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Source: Zoological Science, 40(6) : 444-454

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zs230045

ZOOLOGICAL SCIENCE **40**: 444–454 (2023)

In Vitro Phagocytosis of Different Dinoflagellate Species by Coral Cells

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Coral-dinoflagellate symbiosis is a unique biological phenomenon, in which animal cells engulf single-celled photosynthetic algae and maintain them in their cytoplasm mutualistically. Studies are needed to reveal the complex mechanisms involved in symbiotic processes, but it is difficult to answer these questions using intact corals. To tackle these issues, our previous studies established an in vitro system of symbiosis between cells of the scleractinian coral *Acropora tenuis* and the dinoflagellate *Breviolum minutum*, and showed that corals direct phagocytosis, while algae are likely engulfed by coral cells passively. Several genera of the family Symbiodiniaceae can establish symbioses with corals, but the symbiotic ratio differs depending on the dinoflagellate clades involved. To understand possible causes of these differences, this study examined whether cultured coral cells show phagocytotic activity with various dinoflagellate strains similar to those shown by intact *A. tenuis*. We found that (a) *A. tenuis* larvae incorporate *Symbiodinium* and *Breviolum*, but not *Cladocopium*, and very few *Effrenium*, (b) cultured coral cells engulfed all four species but the ratio of engulfment was significantly higher with *Symbiodinium* and *Breviolum* than *Cladocopium* and *Effrenium*, (c) cultured coral cells also phagocytosed inorganic latex beads differently than they do dinoflagellates. It is likely that cultured coral cells preferentially phagocytose *Symbiodinium* and *Breviolum*, suggesting that specific molecular mechanisms involved in initiation of symbiosis should be investigated in the future.

Key words: Coral-dinoflagellate symbiosis, in vitro system, phagocytosis, different clades of Symbiodiniaceae, latex beads

INTRODUCTION

Coral-dinoflagellate symbiosis is unique, in that animal cells engulf algal cells and maintain them in the cytoplasm, an extraordinary mutualism between taxonomically distant organisms (Baker, 2003; Davy et al., 2012; Weis, 2019). Corals are multicellular animals with two germ layers, ectoderm and endoderm, and gastrodermis cells of the endoderm harbor dinoflagellate symbionts in their cytoplasm. Dinoflagellates of the family Symbiodiniaceae (LaJeunesse et al., 2018) are single-celled yellow-brown algae. These algae exhibit two major states in life: thecate, motile cells with flagella and globular, immobile coccoid cells that proliferate mitotically (LaJeunesse et al., 2012). Symbiotic algae are approximately 10 μm in diameter. Thus, in this mutualism, single algal cells are situated in the cytoplasm of animal cells approximately 20–30 μm in diameter. Corals are thought to provide shelter for their algal symbionts, which supply most of their photosynthetic products to the host corals (Yellowlees et al., 2008). The symbiosis is estimated to have become obligatory more than 200 million years ago

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doi:10.2108/zs230045

(Stanley and Fautin, 2001; Shinzato et al., 2011).

Taking advantage of supplements from symbiotic algae, scleractinian (stony, reef-building) corals produce massive, rocky reefs by depositing calcium carbonate skeletons. Coral reefs harbor an estimated one-third of all described marine species though coral reefs occupy only 0.2–0.3% of the ocean surface (Reaka-Kudla, 2001; Wilkinson, 2008). Reefs support the most biodiverse ecosystems in the oceans, which also support human life in tropical and subtropical countries by virtue of fisheries, tourism, and culture (Reaka-Kudla, 2001; Wilkinson, 2008; Spaldomga et al., 2017). Coral reefs, however, are in crisis due to environmental changes, including increased seawater temperatures, acidification, and pollution, mainly caused by human activities. These stresses cause collapse of coral-dinoflagellate symbioses, resulting in white, dead reefs, an event known as coral bleaching, which leads to loss of coral reefs and all the species they support (Hoegh-Guldberg et al., 2007; Hughes et al., 2017; Sully et al., 2019). Since conservation of coral reefs is one of the most urgent environmental crises facing humanity, cellular and molecular mechanisms involved in coral-dinoflagellate symbiosis are important research subjects not only in terms of basic zoology, but also in regard to sustainability of our planet.

Coral-zoothanthella symbioses can be established through several processes, each of which involves complicated mechanisms. Four fundamental, interrelated processes are (1) mutual partner recognition, (2) onset of symbiosis, (3) maintenance and dynamic homeostasis, including modulation of coral host inert immunity, and (4) dysbiosis, which leads to bleaching (Davy et al., 2012; Weis, 2018). Many studies have attempted to explore cellular and molecular mechanisms of coral-dinoflagellate endosymbiosis (Loh et al., 2001; Wakefield and Kempf, 2001; Baker, 2003; Dove, 2004; Hirose et al., 2008; Biquand et al., 2012; Davy et al., 2012; Yamashita et al., 2013, 2014, 2018; Bockel and Rinkevich, 2019; Yuyama et al., 2018; Weis, 2019; Yoshioka et al., 2020, 2021; Rosset et al., 2021). Recently, our knowledge of these mechanisms has been advanced by studies using the sea anemone *Exaiptasia pallida* (Weis, 2019), the soft coral *Xenia* sp. (Hu et al., 2020), and the stony coral *Stylophora pistillata* (Levy et al., 2021) at the single-cell level (Sebe-Pedros et al., 2017). However, many questions remain regarding molecular mechanisms of symbiosis, especially recognition mechanisms involved in the initial contact of animal and algal cells, which enable subsequent dinoflagellate endocytosis and maintenance of endosymbiosis (Davy et al., 2012).

To tackle questions of symbiosis, many studies have attempted to produce in vitro culture lines of stony coral cells, by which mechanisms of coral-dinoflagellate symbiosis might be investigated. However, so far, most attempts have only reached the primary culture state, but have not achieved a stable, cryo-preservable system (Rinkevich, 2011; Domart-Coulon and Ostrander, 2016; Nowotny et al., 2021). However, we developed stable in vitro coral cell lines originating from planula larvae of the scleractinian coral *Acropora tenuis* (Kawamura et al., 2021a). Most lines are polyclonal and comprise different types of cells, including gastrodermis cells, digestive cells, secretory cells, etc. The IVB5-line is mostly composed of dark, flattened, amorphous cells, which have properties of endoderm, and which engulf algal cells, actively shuffling cell membranes, filopodia, and lamellipodia. Endocytosis was completed within 5 min after coral inoculation with dinoflagellates (Kawamura et al., 2021b), confirming the occurrence of the fast phase of coral-zoothanthella symbiosis in vitro. However, it remains to be determined whether cultured cells retain symbiotic properties with different algal strains, as natural larval cells have. Therefore, the present study examined in vitro incorporation of different algal species by cultured coral cells.

MATERIALS AND METHODS

Cultured IVB5 cell-line of *Acropora tenuis*

Details of production of stable in vitro cell-culture lines originating from planula larvae of the coral *Acropora tenuis* are described in Kawamura et al. (2021a). IVB5 is one of 20 cryo-preservable lines and has been cultured in basic seawater medium consisting of natural seawater, one-fifth volume of H_2O, 10 mM HEPES (final pH 6.8), and the antibiotics penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL). Immediately before experiments, the basic medium was mixed with Dulbecco's modified Eagle's medium (DMEM) containing 15% fetal bovine serum at a ratio of 9:1. The IVB5-line is polyclonal, but major components are dark, flattened, amorphous cells (FACs), 20–40 μm in length (Fig. 1A). FACs are considered interchangeable with spherical, brilliant cells (Fig. 1B). When dark amorphous cells were resuspended in a 24-well multiplate filled with growth medium, they became flat and extended lamellipodia and filopodia with moderate locomotor activity. These flattened amorphous cells likely have properties of gastroderm cells (Kawamura et al., unpublished) and they exhibit in vitro symbiosis with algal cells (Kawamura et al., 2021b).

Symbiodiniaceae strains, culture, and cell size measurements

Five culturable strains of four species of the family Symbiodiniaceae (Coffroth and Santos, 2005; Yamashita and Koike, 2013; Jeong et al., 2014; LaJeunesse, 2017; LaJeunesse et al., 2018) were used in this study: *Symbiodinium microadriaticum* (culture ID, AJIS2-C2), *S. microadriaticum* (culture ID, CCMP828), *Breviolum minutum* (Mf1.05b), *Cladocopium* sp. (Y103), and *Effrenium voratum* (GY-H50-E) (Fig. 2; and see Supplementary Table S1). All strains were pre-cultured at 20°C with standard IMK medium (Sanko Jyunyaku, Tokyo) using an incubator (SANYO MIR-554) under 12 h:12 h dark and light conditions using a fluorescent lamp producing 20 μmol/m^2/s (Kawamura et al., 2021b).

Identities of strains were reconfirmed by sequencing ITS2 parts of their genomes according to the method of Stat et al. (2009) (see Supplementary Figure S1). The primer sequences used were itsD forward: GTGAATTGCAGAACTCCGTG and its2rev2 reverse: CCTCCGCTTACTTATATGCTT. Amplified DNAs were sequenced with a SeqStudio Genetic Analyzer (Applied Biosystems). The sequences obtained were compared to previously determined sequences to confirm identity. Draft genomes of the strains AJIS2-C2, Mf1.05b, and Y103 were available (Shoguchi et al., 2013, 2018; Yoshioka et al., 2021) (see Supplementary Table S1). CCMP828 and GY-H50-E, for which draft genomes are not available, were characterized by partial sequencing of 28S rDNAs on a SeqStudio Genetic Analyzer (Applied Biosystems). A database for BLASTN: GeoSymbio_ITS2_LocalDatabase (4).fasta (https://sites.google.com/site/geosymbio/downloads) (Franklin et al., 2012) was also used for comparison. Details of the strains are as follows.

Symbiodinium microadriaticum strain AJIS2-C2 (ITS2 type A1] (Fig. 2): This strain was originally isolated from the coral *Acropora* sp. at Ishigaki Island, Okinawa, Japan (Yamashita and Koike, 2013). Sequences showed that this strain is ITS2 type A1 (see Supplementary Figure S1A). It was maintained in the laboratory of Dr. Hiroshi Yamashita at the Fisheries Technology Institute, Japan

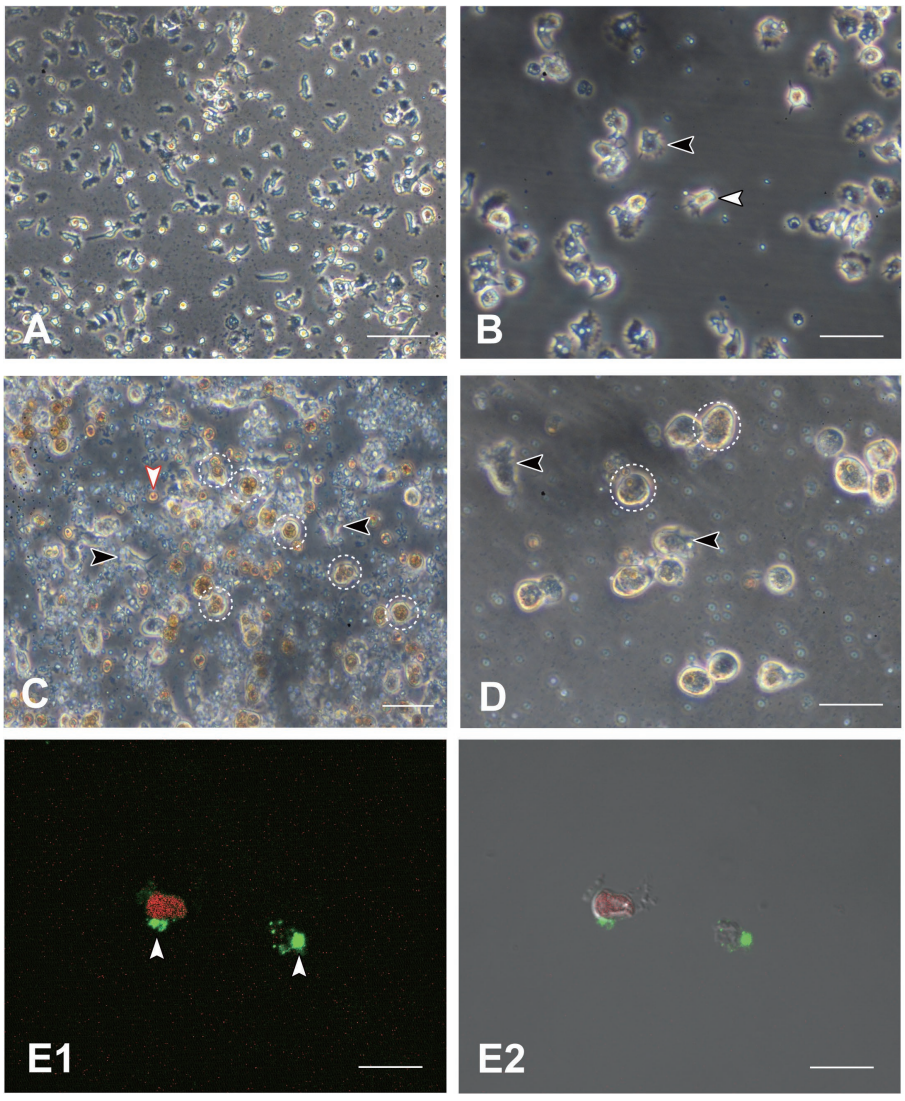

Fig. 1. Configuration of the coral cell line, IVB5. **(A)** Lower magnification of flattened amorphous cells (FACs). Bar, 100 μm. **(B)** FACs (black arrowheads) and spherical brilliant cells (white arrowhead) are considered functionally interchangeable. Bar, 50 μm. **(C)** Coral cells 3 days after algal inoculation. Broken circles show "fatty cells" engulfing algae. Black arrowheads show FACs. Red arrowhead shows an alga. Bar, 50 μm. **(D)** Higher magnification of "fatty cells" (broken circle). Black arrowheads show FACs. Bar, 50 μm. **(E)** AtSnail immunocytochemistry of FACs 2 hours after algal inoculation. Bars, 20 μm. (E1) Dark field image. White arrowheads show nuclei with Snail-expression (green). Alga emits red autofluorescence. (E2) Fluorescent image merged with bright field image.

Fisheries Research and Education Agency, and then transferred to the Marine Genomics Unit at Okinawa Institute of Science and Technology Graduate University (OIST) (Yoshioka et al., 2021).

Symbiodinium microadriaticum strain CCMP828 (ITS2 type A1) (Fig. 2): This strain was originally isolated from the jellyfish *Cassiopeia xamachana* and was obtained from the National Center for Marine Algae and Microbiota (NCMA), Boothbay Harbor, Maine (https://ncma.bigelow.org/CCMP828). Sequences showed that this strain is ITS2 type A1 (see Supplementary Figure S1B).

Breviolum minutum [ITS2 type B1) (Fig. 2): *B. minutum* (LaJeunesse et al., 2018), previously named *Symbiodinium minutum* (LaJeunesse et al., 2012), was originally harbored by the Caribbean coral *Orbicella faveolata* (previously *Montastraea faveolata*), maintained in the laboratory of Dr. Mary Alice Coffroth, at State University at New York, Buffalo, United States and then in the MGU at OIST and Sekida's laboratory of Kochi University. The culture ID of this strain is Mf1.05b (McIlroy and Coffroth, 2017), and it is currently provided through National Institute of Environmental Science, Tsukuba, Japan under https://mcc.nies.go.jp/strainList.do?strainId=3806 upon request. For sequences for ITS2 type B1, see Supplementary Figure S1C.

Cladocopium sp. (ITS2 type C92) (Fig. 2): *Cladocopium* sp. (LaJeunesse et al., 2018) was originally derived from the cardiid clam *Fragum* sp. in Okinawa, Japan, and was isolated by Prof. Terufumi Yamasu at the University of the Ryukyus. The culture ID of this strain is Y103. It has been maintained in the laboratory of Prof. Michio Hidaka at the University of the Ryukyus, and then transferred to laboratories at OIST and Kochi University. This strain is currently provided through the National Institute of Environmental Science, Tsukuba, Japan under https://mcc.nies.go.jp/strainList.do?strainId=4075 upon request. For sequences of ITS2 type C92, see Supplementary Figure S1D.

Effrenium voratum (ITS2 type E1) (Fig. 2): *E. voratum* (LaJeunesse et al., 2018) was originally derived from the East China Sea and maintained as a monoclonal line in the laboratory of Dr. Hongwei Zhao at the College of Ecology and Environment & State Key Laboratory of Marine Resource Utilization in South China Sea of Hainan University and then transferred to OIST and Kochi University. The culture ID of this strain is GY-H50-E. For sequences of ITS2 type E1, see Supplementary Figure S1E.

Measurement of cell size

Symbiodiniaceae strains in culture exhibit three types of morphology: mastigote (motile, with flagella), coccoid (spherical, no flagella), and doublet (dividing, no flagella) (Jeong et al., 2014). Cell size was determined with images of the coccoid stage (Fig. 2). Cells were photographed under differential interference contrast (DIC) imaging using a Zeiss Axio Imager Z1 microscope equipped with an AxioCam digital camera (Zeiss, Jena, Germany). Ten individuals were measured manually using Adobe Photoshop CS6 (version 13.0), and mean cell sizes were calculated.

In vivo experiments with incorporation of dinoflagellates by coral larvae

Five strains of Symbiodiniaceae for infection tests were cultured in the MGU at OIST (Shoguchi et al., 2018). *Symbiodinium microadriaticum* AJIS2-C2 has been maintained in Daigo's IMK medium (Sanko Jyunyaku, Tokyo), plus three antibiotics, ampicillin (100 μg/mL), kanamycin (50 μg/mL), and streptomycin (50 μg/mL). The culture medium of the other four Symbiodiniaceae strains included artificial seawater containing 1x Guillard's (F/2) marine-

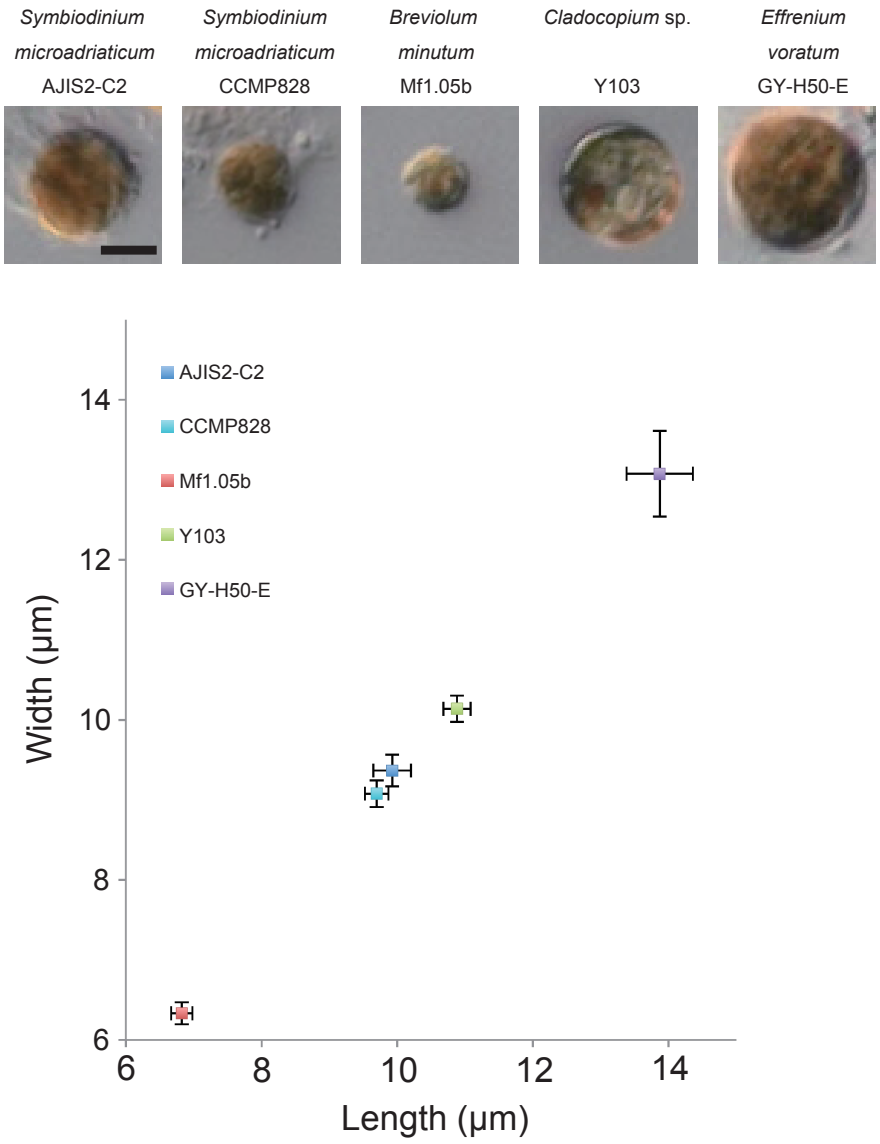

Fig. 2. Five strains of four species of the family Symbiodiniaceae used in this study. Morphology of immobile coccoid cells is shown above, and average cell sizes (length and width) are shown with bars representing standard errors. *Symbiodinium microadriaticum* AJIS2-C2, *S. microadriaticum* CCMP828, *Breviolum minutum* Mf1.05b, *Cladocopium* sp. Y103, and *Effrenium voratum* GY-H50-E. See Supplementary Table S1 for further information of dinoflagellate strains.

water enrichment solution (Sigma-Aldrich) with three antibiotics. Pre-cultures for infection experiments were maintained for more than 1 week without antibiotics.

Co-cultures involving *Acropora* larvae and Symbiodiniaceae were started at larval ages of 8.5 and 12.5 days. *Acropora* larvae were kindly provided in early June of 2019 by Mr. Shuichi Mekaru at Onna Fisheries Co., Onna, Okinawa. ~80 larvae were cultured with 40 mL of filtered seawater in a petri dish. Each Symbiodiniaceae strain was added to the dishes at ~600 cells/mL final density. Control petri dishes without cultured Symbiodiniaceae cells were prepared to confirm the absence of contamination. Co-culture dishes were put into an incubator CLE-305 (TOMY, Japan) set at 25°C and ~20 μmol/m^2/s (12 h light:12 h dark cycle) (Cumbo et al., 2013). After infection for 3 to 12 days, alga-infected larvae were detected by observation of 10 embryos under a Zeiss Axio Imager Z1 microscope (Zeiss, Germany). Observed larvae were trapped between a glass slide and a coverslip and were prevented from swimming. Counts of algal cells within larval bodies were performed by mildly crushing larvae with coverslips. The percentage of Symbiodiniaceae-infected larvae was calculated as described in Yamashita et al. (2018).

Observation of in vitro symbiosis

A 200–250-μL drop of culture medium containing dinoflagellates or latex beads was added to each well of a 24-well multiplate, which contained approximately 1 mL of medium for culturing cells of the IVB5 line. A 24-well multiplate that contained cultured coral cells and algae was put in a translucent, moist container and exposed to natural lighting at 20–22°C throughout culture and observation. Immediately after addition, interactions between the coral cells and dinoflagellates were observed using an inverted microscope (Olympus CKX41) equipped with a color digital camera (WRAYMER SR300) (Kawamura et al., 2021b). Photos obtained by consecutive observation with time-lapse video (2–3-s interval) was converted to a time-lapse video using iPhoto. Pictures and videos were also taken with an ordinary microscope (Nikon Eclipse 80i) equipped with a differential interference contrast (DIC) apparatus.

We also examined whether coral cells retained the capacity for inoculation with dinoflagellates after they were cultured for several days since the first phagocytosis. In order to distinguish dinoflagellates used for the first and second inoculations, dinoflagellates of the second inoculation were labelled with DiI. The labelling was carried out by immersing dinoflagellates in a solution that contained 0.02% DiI in sterile seawater (Setareh Biotech) for 10 min.

Non-ionic latex beads

This study also examined whether cultured coral cells engulf inorganic materials such as latex beads. Microspheres called latex beads or latex particles were purchased from Fisher Scientific (Catalog ID, N37464). They are spherical particles, 10 μm in diameter, in the colloidal size range and are formed from an amorphous polymer, such as polystyrene.

Immunocytochemistry

Approximately 5 × 10^6 FAC cell suspension was fixed in 2% paraformaldehyde for 15 minutes at room temperature. After quenching with 200 mM glycine in phosphate-buffered saline (PBS) for 5 minutes, the cell suspension was incubated for 30 minutes in the blocking solution containing 2.5% skim milk, 0.25% blocking reagent (Roche, Mannheim, Germany), 0.5% nonidet p-40, and 0.2% Triton X-100. Rabbit anti-AtSnail antibody (Kawamura et al., 2021a) was diluted 1000-fold with 0.1% Tween 20 in PBS (PBST). It was incubated with the cell suspension overnight at 4°C. Goat anti-rabbit IgG labeled with fluorescein isothiocyanate (FITC) (Vector Laboratory, Burlingame, CA, USA) was diluted 200-fold with PBST. Samples were stained with the secondary antibody for 0.5 h at

room temperature. They were observed under a confocal microscope (ECLIPSE C1si system; Nikon, Japan).

RESULTS

Coral cell symbiosis with different Symbiodiniaceae strains

Coral cells of dark, flattened morphology actively extended and retracted pseudopodia and showed elevated locomotor activity soon after algae were added to culture dishes (Kawamura et al., 2021b). Coral cells endocytosed symbiotic algae within 30 min, after which, algae were surrounded by host cytoplasmic membrane (Sekida et al., unpublished). Cultured cells that engulfed algal cells changed their morphology from a dark flattened form to a spherical 'fatty' form (Fig. 1C, D). Cells that engulfed algae as well as the not-engulfed algae showed positive signals of AtSnail antibody of *A. tenuis* in the nucleus, indicating that the cultured cells that engulfed algae originated from the coral (Fig. 1E1, E2). Bacteria-like microorganisms are often observed in vacuoles of coral cell cytoplasm (Sekida et al., unpublished), suggesting that coral cells endocytose micro-organic materials. Our previous study showed that approximately 45% of IVB5 cells incorporated *B. minutum* within 24 h after inoculation (Kawamura et al., 2021b). To determine whether coral cells engulf dinoflagellates of other genera or stains of Symbiodiniaceae, in vitro endocytosis was examined using four additional strains, *Symbiodinium microadriaticum* AJIS2-C2 and CCMP828, *Cladocopium* sp. Y103, and *Effrenium voratum* GY-H50-E (Fig. 2, and see Supplementary Table S1). In addition, as a control, we examined phagocytosis of these strains by intact planula larvae.

(a) Engulfment in vivo: Yamashita et al. (2014) reported that planula larvae of *A. tenuis* incorporated clade A (*Symbiodinium*) most effectively (60–100% of larvae), clade D (*Durusdinium*) less effectively (20–100%), and clade B (*Breviolum*) least effectively (10–40%), but larvae did not incorporate clade C (*Cladocopium*). We examined incorporation rates of different Symbiodiniaceae species at two larval stages, 8.5 and 12.5 days after fertilization, and two

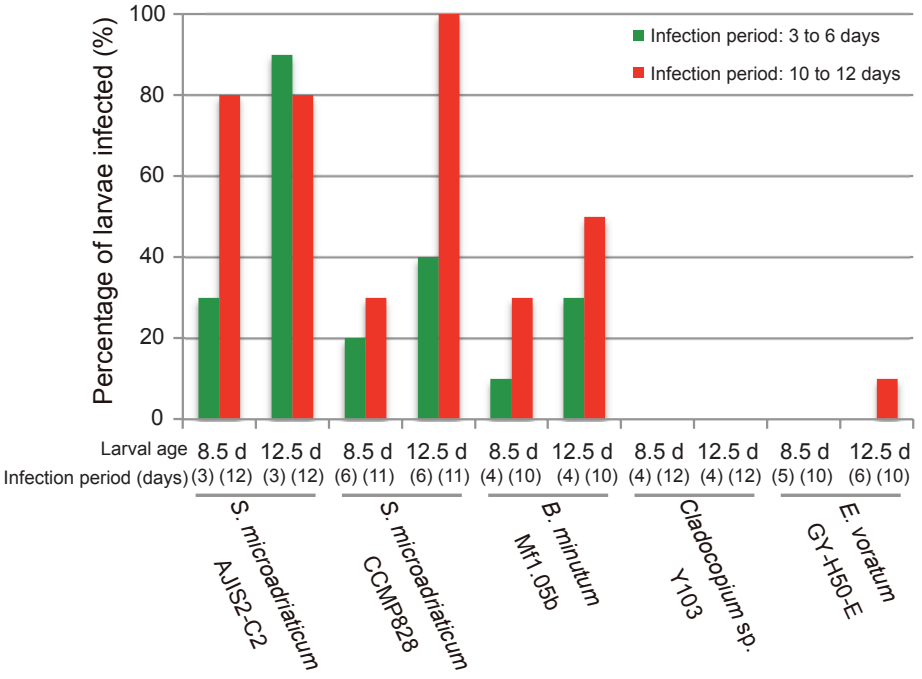

Fig. 3. Engulfment ratios when *Acropora tenuis* planula larvae were mixed with dinoflagellates in vivo. From left to right: *Symbiodinium microadriaticum* AJIS2-C2, *S. microadriaticum* CCMP828, *Breviolum minutum* Mf1.05b, *Cladocopium* sp. Y103, and *Effrenium voratum* GY-H50-E. Dinoflagellates were added to larvae 8.5 and 12.5 days after fertilization. Ratios of larvae that engulfed dinoflagellates were scored at 3–6 days (green bars) and 10–12 days (red bars) after mixing the two organisms.

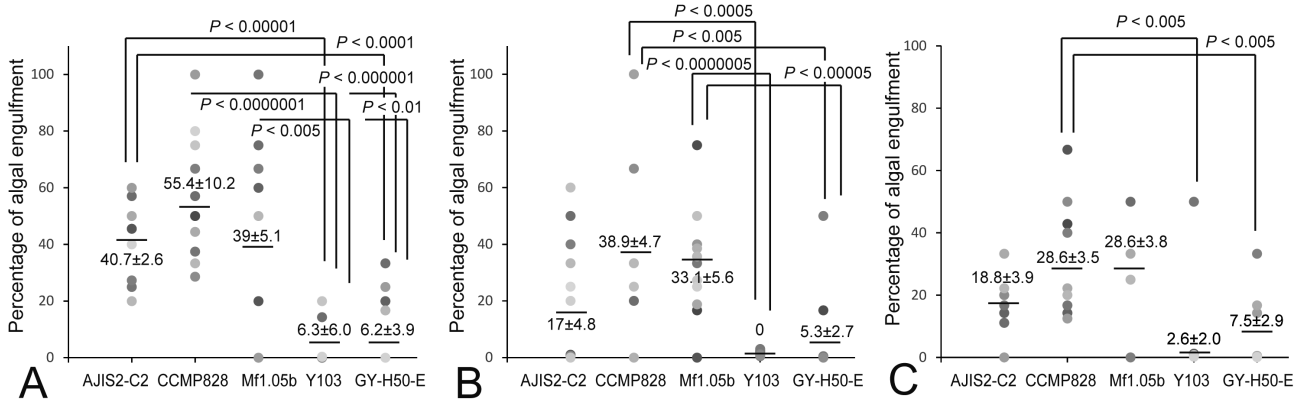

Fig. 4. Scatterplots showing the infection rate of cultured IVB5-line cells that incorporated five species of dinoflagellates in vivo. From left to right: *Symbiodinium microadriaticum* AJIS2-C2, *S. microadriaticum* CCMP828, *Breviolum minutum* Mf1.05b, *Cladocopium* sp. Y103, and *Effrenium voratum* GY-H50-E. **(A, B, C)** One day, 2 days, and 3 days after mixture of the two organisms. Results are expressed as the mean ± standard deviations of more than 10 measurements. *P*-values were calculated with a two-tailed Student's *t*-test.

observation periods, 3–6 and 10–12 days after addition of dinoflagellates (Fig. 3). Under our experimental conditions, *A. tenuis* planula larvae incorporated *Symbiodinium* AJIS2-C2 and CCMP828 and *Breviolum* Mf1.05b. Approximately 70%, 50%, and 30% of larvae incorporated *Symbiodinium* AJIS2-C2 and CCMP828, and *Breviolum*, respectively (Fig. 3). In contrast, *A. tenuis* planula larvae did not engulf *Cladocopium* sp. Y103 (Fig. 3). Larvae at 3–6 days were not infected with *Effrenium* GY-H50-E, but approximately 10% of 12.5-day larvae engulfed *Effrenium* (Fig. 3). In general, 12.5-day larvae exhibited higher ratios of incorporation than 8.5-day larvae (Fig. 3). Larvae incorporated more algae at longer periods of infection (10–12 days after infection) than at shorter periods (3–6 days) (Fig. 3). These results were comparable to those of Yamashita et al. (2014).

(b) Engulfment in vitro: Cultured IVB5-line cells incorporated all four clades of dinoflagellates, but the infection ratio differed significantly between *Symbiodinium*/*Breviolum* and *Cladocopium*/*Effrenium* (Fig. 4). Approximately 41%, 55%, and 40% of cultured cells endocytosed *Symbiodinium* AJIS2-C2 (Fig. 4) and CCMP828 (Figs. 4; 5A1, A2) and *Breviolum* (Figs. 4; 5B1, B2), respectively, within 1 day of mixture. CCMP828 always showed the highest rate of uptake among the five strains (Fig. 4). In contrast, the rates of *Cladocopium* and *Effrenium* incorporation were quite low; only 6% of cultured cells engulfed *Cladocopium* (Figs. 4; 5C1, C2) and *Effrenium* (Figs. 4; 5D1, D2).

In addition, a group constituted of *Symbiodinium*/*Breviolum* and another group of *Cladocopium*/*Effrenium* showed different alga-host cell interactions after incorporation. *Symbiodinium* and *Breviolum* provoked an immediate response of cultured cells, in which cultured cells engulfed these species as soon as 5 min after inoculation. One day later, algal uptake plateaued (Fig. 5A1, B1), and then gradually decreased (Figs. 4; 5A2, B2). For example, approximately 40% of coral cells engulfed *Breviolum* within 1 day and this symbiotic state continued for at least 1 week (Figs. 4, 5B). The symbiotic state, however, did not extend beyond 10 days, and most coral cells with *Breviolum* disappeared within 20 days. Similarly, *Symbiodinium* AJIS2-C2 disappeared from host cells after 4 weeks and CCMP828 after 5 weeks (Figs. 4, 5A2). Host coral cells gradually recov-

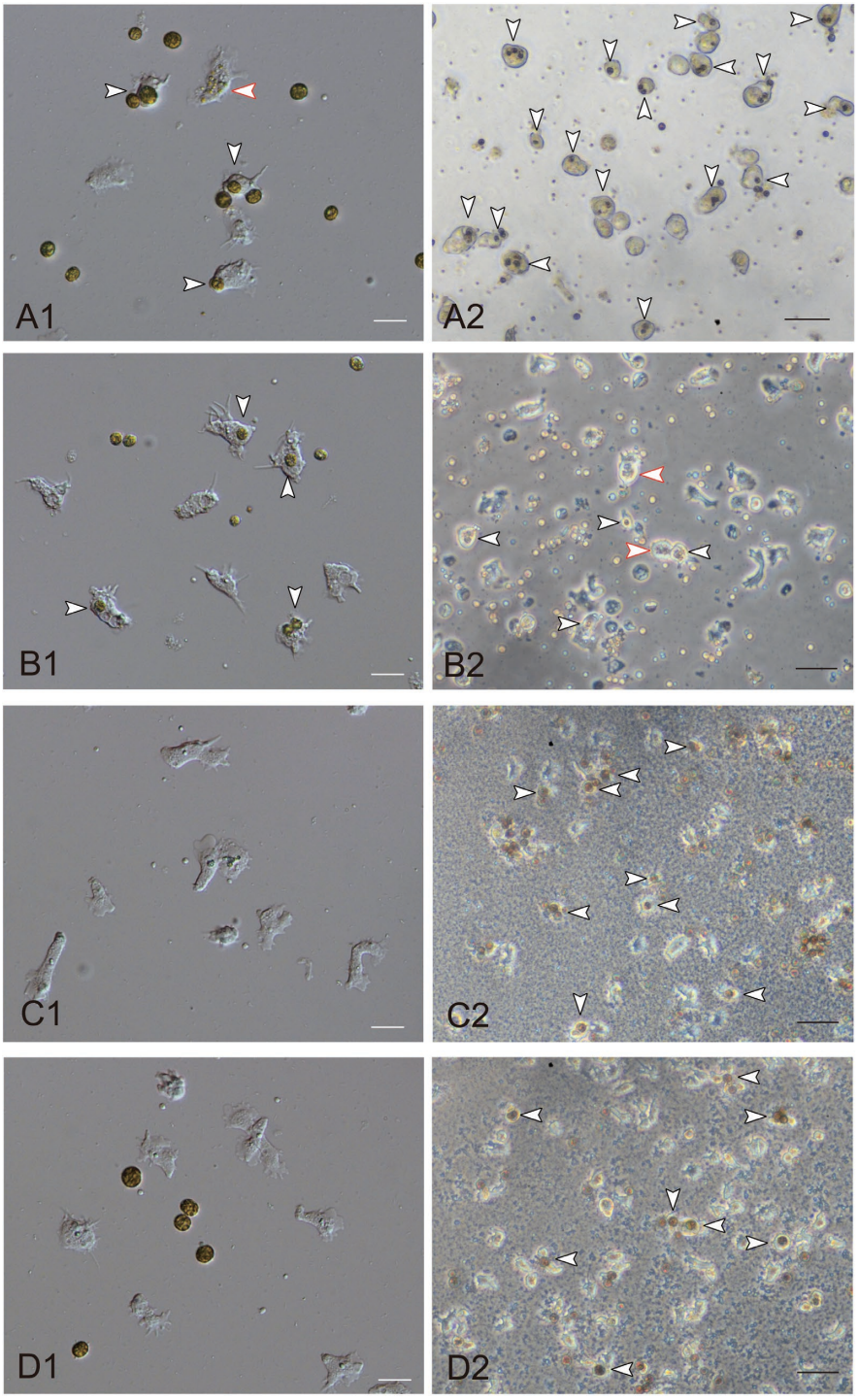

Fig. 5. Differential affinity between algal species and coral cells. **(A)** *Symbiodinium microadriaticum* CCMP828. **(B)** *Breviolum minutum* Mf1.05b. **(C)** *Cladocopium* sp. Y103. **(D)** *Effrenium voratum* GY-H50-E. White arrowheads show coral cells engulfing algae. Red arrowheads show fragmented algae in a coral cell body. **(A1–D1)** One day after inoculation. Nomarski differential interference contrast microscopy. Bars, 20 μm. **(A2, B2)** Three days after inoculation. Inverted phase contrast microscopy (IPC). Bars, 50 μm. **(C2, D2)** 48 days after inoculation, IPC. Bars, 50 μm.

ered the dark flattened morphology (Fig. 5A1). This is likely caused by host cell exocytosis or digestion of algal cells since fragments of dinoflagellates were found in the coral cell cytoplasm (Fig. 5A1, B2).

On the other hand, in the case of *Cladocopium* and *Effrenium*, incorporation occurred at a very low rate until the third day of infection (Fig. 4; Fig. 5C1, D1). Then, host cells with *Cladocopium* and *Effrenium* slightly increased in number until 2 weeks, and up to 50 % by 3 weeks of inoculation (data not shown). Algae were maintained by cultured cells at such an incorporation ratio for more than 7 weeks after inoculation (Fig. 5C2, D2). Host cells retained a spherical form (Fig. 5C2, D2).

(c) Capacity for algal engulfment of coral cells that once lost symbiotic dinoflagellates: As mentioned above, infection was maintained for 4 weeks for *Symbiodinium* and 2 weeks for *Breviolum*, and likely dissolved thereafter. We examined the capacity of cultured cells for phagocytosis after disappearance of algae from the first inoculation. Namely, additional algae were supplied to dark flattened cells 31 days after the primary inoculation of *Symbiodinium* CCPM828 (Fig. 6A2) and 51 days after the primary inoculation of *Breviolum* (Fig. 6B1). One day later, infection was re-established by host cells (Fig. 6A2, B2). Thus, phagocytosis by coral cells can occur if algae are available to host cells at least 1 month after the first inoculation.

We further examined whether in vitro cultured cells having algae in the cytoplasm continue to incorporate additional algae if they are available. Fresh algae were labeled with DiI (Fig. 6C1) and administered to cultured cells 5 days after the first administration of algae (Fig. 6C1). In a few hours, unlabeled and labeled algae were found in single cultured cells (Fig. 6C1). Three days after the secondary inoculation, most cultured cells still had DiI signals, and some cells had remnant DiI signals without algae (Fig. 6C2). These signals would have come from incorporated algae, suggesting algal digestion by coral cells or exocytosis of incorporated algae by coral cells.

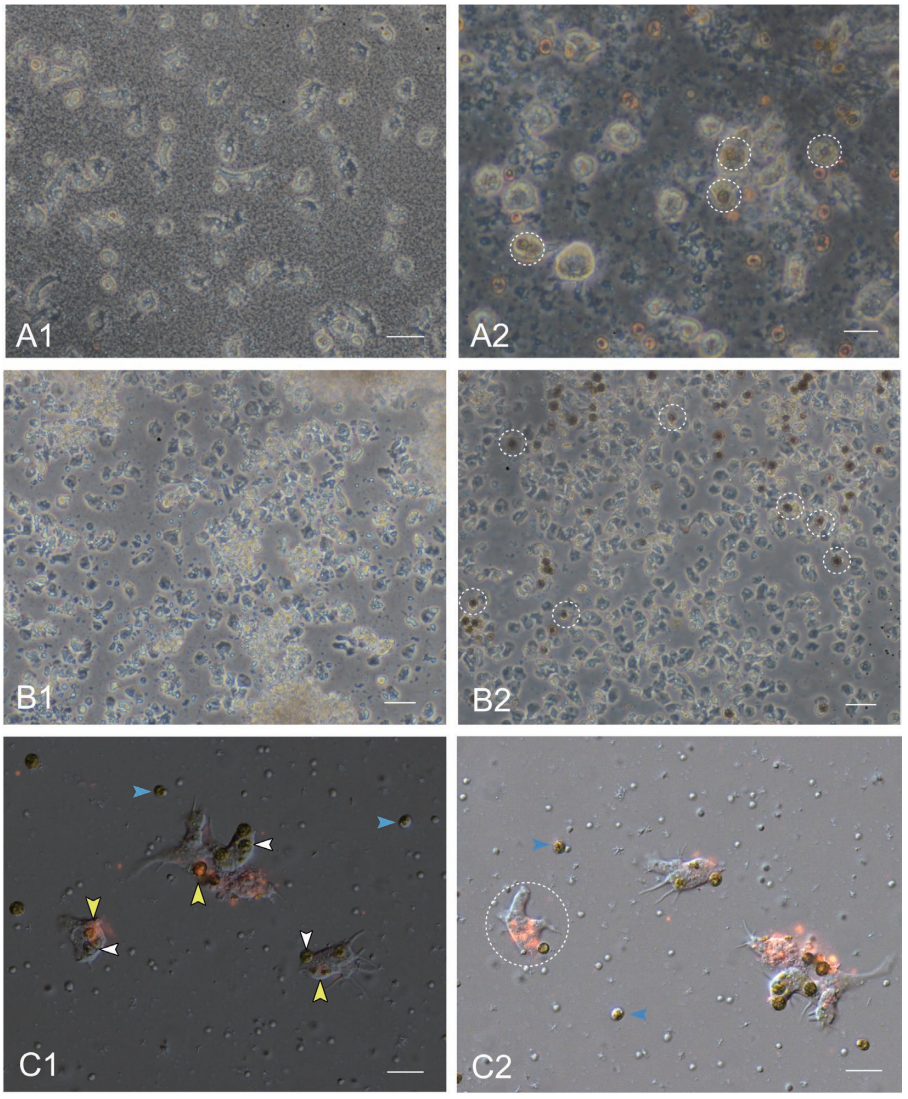

Fig. 6. Effect of additional algae applied to coral cells that lost dinoflagellates after 2 weeks in a symbiotic state. **(A)** *S. microadriaticum* CCMP828. **(B)** *B. minutum* Mf1.05b. **(A1)** 31 days after the primary inoculation of algae. Bar, 50 μm. **(A2)** One day after the secondary inoculation. Broken circles show coral cells engulfing algae. Bar, 20 μm. **(B1)** 51 days after the primary inoculation of algae. Bar, 50 μm. **(B2)** One day after the secondary inoculation. Bar, 50 μm. **(C)** Secondary engulfment of *S. microadriaticum* CCMP82 marked with DiI (yellow arrowhead). White arrowheads show dinoflagellates engulfed after the primary inoculation. Blue arrowheads show algae not incorporated. Bars, 20 μm. **(C1)** A few hours after inoculation. **(C2)** Three days after inoculation showing coral cells that secondarily engulfed algae. White broken circle shows a coral cell that exhibits remnant DiI fluorescent signals without algae.

In vitro endocytosis of coral cell with latex beads

Finally, we examined whether coral cells respond in vitro to inorganic materials such as latex beads and engulf them in the same way as they do symbiotic dinoflagellates. Since dinoflagellates range from 7 to 14 μm (Fig. 2), we selected latex beads of 10 μm and compared responses of coral cells toward beads with responses toward live dinoflagellates. Latex beads did not cause elevated locomotor activity of coral cells as seen in inoculation with *Symbiodinium* and *Breviolum*. In controls, 1 day after application, many cultured cells had engulfed *Symbiodinium* and *Breviolum* (Figs. 7A, 8A). On the other hand, latex beads were attached to surfaces of coral cells and in several cases, they were found in

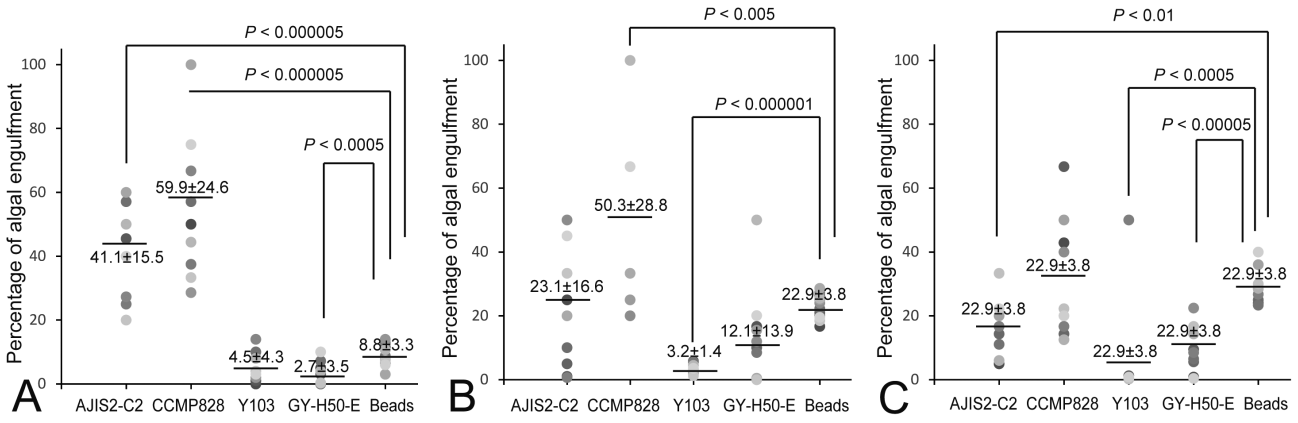

Fig. 7. Scatterplots showing the incorporation rate of latex beads in cultured IVB5 cells. As controls, *Symbiodinium microadriaticum* AJIS2-C2, *S. microadriaticum* CCMP828, *Cladocopium* sp. Y103, and *Effrenium voratum* GY-H50-E were used. **(A, B, C)** One day, 2 days, and 3 days after mixture of the two organisms. Results are expressed as the mean ± standard deviations of more than 10 measurements. *P*-values were calculated with a two-tailed Student's *t*-test.

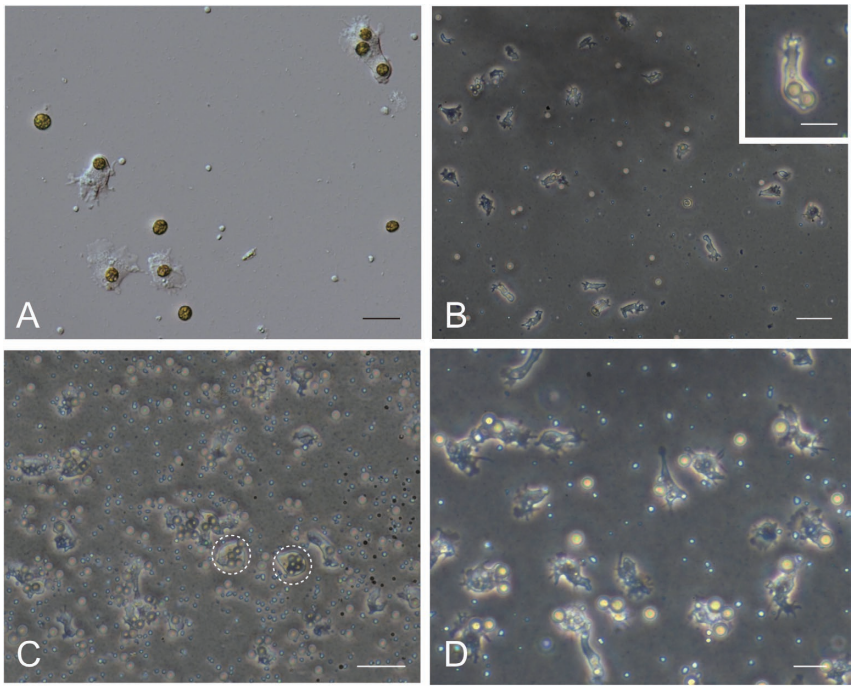

Fig. 8. In vitro coral cell phagocytosis of latex beads. **(A)** Control, 1 day after mixing IVB5 coral cells with algae. Bar, 20 μm. **(B)** Latex beads mixed with coral cells, 1 day after mixing. Approximately 10% of cells had endocytosed beads. Bar, 50 μm. (Inset) Enlargement of a cell that engulfed two beads. Bar, 20 μm. **(C)** Two days after coral cell-bead mixing. Broken circles show coral cells engulfing several beads. Bar, 50 μm. **(D)** Three days after coral cell-bead mixing. Most cells were dark and flattened, having pseudopodia for motility. This contrasts with endocytosis of dinoflagellates, in which coral hosts became spherical (see Fig. 1C, D). Bar, 50 μm.

cell bodies (Fig. 8B). Approximately 10% of cells engulfed beads within 1 day (Fig. 7A). The number of beads associated with single coral cells increased by 2 days of bead application (Figs. 7B, 8C); however, unlike algal uptake, the number of coral cells with incorporated beads did not increase significantly thereafter (Figs. 7C, 8D). In addition, coral cells that endocytosed beads remained dark and flattened and did not become spherical (Fig. 8D). It is highly likely that coral cells are able to endocytose inorganic beads in a different manner from endocytosis of symbiotic dinoflagellates.

DISCUSSION

Members of the family Symbiodiniaceae (Dinoflagellate) have been categorized into several clades or genera based on their morphological and genetic features and recently LaJeunesse et al. (2018) re-classified them into nine clades, A–I, using new data. However, Yorifuji et al. (2021) reported a new clade of Symbiodiniaceae, suggesting that the family is composed of 10 or more clades. Of these, *Symbiodinium* (Clade A), *Breviolum* (Clade B), *Cladocopium* (Clade C), and *Durusdinium* (Clade D), are major symbionts of scleractinian corals (LaJeunesse et al., 2018). Although the symbiotic specificity or preference between coral species and dinoflagellate species is complex, *A. tenuis* planula larvae preferentially incorporate *Symbiodinium*, while larvae phagocytose *Breviolum* and *Durusdinium*, but not *Cladocopium* (Yamashita et al., 2014, 2018). Under our experimental conditions, *A. tenuis* larvae preferentially incorporated *Symbiodinium* and *Breviolum*, but not *Cladocopium* (Fig. 3), and these results are comparable to those of previous studies (Yamashita et al., 2014). On the other hand, the symbiotic preference of *A. tenuis* with different clades of Symbiodiniaceae changes after metamorphosis to mature adults, since *A. tenuis* adults preferentially host *Cladocopium* (Shinzato et al., 2018; Satoh et al., 2021) and *Durusdinium* (Yuyama et al., 2018). Although this issue was beyond the scope of this study, it is an interesting research subject for future studies.

LaJeunesse et al. (2018) found that *Effrenium* (Clade E)

consists of one species, the exclusively free-living (non-symbiotic) *E. voratum.* Under our experimental conditions, 10% of larvae 10–12 days post fertilization phagocytosed *E. voratum*. After the mixture experiment, we checked the ITS2 sequence of this dinoflagellate and confirmed that it was *E. voratum*. However, it remains unknown whether this incorporation is followed by symbiosis, or is simple engulfment, followed by algal exocytosis.

Symbiotic preferences between corals and dinoflagellates have been discussed in relation to the size of dinoflagellates (Biquand et al., 2017). In *A. tenuis* – Symbiodiniaceae symbiosis, Biquand et al. (2017) compared symbiont infectivity among various *Symbiodinium* strains with a size range of 7.3 μm (small), 9.4 μm (medium), and 10.5 (large), and found that larger *Symbiodinium* are less likely to establish symbiotic relationships with host *A. tenuis*, suggesting that cell size might affect symbiosis rate. Cell size may not always be important in establishment of symbiosis, at least when compared among different species of Symbiodiniaceae. Dinoflagellates used in this experiment were approximately 9–10 μm for *S. microadriaticum*, 7 μm for *B. minutum*, 11 μm for *Cladocopium* sp., and 14 μm for *E. voratum* (Fig. 2). The incorporation rate was higher with *S. microadriaticum* (9–10 μm) than *B. minutum* (7 μm) (Fig. 2). Therefore, it is likely that partner recognition of corals and dinoflagellates is the primary factor enabling symbiosis, and that algal cell size may serve as a secondary factor among species of the same genus.

The main objective of this study was to examine what responses cultured cells of *A. tenuis* planula larvae show when mixed with different species of Symbiodiniaceae and different strains of *Symbiodinium*, although we could not examine *Durusdinium* (D), since stains of this clade were not available in the year of our in vitro experiment. Do clustered cells exhibit symbiont preferences in vitro similar to those of planula larvae in vivo? Results of this study suggest that three incorporation states or properties of coral cells depend on target subjects.

Differences were observed in endocytosis of dinoflagellates and latex beads by coral cells in culture. Incorporation of dinoflagellates by IVB5 cells is followed by morphological changes of host cells. Their shapes changed from a dark flattened form with locomotive activity to a spherical fatty form with less motility (Fig. 1). This change was not seen with latex beads (Fig. 8). This suggests that coral cells exhibit phagocytotic activity to various objects in the correct size range. This might be an intrinsic physiological property of coral cells. Moreover, coral cells recognize dinoflagellates as symbiosis targets, which does not occur with latex beads. This property requires biological communication or recognition between these taxonomically divergent organisms. We think that this finding might apply not only to cultured cells in vitro but also in vivo. Therefore, these results should be confirmed in vivo in the future.

The third property of coral cells in relation to symbiosis is deduced from differences in coral cell responses to *Symbiodinium*/*Breviolum* vs. *Cladocopium*/*Effrenium*. When *Symbiodinium*/*Breviolum* were added to culture medium, coral cells responded quickly and engulfed them in as little as 5 min after mixture. In contrast, the initial response of cultured cells against *Cladocopium*/*Effrenium* was quite slow. In addition, the infection rate with *Symbiodinium*/*Breviolum* was significantly higher than that with *Cladocopium*/*Effrenium* (Fig. 4). Behavior of coral cells with engulfed dinoflagellates also differed between the two groups. That is, the interaction of corals cells with engulfed algal cells was active with *Symbiodinium*/*Breviolum*, but not with *Cladocopium*/*Effrenium*. These differences may be caused by coral cell selection preferences against different species of dinoflagellates as targets of phagocytosis and/or symbiosis. In other words, additional mechanisms are required for larval cells to interact with *Symbiodinium*/*Breviolum*, which are not seen with *Cladocopium*/*Effrenium*.

However, these observations raise a question about recognition of *Symbiodinium* and *Breviolum*. Are shared mechanisms or molecules used for the two species, or different and specific mechanisms or molecules used for each of these two species? Coral–dinoflagellate symbiosis involves at least four interrelated processes: partner recognition, onset of symbiosis, maintenance and dynamic homeostasis, and dysbiosis. Cellular and molecular mechanisms involved in each process should be investigated (reviewed by Davy et al., 2012; Weis, 2019). Our question is associated with the first of the four. Identification of cellular and molecular mechanisms involved in these processes is challenging when intact symbionts are used as experimental systems (Wood-Charlson et al., 2006; Kuniya et al., 2015). As discussed above, this study identified three properties of coral cells in the first process of symbiosis: intrinsic phagocytotic activity in general, recognition of Symbiodiniaceae as phagocytotic targets, and preferential phagocytosis of specific dinoflagellates for symbiosis. Therefore, the IVB5-line serves as an in vitro system for studying cellular and molecular mechanisms involved in coral cell symbiosis with photosynthetic dinoflagellates.

ACKNOWLEDGMENTS

We thank Mr. Shunichi Mekaru for proving us *Acropora tenuis* larvae and Kanako Hisata for help in preparation of manuscript. Dr. Steven Aird is acknowledged for his help in editing of the manuscript. This work was funded by an OIST collaboration with Kochi University and in part by JSPS KAKENHI Grant number 22H03794 to KN, ES, KK, SF and NS.

COMPETING INTERESTS

The authors have no competing interests.

AUTHOR CONTRIBUTIONS

KK and NS conceived and designed study. KK, SS, SF, and NS carried out in vitro symbiosis observations. ES, HN, HZ, YS, PF, HY, and SS cultured zooxanthellae. ES, KN and HN performed in vivo incorporation of coral larvae. KK and NS prepared the manuscript and all authors commented on it.

DATA AVAILABILITY

Sequences for ITS2 and 28S rRNA of the *E. voratum* GY-H50-E are available at DDBJ/GenBank/EMBL under accession numbers LC639190 and LC639191, respectively.

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(Received May 29, 2023 / Accepted September 14, 2023 / Published online November 29, 2023)