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Do symbiotic dinoflagellates secrete lipid droplets?

Abstract—We investigated the claim that symbiotic dinoflagellates from corals and sea anemones release lipid droplets in vitro. Putative lipid droplet secretion in vitro is taken as evidence for lipid translocation from algae to host in hospite in symbiotic cnidarians. We confirmed that symbiotic algae isolated from the tropical sea anemone *Condylactis gigantea* exhibited surface "blebs" and attached droplets. The surface entities stained positively with the DNA-specific fluorochrome Hoechst 33258 and with the chromosome stain lacto-aceto-orcein, but not with the lipid lysochrome Sudan Black B. In contrast, bona fide lipid droplets isolated from *C. gigantea* gonad stained positively with Sudan Black B. The data support the interpretation that droplets attached to isolated symbiotic dinoflagellates are host cell nuclei and are consistent with ultrastructural data published elsewhere.

A wide range of marine cnidarians (e.g. corals, sea anemones, gorgonians, etc.) are hosts to symbiotic dinoflagellates. The algae reside within vacuoles in cells of the host endoderm (Gates et al. 1992). They carry out photosynthesis in hospite at rates comparable to cultured or free-living species, but their specific growth rates in steady state populations are relatively low (Berner et al. 1993). This is because photosynthesis and growth are uncoupled in cells in symbiosis. Up to 90% or more of the carbon fixed photosynthetically is released from the algae and translocated to the host cells where it is used for metabolism and growth (Muscatine et al. 1984; Davies 1991).

The nature of the products fixed and released by the algae has received modest attention, but at least one aspect—the release of lipid—remains controversial. The controversy arises from three sets of observations.

First, translocation was initially investigated by observing the nature of ^{14}C-labeled products released to the ambient medium by symbiotic dinoflagellates removed from their host and maintained for short periods in vitro with $H^{14}CO_3^-$ in the light. Algae from the giant clam *Tridacna crocea,* the coral *Pocillopora damicornis,* and the Pacific coast sea anemone *Anthopleura elegantissima* incubated for 30 min to 4 h released up to 60% of the fixed carbon, including substantial amounts of [^{14}C]glycerol (Muscatine 1967; Trench 1971*b,c*). Translocation of glycerol in hospite was supported by the observation that deacylated lipid from *P. damicornis* and *A. elegantissima* animal tissue contained substantial labeled glycerol (Muscatine and Cernichiari 1969; Trench 1971*a*).

Second, in studies of [^{14}C]acetate metabolism of the coral *Pocillopora capitata* from the Revillagigedo Islands, Mexico, Patton et al. (1977) found that uptake of [^{14}C]acetate was enhanced in the light. They proposed that light-driven synthesis of fatty acids by symbiotic algae was followed by translocation of fatty acids or lipid from algae to host. A similar pattern of incorporation of [^{14}C]acetate by *A. elegantissima* was observed by Blanquet et al. (1979). There was a significant shift in acetate incorporation from polar lipid in the dark to triglyceride and wax ester in light. Increased label in animal triglyceride was explained by translocation of either triglycerides or free fatty acids from algae to host cells (*see also* Harland et al. 1991, 1992).

Third, using $H^{14}CO_3^-$ and [^{14}C]acetate tracers, Patton et al. (1983) found that the bulk of ^{14}C was located in fatty acyl moieties of coral animal triglycerides, and not in glycerol moieties as had been reported earlier. They then advanced the interpretation that (p. 121), "photosynthetically fixed carbon is immediately synthesized into lipid which is then translocated to the host." Later, Battey and Patton (1984, 1987) confirmed glycerol translocation in *Condylactis gigantea* but noted also that glycerol is quickly metabolized by this animal

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host and so accounts for the paucity of labeled glycerol in animal triglyceride in this particular test cnidarian.

The idea that lipid is translocated directly was initially based on interpretation of the nature, size, and occurrence of certain droplets attached to the algae isolated from the coral *Stylophora pistillata.* Thus, small vesicles in close and protracted association with symbiotic algae were described as "externally blebbing fat droplets" (Patton and Burris 1983; Kellogg and Patton 1983). "Fat droplets were distinguished from other algal cell protrusions with phase contrast optics. Fat droplets appear as white spheres while other structures appeared grey" (Patton and Burris 1983, p. 132). The evidence that *attached* droplets were lipid was based solely on appearance under phase contrast microscopy. Fat droplets which were analyzed chemically were neither attached to algae nor were they necessarily the same as those attached to the algae. Patton and Burris (1983) rightly concluded that (p. 136) "evidence for algal lipid secretion is still indirect."

In a companion paper, Kellogg and Patton (1983) used the tropical sea anemone *C. gigantea* as a "model coral polyp." Although they showed photomicrographs of algae with attached droplets, they mainly addressed the size and chemical nature of "extra-algal droplets" isolated from tentacles by homogenization. They assumed that these droplets were the same as those attached to the algae because the former were abundant in tentacles where the algae reside. Kellogg and Patton stated that the actual secretion (exocytosis) of lipid was not observed, possibly because it may occur only in hospite.

In view of the uncertainty surrounding this putative phenomenon and the lack of critical evidence, we have reinvestigated the secretion of lipid droplets by symbiotic dinoflagellates. In particular, we have sought to confirm Kellogg and Patton's (1983) observation that lipid droplets are secreted in vitro by freshly isolated symbiotic algae from *C. gigantea.* In this study we confirm their report of "droplets" attached to freshly isolated symbiotic algae. However, specific staining techniques indicate that the attached droplets are DNA-containing entities, most likely host cell nuclei—not lipid droplets.

C. gigantea was collected in the vicinity of Long Key Marine Laboratory, Long Key, Florida. Anemones were shipped by air to UCLA where they were placed in running seawater aquaria and kept at 26°C. Three 40-W fluorescent tubes delivered an irradiance of 80 μmol quanta $m^{-2} s^{-1}$ on a 12 : 12 L/D cycle. Anemones were fed three times weekly on frozen shrimp.

We obtained algae for visualization and staining as follows. Tentacles (2–4 cm) were grasped with forceps, severed with scissors, and immediately either homogenized in artificial seawater in a Teflon-glass tissue homogenizer, followed by centrifugation (1,500 rpm, 2 min) to obtain a pellet enriched in algae, or macerated in Ca^{2+}-free artificial seawater according to the method of Gates and Muscatine (1992). Samples of gonad were either excised and squashed on a microscope slide or homogenized at 0°C and centrifuged (13,000 × *g,* 4°C, 25 min) to obtain bona fide lipid droplets. Lipid droplets at the surface of the preparation were collected with a Pasteur pipette.

Cells and droplets were carefully transferred with a fine-bore mouth pipette to slides coated with poly-L-lysine (mol wt > 70,000, 1% in distilled water). Cells on some slides were stained with the DNA-binding fluorochrome Hoechst 33258 (Sigma) as described by Gates and Muscatine (1992). Others were fixed briefly in 5% buffered Formalin, rinsed in distilled water, dehydrated by brief immersion in an ethanol series (10, 20, 30, 50, 70, 90%), and then stained with the lipid lysochrome Sudan Black B (Baker 1958) in 95% ethanol for 30 min. Slides were then rinsed three times in 90% ethanol, counterstained for 30 min with lacto-aceto-orcein, which in acid solution stains chromosomes (Baker 1958), rinsed in water, hydrated in the ethanol series, and mounted in water for microscopy.

Size (diameter) and number of droplets were determined respectively with a calibrated ocular micrometer and a hemacytometer.

Preparations were viewed under brightfield, phase contrast, or epifluorescence microscopy with an Olympus BH-2 microscope at 40×, 50×, and 100× (oil immersion). Cells were photographed with a D Plan APO 100 UV oil immersion objective lens, Kodak 35-mm Ektachrome film (Daylight, ASA 200), and the BH-2 automatic exposure meter.

Homogenates of *C. gigantea* contained large

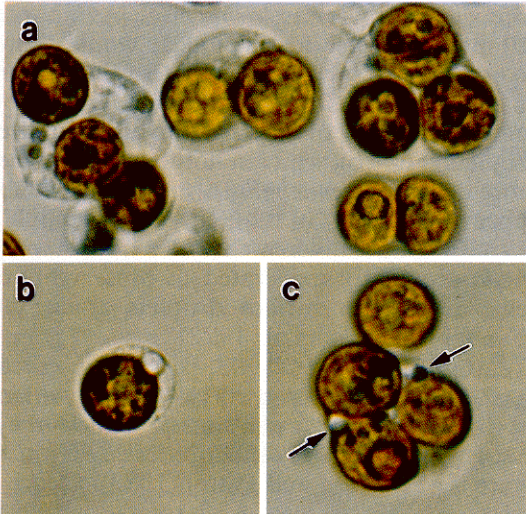

Fig. 1. Symbiotic dinoflagellates within host cells obtained by homogenization of tentacles of *Condylactis gigantea*. Brightfield, oil immersion, 3,800×, details in text.

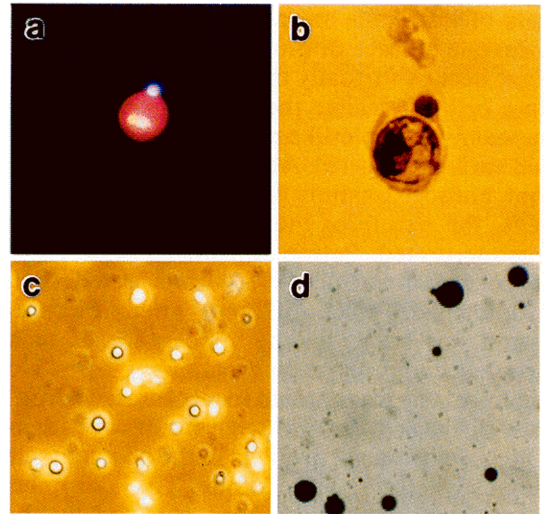

Fig. 2. [a.] One symbiotic dinoflagellate within a *Condylactis gigantea* host cell showing the host cell nucleus stained with Hoechst 33258. Epifluorescence, 3,800×. [b.] As in panel a, but stained with lacto-aceto-orcein. Brightfield, 3,800×. [c.] Lipid droplets from *C. gigantea* gonad, phase contrast, unstained, 1,520×. [d.] As in panel c, but stained with Sudan Black B. Brightfield, 1,520×.

numbers of intact host cells, each containing from one to three algae (Fig. 1a). This result was unexpected as homogenization of other tropical anemone species (e.g. *Aiptasia pulchella*) usually destroys host cells and leaves algae free in suspension. Of 3,390 "single" algae in 32 preparations from 10 different *C. gigantea* tentacle homogenates, 21.22% exhibited spherical surface droplets. Of these cells, >99.9% had only one droplet per cell, as shown in Fig. 1b. In many cases, the host cell plasma membrane which enclosed both the algal cell and adjacent droplet could be clearly discerned (Fig. 1b). When algal cells appeared as doublets or triplets, droplets often occurred at the interstices of the cells (Fig. 1c, at arrows). Droplets had a diameter of 2.63 ± 0.72 μm ($\bar{X} \pm$ SD; range, 1.8–4.4 μm; $n = 48$). Only occasionally did we observe additional droplets of varying size adjacent to algae. None reacted with the specific cytological stains used here. We interpret these as inclusions of unknown type within host cells.

Algae with a single attached droplet were reminiscent of profiles observed in our laboratory when host endoderm cells were released from the tropical anemone *A. pulchella* by maceration. Evidence from specific staining techniques and from scanning and electron microscopy of individual cells indicated that such droplets were host cell nuclei (Gates and Muscatine 1992; Gates et al. 1992).

Cells were stained with the DNA-specific stain Hoechst 33258 to determine if droplets attached to *C. gigantea* algae obtained by homogenization were host cell nuclei. Of 2,545 cells in 24 preparations from 10 tentacles, 23.10% had droplets. 100% were Hoechst positive (Fig. 2a). These stained droplets had a diameter of 2.74 ± 0.73 μm ($\bar{X} \pm$ SD; range, 1.4–4.4; $n = 100$); droplets of 2.3- and >3.4-μm diameters were most frequent.

Cells from homogenates and from macerated tissues were fixed on slides and exposed to Sudan Black B to determine whether attached droplets reacted with this lipid lysochrome. Attached droplets were Sudan Black B negative, but stained positively with a lacto-aceto-orcein counterstain (Fig. 2b), indicating the presence of chromosomes. In contrast, bona fide lipid droplets from gonad preparations (Fig. 2c) stained positively with Sudan Black B (Fig. 2d). These droplets had a diameter of 2.71 ± 1.39 μm ($\bar{X} \pm$ SD; range, 1.0–7.0; $n = 99$).

Our observations show that when *C. gigantea* tentacle tissue is homogenized, large num-

bers of host cells are released which contain from one to three or more algae. Typically, the algae occupy virtually the entire volume of the host cell and cause the host cell nucleus to appear as a dropletlike prominence at the algal cell surface (Fig. 1b). As it is logical to assume that homogenization has destroyed the host cells, the nature of the droplet can easily be misinterpreted. Patton and Burris (1983) and Kellogg and Patton (1983) interpreted such droplets as droplets of lipid. Their interpretation was based on the appearance of such droplets under phase contrast microscopy. No chemical test was applied to algal surface droplets, but wax ester and triglyceride was identified in extra-algal droplets in the host tentacle tissues where the algae reside. These droplets were assumed to have been secreted by the algae. The misinterpretation of attached droplets as consisting of lipid was reinforced by the observation of Kellogg and Patton (1983, p. 137) that "the diameter of the extra-algal droplets (3.4 ± 0.7 μm) was similar to that of droplets observed to protrude from isolated . . . [algae]."

Our data reveal that droplets attached to algae from *C. gigantea* (and which appear to be identical to those figured by Kellogg and Patton) reacted positively with stains specific for DNA (Hoechst 33258) and chromosomes (lacto-aceto-orcein) (Fig. 2a,b). In contrast, although Sudan Black B stained free lipid droplets positively (Fig. 2d), it did not react with any droplets attached to the surface of the algae. We conclude from these data and from ultrastructural criteria presented elsewhere (Gates et al. 1992) that the surface droplets on algae from *C. gigantea* are host cell nuclei contained within the host cell plasma membrane and held adjacent to the algae.

The identification of surface droplets as host cell nuclei rather than lipid droplets is consistent with a range of features described by Patton and Burris (1983) and Kellogg and Patton (1983). For example, only 5–13% of the algae isolated from the coral *S. pistillata* exhibited "attached lipid droplets" at any given time (Patton and Burris 1983). This observation can be explained by the random orientation of cells in microscope preparations. Nuclei beneath a cell would not be seen when the cell was viewed from above. Moreover, host cells damaged by homogenization release many algae. As the host cell nuclei are also lost, the algae would show no evidence of surface droplets. Similarly, our observations of *C. gigantea* homogenates reveal that ~21% of the algae exhibit surface droplets (Fig. 1b), while in homogenates of the more delicate *A. pulchella,* algae with droplets appear relatively infrequently (our unpubl. obs.). In contrast, when tentacles from *A. pulchella* are gently macerated to preserve host cells, algae with surface droplets appear in abundance (Gates et al. 1992).

Patton and Burris (1983, p. 134) observed that "cells with fat droplets [were] held within [a] limiting outer membrane," which they thought was the algal cell amphiesma (*see also* figure 7 of Kellogg and Patton 1983). Clearly, the more likely alternative interpretation is that the limiting outer membrane is the host cell plasma membrane, as shown in Fig. 1b. This would explain why their attempts to dislodge droplets were unsuccessful. Thus Kellogg and Patton (1983, p. 142) remarked that "the close association of lipid droplets with zooxanthellae . . . was quite strong and not disrupted by the isolation procedure that was used for the extra algal droplets." Identification of droplets as host cell nuclei also explains why droplets were never observed to be secreted, grow, or separate from algae.

As to "droplets . . . seen in the crevices of doublets," host cell nuclei are typically found at interstices where doublet or triplet cells contact each other (Fig. 1c: *see also* figures 2 and 4 of Gates et al. 1992). Finally, the absence of droplets attached to algae from *Tridacna* sp. would be consistent with the observation that these algae are apparently not intracellular (Norton et al. 1992).

Kellogg and Patton (1983) reported that some cells had multiple droplets attached at various points. We speculate that the attached pair of droplets shown in their figure 2b may represent a recently divided host cell nucleus. We have observed such profiles in *A. pulchella* cells after maceration and staining with Hoechst 33258.

The observations presented here address the identity of droplets attached to symbiotic algae in vitro. As it now appears doubtful that these are secreted lipid droplets, generalizations on translocation of lipid in hospite, based on se-

cretion of lipid droplets in vitro, may be invalid.

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