Symbiosis, 7 (1989) 117–126
Balaban, Philadelphia/Rehovot

Is Acid Tolerance of Symbiotic *Chlorella in vitro* an Indicator of pH in Intracellular Perialgal Vacuoles of *Hydra viridis*?

M. RAHAT*, E. KESSLER** and V.A.R. HUSS**
**Department of Zoology, The Hebrew University of Jerusalem Jerusalem, Israel 91904*
***Institut für Botanik und Pharmazeutische Biologie der Universität Staudtstrasse 5, D-8520 Erlangen, FRG*

Received July 20, 1988; Accepted April 13, 1989

Abstract

Forty-six strains of 15 taxa of *Chlorella* having 11 known physiological and biochemical characters were examined for their ability to form stable symbioses with a Swiss strain of aposymbiotic *Hydra viridis*. Eighteen strains of 6 taxa: *C. saccharophila* var. *ellipsoidea*, *C. saccharophila* var. *saccharophila*, *C. fusca* var. *vacuolata*, *C. kessleri*, *C. luteoviridis*, and *C. protothecoides* formed stable symbioses with this hydra. Six strains persisted in the cells of hydra for several weeks before they disappeared, and 22 strains were lost from the hydra within 6 days. Among the 11 physiological and biochemical characteristics known in these *Chlorella* species, it was only their ability to grow *in vitro* at or below a pH of 4.0 that correlated with the ability to live as endosymbionts in hydra.

These results suggest that acid tolerance of the algae is a prerequisite for symbiosis with hydra. The fact that some strains which grew at pH 3.5–4.0 did not form a stable symbiosis, however, indicates that growth at low pH is not the only characteristic needed for the successful establishment of symbiosis with aposymbiotic hydra.

Keywords: acid tolerance, *Chlorella*, *Hydra viridis*, symbiosis

1. Introduction

The green *Hydra viridis* hosts *Chlorella*-like algae in its endodermal cells which cannot be cultured *in vitro* (Park et al., 1967; Pardy, 1974). The physiological characteristics of these algae as separate self-sufficient organisms are therefore unknown. However, many free-living species of *Chlorella* have been studied for their physiological and biochemical characteristics (Kessler, 1982, 1987; Kessler et al., 1988).

Aposymbiotic *H. viridis* from which "native" symbiotic algae have been removed, can form stable symbioses with some strains of free-living chlorellae but not with others (Rahat and Reich, 1984, 1985). For the "native" symbiotic chlorellae it has been speculated that their interaction with the host is based on an excretion of maltose by the algae in an acid perialgal vacuole (Cernichiary et al., 1969; Douglas and Smith, 1984). To date however, no direct measurements of pH in the perialgal vacuole of hydra have been reported. We describe here correlations between *in vitro* acid tolerance of free-living chlorellae and their ability to live as endosymbionts in cells of *H. viridis*.

Algae

The strains of *Chlorella* used in our experiments are listed in Table 1; most of them are from the collection of algae at Göttingen (numbers 211–) and Cambridge (numbers 211/). All these species can be identified by well defined chemo-taxonomic characteristics (Kessler, 1982, 1987; Kessler et al., 1988; Rahat and Reich, 1984, 1985).

Stock cultures of these algae were maintained on BBM+/agar slants (Rahat and Reich, 1985), or on the media of Kessler and Czygan (1970), or Kessler and Zweier (1971), solidified with 1.5% agar.

Measurement of acid tolerance in vitro

For the determination of acid tolerance, the algae were grown in 250 ml glass tubes under continuous illumination of ~6000 lx from white fluorescent lamps, at 25°C in a water thermostat (Kniese, Marburg-Marbach) and gassed with air + 2% CO_2 (Kessler et al., 1988).

The culture media (pH 6.4) of Kessler and Czygan (1970) with nitrate, or Kessler and Zweier (1971) with ammonium + thiamine for *C. protothecoides*, were acidified with dilute HCL in steps of 0.5 units to pH 2.0–6.0. After inoculation from agar slants algal growth was observed for two weeks. The

Table 1. Symbiotic and nonsymbiotic *Chlorella* strains and their acid tolerance*

Strain	Species	Symbiotic states	Lowest pH for growth *in vitro*	Strain	Species	Symbiotic states	Lowest pH for growth *in vitro*
211–1a	*C. saccharophila* var. *ellipsoidea*	Symbiotic	2.0	211/1e	*C. vulgaris*	Nonsymbiotic	3.5
				211/21	"	"	4.0
211–1d	*C. sacch.* var. *saccharophila*	"	2.0	UTEX 130	"	"	4.0
211–9a	"	"	2.0	CE 76	"	"	4.0
211–1f	"	"	2.5	NC64A(M)	"	"	4.0
211–9b	"	"	3.0	2	"	"	4.0
3.80	*C. sacch.* var. *ellipsoidea*	"	3.0	211–11b	"	"	4.0
211–2a	*C. luteoviridis*	"	3.0	211/8k	*C. sorokiniana*	"	4.0
211–11g	*C. kessleri*	"	3.0	211–8k	"	"	4.0
NC64A(P)	"	"	3.0	211–11k	"	"	4.0
211/11n	*C. fusca* var. *vacuolata*	"	3.0	211–32	"	"	4.0
211/8p	"	"	3.5	211–34	"	"	4.0
211/8b	"	"	3.5	211–40a	"	"	4.0
211-8b	"	"	3.5	211–40b	"	"	4.0
Fs	*C. protothecoides*	"	3.5	211–40c	"	"	4.0
211/6	"	"	4.0	C–1.1.8	"	"	4.0
211/11a	"	"	4.0	211/11c	"	"	4.5
211/7a	"	"	4.0	Sless 1	"	"	4.5
211-7a	"	"	4.0	Prag A 14	"	"	4.5
				211–11d	"	"	4.5
343	*C. fusca* var. *fusca*	Persistent	4.0	211–14a	*C. zofingiensis*	"	5.5
211–11r	*C.* sp.	"	4.0	211/8e	*C. homosphaera*	"	6.0
211–30	*C.* sp.	"	4.0				
232/1	*C. fusca* var. *rubescens*	"	4.5				
1–9–30	*C. sorokiniana*	"	5.0				
C–1.1.9	*C. minutissima*	"	5.5				

*For further explanation of this Table see Methods and Table 2.

acid limit of growth was defined as the lowest pH permitting at least slow but continuous growth, measured as dry weight, within this period (cf. Kessler, 1965).

Hydra

H. viridis of the Swiss strain used in the present experiments were grown in M solution (Lenhoff and Brown, 1970), under continuous illumination of ~2500 lx, at 20±2°C and fed three times a week with freshly hatched larvae of *Artemia* sp.

For the infection of aposymbiotic hydra with the respective strains of chlorellae, 4 to 5 day old larvae of *Artemia* sp. were used as vectors (Rahat and Reich, 1986b).

2. Results

Formation of symbioses

As shown in Tables 1 and 2, 18 strains of Chlorellae which belong to six taxa (Kessler et al., 1988), could form stable symbioses with the Swiss *H. viridis*, six strains persisted in the cells of the hydra for several weeks before they were lost, and 22 were lost from the hydra within 6 days.

Growth in acid media

Tables 1 and 2 show also the lowest pH at which the various chlorellae could grow *in vitro*. Table 2 sums up the numbers of symbiotic, persistent and non-symbiotic strains and their acid tolerance.

3. Discussion

Why is it that only certain algal/hydra associations are met in nature? What is it that enables the initiation of new "living together" by algal colonization of hydra cells? The latter question may be actually asked for the colonization of any biotop by a given organism (Rahat and Reich, 1986a).

The present day symbioses comprise partners that have coevolved for many years (Rahat, 1985b), and are the result of a mutual selection that is reflected in characteristics of the "native" symbiotic algae, e.g. maltose excretion (Cernichiary et al., 1969), and their inability to grow *in vitro*.

Table 2. Acid tolerance (lowest pH limit of growth *in vitro*) of *Chlorella* strains that can or cannot form stable symbioses with *Hydra viridis*

pH limit	Symbiotic (a)	Persistent (b)	Non-symbiotic (c)
2.0	3	0	0
2.5	1	0	0
3.0	6	0	0
3.5	4	0	1
4.0	4	3	15
4.5	0	1	4
5.0	0	1	0
5.5	0	1	1
6.0	0	0	1

(a) *Symbiotic*: Algae reproduce in the cells of the hydra and are passed on to subsequent generations of buds. Rate of 'greening' of hydra differs with different chlorellae. Some hydra get green to the naked eye within 1–2 weeks, in other strains several weeks are required, or the chlorellae can be only seen with a dissection microscope.

(b) *Persistent*: Some algae might be found in the hydra even several weeks after infection, but they are slowly diluted out and no stable symbiosis is formed.

(c) *Non-symbiotic*: Six days after infection no algae are found in the hydra.

In our study we tried to identify characteristics of free-living chlorellae that can survive in the cells of the Swiss strain of *H. viridis* and form stable symbioses with this hydra.

In nature all susceptible "symbiotic" hydra are green, as they have been infected by algae some time or other (Rahat, 1985b; Rahat and Reich, 1984, 1986b, 1987). In the laboratory however, we can study the factors needed for the establishment of a "new" symbiosis by infection of aposymbiotic hydra with living algae.

Algae have been used as ecological indicators in open habitats (Schubert, 1984). Symbiotic algae might thus similarly be used as indicators of bio-ecological differentiation of algae-hosting vacuoles and serve as a unique tool or probe for the study of the intracellular environment. The "calibration" of such a tool would be achieved by the *in vitro* culture of the endosymbiotic algae in a defined synthetic medium. Any factor, or its equivalent, required by the algae *in vitro* would obviously be available to them in the *in vitro* intracellular algae-hosting vacuole (Rahat and Reich, 1985). A study of *in vitro* growth requirements of chlorellae that can form stable symbioses with

hydra, should thus disclose the identity of the required growth factors and conditions present or absent in the respective perialgal cell vacuoles.

Rees (1987), stated that "establishment of the symbiosis results in little or no alteration in host biochemistry. It is simply as though the host were largely oblivious to its symbiont's presence and continued to be an 'aposymbiotic' animal". The inoculation of an alga into a fresh medium *in vitro* and the entry by phagocytosis of an alga into a cell's vacuole might thus be regarded to be analogous (Rahat and Reich, 1986b). In both cases it is the immediate conditions and the composition of the medium that decide about the survival of the alga and its future growth in the new environment.

The intravacuolar pH in a phagosome seems to be of major importance for the survival of an enclosed organism. Heiple and Taylor (1982), have suggested for the ameba *Chaos carolinensis* that "phagosomal and pinosomal pH changes may be required for lysosomal fusion and may be involved in regulation of lysosomal enzyme activity". McNeil et al. (1983), have concluded that "acidification of phagosomes by *Ameba proteus* is initiated...prior to phagosome-lysosome fusion...endosomal acidification (being) a transmembrane signal in regulating endosome-lysosome formation".

Lowering the pH in a phagosome seems thus to be the mechanism which results in digestion of intracellular intruders. To raise the pH in the phagosome is a means to avoid digestion by the host (Black et al., 1986). Accordingly, algae that can raise the pH in the phagosome and survive phagocytosis, might later resume growth and form stable symbioses. However, although the effect of algae on the environment through their metabolites is known (Lucas, 1961), we do not know if algae do change the pH in the hydra cell vacuole.

Some algae may survive the critical stage of phagocytosis and the effect of vacuolar environment (Hohman et al., 1982), but apparently cannot reproduce at a rate sufficient to compensate for their dilution by the dividing host cells (Rahat, 1985a). This can explain prolonged persistence of algae that are eventually lost from the hydra.

Jolley and Smith (1980), have stated that *Chlorella* strains with sporopollenin in their cell wells, and strains with mesotrophic nutrition, seem to be more acceptable as symbionts in hydra. Our results, however, do not support this assumption: among the six symbiotic species (Kessler et al., 1988), only *C. fusca* var. *vacuolata* is able to produce secondary carotenoids (a property correlated with the presence in the cell wall of sporopollenin; Atkinson et al., 1972; Burczyk, 1987), and only *C. protothecoides* is mesotrophic, i.e. requires reduced sources of nitrogen for growth and is unable to reduce nitrate.

The overlapping range of pH for the symbiotic, non-symbiotic and persistent algae (Tables 1 and 2), shows that it is not pH alone that enables long-lasting symbiosis. Evidently, another species-specific property is required.

Our results show that of the 11 known characteristics of free-living chlorellae (Kessler, 1982, 1987; Kessler et al., 1988), acid tolerance is required to enable them to live as endosymbionts in hydra. May we conclude that the perialgal intracellular "medium" is acid?

There seem to be contradictory data for the algal/hydra interactions (Rahat and Reich, 1987), and for the vacuolar pH and related phenomena.

1. Some native symbiotic chlorellae are supposed to excrete maltose into the host vacuole at a pH of 4–5 (Cernichiary et al., 1969; Mews and Smith, 1982; Douglas and Smith, 1984).
2. Maltose production by these chlorellae is supposed to be essential for the establishment of this symbiosis (McAuley and Smith, 1982; Mews and Smith, 1982).
3. The same chlorellae are supposed to be unable to grow at the above low pH (Douglas and Smith, 1984).
4. A low vacuolar pH enhances phagosome-lysosome fusion that may result in the digestion of the chlorellae in the algae-containing phagosome (Heiple and Taylor, 1982; Dean et al., 1984; McNeil et al., 1983).
5. Our present results show that only chlorellae that can grow *in vitro* at a low pH can form stable symbioses with hydra.

According to the above circumstantial evidence, what is the pH in the algae-hosting vacuole? We conclude that a direct measurement of the pH in the perialgal vacuole is needed to resolve the apparent paradox and enable a reexamination of the above data.

Acknowledgement

Our thanks are due to Mrs. Edith Weitemeyer for excellent technical assistance. This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

REFERENCES

Atkinson, A.W., Gunning, B.E.S., and John, P.C.L. 1972. Sporopollenin in the cell wall of *Chlorella* and other algae: ultrastructure, chemistry and incorporation of ^{14}C-acetate, studied in synchronous cultures. *Planta* **107:** 1–32.

Black, C.M., Palieschesky, M., Beaman, B.L., Donovan, R.M., and Goldstein, E. 1986. Acidification of phagosomes in murine macrophages: blockage by *Nocardia asteroides*. *J. Inf. Dis.* **154:** 952–958.

Burczyk, J. 1987. Biogenic relationships between ketocarotenoids and sporopollenins in green algae. *Phytochem.* **26:** 113–119.

Cernichiary, E., Muscatine, L., and Smith, D.C. 1969. Maltose excretion by the symbiotic algae of *Hydra viridis*. *Proc. R. Soc. Lond. B.* **173:** 557–576.

Dean, R.T., Jessup, W., and Roberts, C.R. 1984. Effect of exogenous amines on mammalian cells with particular reference to membrane flow. *Biochem. J.* **217:** 27–40.

Douglas, A.E. and Smith, D.C. 1984. The green hydra symbiosis VIII. Mechanisms in symbiont regulation. *Proc. R. Soc. Lond. B.* **221:** 291–319.

Heiple, J.M. and Taylor, D.L. 1982. pH changes in pinosomes and phagosomes in the ameba *Chaos carolinensis*. *J. Cell Biol.* **94:** 143–149.

Hohman, T.C., McNeil, P.L., and Muscatine, L. 1982. Phagosome-lysosome fusion inhibited by algal symbiont of *Hydra viridis*. *J. Cell Biol.* **94:** 56–63.

Jolley, E. and Smith, D.C. 1980. The green hydra symbiosis. II. The biology of the establishment of the association. *Proc. R. Soc. Lond. B.* **207:** 311–333.

Kessler, E. 1965. Physiologische und biochemische Beitrage zur Taxonomie der Gattung *Chlorella*. I. Saureresistenz als taxonomisches Merkmal. *Arch. Mikrobiol.* **52:** 291–296.

Kessler, E. 1982. Chemotaxonomy in the Chlorococcales. In: *Progress in Phycological Research*. Vol. 1. F.E. Round and D.J. Chapman, eds. Elsevier, Amsterdam, pp.111–135.

Kessler, E. 1987. Separation of *Chlorella ellipsoidea* from *C. saccharophila* (Chlorococcales): no growth on mannitol and cadmium sensitivity. *Plant Syst. Evol.* **157:** 247–251.

Kessler, E. and Czygan, F.C. 1970. Physiologische und biochemische Beitrage zur Taxonomie der Gattung *Chlorella*. IV. Verwertung organischer Stickstoffverbindungen. *Arch. Mikrobiol.* **70:** 211–216.

Kessler, E., Huss, V.A.R., and Rahat, M. 1988. Species-specific ability of *Chlorella* strains (*Chlorophyceae*) to form stable symbioses with *Hydra viridis*. *Plant Syst. Evol.* **160:** 241–246.

Kessler, E. and Zweier, I. 1971. Physiologische und biochemische Beiträge zur Taxonomie der Gattung *Chlorella*. V. Die auxotrophen und mesotrophen Arten. *Arch. Mikrobiol.* **79:** 44–48.

Lenhoff, H.M. and Brown, R.D. 1970. Mass culture of hydra: an improved method and its application to other aquatic invertebrates. *Lab. Anim.* **4:** 139–154.

Lucas, C.E. 1961. On the significance of external metabolites in ecology. *Symp. Soc. Exp. Biol.* **15:** 190–206.

McAuley, P.J. and Smith, D.C. 1982. The green hydra symbiosis. V. Stages in the intracellular recognition of algal symbionts by digestive cells. *Proc. R. Soc. Lond. B.* **216:** 7–23.

McNeil, P.L., Tanasugarn, L., Meighs, J.B., and Taylor, D.L. 1983. Acidification of phagosomes is initiated before lysosomal enzyme activity is detected. *J. Cell Biol.* **97:** 692–702.

Mews, L. and Smith, D.C. 1982. The green hydra symbiosis. VI. What is the role of maltose transfer from algae to animal? *Proc. R. Soc. Lond. B.* **216:** 397–413.

Pardy, R.L. 1974. Some factors affecting the growth and distribution of the algal endosymbionts of *Hydra viridis*. *Biol. Bull.* **147:** 105–118.

Park, H.D., Greenblatt, C.L., Mattern, C.F.T., and Merril, C.R. 1967. Some relationships between *Chlorohydra*, its endosymbionts and some other chlorophyllous forms. *J. Exp. Zool.* **164:** 141–162.

Rahat, M. 1985a. Competition between chlorellae in chimeric infections of *Hydra viridis*: the evolution of a stable symbiosis. *J. Cell Sci.* **77:** 87–92.

Rahat, M. 1985b. Origin and evolution of symbiosis in the green hydra. *Arch. Sc. Geneve.* **38:** 385–399.

Rahat, M. and Reich, V. 1984. Intracellular infection of aposymbiotic *Hydra viridis* by a free living *Chlorella* sp.: initiation of a stable symbiosis. *J. Cell Sci.* **65:** 265–277.

Rahat, M. and Reich, V. 1985. Correlations between characteristics of some free living *Chlorella* sp. and their ability to form stable symbioses with *Hydra viridis*. *J. Cell Sci.* **74:** 257–266.

Rahat, M. and Reich, V. 1986a. Algal/hydra symbiosis — a topic in ecology. In: *Environmental Quality and Ecosystem Stability*. Z. Dubinsky and Y. Steinberger, eds. Vol. 3. Bar Ilan University Press, Ramat Gan, Israel, pp. 331–337.

Rahat, M. and Reich, V. 1986b. Algal endosymbiosis in brown hydra: Host/symbiont specificity. *J. Cell Sci.* **86:** 273–286.

Rahat, M. and Reich, V. 1987. A reevaluation of alleged requirements for the establishment of algal/hydra symbioses and host/symbiont specificity — preadaptation and vacuolar ecology. *Endocyt. C. Res.* **4:** 13–23.

Rees, T.A.V. 1987. The green hydra symbiosis and ammonium I. The role of the host in ammonium assimilation and its possible regulatory significance. *Proc. R. Soc. Lond. B.* **229:** 229–314.

Schubert, E. (ed.) *Algae as Ecological Indicators*. Academic Press, New York.