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EXTREMELY PSYCHROPHILIC MICROALGAE ISOLATED FROM THE ANTARCTIC OCEAN

Hidetoshi OKUYAMA[1], Kazuhiro KOGAME[1], Makoto MIZUNO[2], Wataru KOBAYASHI[1], Hajime KANAZAWA[1], Shuji OHTANI[3], Kentaro WATANABE[3] and Hiroshi KANDA[3]

[1]*Department of Biology, Faculty of Science, Hokkaido University, Kita-ku, Sapporo 060*
[2]*Department of Bioproduction, Faculty of Bioindustry, Tokyo University of Agriculture, Yasaka, Abashiri 099–24*
[3]*National Institute of Polar Research, 9–10, Kaga 1-chome, Itabashi-ku, Tokyo 173*

Abstract: Two psychrophilic strains of unicellular algae were isolated from seawater samples collected in the Antarctic Ocean. The one (strain A) was a species of *Cymbella* (Bacillariophyceae) and the other (strain B) was a species that belonged to the class Prymnesiophyceae (a prymnesiophyte). Optimum temperatures for growth of both the algae were between 2.5°C and 5°C. Upper limit temperatures for growth of strain A (*Cymbella* sp.) and strain B were between 12.5°C and 15°C and between 7.5°C and 10°C, respectively. We suggest that these algae should be categorized into extremely psychrophilic algae from their significantly lower cardinal temperatures for growth.

1. Introduction

Temperature is one of the important factors that determine the general geographical distribution of certain algae (SOEDER and STENGEL, 1974; YANAGITA, 1990). Since the latter half of the 19th century many biologists have navigated the Antarctic Ocean and have reported a large number of phytoplanktons obtained there (FUKASE, 1962; HASLE, 1969; FUKUCHI, 1980). However, their reports on phytoplanktons have been limited to the results of taxonomical and ecological studies. Most planktonic algae from the Antarctic Ocean are considered to be psychrophilic and stenothermic (SOEDER and STENGEL, 1974). At the present time the biochemical bases of such properties of psychrophilic algae have been poorly examined. In this study an attempt is made to isolate psychrophilic microalgae from seawater of the Antarctic Ocean as the first step to elucidate their psychrophily biochemically.

2. Materials and Methods

2.1. Samples

Seawater samples were collected in the Antarctic Ocean (56°50′S, 149°56′E) on March 15, 1989 by S. OHTANI during the 30th JARE cruise of the SHIRASE. The

samples, of which temperature was 3°C, were kept under 4°C during transportation.

2.2. Cultivation and isolation of algae

After 400 ml of seawater samples were centrifuged at 5000 rpm for 10 min at 4°C, about 390 ml of seawater was discarded by decantation. The remaining samples (10 ml) were mixed, and transferred to 90 ml of PES medium (PROVASOLI, 1968) that contained 48 U/ml potassium penicillin G and 22 U/ml streptomycin sulfate. The samples were cultured at 4°C under continuous illumination at 1000 lx with day-light type fluorescent lamps.

The culture medium turned tinged with yellowish brown with time. After 4–8 weeks from the inoculation, microalgae were isolated by repeated pipetting. Resultantly two kinds of axenic unicellular algae were obtained. The one was a diatom and was tentatively designated as strain A. The other was designated as strain B. Isolated organisms were grown in 500-ml flasks with 400 ml of PES medium at 5°C in a 16 h light/8 h dark regime under the illumination with day-light fluorescent lamps at 3000 lx.

2.3. Microscopy

Cells of strain A (a diatom) were preserved in 3% formalin in seawater. This preserved material was examined for light microscopy. For scanning electron microscopy (SEM), the preserved material was treated with conc. HNO_3 for 48 hours at room temperature and washed with distilled water. The acid-treated material was air dried, coated with gold, and examined with scanning electron microscopes Hitachi S510 and JEOL JSM-T20.

Cells of the other isolate, strain B, were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, that contained 0.25 M sucrose. The post-fixation was carried out with 1% OsO_4. The fixed material was dehydrated in an ethanol series and dried in a critical point dryer with liquid CO_2. The dried material coated with gold was used for SEM, as described above.

2.4. Pigment analysis

The separation and identification of the major chlorophylls from materials was carried out by thin-layer chromatography (TLC) with precoated silica gel plates (code no. 5721, Merck). Pigments were extracted from the frozen materials with ice-cold acetone and aliquots of the extracts were subjected to TLC. The following two solvent systems were used: Solvent I, chloroform/methanol/28% NH_4OH in water (80: 20:4, vol/vol); Solvent II, isooctane/2-propanol (20: 1, vol/vol). The pigments were identified by comparing their R_f values with those of standards. Furthermore, the spot of each pigment was extracted from silica gel with a mixture of chloroform, methanol, and water (1: 1: 0.9, vol/vol). The light-absorption spectrum of each extracted pigment dissolved in ethyl ether and acetone was compared with those of standards. *Petalonia fascia* (Scytosiphonales, Phaeophyceae) was used as the source material for standard chlorophylls *a* and *c*.

2.5. Measurement of growth

The alga was grown statically as preculture in a 400 ml medium in a 500 ml-flask at 5°C under 3000 lx for 3 to 4 weeks. Since the alga grew on an inner surface of the flask, it was detatched with a sterile pipette. Then, 0.5 ml of suspensions that contained several flocks of the alga was inoculated to 19.5 ml of PES medium in a 50-ml flask, and they were incubated at the temperature range from 2.5°C to 15°C, under the same light conditions as those of the preculture. At suitable intervals cultures were subjected to sonic vibration for 2 sec to disperse cells, and then turbidity of cell suspension was estimated at 750 nm.

The specific growth rate, k, was calculated from the formula:

$$k=\frac{2.303\,(\log x_2-\log x_1)}{t_2-t_1}\ \text{week}^{-1},$$

where x_1 and x_2 are the average turbidities of three cultures at time t_1 and t_2, respectively.

3. Results and Discussion

3.1. Microscopy

Cells of strain A (a diatom) were semicircular with round ends in valve view (Fig. 1A) and rectangular in girdle view (Fig. 1B). Valves were 3.9–4.8 μm long and 1.5–2.1 μm wide. A narrow and straight axial area was located in the ventral side of valve (Fig. 1C). Raphe was straight. A distal outer raphe fissure slightly curved to dorsal side and a proximal outer raphe fissure did not curve (Fig. 1C). A single stria was composed of a single row of small round pores (Fig. 1C). Striae were parallel in the middle of valve and radiate at the end (Fig. 1C). Striae density was 60–80 striae in 10 μm along the margin near the middle of valve. A valve with the density of 70 striae in 10 μm was most frequently observed. A valve whose dorsal striae in the middle of valve become shorter was sometimes observed. Inner axial area thickened (Fig. 1D). A distal inner raphe fissure did not end in a helictoglossa (Figs. 1D and 1E). Both distal and proximal ends of an inner raphe fissure did not curve (Fig. 1E).

Distinction between the genera *Amphora* and *Cymbella* was described by KRAMMER (1980). *Cymbella* is lacking hyaline area between the residuum side and the raphe side, which is a significant difference between the two genera (KRAMMER, 1980). This characteristic as well as the structure of stria supports the idea that the present diatom, strain A, belongs to the genus *Cymbella*, although the morphology of outer and inner raphe fissures was different from the characteristic of the *Cymbella*. Strain A was unique in its small cell size and high striation density among diatoms belonging to the genus *Cymbella*. In the present study it was tentatively designated as *Cymbella* sp. strain A.

Strain B had motile and non-motile stages. The motile cell (Fig. 2A) was somewhat spherical in shape and was 3 to 6 μm in diameter. It had two subequal flagella on the top (Fig. 2A). The haptonema was found between the two flagella by SEM (Fig. 2D), although it could not be observed by light microscopy (see Fig. 2A).

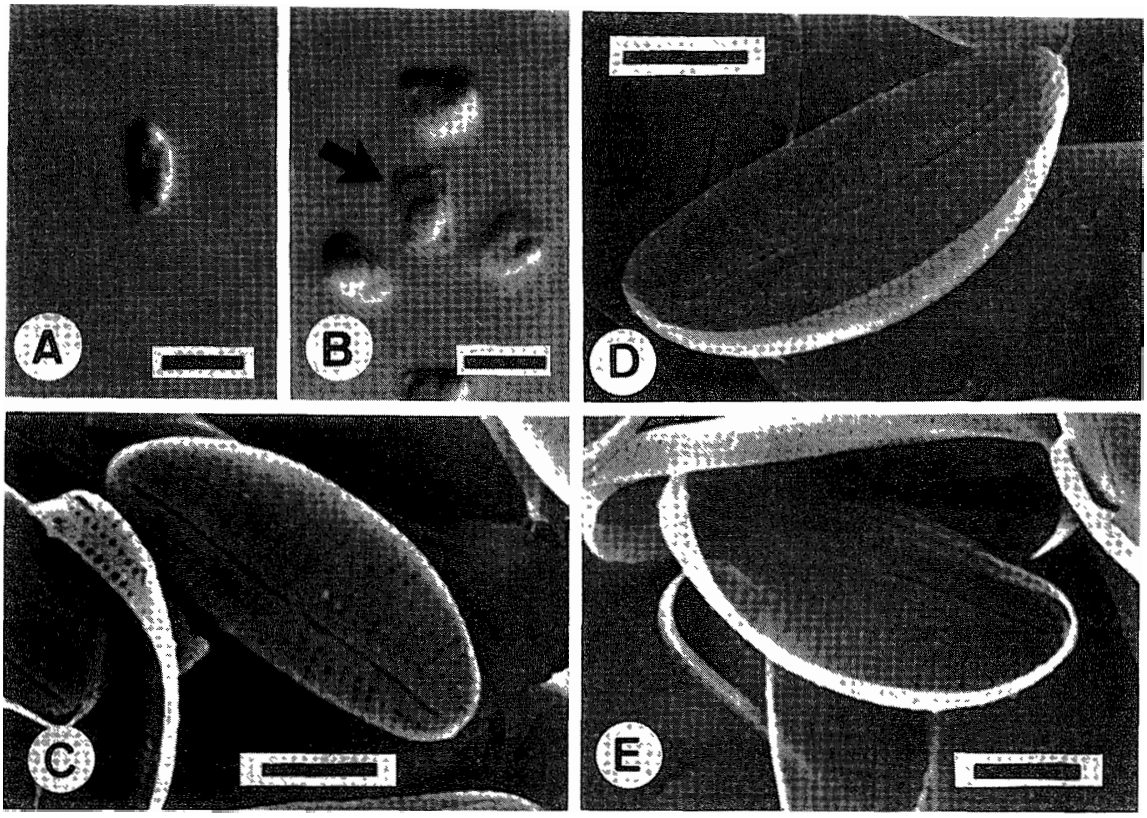

Fig. 1. Micrographs of strain A (diatom). A, Ligth micrograph of a cell valve view. B, Light micrograph of cells. An arrow shows a cell in girdle view. C, Scanning electron micrograph of outer surface of valve. D and E, Scanning electron micrographs of inner surface of valve. Scales in panels A and B, and C–E indicate 5 μm and 1 μm, respectively.

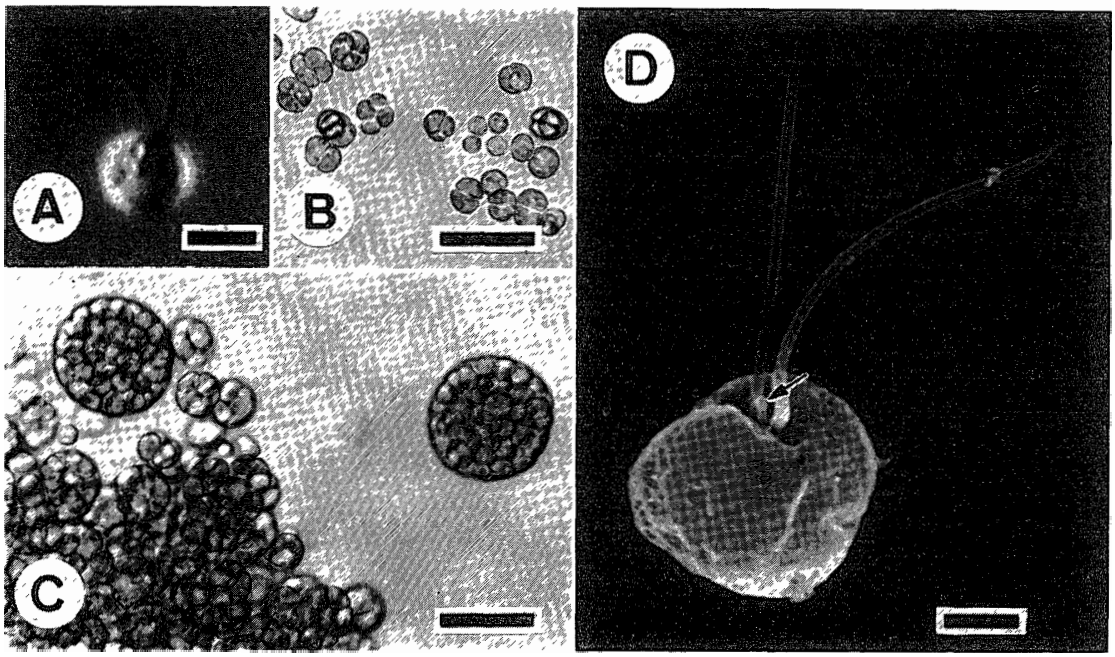

Fig. 2. Micrographs of strain B (prymnesiophyte). A, Light micrograph of a motile cell. B, Light micrograph of non-motile cells. C, Light micrograph of spherical masses of non-motile cells. D, Scanning electron micrograph of a motile cell. An arrow shows a reduced haptonema. Scales in panels A, B and C, and D indicate 5 μm, 30 μm, and 1 μm, respectively.

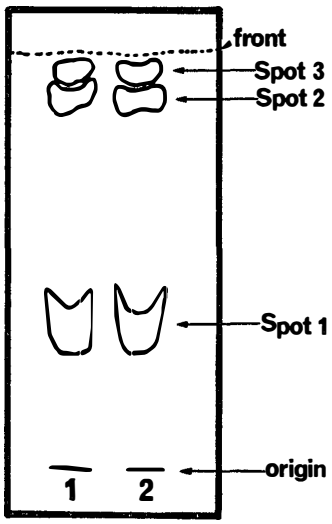

Fig. 3. Thin-layer chromatograms of pigments prepared from cells of strain B. Lanes 1 and 2, pigments prepared from cells of strain B and Petalonia fascia, *respectively. Development was carried out with solvent I (see text).*

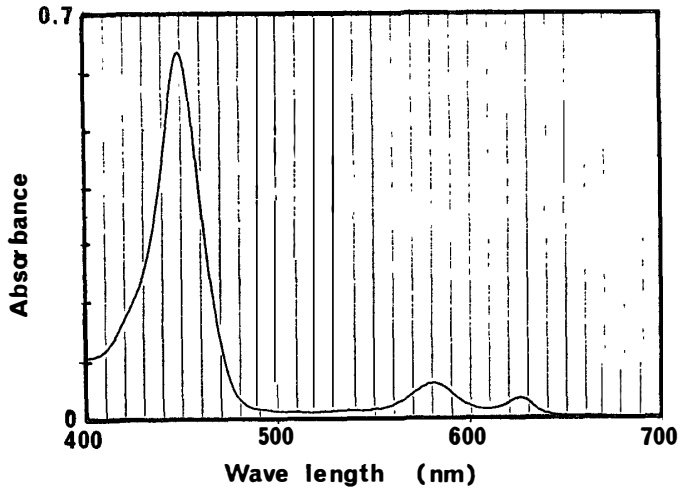

Fig. 4. The absorption spectrum of chlorophyll c fraction prepared from cells of strain B. (see text).

Non-motile cells of strain B multiplied by division and formed spherical masses on the inner surface of the culture flask (Figs. 2B and 2C). About five days after the transfer of non-motile cells to fresh medium, motile cells appeared. However, the motile cells disappeared age-dependently and could not be observed after two weeks from the inoculation.

The presence of a haptonema, in addition to two apical and subequal flagella, (see Fig. 2D) indicates that strain B belongs to the Prymnesiophyceae. According to PARKE and DIXON (1976), the reduced haptonema is characteristic of prymnesiophytes that belong to the order Isochrysidales. Thus, strain B was treated as a member of Isochrysidales and designated as prymnesiophyte strain B in this study. Further characterization for identification of strain B is in progress.

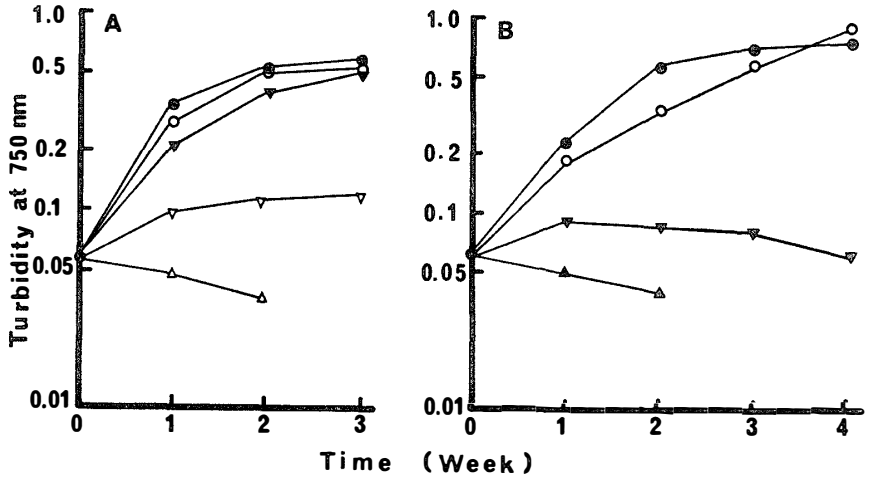

Fig. 5. Effect of temperature on the growth of strains A and B. ○, *2.5°C;* ●, *5°C;* ▼, *7.5°C;* ▲, *10°C;* ▽, *12.5°C;* △, *15°C. A, Strain A. B, Strain B.*

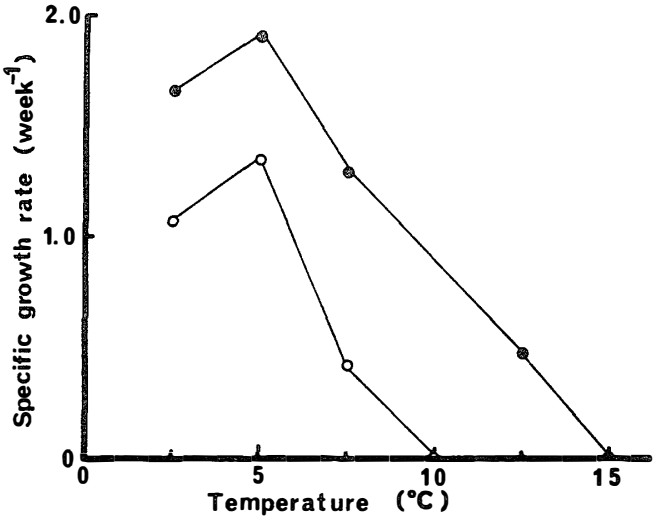

Fig. 6. Specific growth rates of strains A and B. ●, *Strain A.* ○, *Strain B.*

3.2. Pigment analysis

The TLC profile of acetone extracts of cells shows that strain B has 3 major spots (Fig. 3). Spots 1 (pale green), 2 (orange) and 3 (blue green) on lane 1 were identified as chlorophyll *c*, carotenoid and chlorophyll *a*, respectively, by comparing their R_f values with those of standard pigments (Lane 2) which were prepared from an authentic *Petalonia fascia* (phaeophyte). The same result was obtained in a different solvent (Solvent II) for development (data not shown). The absorption spectrum of chlorophyll *c* fraction of strain B was typical one of chlorophyll *c* (Fig. 4, see ROWAN, 1989).

3.3. Temperature range and kinetics for growth

The growth curves and the specific growth rates of strains A and B at different temperatures are shown in Figs. 5 and 6. Strain B could not grow at 10°C at all and exhibited only poor growth at 7.5°C (Fig. 5B). Accordingly, the true upper

limit temperature for growth is between 7.5 and 10°C. The optimum temperature for growth is between 2.5 and 5°C where growth reached plateau over 3 weeks. The specific growth rate of strain B at 2.5°C was 80% of that at 5°C. Strain A also grew well in a temperature range from 2.5°C to 7.5°C, but not 15°C at all (Fig. 5A). The true upper limit temperature of this organism is between 12.5 and 15°C. Generation time (day) of each strain at various temperatures is as follows: 2.5°C, 2.92; 5°C, 2.55; 7.5°C, 3.76; 12.5°C, 9.90 for strain A; 2.5°C, 4.59; 5°C, 3.59; 7.5°C, 11.83 for strain B. From kinetics for growth of these two algae shown in Fig. 6 it is likely that they can grow at or below 0°C.

VAN BAALEN and O'DONNELL (1983) isolated five species of psychrophilic diatoms from ice and water samples from the ice-edge in the Bering Sea. Recently YANAGITA (1990) listed three species of psychrophilic algae, *Chlamydomonas* sp. (green alga), *Fragilaria sublinearis* (diatom) and *Koliella tatrae* (green alga). In these cases the term psychrophiles is used according to MORITA (1975) as those organisms whose upper limit temperature for growth is 20°C, optimum temperature below 15°C, and lower limit 0°C or below. The psychrophilic algae mentioned above except for *K. tatrae* have optimum temperatures for growth above 10°C. *K. tatrae* has optimum temperature for growth at 4°C and upper limit at 10°C (HINDAK and KOMAREK, 1968). Optimum and upper limit temperatures of our isolates for growth are significantly lower than the corresponding cardinal temperatures defined by MORITA. We suggest that organisms that have optimum and upper limit temperatures for growth less than 5°C and below 15°C, respectively, should be categorized into extremely psychrophilic microorganisms. KATAHIRA *et al.* (1990) isolated a psychrophilic diatom, *Navicula* sp., from the Antarctic Ocean. However, this diatom does not belong to extreme psychrophiles, because it has an optimum temperature for growth at about 10°C. Although JEFFREY and WRIGHT (1987) described two Antarctic strains of *Phaeocystis pouchetii* (prymnesiophyte), cardinal temperatures for growth of those organisms were not determined. This is the first case that the diatom (strain A) and the prymnesiophyte (strain B) are classified into extreme psychrophiles.

Psychrophilic bacteria have been focused for elucidation of biochemical mechanisms of psychrophiles (SOEDER and STENGEL, 1974; MORITA, 1975; OKUYAMA *et al.*, 1990), but psychrophily of eukaryotes is poorly known. The fact that algae contain very high level of long-chain poly-unsaturated fatty acids as fatty acyl moieties of membrane lipids (WOOD, 1974) urges us to examine the relationship between psychrophily and such fatty acids. Two extremely psychrophilic strains obtained in this study, *Cymbella* sp. strain A and a prymnesiophyte strain B, may be suitable organisms for this purpose.

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