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Trying to cultivation of *Dinophysis caudata* (Dinophyceae) and the appearance of small cells

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Abstract: *Dinophysis* species have been reported to be involved in DSP incidents but the biology of these mixotrophic dinoflagellates is poorly understood due to unsuccessful attempts at cultivation. In this report, we attempted the cultivation of *D. caudata* obtained from Ogamae Bay (Oita prefecture, Japan) with certain kinds of organisms as food. The organisms were three phytoplankton species (*Thalassiosira* sp., *Chroomonas* sp. and an eukaryotic picophytoplankton), and these cells were crushed by ultrasonic treatment and frozen (−30°C). As a result, the addition of food was apparently effective for *D. caudata* growth. *D. caudata* continued to grow by binary fission until 22 days after the inoculation and increases in cell numbers were especially seen after the food was added. The largest number of *D. caudata* cells obtained from a single cell was 28, and we could keep them alive for at least 2 months. In the succeeding scale-up cultivation, all *D. caudata* cells were transferred from a 1-ml culture in the well of a microplate to a 10-ml culture medium in an Erlenmeyer flask. In one case, *D. caudata* cells were filled with dense plasmatic contents in spite of 46 days of incubation, and the size and shape were the same as for normal natural cells. In the other case of flask cultivation, small cells of *D. caudata* (*D. diegensis*-like cells and *D. diegensis*-like small cells) appeared, and one of them had two trailing flagella. The appearance of these small cells in the culture of *D. caudata* clearly indicates that they are each form of *D. caudata* and cannot be considered separate species.

Key words: *Dinophysis caudata*, diarrhetic shellfish poisoning, food organisms, mixotrophy, *Dinophysis diegensis*-like cell

Introduction

The dinoflagellate genus *Dinophysis* Ehrenberg includes several species which cause diarrhetic shellfish poisoning (DSP). Species of *Dinophysis* occur in different tropical and temperate marine waters (Hallegraeff & Lucas 1988; Hallegraeff 1993). Much of what is known of the ecology, biology and toxicology of *Dinophysis* comes from studies on natural populations because it is not yet possible to culture any of the species of *Dinophysis* for extended periods in the laboratory (Ishimaru et al. 1988; Sampayo 1993; Maestrini et al. 1995; Subba Rao 1995; Delgado et al. 1996; Uchida et al. 1999; Reguera & González-Gil 2001).

There has been no study that tried to cultivate *D. caudata* Saville-Kent despite that this is a species responsible for DSP. *D. caudata* is a photosynthetic species and contains chloroplasts. Nishitani et al. (2002) found food vacuoles in *D. caudata* cells, suggesting that a phagotrophic feeding mechanism exists. Also in other species, Jacobson & Andersen (1994) found food vacuoles in photosynthetic species of *D. acuminata* Claparède et Lachmann and *D. norvegica* Ehrenberg. Based on these findings, it has generally been recognized in recent years that mixotrophy is a common nutritional mode in the genus *Dinophysis*, implying that they ingest food organisms. Heterotrophy presumably functions as a survival strategy during the time when photosynthesis cannot support continuing growth in mixotrophic species.

Based on our investigations for several years, we found that picophytoplankton cells often attached to the cell surface of *Dinophysis* spp. (Imai & Nishitani 2000), and empirically noticed that the addition of food organisms, espe-

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cially pico-sized phytoplankton, was effective for cultivation of *Dinophysis*. Here we report (1) cultivation results of *D. caudata* obtained by addition of certain kind of foods, and (2) that small cells of *D. caudata* appeared during laboratory cultivation experiments.

Materials and Methods

A water sample was collected from the surface (0 m) of Ogamae Bay (32°47′N, 131°55′E), Oita Prefecture, Kyushu, Japan, on January 16, 2002, and transported to the laboratory in one day. After cell concentration by the use of a plankton net (mesh size $\phi 10\,\mu$m), *D. caudata* cells were individually isolated with microcapillary pipettes under an inverted microscope (Nikon TE300). Each cell was washed two to three times with seawater filtered through a 0.1-μm pore Nuclepore filter and finally inoculated into a microplate well (Corning, 48 well) containing 1 ml of culture medium. We used the modified SWM-3 culture medium (Chen et al. 1969; Imai et al. 1996) and totally diluted it to 1/50 with filtered seawater (0.1 μm) from the station where the water sample was collected. All cultures were started from a single *D. caudata* cell per well with 1 ml of medium and incubated at a temperature of 25°C under irradiance of 45 μmol photons $m^{-2}\,sec^{-1}$ with a 14 h light 10 h dark photocycle. The microalgae used as food organisms were *Thalassiosira* sp. (valve diameter: 5–7 μm), *Chroomonas* sp. (length: 6–8 μm) and an eukaryotic picophytoplankton (diameter: 1.5–2.5 μm) (Fig. 1A–C). *Thalassiosira* sp. was isolated from Maizuru Bay in 1998, *Chroomonas* sp. from an intertidal flat of Hyogo Prefecture in 2001, and the eukaryotic picophytoplankton from Hiroshima Bay in 2001. These strains are not axenic. Food concentrations were 60000 cells in *Thalassiosira* sp., 4000 cells in *Chroomonas* sp. and 12000 cells in the picophytoplankton per well, respectively, per addition. These three phytoplankton species are good as food for *Dinophysis* given the results of our culture experiments for *D. acuminata* and *D. fortii* Pavillard for several years (Nishitani et al. unpublished data). Cells of food organisms grown in the modified SWM-3 medium were centrifuged, washed, and suspended in filtered seawater, and then some food organisms were crushed with ultrasonic treatment and freezing (−30°C) before their addition to *Dinophysis* cells. In the two cases with the best results, 24 days after the inoculation, all *D. caudata* cells were transferred from a well to an Erlenmeyer flask (containing 10 ml of new culture medium with new food organisms) for the trial of further cultivation of *D. caudata*.

Results

In the experiment, eight *D. caudata* cells were isolated from the sample seawater. Figure 2 shows the results of microplate culture experiments with crushed and frozen food (Fig. 2-A, B, C), live food (Fig. 2-D) and no food (Fig. 2-E). In Fig. 2-A and B, the largest number of *D. caudata* cells obtained from a single cell was 28 after incubation for 22 days. This value is equivalent to a growth rate of 0.22 divisions day^{-1}. In the case of Fig. 2-A, eukaryotic picophytoplankton, *Thalassiosira* sp. and *Chroomonas* sp. were added to *D. caudata* cells. In the case of Fig. 2-B, only eukaryotic picophytoplankton was added. In the experiments of Fig. 2-A and B, *D. caudata* cells were actively swimming in the wells, and morphological changes (deformation, dimorphism, couplets, putative cysts) were not observed. In the case of Fig. 2-C, *D. caudata* grew to 7 cells with the addition of only *Thalassiosira* sp., and grew to 25 cells with the simultaneous addition of *Thalassiosira* sp. and *Chroomonas* sp. In the case of Fig. 2-D, live food, the eukaryotic picophytoplankton cells used in Fig. 2-A, were added and *D. caudata* grew slowly in the microplate well. At the late phase of cultivation in Fig. 2-D, the growth of *D. caudata* seem to be inhibited by the over growing of food organisms (picophytoplankton). In the case of Fig. 2-E with no addition of food (control), *D. caudata* grew with at most

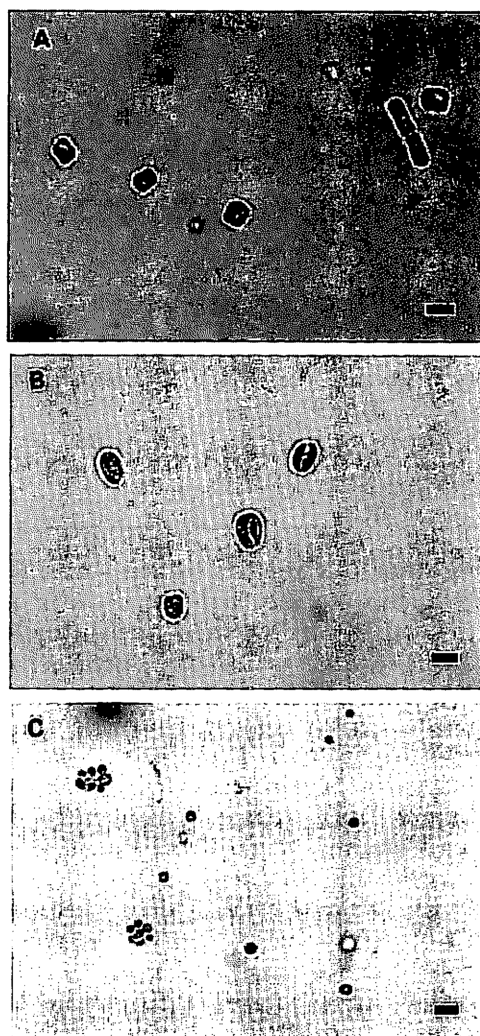

Fig. 1. Light micrographs of food organisms. A: *Thalassiosira* sp. B: *Chroomonas* sp. C: eukaryotic picophytoplankton. All scale bars represent 5 μm.

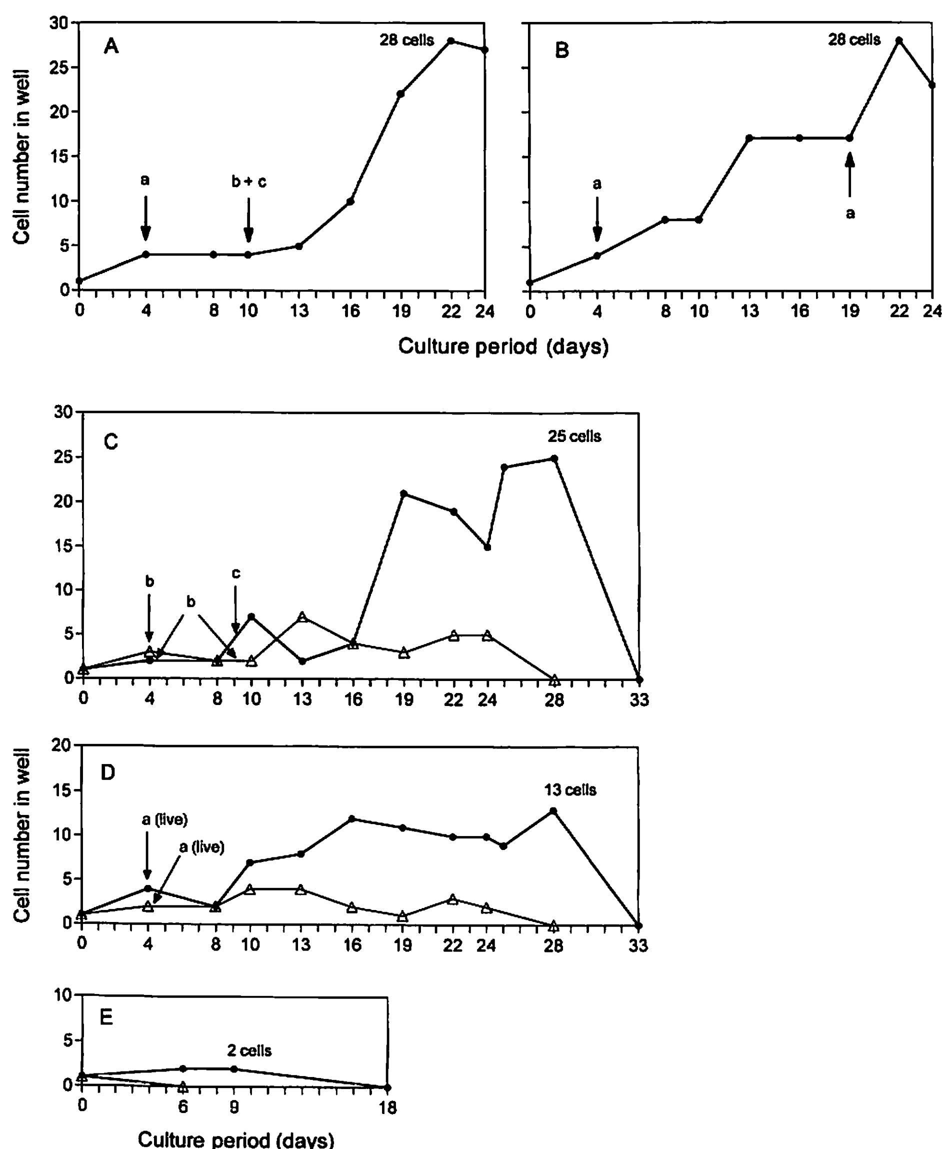

Fig. 2. Growth of *D. caudata* from a single cell in a microplate well with (A–D) and without (E) the food organisms. A: supply of eukaryotic picophytoplankton crushed and frozen at −30°C, B: supply of *Thalassiosira* sp. crushed and frozen at −30°C, C: supply of *Chroomonas* sp. crushed and frozen at −30°C, D: supply of eukaryotic picophytoplankton without treatment by crushing and freezing (live). In the case of D and E, *D. caudata* were cultivated in duplicate (● and △).

2 cells obtained from a single cell in microplate wells.

In further culture experiments in Erlenmeyer flasks, *D. caudata* cells were transferred from each well to each flask, and simultaneously the new foods (the eukaryotic picophytoplankton+*Thalassiosira* sp.+*Chroomonas* sp.) were added in Fig. 2-A and only the eukaryotic picophytoplankton in Fig. 2-B. In the case of Fig. 2-A, *D. caudata* cells were filled with dense plasmatic contents in spite of 46 days incubation, and the size and shape remained normal (Fig. 3-A). However these cells became thinner in the flask, with reduced plasmatic contents, and could be kept alive for at least 2 months from the start of cultivation in the microplate well.

In the flask culture experiment using the cells of the microplate well of Fig. 2-B, cultured with only eukaryotic picoplankton, small cells of *D. caudata* appeared (Fig. 3-B, C, D). At one week after the transfer to the flask, several pairs of dimorphic cells (*D. diegensis*-like cells, ca. 63 μm in length) (Reguera & González-Gil 2001) were observed (Fig. 3-B). One of the *D. diegensis*-like cells had two trailing flagella, which is presumably a planozygote (Fig. 3-C). Thereafter, however, we could not confirm the formation of cysts or the further division of the possible planozygote. After a further one week incubation in the flask, several *D. diegensis*-like small cells (Reguera & González-Gil 2001) appeared (Fig. 3-D), and these small cells (ca. 43 μm in length) swam faster than the *D. caudata* cells of normal size (ca. 83 μm in length). In both flask cultivations, the

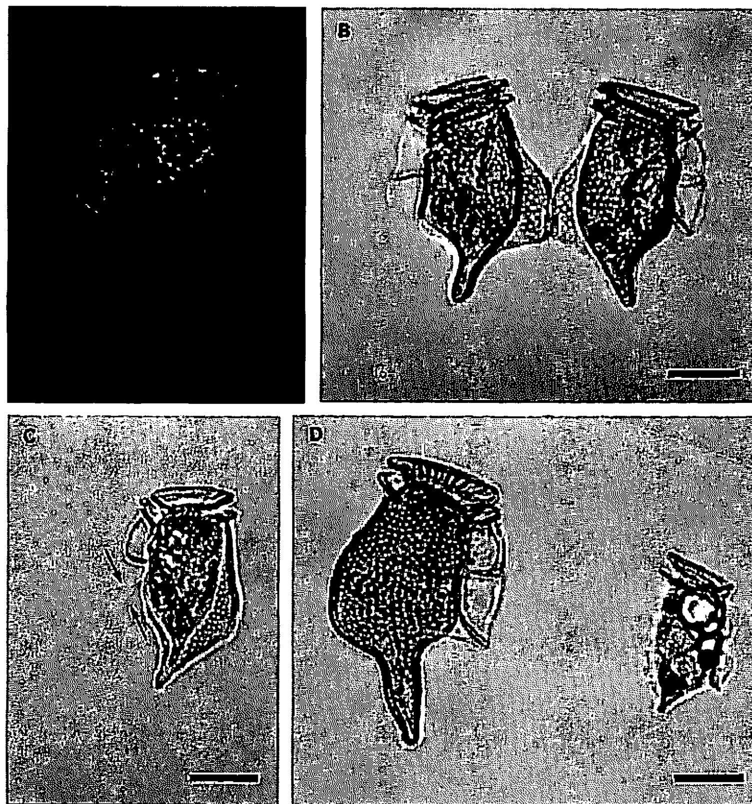

Fig. 3. *D. caudata* cells cultivated in Erlenmeyer flasks. All scale bars represent 20 μm. A: A cell filled with dense plasmatic contents after incubation for 46 days. B: A pair of dimorphic cells resulting from a depauperating division of *D. caudata*, with newly formed *D. diegensis*-like cells. C: A dimorphic *D. diegensis*-like cell with two trailing flagella. The flagella are indicated by arrows. D: Normal size cell of *D. caudata* (left) and the small cell (*D. diegensis*-like small cell, right).

numbers of *Dinophysis* cells did not apparently increase. In the case of flask cultivation using the cells from Fig. 2-B, more than 4 cells of *D. caudata* and more than 15 *D. diegensis*-like small cells could be observed 41 days after the inoculation to the microplate well. With our observations, the enlargement of small cells could not be confirmed.

Discussion

Concerning the cultivation of *Dinophysis* spp., reports are few because no species have been successfully cultivated. Ishimaru et al. (1988) demonstrated that a single cell of *D. fortii* cultured with a cryptomonad *Plagioselmis* sp. grew to a maximum of 22 cells in 3 weeks. Maestrini et al. (1995) reported that a single cell of photosynthetic *Dinophysis* (*D.* cf. *acuminata*, *D. acuta* Ehrenberg and *D. sacculus* Stein) cultured with bacteria, picoplankton and nanoplankton grew to at most 16 cells in 4 months. Therefore, the results (single cell to 28 cells) obtained in this study seem to be the best so far, although they are not sufficient to be regarded as a culture in the full sense.

The experimental conditions used in this study were based on our experience with culture experiments on *Dinophysis* species for several years. Concerning the timing of food addition, a delay (several days) before the food was supplied was more effective for the growth of *Dinophysis* species than the immediate addition of food after *Dinophysis* isolation. In our previous culture experiments, live food was not effective and so the organisms were crushed by ultrasonic treatment and freezing to prevent the possible effects of over growing. The degree of crushing of the foods differed between the phytoplankton species. The cryptophyte *Chroomonas* sp. can be easily crushed (more than 90%), but small phytoplankton (eukaryotic picophytoplankton and the diatom *Thalassiosira* sp.) were difficult to crush (crushing ratios were less than 30%). It is important to confirm the effects of the crushing of food organisms. The smaller particles may be more easily ingested since the flagellar pore of *D. caudata* is ca. 3–5 μm in our observation. Although actual phagotrophic behavior could not be observed, the ingestion of food organisms through the flagellar pore is considered likely (Lucas & Vesk 1990; Berland et al. 1995). As another possibility, *D. caudata* might use the organic substances eluted from crushed food organisms. In further experiments, it must be confirmed which substance is effective, crushed pieces or filtrate.

In this study, the possibility of bacterial effects on *Dinophysis* cultivation can not be entirely excluded, because none of the three cultures of food microalgae were axenic.

There is a paucity of studies on the relationship between *Dinophysis* and bacteria. However, we consider that the growth of *Dinophysis* can not be fully supported only by the ingestion of such small bacteria as compared with *Dinophysis* from the viewpoint of biomass. But there is a possibility of the production of some growth-promoting substances by bacteria.

To establish a scale-up culture of *D. caudata*, cells were transferred to 10 ml of culture medium in flasks supplied with the food organisms. In the flask, a cell with two trailing flagella, which is considered to be a planozygote, was observed. It is highly possible that the cell with two trailing flagella is a process of the life cycle of *D. caudata* although sexual conjugation was not observed in this study. In other *Dinophysis* species, the existence of a planozygote was reported in *D.* cf. *acuminata* (McLachlan 1993), *D. sacculus* (Delgado et al. 1996) and *D. pavillardi* Schroeder (Giacobbe & Gangemi 1997). The presence of dimorphic cells has also been reported in *D. caudata* based on natural samples (Moita & Sampayo 1993, Reguera et al. 1995, Reguera & González-Gil 2001). A dimorphic cell has one large hypothecal plate inherited from the maternal cell (*D. caudata*) and a small newly formed one during cell division (Fig. 3-B). Thereafter, the dimorphic cell becomes a *D. diegensis*-like cell, and further, the cell forms a smaller dimorphic cell during further cell division. As a result, a *D. diegensis*-like small cell appeared (Fig. 3-D). These small cells were not observed in culture in the microplate wells. Therefore, the transfer from the microplate well to the flask might induce small cell formation. The appearance of small cells in trials of laboratory cultivation was also reported in *D. fortii* (Uchida et al. 1999), *D. sacculus* (Delgado et al. 1996) and *D. pavillardi* (Giacobbe & Gangemi 1997). This phenomenon was considered a sexual process, and the small cells may have differentiated into gametes although further cytological study is necessary to clarify the life cycle of *Dinophsis*.

In our observation of culture experiments, photosynthetic *Dinophysis* cells gradually lost their photosynthetic pigments and got paler after the incubation period of 1 month. In this study, *D. caudata* kept their pigments for rather a long period with food organisms of eukaryotic picophytoplankton. This eukaryotic picophytoplankton emitted red autofluorescence under blue-light excitation, but identification is extremely difficult by normal microscopy. Various species of eukaryotic picophytoplankton have been reported so far, and these species generally belong to the Chlorophyceae, Prasinophyceae and Eustigmatophyceae (Johnson & Sieburth 1982, Takahashi & Hori 1984, Stockner & Antia 1986).

To establish a culture of *Dinophysis*, it is essential to (1) improve the method and timing of the transfer of *Dinophysis* cells from small-scale culture vessels such as microplate wells to large-scale ones such as flasks to increase cell numbers and to keep them for longer periods, (2) search for the most suitable food organism, and the additive concentrations and the timing of supply, and (3) set the optimal culture conditions (i.e. light intensity, temperature, salinity etc.). Although the result obtained in this study is not complete, it might be the first step to establishing *Dinophysis* cultures in the full sense successfully.

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