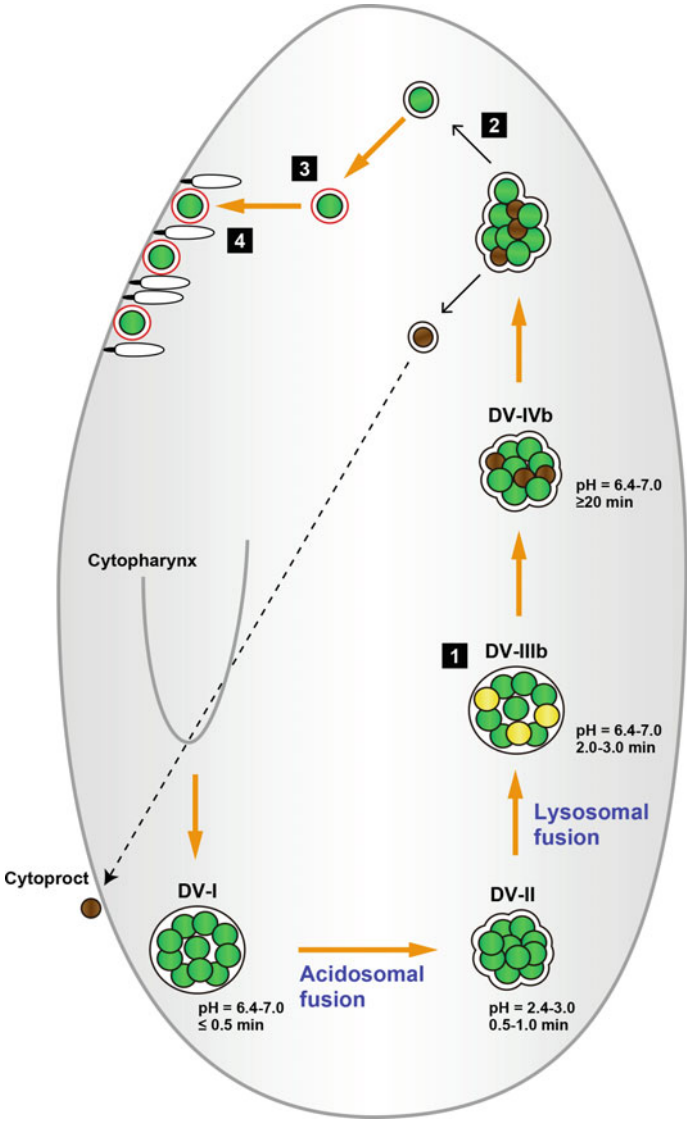

Fig. 16.5 Schematic representation of the algal reinfection process. Using pulse label and chase method, four important cytological events necessary to establish endosymbiosis were clarified. About 3 min after mixing with algae-free *P. bursaria* cells and isolated symbiotic algae, some algae acquire resistance to the host lysosomal enzymes in the DVs. About 30 min after the mixing, the algae start to escape from the DVs as a result of the budding of the membrane into the cytoplasm. About 45 min after the mixing, the DV membrane enclosing SGC differentiates to the PV membrane, which provides protection from lysosomal fusion. Then, the SGC localizes beneath the host cell cortex. About 24 h after the mixing, the SGC starts to increase by cell division and establishes endosymbiosis. Modified from Kodama and Fujishima (2005)

16.3.2 Four Important Events in Re-establishment of Endosymbiosis

Four important cytological events needed for establishing endosymbiosis and their timings in the infection process were clarified on the basis of the DV stages as described above (Kodama 2013; Kodama and Fujishima 2005, 2007, 2008, 2009a, b, c, 2010a, b, 2011, 2012a, b, 2014; Kodama et al. 2007, 2011). These four cytological events are described below.

16.3.2.1 Event One

After the lysosomal fusion to the DVs, some algae show temporary resistance to the host's lysosomal enzymes in the DV-IIIb and DV-IVb, even when the digested ones coexist. This phenomenon depends on photosynthetic activity of the isolated algae before mixing with *P. bursaria*. When the isolated algae were kept constantly under dark (DD) conditions for 24 h, almost all algae were digested in the DV. The detailed results were shown in Sect. 16.3.3. Thus, it can be said that the symbiotic algae do not prevent acidification and lysosomal fusion of the host's DV during the re-establishment of endosymbiosis (Kodama and Fujishima 2005).

16.3.2.2 Event Two

Thirty minutes after the mixing algae-free *P. bursaria* and isolated symbiotic algae, the algae start to escape from DV-IVb vacuoles as the result of budding of the membrane into the cytoplasm. Both living and digested algae bud from the DVs of *P. bursaria* (Kodama and Fujishima 2005). *Saccharomyces cerevisiae* cells and polystyrene latex beads of a diameter of 3 μm or greater were able to bud, too (Kodama and Fujishima 2012b). However, this budding is not observed when India ink, 0.81 μm diameter polystyrene latex beads, or food bacteria (*Klebsiella pneumonia*) were ingested into the DVs (Kodama and Fujishima 2005). These results suggest that *P. bursaria* can recognize the diameter of the contents of the DVs, and that those with a diameter of about 3 μm or greater can escape from the DV by the budding of the DV membrane. Because Dynasore, a dynamin inhibitor, greatly inhibited DV budding, dynamin might be involved in this process.

16.3.2.3 Event Three

After the budding from the DV-IVb vacuole, the DV membrane enclosing SGC differentiates into the PV membrane, which provides protection from lysosomal fusion (Kodama and Fujishima 2005, 2009a, b). To understand the timing of differentiation of PV from the host DV, algae-free *P. bursaria* cells were mixed with

isolated symbiotic algae for 1.5 min, washed, chased, and fixed at various times after mixing. Then, lysosomal enzyme, acid phosphatase (AcPase) activity in the vacuoles enclosing the algae was detected using Gomori's staining (Gomori 1952). This activity appears in 3 min-old vacuoles; all DVs containing algae demonstrate the activity at 30 min. Algal budding from the DVs begins at 30 min as described above. In the budded membrane, each alga is surrounded by a layer of Gomori's thin positive staining. The vacuoles involving a SGC move quickly and attach immediately beneath the host cell cortex. The first SGC and the first attachment of the SGC beneath the host cell cortex, respectively, occur at 30 and 45 min after mixing. These results suggest that differentiation of the PV membrane occurs within 15 min after the algal budding from the host DV (Kodama and Fujishima 2009c). We have succeeded in developing monoclonal antibodies (mAb)s specific for the DV membrane of *P. bursaria*. These mAbs do not react with the PV membrane, which containing SGC(s). This indicates that both membranes are substantially different (Fujishima and Kodama, unpublished data).

16.3.2.4 Event Four

The SGC(s) wrapped by the PV membrane localize beneath the host cell cortex (Kodama 2013; Kodama and Fujishima 2005, 2011, 2013). Both many trichocysts and mitochondria also localize in this area (Fujishima and Kodama 2012). Gomori's staining showed that the AcPase activity is low in this area (Kodama and Fujishima 2008, 2009b). These observations reflect the possibility that the PV membrane might have no capability for protection from lysosomal fusion, but can avoid lysosomal fusion by binding to the mitochondoria, trichocysts or unknown structures near the host cell cortex to localize at the area of the cell where primary lysosomes are usually missing. To confirm this possibility, preexisting trichocysts beneath the host cell cortex were removed from *P. bursaria* cells through treatment with lysozyme, thereby reducing the AcPase activity-negative area and exposing the PVs to the AcPase activity-positive area, and examined whether the PV's protection from the lysosomal fusion is still achieved or not. The trichocyst-free cell reduced the AcPase activity-negative cortical layer to less than 3 μm depth at the dorsal cortex. However, even if a part of the algal cell had been exposed in the AcPase activity-positive area, the algae were able to attach beneath the host cell cortex and to protect it from lysosomal fusion (Kodama and Fujishima 2009b). This is the first evidence to demonstrate that the PV membrane can give protection from host lysosomal fusion, and that the PV membrane does not require trichocysts for intracellular localization, because the PV membrane could localize the trichocyst-free cell cortex. This result suggests the possibility that the mitochondria anchor the PV membrane near the host cell cortex (Kodama and Fujishima, unpubl. data). Schematic representation of algal reinfection process and four important events in re-establishment of endosymbiosis is summarized in Fig. 16.5.

16.3.3 Algal Resistance to the Host Lysosomal Enzymes

During the algal infection process, the first hurdle for the algae is acquisition of resistance to the host's lysosomal enzymes in the DV as the event one (Kodama and Fujishima 2005). In the event one, some of the algae are not digested in DVs that had been fused with the host lysosomes even in the presence of others that are being digested in a same DV (Figs. 16.6 and 16.7). This differential fate of algae in the same DV is not an inherent property of the algae because this phenomenon occurs

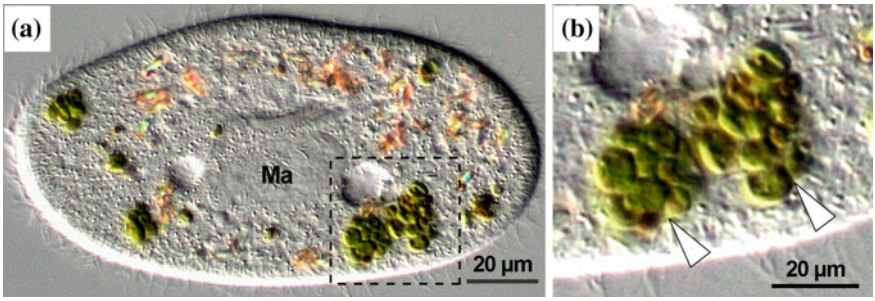

Fig. 16.6 Photomicrograph of algae-free *P. bursaria* 1 h after mixing with isolated symbiotic algae. **b** Shows highly magnified images of the square enclosed area in **a**. As shown by the white arrowhead in **b**, some algae were not digested even if coexisted with the digested brown ones in the same DVs after lysosomal fusion. *Ma* Macronucleus. From Kodama and Fujishima (2010b)

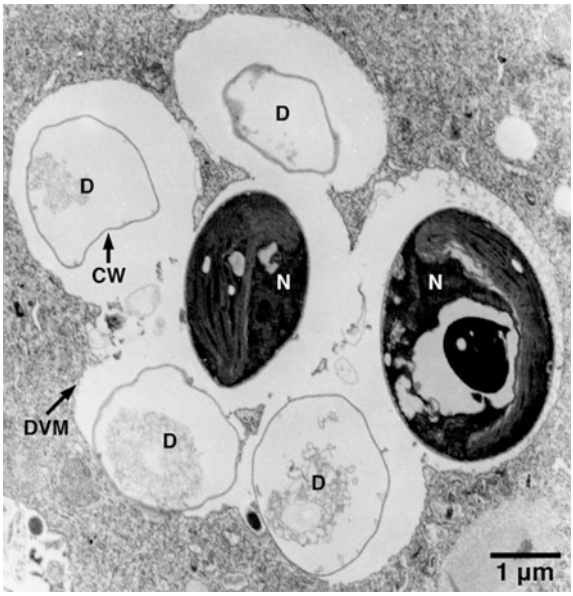

Fig. 16.7 Transmission electron micrograph of a DV-IVb. Three hours after mixing with algae and algae-free *P. bursaria* cells, algae-ingested cells were fixed for transmission electron microscopy. Partially digested (D) and nondigested (N) algae are enclosed in the same DV. The nondigested algae are not separated from the digested algae by a membrane representing a PV membrane. DVM, DV membrane; *CW* Cell wall. From Kodama and Fujishima (2010a)

even with clonal symbiotic algae. Furthermore, this algal fate is independent of the algal cell cycle stage and location of the algae in the DV. Moreover, this resistance to digestion is not related to the algal protein synthesis (Kodama et al. 2007). Gu et al. (2002) showed that degeneration of the symbiotic *Chlorella* under DD conditions is induced by the host lysosomal fusion to PV membranes. This report suggests that the photosynthetic activity and/or related cellular processes of the algae play important functions in protection from the lysosome fusion to the PV membrane (Kodama and Fujishima 2014).

16.3.3.1 Effects of Various Treatments of Isolated Symbiotic *Chlorella Variabilis* Before Mixing with Algae-Free *P. bursaria*

Most of the isolated symbiotic *C. variabilis* incubated under constant light (LL) conditions for 24 h were able to resist digestion in the host DV. The undigested algae then bud from the DVs, and the algae localized beneath the host cell cortex to establish endosymbiosis with algae-free *P. bursaria* cells as shown above and Fig. 16.8a (Kodama and Fujishima 2005, 2012a, b, 2014). However, by incubation of isolated symbiotic algae under DD conditions for 24 h before mixing with the host cells, most of the algae lost the capability of resistance to the host lysosomal enzymes in the DV. Only a few algae are able to avoid digestion and could be localized beneath the host cell cortex after budding from the DVs (Fig. 16.8b, arrowheads). We looked for morphological differences of the vacuole in LL-incubated, LL-incubated with photosynthesis inhibitor DCMU, and DD-incubated algae by staining with LysoSensor Yellow/Blue DND-160 (LysoSensor) (Fig. 16.9). In live cells, LysoSensor accumulates in acidic vacuoles of plant cells (Swanson et al. 1998), and exhibits predominantly yellow fluorescence. As presented in Fig. 16.9b, several

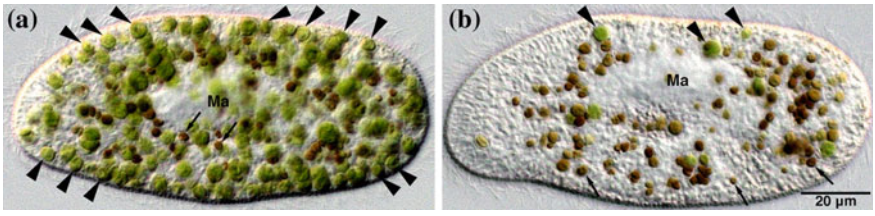

Fig. 16.8 Photomicrographs of algae-free *P. bursaria* cells after mixing with LL- (**a**) or DD- (**b**) incubated algae for 24 h. Both cells were mixed and kept under LL or DD conditions. As shown in (**a**), many LL-incubated algae showed resistance to the host lysosomal enzymes, and the undigested green algae localized beneath the host cell cortex (**a**, *arrowheads*). On the other hand, most of the DD-incubated algae were digested, and the algal color changed from green to brown (**b**, *arrows*). Few algae were able to avoid digestion and establish endosymbiosis (**b**, *arrowheads*). These results show that the algal incubation under LL conditions before ingestion by the alga-free *P. bursaria* cells is necessary to prevent algal digestion. Arrowhead, undigested SGC(s) localized beneath the cortex; arrow, digested brown alga; *Ma* Macronucleus. From Kodama and Fujishima (2014)

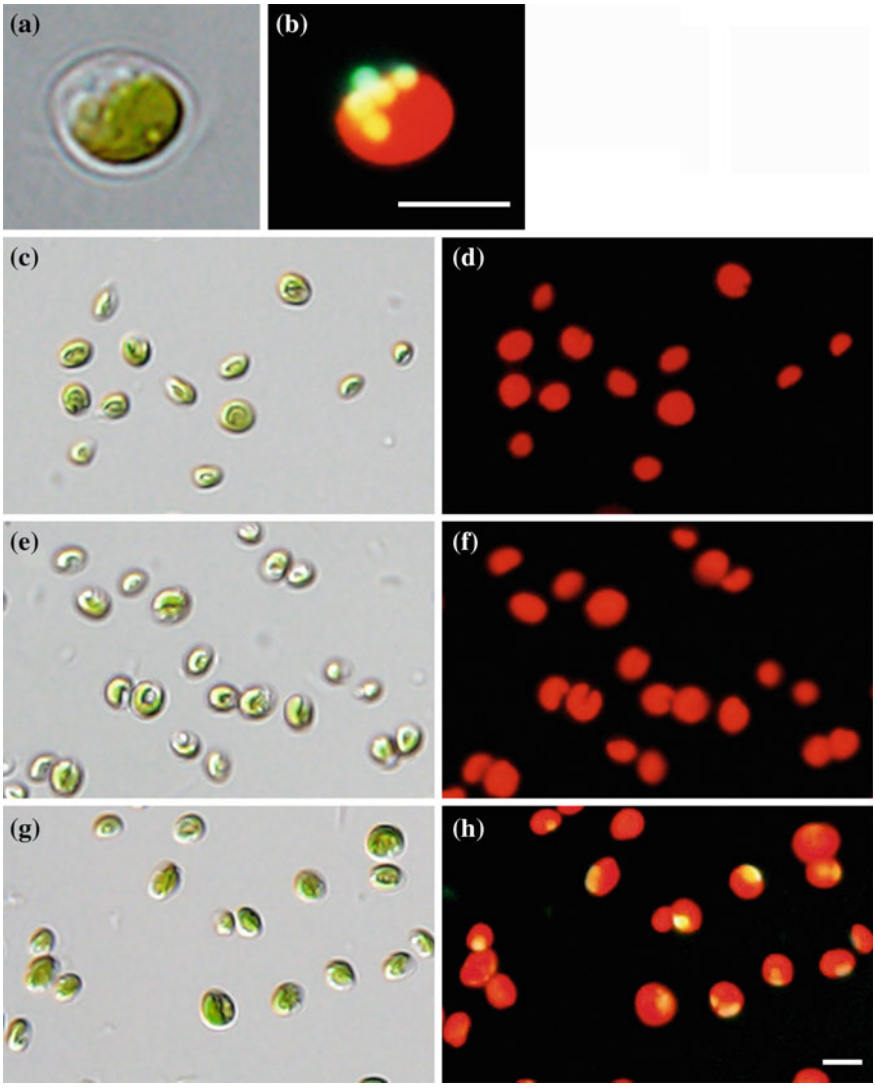

Fig. 16.9 DIC photomicrographs of LL-incubated (**c**), LL-incubated with 10^{-5} M DCMU (**e**), and DD-incubated (**a** and **g**) algae, and fluorescence photomicrographs of LysoSensor-treated LL-incubated (**d**), LL-incubated with 10^{-5} M DCMU (**f**), and DD-incubated (**b** and **h**) isolated symbiotic algae. LysoSensor accumulates in acidic vacuoles, and shows yellow fluorescence (**b**). These figures show that the DD-incubated algae have many yellow fluorescence vacuoles (**h**), more than those incubated under LL conditions with (**f**) or without (**d**) DCMU. The red color shows chlorophyll autofluorescence in the chloroplast. *Scale bars* 5 μm. From Kodama and Fujishima (2014). (Color figure online)

small spherical vacuoles with yellow fluorescence were observed in the algae. No differences in the algal color, shape or volume in LL-incubated (Fig. 16.9c), LL-incubated with 10^{-5} M DCMU (Fig. 16.9e), or DD-incubated (Fig. 16.9g) algae were observed using DIC microscopy. However, fluorescent microscopy clearly revealed that the number of vacuoles in the DD-incubated algae (Fig. 16.9h) increased more than those in algae incubated under LL conditions with (Fig. 16.9f) or without (Fig. 16.9d) DCMU. Kuchitsu et al. (1987) reported that the number of the vacuoles increases in the algal cells at the stationary phase of growth compared with the cells in the log phase of growth. Furthermore, it has been shown that the vacuole volume becomes extremely large after a long period of sugar starvation in the plant cell (Yu 1999). Taken together, algal starvation induced by the inhibition of photosynthesis under the DD conditions might be a cause of the vacuole development. Although the reason why the alga with the vacuole is digested preferentially in the host DV remains unknown, our results suggest that whether the algae are digested or not in the host DVs can de determined by staining the algae with LysoSensor. Figure 16.10 shows schematic representation of the algal digestion patterns in the DVs after the various treatments before mixing with algae-free *P. bursaria.* Our results show that a few of the algae were able to establish endosymbiosis with algae-free *P. bursaria* cells when the algae were incubated under DD conditions.

16.3.4 Transcriptome Analysis Between Algae-Free and -Bearing P. bursaria *Cells*

Despite the importance of *P. bursaria-Chlorella* sp. endosymbiosis as shown above, genomic resources had not been identified for *P. bursaria*. Therefore, we compared gene expressions through RNA-Seq analysis and de novo transcriptome assembly of algae-free and algae-bearing host cells (Kodama et al. 2014). To expedite the process of gene discovery related to the endosymbiosis, we have undertaken Illumina deep sequencing of mRNAs prepared from algae-bearing and algae-free *P. bursaria* cells. We assembled the reads de novo to build the transcriptome. Sequencing using Illumina HiSeq 2000 platform yielded 232.3 million paired-end sequence reads. Clean reads filtered from the raw reads were assembled into 68,175 contig sequences. Of these, 10,557 representative sequences were retained after removing *Chlorella* sequences and lowly expressed sequences. Nearly 90 % of these transcript sequences were annotated by similarity search against protein databases. Hsp70 and glutathione S-transferase (GST) genes were up-regulated and down-regulated as shown by the positive and negative values of logFC, respectively, in algae-bearing cells compared to algae-free cells (Table 16.1). Of the 10,557 unigenes, 8 were annotated as Hsp70 with logFC of −1.3 to 5.6, with a median of 0.92.

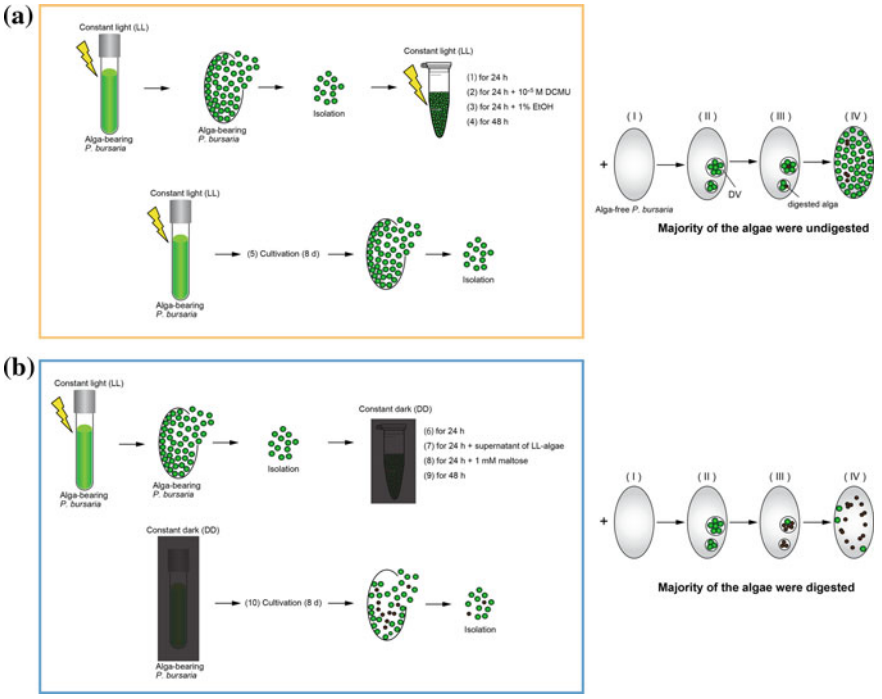

Fig. 16.10 Schematic representation of the algal digestion patterns in the DVs. Under LL conditions (**a**), the isolated symbiotic algae from LL-incubated algae-bearing *P. bursaria* cells were incubated for 24 h (*1*), for 24 h with 10^{-5} M DCMU (*2*), for 24 h with 1 % ethanol (EtOH) (*3*), for 48 h (*4*), and for 8 days (*5*) after (*1–4*) and before (*5*) isolation from algae-bearing *P. bursaria* cells. After mixing with algae-free *P. bursaria* cells (**a** *I*), some algae were ingested from the host cytopharynx and were enclosed in the DVs (**a** *II*). After the lysosomal fusion with the DVs, few algae were digested (*brown alga* in **a** *III*), but most of the algae showed resistance to the host lysosomal enzymes and were not digested (*green algae* in **a** *III*). Finally, most of the algae ingested in the DVs were able to establish endosymbiosis with algae-free *P. bursaria* cells (**a** *IV*). On the other hand, under the DD conditions (**b**), the isolated symbiotic algae from LL-incubated algae-bearing *P. bursaria* cells were incubated for 24 h (*6*), for 24 h with supernatant of LL-incubated algae (*7*), for 24 h with 1 mM maltose (*8*), for 48 h (*9*) and for 8 days (*10*) after (*6–9*) and before (*10*) isolation from algae-bearing *P. bursaria* cells. After mixing with algae-free *P. bursaria* cells (**b** *I*), some algae were enclosed in the DVs as with the LL-incubated algae (**b** *II*) as shown in (**a** *II*). After the lysosomal fusion, most of the algae were digested (*brown alga* in **b** *III*) and a few algae showed resistance to the host lysosomal enzymes (*green alga* in **b** *III*). Most of the algae were digested and excreted from the host cytopharynx (**b** *IV*). From Kodama and Fujishima (2014)

16.3.4.1 Glutathione S-transferase

It is conceivable that photo-oxidative stress is greater in algae-bearing *P. bursaria* cells than in algae-free ones. To determine whether oxidative stress and UV-induced photo-oxidative stress are greater in algae-bearing *P. bursaria* cells than in algae-free ones, Hörtnagl and Sommaruga (2007) examined the level of

Table 16.1 Transcripts encoding glutathione S-transferase and heat shock 70 kDa protein in *P. bursaria*

Trinity transcript name	Annotation from the SwissProt database	logFC
Heat shock 70 kDa protein		
comp43044_c0	sp|P09446|HSP7A_CAEEL|Heat shock 70 kDa protein A OS = *Caenorhabditis elegans*	5.601
comp36402_c4	sp|P09446|HSP7A_CAEEL|Heat shock 70 kDa protein A OS = *Caenorhabditis elegans*	4.183
comp36402_c6	sp|P14834|HSP70_LEIMA|Heat shock 70 kDa protein (Fragment) OS = *Leishmania major*	1.975
comp36402_c1	sp|Q9S9N1|HSP7E_ARATH|Heat shock 70 kDa protein 5 OS = *Arabidopsis thaliana*	1.555
comp37280_c1	sp|P37899|HSP70_PYRSA|Heat shock 70 kDa protein OS = *Pyrenomonas salina*	0.287
comp43771_c0	sp|P09446|HSP7A_CAEEL|Heat shock 70 kDa protein A OS = *Caenorhabditis elegans*	–0.594
comp41901_c0	sp|Q9S7C0|HSP7O_ARATH|Heat shock 70 kDa protein 14 OS = *Arabidopsis thaliana*	–1.076
comp41912_c0	sp|F4JMJ1|HSP7R_ARATH|Heat shock 70 kDa protein 17 OS = *Arabidopsis thaliana*	–1.337
comp43044_c0	sp|P09446|HSP7A_CAEEL|Heat shock 70 kDa protein A OS = *Caenorhabditis elegans*	5.601
Glutathione S-transferase		
comp37410_c0	sp|P78417|GSTO1_HUMAN|Glutathione S-transferase omega-1 OS = *Homo sapiens*	–0.119
comp32377_c0	sp|Q9ZRT5|GSTT1_ARATH|Glutathione S-transferase T1 OS = *Arabidopsis thaliana*	–0.288
comp36943_c0	sp|Q9ZVQ3|GSTZ1_ARATH|Glutathione S-transferase Z1 OS = *Arabidopsis thaliana*	–0.748
comp37841_c0	sp|Q9ZRT5|GSTT1_ARATH|Glutathione S-transferase T1 OS = *Arabidopsis thaliana*	–0.851
comp36483_c0	sp|Q9ZRT5|GSTT1_ARATH|Glutathione S-transferase T1 OS = *Arabidopsis thaliana*	–1.557
comp35816_c1	sp|P78417|GSTO1_HUMAN|Glutathione S-transferase omega-1 OS = *Homo sapiens*	–1.564
comp36242_c0	sp|P16413|GSTMU_CAVPO|Glutathione S-transferase B OS = *Cavia porcellus*	–5.749

oxidative stress by assessing reactive oxygen species with two fluorescent probes (hydroethidine and dihydrorhodamine 123) by flow cytometry. Their results indicated that oxidative stress is higher in algae-free *P. bursaria* cells than in algae-bearing one. Our results showed that expression levels of GST genes in algae-free cells were down-regulated than that in algae-bearing cells (Kodama et al. 2014). This enzyme is related to protect cells from oxidative stress as shown by

McCord and Fridovich (1969), Veal et al. (2002), and our results agreed with the results of Hörtnagl and Sommaruga (2007).

16.3.4.2 Hsp70

Furthermore, it is known that *Paramecium* cell acquires heat-shock resistance by infection of endonucler symbiotic bacteria *Holospora* as shown above (Hori et al. 2008; Hori and Fujishima 2003), and osmotic-shock resistance (Smurov and Fokin 1998). Hori and Fujishima (2003) found that *H. obtusa*-bearing paramecia expressed high levels of *hsp70* mRNA even at 25 °C. Algae-bearing cells show a higher survival ratio against 0.5 mM nickel chloride, high temperatures (42 °C), and 150 mM hydrogen peroxide than the algae-free cells (Kinoshita et al. 2009; Miwa 2009). We found that most of isoforms of the *hsp70* transcripts showed up-regulation by algal infection (Kodama et al. 2014). This up-regulation may be related to the host's tolerance to environmental fluctuations.

16.4 Conclusion

Recently, we succeeded draft genome sequences of three *Holospora* species, *H. obtusa*, *H. undulata*, and *H. elegans* (Dohra et al. 2013, 2014). Furthermore, whole transcriptome analysis between algae-free and algae-bearing *P. bursaria* was succeeded (Kodama et al. 2014). We can expect that these data enable us to understand the molecular mechanisms for establishments of the primary and the secondary symbioses and for the host evolutionary adaptation to global climate change.

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