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Brief report

# An experimental test of the symbiosis specificity between the ciliate *Paramecium bursaria* and strains of the unicellular green alga *Chlorella*



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## Summary

**The ciliate *Paramecium bursaria* living in mutualistic relationship with the unicellular green alga *Chlorella* is known to be easily infected by various potential symbionts/parasites such as bacteria, yeasts and other algae. Permanent symbiosis, however, seems to be restricted to *Chlorella* taxa. To test the specificity of this association, we designed infection experiments with two aposymbiotic *P. bursaria* strains and *Chlorella* symbionts isolated from four *Paramecium* strains, seven other ciliate hosts and two *Hydra* strains, as well as three free-living *Chlorella* species. *Paramecium bursaria* established stable symbioses with all tested *Chlorella* symbionts of ciliates, but never with symbiotic *Chlorella* of *Hydra viridissima* or with free-living *Chlorella*. Furthermore, we tested the infection specificity of *P. bursaria* with a 1:1:1 mixture of three compatible *Chlorella* strains, including the native symbiont, and then identified the strain of the newly established symbiosis by sequencing the internal transcribed spacer region 1 of the 18S rRNA gene. The results indicated that *P. bursaria* established symbiosis with its native symbiont. We conclude that despite clear preferences for their native *Chlorella*, the host–symbiont relationship in *P. bursaria* is flexible.**

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## Introduction

Symbioses, in general, are defined as two or more species living together in beneficial coexistence (e.g. Margulis and Fester, 1992; Douglas, 1995). This type of mutualistic interaction plays an important role in maintaining populations living under precarious environmental conditions (Margulis and Fester, 1992; Dolan and Pérez, 2000). In symbiotic ciliates, potential phototrophic endosymbionts are ingested by the host, but are able to escape digestion (Görtz, 1996). Such mixotrophic organisms combine the advantages of a heterotrophic nutrition mode with autotrophic energy gain, either through sequestered chloroplasts (kleptoplasts) or through algal symbionts (Dolan, 1992; Jones, 1994). An example of the latter is one of the classical symbioses, the mutualistic relationship between the ciliate *Paramecium bursaria* (Hymenostomatia) and the unicellular green alga *Chlorella* (Trebouxiophyceae) (Karakashian, 1975; Reisser, 1986). This symbiosis seems to represent a permanent association with hereditary symbionts (Siegel, 1960), where each algal cell is enclosed in an individual host vacuole, i.e. the perialgal vacuole. The exclusive mutualistic relationship of *P. bursaria* with 'zoochlorellae' under natural conditions has long been considered as a fact, but natural populations of aposymbiotic *P. bursaria* have been recently reported (Tonooka and Watanabe, 2002).

*Paramecium bursaria* is known to be easily invaded by various potential symbionts/parasites such as bacteria, yeasts and algae (Görtz, 1982; Omura *et al*., 2004). Although an infection with different algal symbionts is possible (Weis, 1978; Schulze, 1951), permanent establishment of a stable symbiosis seems to be restricted to *Chlorella*. Molecular phylogenetic analyses of the endosymbionts of *P. bursaria* have identified several *Chlorella* strains involved (Huss *et al*., 1989; Nishihara *et al*., 1998; Hoshina *et al*., 2004; 2005). Different *Chlorella* species have been found to have distinct suitabilities for the establishment of stable symbioses in *P. bursaria* (Hirshon, 1969; Weis, 1978) and infection rates have been shown to be affected by specificity of host and potential symbiont,

**Table 1.** Origin of the 16 *Chlorella* strains and summary of infection experiments with two aposymbiotic *P. bursaria* strains.

| Algal strain | Natural host | Origin | Infection[a] | | Uncorrected p-distance[b] | Accession number[c] |
|---|---|---|---|---|---|---|
| | | | PbPIBw | PbKM2w | | |
| *Chlorella* Stokesia PIB | *Stokesia vernalis* | Piburger See, Austria | + | + | 0.1984 | EF030572 |
| *Chlorella* Pelagodileptus PIB | *Pelagodileptus trachelioides* | Piburger See, Austria | + | + | 0.2040 | EF030573 |
| *Chlorella* Stentor PIB | *Stentor polymorphus* | Piburger See, Austria | + | + | 0.2006 | EF030575 |
| *Chlorella* Teuthophrys PIB | *Teuthophrys trisulca trisulca* | Piburger See, Austria | + | + | 0.2066 | EF030574 |
| *Chlorella* Uroleptus PIB | *Uroleptus* sp. | Piburger See, Austria | + | + | 0.2017 | EF030571 |
| *Chlorella* Askenasia PIB | *Askenasia chlorelligera* | Piburger See, Austria | + | + | 0.2012 | EF030576 |
| *Chlorella* Askenasia GKS | *Askenasia chlorelligera* | Gossenköllesee, Austria | + | + | 0.1978 | EF030577 |
| *Chlorella* PbPIB | *Paramecium bursaria* | Piburger See, Austria | + | + | – | EF030582 |
| *Chlorella* PbW | *Paramecium bursaria* | Wildbichl, Austria | + | + | 0 | EF030583 |
| *Chlorella* OCh | *Paramecium bursaria* | Karelia, Russia | + | + | 0 | EF030578 |
| *Chlorella* PbKM2 | *Paramecium bursaria* | Shimane, Japan | + | + | 0.1767 | EF030584 |
| *'Chlorella' Hydra* KRAN | *Hydra viridissima* | Innsbruck, Austria | – | – | x | x |
| *'Chlorella' Hydra* multiheaded (MH) | *Hydra viridissima* | Florida, USA | – | – | x | x |
| *'Chlorella minutissima'* CCAP211/52 | free-living | CCALA/New Zealand | – | – | 0.2759 | EF030581 |
| *Chlorella sorokiniana* Praha A14 | free-living | CCALA/Czech Republic | – | – | 0.2902 | EF030580 |
| *'Chlorella' saccharophila* 211–1a | free-living | SAG/Germany | – | – | 0.4610 | EF030587 |

**a.** Infection success; (+), accepted as symbiont; (–), not accepted as symbiont.
**b.** Phylogenetic (uncorrected p-) distances of a 412-bp gene segment including the ITS-1 region of infected algal strains to *P. bursaria* PIB native symbionts (EF030582).
**c.** NCBI accession numbers of a 412-bp gene segment including the ITS-1 region as reported in Summerer and colleagues (submitted). Except for *Hydra viridissima* (Cnidaria) all other natural hosts are ciliates.
PbPIBw, aposymbiotic *P. bursaria* isolated from Piburger See, Austria; PbKM2w, aposymbiotic *P. bursaria* strain KM2 isolated in Japan.

such as recognition of surface antigens, or by physiological conditions of the partners involved (Karakashian and Karakashian, 1973; Weis, 1979; Nishihara *et al*., 1998).

Despite numerous studies on the ciliate-*Chlorella* system, the mechanisms and constraints of infection and establishment of symbiosis are still not fully understood. For example, none of the reports on *Chlorella* symbioses has mentioned phylogenetic relatedness of compatible symbionts. In a previous study on the phylogeny of symbiotic *Chlorella,* we identified several strains of *in hospite Chlorella* (i.e. in the host) among different symbiotic ciliate species, but also within one single species from different habitats (Summerer *et al*., submitted).

In this study, we designed infection experiments with two aposymbiotic strains of *P. bursaria* to test (i) whether infection ability reflected phylogenetic relationships and (ii) whether the aposymbiotic host selected for its native *Chlorella* or not when offered two other compatible strains.

### Infection ability of aposymbiotic *P. bursaria* by native and non-native strains of *Chlorella*

For these experiments, two aposymbiotic strains of *P. bursaria* (PbKM2w and PbPIBw) and 16 different *Chlorella* strains were used (Table 1). As symbiont density and contact time are known to play a role in successful infection (Weis and Ayala, 1979), we selected conditions favouring this process. Thus, after 7 days of coculturing aposymbiotic *P. bursaria* with dense *Chlorella* cultures (10 000 algae/*Paramecium* cell), we found 11 infective out of 16 *Chlorella* strains tested (Table 1). We considered successfully infected *P. bursaria*, those that did not show any differences in appearance (Fig. 1A and B) or behaviour compared with native strains and maintained a stable symbiosis for several months. Although all *Chlorella* strains were initially ingested and retained in food vacuoles (Fig. 1C), and sometimes also digested by *P. bursaria*, only the 11 infective *Chlorella* strains were able to establish a symbiosis in both ciliate strains.

Both strains of *P. bursaria* were easily infected with their native *Chlorella* and with those of all other investigated *P. bursaria* strains, no matter whether they belonged to the 'northern' (i.e. Europe: PbPIB, PbW and OCh) or the 'southern' (i.e. Japan: PbKM2) ecotype (*sensu* Kvitko *et al*., 2001). These results are in contrast to those of Weis (1978), who found *Chlorella* isolates of *P. bursaria* that did not infect aposymbiotic paramecia. However, these authors used different host and symbiont strains than the ones tested in this study. Moreover, we believe the much longer incubation times used in our study compared with only 20 h in Weis (1978) study, might also explain this discrepancy.

An infection with the symbionts of two *Hydra viridissima* strains failed in spite of multiple experiments with different cell densities, symbiont preparations and ex-symbiotic 'storage' times of *Hydra* symbionts (Table 1). Unsuccess-

 

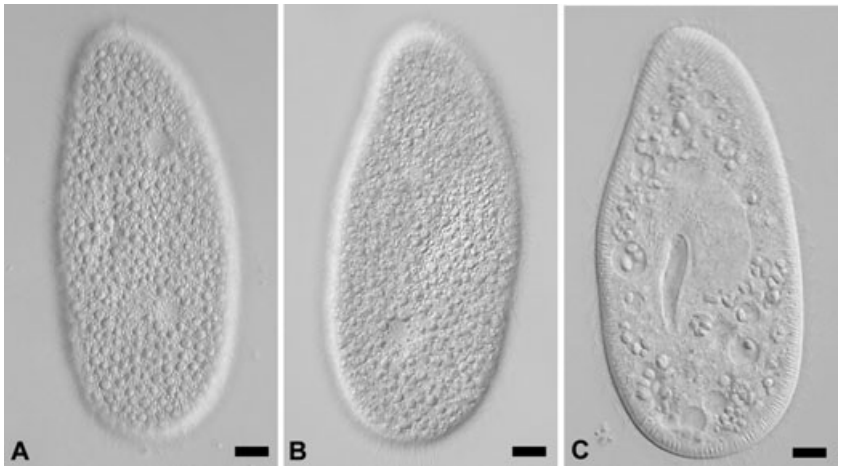


**Fig. 1.** Results of infection experiments of aposymbiotic *P. bursaria* showing the native symbiosis of *P. bursaria* KM2 (A) and examples of a successful (B) and an unsuccessful (C) infection after 7 days of coculturing. Note, that *Chlorella* is also ingested into food vacuoles in unsuccessful pairings. Scale bar: 10 μm.

ful infection pairings also occurred with three free-living *Chlorella* taxa (Table 1). The ability of *Chlorella* to synthesize maltose and to tolerate acidic environments, as well as the possession of a specific recognition mechanism have been identified as prerequisites for establishing a symbiosis (Huss *et al*., 1993; Weis, 1979). Although we did not test those characteristics, Douglas and Huss (1986) and Huss and colleagues (1993) stated that successful endosymbionts only occur within lineages of algae which are predisposed for symbiosis. Thus, our finding of free-living *Chlorella* being incompatible for infection was not unexpected.

We used the sequences of the internal transcribed spacer region 1 (ITS-1) of the 18S rRNA gene of *Chlorella* strains reported by Summerer and colleagues (submitted) and calculated the phylogenetic distances using the software PAUP*4.0b6 (Swofford, 2000) to test whether there was a relationship to their infection ability. Compatible *Chlorella* from the same *Paramecium* ecotype (Kvitko *et al*., 2001) had identical DNA sequences, but those from the 'southern' ecotype (PbKM2) showed a sequence difference in the ITS-1 region of 17.67% (Table 1). Compatible *Chlorella* strains of other ciliate species had sequence divergences below 21% (19.78% to 20.66%). By contrast, the incompatible free-living taxa diverged by more than 27% in the same region (Table 1).

A close phylogenetic relationship of prospective symbionts could be a prerequisite for infection. It is likely that closely related algae have similar physiological properties and that *P. bursaria* and their symbionts have to be well adapted to each other interacting in multiple ways. An example of this tight coupling besides nutritional cooperations is that *P. bursaria* is able to synchronize the symbionts cell division to its own by regulating the cell cycle of endosymbiotic *Chlorella* and keeping the algal population constant until the host cell enters cytokinesis (Kadono *et al*., 2004). Nevertheless, we did neither have free-living *Chlorella* differing less than 27% nor find ciliate symbionts with more than 21% sequence divergence to conclude whether incompatibility was exclusively dependent on phylogenetic distance.

From the analysis of the scientific literature it was unclear, whether specific ciliate strains accept specific *Chlorella* symbionts or not. For example, Weis (1978) suggested that the syngenic identity of *P. bursaria* has nothing to do with their acceptance of specific *Chlorella* symbionts, whereas Hoshina and colleagues (2005) argued that the types of *Chlorella* symbionts depended on their *Paramecium* hosts' lineages. We showed that *Chlorella* isolated from four different *P. bursaria* strains were compatible to both host strains tested. These results indicate that besides from infectivity, additional factors play a role in the specific composition of the symbiosis. Specific adaptation of symbiotic partners to different environmental conditions, such as exposure to elevated solar UV radiation or temperature, could be another reason for finding distinct algal symbionts in natural ciliate populations (Kvitko *et al*., 2001; Sommaruga *et al*., 2006; Summerer *et al*., submitted). Thus, the actual availability of a necessarily compatible *Chlorella* symbiont in the respective environment seems to be important. Likewise, a shift in the composition of symbionts is known to occur in marine *Symbiodinium* (Dinophyceae) associations after environmental change and bleaching events (Baker, 2003).

One common feature observed in infection experiments of all investigated *Chlorella* strains was the uptake of several algal cells together into food vacuoles of *P. bursaria* in the course of minutes (Fig. 1). It is known that *P. bursaria* efficiently ingests algae other than their *Chlorella* symbionts (Hirshon, 1969; Karakashian and Karakashian, 1973). For example, in our case, *P. bursaria* was able to live in axenic conditions on the free-living strain *Chlorella saccharophila* for months (M. Summerer, pers. observation). A certain number of infective *Chlorella*

 

strains escaped from the food vacuole and were found individually enclosed in perialgal vacuoles, mainly distributed at the periphery of the *Paramecium* cell (Fig. 1). This phenomenon has been observed in previous studies (e.g. Karakashian, 1975). However, the peripheral region of *P. bursaria* cells can also be infected by bacteria and yeasts, which are not considered to be beneficial symbionts (Görtz, 1982), so that the accumulation of ingested *Chlorella* near the cell surface seems to be a prerequisite for the establishment of a symbiosis and not its result (Omura *et al*., 2004). Moreover, Kodama and Fujishima (2005) recently demonstrated that potentially symbiotic algae were able to escape from the paramecia's food vacuole after the fusion of acidosoms and lysosoms and succeeded to establish endosymbiosis.

### Mixed infection experiment

We also assessed the host–symbiont specificity in a mixed infection experiment using the native *Chlorella* strain of *P. bursaria* PbPIBw isolated from Piburger See, and two other compatible *Chlorella* strains including one from another hymenostome ciliate, *Stokesia vernalis*, isolated from the same lake, and *Askenasia chlorelligera* isolated from another lake (Gossenköllesee). These two non-native *Chlorella* strains had very similar phylogenetic distances to the native symbiont (Table 1).

The ITS-1 sequences of all three infection replicates turned out to be identical to each other and to the native *Chlorella* symbionts of *P. bursaria* PbPIB. Certainly, we cannot exclude the presence of a minimal number of actually different symbionts in our samples which potentially were not amplified by polymerase chain reaction (PCR). However, from a mixture of three compatible symbionts, *P. bursaria* at least largely preferred their native *Chlorella* to establish a symbiosis. Although previous studies on the symbiosis of *P. bursaria* have identified variations in the infection abilities of different algal symbionts (i.e. Schulze, 1951; Weis, 1978), our results are the first to evidence a highly sophisticated degree of specific symbiont recognition in *P. bursaria.* Likewise, Nishiguchi and colleagues (1998) found a pronounced dominance of the native symbionts in experimental colonization of the squid *Euprymna scolopes* with different luminous bacteria (*Vibrio fisheri*). Moreover, these authors also found a coherency between colonization competency of prospective symbionts and the phylogenetic relatedness of the partners.

In natural environments, species-specific symbioses with optimized adaptations of both partners, seem to be the rule in *P. bursaria*, however, the successful establishment of a mutualistic relationship with several *Chlorella* symbionts even from other ciliate species is possible. Our findings indicate that despite clear preferences for their native *Chlorella*, the host–symbiont relationship in *P. bursaria* seems to be flexible enough to adapt to environmental change by accepting non-native *Chlorella* at least under experimental conditions.

### Experimental procedures

The ciliates and their symbionts were collected, identified and isolated as described elsewhere (Sonntag *et al*., 2006; Summerer *et al*., submitted). *Chlorella*-free (i.e. aposymbiotic) clones of *P. bursaria* were obtained by growing the symbiotic ciliates in permanent darkness for several weeks to months at 20°C on a bacterial diet supported by a lettuce culture medium enriched with 1.5% Chalkley's medium. The absence of symbiotic algae was regularly checked with an Olympus microscope (BX50).

*Chlorella* symbionts were grown in Woods Hole MBL medium (Guillard and Lorenzen, 1972), except for *P. bursaria* symbionts, which were cultured in Tamiya medium (Tamiya, 1957) or freshly isolated. The *Chlorella* of *H. viridissima* were freshly isolated from their hosts by mechanical disruption and centrifugation. These *Chlorella* did not grow ex-symbiotically, but survived long enough in liquid Woods Hole MBL medium to perform infection experiments. The aposymbiotic strains of ciliates and the *Chlorella* strains were grown in light/temperature controlled chambers under a 16:8 h light : dark photoperiod and at 17–20°C.

For the mixed infection experiment, the different *Chlorella* strains were concentrated by gentle centrifugation (8000 *g*, 5 min) and mixed in a 1:1:1 ratio (final density of *Chlorella* cells ~$10^6$ ml$^{-1}$) in Woods Hole MBL medium. Then, aposymbiotic *P. bursaria* PbPIBw (*Chlorella* : host ratio 10 000:1) were added for infection and after 24 h, not ingested algae were removed by filtration through a nylon net (20 μm mesh size). Then, the infected *P. bursaria* were cultured in sterile Woods Hole MBL medium enriched with 30 mg l$^{-1}$ yeast extract, in 100-ml Schott glass bottles for 10 days. The infection experiment was performed in triplicates.

To identify which *Chlorella* strain was found in the newly established symbiosis, infected ciliates were filtered through a nylon net (20 μm mesh size) to remove free *Chlorella* cells. About 30 000 *P. bursaria* per infected culture (triplicates) were harvested by centrifugation (3000 *g*, 5 min) and the pellet was frozen in liquid nitrogen. Total DNA was extracted by a commercial DNA extraction kit (DNeasy® Plant Mini Kit/Quiagen) according to the manufacturer's directions, with the following modifications: 600 μl of precooled (0–4°C) P1-buffer and 20 μl of RnaseA (20 g l$^{-1}$) were added to the frozen sample pellet and the mixture was sonicated on ice for 3 min at 42 W to ensure the rupture of the algal cell walls. After this step, we followed the standard extraction protocol. We amplified both strands of the ITS-1 using the following primers 5′-GGAGAAGTCGTAACAAGGTTTCCG-3′ and 5′-ATCCTGCAATTCACACCAAGTATCG-3′ (Huss *et al*., 2002). Polymerase chain reaction amplification was performed on a Techne PHC-3 Thermal Cycler. An initial denaturation step of 94°C for 5 min was followed by 35 cycles under the following conditions: 45 s denaturation at 94°C, 45 s annealing at 53°C and 60 s extension at 72°C, followed by a final extension step of 72°C for 5 min. To check the density of amplified DNA fragments, 2-μl aliquots of the PCR prod-

 

ucts were separated on a 1% TAE-agarose gel in TAE buffer and stained with ethidium bromide. The amplification products were purified with ExoSAP-IT®/USB-Corporation before direct sequencing (Sanger *et al*., 1977) with an ABI PRISM Big Dye Terminator Cycle Sequencing Kit using an ABI 3100 DNA sequencer, according to the manufacturer's instructions. The resulting 412-bp gene segments including the ITS-1 region were individually checked and aligned using the computer program ClustalX (Thompson *et al*., 1994).

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