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THERE IS AN ECOLOGICAL BASIS FOR HOST/SYMBIONT SPECIFICITY IN *CHLORELLA/HYDRA* SYMBIOSES

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Summary: The *Hydra/Chlorella* symbiosis is an important model system for examining host/symbiont specificity in alga/invertebrate systems. The capability of free-living *Chlorella* to establish symbioses correlates with tolerance of growth to low pH. This agrees with preliminary evidence that pH levels in perialgal vacuoles in aposymbiotic hydra drop to values of 3.5 - 4.0 within several hours after infection. In contrast, excretion of maltose or other sugars and the binding capacity of several lectins is unrelated to infectivity. Formation of successful endosymbioses also appears to be species-specific. Small subunit rRNA sequence comparisons showed that native symbionts of *H. viridissima* descend from at least two symbiotic events. Symbionts of the 'European'-, 'Swiss'- and 'Jerusalem' strain are closely related to the *C. vulgaris/sorokiniana* group, and Strain HvT from Israel is very similar to *C. protothecoides.* We conclude from the above data that ecological factors given by the environment inside the phagosomes of the hydra determine host/symbiont specificity rather than 'recognition' processes.

INTRODUCTION

Algae/hydra symbiosis has been described as an active host/symbiont interaction. Muscatine et al. (1) have suggested that a successful establishment of symbiosis is the result of "very specific recognition between the algae and hydra". In turn, recognition of symbiotic algae and regulation of their number in the cells of hydra have been claimed to depend on the ability of the algae to excrete maltose (2,3,4,5), and on specific markers on the algal cell wall (6,7). Since the first algae that colonized hydra cells must have been free-living (8), they must have fortuitously established specific recognition elements.

A competing hypothesis is that algae/hydra symbiosis is based on 'ecological' para-meters (9). Hydra prey on small filter-feeding invertebrates, e.g. small crustaceans, that filter-feed on unicellular algae. Algae present in the gut of the prey, are ingested and phagocytosed by the digestive cells of the hydra together with food particles. The algae colonize the hydra through the latter's prey, there is competition between the introduced algae (10), and territoriality of the established symbionts may prevent further infection (11).

Symbiotic abilities of free-living *Chlorella* species

The native symbiont of *Hydra viridissima* can be removed by various methods (cf. 12). The aposymbiotic (= without symbionts) hydra are then available for reinfection experiments exploring the ability of different free-living algae to colonize their cells. Rahat and Reich (13) were able to introduce various laboratory strains of *Chlorella* into aposymbiotic hydra and observed that some of them formed stable symbioses whereas others were expelled or digested. This raised the question: why can some algae live in the cells of *Hydra* while others cannot?

In the symbiotic and non-symbiotic free-living strains of *Chlorella,* a clear-cut corre-lation exists between their ability to colonize cells of *H. viridissima* and their ability to grow *in vitro* in nutrient-enriched media (13). This correlation was assumed to be related to the eco-physiological conditions in the algae-hosting vacuoles (= phagosomes of the hydra's digestive cells), and to the nutrient requirements of the respective algae.

At this point, it was of interest to see whether the ability for symbiosis is a species-specific characteristic and shows any correlations with known physiological or biochemical characters (14,15,16) of the respective *Chlorella* species. This might lead to new insights into the physiological basis of symbiosis, i.e. the specific requirements of life in the rather special environment of the vacuoles of *Hydra.*

Sixteen *Chlorella* strains previously studied in Jerusalem (13) were identified according to 8 physiological and biochemical characters, and 30 previously assigned strains (14) were examined for their symbiotic properties. The 46 *Chlorella* strains belong to 15 clearly defined taxa, and the ability for symbiosis was found to be a species-specific character. Six taxa were able to form a stable symbiosis, while the other nine taxa were unable to do so (19). Among the 11 known physiological and biochemical characters of the 15 species (hydrogenase, secondary carotenoids, nitrate reduction, thiamine requirement, gelatine liquefaction, starch hydrolysis, lactate fer-mentation, growth on mannitol, and acid, salt, and temperature tolerances of growth), only acid tolerance of growth seems to be correlated with their symbiotic abilities (Table 1). All symbiotic species were able to grow at or below pH 3.5, whereas the

Table 1. Ability of symbiosis with *Hydra viridissima* and acid tolerance of growth of 15 *Chlorella* species.

Species	Symbiosis	Limit at low pH
C. saccharophila (KRÜGER) MIGULA	+	2.0-3.0
C. ellipsoidea GERNECK	+	2.0-3.0
C. kessleri FOTT ET NOVÁKOVÁ	+	3.0
C. luteoviridis CHODAT	+	3.0
C. *fusca* var. *vacuolata*[1] SHIHIRA ET KRAUSS	+	3.0-3.5
C. *protothecoides* KRÜGER	+	3.5-4.0
C. vulgaris BEIJERINCK	-	3.5-4.0
C. *sorokiniana* SHIHIRA ET KRAUSS	-	4.0-5.0
C. *lobophora* ANDREYEVA	-	4.0
C. mirabilis ANDREYEVA	-	4.0
C. fusca var. *fusca*[1] SHIHIRA ET KRAUSS	-	4.0
C. fusca var. *rubescens*[1] (DANGEARD) KESSLER ET AL.	-	4.5
C. zofingiensis DÖNZ	-	5.5
C. minutissima FOTT ET NOVÁKOVÁ	-	5.5
C. homosphaera SKUJA	-	6.0

[1]According to molecular data (DNA hybridization, rRNA sequences), these taxa belong to the genus *Scenedesmus* (17,18).

non-symbiotic taxa are growth-limited below pH 4 (19,20). In this connexion it is interesting to note that some zoochlorellae isolated from *Hydra* excrete large quantities of maltose in acid media below pH 4, and this phenomenon is supposed to be essential for the establishment of symbiosis (2,3,5,21). The intracellular vacuoles of *Hydra* which contain the symbiotic algae might therefore be a rather acidic environment (20).

Excretion of sugars by *Chlorella*

The excretion of three sugars, i.e. maltose, glucose, and glucose-6-phosphate, was studied in 38 strains of *Chlorella* (22). Typical results for representative strains of 7 symbiotic and 8 non-symbiotic taxa are shown in Table 2. It is evident that maltose, so far considered to be of special importance for symbiosis (2,3,5,21), is by no means the predominant sugar. In fact, among the species capable of symbiosis, only *C.* spec. (= *"C. paramecii"*, a symbiotic strain isolated from *Paramecium bursaria* (cf. 23) produces very large quantities of maltose, but with an optimum at pH 5.5-6.0

rather than below pH 4. In most symbiotic species, glucose-6-phosphate is the main sugar component, but the amounts excreted are comparatively small (Table 2).

Table 2. pH-dependent sugar excretion by *Chlorella* strains capable and incapable of symbiosis with *Hydra viridissima.* Highest rates (x 10^{-10}g · ml^{-1} · h^{-1} per 10^6 cells) at the respective pH optima. Maltose = mal, glucose = glu, glucose-6-phosphate = glu6ph.

Species	Strain	Symb.	Predominant sugar	Highest excretion	pH optimum
C. saccharophila	211-9a	+	glu6ph	1.1	2.5 -4.5
C. ellipsoidea	3.80	+	glu6ph	1.6	2.5 -6.0
C. kessleri	211-11g	+	glu6ph	4.9	6.0
C. luteoviridis	211-2a	+	glu6ph	3.4	2.5 -6.0
C. fusca var. *vacuolata*	211-8b	+	glu	2.6	2.5
C. protothecoides	211-7a	+	glu6ph, mal	0.7, 0.6	2.5 -3.5
C. spec. (="C. *paramecii")*	211-6	+	mal	86	5.5 -6.0
C. vulgaris	211-1 lb	-	mal	33	2.5
C. sorokiniana	211-8k	-	mal, glu	0.9, 0.9	2.5
C. lobophora	750-I	-	mal, glu	14,7	2.5
C. mirabilis	748-I	-	mal	90	2.5 -3.5
C. fusca var. *fusca*	343	-	glu6ph	3.2	5.0 -6.0
C. fusca var. *rubescens*	232/1	-	glu6ph, mal	1.9, 1.9	2.5
C. zofingiensis	211-14a	-	mal	3.5	2.5
C. minutissima	C-1.1.9	-	mal	5.3	5.0-6.0

Among most non-symbiotic taxa, maltose is the predominant sugar with *C. vulgaris* and *C. mirabilis* excreting very large quantities, especially in rather acid media (Table 2). This phenotype is considered to be typical for symbiotic algae (2,3,5,21), but the data of Table 2 show that there is no obvious connection between formation of symbiosis and the release of the sugars studied here. Glucose-6-phosphate is the predominant sugar in most symbiotic, but also in some non-symbiotic species. On the other hand, the excretion of maltose prevails in most non-symbiotic taxa, but also in one symbiotic taxon. Likewise, producers of very large amounts of maltose are found among the symbiotic and the non-symbiotic species, and the same is true for strains which show only very low rates of excretion. In addition, optimum sugar release at

high and at low acidities occurs both among the symbiotic and the non-symbiotic species.

The time course of sugar excretion after transfer into medium with the pH of the limit of growth, did not exhibit any relationship with the symbiotic abilities of the *Chlorella* species, either. Some strains of symbiotic and non-symbiotic taxa initially display high rates of sugar excretion with lower rates later on. Other strains of both categories show very little release at first with increasing rates later (22).

Thus, our results do not provide any support for the assumption that the excretion of maltose is an important prerequisite for the *Hydra/Chlorella* symbiosis, and that it occurs predominantly in a rather acidic environment at or below pH 3.5.

Lectin-binding characteristics of symbiotic and non-symbiotic *Chlorella*

The first encounter between engulfed algae and the potential host cell is through their cell wall and membranes. Treatment of algal symbionts with lectins before host infection significantly decreased the number of symbionts taken up by the host (24,25,26). This indicated a possible involvement of surface glycoconjugates in phagocytosis and in the establishment of algal symbioses. We looked therefore for a correlation between lectin-binding characteristics and 'symbiocity' of the chlorellae. Six different lectins were examined for their agglutination and binding properties with symbiotic and non-symbiotic chlorellae. Agglutination and lectin-binding characteristics were found to be species-specific but there was no correlation with the ability or inability of the respective *Chlorella* to form symbiosis with *Hydra.* The native symbionts differed in their agglutination properties from the cultured strains, but no clear correlation between the ability of the latter to form symbiosis and the agglutination with lectins was found (26,27).

The intracellular pH in the perialgal vacuoles

The fate of ingested algae is decided in the phagosomes. Phagosome/lysosome fusion is a mechanism by which cells defend themselves against invasion of foreign organisms, and phagosomal pH regulates the release of lysosomal enzymes into the phagosome. Invaders into a cell are supposed to be digested at a low phagosomal pH, and raising the pH by the invader is a means to avoid digestion (28,29,30). On the other hand, all 'symbiotic' free-living *Chlorella* were found to tolerate a low pH of 3.5 or less (Table 1) indicating that this might be a prerequisite for survival of potential symbionts if the perialgal vacuoles are acidic. In *Hydra,* however, no direct study of the intra-phagosomal pH has yet been published.

Using an ACAS 570 Interactive Laser Cytometer we applied the Fluorescence Ratio Microscopy technique to determine the intraphagosomal pH and its kinetics for

the first 24 hrs after infection. Preliminary results show that 2 h after infection of symbiotic algae, the phagosomal pH is 3.5 - 6.0. Five and 24 hours later there is a tendency towards acidification to a pH of 3.5 - 4.0. When non-compatible algae are infected into hydra, the pH tends to be somewhat less acidic. Further study is needed to correlate these data with the compatibility of the cosymbionts.

In a recent study of the 'European' strain of *H. viridissima* the acidity of the perialgal space in the phagosomes has been questioned (31), but no conclusive data were provided.

Taxonomic affiliation and phylogenetic position of native algal symbionts

In contrast to the free-living *Chlorella* strains that were experimentally shown to form stable symbioses with aposymbiotic 'Swiss' hydra (19), the native symbionts of *Hydra viridissima* seem to be better adapted to their specific environment inside the perialgal vacuoles. This can be recognized by the degree of colonization of the host. Native symbionts usually are homogeneously distributed in the digestive cells of *Hydra* turning them entirely green. Artificially infected 'symbiotic' free-living algae usually inhabit only parts or even spots of the hydra. Competition experiments of free-living 'symbiotic' *Chlorella* and isolated native symbionts simultaneously infected into aposymbiotic hydra have shown that the latter eventually succeed in colonizing the hydra (10). It seems therefore that native symbionts share a relatively long evolutionary history with their host resulting in the selection and survival of algae most suitable to live in this specific environment. This raises the questions whether all native symbionts found today in green hydra are descendants of a single and early symbiotic event (monophyletic origin) or of multiple events (polyphyletic origin), and how these symbionts are related to free-living *Chlorella* species.

The taxonomy of zoochlorellae that live in *Hydra* has been hampered by the apparent inability to culture the symbionts *in vitro,* i.e. outside their host cells (32,33). There are two reports about the successful cultivation of *H. viridissima* symbionts (34,35), but both cannot rule out contamination with *Chlorella* cells e.g. adhering to the sticky pedal disc of the hydra. After Brandt's original description of the symbionts as a new genus *Zoochlorella* (36), Pardy (37) compared morphological characteristics of the symbionts of different *H. viridissima* strains. He found that the symbionts from the 'Florida' strain and 'English' strain of green hydra differ in at least three respects: chloroplast morphology, the appearance of the polyphosphate bodies, and the presence of a pyrenoid in the 'English'- but not in the 'Florida' strain. No taxonomic conclusions were drawn from this study because of the considerable morphological plasticity of the symbionts.

In a previous paper we showed that the symbionts of three *Hydra* strains, the 'European'- (Esh), 'Swiss'- (Ssh), and 'Jerusalem' (Jsh) symbiotic *Hydra* can be

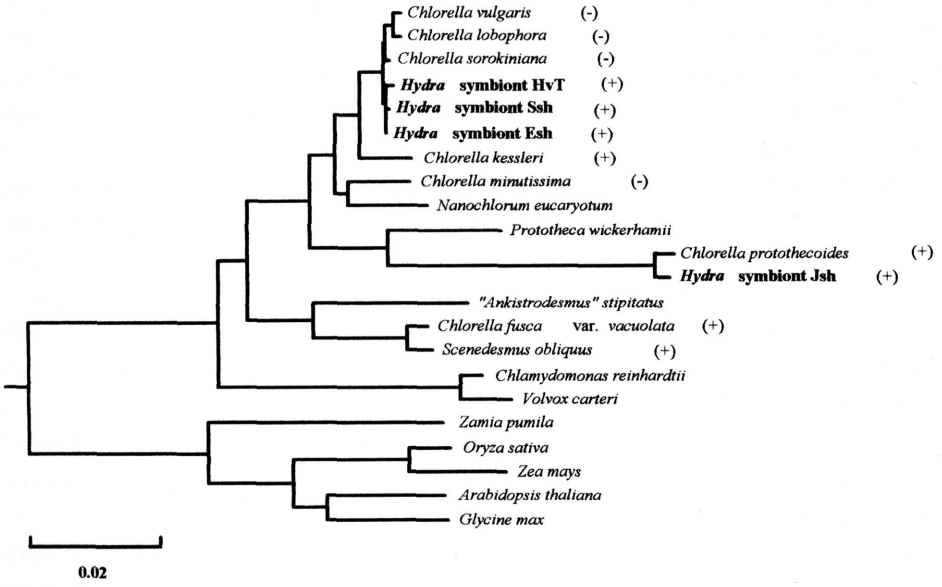

Figure 1. Phylogenetic relationships of native symbionts of *Hydra viridissima* to free-living *Chlorella* species. The tree is inferred from distance data of complete SSU rRNA sequences (cf. 17). Sequences from reference organisms are from the EMBL Data Library. The capability to initiate a stable symbiosis with the 'Swiss' strain of *H. viridissima* is indicated in brackets, if determined. The scale indicates fixed mutations per sequence position.

differentiated by their respective cell morphology, DNA base composition, and sensitivity of their cell wall against SDS (38). We have now investigated these symbionts with respect to their small subunit ribosomal RNA (SSU rRNA) similarities, including the symbiont of one more strain of *H. viridissima* isolated in Israel (HvT). The algae were separated from host tissue, and DNA isolated and purified as described by Huss et al. (38). SSU rRNA genes were amplified by PCR, and sequenced either from M13 templates (17), or directly from single stranded PCR products isolated with magnetic beads according to the instructions provided by the manufacturer (DYNAL). The complete SSU rRNA sequences of Esh, HvT, Jsh and Ssh as well as of *Chlorella lobophora* ANDRRYEVA 750-I (V.M. Andreyeva, St. Petersburg, GUS) and *C. sorokiniana* SHIHIRA ET KRAUSS SAG 211-8k (type strain of the Sammlung für Algenkulturen in Göttingen, FRG) that served as reference organisms, were determined and are available from the EMBL Data Library (Accession Numbers X72706, X72707, X72708, X72854, X63504, X62441). A phylogenetic tree (Fig. 1) was constructed from 1722 unambiguously aligned nucleotide positions by the distance matrix program of G.J. Olsen (cf. 17).

As shown in Fig. 1, the *Hydra* symbionts Esh, Ssh and HvT are very closely related to each other. The SSU rRNA sequences of symbionts from the 'European' hydra which was isolated in the U.K. and sometimes is also called the 'English' hydra (39), and from the 'Swiss' strain isolated in Switzerland, are distinguished by a single nucleotide substitution while the symbiont of strain HvT isolated in Israel is characterized by two more mutations. According to SSU rRNA sequence similarities, the most closely related free-living *Chlorella* species is *C. sorokiniana* with two, three and four differences compared to Esh-, Ssh- and Hvt symbionts, respectively. *C. sorokiniana,* on the other hand, is closely related to *C. vulgaris* and *C. lobophora* (Fig. 1), a species described by V.M. Andreyeva (40). This group, together with *C. kessleri,* comprises the 'true' chlorellae while other *Chlorella* species are much more distantly related and belong to different genera and even families (17). Fig. 1 implies a common origin of the three symbionts at a time close to the separation of *C. sorokiniana* and *C. vulgaris/lobophora.* However, the sequence differences are too minimal to be statistically relevant for the indicated branching order.

A close relationship to the *C. sorokiniana /vulgaris* group has previously been demonstrated for *Chlorella* symbionts from *Paramecium bursaria* (Ciliata) (18,41,42), *Acanthocystis turfacea* (Rhizopoda, Heliozoa) (23), and *Spongilla fluviatilis* (Porifera) (43). It seems, therefore, that this group is particularly predisposed for symbiotic interactions with a variety of invertebrates as suggested by Douglas & Huss (41). However, as shown by Kessler et al. (19) and indicated in Fig. 1, none of the free-living strains of the *C. vulgaris/sorokiniana* group is able to establish symbiosis with *H. viridissima.* This result, based on the study of only one *Hydra* host strain, Ssh, may simply indicate that minimal changes in physiological or any other properties are sufficient to determine success or failure in colonizing a potential host. This view is supported by the observation that the competence for initiating symbiosis with *H. viridissima* is not restricted to a phylogenetically coherent group of algae but is more or less irregularly found in different lineages of chlorococcalean algae (Fig. 1).

The symbiont of the 'Jerusalem' strain of *H. viridissima,* Jsh, is morphologically different from Esh- and Ssh symbionts, and has a significantly distinct DNA base composition (38). The rRNA sequence now confirms that the Jsh symbiont is not related to the other symbionts but instead to *C. protothecoides* (Fig. 1), a mesotrophic and auxotrophic *Chlorella* species (44). Again, with 10 nucleotide exchanges in its SSU rRNA, the relationship to a free-living species is rather close since the rRNA gene of *C. protothecoides* evolves in a tachytelic fashion, meaning that it accumulates mutations much more rapidly than do most other organisms. This behaviour is indicated in Fig. 1 by the long branch leading to *C. protothecoides* and the Jsh symbiont (cf. 17). Most of the nucleotide substitutions between both algae occur in

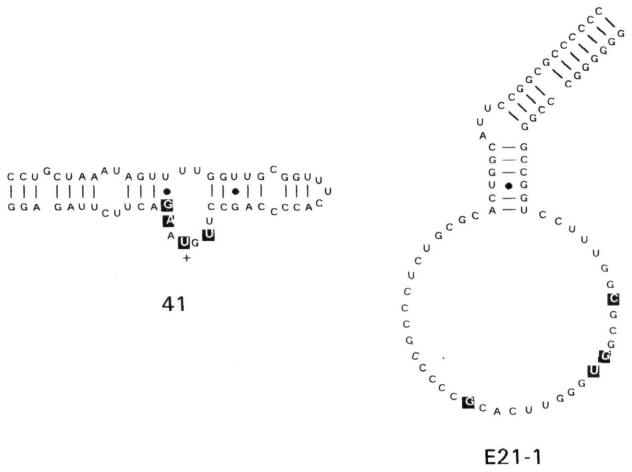

Figure 2. Small subunit rRNA secondary structure model of helices 41 and E21-1 from the 'Jerusalem' hydra symbiont. 8 out of a total of 10 mutations are located in the above two regions that are known to be hypervariable. The large loop of helix E21-1 results from an insertion of 26 nucleotides compared with other *Chlorella* species, and is unique to *C. prototheco*ides (17). Reversed letters indicate base substitutions compared with *C. prototheco*ides. Insertion of a nucleotide is shown by +.

two hypervariable regions of the SSU rRNA gene which define helix 41 and the loop of helix E21-1 (Fig. 2). In *C. prototheco*ides, the loop of helix E21-1 is characterized by a unique insertion of 26 nucleotides found only in this organism (17) and in the Jsh symbiont. This may be regarded as another evidence of their close phylogenetic relationship.

CONCLUDING REMARKS

In conclusion, our data show that the native *Hydra* symbionts result from at least two recent but independent symbiotic events. From our infection experiments of aposymbiotic *Hydra* with free-living *Chlorella* (13,19,20,45) and from a similar study by Jolley & Smith (39) it appears that new symbioses may be easily initiated. However, the competition experiments described above show that better adapted native symbionts eventually displace the 'foreign invader' (10). The symbionts usually are transmitted to the progeny of *Hydra* both asexually by budding and sexually within eggs (11). Ingestion of suitable algae by hydra that for any reason have become aposymbiotic, seems to be the only way for establishing a new symbiosis. This is probably a rare event and has never been reported from nature.

Garber (46) has shown that the host of any symbiotic interaction must serve as a 'growth medium' for the symbiont. When nutrition-deficient, but otherwise virulent mutants of animal and plant pathogens were injected into their host, they did not grow unless the missing nutrient was supplied. Garber concluded that "It is feasible that the specificity of certain parasites for specific tissues or organs may reflect a relationship between the nutritional demand of the parasite and availability of the required compound in these tissues or organs". The absence of specific growth factors in a host might thus impart immunity against invaders that cannot survive without them, as it similarly prevents colonization of abiotic habitats. This hypothesis apparently applies to hydra and other lower organisms lacking lymphocytes and an immune system such as found in vertebrates.

Experimental symbioses formed in the laboratory support this 'ecological' hypothesis. Distribution pattern and number of algae per hydra cell differ in *H. viridissirna* infected with free-living strains of *Chlorella* (13), and in 'brown' hydra infected with *Symbiococcum hydrae,* as they do in the native symbioses (47). This shows different 'carrying capacities' of hydra cells for different algae. An extreme case in point is seen in the Maxi strain of *H. magnipapillata.* In this hydra, numerous symbiococci are hosted in the hypostomal cells while only few algae are found in the rest of the polyp. A plausible explanation would be that the intravacuolar environment in the hypostomal cells of the Maxi strain supports algal growth while that in other digestive cells of this hydra does not.

As a simple working hypothesis for further study, the "Test Tube Hypothesis" is suggested (48): "Algae ingested into the digestive vacuole of a hydra cell are subjected to a selection similar to that occurring in any open abiotic habitat in nature, or in a medium in a test tube. Algae that are preadapted to live and reproduce in the given environment or medium will remain successful colonizers, exposed to mutual selective coevolution towards the formation of a stable symbiosis".

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