Mechanisms for Establishing Primary and Secondary Endosymbiosis in Paramecium

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Complete List of Authors:	Fujishima, Masahiro; Yamaguchi University, Joint Faculty of Veterinary Medicine Kodama, Yuuki; Shimane University, Institute of Agricultural and Life Sciences, Academic Assembly
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Mechanisms for Establishing Primary and Secondary Endosymbiosis in *Paramecium*

Masahiro Fujishima[a] & Yuuki Kodama[b]

a Joint Faculty of Veterinary Medicine, Yamaguchi University, Yoshida 1677-1, Yamaguchi 753-8515, Japan
b Institute of Agricultural and Life Sciences, Academic Assembly, Shimane University, Nishikawatsu-cho 1060, Matsue 690-8504, Japan

Correspondence
M. Fujishima, Joint Faculty of Veterinary Medicine, Yamaguchi University, Yoshida 1677-1, Yamaguchi 753-8515, Japan.
Telephone/FAX number: +81 83-933-5712; e-mail: fujishim@yamaguchi-u.ac.jp

ABSTRACT

Primary (eukaryote and procaryote) and secondary (eukaryote and eukaryote) endosymbiosis are driving forces in eukaryotic cell evolution. These phenomena are still contributing to acquire new cell structures and functions. To understand mechanisms for establishment of each endosymbiosis, experiments that can induce endosymbiosis synchronously by mixing symbionts isolated from symbiont-bearing host cells and symbiont-free host cells are indispensable. Recent progress on endosymbiosis using *Paramecium* and their endonuclear symbiotic bacteria *Holospora* or symbiotic green alga *Chlorella* has been remarkable, and providing excellent opportunities for elucidating host-symbiont interactions. These organisms are now becoming model organisms to know the mechanisms for establishing primary and secondary endosymbiosis. Based on experiments of many researchers, we introduce, how these endosymbionts escape from the host lysosomal fusion, how they migrate in the host cytoplasm to localize specific locations within the host, how their species specificity and strain specificity of the host cells are controlled, how their life cycles are controlled, how they escape from the host cell to infect more young host cell, how they affect to the host viability and to gene expression, what kind of substances are needed in these phenomena, and what changes had been induced in the symbiont and the host genomes.

Abbreviations: AcPase, acid phosphatase; AF, activated form; DIC, differential interference contrast; DV, digestive vacuole; HLB, *Holospora*-like bacteria; IF, infectious form; mAb,

monoclonal antibody; PV, perialgal vacuole; RF, reproductive form; TEM, transmission electron microscope

Keywords
Candidatus; *Chlorella*; genome plasticity; *Holospora*; infection; *P. caudatum*; *P. bursaria*; symbiosome

INTRODUCTION

Many endosymbiotic organisms in the genus *Paramecium* have been described (Fokin 2004; Fokin 2012; Fokin and Görtz 2009; Hoshina et al. 2010; Kzokoli et al. 2016; Potekhin et al. 2021; Schrallhammer and Potekhin 2020; Serra et al. 2016; Siegel and Karakashian 1959). Among them, the Gram-negative bacteria *Holospora* spp. and the symbiotic green alga *Chlorella* spp. are used in studies of cell-cell interactions, adaptability to the host environment, and eukaryotic cell evolution. *Holospora* spp. cannot grow in ordinary culture media outside of host cells because of their reduced genome size (Dohra et al. 2013, 2014; Garushyants et al. 2018). Currently nine *Holospora* spp. have been discovered in the *Paramecium* genus (Fokin and Görtz 2009; Potekhin et al. 2018). Furthermore, several *Holospora*-like bacteria (HLB) have been discovered in *Paramecium* and *Frontonia* (Fokin et al. 2019). The infectious form (IF) of *Holospora* spp. invades the target nucleus, a macronucleus, or a micronucleus via the host digestive vacuole (DV), as shown in studies mixing aposymbiotic host cells and IFs isolated from *Holospora*-bearing host cells (Fujishima 2009). Then, infection processes were observed under a light microscope (Fujishima and Kodama 2012, 2014). *Holospora* spp. are the only known organisms with the ability to distinguish between two kinds of host nuclei. Therefore, the molecules responsible for this ability have received the attention of many researchers. Apparently, these bacteria can detect differences in nuclei originating from a common fertilization nucleus. *Holospora* spp. are sometimes thought to be harmful parasites because their overgrowth depresses host growth and eventually kills the host. However, *Holospora* changes the expression of various genes in the host, which allows the host to acquire various stress resistances (Fujishima et al. 2005; Hori and Fujishima 2003; Hori et al. 2008; Nakamura et al. 2004; Smurov and Fokin 1998). Therefore, *Holospora* and *Paramecium* provide a good opportunity to investigate the process of the transition from harmful parasitic bacteria to beneficial symbiotic bacteria, similar to the process that occurred during the evolution of eukaryotic cells by endosymbiosis.

P. bursaria has a large number of symbiotic green algae *Chlorella* spp. in the cytoplasm, which are wrapped in a perialgal vacuole (PV) membrane derived from the host DV membrane. The PV membrane corresponds to the symbiosome membrane and protects the alga from

digestion by host lysosomal fusion (Kodama and Fujishima 2009b). Both *P. bursaria* and the symbiotic algae retain the ability to divide without a partner. Reestablishment of secondary endosymbiosis between the aposymbiotic host cells and symbiotic *Chlorella* cells isolated from alga-bearing host cells can be induced synchronously by pulse-labeling the symbiotic *Chlorella* cells, chasing for various times (Kodama and Fujishima 2005), and observing the infection processes using a light and a transmission electron microscope (TEM) (Fujishima and Kodama 2012, 2014; Kodama and Fujishima 2009a, 2010, 2016). Four important cytological events are needed to establish secondary endosymbiosis and their timing in the infection process (Kodama and Fujishima 2005, 2009c, 2010, 2011a, 2011b, 2016). *P. bursaria* is now becoming a model organism for studying the induction of secondary symbiosis. Furthermore, the nuclear genomes of the symbiotic *C. variabilis* (Blanc et al. 2010) and the host *P. bursaria* are known (Cheng et al. 2020).

In the first half of this article, we discuss the geographic origin of primary endosymbiosis between *Paramecium* and *Holospora* or *Holospora*-like bacteria (HLB). We also discuss species specificity, nucleus specificity of *Holospora* and HLB, the life cycle, molecules controlling infection by *Holospora*, how *Holsopora* exerts an impact on the host cell, and the *Holospora* genome. In the latter half of this article, features of the PV membrane, the re-establishment process of secondary endosymbiosis in *P. bursaria*, and genomic studies in *P. bursaria* and symbiotic *C. variabilis* are described. Indeed, *Holospora* and *Chlorella* provide an excellent opportunity for us to elucidate infection processes and to analyze phenomena leading to the evolution of eukaryotic cells. Because these endosymbiotic organisms can be easily isolated from the host homogenate and the reinduction of the endosymbiosis can be induced synchronously with a large number of cells.

GEOGRAPHIC ORIGIN OF PRIMARY ENDOSYMBIOSIS BETWEEN *PARAMECIUM* AND *HOLOSPORA* OR *HOLOSPORA*-LIKE BACTERIA

Holospora spp. are endonuclear symbionts of ciliate *Paramecium* spp. (Fokin 1989, 1991; Fokin and Görtz 2009; Fokin and Sabaneyeva 1993; Gibson et al. 1986; Hafkine 1890; Lanzoni et al. 2016; Ossipov 1973; Ossipov et al. 1975, 1980; Preer 1969; Preer and Preer 1982). *Holospora* species used in these papers are belonging to the order Rickettsiales (Amann et al. 1991; Lang et al. 2005; Potekhin, et al. 2018) and shown in Table 1. Fig. 1 shows photographs of the most commonly used *H. obtusa*- and *H. undulata*-bearing host *P. caudatum*. *Holospora*-bearing or HLB-bearing paramecia are so far collected in low-temperature areas such as the White Sea coast of Russia, Northern Europe, and the Kamchatka Peninsula, but not from domains where a high temperature of more than 30 ℃ are constantly maintained. Serra et al (2016) summarized places in a map where *Holospora* spp. and HLBs had been ever collected,

and reported retrieval of a new HLB from *P. multimicronucleatum*, "*Ca.* Gortzia infectiva" in *P. jenningsi* and also *H. obtusa* in *P. caudatum* from the south of India. This is the first finding of the *Holospora* genus at the lowest latitude ever reported. However, as shown in their paper, the location of the collection is at higher elevation with a moderate climate. As far as we know *Holospora*-bearing *Paramecium* spp. have not been collected in areas of the tropics where temperatures more than 30 ℃ are constantly maintained. This is consistent with the fact that the infectivity of *H. obtusa* is easily inactivated by incubation at 30 ℃ for four days or at 40 ℃ for five min (Fujishima et al. 1991). *Holospora* spp. and HLBs are found in the northern hemisphere, but not near the equator or in the southern hemisphere. This suggests that the first endosymbiosis between *Paramecium* and *Holospora* or HLBs might have begun in the cold regions of the northern hemisphere, followed by species dispersion southward.

SPECIES AND NUCLEUS SPECIFITY OF *HOLOSPORA* AND HLB

As shown in Table 1, *Holospora* spp. and HLBs show species and nuclear specificity in their habitats. Among them, *H. bacillata* and *H. caryophila* can be found in the macronuclei of several *Paramecium* spp. *H. bacillata* was originally discovered in the macronucleus *P. calkinsi* (Fokin and Sabaneyeva 1993), but later in *P. nephridiatum* too (Fokin 1989). Reproducibility of this result was confirmed by Görtz and Schmidt (2005). *H. caryophila* was originally discovered in the macronuclei of *P. biaurelia* (Preer 1969; Preer and Preer 1982) and *P. caudatum* (Görtz 1987), but was later found to be experimentally maintained in the macronuclei of other *P. aurelia* spp. (Castelli et al. 2015; Potekhin et al. 2018).

Holospora spp. can distinguish between the nuclear envelopes of the host macro- and micronucleus (Fujishima and Kawai 2004), invade a specific nucleus via the host DV within 10 min (Fujishima and Görtz 1983), and multiply in the target nucleus. Fujishima and Fujita (1985) called bacterial invasion into the host target nucleus an "infection". Fujishima and Fujita (1985) and Fujishima (1986) found that *H. obtusa* can infect *P. caudatum*, *P. multimicronucleatum,* as well as 14 *P. aurelia* species and *P. jenningsi*, and that stable maintenance for more than five days was achieved only in specific *P. caudatum* strains. *H. obtusa* cells, which have invaded the macronucleus of species other than *P. caudatum*, aggregate in the nucleus and then are synchronously expelled from the nucleus (Fokin et al. 2005; Fujishima 1986; Fujishima and Fujita 1985). Synchronous elimination of the infected bacteria suggests that infection with *H. obtusa* induces an unknown host defense mechanism against the infecting *Holospora*. Thus, *H. obtusa* infection can be induced in the macronuclei of species closely related to *P. caudatum*. However, *H. obtusa* maintenance in the host nucleus is controlled by the host genotype (Fujishima and Mizobe 1988), like *Caedibacter taeniospiralis* in *P. aurelia* species (Preer et al. 1974). Thus, infection and maintenance are independently-controlled phenomena (Fujishima and

Fujita 1985). These findings also suggest that the nuclear envelopes of macro- and micronuclei qualitatively differentiate during differentiation from a fertilization nucleus. To determine the timing of differentiation, Fujishima and Görtz (1983) mixed IFs of *H. obtusa* and *P. caudatum* exconjugants at various differentiation stages of macronuclear anlagen and found that the infectability against *H. obtusa* was acquired by four of the eight post-zygotic nuclei as soon as the four nuclei differentiated into macronuclear anlagen. Fragments of an old macronucleus in the exconjugant cell also showed infectability against *H. obtusa.*

Skovolodkin et al. (2001) transferred micronucleus-specific *H. undulata* and macronucleus-specific *H. obtusa* cells from the donor *P. caudatum* micro- or macronuclei to recipient *P. caudatum* macronuclei by microinjection. Infectious forms (IFs) of both *Holospora* spp. could not differentiate into reproductive form (RF) cells in the recipient macronucleus and were deleted into the cytoplasm. In normal infection, IFs differentiate into activated form (AF) cells by acidification in the host DVs. AF cells appear darker than IF cells under phase-contrast microscopy (Görtz and Wiemann 1989). Infected AF cells in the target nucleus soon acquire an affinity for nuclear chromatin (Görtz 1983) and the bacterial outlines become obscure. However, injected IFs remain distinct and do not form constrictions for binary fission in macronuclei. The finding that acidification of the isolated IFs induces AF-specific morphology and protein synthesis (Kawai and Fujishima 1997) suggests that IFs cannot differentiate into RFs unless they infect the nucleus after differentiation into the AF through the host DV. Injected *H. obtusa* and *H. undulata* RFs divide in the recipient macronucleus, while *H. undulata* RFs are expelled within five days after injection, indicating that micronucleus-specific *H. undulata* cannot be maintained in the macronucleus, even if RFs are injected into the macronucleus of a genetically-identical recipient cell.

HOLOSPORA LIFE CYCLE

Holospora spp. show morphologically and functionally different forms in their life cycle: reproductive form (RF) (1.5-2 µm long in *H. obtusa*), infectious form (IF) (10-15 µm long in *H. obtusa*), an intermediate form during differentiation from the RF to the IF, and the activated form (AF) (Fujishima et al. 1990a; Fujishima 2009) (Fig. 2). *Holospora* spp. exist as RFs and grow by binary fission in the host nucleus when the host cells are in the log phase. The RF ceases binary fission and differentiates into the IF through intermediate forms when the host cell division is interrupted by starvation or inhibition of host protein synthesis with emetine in the presence of *Holospora* protein synthesis (Fujishima et al. 1990a). This phenomenon indicates that proteins synthesized by the host and RF cells are essential for RF multiplication, and that *Holospora* protein synthesis is needed for IF differentiation. This may explain why protein spots from RF and IF differ by more than 65% on Two-dimensional sodium dodecyl sulfate polyacrylamide gel

electrophoresis (2D-SDS-PAGE) (Fujishima et al. 1990a). During IF differentiation, the RF elongates, increases buoyant density from 1.09 g/ml to 1.16 g/ml (IF) (Fujishima et al. 1990a), and changes the outer membrane surface morphology. The bacterial outer membrane is an asymmetric bilayer composed mainly of phospholipids in the inner leaflet and lipopolysaccharide (LPS) in the outer leaflet. A space between the cell membrane and the outer membrane is called as a periplasm. Bacteria with a buoyant density of 1.09 g/ml have a rough surface, while those with a buoyant density of 1.16 g/ml have an smooth surface except for one end (Fujishima et al. 1990b). The IF has a distinctive structure. Approximately one-half consists of the cytoplasm, and the other half is a periplasmic lumen with an electron-translucent tip (Dohra and Fujishima 1999; Dohra et al. 1994; Fujishima and Hoshide 1988; Görtz 1980; Görtz and Wiemann 1989; Görtz et al. 1989; Iwatani et al. 2005; Abamo et al. 2008). The electron-translucent tip of the IF corresponds to the rough tip. These three regions can be identified using Nomarski- and phase-contrast optics (Dohra and Fujishima 1999; Görtz and Dieckmann 1980). Under a phase-contrast microscope, the cytoplasmic region appears refractile, but the periplasmic region, including the electron-translucent tip, appears dark (Dohra and Fujishima1999; Görtz and Dieckmann 1980). When the IFs are mixed with aposymbiotic host cells, they are ingested into host DVs and differentiate into AFs.

Isolation of *Holospora* from host cell homogenates was first achieved in *H. caryophila* from *P. biaurelia* (Preer 1969). Later, *H. obtusa* (Freiburg 1985; Fujishima and Nagahara 1985: Fujishima et al. 1990a), *H. elegans* (Schmidt et al. 1987), *H. recta* (Kawai and Fujishima 1996), and *H. undulata* (Timofeyeva and Rautian 1997) were isolated from host homogenates or isolated nuclei. Cryopreservation of isolated IFs was first achieved in *H. obtusa* from *P. caudatum* using 10% (v/v) Dimethyl sulfoxide or 10% (v/v) glycerol (Fujishima et al 1991). After thawing, the IFs exhibited infectivity and proliferation ability after differentiation into RFs in macronuclei. Similarly, cryopreservation of isolated IFs was successful in *H. recta* (Kawai and Fujishima 1996), *H. undulata* (Millot and Kaltz 2006), and *H. elegans* (Fujishima and Kawai., unpubl. data) (Table 1).

When isolated IFs are mixed with paramecia, they are engulfed into host DVs, escape using the electron-translucent tip, move toward the target nucleus via host actin polymerization (Fujishima 2009; Fujishima et al. 2007; Iwatani et al. 2005; Sabaneyeva et al. 2009), and penetrate the target nuclear envelope with the electron-translucent tip (Fujishima 2009; Fujishima and Fujita 1985; Fujishima and Kawai 2004; Görtz and Wiemann 1989; Iwatani et al. 2005). This special tip is designated as the “invasion tip” (Iwatani et al. 2005). IF’s infectivity is lost by treatment with α-mannosidase (Fujishima et al. 1991). *P. caudatum* lysosomes have α-mannosidase activity (Fok and Allen 1988). Therefore, the bacteria must escape from DVs within 8 min after being engulfed, because lysosomal fusion occurs 8 min after mixing (Fok and

Allen. 1988). The first bacterial infection of the host macronucleus occurs within 10 min (Fujishima and Görtz 1983). Most of the AFs in the DVs that fail to escape are partially digested and eventually expelled from the host cytoploct.

During differentiation from the RF to the IF, two 4',6-diamidino-2-phenylindole (DAPI)-positive nucleoids form in *H. obtusa* (Fujishima et al. 1990a, Kawai and Fujsihima 1996). *H. elegans* and *H. undulata* also form two nucleoids in IF (M. Fujishima, unpublished observations). In *H. recta*, however, a large nucleoid forms near the IF periplasmic region (Kawai and Fujishima 2000). Additionally, the number and shape of the nucleoids in the IF of *H. obtusa* differs among strains (Fujishima and Görtz., unpubl. observ.).

When host cells grow by binary fission, IFs of *H. obtusa* in the host macronucleus collect in a connecting piece of the dividing macronucleus. They are then freed from the nucleus and are eventually expelled from the host cytoplasm (Wiemann 1989). The RF's outer membrane has a stronger affinity for host chromatin than that of the IF, so the RFs remain in each daughter nucleus (Ehrsam and Görtz 1999; Fokin et al. 1996; Görtz et al. 1992; Wiemann 1989). This connecting piece containing IFs appears in *H. obtusa*, *H. undulata*, *H. elegans*, *H. recta*, *H. acuminata*, and *H. curviuscula*, but not in *H. caryophila*, *H. bacillata*, *H. curvata*, or Ca. *H. parva* (Fokin and Görtz 2009; Potekhin et al. 2018). When the macronucleus is filled with many IFs, the host cell ceases cell division and eventually dies. The IFs that appear outside the host cell can then infect new host cells. Because *Paramecium* have a limited life span and *Holospora* cannot grow outside host cells, IFs must escape from the host to infect younger host cells. Therefore, the differing nature of the IF and RF outer membrane is indispensable for *Holospora* survival.

MOLECULES CONTROLLING THE *HOLOSPORA* INFECTION PROCESS

Infection by *H. obtusa* consists of six stages: (1) Infectious form (IF) engulfment into the host DV-I (Fujishima and Görtz 1983), (2) Activated form (AF) differentiation in acidified DV-II (Fujishima et al. 1990a; Fujishima and Kawai 1997) and the partial exposure of an invasion tip lumen-specific 89-kDa protein in the invasion tip (Iwatani et al. 2005), (3) AF escape from the DV-II to the host cytoplasm before host lysosomal fusion (Fujishima et al. 2007), (4) intracellular AF migration mediated by host actin polymerization (Fujishima 2009; Fujishima et al. 2007; Sabaneyeva et al. 2009), (5) recognition of a target nuclear envelope by specific binding between lipopolisaccharides on the outer membranes of both AF and IF and unknown receptors on the target nuclear envelope (Fujishima and Kawai 2004), and (6) penetration of the nuclear envelope with the invasion tip (Iwatani et al. 2005) (Fig. 1). Iwatani et al. (2005) developed a monoclonal antibody (mAb) specific for the invasion tip lumen protein. The antigenic protein was electro-eluted from a spot of 2D-SDS-PAGE gel of *H. obtusa* IFs, and

analyzed to determine the partial amino acid sequence and to identify the corresponding gene. The deduced amino acid sequence contains two actin-binding motifs near the N-terminus. Indirect immunofluorescence microscopy with antibodies against the 89-kDa protein and host actin 1-1 showed that five epitopes of the 89-kDa protein were present in the lumen of the invasion tip, while some epitopes translocated outside the outer bacterial membrane when the IFs were engulfed into the host digestive vacuole (DV). AFs in the cytoplasm keep the 89-kDa protein outside the tip, and host actins accumulate around the 89-kDa proteins immediately after bacterial escape from the DV. When the AF penetrates the host macronuclear envelope, the 89-kDa proteins and host actins remain at the entry point, even if the AF completely invades the nucleus (Iwatani et al. 2005; Fujishima 2009; Fujishima et al. 2007). Similar actin-based *H. obtusa* motility was also observed by Sabaneyeva et al. (2009). Electron microscopy showed the formation of fine fibrous structures presumed to be 89-kDa proteins and host actin fibers that co-localize with mAbs-labeled regions of the bacterium. Thus, the 89-kDa protein and the host actin play a role in *Holospora* escape from host DV-II, migration in the host cytoplasm, and invasion into the target macronucleus (Fig. 3). However, it remains unclear how AFs penetrate the host DV-II membrane and the host macronuclear envelopes.

On the other hand, based on TEM observations, Görtz (1996) proposed a hypothesis for *H. obtusa*'s invasion into the target macronucleus. Namely, AF of *H. obtusa* appears in the host cytoplasm with lapping by the host DV membrane, where the membrane vesicles are further surrounded by the host endoplasmic reticulum cistern in the host cytoplasm. Then, the first membrane derived from the DV membrane degenerates, and the AF becomes lapped with a double membrane derived from the host endoplasmic reticulum cisternae. Invasion of the AF into the macronucleus is mediated by fusion between two kind of double membranes: a double membrane lapping the AF and a macronuclear membrane. However, observation of Iwatani et al. (2005) that the AF appears in the host cytoplasm without being lapped with any membrane and moves by a help of the AF's 89-kDa protein and the host actin cannot be explained.

IMPACT OF *HOLOSPORA* ON THE HOST CELL

Numerous IF cells in the host macronucleus inhibit host cell division and eventually kill the host. Görtz and Fujishima (1983) showed that *H. elegans*-bearing *P. caudatum* cannot give rise to new functional macronuclei after conjugation. Therefore, *Holospora* species are considered parasitic bacteria. However, *Holospora*-bearing paramecia can survive longer at 10 °C compared to genetically identical *Holospora*-free cells (Fujishima., unpubl. observ.) and can acquire temperature-shift resistance if the bacteria in the nucleus are RF cells (Fujishima et al. 2005). Smurov and Fokin (1998) showed that *Holospora*-bearing *P. caudatum* acquires osmotic shock resistance. *H. obtusa* and *H. elegans* enhance expression of heat shock protein gene *hsp70* in host

cells (Hori and Fujishima 2003, Hori et al. 2008). Differential display reverse transcription PCR showed that *H. obtusa* changes the expression of various genes in the host *Paramecium* after endosymbiosis (Nakamura et al. 2004). A periplasmic 63-kDa protein from *H. obtusa* is one cause underlying alterations in host gene expression, because this protein is synthesized soon after infection and is secreted outside the bacterium to bind to the host chromatin (Abamo et al. 2008). Other periplasmic proteins are secreted from bacteria during early infection (Dohra et al. 1994; 1997). Dohra et al. (1998) showed that the bacterial chaperon, *groEL* homolog is highly expressed in *H. obtusa* RF, even at 25 °C, and may import host proteins or reduce damage from various stresses such as heat shock by protecting essential *Holospora* and host cell proteins.

As shown before, maintenance of the infected *H. obtusa* in the macronucleus is achieved only by specific *P. caudatum* strains (Fujishima and Fujita 1985). Skovorodkin et al. (2001) observed appearance of vacuolar structures of various sizes before disappearance of *H. obtusa* from the host macronucleus in both or alternate nuclei. Similar vacuolar structures are also reported in the micronucleus of a *P. caudatum* strain, which cannot maintain the infected *H. undulata* in the nucleus (Skovorodkin and Fokin 1991). Functions of this vacuolar structure are unknown. On the other hand, Fokin and Skovorodkin (1997) revealed by micronuclear transplantation that the host's ability to maintain *H. undulata* within the micronucleus is controlled by the macronucleus rather than the micronucleus.

THE *HOLOSPORA* GENOME

Up to now, four *Holospora* genomes have been sequenced: *H. obtusa*, *H. elegans*, *H. undulata* (Dohra et al. 2013, 2014), and *H. curviuscula* (Garushyants et al. 2018). The genome sizes of *H. obtusa, H. elegans*, *H. undulata*, and *H. curviuscula* are 1.33, 1.27, 1.40, and 1.72 Mb, respectively. Their respective GC content is 35.2, 36.0, 36.1, and 37.6%. The number of predicted genes of these bacteria is 1117, 1212, 1224, and 1594, respectively. Dohra et al. (2014) showed that common orthologous genes yield 572 single-copy core genes shared by *H. obtusa*, *H. elegans*, and *H. undulata*. *Holospora* rely on the host for energy production. Garushyants et al. (2018) showed that these four species are missing most amino acid synthesis pathways, This indicates that *Holospora* needs to import most amino acids from their hosts. Furthermore, *Holospora*, like *Rickettsia*, cannot synthesize purines and pyrimidines, although they can convert uridine triphosphate (UTP) to nucleoside triphosphate (NTP). *Holospora* needs to import all nucleoside triphosphates from the host cell. Additionally, *Holospora* has genes for ribonucleotide reductases, so they can convert ribonucleotides to deoxyribonucleotides and can use these nucleotides as an energy source (Garushyants et al. 2018). *Holospora* spp. are missing genes involved in glycolysis and the citric acid cycle, except for genes encoding malate dehydrogenase, although they have the pyruvate dehydrogenase complex and can convert

pyruvate to acetyl-CoA, acetoacetyl-CoA, and acetoacetate to produce ATP. F_1F_0-ATPase is also missing (Garushyants et al. 2018).

Holoposra spp. maintain genes mediating protein secretion or translocation (Garushyants et al. 2018). This ability may underlie the translocation of the 89-kDa protein from the *H. obtusa* invasion tip to the outer membrane during early infection (Iwatani et al. 2005). In addition, the 5.4-, 15-, and 39-kDa periplasmic proteins secreted by *H. obtusa* during infection may be controlled by secretory genes (Dohra et al. 1997; Fujishima et al. 1997; Abamo et al. 2008). Micronucleus-specific *H. undulata* and *H. elegans* are recognized as different species based on RF and IF cell morphology (Hafkin 1890; Fokin and Görtz 2009; Fujishima 2009). However, comparing the *16S rRNA* genes of these two species revealed a single nucleotide difference. This result suggests that these two species may be variant strains derived from the same species (Garushyants et al. 2018). Recent phylogenetic analysis of *Holospora* spp. also showed that *H. elegans* and *H. recta* should be considered subspecies of *H. undulata* (Wackerow-Kouzova and Myagkov 2021).

SECONDARY ENDOSYMBIOSIS BETWEEN *CHLORELLA* SPECIES AND *PARAMECIUM BURSARIA*

Extant symbioses illustrate symbiosis as a mechanism of evolutionary innovation. One example is the endosymbiotic system between *P. bursaria* and *Chlorella* sp. (Margulis 1993). *P. bursaria* maintain several hundred endosymbiotic algae in their cytoplasm (Fig. 4A and C). Recent molecular analyses revealed that nearly all *P. bursaria* contain symbiotic algae belonging to either the so-called American or European group and described the symbionts as *Chlorella variabilis* Shihira *et* Krauss and *Micractinium reisseri* Hoshina, Iwataki *et* Imamura sp. nov., respectively (Chlorellaceae, Trebouxiophyceae) (Hoshina et al. 2010). Symbiotic algae are transmitted to both daughter cells during cell division and are retained during conjugation (Karakashian 1963). The symbiotic algal population size in the host cell is maintained at a constant level during growth in the presence of light and nutrients (Iwai et al. 2016).

The association of *P. bursaria* with *Chlorella* spp. is mutually beneficial. The host supplies the algae with nitrogen components and CO_2 (Albers and Wiessner 1985; Reisser, 1980), and algal carbon fixation is enhanced in the host cell (Kato and Imamura 2009). The host protects algae in the perialgal vacuole (PV) from infection by the *Chlorella* virus (Kawakami and Kawakami 1978; Van Etten et al. 1985; Yamada et al. 2006, see the next section in this review). Meanwhile, the algae supply the host with maltose, a photosynthetic product (Brown and Nielsen 1974; Reisser 1976), and provides an oxygen supply for host respiration (Reisser, 1980). Additionally, maltose from symbiotic *Chlorella* is closely related to circadian rhythms, including

host mating reactivity and photoaccumulation (i.e., light-harvesting) (Miwa 2009). Furthermore, maltose plays a role in various stress resistances against high-temperature and chemicals in algae-bearing *P. bursaria* cells (Miwa 2009). Because free-living *Chlorella* cannot release their photosynthetic sugar products, maltose release may be an essential factor in establishing endosymbiosis (Weis 1979). Algae-bearing *P. bursaria* grow better than algae-free cells (Görtz 1982; Karakashian 1963) because digestion of algae allows host cells to survive under starvation conditions (Kodama and Miyazaki 2021).

Despite the mutually beneficial relationships between *P. bursaria* and symbiotic algae, their relationship is facultative mutualism because algae-free *Paramecium* cells (i.e., white cells) and isolated symbiotic algae can grow without a partner. Algae-free *P. bursaria*, as shown in Fig. 4B, can be easily produced from algae-bearing cells by rapid fission (Jennings 1938), cultivation in darkness (Karakashian 1963), X-ray irradiation (Wichterman 1948), or by treatment with 3- (3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a photosynthesis inhibitor (Reisser 1976), by paraquat (Hosoya et al. 1995), or cycloheximide (Kodama and Fujishima 2008; Kodama et al. 2007; Weis 1984). Algae-free *P. bursaria* can reassociate in original or novel combinations by mixing them together (Siegel and Karakashian 1959; Pringsheim 1928). Therefore, *P. bursaria* is a model organism for studying cell-to-cell interactions and the evolution of eukaryotic cells through secondary endosymbiosis (Kodama et al. 2014; Kodama and Fujishima 2016).

FEATURES OF THE PERIALGAL VACUOLE MEMBRANE

Each symbiotic alga is surrounded by a PV membrane (Fig. 4F) and is attached to the host cell cortex (Fig. 4C and 4E). Fig. 4D and E show the cell membranes of algae-free (Fig. 4D) and algae-bearing (Fig. 4E) *P. bursaria*. Numerous spindle-shaped trichocysts are observed on the cell membrane. The trichocysts of algae-bearing *P. bursaria* are pushed aside by symbiotic algae enclosed in the PV membrane (Kodama and Fujishima 2009b, 2011a, Fig. 4E, arrowheads). The PV membranes are derived from host DV membranes, but neither the DV membrane nor the crystals are localized beneath the cell cortex (Fig. 4D).

We observed symbiotic *C. variabilis* during algal cell division in the PV membrane. As shown in Fig. 4G and H, the algal mother cell wall is discarded from the PV membrane, which encloses the symbiotic alga. When symbiotic algae divide, the PV membrane must increase because symbiotic algae without a PV membrane cannot be observed using transmission electron microscope (TEM). When symbiotic algae divide in the PV membrane, the coiled mother cell wall fragment is pinched off from the proto-PV membrane. Because we never observed the mother cell walls stored in the *Paramecium* cytoplasm by TEM (data not shown), and many coiled cell walls are observed at the bottom of the test tubes for *P. bursaria* culture, it seems that the old cell walls are selectively isolated from the PV by unknown mechanism and eventually

excreted outside the host cell without being digested by the host digestive vacuole (Kodama and Fujishima 2012a).

The PV membrane prevents host lysosomal fusion (Gu et al. 2002; Kodama and Fujishima 2009a). Similar to the symbiotic relationship between *P. bursaria* and *Chlorell*a spp., symbionts or parasites that are protected from host lysosomal fusion are observed in the ciliate *Climacostomum virens* (Reisser 1984), the cnidarian *Hydra viridis* (O'Brien 1982; Hohman et al. 1982), and the apicomplexan *Toxoplasma gondii* (Scholtyseck and Piekarski 1965). The endosymbiotic X-bacteria in *Amoeba proteus* (Ahn et al. 1990), symbiotic *Chlorella variabilis* in *Stentor pyriformis* (Hoshina et al. 2021), the dinoflagellate *Symbiodinium microadriaticum* (Fitt and Trench 1983), and *Mycobacterium tuberculosis* (Deretic and Fratti 1999; Sinai and Joiner 1997) are also enclosed in a vacuole, forming so-called symbiosomes derived from the host DV membrane. PV membranes are universal and important for establishing and maintaining endosymbiosis. The PV membranes: (1) encloses a single algal cell (Karakashian and Rudzinska 1981); (2) is associated with the algal cell wall and directly contacts the host mitochondrial membrane (Song et al. 2017); (3) diameter does not vary, except during the algal cell division (Reisser 1992); (4) does not participate in host cyclosis, but is located immediately beneath the host cell cortex (Kodama and Fujishima 2005; Reisser 1986); (5) particle density and distribution show few signs of endocytotic or exocytotic activity (Meier et al. 1984); and (6) contains an active proton pump and establishes a gradient that drives maltose transport from the symbiotic algae to the host cell (Schüßler and Schnepf 1992).

Cycloheximide preferentially inhibits protein synthesis in the symbiotic algae, but only slightly inhibits host protein synthesis. The PV membrane swells synchronously when alga-bearing *P. bursaria* are treated with cycloheximide. Transmission electron microscopy revealed a wide space between the PV membrane and the algal cell wall, and degeneration of the algal ultrastructure. After synchronous PV membrane swelling, the membrane detaches from the host cell cortex and the symbiotic algae are digested by host lysosomal fusion. These phenomena are induced only under constant light conditions, suggesting that algal proteins synthesized during photosynthesis may play important roles in preventing PV membrane expansion, keeping the PV membrane close to the host cell cortex, and preventing lysosomal fusion with the PV membrane (Kodama and Fujishima 2008; Kodama et al. 2011).

Recent studies show that the DV and PV membranes have distinct properties. We developed monoclonal antibodies (mAbs) specific to the *P. bursaria* DV membrane. The mAbs do not react with the PV membrane, indicating that the membranes are substantially different (Fujishima and Kodama., unpubl. data). Recently, Aoyagi et al. (2019) showed the localization of oligosaccharides in close proximity to symbiotic *C. variabilis* in *P. bursaria*. The oligosaccharides were detected in samples containing PV membrane-bound algae, and were

absent in isolated symbiotic algae and DV membrane-bound algae. These results provide important new insights into the chemistry of DV and PV membranes.

RE-ESTABLISHMENT OF SECONDARY ENDOSYMBIOSIS IN *P. BURSARIA*

An experiment on reinfecting algae-free *P. bursaria* cells with *Chlorella* was reported in 1959 by Siegel and Karakashian. However, the details of the algal reinfection process remained unknown. The reinfection of algae-free *P. bursaria* cells with *Chlorella* spp. was conducted via phagocytosis by the host. To investigate the algal reinfection process, we observed the fate of the algae ingested in host DVs. Algae-free cells were mixed with isolated symbiotic algae for 1.5 min, washed, chased, and fixed after mixing. Then, the cells were observed by light and transmission electron microscopy with or without Gomori's staining to detect lysosome-specific acid phosphatase (AcPase) activity (Gomori 1952). The experimental procedure of Gomori's staining method is shown in Fig. 5A and typical light microscopic images are shown in Fig. 5B. During the algal reinfection process, we found that four important cytological events are needed to establish endosymbiosis (Fujishima and Kodama 2014; Kodama and Fujishima 2009c, 2016).

First, algae must be resistant to host acidosomal and lysosomal fusion to DVs. The DVs observed during the algal reinfection process were classified into four different stages (DV-I, DV-II, DV-III, and DV-IV) based on their morphology and pH referred to Fok and Allen (1988) (Fig. 5B). After mixing with algae-free cells, one or several algae pass through the host cytopharynx and are endocytosed as DV-I (Fig. 5B-a). The DV-I vacuole has a rounded membrane containing only green algae, and is clearly visible. Acidified and condensed DV-IIs appear 0.5–1 min after mixing (Fig. 5B-c). The vacuole membrane is barely visible. After the acidosome-DV fusion, the algae do not change and retain a green color. Even when *Chlorella* sp. are isolated from *P. bursaria* and treated with acidic buffer for 1 h, they did not lose their infectivity (Kodama and Fujishima, unpubl. data). Therefore, algae can likey resist acidosomal fusion to the DV. Acidic pH stimulates maltose release from isolated symbiotic algae (Kamako and Imamura 2006; Shibata et al. 2016), so acidosomal fusion to the algae-enclosing DVs may establish endosymbiosis. Lysosomal fusion occurs at 2–3 min, accompanied by increased intravacuolar pH, and a swollen DV-III appears. In DV-III, the vacuole membrane is visible and the algae are digested, leaving a faint yellow or green color. The DV-III stage is further classified into three substages: DV-IIIa contains green algae only; DV-IIIb contains faint yellow and green algae (Fig. 5B-e); and DV-IIIc contains faint yellow algae only. In the final stage, DV-IV, the vacuolar size is again reduced, rendering the vacuolar membrane barely visible. The algae are undigested green, digested brown, or both. This vacuole is observed in cells after 20-30 min. DV-IV is 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. Some algae in

DV-IIIb and DV-IVb are not digested and retain their green color, even in the presence of digested algae. This phenomenon depends on the photosynthetic activity of the isolated algae before mixing with *P. bursaria*. When symbiotic algae isolated from algae-bearing paramecia are kept under constant dark conditions for 24 h before mixing with algae-free paramecia, almost all algae are digested in the host DVs. As a result, these algae cannot re-establish endosymbiosis (Kodama and Fujishima 2014).

Second, the algae bud from the DV membrane into the cytoplasm. As shown in Fig. 5B-g, condensed DV-IVs appear at 20-30 minutes. Thirty minutes after mixing algae-free *P. bursaria* and isolated symbiotic algae, the algae start to escape from DV-IVb vacuoles due to membrane budding into the cytoplasm (Fig. 5B-i). Both undigested and digested algae bud from the DVs (Kodama and Fujishima 2005). *Saccharomyces cerevisiae* and polystyrene latex spheres with a diameter of 3 μm or larger are also able to bud (Kodama and Fujishima 2012b). However, this budding is not observed when India ink, 0.81-μm diameter polystyrene latex spheres, or food bacteria were ingested into the DVs (Kodama and Fujishima 2005). These results suggest that *P. bursaria* can recognize the DV diameter. The dynamin inhibitor Dynasore greatly inhibits DV budding, suggesting that dynamin might be involved in this process (Kodama and Fujishima 2012b). Recently, we determined that the budding DV membrane can recognize symbiotic alga by eliminating other small microspheres in the same DV. To clarify the budding process, we mixed 0.20-μm fluorescent-labeled microspheres with isolated symbiotic algae during re-endosymbiosis. Microsphere fluorescence was not observed near the PV membrane, as expected, and budding DVs contained both undigested green and digested algae. Additionally, the algal re-endosymbiosis rate was significantly reduced in the presence of microspheres. These observations show that *P. bursaria* allows algal budding only from the DVs without microspheres, and this process requires close contact between the DV membrane and the algal cell wall (Kodama and Sumita 2022).

Third, the DV membrane differentiates into the PV membrane. After algal budding from the DV-IVb membrane, DV membranes enclosing an undigested green alga differentiate into PV membranes, which provide protection from lysosomal fusion (Kodama and Fujishima 2005, 2009a,b). To understand the timing of PV differentiation from the host DV, algae-free *P. bursaria* cells were mixed with isolated symbiotic algae, washed, chased, and fixed. Then, the lysosomal AcPase activity in the vacuoles enclosing the algae was analyzed using Gomori's staining (Fig. 5B). Neither DV-I nor DV-II show AcPase activity (Fig. 5B-b, 5B-d), while AcPase activity appears in 3 min-old DV-III (Fig. 5B-f). All DVs containing algae demonstrate activity at 30 min of DV-IV formation (Fig. 5B-h). Algal budding from the DVs begins at 30 min, as described above. In the budding membrane, each alga is surrounded by a layer of thin, positive Gomori's staining (Fig. 5B-j). The algae-containing vacuoles quickly move and attach

immediately beneath the host cell cortex (Fig. 5B-k). These vacuoles show no AcPase activity (Fig. 5B-l). The appearance of the first budding alga from DV-IV and the attachment beneath the host cell cortex occurred at 30 and 45 min after mixing, respectively. These results suggest that differentiation of the PV membrane occurs within 15 min after algal budding from the host DV (Kodama and Fujishima 2009c).

Fourth, symbiotic algae within the PV membrane localize beneath the host cell cortex (Kodama 2013; Kodama and Fujishima 2005, 2011a, 2013, Fig. 4E). Many trichocysts and mitochondria are also localized in this area (Fujishima and Kodama 2012). Gomori's staining showed that scant AcPase activity was observed in this area (Kodama and Fujishima, 2009b). These observations raise the possibility that the PV membrane might not protect against lysosomal fusion, but avoid lysosomal fusion by binding to a lysosome-free area. To confirm this possibility, we removed preexisting trichocysts beneath the *P. bursaria* cell cortex by treatment with lysozyme, thereby reducing the AcPase activity-negative area and exposing the PVs to AcPase, and examined whether PVs can protect against lysosomal fusion. The trichocyst-depleted cells reduced the AcPase activity-negative cortical layer to less than 3 μm in the dorsal cortex. However, even if a part of the PV membrane was exposed in the AcPase activity-positive area, the algae attached to the host cell cortex, which protected it from lysosomal fusion (Kodama and Fujishima 2009b). This is the first evidence to demonstrate that the PV membrane can protect against host lysosomal fusion and that the PV membrane does not require trichocysts for intracellular localization. A schematic representation of the algal reinfection process and the four important cytological events that re-establish endosymbiosis are summarized in Fig. 6.

Symbiotic or parasitic organisms have mechanisms to escape from host lysosomal digestion: 1) resistance to the host lysosomal enzymes inside the DV or endosome; 2) escape from the DV or endosome; and 3) blocking lysosomal fusion. Our data indicate that *Chlorella* has a complicated mechanism for avoiding digestion. First, an alga acquires temporary resistance to host lysosomal enzymes in the DV. Then, the alga escapes from the DV. Third, the alga is wrapped with a PV membrane. Finally, the alga localizes to the host cell cortex. Recent genomic results have enabled us to understand the molecular mechanisms underlying the establishment of secondary symbiosis and host evolutionary adaptation (Kodama et al. 2014).

GENOMIC STUDIES IN *P. BURSARIA* AND *CHLORELLA* SPECIES

In 2014, we performed transcriptome analysis (RNA seq) using symbiont-bearing and symbiont-free *P. bursaria* to investigate the genes related to endosymbiosis. We compared gene expression between algae-bearing and alga-free *P. bursaria* to elucidate the genetic control underlying the establishment of secondary symbiosis. Of the differentially-expressed genes, those encoding glutathione S-transferase, trans-2-enoyl-CoA, aminotransferases, and ribosomal proteins were

downregulated, while genes for Hsp70, transcriptional activator Myb-related proteins, and signal transduction histidine kinase were upregulated in symbiont-bearing *P. bursaria*. Our data provide a comprehensive sequence resource for future studies of *P. bursaria* (Kodama et al. 2014).

Cheng et al. (2020) sequenced and annotated the functional macronuclear genome of four *P. bursaria* strains. For strain Dd1g, the total genome size was 26.8 Mb, the GC content was 28.8%, and the number of genes was 15,101. Of these, 3,773 genes were absent in other *Paramecium* species (i.e., *P. candatum* and *P. tetraurelia*). To gain insights into *P. bursaria* physiology, they compared the functional gene set of *P. bursaria* with those of two other sequenced *Paramecium* genomes, *P. caudatum* and *P. tetraurelia*. Six gene families were highly duplicated only in *P. bursaria*, including the WD40 domain, which is related to legume-Rhizobium symbioses and domain catalytic (TLDc) domain-containing proteins, which primarily function as antioxidants to protect cells from reactive oxygen species. Expansion of the TLDc domain-containing gene in *P. bursaria* may be an adaptation to oxidative stress. Expression of *Paramecium* surface antigen domain-containing genes is often induced by environmental changes, but it remains unclear why *Paramecium* surface antigen-containing, uncharacterized protein family (UPF) 0047 domain-containing, and protein kinase domain-containing genes are highly duplicated in *P. bursaria*. These results will facilitate genomic studies on the mechanism of eukaryotic cell evolution and biodiversity through endosymbiosis.

Regarding symbiotic *Chlorella* sp., Blanc et al. (2010) sequenced the 46-Mb nuclear genome of *C. variabilis* strain NC64A. This strain was isolated from *P. bursaria* nearly 50 years ago and was cultivated outside the host (Karakashian et al. 1968). We confirmed that NC64A is infective to algae-free *P. bursaria*, but the infection rate depends on the culture growth (Kodama and Fujishima 2016). A genomic study of NC64A revealed the expansion of protein families that may participate in adaptation to symbiosis (Blanc et al. 2010). The number of amino acid transporters was significantly increased in NC64A. Some of these transporters may be expressed in a symbiotic environment. This observation is consistent with previous studies that suggest that *Chlorella* symbionts, including NC64A, possess an efficient system for importing amino acids from the host and can use amino acids as a source of nitrogen (Kato et al. 2006). Experimental evidence suggests that the cell wall of *Chlorella* species, including NC64A, contains glucosamine polymers such as chitin and chitosan (Kapaun and Reisser 1995). Chitin is a natural component of fungal cell walls and arthropod exoskeletons, and is not normally present in green algae. Although the origin of chitin and its derivatives in the *Chlorella* genus remains an enigma, the ability of *Chlorella* to produce chitinous cell walls likely resulted from horizontal gene transfer from algal viruses, prokaryotes, or fungi. Chitin may also participate in the recognition process of algae-free *P. bursaria* by symbiotic *Chlorella.*

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FIGURE LEGEND

Fig. 1. Differential interference contrast images of *Paramecium caudatum*. (**A**) A macronucleus with *Holospora obtusa*. Straight rod-shaped structures in the macronucleus are infectious forms of *H. obtusa.* (**B**) A micronucleus with *H. undulata*. Spiral structures in the micronucleus are infectious forms of *H. undulata*. Ma, macronucleus; Mi, micronucleus. Refractile structures in the cytoplasm = crystals. Scale bar = 10 μm.

Fig. 2. *Holospora* life cycle. Classification of digestive vacuoles (DVs) was described by Fok and Allen (1988). Spherical DV-Is differentiate to condensed and acidified DV-IIs by fusion of acidosomes (i.e., early endosomes, Ref. Allen and Fok 1988) and evagination of the DV membrane. The DV-IIs differentiate into swollen DV-IIIs by fusion with primary lysosomes. Undigested materials remain in DV-IVs, which do no more show acid phosphatase activity. The DV-IVs fuse with the cytoproct and discharge their contents. Infectious-form (IF) *Holospora* differentiates to the activated form (AF) in the acidified DVs. Some AFs escape into the cytoplasm from the acidified DVs during the transition from DV-1 to DV-II using an invasion tip and migrate toward their target nuclei with using host actin polymerization. The AF penetrates the target nuclear envelope with the invasion tip. After infection, the bacterium differentiates into the reproductive form (RF). During this process, the bacterium decreases its buoyant density from 1.16 to 1.09 g/ml. The RFs halt binary fission and differentiate into the IF. The IFs are freed from the host cells (see text). Acridine orange stains the DV-II orange, the DV-III yellow, and other DVs yellow-green, thus indicating raising luminal pH. Ma, macronucleus; Mi, micronucleus. Updated from Fujishima (2009).

Fig. 3. Role of 89-kDa periplasmic proteins and host actin in infection by *H. obtusa*. Schematic representation by indirect immunofluorescence microscopy with a monoclonal antibody mAb IF-4-2 specific for 89-kDa protein in an invasion tip of the IF and a mAb actin 1-1 specific for *P. caudatum* actin and by transmission electron microscopy. (**A**) The 89-kDa proteins are localizing in the invasion tip lumen. (**B**) N-terminal of the 89-kDa protein with actin-binding motifs translocates outside the tip by acidification of the host DV and the IF differentiates to the AF. Because antibodies hardly enter the periplasm of *Holospora* without brief sonication or

treatment with 20 mM NaOH, the 89-kDa protein of IF cannot be labeled by indirect immunofluorescence microscopy. However, the invasion tip of the AF can be labelled without permeabilization, because a part of the 89-kDa protein is exposed outside the tip of the AF. As shown in **B**, the invasion tip of the AF becomes closed to the DV membrane by contraction of the DV membrane. In such DV, many fine fibers can be observed by electron microscopy between the invasion tip and DV membrane. Since immunofluorescence of the 89-kDa protein and the fibrous structures appear at exactly the same location, fibrous structures seem to involve the 89-kDa proteins to work as a scaffold, and may provide a driving force for escape of the AF from the DV. Furthermore, small vacuole like structures accumulate outside of the extrusion of the DV. These structures may assist the AF in leaving from the DV. (**C**) The AF escapes from the acidified DV without being surrounded by the DV membrane. Immediately after leaving the DV, host actins accumulate around the invasion tip and the AF moves into the host cytoplasm. Outside the invasion tip (fibrous structures) can be labeled by both anti-89-kDa and anti-actin 1-1 mAbs. Also, electron microscopy showed that the tip is covered with fibrous structures in the host cytoplasm. (**D**) When the AF invades the host macronucleus, the 89-kDa proteins, host actin and the fibrous structures remain at the entry point of the macronuclear envelope. Ma, macronucleus. Updated from Iwatani et al. (2005) and Fujishima (2009).

Fig. 4. Differential interference contrast (DIC) and transmission electron microscopic (TEM) images of *P. bursaria*. Ma, macronucleus; Chl, chloroplast; Cy, cytopharynx; Tc, trichocyst; c, crystals; d, digestive vacuole; PV, perialgal vacuole membrane; CW, cell wall; Mt, mitochondrion; mcw, mother cell wall; dcw, daughter cell wall. (**A**) Endosymbiotic algae *Chlorella variabilis* strain 1N-bearing *P. bursaria* strain Yad1g1N. (**B**) Algae-free *P. bursaria* strain Yad1w (w means white). (**C**) TEM image of *P. bursaria*. Note that symbiotic algae localize beneath the host cell cortex with host trichocysts. (**D**) and (**E**) Enlarged DIC images of the cell cortices of algae-free (**D**) and algae-bearing (**E**) *P. bursaria*. The symbiotic algae push the host trichocysts aside and were fixed beneath the host cell cortex (arrowhead). (**D**) Neither DV membranes nor crystals localize between host trichocysts. Brownish structures = crystals. (**F**) TEM image of mature symbiotic *C. variabilis* inside the PV membrane. (**G**) TEM image of *C. variabilis* during cell division. Some coiled fragments of the mother cell wall are observed inside the PV membrane. Two daughter cells with coiled fragments of the mother cell wall are observed. (**H**) The magnified image of the square area in (**G**) showing a coiled fragment of the mother cell wall separated by pinching off from the PV membrane (arrows in **H**). Ma, macronucleus; Chl, chloroplast; Cy, cytopharynx; Tc, trichocyst; c, crystals; d, digestive vacuole; PV, perialgal vacuole membrane; CW, cell wall; Mt, mitochondrion; mcw, mother cell wall; dcw, daughter cell wall. Scale bars: 20 μm (**A** and **B**), 7 μm (**C**), 10 μm (**D** and **E**) and 500 nm

(**F**-**H**). (**C** and **F**) from Kodama and Fujishima (2010). (**D** and **E**) from Kodama and Miyazaki (2021). (**G** and **H**) from Kodama and Fujishima (2012a).

Fig. 5. (**A**) Typical light microscopy images after the following Gomori's staining method. 1) 500 ml aliquot of the cell suspension was fixed by mixing with an equal volume of the fixative (4.0% (w/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, containing 8% (w/v) sucrose at 4 ℃.) for 30 min at 4 ℃. 2) The cells were washed with 1 ml of the wash buffer (0.1 M cacodylate buffer, pH 7.2 containing 8% (w/v) sucrose) from 30 min to overnight at 4 ℃ (the buffer was changed three times). 3) The cells were then incubated in 1 ml of Gomori stain solution (3 mM Pb $(NO_3)_2$, 10 mM Na-β-glycerophosphate (a substrate for the AcPase), 8% (w/v) sucrose, 0.05M acetate buffer, pH 5.0) for 1 h at 37 ℃. 4) For control experiments, cells were incubated in Gomori stain solution containing 0.01M sodium fluoride (NaF, an inhibitor of the AcPase), or a solution lacking Na-β-glycerophosphate. 5) The cells were washed three times with the wash buffer (0.05 M acetate buffer of pH 5.0 containing 8% (w/v) sucrose) for 10min at room temperature. 6) The cells were incubated in 1 ml of 0.05% (w/v) $(NH_4)2S$ for 1 min at room temperature. 7) The cells were washed three times with 1 ml of distilled water for 3 min each at room temperature and observed using a differential interference contrast (DIC) microscope. Acid phosphatase (AcPase) activity of the DV membrane is shown as black granules (left). Negative control experiments (with NaF, an AcPase inhibitor or in cells lacking Na-β-glycerophosphate, an AcPase substrate) showed no granules (middle and right, respectively). Scale bar, 10 μm. (**B**) DIC micrographs of reinfection by symbiotic *C. variabilis* in algae-free *P. bursaria*. Algae-free paramecia were mixed with isolated algae and fixed at 0.5 min (a and b), 1 min (c and d), 10 min (e and f), 30 min (g and h), and 3 h (i–l) after mixing. Cells non-treated with Gomori solution: a, c, e, g, i, and k. Cells treated with Gomori solution: b, d, f, h, j, and l. DV-I, a and b; DV-II, c and d; DV-IIIb, e, and f; DV-IVb, g and h. Panels (i) and (j) show an alga escaping by budding from the DV-IVb membrane (red arrowhead). Insets of (i) and (j): enlarged photomicrographs of the escaping alga. (k and l): algae attached immediately beneath the host cell cortex (black arrows). DV-I (b) and DV-II (d) are AcPase-negative, while DV-IIIb (f) and DV-IVb (h and j) are AcPase-positive. The green alga escaping from the host DV (j), which are located immediately beneath the host cell cortex (l, black arrows) are AcPase-negative. Scale bars, 10 μm (l), and 2 μm (inset in j). (**B**) is from Kodama and Fujishima (2009a). See section RE-ESTABLISHMENT OF SECONDARY ENDOSYMBIOSIS IN *P. BURSARIA* for detailed descriptions of each stage of DVs.

Fig. 6. Schematic representation of algal reinfection. Four important cytological events (labeled 1-4) are needed to establish endosymbiosis (Kodama and Fujishima 2005). (**1**) About 3 min after

mixing of algae-free *P. bursaria* cells with isolated symbiotic algae, some algae in DVs acquire resistance to the host lysosomal enzymes. (**2**) About 30 min after mixing, the algae escape from the DVs by budding of the DV membrane. This budding is completely inhibited by dynamin inhibitor Dynasore. (**3**) About 45 min after mixing, the DV membrane enclosing a single undigested green alga differentiates into the PV membrane (red), which provides protection from the host lysosomal fusion. (**4**) Then, the alga localizes beneath the host cell cortex by adhesion of the PV membrane and outer membrane of the host mitochondrial outer membrane. About 24 h after mixing, the alga beneath the host cell cortex begins cell division and establishes endosymbiosis. (Updated from Kodama and Fujishima 2009a)

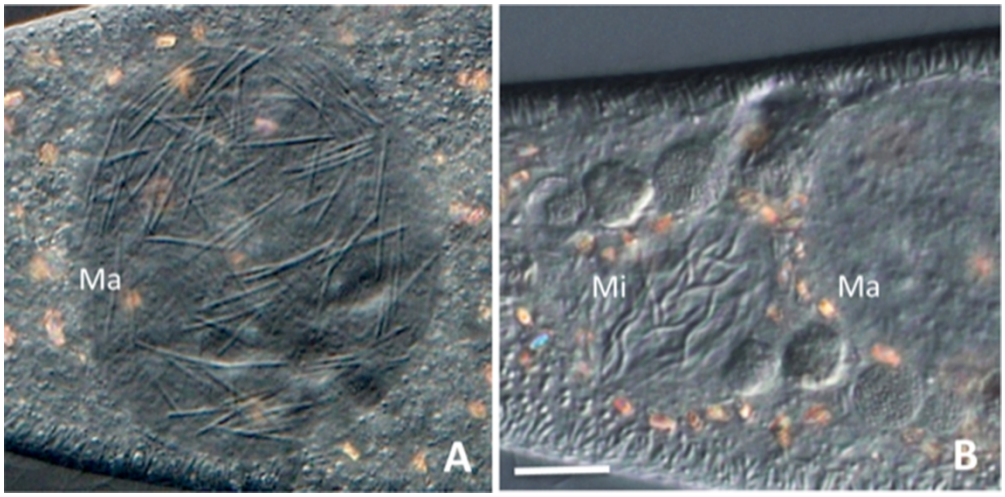
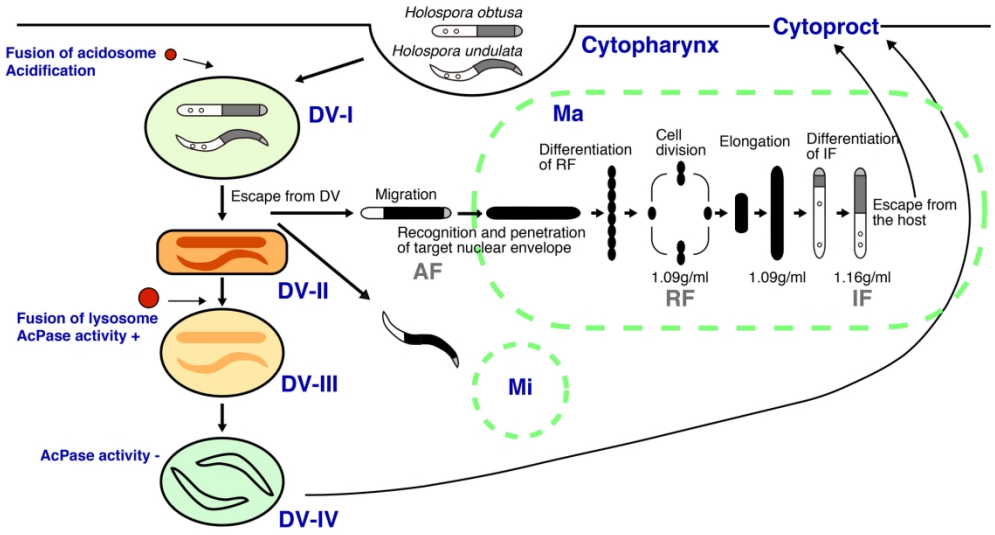


Fig. 1. Differential interference contrast images of *Paramecium caudatum*.



Holospora life cycle.

180x97mm (300 x 300 DPI)

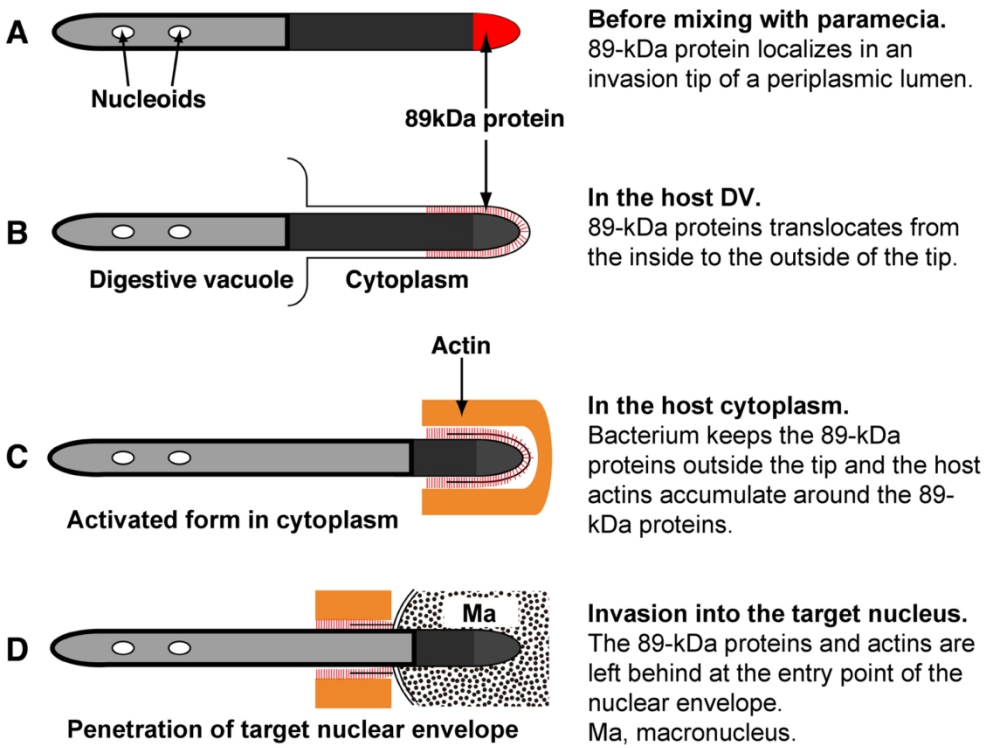


Fig. 3. Role of 89-kDa periplasmic proteins and host actin in infection by *H. obtusa*.

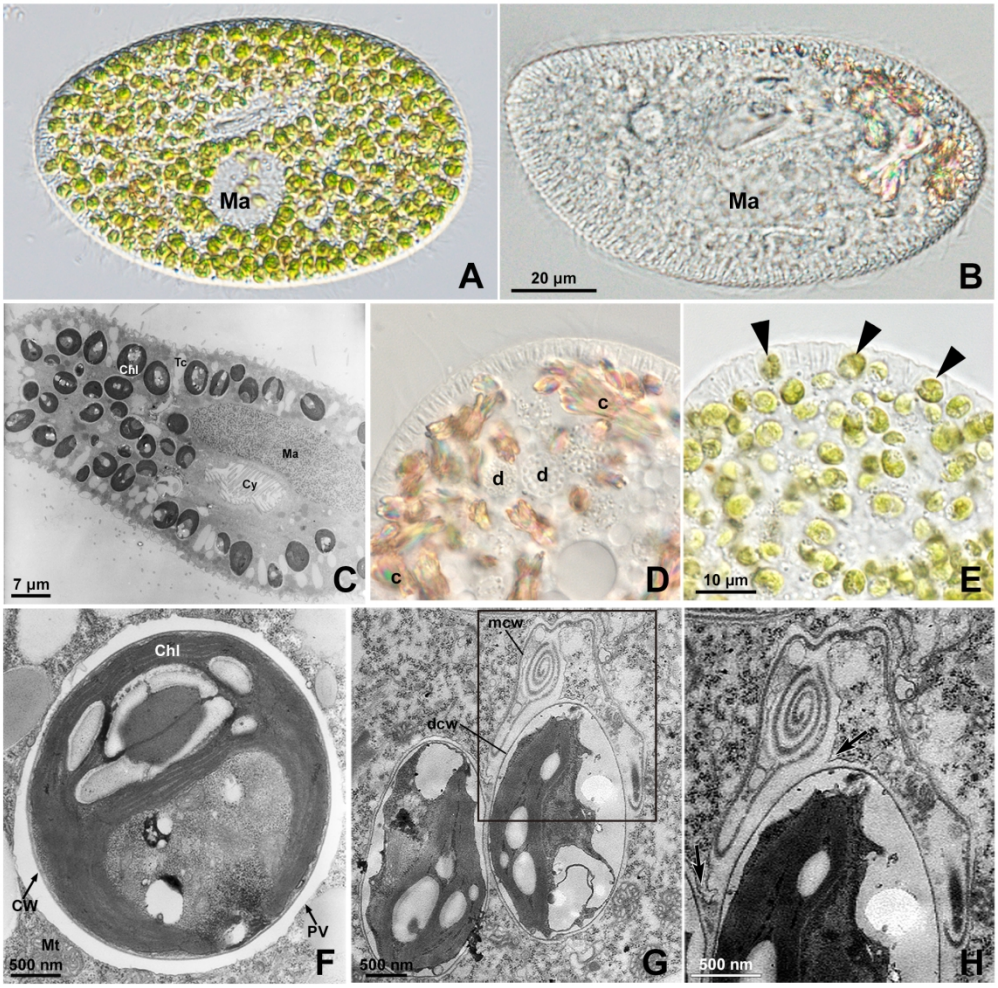


Fig. 4. Differential interference contrast (DIC) and transmission electron microscopic (TEM) images of *P. bursaria*.

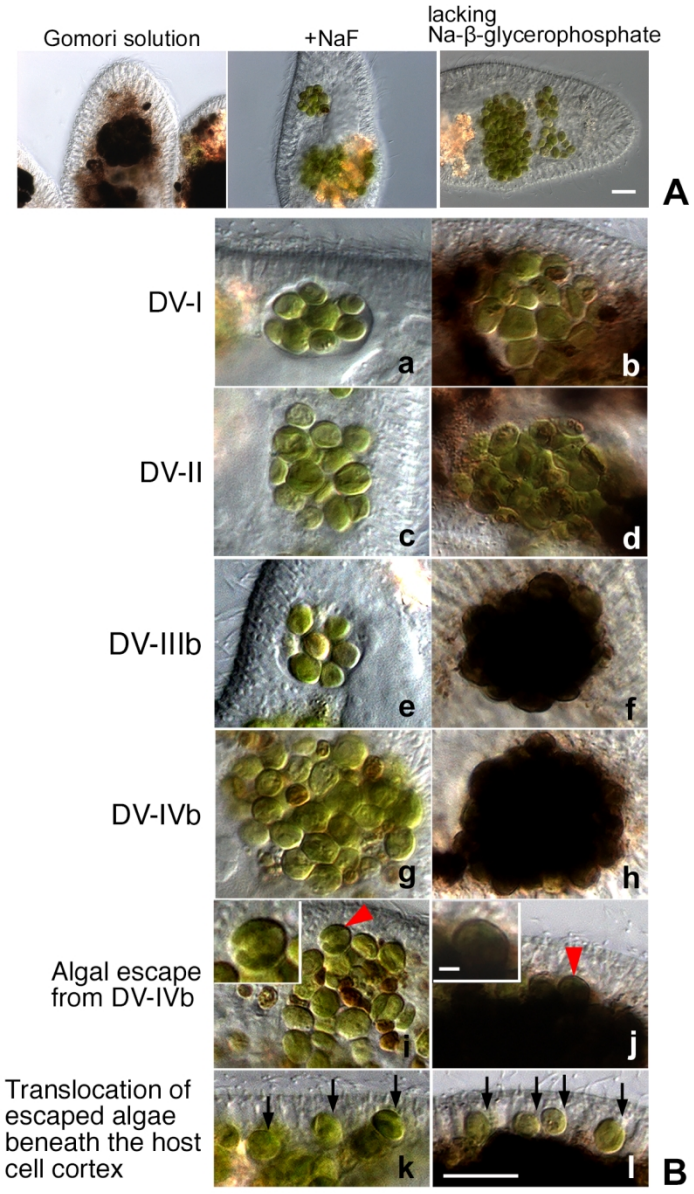

Gomori solution
+NaF
lacking
Na-β-glycerophosphate
A
DV-I
DV-II
DV-IIIb
DV-IVb
Algal escape
from DV-IVb
Translocation of
escaped algae
beneath the host
cell cortex
a
b
c
d
e
f
g
h
i
j
k
l
B

Fig. 5.

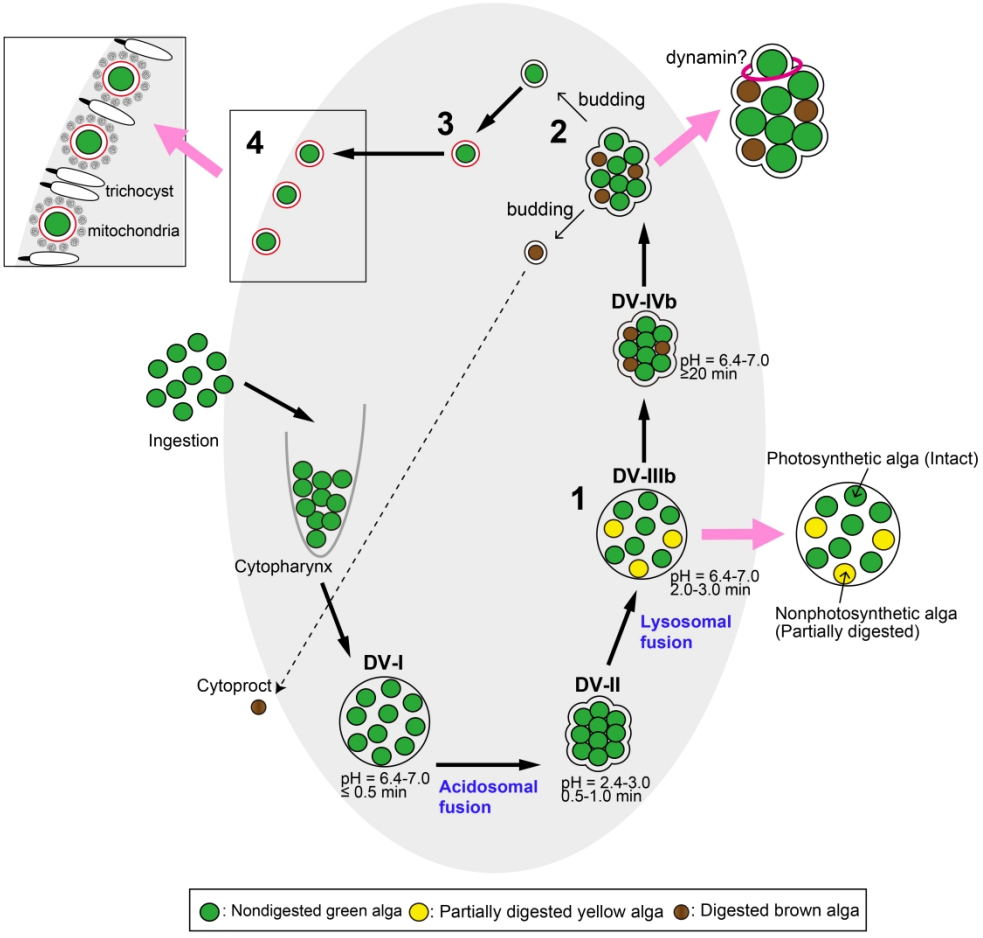


Fig. 6. Schematic representation of algal reinfection.

180x170mm (600 x 600 DPI)

Table 1. Species specificity and nucleus specificity of *Holospora* spp. and *Holospora*-like bacteria (HLB).

Holospora and HLB	Habitat	Host ciliate	References	Cryopreservation
*Holospora obtusa**1	Ma	*Paramecium caudatum*	Hafkin 1890; Gromov and Ossipov 1981; Görtz and Schmidt 2005	Fujishima et al. 1991
H. elegans	Mi	*P. caudatum*	Hafkin 1890; Gromov and Ossipov 1981; Görtz and Dieckmann 1980; Görtz and Schmidt 2005	Fujishima 2009
H. undulata	Mi	*P. caudatum*	Hafkin 1890; Ossipov 1973; Gromov and Ossipov 1981; Görtz and Schmidt 2005	Millot and Kaltz 2006
H. recta	Mi	*P. caudatum*	Fokin 1991; Görtz and Schmidt 2005	Kawai and Fujishima 1996
H. curviuscula	Ma	*P. bursaria*	Hafkin 1890; Gromov and Ossipov 1981; Görtz and Schmidt 2005; Rautian and Wackerow-Kouzova 2013	
H. acuminata	Mi	*P. bursaria*	Hafkin 1890; Ossopov et al. 1980; Rautian and Wackerow-Kouzova 2013	
H. bacillata	Ma	*P. calkinsi*	Fokin and Sabaneyeva 1993;	

			Görtz and Schmidt 2005	
	Ma	*P. nephridiatum*	Fokin 1989; Görtz and Schmidt; 2005	
H. curvata	Ma	*P. calkinsi*	Fokin and Sabaneyeva 1993	
H. caryophila	Ma	*P. caudatum*	Görtz 1987	
	Ma	*P. primaurelia*	Potekhin et al. 2018	
	Ma	*P. biaurelia*	Preer 1969; Preer and Preer 1982	
	Ma	*P. triaurelia*	Potekhin et al. 2018	
	Ma	*P. tetraurelia*	Potekhin et al. 2018	
	Ma	*P. sexxaurelia*	Potekhin et al. 2018	
	Ma	*P. octaurelia*	Castelli et al. 2015	
	Ma	*P. novaurelia*	Potekhin et al. 2018	
	Ma	*P. tredecaurelia*	Potekhin et al. 2018	
	Ma	*P. quadecaurelia*	Potekhin et al. 2018	
	Ma	*P. jenningsi*	Potekhin et al. 2018	
	Ma	*P. multimicronucleatum*	Potekhin et al. 2018	
	Ma	*P. putrinum*	Potekhin et al. 2018	
Candidatus Holospora parva	Ma	*P. chlorelligerum*	Lanzoni et al. 2016	
Ca. Gortzia yakutica	Ma	*P. putorinum*	Beliavskaia et al. 2020	
Ca. Gortzia infectiva *2	Ma	*P. jenningsi*	Boscaro et al. 2013	
Ca. Gortzia shahrazadis	Ma	*P. multimicronucleatum*	Serra et al. 2016	
Ca. Hafkinia simulans	Ma	*Frontonia salmastra*	Ferrantini et al. 2007; Fokin et al. 2019	

Ma, macronucleus. Mi, micronucleus.

*1 Temporary macronuclear invasion is known in *P. multimicronucleatum*, 14 species of *P. aurelia* complex, and *P. jenningsi* but not maintained in the nucleus (Fujishima and Fujita, 1985; Fujishima1986).
*2 Temporary macronuclear invasion is known in *P. quadecaurelia*, *P. schewiakoffi*, *P. sonneborni*, *P. caudatum* (Boscaro et al. 2013)

Recent phylogenetic analysis showed that *H. elegans* and *H. recta* should be considered subspecies of *H. undulata* (Wackerow-Kouzova and Myagkov 2021). Furthermore, from phylogenetic relationship among *H. caryophila* and other *Holospora* species, *H. caryophila* is proposed to establish a new genus within the family *Holosporaceae* as *Preeria caryophila* comb. nov. (Potekhin et al 2018).