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## Purification of A Virus-like Particle Causing Growth Suppression of *Alexandrium catenella*

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### Abstract

A virus-like particle, which affected negative growth of *Alexandrium catenella*, was purified from a culture supernatant of growth-suppressed *A. catenella*. The virus particles were rounded shape with  $70 \pm 3$  nm in diameter. The virus was also estimated as non-lipid structured RNA virus because of loss of the infectivity by RNase and Proteinase K treatment, but no change in the infectivity by DNase and ether treatment.

**Key words:** *Alexandrium catenella*, growth suppression, virus particle, purification, RNA virus

### Introduction

The existence of 18 growth suppression agents for marine phytoplankton *Alexandrium catenella*, *Gymnodinium mikimotoi* and *Tetraselmis* sp. were observed in seawater samples collected at the mouth of Funka Bay from September to October in 1993 to 1995 (Onji et al., 1999; Onji et al., 2000). The growth suppression agents were characterized as virus-like agents with following physical and chemical traits; transferable, 0.05- $\mu$ m to 0.10- $\mu$ m in the size, unstable at 50°C, tolerance to ether treatment, unstable at pH 5.0, resistance to DNase treatment, sensitivity to RNase treatment, sensitivity to UV irradiation ( $5.0 \times 10^4 \mu\text{W} \cdot \text{sec}/\text{cm}^2$ ) and titration of  $10^{-1}$ . Furthermore, proteinase K susceptibility and the ultracentrifugation profile were different among *A. catenella*, *Tetraselmis* sp. and *G. mikimotoi*'s growth suppression agents, at least three types of virus or viroid might be the main body of the agents (Onji et al., 2000). In this paper, we tried to purify the *A. catenella*'s growth suppression agents, and an electron microscopic observation revealed the presence of virus-like particles in sucrose-gradient purified fraction.

### Materials and Methods

#### Culture of Phytoplankton

An axenic culture of *Alexandrium catenella* strain TN-7 was used in this study and cultured in f/2 medium (Watanabe and Nozaki, 1994) under cool white light

irradiation at about  $45.6 \mu\text{mol photon m}^{-2} \text{s}^{-1}$  with a 14:10 LD cycle at 20°C. Cell number of *A. catenella* in each culture was counted using a hemocytometer (Erma) as described by Onji et al. (1999).

#### *A. catenella* Growth Suppression agents

In previous paper (Onji et al., 1999; Onji et al., 2000), transferable samples collected at 1993 to 1995 showing the growth suppression effect were found and tentatively characterized (Onji et al., 1999; Onji et al., 2000). These infective agents continued to propagate in the same phytoplankton used for the screening as the host. The agents were dispensed in 2-ml subsample into 2.5-ml tubes and kept at  $-80^\circ\text{C}$  before use. The growth suppression rate was calculated as described by Onji et al. (1999; 2000) and growth suppression rate less than 30% was defined as negative growth suppression. ACO2893, ACO1794 and ACS2995 were used for further purification in this study.

#### Concentration and purification of the virus-like agent

A growth suppression agent was concentrated to 3 ml from 1200 ml supernatant by ultra-centrifugation at  $10,000 \times g$  for 4 h at 2 times. The 3 ml of concentrated fraction was purified for the sucrose step gradient ultra-centrifugation ( $10,000 \times g$  for 1.5 h, 10, 20, 30, 40% w/v in filtrated TE buffer (10 mM Tris-1 mM EDTA, pH 8.0)), however no virus bands were observed. Therefore this gradient dispensed equally in 4 fractions from upper to bottom. Each fraction was rinsed with TE buffer and inoculated to a fresh culture of *A. catenella* TN-7,

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and the phytoplankton growth suppression effects of each fraction were observed.

The cells of phytoplankton that growth suppressed were collected by centrifugation (1,200×g) for 15 min at 4°C. The cells were then washed 2 or 3 times with f/2 medium and suspended in 2 ml of f/2 medium. The cells of each phytoplankton were disrupted by ultrasonication (Otake, 30 sec. by 6 times under 100 W) as they were chilled on ice. After removal of cell debris by centrifugation (15 min. at 1,200×g), the cell-free extract was obtained and filter-sterilized with a 0.45 μm pore-size membrane filter. The cell-free extract was also purified as previous methods. No virus band was observed. Therefore this gradient dispensed equally in 4 fractions from upper to bottom. Each fraction was rinsed with TE buffer and inoculated to a fresh culture of *A. catenella* TN-7, and the phytoplankton growth suppression effects of each fraction were observed.

#### Electron microscopy

Sucrose-gradient purified fraction showing the growth suppression effect was dropped on collodion carbon-coated copper grids for 15 min. The grid was then stained with 2% aqueous uranyl acetate, examined and photographed at 75 kV using a Hitachi H-700 transmission electron microscope. Size of virus particles was estimated from photographic images of negatively stained virus particles.

#### Acridine Orange and DAPI (4', 6-diamidino-2-phenylindole) staining

To examine the type of nucleic acid in virus-like particles, sucrose-gradient purified fraction showing the growth suppression effect was stained with DAPI and acridine orange solution, and observed type of stained nucleic acid using an epifluorescence microscope (Axioskop H, Zeiss). One-tenth volume of 50 μg/ml DAPI (Kanto Chemical Co.) or 500 μg/ml acridine orange (Kanto Chemical Co.) solutions was added into the purified fraction and stained for 5 min, respectively.

The staining-dye was dissolved in autoclaved distilled water and stored in a plastic tube (YS-5, Toyo Kizai).

#### Characterization of the Virus-like Particles

Nuclease sensitivity, Proteinase K sensitivity and ether tolerance to the purified virus-like particles were characterized by the methods described in a previous paper (Onji et al. 1999).

#### Results and Discussion

On the basis of previous characterization of the phytoplankton growth suppression agent, it was suggested that *A. catenella* growth suppression agents were virus-like agents and were infected to *A. catenella* cells (Onji et al. 1999; Onji et al. 2000). However, the main body of the growth suppression agent was not yet observed due to the low titer (Onji et al. 1999; Onji et al. 2000).

To concentrate the main body of the agent, a concentration efficiency of the virus-like agents by ultracentrifugation and ultrafiltration was compared. The concentration fold by ultracentrifugation was 13 fold, and the fold was higher than that by ultrafiltration method (Table 1). These results showed that ultracentrifugation was better method for the concentration of the agents than ultrafiltration (Table 1).

The concentrates obtained by ultracentrifugation were used for further purification of the growth suppression agent of *A. catenella* in the culture supernatant and the cell-free extract. The concentrate was applied to a sucrose step gradient centrifugation, but no bands containing infective virus was observed. Then, this gradient dispensed equally in 4 fractions and the phytoplankton growth suppression effects of each fraction were observed. It was observed that the 3rd fraction in the tube affected the growth of the plankton (Figs. 1 and 2). Therefore it was positive result that the agents were infected in the cell and grown intracellular.

Virus-like particles with 70±3 nm in diameter obser-

Table 1. Concentration efficiency of phytoplankton growth suppression virus-like agent (PGSVLA)

PGSVLA	Fraction	Volume (ml)	Titer	GSR* <sup>1</sup> (%)	Activity* <sup>2</sup>	Folds* <sup>3</sup>	Yield (%)* <sup>4</sup>
ACO2893	Culture supernatant	1,200	5	36.1	180.5	1.00	100
	Ultracentrifugation	60	15	40.8	612	3.39	17.0
	2nd-ultracentrifugation	3	50	46.3	2,315	12.8	3.2
	Culture supernatant	120	5	34.3	171.5	1.00	100
	Ultrafiltration	15	10	45.7	457	2.66	33.3

\*<sup>1</sup> Growth suppression rate.

\*<sup>2</sup> Activity was calculated from titer and GSR.

\*<sup>3</sup> Folds were calculated as concentration efficiency of activity.

\*<sup>4</sup> Yield was calculated with volume and activity.

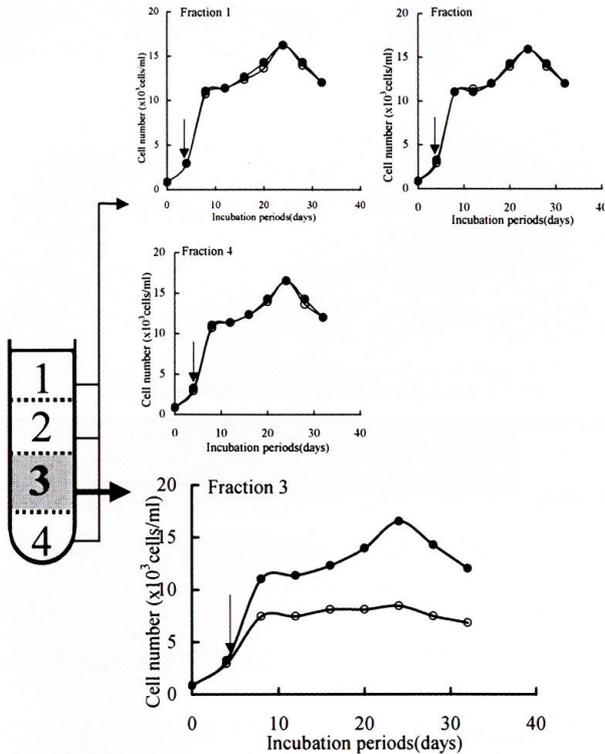


Fig. 1. Purification of the growth suppressed agents for *A. catenella* TN-7 (ACO2893) from the culture supernatant by sucrose-gradient centrifugation. Closed symbols indicate the control culture, and open symbols indicate the experimental culture inoculated with a fraction after the sucrose gradients concentration on the bottom fraction. Downward-directed arrow indicates the inoculation time.

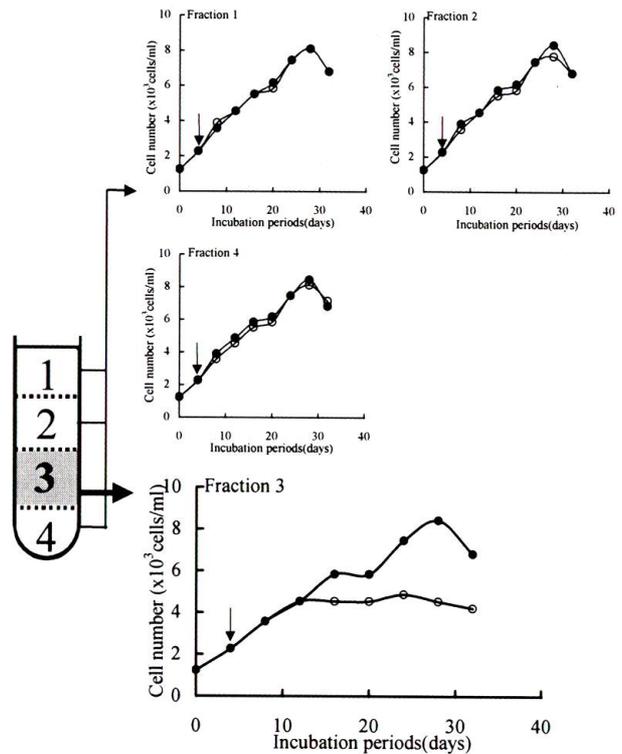


Fig. 2. Purification of the growth suppressed agents for *A. catenella* TN-7 (ACO2893) from the cell-free extract by sucrose-gradient centrifugation. Closed symbols indicate the control culture, and open symbols indicate the experimental culture inoculated with a fraction after the sucrose gradients concentration on the cell free extract. Downward-directed arrow indicates the inoculation time.

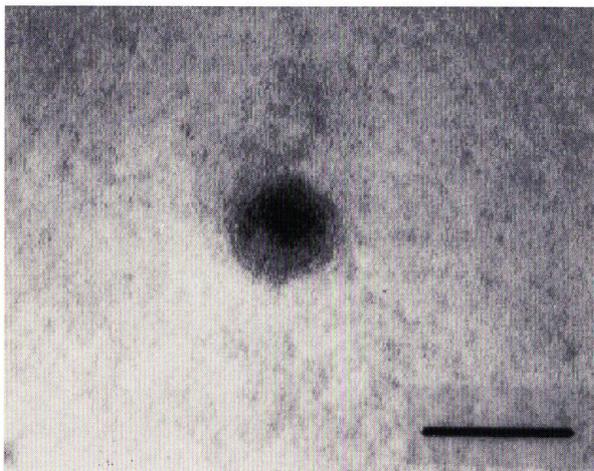


Fig. 3. Electron micrograph of virus-like particle from the purified fraction by sucrose-gradient centrifugation of the growth suppression agent (ACO2893). Scale bar : 100 nm.

ved in the purified fraction (Fig. 3). A growth suppression effect for *A. catenella* was observed in the purified fraction. These results suggest that the virus-like parti-

cle is main body of growth suppression for *A. catenella*. Moreover, the fraction was influenced by RNase and Proteinase K treatment and not affected by DNase and ether treatment (Table 3). Additionally, the virus-like particles was stained with acridine orange, that means the virus has RNA genome, but not stained with DAPI (Fig. 4). Therefore, it is believed that the agent may be non-lipid structured RNA virus.

A virus infected to the green algae *Chara corallina*, that is a freshwater phytoplankton taxonomically close to a land plant, have been found and isolated. *Chara corallina virus* (CCV) was known to be rod shaped and closely resembled to rod shaped RNA viruses infected to higher plants. The CCV was also recognized as the first RNA virus infecting phytoplankton (Stotnicki et al. 1976). It will be still needed to get the evidence which the virus-like particles have RNA genome. Further work is also required to elucidate the biology of the virus in marine environments.

Table 2. Characteristics of the positive growth suppressed fraction obtained by sucrose gradient centrifugation

PGSVLA	Growth suppression effect*			
	DNase	RNase	Proteinase K	Ether
(A) purified fraction from supernatant				
ACO2893	— (35.9)	+ (3.8)	+ (3.7)	— (36.2)
ACO1794	— (36.0)	+ (4.0)	+ (3.7)	— (36.2)
ACS2995	— (40.0)	+ (4.0)	+ (3.7)	— (36.2)
(B) purified fraction from cell-free extract				
ACO2893	— (34.6)	+ (3.8)	+ (5.5)	— (33.3)
ACO1794	— (34.6)	+ (0.0)	+ (5.5)	— (36.2)
ACS2995	— (34.6)	+ (0.0)	+ (5.5)	— (39.1)

+ : Suppression was observed.

— : No effect (&lt;10% growth suppression effect.)

\*Growth suppression rate (%) are given in parentheses.

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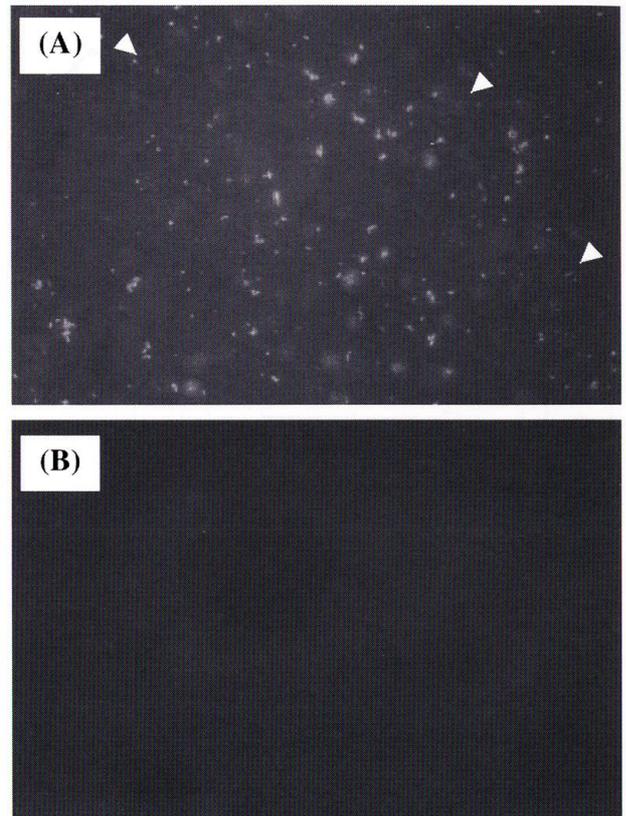


Fig. 4. Micrographs of virus-like particles from the purified fraction by sucrose gradients of the growth suppression agent (ACO2893) stained Acridine Orange (A) and DAPI (B). Arrows indicate the virus particles.