Symbiosis, 10 (1991) 95–113
Balaban, Philadelphia/Rehovot

Structural Aspects of Prochloron-Tunicate Symbiosis

HEWSON SWIFT and DEBORAH L. ROBERTSON
Department of Molecular Genetics and Cell Biology
The University of Chicago, 1103 East 57th Street, Chicago, IL 60637, USA
Tel. 312-702-8041

Received May 27, 1990; Accepted July 22, 1990

Abstract

The colonial didemnid ascidian *Lissoclinum patella* harbors numerous cells of the symbiotic prokaryote alga *Prochloron*. We have collected colonies of *L. patella* from the reef flats of Palau, West Caroline Islands, and observed their normal growth patterns, motility and regenerative properties. Colonies differ markedly in structure depending on the conditions of illumination. *L. patella* possesses extensive regions of tunic (matrix) which surrounds the zooids and may comprise 80% of the colony mass. The matrix contains an extensive population of extra zooidal fibroblast-like cells that possess abundant secretory granules and lysosomes. We conclude that the continual motility and regenerative properties of *L. patella* colonies depend in part upon the continual secretion and resorption of the matrix. We propose that the structure of host colonies may be modified in ways that provide optimal growth conditions for the symbionts.

Keywords: *Prochloron*, *Lissoclinum*, Tunicate symbiosis, Tunicate motility, tunic restructuring

1. Introduction

The small colonial ascidians of the family Didemnidae are of particular interest because they are hosts to the unique prokaryote alga, *Prochloron*. Although didemnids are widely distributed in both temperate and tropical seas around the world, the association with *Prochloron* is found exclusively in warm tropical waters. Much has been written about the physiology and morphology of

0334-5114/91 /$03.50

Prochloron (see Lewin and Cheng, 1989 for review), but far less is known about the biology of its hosts. This paper considers several aspects of the biology of one didemnid species, *Lissoclinum patella*, with particular reference to host adaptations that appear to provide the conditions for vigorous growth of the symbiont.

L. patella is abundant in certain South Pacific reef flats, and the symbiotic algal cells occur free in host cavities, being readily extracted in large numbers merely by squeezing host colonies. It has thus been the host species of choice for a number of physiological and molecular studies (Alberte, 1989). *Prochloron* has also been described as a symboint of about 40 other ascidians, all but a few belonging to the didemnid family (Cox, 1986). These other hosts for the most part form much smaller colonies, and the symbionts are either sparsely distributed on outer surfaces only, or are for structural reasons more difficult to extract. Although the basic zooid morphology is similar among all didemnids, the growth patterns, colony size and tissue consistency all show marked differences. For example, the colonies of *Didemnum molle*, also abundant in the South Pacific, are much smaller, spherical instead of flattened, with a semiliquid mucin-like matrix that makes extrusion of symbiotic algae almost impossible, and in *Tridemnum* species the symbionts are embedded in semisolid matrix material. The findings discussed here for the *L. patella-Prochloron* symbiosis are thus not readily applicable to other species. In addition,there are great differences in size and structure of *Prochloron* cells from different hosts, almost certainly deserving of designation as separate species (Cox, 1986; Swift, 1989).

For reasons still unclear (in spite of numerous attempts) it has been impossible to grow *Prochloron* cells free from their living hosts. Efforts to transport the host organisms from the South Pacific to laboratories in the United States have also been unsuccessful. It is apparent that *Lissoclinum* and *Didemnum* colonies are sensitive to thermal changes, particularly at temperatures below their normal environmental range of 26–29°C (Alberte et al., 1987). For these reasons, our observations on the growth and behavior of living colonies had to be made during relatively short visits to Palau where the didemnid-plus-*Prochloron* associations are abundant. Laboratory experiments using freshly collected colonies were made possible through the use of the excellent facilities developed for the giant clam (*Tridacna*) project at the Micronesian Mariculture Demonstration Center in Koror, Palau.

Our observations indicate that a complex relationship exists between *Prochloron* and its *L. patella* host; the host colonies may continually undergo behavioral and structural modifications in response to environmental changes in ways that presumably provide adequate growth conditions to the symbionts.

A colony of hundreds of zooids behaves essentially as a single individual, dependent for its plasticity on its large population of extra- zooidal cells that are responsible for the continual synthesis of new matrix and the resorption of the old extracellular material. Although the *Prochloron* symbionts are extracellular to host tissues, when new colonies of *L. patella* arise by asexual reproduction they are already infected with symbiotic algae. Sexual reproduction occurs as well, producing tadpole larvae which carry special folds for attachment of *Prochloron* cells (Kott, 1980; Swift, 1989). These modifications, behavioral, structural and probably physiological as well, suggest that the symbiotic association between *Prochloron* and *Lissoclinum* has doubtless developed over long periods of evolutionary time.

2. Materials and Methods

Observations on living colonies of *Lissoclinum patella* were made in Palau, West Caroline Islands (Lat 7°25′N; Long. 134°30′E), on expeditions during 1984, 1986, 1988 and 1990. Specimens used in morphological studies were collected from reef flats in the Kamori Channel, and transported in seawater to the Microesian Mariculture Demonstration Center (MMDC), Koror, Palau. Experiments examining colony growth and movements were conducted in large cement tanks at MMDC equipped with running seawater. Tissue samples for electron microscopy were fixed in 4% glutaraldehyde and phosphate buffer (pH 7.4) or in 4% formaldehyde (generated from paraformaldehyde) in buffered seawater, for transfer to Chicago, IL and later postfixation in 1% osmium tetroxide. Material for scanning electron microscopy was critical point-dried from amyl acetate and vapor coated with gold-palladium.

Prochloron cells were expressed from *L. patella* by squeezing colonies, allowing algal cells to pass through a 470 μm macrofilter (Spectrum) and into seawater buffered with 200 mM bicine (pH 7). Algal cells were frozen in a refrigerator freezer and brought back frozen to Chicago. DNase activity in fluid surrounding expressed cells was determined by incubating *Hind*III-digested phage Lambda DNA in the fluid at 37°C for 1 hr. These samples were run in a 0.6% agarose gel and stained with ethidium bromide. Acid phosphatase in *L. patella* tissues was demonstrated in formalin-fixed cryostat sections with a diazo coupling dye (fast garnet GBC-Sigma Diagnostics) at pH 4.5. Polysaccharides were demonstrated with the periodic-acid-Schiff (PAS) reaction: formalin fixed paraffin embedded tissue sections were hydrolyzed 10 min in 10 mg ml^{-1} periodic acid in sodium acetate-buffered 70% ethanol, and stained with the Schiff reagent.

3. Results

Growth patterns

In the study site, *L. patella* grew among coarse sand and coral rubble on reef flats in the upper subtidal zone. Colonies were only rarely exposed at the lowest tides. They were often present in large multiple clusters, 1 to 3 m across, composed of many individual colonies, although isolated single colonies were also present. Colonies were usually attached to and intermingled with mats of calcareous algae (*Halimeda* sp.), and also were often shaded with stands of seagrass (*Enhalus* sp.). Some colonies grew around stems of seagrass, wrapped around plant bases, but also surrounding calcareous sponges or on surfaces of dead coral. The large clusters usually contained a few big individual colonies, 15 to 20 cm across. These were highly irregular in outline, with several peripheral projections where new colonies, 2–5 cm across, apparently budded off the parent colony to form new colonies by asexual reproduction. Buds in all stages of separation were seen, some attached only by narrow strands of tunic material. Colonies growing close together did not fuse, but maintained their individuality in regions of contact. The large cluster pictured in Fig. 1 shows peripheral buds and an extensive region of contact indicated by parallel peripheral ridges bordering each colony.

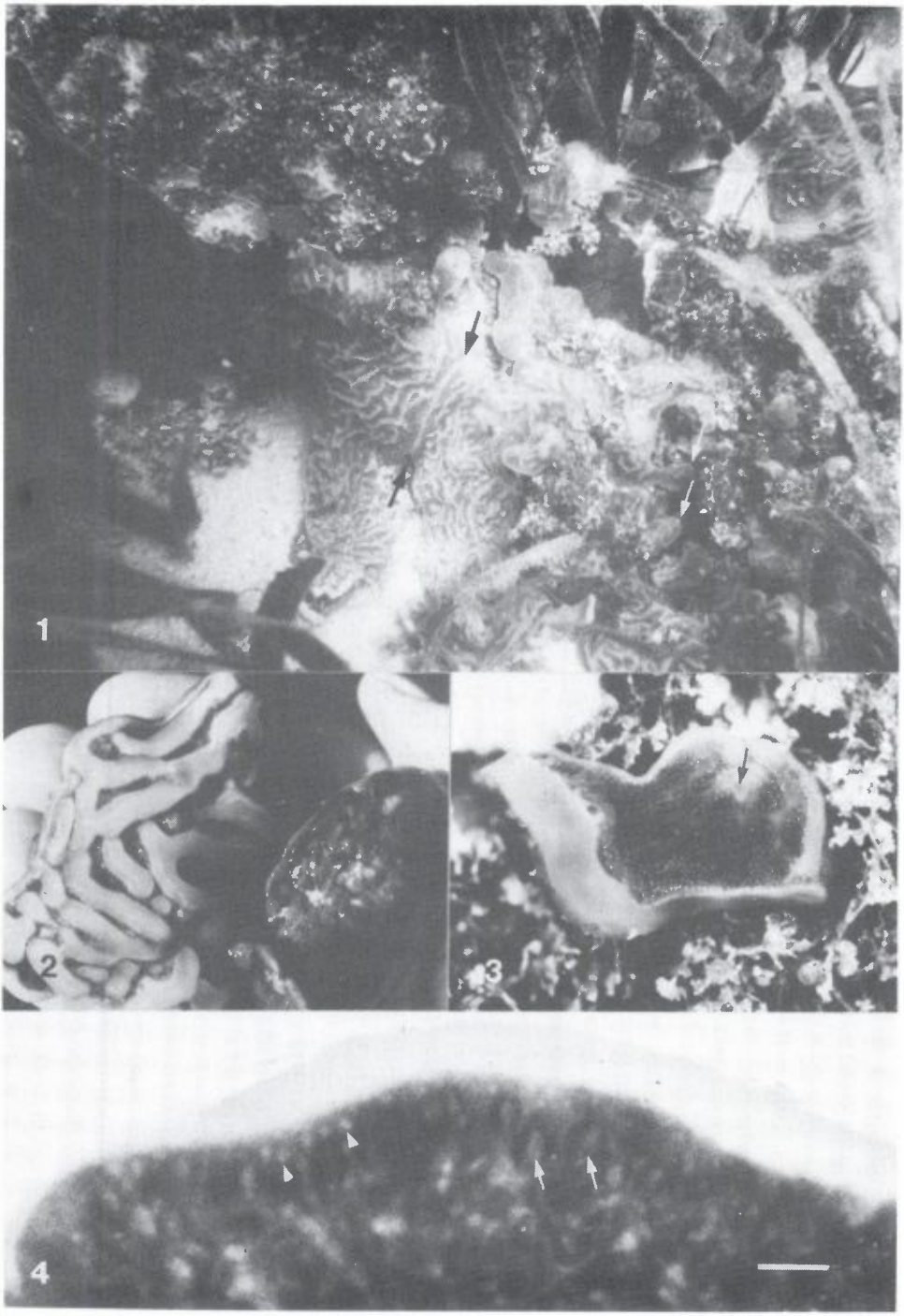
Wide morphological diversity occurred among separate colonies in a single cluster, or even in different regions of the same colony (Alberte et al., 1986). Colonies buried among the lawns of *Halimeda*, where sunlight was strongly attenuated, were dark green, with a smooth upper surface. The layer of tunic matrix over the individual zooids was thin (often less than 100 μm) so that *Prochloron* cells were close to the upper surface. Most colonies were partially shielded from direct sunlight by surrounding vegetation, but those in

Figure 1. An area of the reef flat about 0.8 m across, showing a number of individual colonies of *Lissoclinum patella*. Bases of seagrass (*Enhalus*) are present at left and top center. A clump of calcareous alga (*Halimeda*) is at the upper left. Black arrows show a place where two large *L. patella* colonies are in contact but not fusing. Asexual budding to form new colonies is shown by white arrows.

Figure 2. A heavily ridged colony of *L. patella* at left and a dark-grown colony at the right to demonstrate morphological extremes of colonies subjected to different conditions of illumination. Slightly less than life size (0.8×).

Figure 3. A small colony growing on *Halimeda*, showing a prominent region of extrazooidal matrix. Small white dots mark sites of incurrent siphons surrounded by calcareous spicules. Excurrent siphon (arrow) (0.8×).

Figure 4. Edge of a small colony illuminated from behind to show dark areas of *Prochloron* cells delimiting mature zooids (arrows). New asexually formed zooids are forming at colony margin (arrowheads). (About 10×. Size bar 1 mm).


1
2
3
4

unshaded regions were white with prominent ridges on the upper surface. The more prominent ridges were up to 5 mm thick and 6–8 mm wide. The separate zooids were variable in size, depending on maturity. The largest were approximately 0.3 × 2 mm in size, surrounded by an extensive cloacal cavity. Incurrent siphons extended from the upper colony surface through the upper matrix layer. These were short (0.1–0.2 mm) in the dark-grown colonies, and much longer where they extended through a thicker matrix layer. Incurrent siphons were demarcated by small clusters of surrounding calcareous spicules. Siphons were more evenly spaced in dark-grown colonies, but located only between the ridges of light-grown colonies (compare Figs. 2 and 3). In living specimens the pharyngeal baskets of the zooids were surrounded by the cloacal cavities. *Prochloron* cells were concentrated in the cloacal cavities, and in specimens illuminated from below the green algal cells could be seen to outline and surround the colorless baskets of each zooid. Zooids were largest in central parts of the colony (Fig. 4, arrows) and were much smaller in the young developing zooids at the growing edges (Fig. 4, arrowheads).

Motility

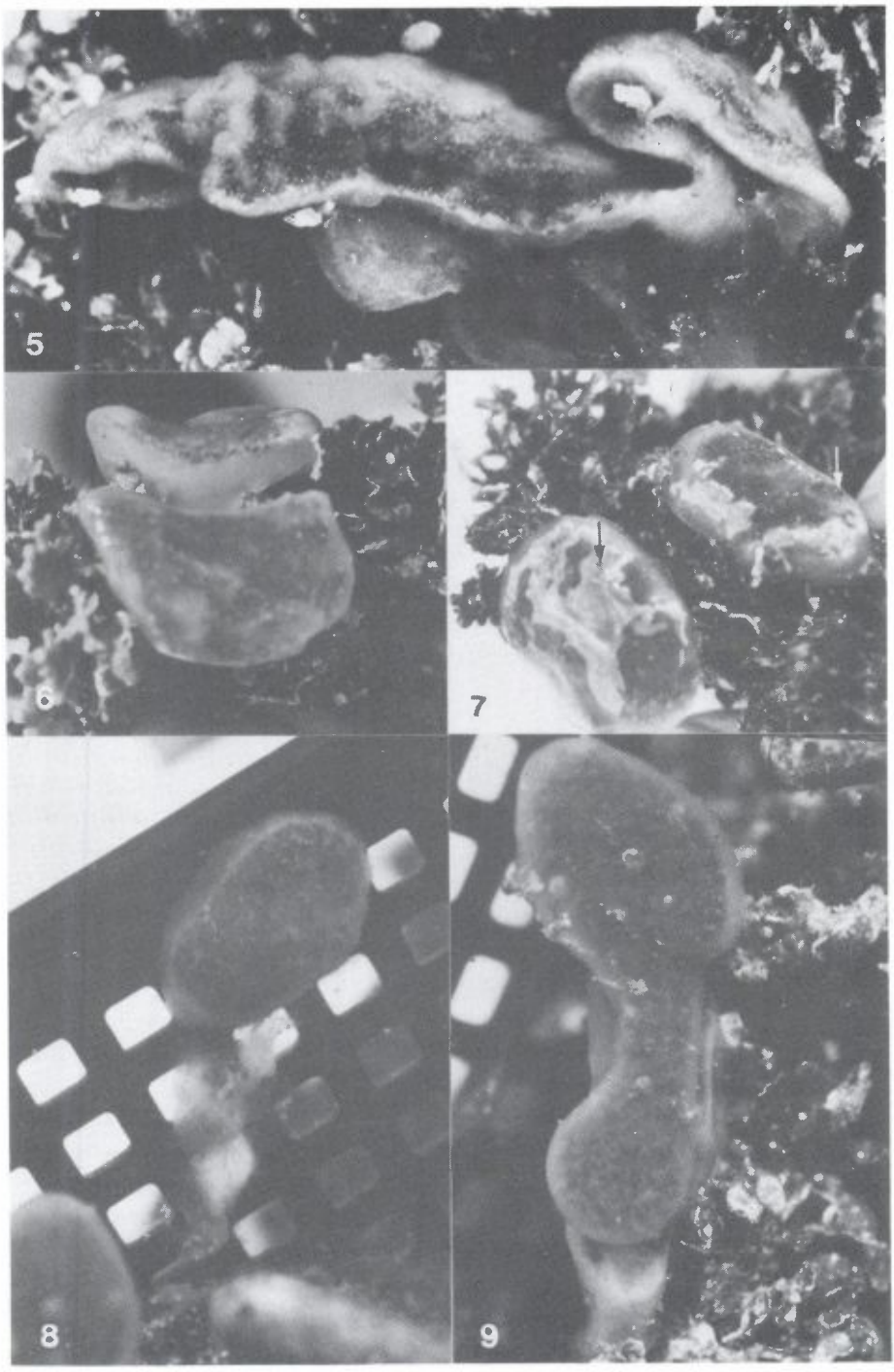
Most of the mass of the colony (in some cases up to an estimated 80%) occurred outside the zooids in the tunic (or matrix). In dark-grown colonies the zooids and their associated algal cells formed a narrow layer of green material, with a thin upper matrix layer and a much thicker layer below (in some cases up to 2 cm thick). This matrix was cartilage-like, flexible but tough. It could attach the colony to a smooth substrate (as in plastic containers) but in the natural habitat it conformed to the rough surfaces of the substrate. Whole blades of calcareous algae were sometimes embedded within it, as were rough margins of coral fragments. Colonies thus become firmly anchored to the reef by the surrounding properties of the tunic.

When large flat colonies were physically removed from their normal attachments and inverted, they tended to fold, with the zooid surface outward, over a period of a few hours (Fig. 5). The colonies eventually established new

Figure 5. A colony 24 hr following its removal from a flat substrate, placed with its upper surface down. The colony has folded over, and is in the process of righting. (About life size).

Figures 6 & 7. A small colony cut in two pieces (left) and the same colony 24 hr later (right). A new excurrent siphon has formed (white arrow) and cut edges have been overgrown. (about 1.5×).

Figures 8 & 9. Two migrating colonies, showing trails of sloughed matrix left behind. Colonies were in attenuated oblique illumination for 72 hr and moved towards region of brighter light. (about life size).


5
6
7
8
9

contacts with the substrate, and acquired a nearly normal reorientation over a period of 4 days. When small colonies were cut into two pieces, one with an excurrent siphon and one without, the cut surfaces healed over during the subsequent 24 hr and a new excurrent siphon formed (Figs. 6 and 7.).

To examine directly the response of *Lissoclinum* colonies to light, colonies were placed in plastic meshwork containers, and shielded from direct light by a concrete slab. This treatment reduced the light level from a range of 6,000–8,000 μE $m^{-2}s^{-1}$ to 300–500 μE $m^{-2}s^{-1}$ and provided oblique illumination from one edge of the container. Two young colonies (2–4 cm in diameter) moved toward the illuminated area at rates from 0.8 to 2.2 cm per 24 hr. This movement was accomplished by the addition of new matrix at the advancing edge of the colony, leaving behind a clear region of matrix devoid of zooids (Figs. 8 and 9). At first the matrix on the trailing edge had the same consistency as normal tunic material, but later it became flaccid and was sloughed from the colony. Colony movement thus did not entail the slipping of the lower matrix over the substrate, but rather concerned the synthesis of a region of new matrix with the zooids advancing into it. In this way, colonies apparently can move over the roughest surfaces while continually maintaining their attachment.

Spicules

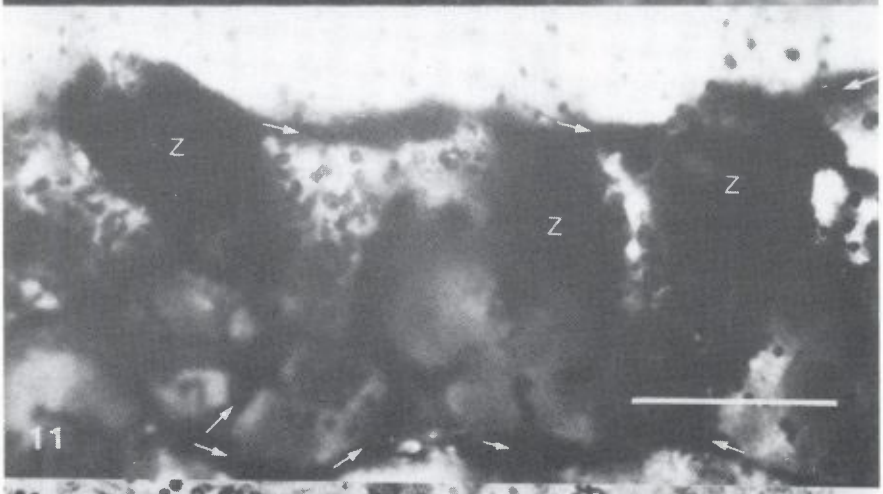
The matrix or test of *L. patella* contained numerous stellate calcareous crystals. These appeared to form intracellularly since small spicules were frequently observed surrounded by a narrow rim of cytoplasm. They were most numerous on the upper surface, but occurred throughout the matrix in both dark- and light-grown colonies. The spicules were refractile and scattered light in colonies viewed by indirect light (Fig. 10), but appeared dark with translucent

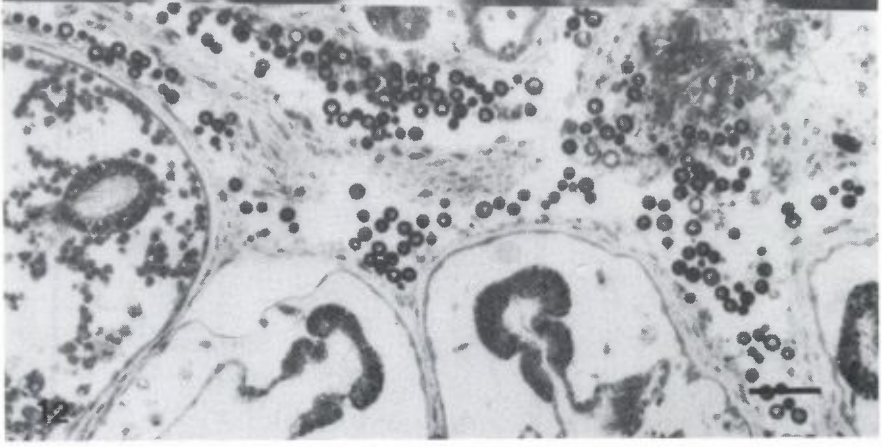
Figure 10. A razor blade section of an unfixed section, in indirect (reflected) illumination, showing the upper surface of a light grown colony and underlying zooids. Incurrent siphons extend from the upper surface to the pharyngeal baskets, here surrounded by calcareous spicules, but largely avoid the thickened region of the ridge. Spicules are concentrated below the central ridge. Canals are shown at arrows. (About 8×. Size bar 1 mm).

Figure 11. A region similar to Fig. 10, but lit by transillumination. Dark areas are concentrations of *Prochloron* cells which surround the zooids (Z) and crowd the interconnecting channels (arrows). Spicules appear dark. (About 27×. Size bar 1 mm).

Figure 12. Epon-embedded tissues, cut at 1 μm and stained with thionin to show numerous cells of *Prochloron* present in channels between zooids. The two central zooids show sections through the endostyle. (About 90×. Size bar 100 μm).


10


Z
Z
Z
11


12

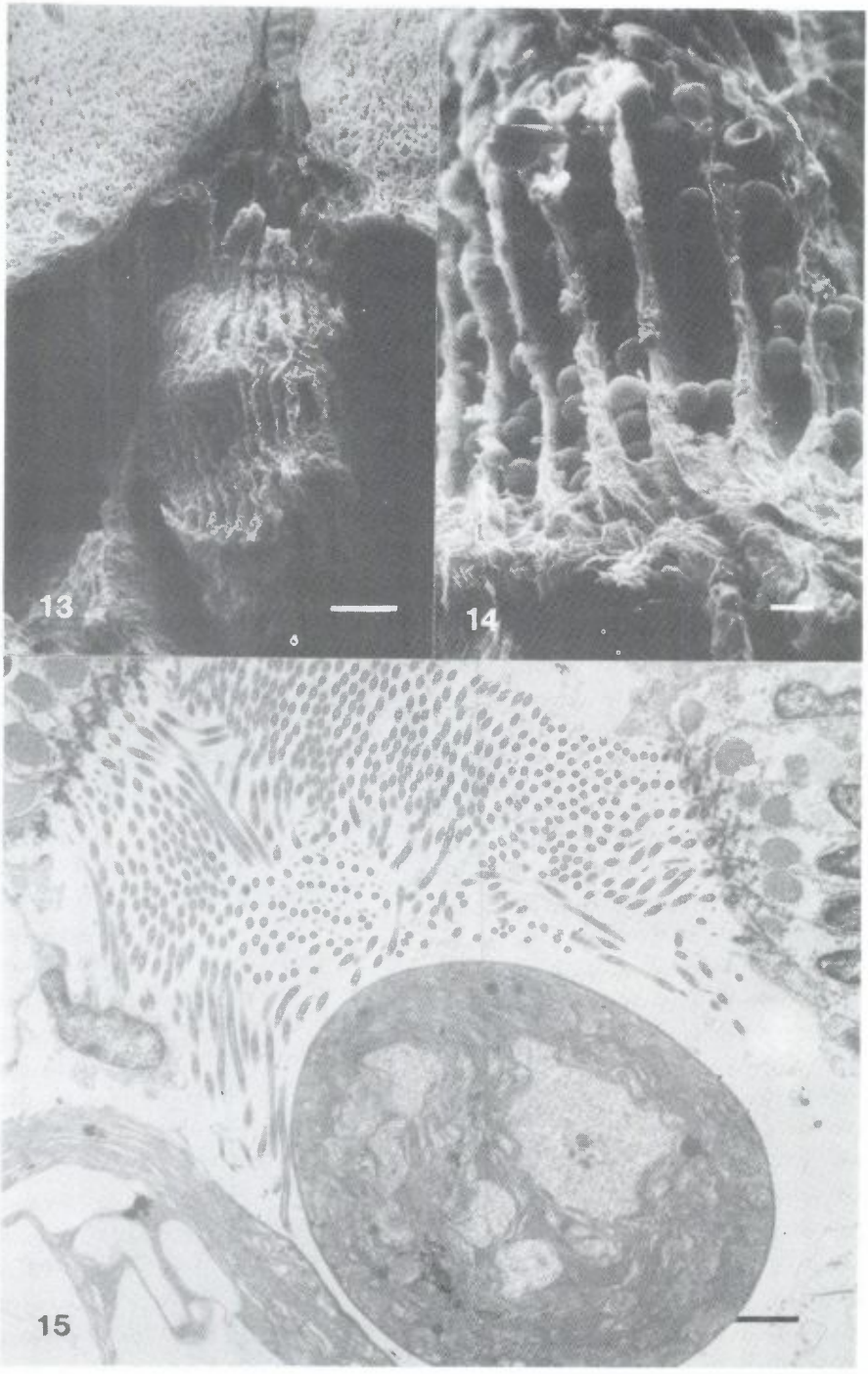

13
14
15

Figure 13. Pharyngeal basket of a zooid as seen by scanning electron microscopy, showing stomata and part of the incurrent siphon surrounded by bands of smooth muscle. Note the fibrous sponge-like texture of the dried matrix at the upper corners, and the extensive cloacal spaces surrounding the pharynx. (90×. Size bar 100 μm).

Figure 14. Another zooid showing cells of *Prochloron* inside the stomata. (About 580×. Size bar 10 μm).

Figure 15. Electron micrograph of a thin section through a portion of the pharyngeal basket, showing two ciliated pharyngeal bars (upper corners), numerous sections of cilia and microvilli, and a cell of *Prochloron* in the stomatal space. (8,000×. Size bar 1 μm).

illumination. They were concentrated beneath the ridges, and possibly could attenuate light availability to algal cells (Olson, 1983), but we obtained no evidence as to whether their number is modulated by light intensity.

Pharyngeal sacs and connecting channels

Zooids were interconnected by a complex series of channels that ramify throughout the colony. Figure 11 shows a razor blade section (about 2 mm thick) of an unfixed colony lit from behind. The cloacal spaces are demonstrated merely by the presence of algal cells, which surround the pharyngeal baskets of individual zooids (labeled Z in Fig. 11). The channels connect the peripharyngeal spaces at both their upper and lower margins (arrows) and form a network around the abdominal regions. These channels can be seen containing numerous spherical cells of *Prochloron.*

The structures of the pharyngeal sac and branchial stomata are shown in Figs. 13 and 14, as seen by scanning electron microscopy. Algal cells surround the stomatal slits but only rarely are found in the digestive tract. In Fig. 15, a *Prochloron* cell is seen by transmission electron microscopy among cross sections of cilia that line the inner surfaces of the pharngeal bars and provide the circulation of seawater through the tubular system. Figure 16, of a thin plastic section viewed by light microscopy, is another view of *Prochloron* cells between the bars of the branchial basket.

Endosymbiotic bacteria

Although *Prochloron* cells in *L. patella* always appeared to lie in cloacal spaces, there was an abundant collection of bacteria within the gelatinous extracellular matrix. Bacteria are visible in the cells of the branchial bars (Fig. 16, arrows) and also in the matrix material lining the incurrent siphons. At least two different bacterial species appear to be present, as indicated by their markedly different size (Fig. 17).

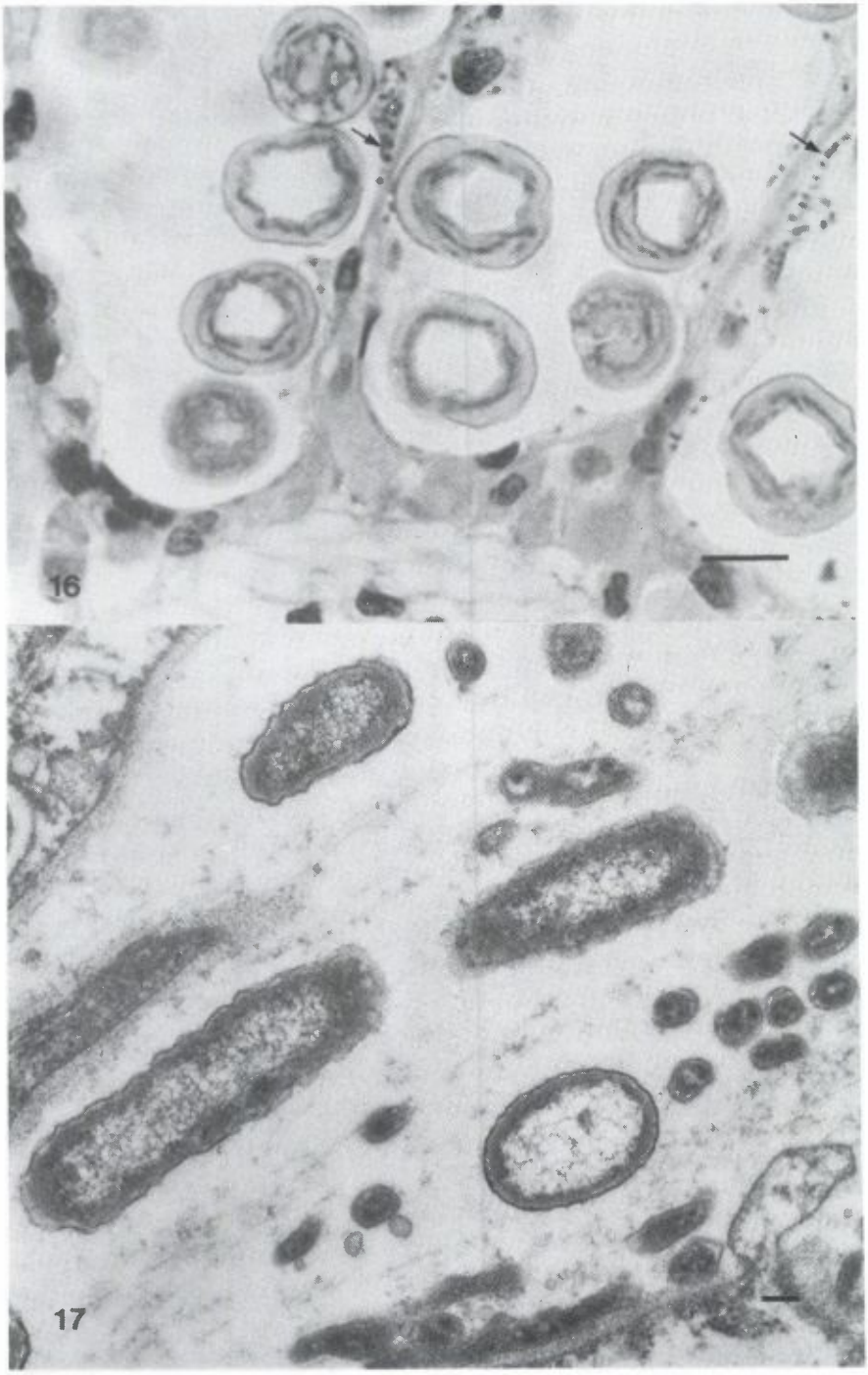

16
17

Figure 16. *Prochloron* cells in stomatal openings, as seen in light microscopy of plastic embedded tissue, stained for nucleic acids by azure B. Numerous bacterial cells are visible within areas of matrix (arrows). 1,200×. Size bar 10 μm.)

Figure 17. Electron micrograph of an area of matrix surrounding an incurrent siphon. Bacterial cells of two different sizes are evident. (50,000×. Size bar 0.1 μm).

Cells of the tunic

The tunic contained numerous isolated fibroblast-like cells distributed throughout the matrix, being most numerous along the upper surface (Fig. 25). Electron micrographs demonstrate prominent inclusions within these cells of two kinds: oval secretory granules of uniform electron density, sometimes showing regions of dense irregular granules, and structures resembling typical lysosomes (Figs. 18–20). The secretory granules appear to be forming within the fibroblast-like cells, since endoplasmic reticulum and Golgi membranes typical of secretory cells are present. In some cells, indented pockets of the cell membrane, filled with diffuse material almost certainly indicate regions where secretory granules have emptied by exocytosis into the extracellular matrix (Figs. 18 and 20). By light microscopy the oval inclusions are seen to be strongly PAS positive, and thus contain polysaccharides capable of hydrolysis with periodic acid (Fig. 23).

Lysosomes show the irregular structure characteristic of these organelles. Some lysosome inclusions were also PAS positive, but stained less intensely than the secretory granules (Fig. 23) while others contained bacteria (Fig. 19). The lysosomes showed a positive reaction for acid phosphatase, as demonstrated on frozen sections stained with a diazo dye (Fig. 24), and in this respect are typical of lysosomes of other animal cells.

When colonies of *L. patella* were squeezed and the exudate passed through a macrofilter to extract *Prochloron*, the algal cells were at first highly refractile, but soon shrank slightly and became more transparent, a change probably associated with cell death (Fig. 21). The freshly expressed fluid had a low pH, and the presence of DNase activity was readily apparent (Fig. 22). The addition of EDTA inhibited the degradation of the DNA, presumably by chelating magnesium ions, and supported the hypothesis that the degradation was due to enzyme activity (compare lane b to lanes c and e; Fig. 22). The fluid material also appears to contain large amounts of phenolic compounds, presumably from *Prochloron* (Barclay et al., 1987), which may cause DNA fragmentation. Polyvinyl pyrrolidone (PVP) binds phenolic compounds, but the addition of 1% PVP did not have an effect on the DNase activity in these experiments (compare lane b to lane f; Fig. 22). It seems likely that both the high acidity and DNase activity derive from the lysosomes of the tunic fibroblasts.

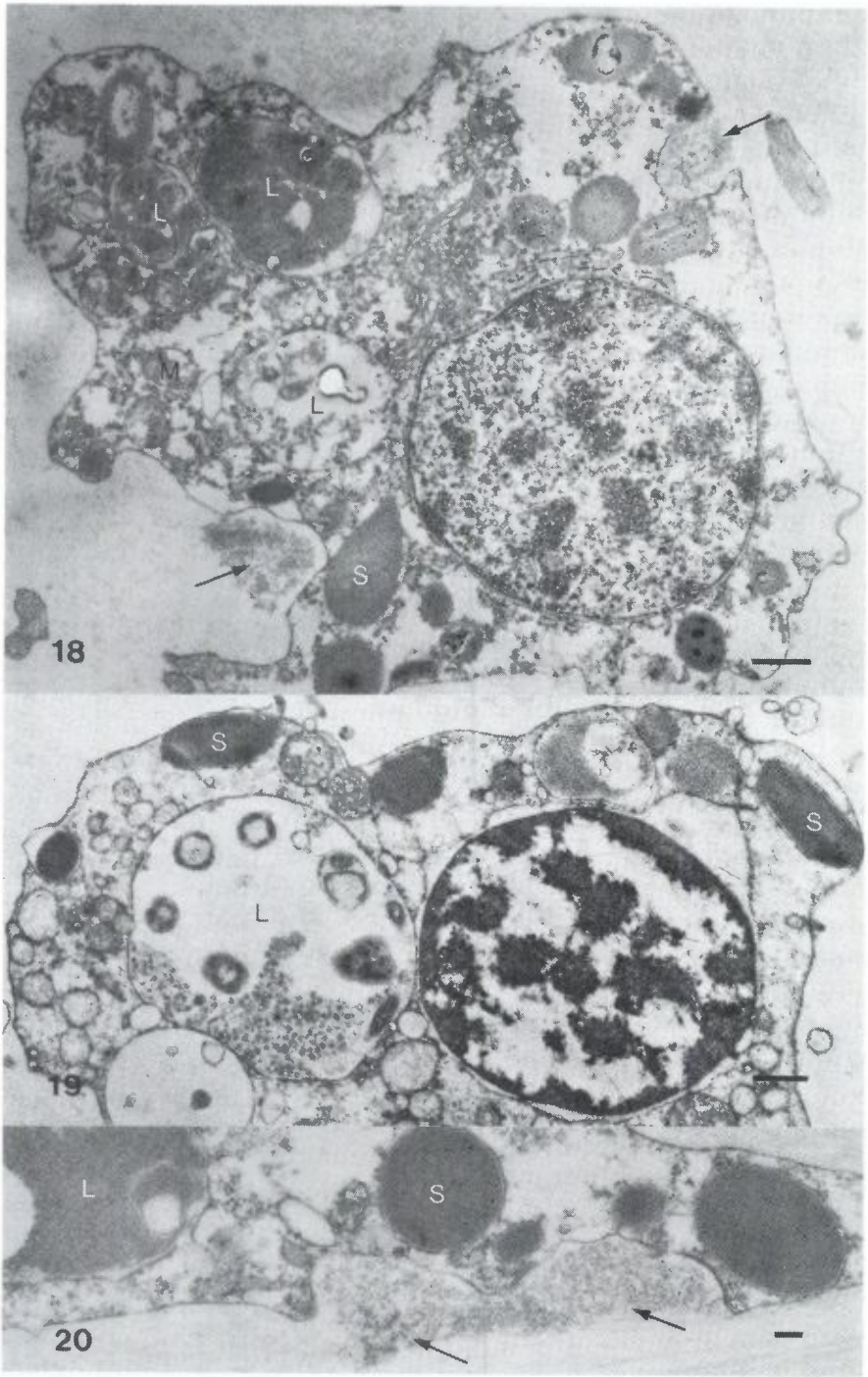

L
L
M
L
S
18
S
S
L
19
L
S
20

Figure 18. Fibroblast-like cell from the extrazooidal matrix, showing several lysosomes (L) secretion granules (S), and mitochondria (M). Indentations in the cell membrane filled with fibrous material (arrows) are sites where secretory granules appear to have emptied by exocytosis. (7,500×. Size bar 1 μm).

Figure 19. Another fibroblast-like cell showing secretory granules (S) and a large lysosome containing bacteria. (7,500×. Size bar 1 μm).

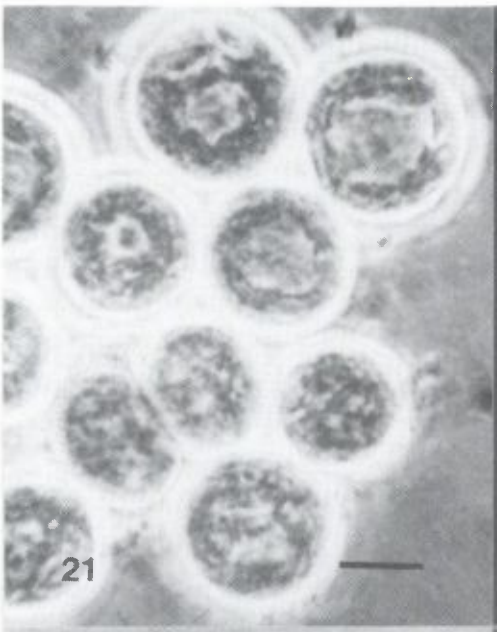
Figure 20. Part of another fibroblast-like cell, showing a large lysosome (L) filled with homogenous material, and an indented region where secretion granules (arrows) appear to have emptied into the extracellular matrix. (35,000×. Size bar 0.1 μm).

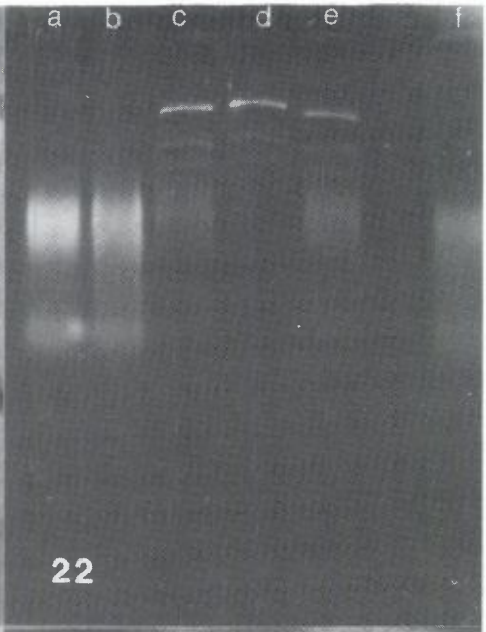
4. Discussion

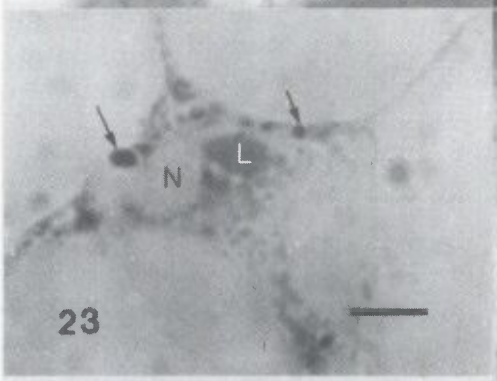
Colonies of *L. patella*, unlike many sessile marine invertebrates, appear to undergo structural changes in response to altered environmental conditions of illumination and orientation. The remarkable regenerative properties of ascidians and their extensive asexual reproduction have been frequently described (Azema, 1937; Berrill, 1950; Freeman, 1971), and in this respect *L. patella* also shows a rapid regenerative healing of cut surfaces. Our studies also indicate that many of the reparative properties of *L. patella* colonies are associated with the activity of extrazooidal cells both in the secretion of new matrix material and in the endocytosis and intracellular digestion of old matrix by the lysosomal system. We have followed Andrew (1962) in referring to these extrazoodial cells of the matrix as "fibroblast-like" cells. In other tunicates, the origin of matrix cells from blood elements has been described (Smith, 1970), but currently we have no evidence that the fibroblast-like cells of *Lissoclinum* derive from blood cells.

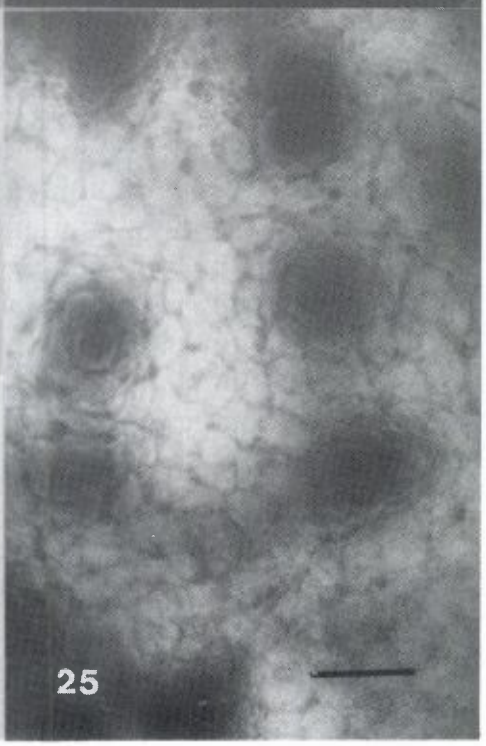
It appears likely that during the isolation of *Prochloron* cells from *L. patella*, by squeezing the colonies and passing the exudate through macrofilters, the expressed cell suspensions become contaminated with damaging hydrolytic enzymes from the fibroblast-like cells of the matrix. Although these algal cells have been suitable for physiological studies (Alberte et al., 1986; and in prep.), *Prochloron* cells extracted by a more gentle lavage of host cloacal cavities may be better candidates for future growth experiments and studies of molecular biology.

Of importance to our understanding of the nature of *L. patella-Prochloron* symbiosis are the behavioral modifications of the host organisms that seem to optimize the conditions for growth of the symbiont. Our results suggest that *L. patella* may actively optimize conditions for algal growth, both by moving to regions of proper illumination through the synthesis of new matrix in the preferred direction and by modifying the structure of the tunic covering the upper surface of the colony. It is apparent that optimal conditions for algal growth within the host are met by reduced rather than direct sunlight


21


a b c d e f
22


N
L
23


25

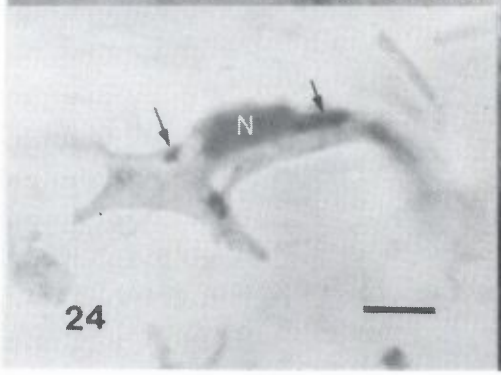

N
24

Figure 21. *Prochloron* cells expressed from *L. patella* into glutaraldehyde, and viewed by phase microscopy to show the large central vacuole surrounded by thylakoids. A dividing cell is at lower left. (1,000×. Size bar 10 μm).

Figure 22. Demonstration of DNase activity in the fluid surrounding *Prochloron* cells which have been isolated from *L. patella* by squeezing. Lane d is of DNA from phage λ as digested with restriction enzyme *Hind*III. Lane a is fluid alone, containing diffuse bands, probably of DNA fragments from lysed cells. Lane b also contains phage λ, but the DNA bands shown in lane d have been digested. Lane c contains phage DNA and also 0.25 M EDTA. Lane e is similar but with 0.17 M EDTA. Lane f contained 1% polyvinyul pyrrolidone, to inactivate phenols (0.6% agarose gel stained with ethidium bromide).

Figure 23. Fibroblast-like matrix cell stained for polysaccharides by the PAS reaction, demonstrating that the secretory granules (arrows) contain polysaccharides. The nucleus (N) is unstained. (900×. Size bar 10 μm).

Figure 24. Fibroblast-like matrix cell stained for acid phosphatase. Lysosomes (arrows) are strongly positive. The nucleus (N) has been counterstained with hematoxylin. (850×. Size bar 10 μm).

Figure 25. Unfixed and unstained section of the upper surface of a colony viewed of phase microscopy, showing incurrent siphons and the surrounding network of extrazooidal fibroblast-like cells of the matrix. (About 120×. Size bar 100 μm).

(Alberte et al., 1986). Photosynthesis-irradiance relationships for *Prochloron* cells isolated from *L. patella* showed maximum rates of oxygen evolution at about 575 μE $m^{-2}s^{-1}$ for cells from colonies grown in high light, but only 175 μE $m^{-2}s^{-1}$ for cells from colonies grown in low-light conditions. In the latter cells, higher levels resulted in photoinhibition (Alberte et al., 1986). In our studies, heavily ridged colonies of *L. patella* survived in direct sunlight, but less-ridged, green colonies from heavily shaded regions of *Halimeda* mats acquired extensive bubbles in the cloacal cavities when moved into direct sunlight, and in many cases became detached and floated to the surface.

Our results are similar to the morphological changes (Olson, 1980) and movement patterns reported for other didemnids harboring *Prochloron* (Birkeland et al., 1981; Cowan, 1981; Thinh et al., 1981; Olson, 1983; 1986). However, further experiments are necessary to delineate adaptations of the host-symbiont relationship from general ascidian movements (Carlisle, 1961) and morphological modifications that protect the host from damaging ultraviolet radiation (Jokiel, 1980). The preliminary findings on the growth patterns, histology and cytochemistry of *L. patella* reported here clearly suggest a variety of experiments for the future exploration of this symbiotic system.

Acknowledgements

The authors are indebted to Ralph Lewin and Lanna Cheng for extensive guidance and support during field studies in Palau. We also wish to thank

Gerald Heslinga for permission to use facilities of the Micronesian Mariculture Demonstration Center in Koror. Our studies were supported by Grant N-00014-88K-0258 from the Office of Naval Research to H.S. and by Grant OEC-8901731 from the National Science Foundation to Lanna Cheng and Ralph A. Lewin. D.L.R. was supported by Training Grant GM07183 from the National Institutes of Health.

REFERENCES

Alberte, R.S., Cheng, L., and Lewin, R.A. 1986. Photosynthetic characteristics of *Prochloron* sp./ascidian symbioses. I. Light and temperature responses of the algal symbiont of *Lissoclinum patella*. *Mar. Biol.* **90:** 575–587.

Alberte, R.S., Cheng, L., and Lewin, R.A. 1987. Characteristics of *Prochloron*/ascidian symbioses. II. Photosynthesis-irradiance relationships and carbon balance of associations from Palau, Micronesia. *Symbiosis* **4:** 147–170.

Alberte, R.S. 1989. Physiological and cellular features of *Prochloron*. In: *Prochloron, a Microbial Enigma*. R.A. Lewin and L. Cheng, eds. Chapman and Hall, London, pp. 31–52.

Andrew, W. 1962. Cells of the blood and coelomic fluids of tunicates and echinoderms. *Am. Zool.* **2:** 285–297.

Azema, M. 1937. Recherches sur le sang et l'excretion chez les Ascidies. *Annales de l'Institut Oceanographique de Paris* **17:** 1–150.

Barclay, W.R., Kennish, J.M., Goodrich, V.M., and Fall, R. 1987. High levels of phenolic compounds in *Prochloron* species. *Phytochemistry* **26(3):** 739–747.

Berrill, N.J. 1950. *The Tunicata*. Bernard Quaritch, Ltd., London, p. 354.

Birkeland, C., Cheng, L., and Lewin, R.A. 1981. Motility of didemnid ascidian colonies. *Bull. Mar. Sci.* **3(1):** 170–173.

Carlisle, D.B. 1961. Locomotory powers of adult ascidians. *Proc. Zool. Soc. Lond.* **136:** 142–146.

Cowan, M.E. 1981. Field observations of colony movement and division of the ascidian *Didemnum molle*. *Mar. Ecol. Prog. Ser.* **6:** 335–337.

Cox, G. 1986. Comparison of *Prochloron* from different hosts. I. Structural and ultrastructural characteristics. *New Phytol.* **104:** 429–445.

Freeman, G. 1971. A study of the intrinsic factors which control the initiation of asexual reproduction in the tunicate *Amaroucium constellatum*. *J. Exp. Zool.* **178(4):** 433–456.

Jokiel, P. 1980. Solar ultraviolet radiation and coral reef epifauna. *Science* **207:** 1069–1071.

Kott, P. 1980. Algal-bearing didemnid ascidians in the Indo-West-Pacific. *Me. Qd Mus.* **20(1):** 1–47, pls. 1–4.

Lewin, R.A. and Cheng, L. 1989. *Prochloron, A Microbial Enigma.* Chapman and Hall, London, p. 129.

Olson, R.R. 1986. Light-enhanced growth of the ascidian *Didemnum molle/ Prochloron* sp. symbiosis. *Mar. Biol.* **93:** 437–442.

Olson, R.R. 1983. Ascidian-*Prochloron* symbiosis: The role of the larval photoadaptations in midday larval release and settlement. *Biol. Bull.* **165:** 221–240.

Smith, M.J. 1970. The blood cells and tunic of the ascidian *Halocynthia aurantium* (Pallas). I. Hematology, tunic morphology, and partition of cells between blood and tunic. *Biol. Bull.* **138:** 354–378.

Swift, H. 1989. The cytology of *Prochloron.* In: *Prochloron, A microbial Enigma.* R.A. Lewin and L. Cheng, eds. Chapman and Hall, London, pp. 31–52.

Thinh, L.V., Griffiths, D.J, and Ngan, Y. 1981. Studies of the relationship between the ascidian *Diplosoma virens* and its associated macroscopic algae. II. Aspects of the ecology of the animal host. *Aust. J. Mar. Freshwater Res.* **32:** 795–804.