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NOTE

Temperature induction of viruses in symbiotic dinoflagellates

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ABSTRACT: Bleaching manifests itself as a loss of symbiotic dinoflagellates (zooxanthellae) and/or chlorophyll from a variety of symbiotic hosts, including corals and sea anemones. Bleaching is known to result from a range of environmental stresses, the most significant of which is elevated temperature; how these stresses elicit a bleaching response is currently the focus of intense research. One consequence of environmental stress that has yet to be considered is viral attack. Here, we have isolated a transferable infectious agent believed to be a virus, from zooxanthellae of the temperate sea anemone *Anemonia viridis*. The infectious agent is induced by elevated temperature. Once induced, the filterable agent can be further propagated without heat induction, thus fulfilling Koch's postulates. We propose that zooxanthellae harbor a latent viral infection that is induced by exposure to elevated temperatures. If such a mechanism also operates in the zooxanthellae harbored by reef corals, and these viruses kill the symbionts, then this could contribute to temperature-induced bleaching.

KEY WORDS: Viruses · Anemones · Coral bleaching · Zooxanthellae · Latent infection

Many species of marine invertebrates, such as corals, sea anemones and giant clams, harbor endosymbiotic dinoflagellates (zooxanthellae) in their tissues. Zooxanthellae release short-term photosynthetic products to their hosts and so are an important source of organic carbon for host metabolism, growth and reproduction (Muscatine 1990). Zooxanthellae are also important in the recycling and conservation of essential nutrients, such as nitrogen (Wang & Douglas 1998), and enhance calcification rates in corals (Davies 1992).

Incidents of coral bleaching, synonymous with the loss of photosynthetic pigments and/or zooxanthellae from the host, have been frequent in recent years (Hoegh-Guldberg 1999). Indeed, the coral bleaching event in 1997 to 1998 was the most geographically extensive and severe in recorded history, and caused significant coral mortality worldwide (Harvell et al. 1999). Associated with these catastrophic ecological events were widespread socio-economic impacts (Obura 1999, Rajasuriya et al. 1999), leading to tremendous public interest in the causes of coral bleaching (Anderson 1999, Pockley 1999, 2000). Increased seawater temperature has been identified as the major culprit for worldwide coral bleaching events (Harvell et al. 1999). However, the mechanism of thermal disruption is still being determined. Current research has investigated processes such as host cell adhesion dysfunction (Gates et al. 1992), bacterial attack (Kushmaro et al. 1997, Banin et al. 2001) and thermal disruption of photosystems I and II (Iglesias-Prieto et al. 1992, Warner et al. 1999, Jones et al. 2000). However, one potential factor that has not been investigated is viral infection.

The importance of viruses in the marine environment has become increasingly apparent over the last decade (Fuhrman 1999). Concentrations of up to 10^8 ml^{-1} have been reported (Bergh et al. 1989) and it is thought that most microbial populations in seawater have viruses that infect them. Although most of these viruses are thought to infect bacteria (Wilcox & Fuhrman 1994), a significant proportion of them are known to infect primary producers, such as *Emiliania huxleyi* (Bratbak et al. 1996) and cyanobacteria (Wilson et al. 1993), and play an active role in the structuring of planktonic communities (Wilson et al. 1998). In this study, we conducted some simple experiments to determine if viruses are involved in temperature-mediated death of zooxanthellae isolated from the temperate anemone *Anemonia viridis*.

Methods. ***Preparation of zooxanthellae:*** Anemones were collected at low tide from intertidal pools on Batten Bay, near Plymouth, UK. Freshly collected anemones were used for each experiment. They were transferred to a 10 l holding tank at 15°C, fed by a coarse filtered flow-through seawater system, and piped into the laboratory from seawater off the coast of Plymouth, UK. Illumination was provided on a 16:8 h light:dark cycle from a 150 W cool aquarium light.

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Zooxanthellae were isolated from anemones by homogenizing up to 12 tentacles in sterile seawater in a tissue grinder. The homogenized suspension was centrifuged at low speed and the supernatant discarded. The process of resuspending the pellet in fresh sterile seawater and centrifugation was repeated until the supernatant was clear of sea anemone debris. Zooxanthellae in the cleaned pellet and subsequent induction experiments were enumerated in a Neubauer improved hemacytometer.

Virus induction and propagation: Zooxanthellae isolated from the anemone were exposed to a heat shock of 32°C for a period of 24 h and then incubated at 15°C for a further 7 d. Following this experiment, lysed zooxanthella suspensions were passed through a 0.2 µm filter, and 100 µl aliquots of the filtrate were added to freshly isolated zooxanthellae, which were then incubated at 15°C *without heat treatment*. During virus induction and propagation, zooxanthellae were incubated in a cooled illuminated incubator (Sanyo) set on a 16:8 h light:dark cycle at the required temperature.

Thin section analysis: Prior to embedding, zooxanthellae were fixed in 4% glutaraldehyde and 1% osmium tetroxide. Fixatives were replaced with sterile seawater after low speed centrifugation to pellet the cells. After dehydration through an acetone series of 25, 50, 75 and 100%, the pelleted cells were embedded in TAAB embedding resin (TAAB Laboratory Equipment Ltd.) and hardened at 60°C for 48 h. Sections were cut on a Reichert-Jung Ultracut microtome and stained with solutions of uranyl acetate and lead citrate on copper grids. Transmission electron microscopy (TEM) was conducted on a JEOL 200CX operated at 160 kV at magnifications up to 150 000×.

Results and discussion. Exposure of zooxanthellae isolated from the temperate sea anemone *Anemonia viridis* to 32°C for a period of 24 h induced cell lysis; most of the isolated zooxanthellae were killed compared to a 15°C control (Fig. 1a). We did observe a decline in numbers in the controls (Fig. 1), however, it is known that zooxanthellae from *Anemonia viridis* are difficult to culture and rarely survive for more than a few months in isolation (Davy 1994). TEM analysis revealed an abundance of small (40 to 50 nm) virus-like particles (VLPs) in the zooxanthella suspensions that had collapsed following heat treatment (Fig. 2). In addition, VLPs of the same size were observed in thin sections of zooxanthellae still harbored by *Anemonia viridis*, after the anemones were exposed to a gradual increase in temperature to 32°C over a 2 wk period (results not shown). It has previously been observed that heat-shock treatment can induce lytic infection of the green alga *Cylindrocapsa geminella* (Hoffman & Stanker 1976). Using thin-sectioning and TEM, these workers demonstrated that viral particles ca 200 to 300 nm in diameter can be produced following a heat shock of 40°C for 6 h.

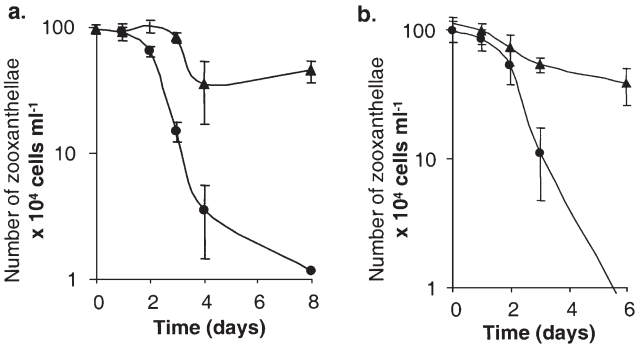

Fig. 1. Induction experiments on zooxanthellae isolated from the sea anemone *Anemonia viridis*. (a) Changes in zooxanthella density following either heat shock for 24 h at 32°C; (b) or following infection by a filtered aliquot from the heat shock lysate, where complete lysis was observed after 6 d (final data point not shown). In each experiment, the control zooxanthellae (▲) were incubated at 15°C on a 16:8 h light:dark cycle. Heat-treated zooxanthellae (●, a) were transferred to control conditions after 24 h. Infected zooxanthellae (●, b) were incubated under control conditions throughout the experiment. Data points are the mean and standard deviation of triplicate counts

Our results indicated that we had induced a latent virus from the zooxanthellae following the heat shock. We were initially concerned that the results were influenced by heat-induced bacteriophages in the non-axenic preparation. However, to rule this out, we employed thin-section analysis of infected zooxanthellae (discussed below, Fig. 3).

During a latent infection, a single virus particle incorporates its nucleic acid into host genomic DNA and

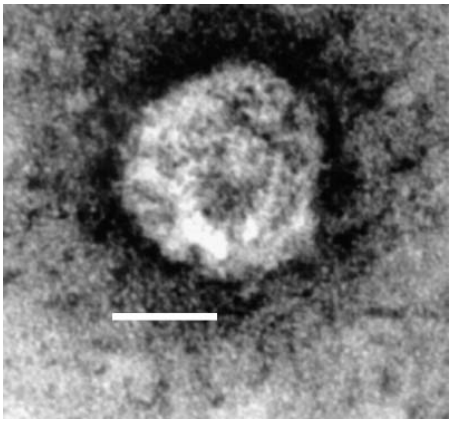

Fig. 2. Transmission electron micrograph of a virus-like particle observed following heat shock treatment of zooxanthellae isolated from the sea anemone *Anemonia viridis*. Non-concentrated supernatant was viewed, following negative staining with uranyl acetate, from lysed, isolated zooxanthellae which had previously been subjected to a heat induction of 32°C for 24 h then incubated at 15°C on a 16:8 h light:dark cycle for 6 d. Scale bar = 25 nm

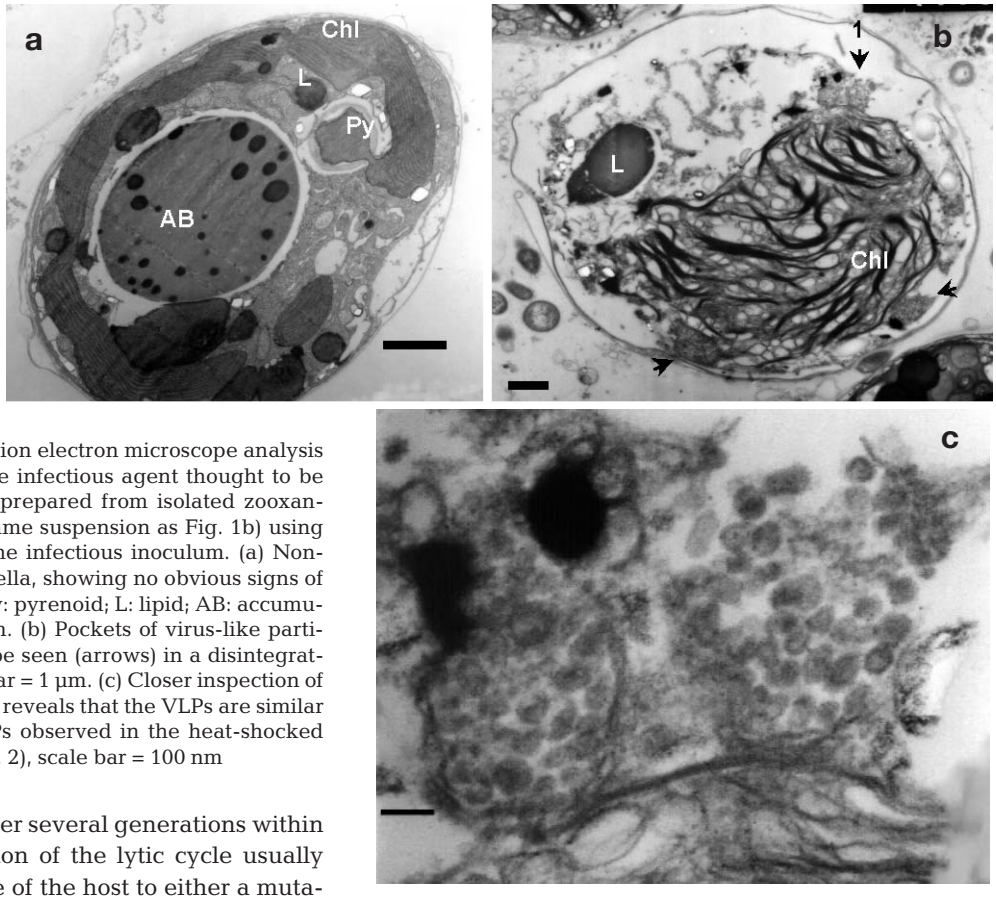

Fig. 3. Thin section transmission electron microscope analysis of zooxanthellae showing the infectious agent thought to be a virus. Thin sections were prepared from isolated zooxanthellae, 6 d post-infection (same suspension as Fig. 1b) using a 0.2 µm filtered lysate as the infectious inoculum. (a) Non-inoculated, control zooxanthella, showing no obvious signs of infection. Chl: chloroplast; Py: pyrenoid; L: lipid; AB: accumulation body, scale bar = 1 µm. (b) Pockets of virus-like particles (VLPs) (viroplasm) can be seen (arrows) in a disintegrating zooxanthella cell, scale bar = 1 µm. (c) Closer inspection of a viroplasm (from b, arrow 1) reveals that the VLPs are similar in size (45 to 50 nm) to VLPs observed in the heat-shocked zooxanthellae (Fig. 2), scale bar = 100 nm

replicates as a provirus over several generations within the host genome. Induction of the lytic cycle usually occurs following exposure of the host to either a mutagenic stimulation such as short wave ultra-violet (UV) light or environmental stresses such as nutrient availability (Scanlan & Wilson 1999) or, classically, increased temperature (Edgar & Lielausis 1964). This exposure eventually leads to host cell lysis and further propagation of progeny viruses in susceptible hosts.

To fulfil Koch's postulates, filtered lysed zooxanthella suspensions were added to freshly isolated zooxanthellae, then incubated *without heat treatment*. Infected preparations started to lyse after 2 d and substantial lysis was evident after 6 d (Fig. 1b). Thin section TEM analysis of infected cells (taken on Day 6, Fig. 1b) revealed that pockets of VLPs (viroplasm) were present in dying zooxanthellae (Fig. 3b). VLPs within the viroplasms (Fig. 3c) were similar in size to those observed after the initial heat shock treatment. In contrast, no VLPs were observed in non-inoculated, control zooxanthellae (Fig. 3a). Thus, Koch's postulates were fulfilled; an infectious agent isolated from a diseased host was used to re-infect healthy host cells that subsequently showed the same disease symptoms. However, host cells that harbor a latent infection usually exhibit superinfection immunity, which makes them resistant to further infection. Our results suggest that not all zooxanthellae in this sea anemone harbor a latent infection (otherwise further propagation of the virus would not have been possible). This could indicate the presence of different zooxanthella types in *Anemonia viridis*, with varying degrees of susceptibility to infection. Evidence for different types of zooxanthellae in *A. viridis* is equivocal (Bythell et al. 1997, Davy et al. 1997a,b), but the simultaneous presence of different zooxanthella taxa has been observed in corals (Rowan & Knowlton 1995, Rowan et al. 1997).

Previous reported attempts to isolate viruses from marine symbiotic algae have been few and unsuccessful. O'Brien et al. (1984) looked at thin sections of dinoflagellates (zooxanthellae) and *Chlorella*-like algae (zoochlorellae) symbiotic with the temperate sea anemone *Anthopleura xanthogrammica*, expecting to find viruses. Their failure to observe any led them to believe that the absence of lytic viruses was a fundamental distinction between marine and freshwater algal-invertebrate symbiosis, though it should be noted that these authors did not look at environmentally stressed individuals. In contrast, viruses have been observed in free-living marine dinoflagellates (cf. symbiotic dinoflagellates). Franca (1976) reported the presence of numerous small virus-like particles (VLPs), approximately 35 nm in diameter, in the cytoplasm of *Gyrodinium resplendens*. Sicko-Goad & Walker (1979) found much larger VLPs (385 nm) in the freshwater

dinoflagellate *Gymnodinium uberrimum*, which were observed budding from a vesicular viroplasmic area. These workers suggested that a latent infection had been induced by changing environmental conditions (not specified).

As stated previously, high seawater temperatures have been blamed for major coral bleaching events (Anderson 1999, Harvell et al. 1999, Pockley 1999, 2000). Given the close taxonomic relationship between the zooxanthellae in *Anemonia viridis* and those of reef corals (Bythell et al. 1997, Davy et al. 1997b), our novel findings raise the possibility that viral infection contributes to bleaching events on coral reefs. We consider this to be an important topic for future research.

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