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Uptake of high molecular weight dextran by the dinoflagellate *Alexandrium catenella*

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ABSTRACT: Carbohydrate macromolecules (dextrans) labeled with fluorescein isothiocyanate (FITC) were taken up by the dinoflagellate *Alexandrium catenella* at a substrate concentration of 5 mg C l^{-1}. The FITC-labeled dextrans appeared in what resembled food vacuoles inside the dinoflagellate cells. Between 5 and 50% of the cells contained fluorescent green vacuoles. *A. catenella* took up dextrans of high molecular weight (2000 kDa) but did not show significant uptake of lower molecular weight (20 kDa) dextrans. The uptake of the 2000 kDa dextran was higher with addition of humic substances to the growth medium and did not seem related to the presence of bacteria. Phagocytosis of fluorescent microspheres (0.36 µm) by *A. catenella* was also investigated. Although aggregation of fluorescent microspheres was observed in the sulcal region of the cells, no evidence was found of phagocytosis of bacterial-size prey by *A. catenella*. These observations show that *A. catenella* has the capacity to take up high molecular weight organic molecules, perhaps by pinocytosis.

KEY WORDS: *Alexandrium catenella* · Dextran · Mixotrophy · Humic substances · Dinoflagellate · Epifluorescence

INTRODUCTION

Many phytoplankton species, including dinoflagellates, are capable of gaining energy through both endocytosis and photosynthesis (Boraas et al. 1988). These species are called mixotrophic. Endocytosis includes 2 major processes: phagocytosis (engulfing of particulate material by a cell) and pinocytosis (uptake of dissolved substances from the medium outside the cell). These processes enable some algal species to obtain energy, macronutrients or vitamins (Gaines & Elbrächter 1987). Phagocytosis by marine and freshwater phytoflagellates is well documented (e.g. Sanders & Porter 1988), and has been shown to occur in various light and nutrient conditions but not necessarily concomitant with photosynthesis. The importance of phagocytosis for growth of mixotrophic phytoplankton is highly variable. Phagocytosis can contribute approximately 3 to 60% of carbon compared to photosynthesis (Bird & Kalff 1987).

Direct utilization of low molecular weight organic compounds such as urea and amino acids in order to obtain nitrogen has been demonstrated for many phytoplankton species (Flynn & Butler 1986, Antia et al. 1991). However, dissolved high molecular weight organic compounds (>1 kDa), such as polypeptides, polysaccharides, and humic substances, are too large to be directly taken up through the cytoplasmic membrane. Instead, ingestion of these molecules must involve a transport mechanism in order for the molecules to pass the cell membrane. Investigations on the utilization of high molecular weight compounds by photosynthetic phytoplankton have involved the characterization of cellular components and their cytological transformations (Klut et al. 1987, 1988). *Amphidinium carterae*, *Prorocentrum micans* and *Dunaliella tertiolecta* have been shown to incorporate lectins and horseradish peroxidase (Klut et al. 1987, 1988). These large organic molecules are presumably taken up by pinocytosis via the pusule system of these flagellates (Klut et al. 1987). The marine chrysophyte *Ochromonas* sp. was also reported to take up a FITC-labeled (fluorescein isothiocyanate labeled) polymer of glucose

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(Cucci et al. 1989). However, the importance of pinocytosis in phytoplankton nutrition still remains unclear.

This paper describes possible mixotrophic behaviour by *Alexandrium catenella*. We investigated (1) phagocytosis, using fluorescent latex beads, and (2) pinocytosis by using fluorescently labeled dextrans. The results show an uptake of dextrans by *A. catenella*, but no uptake of latex beads, indicating that this species has the ability to assimilate dissolved organic macromolecules but not bacteria-sized particles.

MATERIALS AND METHODS

An axenic strain of *Alexandrium catenella* (CCMP 1598) was used in culture experiments to determine ingestion of monodisperse 0.36 µm fluorescent microspheres and uptake of macromolecular polysaccharides (dextrans) labeled with FITC.

Alexandrium catenella was maintained in inorganic (f/10) (Guillard & Ryther 1962) and organic media at 16°C, at a light intensity of 80 µmol m^{-2} s^{-1} (measured with a flat Li-Cor sensor) and a 12 h dark : 12 h light cycle. Organic medium was prepared by enrichment of autoclaved filtered (0.2 µm) seawater with riverine-isolated humic substances (HS) (Carlsson et al. 1993). The addition of HS to the seawater increased the DOC concentration from 1.3 mg C l^{-1} to 10.2 mg C l^{-1} (Carlsson et al. 1998, this issue). Two sets of cultures maintained in these 2 media were enriched with a natural marine bacteria community (Whatman GF/F filtrate) giving a final concentration of 2 to 2.5 × 10^6 bact. ml^{-1}.

Phagocytosis. For studying phagocytosis in *Alexandrium catenella* in inorganic and organic media, triplicate 50 ml bacteria free cultures were incubated in 100 ml teflon flasks in light with fluorescent microspheres (0.36 µm, Molecular Probes, final concentration 1 × 10^7 ml^{-1}). Incubations lasted for 8 h with samples taken at 0, 1, 5 and 8 h. Control samples were fixed with formaldehyde (1% final concentration) before adding the microspheres, but were otherwise treated in the same way as test samples. For detection of fluorescent microspheres in *A. catenella*, samples (5 to 10 ml) were fixed by addition of 50 to 100 µl formaldehyde. Samples were then centrifuged for 10 min at 1200 rpm (220 × *g*). After removing the supernatant, 20 µl from the pellet containing the cells was mounted under a cover slip and the sample was sealed with transparent nail polish.

Uptake of dextrans. Uptake of dextrans by *Alexandrium catenella* grown in axenic or bacteria-enriched inorganic/organic media was studied by incubating 20 ml cultures in triplicate in 25 ml scintillation vials with FITC-labeled dextrans (MW 2000 and 20 kDa) (Sigma). FITC-labeled dextrans were dissolved in autoclaved sterile filtered Milli-Q water and filtered (0.2 µm) before use. Dextrans were added separately at a concentration of 5 mg C l^{-1}. Incubations lasted for 24 h with samples taken at 0, 5, 9 and 24 h. Control samples were fixed with formaldehyde (1% final concentration) before adding the FITC-labeled dextrans. These controls were incubated, sampled and fixed as described above.

To observe uptake of FITC-labeled dextrans by the cells, samples were centrifuged and after removing the supernatant, cells were resuspended in 1.5 ml alkaline (pH = 9 to 10) phosphate buffer saline solution (PBS), since FITC fluoresces most intensely at alkaline pH (Sherr et al. 1993). The cell-PBS mixture was then transferred to 2 ml plastic test tubes and centrifuged for 10 min (2000 rpm; 620 × *g*). Approximately 1 ml of the supernatant was withdrawn carefully with a pipette and the remaining cells were vortexed. A drop (20 µl) of the cell-PBS mixture was mounted as described for the fluorescent microspheres.

Samples were observed using an Olympus BX 50 microscope equipped for epifluorescence [filter sets for UV and blue light (BL) excitation: UV, excitation 360 to 370 nm, barrier filter 420 nm and dichroic mirror 400 nm; BL, excitation 470 to 490 nm, barrier filter 515 nm and dichroic mirror 500 nm]. Axenity of the cultures and bacterial abundance in the treatments with bacteria were checked by staining 2 ml subsamples of the cultures with the DNA-specific fluorochrome DAPI (20 µg ml^{-1} final concentration), filtering the bacteria onto 0.2 µm black polycarbonate filters (Poretics Inc.), and inspecting the samples with UV-light excitation (Porter & Feig 1980).

Dinoflagellates were observed with BL excitation to determine the presence or absence of fluorescent microspheres or yellow-green FITC fluorescence inside the cells. At least 50 to 100 cells were examined in each sample. Since the FITC fluorescence faded after about 20 to 30 s of illumination, the cells were first located on the slide with transmitted white light and then illuminated with BL. The cells were also observed in white transmitted light to check if the theca was damaged. FITC uptake was calculated as the percentage of *Alexandrium catenella* cells with ingested fluorochrome.

RESULTS AND DISCUSSION

Phagocytosis of microspheres in *Alexandrium catenella*

No uptake of fluorescent microspheres by *Alexandrium catenella* was observed in any of the treatments. However, microspheres were found to aggregate in

the sulcus region of the cell or in the vicinity of the sulcus. This has been observed before for *Alexandrium tamarense* and *Alexandrium fundyense* when cells were incubated with a variety of live (5-chloromethyl fluorescein diacetate, CMFDA-labeled) or dead [4',5-(4,6-dichlorotriazine-2-yl) aminofluorescein, DTAF-labeled] bacteria/microflagellates (C. Legrand & D. M. Anderson unpubl. data). Fixation with formaldehyde often causes the egestion of recently ingested particles in flagellates (Sieracki et al. 1987). However, the aggregation of fluorescent microspheres near the sulcus was also observed in live *A. catenella* cells. This suggests that we can probably exclude particle egestion by *A. catenella* as a result of the addition of formaldehyde in the samples.

In the non-living control cultures, there was no accumulation of microspheres in the sulcal region. This suggests that the aggregation of particles in a specific area of the algal cell involves active participation of the cell and is not just a physical process.

The presence of bacteria inside *Alexandrium* sp. cells has been previously reported (Doucette et al.

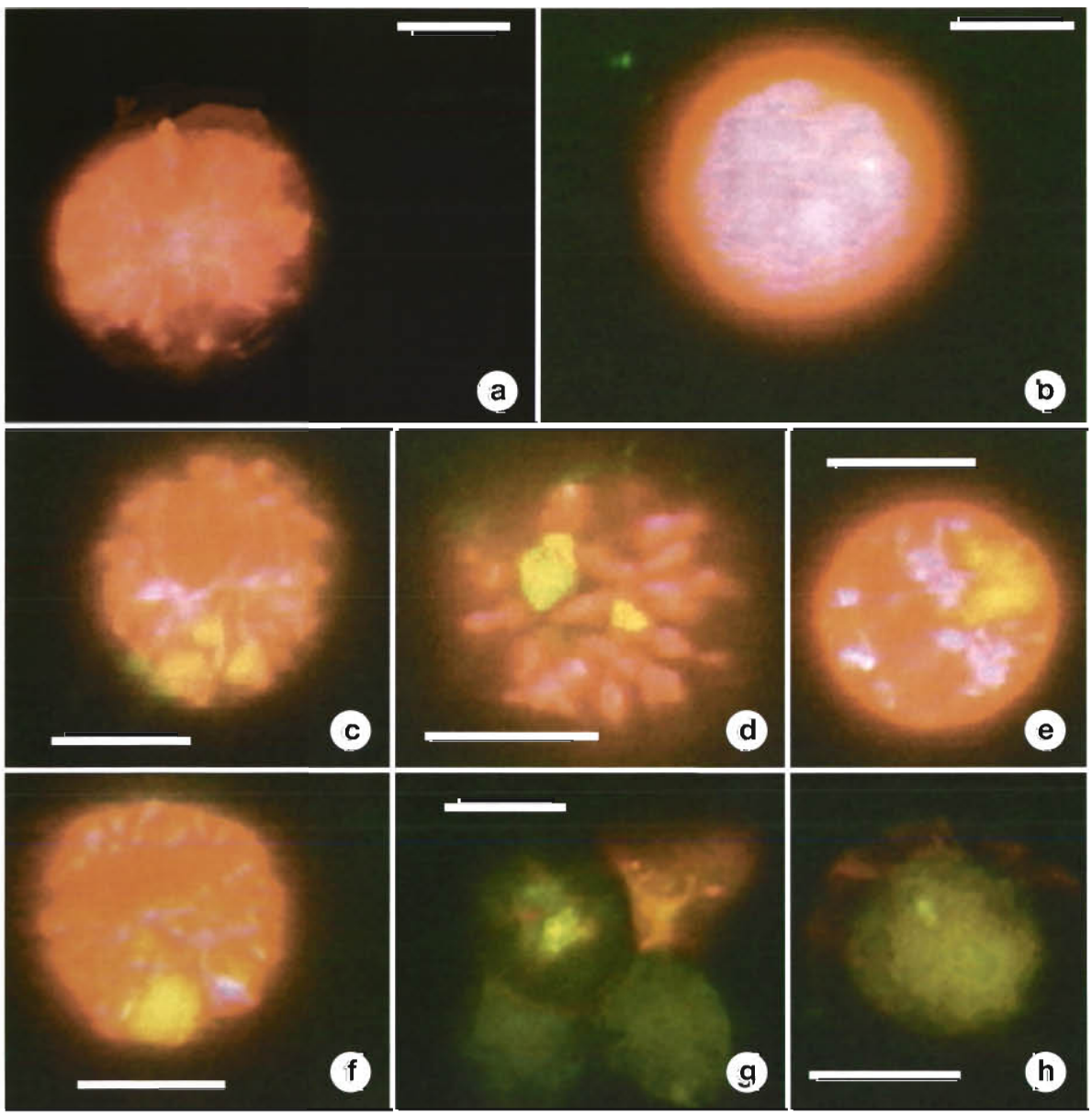

Fig. 1. Epifluorescent micrographs of (a, b) *Alexandrium catenella* cells from the non-living control, (c–f) *A. catenella* cells with ingested FITC-labeled dextrans, (g, h) *A. catenella* cells with a damaged thecas and possible passive diffusion of FITC-labeled dextrans into the cells. Scale bars = 10 µm

1998). However, there is no evidence of phagocytosis of bacteria by *Alexandrium* species, despite several attempts to demonstrate this (D. M. Anderson pers. comm., R. V. M. Jovine pers. comm., C. Legrand unpubl. data). Several studies suggest that bacteria-size prey (e.g. cyanobacteria) are too small to be ingested directly by mixotrophic dinoflagellates (Hansen et al. 1994, Hansen & Nielsen 1997, Legrand et al. 1998). However, by preying on bacterivorous organisms, dinoflagellates could indirectly ingest bacteria (Bockstahler & Coats 1993), as has been demonstrated for predacious ciliates ingesting smaller ciliates (Dolan & Coats 1991). Ciliates and other dinoflagellate prey have also been observed in *Alexandrium ostenfeldii* (Jacobson & Anderson 1996).

Pinocytosis of FITC-labeled dextrans

In all samples where FITC-labeled dextrans were added, a bright background fluorescence was observed. However, this did not interfere with the observations of green vacuoles inside *Alexandrium catenella* cells. Extracellular microaggregates of FITC-dextrans were also observed, suggesting that a physical aggregation of dextrans took place during the incubation. The aggregates were larger when 2000 kDa dextrans were added compared to 20 kDa.

Killed cells from the control cultures fluoresced bright red (Fig. 1a, b) and no green fluorescence was observed inside the majority of these cells. However, a small proportion of cells (<5%) showed traces of green fluorescence inside the cells and were thus counted (Fig. 2). FITC-dextrans taken up by the cells appeared in intracellular green vacuoles and were easily distinguished from the red chlorophyll autofluorescence (Fig. 1c–f). A few of the cells with green vacuoles had a damaged theca, and these were not counted as cells with ingested FITC-dextrans since the uptake of FITC might have been a passive reaction (Fig. 1g, h). Since we do not know if the uptake in these cells was passive, caused by a damaged theca, or if it was caused by an active uptake, the proportion of cells with green vacuoles formed after a active uptake of dextran might have been underestimated by a maximum of 5 to 10%.

Approximately 5% of all *Alexandrium catenella* cells (living or fixed) incubated with 20 kDa FITC-dextrans were observed with green vacuoles (Fig. 2). The lack of significant differences between the non-living controls and the living cells suggests that *A. catenella* is not capable of ingesting the 20 kDa FITC-dextrans. Between 35 and 40% of the *A. catenella* cells maintained in the organic media (+HS) were observed with food vacuoles. Uptake of the high molecular weight (2000 kDa) FITC-dextrans was the same in cultures

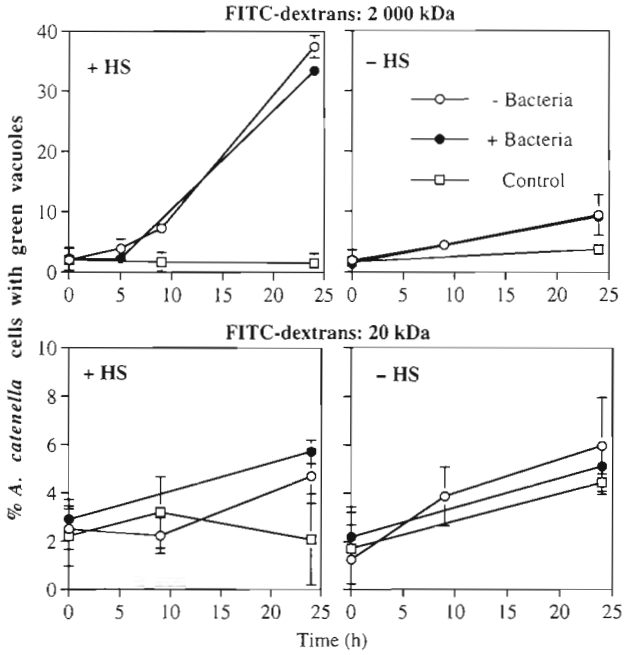

Fig. 2. Percentages (± SD, n = 3) of *Alexandrium catenella* cells that took up FITC-labeled dextrans (5 mg C l^{-1}) during a 24 h incubation period in light. *A. catenella* was maintained in media with (+HS) or without (–HS) additions of humic substances, with (+bacteria) or without (–bacteria) addition of marine bacteria. Controls were killed with formaldehyde before the addition of FITC-labeled dextrans

with or without bacteria (Fig. 2). In the treatments where *A. catenella* was grown with no addition of HS, only 10% of the cells had green vacuoles.

The mixotrophic marine chrysophyte *Ochromonas* sp. has been shown to incorporate 40 kDa FITC-dextrans in food vacuoles by pinocytosis (Cucci et al. 1989). The proportion of *Ochromonas* sp. cells which pinocytized FITC-dextrans measured by flow cytometry ranged from 12 to 50% over a 75 min incubation period. Although the percentages of *Ochromonas* sp. and *Alexandrium catenella* cells ingesting FITC-dextrans are comparable, *Ochromonas* sp. cells have faster uptake rates (50% after 45 min compared to 24 h in *A. catenella*). This may be because some ochromonadales do not have high photosynthetic capacities (Sanders et al. 1990) in comparison with *A. catenella*. Pinocytosis of extracellular organic molecules may thus play a minor role in *A. catenella* normal nutrition.

There was no difference in the percentage of *Alexandrium catenella* with green vacuoles in the presence or absence of bacteria (Fig. 2). The proportion of cells with green vacuoles in all control cultures was <5%. These results suggest that the presence of bacteria had no influence on the utilization of macromolecules by *A. catenella*, indicating that this process is not dependent on the bacteria-phytoplankton inter-

actions. Other studies with heterotrophic flagellates demonstrated an association between the ingestion of FITC-labeled macromolecules of various molecular weight (50 to 2000 kDa) and ingested particles (Sherr 1988, Tranvik et al. 1993). In these experiments, fluorescence was always observed in the same area of the cell in which ingested bacteria or fluorescent microspheres were found, suggesting a phagotrophic uptake of the macromolecules (Tranvik et al. 1993). However, our results provide no evidence for such an association since *A. catenella* did not show any uptake of fluorescent microspheres.

Previous studies with dinoflagellates, chlorophytes and euglenoids reported pinocytosis to be the mechanism of ingestion of high molecular weight organic molecules (Kivic & Vesk 1974, Klut et al. 1987). Algal cells undergoing pinocytosis expand a small vesicle from the plasma membrane and engulf the macromolecules that later accumulate in vacuoles. The ingestion of 2000 kDa FITC-dextrans by *Alexandrium catenella* in our experiment appears to be related to the presence of humic substances in the media. The enhancement of pinocytosis may be the result of an adaptation process to the organic medium since the cells were maintained in these conditions for 7 to 8 d before the addition of dextrans (Carlsson et al. 1998, this issue).

In *Prorocentrum* spp., certain specific cellular components have been described as accumulation or PAS (Periodic Acid Shiff) bodies (Zhou & Fritz 1993). These PAS bodies occur in the cytoplasm and have properties characteristic of eukaryotic lysosomes (Zhou & Fritz 1994). Under nutrient starvation, similar bodies with yellow autofluorescence can be numerous in *Alexandrium catenella*, *A. fundyense*, and *A. tamarense* cells (C. Legrand pers. obs.). These PAS bodies resemble the shape of the green vacuoles in *A. catenella*. Whether or not PAS bodies have a similar function to food vacuoles is still an open question. Jacobson & Anderson (1996) hypothesized that inclusions with yellow autofluorescence in *A. ostenfeldii* may be digested remains of prey which would supply nutrient storage prior encystment.

This study provides evidence that *Alexandrium catenella* is able to take up high molecular weight (2000 kDa) FITC-dextrans and accumulate these macromolecules in vacuoles, perhaps using pinocytosis. Interestingly, the uptake of dextran was triggered by the presence of humic substances and independent of bacterial presence. However, the significance of this utilization of dissolved organic matter in *A. catenella* nutrition, especially in the natural environment, remains unknown.

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