Extremophiles (2000) 4:385–389

ORIGINAL PAPER

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Tissue culture of the deep-sea bivalve *Calyptogena soyoae*

Received: June 1, 2000 / Accepted: October 4, 2000

Abstract Tissue culture for the deep-sea clam *Calyptogena soyoae* (*C. soyoae*) has been examined. Mantle tissue was cultured in Dulbecco's modified Eagle medium that was prepared using artificial seawater supplemented with fetal bovine serum (FBS) and the body fluid of *C. soyoae*. The mantle cells were viable in culture for at least 13 days at 4°C and atmospheric pressure on a polylysine-coated dish, although no cells attached in the body fluid-free culture medium. It was found that mantle cells synthesized DNA and seemed to proliferate under atmospheric conditions.

Key words *Calyptogena soyoae* · Deep-sea clam · Cell culture · DNA synthesis

Introduction

Since the advent of deep-sea vessels opened the door to research on deep-sea organisms, many deep-sea microorganisms have been isolated and characterized. Although increasing attention has been paid to deep-sea multicellular organisms, no one has been successful in culturing them. Primary tissue culture of deep-sea multicellular organisms should provide us with exceptional opportunities to contribute to biotechnology. The present study was therefore undertaken to develop a primary tissue culture method under atmospheric pressure for the deep-sea clam *Calyptogena soyoae* (collected at depths of 1180, 1148, and 1386 m).

Communicated by K. Horikoshi

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The deep-sea clam *Calyptogena soyoae* (*C. soyoae*) was discovered at Sagami Bay, Japan (Okutani and Egawa 1985) and was able to be kept alive in seawater at 4°C and atmospheric pressure for a few days. *C. soyoae* has chemoautotrophic symbiotic bacteria in the gill (Sakai et al. 1987; Ishii et al. 1988; Hashimoto et al. 1994), and may be sustained by the chemoautotrophic consumption of hydrogen sulfide in water released from beneath the seabed (Masuzawa et al. 1992).

Establishment of marine invertebrate cell culture has been severely hampered because of contamination by microorganisms. Another considerable problem occurs if the culture medium contains growth factors. Nevertheless, primary cell cultures from marine clams have been maintained, although only for a short time (Li and Stewart 1966; Cecil 1969; Odintsova and Khomenko 1991; Naganuma et al. 1996; Takeuchi et al. 1994a,b, 1999). These researchers used a seawater-based medium supplemented with animal serum or sera and various antibiotics to cultivate the cells from marine clams. Therefore, the fresh blood-like fluid of *C. soyoae* was added to the culture medium because it is expected to contain some growth factors.

In this study, the mantle cells, carefully removed from bacterial contamination, are plated on a polylysine-coated dish and cultured in a salt-enriched medium containing fetal bovine serum (FBS) and fresh blood-like body fluid of *C. soyoae* at 4°C and atmospheric pressure.

Materials and methods

Animals

Deep-sea bivalves (*Calyptogena soyoae*) were collected by the submersible *Shinkai 2000* (dive 1047, September 20, 1998, 1386 m, 35°05.74′N, 139°20.42′E; and dive 1142, October 9, 1999, 1148 m, 34°59.90′N, 139°13.62′E) and the unmanned submersible *Dolphin 3K* (dive 331, July 1, 1997, 1180 m, 34°59.99′N, 139°13.70′E) operated by the Japan Marine Science and Technology Center from the area of

Sagami Bay, Japan. The deep-sea bivalves were extremely sensitive to increments of temperature; therefore, the bivalves were collected with much deep-sea sediment and were buried deeply. The bivalves were kept alive for 3 days in 4°C artificial seawater under atmospheric pressure.

Materials

Petri dishes (35 × 10 mm tissue culture dishes) were obtained from Falcon (Franklin Lakes, NJ, USA). Poly-D-lysine (500–550 kDa), mouse laminin (35-mm dish), human fibronectin (35-mm dish), and collagen I (35-mm dish) were purchased from Biocoat (Bedford, UK). Penicillin streptomycin fungizone 100× concentrated mixture and FBS were from Bio Whittaker (Walkersville, MD, USA).

Medium

Dulbecco's modified Eagle medium (DMEM; ICN Biomedicals, Aurora, OH, USA), originally formulated for use in a CO_2-free system, was modified as shown in Table 1. Briefly, DMEM powder was dissolved in artificial seawater. Consequently, 1% (v/v) antibiotics (penicillin streptomycin fungizone 100× concentrated mixture), and 10% (v/v) FBS were added to the medium. Medium pH was adjusted by HEPES buffer (pH 7.8).

The blood-like body fluid of *C. soyoae* was extracted by cutting at the adductors with a scalpel, and filtered using a 5 μm pore size; 10% (v/v) fresh body fluid sterilized by a 0.2-μm-pore-size filter was added if the culture medium was supplemented with the body fluid.

Table 1. Modified Dulbecco's modified Eagle medium (DMEM) for primary culture of the mantle cells from the deep-sea clam *Calyptogena soyoae*

Dulbecco's modified Eagle medium (DMEM; powder)		13 400 mg/l
Artificial seawater	NaCl	28 600 mg/l
	KCl	200 mg/l
	HEPES	6000 mg/l
	Na_2EDTA	10 mg/l
	$FeCl_3\ 6H_2O$	0.1 mg/l
	H_3BO_3	2 mg/l
	$MnCl_2\ 4H_2O$	0.4 mg/l
	$ZnCl_2$	0.05 mg/l
	$CoCl_2\ 6H_2O$	0.01 mg/l
	NaBr	10 mg/l
	$SrCl_2$	2 mg/l
	RbCl	0.2 mg/l
	LiCl	0.2 mg/l
	Na_2MoO_4	0.5 mg/l
	KI	0.01 mg/l
Antibiotics	Penicillin	100 000 unit/l
	Streptomycin	100 mg/l
	Fungizone	250 μg/l
FBS		10% v/v
Blood-like body fluid of *C. soyoae*		10% v/v
CO_2		Ambient (pH 7.8)

FBS, fetal bovine serum

Primary culture for *Calyptogena* mantle tissue

To cultivate the cells from *Calyptogena soyoae*, the mantle tissues of *C.* (Fig. 1) were removed and transferred to ice-cold (about 0°–4°C) artificial seawater containing 10 mg/ml kanamycin (Sigma, St. Louis, MO, USA) as soon as possible (about 10–30 s). The artificial seawater containing kanamycin must be filtered. The mantle tissues were washed three times with cold artificial seawater containing 60 mg/l of kanamycin, transferred to 70% ethanol, and dipped for 30–60 s. Because sterilization was not completed by treatment with penicillin and streptomycin exposure, it was strictly necessary to sterilize the tissue by 70% ethanol exposure to exclude animal plankton contamination. The exposure time was, however, set between 30 and 60 s because too much exposure might cause cell toxicity. After sterilization by 70% ethanol, the mantle tissues were rinsed with cold artificial seawater containing 60 mg/l kanamycin another three times, transferred to a petri dish, and chopped to 5 × 5 mm^2. Pieces of the mantle tissue (5 × 5 mm^2) were transferred to polylysine-, laminin-, fibronectin-, collagen I-, or noncoated petri dishes and covered with coverslips. After these procedures, the mantle tissues were cultured in the

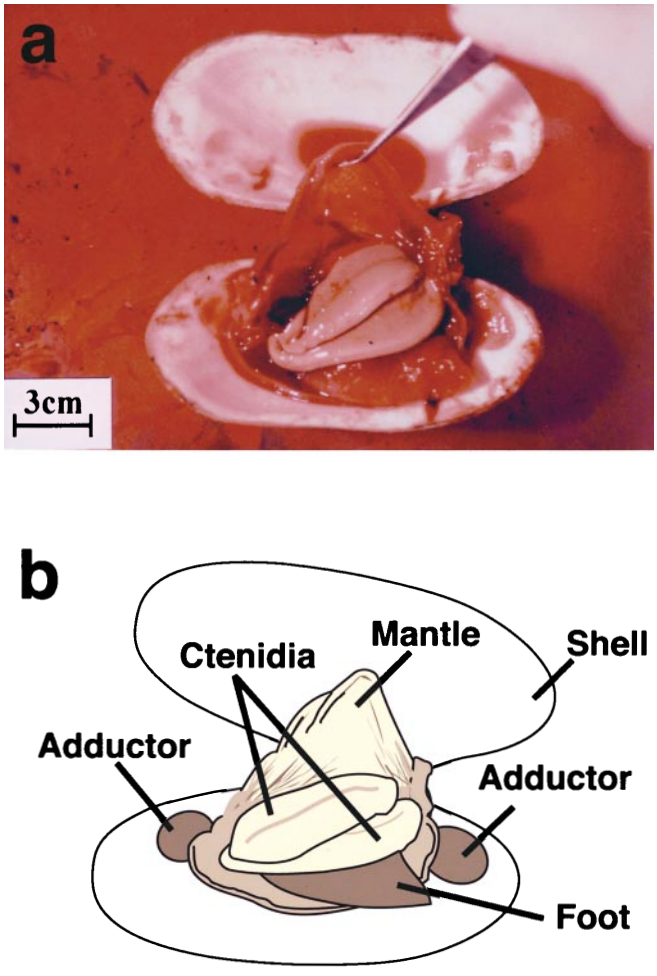

Fig. 1a,b. Internal view of the soft parts of *Calyptogena soyoae*: **a** photograph; **b** schematic illustration

culture medium (Table 1) at atmospheric pressure. Incubation temperature was set to 4°, 8°, 15°, 20°, and 37°C, respectively. The coverslip was removed after 4–7 days of cultivation. The culture medium was changed once or twice a week.

Cell viability was tested by calcein acetoxy methyl ester (calcein AM) and ethidium homodimer-1 (EthD-1) double-staining according to the manufacturer's procedure (live/dead viability/cytotoxicity kit; Molecular Probes, Eugene, OR, USA). Briefly, the mantle cells were incubated with both 2 μM calcein-AM and 4 μM EthD-1 in artificial seawater for 15 min at 4°C. After incubation, the cells were observed under blue excitation light using a fluorescence microscope (model IMT2-RFC; Olympus, Tokyo, Japan).

Measurement of DNA synthesis

DNA synthesis was assayed in mantle tissue culture fragments of $3 \times 3\,mm^2$ using a DNA IdU labeling and detection kit (Takara, Tokyo, Japan) according to the kit procedure. Briefly, mantle tissue fragments ($3 \times 3\,mm^2$) were transferred to a polylysine-coated 96-well plate (Falcon). The tissue fragments were cultured in medium containing either 10 μM 5-iodo-2′-deoxyuridine (IdU) or 0 μM as a control for 48 h at 4°C. After 48-h cultivation, the tissue fragments were washed twice with artificial seawater and were fixed by fixative solution at 37°C for 30 min. After incubation, the fragments were washed three times by PBS and further incubated with blocking solution at 37°C for 30 min. After these treatments, the fragments were reacted with 5 μg/ml anti-IdU antibody conjugated with peroxidase at 37°C for 30 min. The amount of DNA synthesis was measured by the peroxidase activity.

Statistical analysis

Statistical analysis was performed using Student's *t* test (Ichihara 1990). The calculations were performed using Microsoft Excel.

Results

Tissue culture for *Calyptogena soyoae* mantle cells

The mantle tissue fragments ($5 \times 5\,mm^2$), which were carefully removed from bacterial contamination, were plated on a polylysine-, collagen-, laminin-, fibronectin-, or noncoated dish. The mantle tissues were cultured in salt-enriched DMEM containing 10% FBS and 10% fresh blood-like body fluid of *C. soyoae* at 4°, 8°, 15°, 20°, and 37°C under atmospheric pressure. It should be noted that the mantle cells showed adhesion to polylysine-coated dishes only at 4°C (Fig. 2). The mantle cells seemed to proliferate slightly because some mantle cells had two nuclei as a result of mitosis (Fig. 2).

After 14 days of cultivation, the majority of cells were detached from the surface of the polylysine-coated petri dishes and were dead. It is surprising that no cells were attached to collagen I-, laminin-, fibronectin-, and noncoated dishes. When the temperature was elevated above 4°C, almost all the cells were detached and dead. In addition, the cells also detached from the bottom of the polylysine surface in the blood-like body fluid-free medium. The body fluid components as well as temperature were found critical for culturing mantle cells under atmospheric pressure.

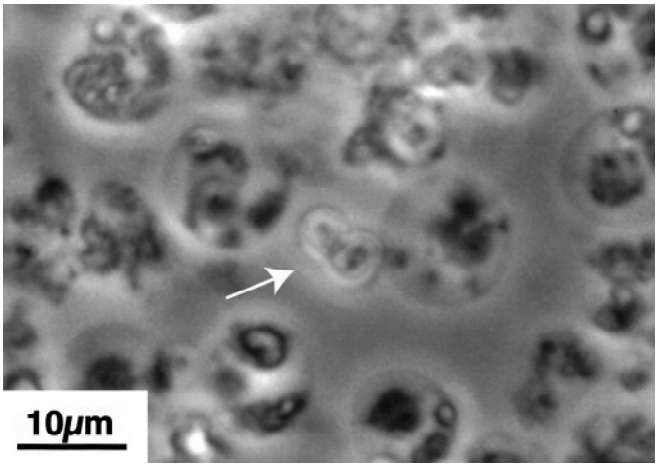

Fig. 2. Mitotic-like mantle cells (*arrow*) cultured for 4 days at 4°C on a polylysine-coated dish

Mantle cell viability test

Cell viability was investigated by double-staining with both calcein-AM and EthD-1 (Figs. 3, 4). After being double-stained, living cells emit green calcein fluorescence because of esterase activity (Figs. 3a, 4c). In contrast, dead cells generate a bright red fluorescence when EthD-1 enters cells with damaged membranes (Fig. 4c). Green calcein fluorescence was detected in the edges of mantle tissue fragments (Fig. 3a). The living cells have a spherical shape and weakly attach to polylysine-coated dishes after coverslip removal and medium change (Fig. 4). The mantle cells look similar in shape and size to Japanese scallop larval cells (Odintsova and Khomenko 1991). These results indicate that the cells could be kept alive under atmospheric pressure.

Proliferation of the cultured cells

We measured DNA synthesis in the mantle cells because some mantle cells had two nuclei as a result of mitosis (Fig. 2). Figure 5 shows the results of measurement of DNA synthesis in $3 \times 3\text{-}mm^2$ mantle tissue fragments. DNA synthesis was detected in the cultures and increased twofold compared with the control ($^*P < 0.05$). This result indicated that the mantle cells could proliferate in salt-enriched medium on a polylysine-coated dish at 4°C.

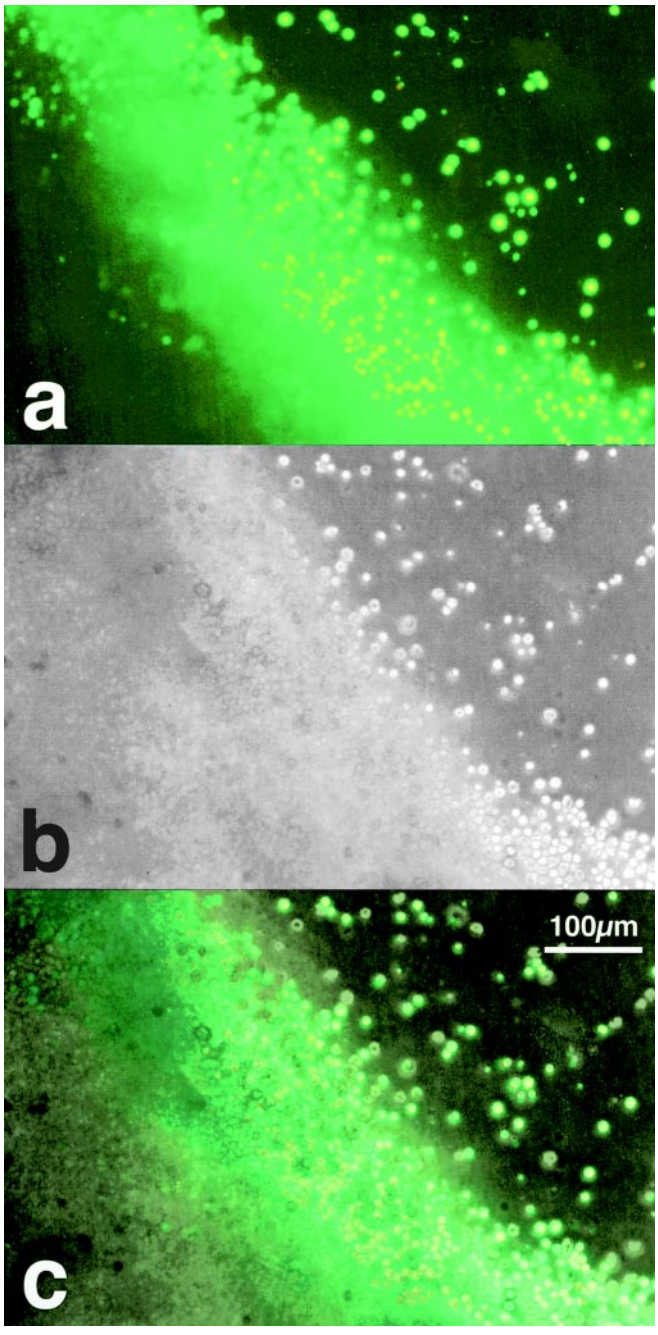

Fig. 3a–c. Mantle tissue of *Calyptogena soyoae* on a polylysine-coated dish cultured at 4°C for 6 days: **a** fluorescence microscopy of live mantle cells; **b** phase-contrast microscopy; **c** superimposed photograph. The cells were incubated with fluorogenic substrate calcein acetoxy methyl ester (calcein-AM) and ethidium homodimer-1 (EthD-1). Living cells emit green calcein fluorescence because of esterase activity. Dead cells generate red fluorescence as a result of EthD-1 entering cells with damaged membranes

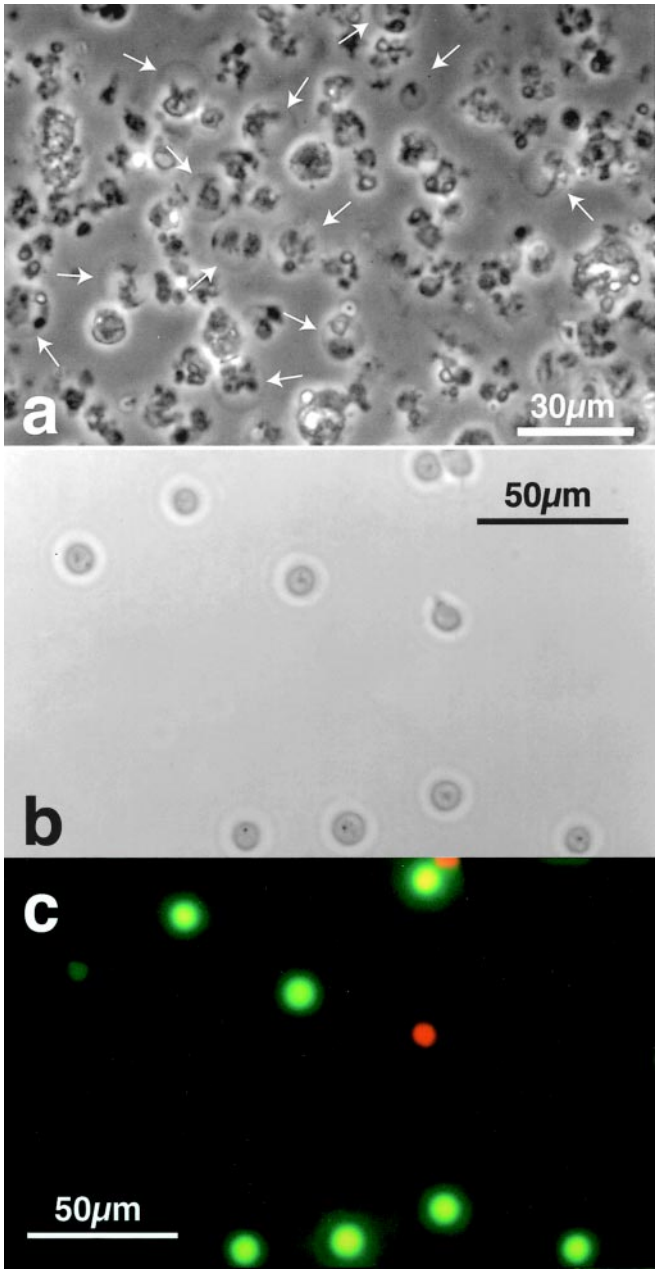

Fig. 4a–c. Mantle cells on a polylysine-coated dish. **a** Cells cultured for 4 days before change of medium; *arrows* indicate where cells have attached to the polylysine-coated dish. **b** Phase-contrast and **c** fluorescence microscopy of cells cultured for 6 days after coverslip removal and medium change. The cells were incubated with fluorogenic substrate calcein-AM and EthD-1. Living cells emit green calcein fluorescence because of esterase activity; dead cells generate red fluorescence as a result of EthD-1 entering cells with damaged membranes

Discussion

It is concluded that the mantle cells from the deep-sea clam *C. soyoae* can be cultured and will synthesize DNA at a low temperature around 4°C when the mantle tissue is sterilized sufficiently and 10% fresh blood-like body fluid of *C. soyoae* is added to the medium. Momma and coworkers observed for 1 year and 9 months the same deep seabed area inhabited by *C. soyoae* (Momma et al. 1995). They reported only small changes in temperature at the seabed, ranging between 2.46° and 3.25°C throughout the year, thus explaining why almost all the mantle cells detached and died when the temperature was elevated above 4°C.

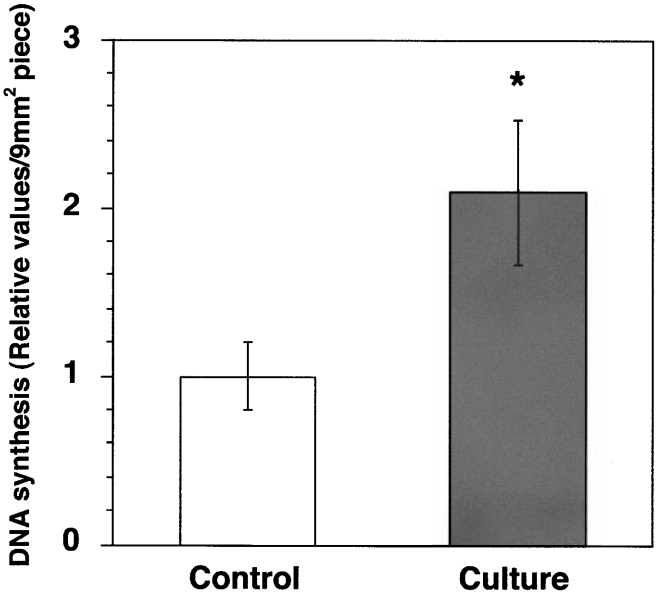

Fig. 5. DNA synthesis in cultures of mantle cells. Mantle cells were cultured in the medium containing either 0 μM (control, *open bar*) or 20 μM (culture, *closed bar*) IdU (5-iodo-2′-deoxyuridine) for 48 h. After incubation, the fragments were reacted with anti-IdU antibody conjugated with peroxidase. The amount of DNA synthesis was measured by peroxidase activity. Values shown are the means ± SEM of three independent experiments (*$P < 0.05$ compared to the control). The data were analyzed by Student's *t*-test (Ichihara 1990)

When the mantle tissues were cultured in body fluid-free medium, no or few live cells were observed. Moreover, almost all the cells died after a few days of culture when –20°C freeze-stored body fluid was added to the medium. These results suggest that cultivation of the mantle cells requires the growth factor(s) in the fresh blood-like body fluid of *C. soyoae*. Because almost all growth factors, including proteins and chemical compounds, can be stored in frozen solutions, this result seems to suggest that the blood-like body fluid contains complement-like material(s) that act as a host defense mechanism and consequently induce cell death.

The deep-sea clam *C. soyoae* has chemoautotrophic symbiotic bacteria in the gill (Sakai et al. 1987; Ishii et al. 1988; Hashimoto et al. 1994); therefore, the chemoautotrophic consumption of hydrogen sulfide might require growth factors. Masuzawa and coworkers reported that *C. soyoae* has a high concentration of heavy metals such as Ag (×250), Cr (×43), Zn (×15), Hg (×12), and Sb (×11) compared with the shallow-water clam *Mytilus edulis* from Matoya Bay, Japan (Masuzawa et al. 1988).

These elements and their degrees of accumulation are very similar to findings in *Calyptogena magnifica* collected from the metal-rich hydrothermal vent on the 21° N East Pacific Rise (Masuzawa et al. 1988; Roesijadi and Crecelius 1984). In addition to the chemoautotrophic consumption of hydrogen sulfide (Masuzawa et al. 1992), *C. soyoae* mantle cells might also need these heavy metals as growth components. Because the collected *C. soyoae* live in seabeds at depths from 1100 to 1400 m, further research should be conducted to elucidate the effective roles of the body fluid on proliferation of the cells under high pressure and low temperature.

References

Cecil JT (1969) Mitoses in cell cultures from cardiac tissue of the surf clam *Spisula solidissima*. J Invertebr Pathol 14:407–410

Hashimoto J, Fujikura K, Fujiwara Y, Sukizaki S (1994) Relationship between life habit of vesicomyid clams and concentration of sulfide in interstitial water, off Hatsushima site, Sagami Bay. JAMSTEC J Deep Sea Res 10:381–388

Ichihara K (1990) Statistics for bioscience–practical technique and theory. Nankoudo, Tokyo

Ishii T, Watanabe M, Ishizuka T, Ohta S, Sakai H, Haramura H, Shikazono N, Togashi K, Minai Y, Tominaga T, Chinzei K, Horikoshi M, Matsumoto E (1988) Geological study with the "SHINKAI 2000" in the West Sagami Bay including *Calyptogena* colonies. JAMSTECTR Deep Sea Res 4:189–218

Li MF, Stewart JE (1966) In vitro cultivation of cells of the oyster, *Crassostrea virginica*. J Fish Res Board Can 23:595–599

Masuzawa T, Handa N, Koyama M (1988) Trace element composition of *Calyptogena soyoae* from a giant clam colony off Hatsushima Island, Sagami Bay, Japan. JAMSTECTR Deep Sea Res 4:225–232

Masuzawa T, Handa N, Kitagawa H, Kusakabe M (1992) Sulfate reduction using methane in sediments beneath a bathyal "cold seep"giant clam community off Hatsushima Island, Sagami Bay, Japan. Earth Planet Sci Lett 110:39–50

Momma H, Mitsuzawa K, Kaiho Y, Iwase R, Fujiwara Y (1995) Long-term deep sea floor observation off Hatsushima Island in Sagami Bay–one year in the *Calyptogena soyoae* clam colony. JAMSTEC J Deep Sea Res 11:249–268

Naganuma T, Akutsu T, Ishida T, Kato C, Horikoshi K (1996) In situ RT-PCR from the β-tubulin mRNA in abalone cells in primary cultures. J Mar Biotechnol 4:75–81

Odintsova NA, Khomenko AV (1991) Primary cell culture from embryos of the Japanese scallop *Mizuchopecten yessoensis* (Bivalvia). Cytotechnology 6:49–54

Okutani T, Egawa K (1985) The first underwater observation on living habit and thanatocoenoses of *Calyptogena soyoae* in bathyal depth of Sagami Bay. Venus (Jpn J Malacol) 44:285–288

Roesijadi G, Crecelius EA (1984) Elemental composition of the hydrothermal vent clam *Calyptogena magnifica* from the East Pacific Rise. Mar Biol 83:155–161

Sakai H, Gamo T, Endo K, Ishibashi J, Yanagisawa F, Kusakabe M, Akagi T, Ishizuka T, Igarashi G, Ohta S (1987) *Calyptogena* colonies on the seabed off Hatsushima, Sagami Bay: a geochemical study with SHINKAI 2000. JAMSTECTR Deep Sea Res 3:75–90

Takeuchi Y, Yamamoto S, Odo S (1994a) Primary and secondary cultures of larval cells of pacific oyster, *Crassostrea gigas*. J Mar Biotechnol 1:171–175

Takeuchi Y, Yamamoto S, Odo S (1994b) Primary culture of larval cells of the mussel, *Mytilus edulis galloprovincialis*. J Mar Biotechnol 2:51–54

Takeuchi Y, Inoue K, Miki D, Odo S, Harayama S (1999) Cultured mussel foot cells expressing byssal protein genes. J Exp Zool 283:131–136