Extremophiles (2001) 5:409–415
Digital Object Identifier (DOI) 10.1007/s007920100213

ORIGINAL PAPER

Sumihiro Koyama · Tetsuya Miwa · Takako Sato
Masuo Aizawa

Optical chamber system designed for microscopic observation of living cells under extremely high hydrostatic pressure

Received: February 1, 2001 / Accepted: May 22, 2001 / Published online: October 11, 2001

Abstract A novel pressure chamber system has been developed for the study of living cells under conditions of extremely high hydrostatic pressure up to 100 MPa (1 atm = 0.101325 MPa). The temperature in the chamber is thermostatically controlled in the range from 2° to 80°C. Two high-pressure pumps are employed for continuous perfusion of the chamber with culture medium and a chemical solution under high hydrostatic pressure conditions. The chamber has a 2-mm-thick glass window 2 mm in diameter, with a minimum working distance of 3.8 mm. The chamber system is designed to be adaptable to a variety of microscopic and imaging techniques. Using this chamber system, we successfully carried out real-time observations of elongated *Escherichia coli* and rounded HeLa cells under pressure.

Key words Optical chamber · Hydrostatic pressure · *Escherichia coli* · HeLa cells

Introduction

Since the discovery of piezophilic and piezotolerant organisms (reviews in Yayanos 1995; Kato and Bartlett 1997; Abe and Horikoshi 2001), much interest has been focused on effects of hydrostatic pressure on a variety of deep-sea and surface-dwelling organisms. Some microorganisms such as *Serratia marinorubra*, *Flavobacterium okeanokoites*, and *Escherichia coli* become extremely elongate under pressure (ZoBell and Cobet 1964; ZoBell and Oppenheimer 1950). Filaments are formed by nearly all bacteria either as a stage in the life cycle or as a result of injury by chemical agents, nutritional deficiencies, or physical conditions (reviewed in Hughes 1956). Hydrostatic pressure of 30 MPa or greater reversibly induces mammalian cells to round up (Landau 1960, 1961; Bourns et al. 1988; Crenshaw et al. 1996). At pressures between 30 and 60 MPa, mammalian cells respread after pressure release and progress normally through the cell cycle (Landau 1960, 1961). Although the relationship between hydrostatic pressure and organisms has been extensively studied (reviews in Yayanos 1995; Bartlett et al. 1995; Kato and Bartlett 1997; Abe et al. 1999; Abe and Horikoshi 2001), microscopic observations that include the application of fluorescent dyes under pressure have been hampered by the lack of a practical technique.

We have developed a pressure chamber system equipped with high-pressure pumps to continuously perfuse the chamber with culture medium in order to provide nutrition and oxygen to the cells under pressures ranging from atmospheric pressure to 100 MPa. Because it is designed for use in studies of thermophiles from the deep-sea bottom, this novel chamber system is also designed to control the temperature within the range from 2° to 80°C. Previously described pressure chambers are generally unsuitable for our purpose, because they lack a means of continuous perfusion with culture medium at pressures up to 100 MPa (Pagliaro et al. 1995; Salmon and Ellis 1975; Besch and Hogan 1995). Several researchers have described chambers that were perfused with medium at modest pressures (Pagliaro et al. 1995; Besch and Hogan 1995), but pressures over 15 MPa were not used.

The chamber system was primarily designed to meet five objectives: (1) it should be operational under extremely high hydrostatic pressures up to 100 MPa; (2) it should allow continuous perfusion of the chamber with medium for cell maintenance; (3) it should allow control of the rate of change of pressure (30 MPa/min); (4) it should allow exposure of the cells to a chemical solution; and (5) it should have mechanical features compatible with a variety of microscope and imaging techniques.

Communicated by K. Horikoshi

S. Koyama (✉) · T. Miwa · T. Sato · M. Aizawa
Frontier Research System for Extremophiles, Japan Marine Science and Technology Center, 2-15 Natsushima-cho, Yokosuka 237-0061, Japan
Tel. +81-468-679691; Fax +81-468-666364
e-mail: skoyama@jamstec.go.jp

M. Aizawa
Department of Biological Information, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama, Japan

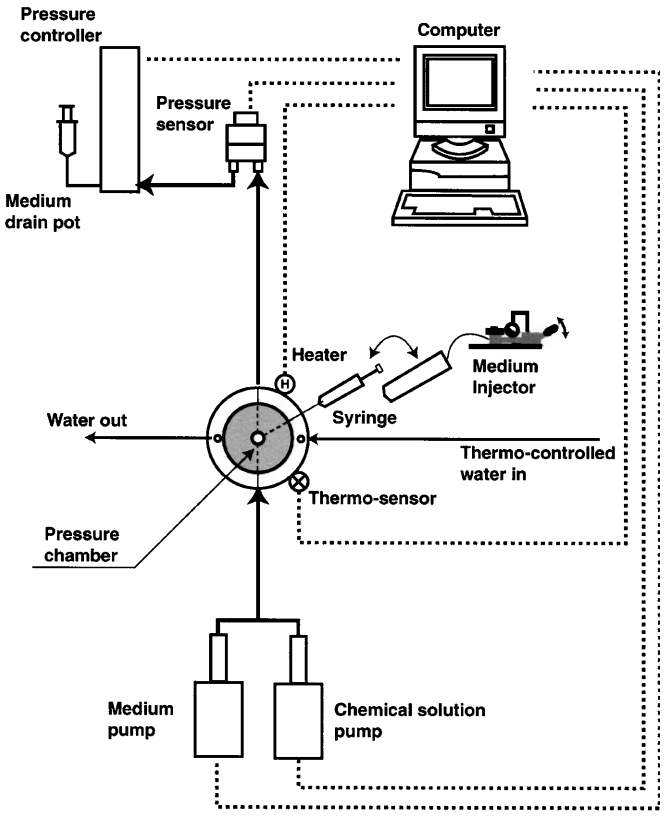


Fig. 1. Diagram of the control system

Here, we describe the details and performance of this novel pressure chamber system using *E. coli* cells and HeLa cells, a human cancer cell line.

Materials and methods

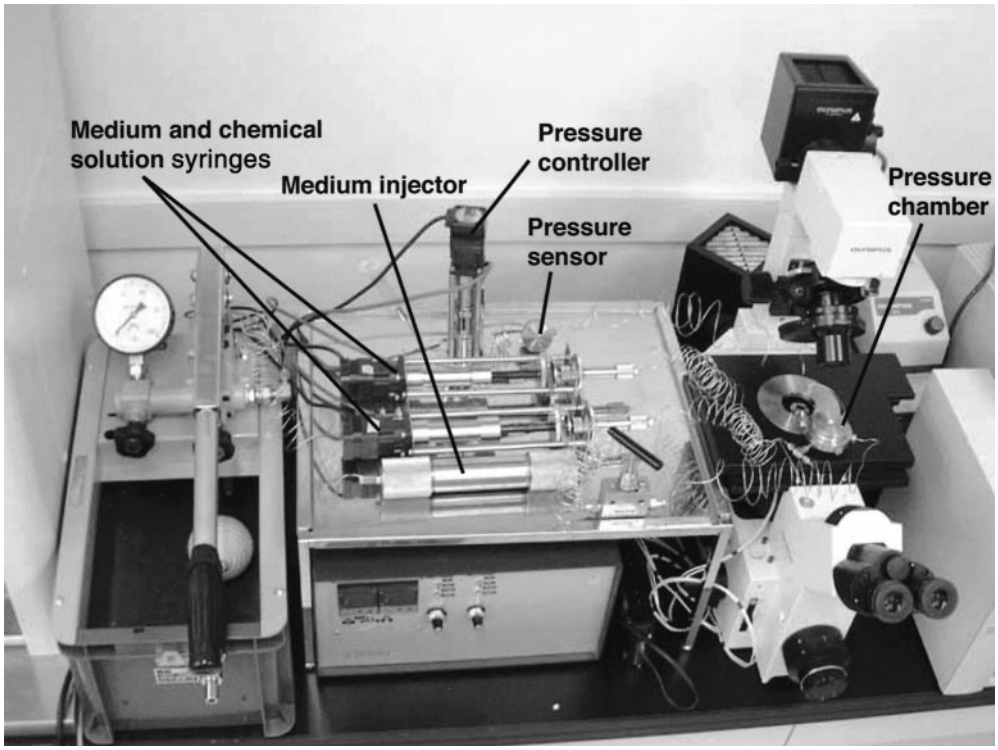
Microscope

The fluorescence and differential interference contrast microscope (IX70) used was purchased from Olympus (Tokyo, Japan). An Olympus color chilled 3CCD camera (M-3204C) was also used.

Chamber design

The pressure chamber system (Figs. 1, 2, 3, 4) was constructed using SUS304 stainless steel. The upper window, 8-mm-thick Pyrex glass, was of conventional design and was weakly screwed onto the upper chamber lid. The optical window, a 2-mm-diameter hole on the lower side of the chamber, consisted of a 2-mm-thick Pyrex cover glass weakly screwed onto the bottom chamber lid. Nitrile O-ring gaskets were employed to tightly seal the pressure chamber by means of the inner high hydrostatic pressure itself, and leakage of the medium was thereby avoided. Cells adhering to the inner surface of the bottom window were within

Fig. 2. Photograph of the assembled control system



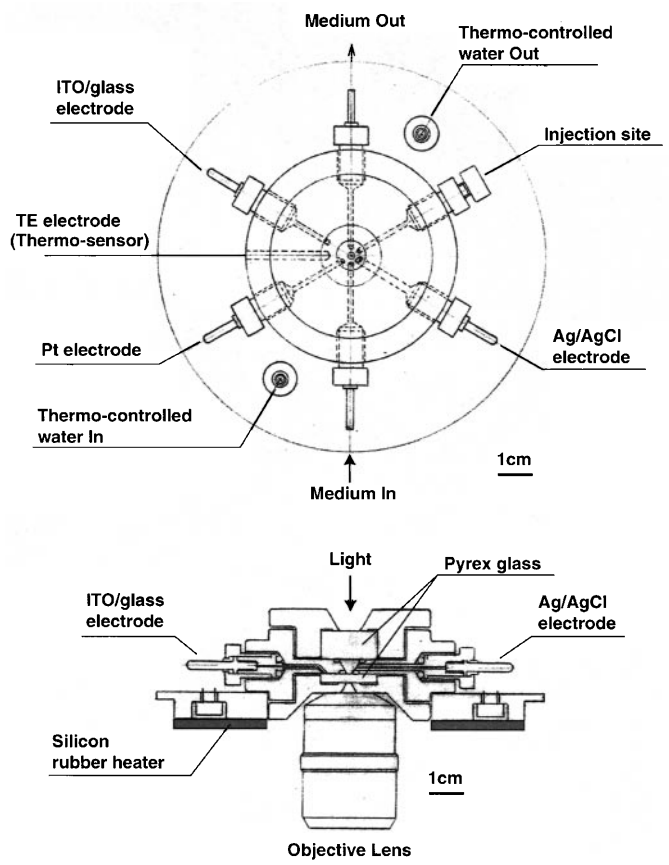


Fig. 3. Detailed drawing of the chamber. The material is SUS304 stainless steel. *TE electrode*, thermoelectric electrode; *ITO*, indium tin oxide

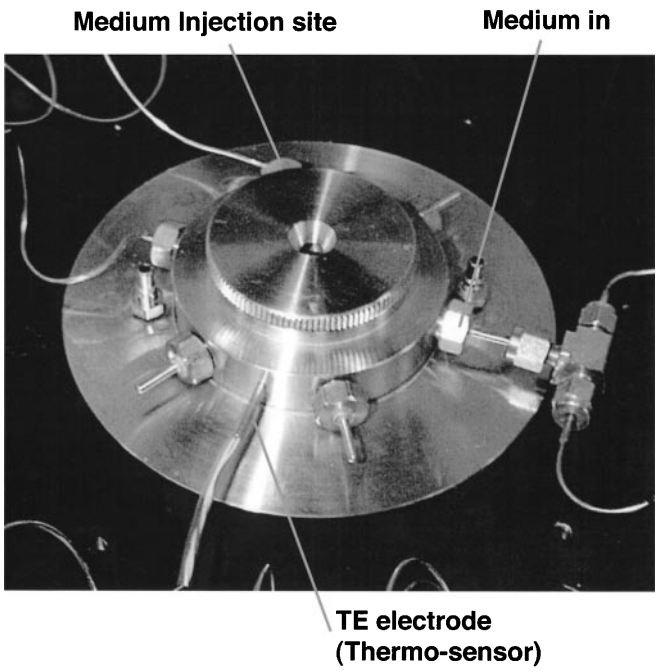


Fig. 4. Photograph of the assembled chamber

working distance (= 3.8 mm) of the objective lens of the inverted microscope. For observation of microorganisms, the cells were injected into the chamber system at an injection site (Figs. 1, 3). An objective lens with a magnification of 20× or greater could focus on the inner surface of the bottom window because of the interaction of the transillumination with the side wall. The chamber, medium ducts, and the medium syringe pumps must be sterilized by autoclaving before use.

Perfusion and pressure control

Delivery of solutions to the chamber was achieved through the action of two 0.75-ml syringe pumps (Figs. 1, 2). The chamber system was designed to allow the use of an increased number of syringes. The rate of compression and the flow rate were controlled by the stepping motors connected to the syringes (Figs. 1, 2). The rate of decompression was controlled by a pressure controller (Figs. 1, 2). Faster compression and decompression could be obtained through the use of a hand pump (Figs. 1, 2), which was connected to the injection site (Fig. 3). The pressure was monitored through a pressure sensor (range up to 101.83 MPa) and controlled using a DOS/V computer (Valuestar NX model VE-40D/5, NEC, Tokyo, Japan) with instrument control software and Windows 98.

Temperature control

The chamber temperature was in the range from 2° to 80°C and was monitored and controlled by a thermo-control system that used a thermoelectric (TE) electrode sensor and a silicon rubber heater to generate thermo-controlled water (Figs. 1, 3).

HeLa cell culture under high hydrostatic pressure

A 2-mm-thick cover glass was coated with 0.1 mg/ml poly-D-lysine (Becton Dickinson Labware, MA, USA) in PBS for 1 h at 37°C. Thereafter, the cover glass was washed with phosphate-buffered solution (PBS). HeLa cells purchased from Dainippon Seiyaku (Tokyo, Japan) were seeded on the cover glass and cultured in Dulbecco's modified Eagle medium (DMEM; ICN Biomedicals, Aurora, OH, USA) supplemented with 10%(v/v) fetal bovine serum (FBS; BioWhittaker, Walkersville, MD, USA), 50 units/ml penicillin, and 50 µg/ml streptomycin (BioWhittaker). The cells were grown for 1–2 days at 37°C in a humidified atmosphere of 5% CO_2.

When the cells had formed a confluent monolayer, the cover glass was placed in the high-hydrostatic pressure chamber (Fig. 2) that was filled with Dulbecco's modified Eagle medium (DMEM). The perfusion medium was adjusted to pH 7 using 1 M HCl under atmospheric CO_2 conditions. The cells were then exposed to hydrostatic pressures up to 50 MPa. Medium perfusion speed was set to 0.8 ml/min.

Real-time observation of *Escherichia coli* cells under pressure

Escherichia coli cells (strain JM109) were precultured in 1 ml of LB medium overnight at 37°C. One hundred microliters of the cultured medium was injected into a pressure chamber, which was filled with fresh LB medium, through an injection site (Figs. 1, 3) using a Hamilton 100-µl microsyringe (Type PT-5, Hamilton, Reno, NV, USA). After these procedures, the *E. coli* cells were exposed to hydrostatic pressure up to 45 MPa with compression speeds of 2.25 MPa/min. The medium perfusion speed was set to 0.8 ml/min.

Real-time observation of *Escherichia coli* and HeLa cells under pressure

Several researchers have reported the effects of rapidly increasing pressure (at a rate of a few MPa/s) on mammalian cells, which were fixed under pressure (Bourns et al. 1988; Crenshaw et al. 1996). In the present study, we examined the effect of slowly increasing the pressure (0.56 MPa/min) on HeLa cells. The HeLa cells had grown to form a confluent monolayer on the 2-mm-thick cover glass under atmospheric pressure conditions. Fig. 6 shows the morphological changes observed in the HeLa cells under elevated

Results

Pressure and temperature regulating performance

The chamber system, illustrated in Figs. 1 and 3, is designed to fit a variety of microscopic and imaging techniques. Table 1 shows the characteristics and performance parameters of the chamber system. The cells can be observed in situ while the chamber is being perfused with the culture medium under extremely high hydrostatic pressure in the range from 0.1 MPa to 100 MPa. The maximum rates of compression and decompression were both approximately 30 MPa/min. The temperature of the medium could be controlled between 2° and 80°C by the TE electrode sensor, the silicon rubber heater, and the thermo-controlled water (Figs. 1, 3). In addition, for stimulating the cells by electrical potential (Koyama et al. 1996, 1997), the chamber system had indium tin oxide (ITO)/glass working, Ag/AgCl reference, and Pt counter electrodes (Figs. 3, 4).

To examine the performance of the pressure chamber system, the stability of the pressure and temperature controls was tested (Fig. 5). Figs. 5a and b show the performance of the computer-controlled hydrostatic pressure levels. Even if the set pressure value was rapidly changed, the pressure in the chamber system followed the set value. When the pressure was set at 70 MPa, the range of pressure fluctuation was 70 ± 2.7 MPa (Fig. 5b). On the other hand, the range of temperature fluctuation was 37 ± 0.3°C when the temperature was set at 37°C (Fig. 5c).

Table 1. Characteristics of the chamber system

Pressure	0.1–100 MPa
Compression speed	Max. approx. 30 MPa/min
Temperature	2°–80°C
Optical window area	3.14 mm^2
Culture volume	Approx. 100 µl
Pump volume	750 µl×2 (or more)
Cover glass thickness	2 mm
Objective lens (Olympus)	20×/0.40 N.A. LCPlanFl
	40×/0.55 N.A. SLCPlanFl

N.A. numerical aperture

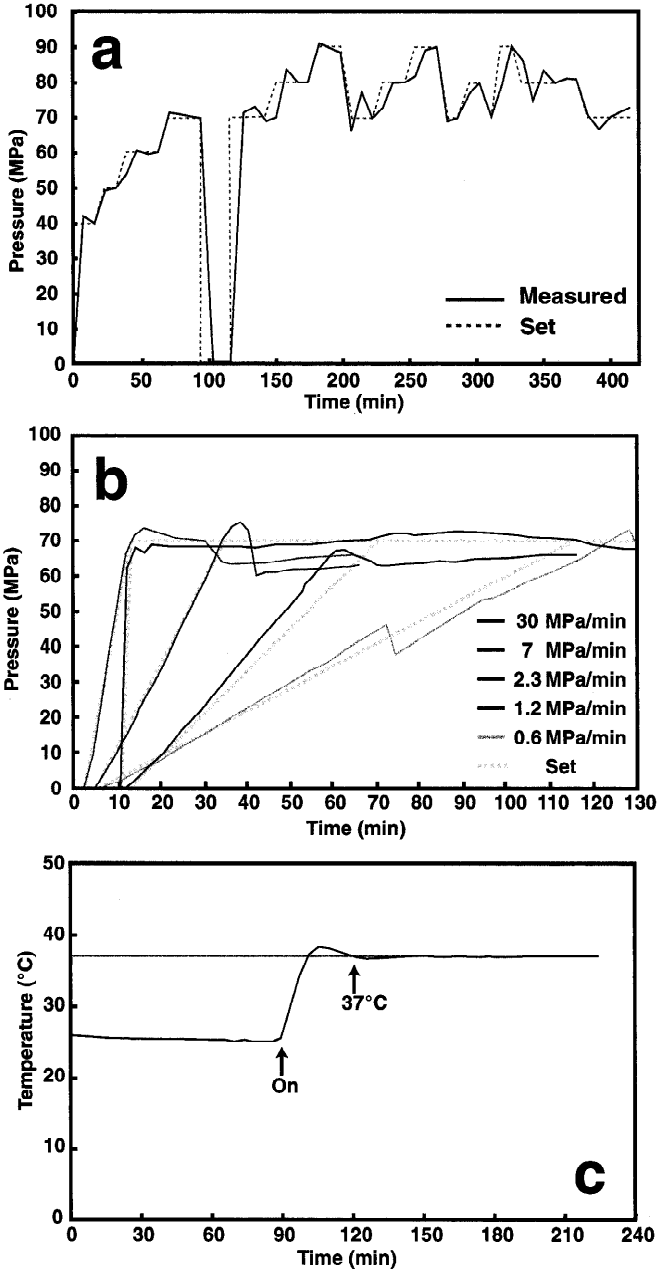


Fig. 5a–c. Stability of the pressure and temperature controls: **a** pressure control, **b** compression rate control, and **c** temperature control

pressure at 37°C. The culture medium was continuously perfused at a rate of 800 µl/min. Even though the rate of compression, 0.56 MPa/min, was extremely slow, a slight change in the morphology of the HeLa cells was observed at 25 MPa and noticeable cell rounding and retraction were observed at over 40 MPa (Fig. 6). After 17 h of 50-MPa pressurization, all of the HeLa cells were disrupted and had died as a result of exposure to high hydrostatic pressure.

To confirm whether microorganisms can also be observed using the pressure chamber system, we investigated the elongation of *E. coli* under pressure. One hundred microliters of *E. coli* cell suspension (strain JM109), which had been grown overnight in LB medium at 37°C, was injected into the pressure chamber using a 100-µl Hamilton syringe and then pressurized up to 45 MPa at a rate of 2.25 MPa/min. To provide nutrition and oxygen to the cells, fresh LB medium was continuously perfused at 800 µl/min and 37°C.

Although no or few elongated cells were found during the pressurization time (Fig. 7a,b) because the cells floated in the medium, we succeeded in observing elongated *E. coli* cells during 20 h of cultivation under 45 MPa pressure (Fig. 7c). This is the first real-time observation of living microorganisms in a high-hydrostatic pressure environment.

Discussion

Here we have described a compact, perfused, and temperature-controlled pressure chamber system, which was specifically used for microscopic observation. Previously described pressure chambers were designed for observation of terrestrial mammalian cells (Pagliaro et al. 1995; Salmon and Ellis 1975; Besch and Hogan 1995). Therefore, these pressure chambers are generally unsuitable for microscopic observation of deep-sea microorganisms and deep-sea multicellular organisms under extreme environmental conditions such as extremely high pressure together with high or low temperatures.

Several researchers have reported that a pressure greater than 30 MPa induces significant morphological changes in mammalian cells (Landau 1960, 1961; Bourns et al. 1988; Crenshaw et al. 1996). In those studies, a hand pump was used, and, therefore, the morphological changes may have been due to the rapid rate of compression. To investigate whether terrestrial cells showing changes in morphology are capable of growth under high hydrostatic pressure conditions, we cultured HeLa cells using our newly developed pressure chamber system. Even though the rate of compression was slow at 0.56 MPa/min, the cell morphology changed substantially at pressures above 25 MPa and all of

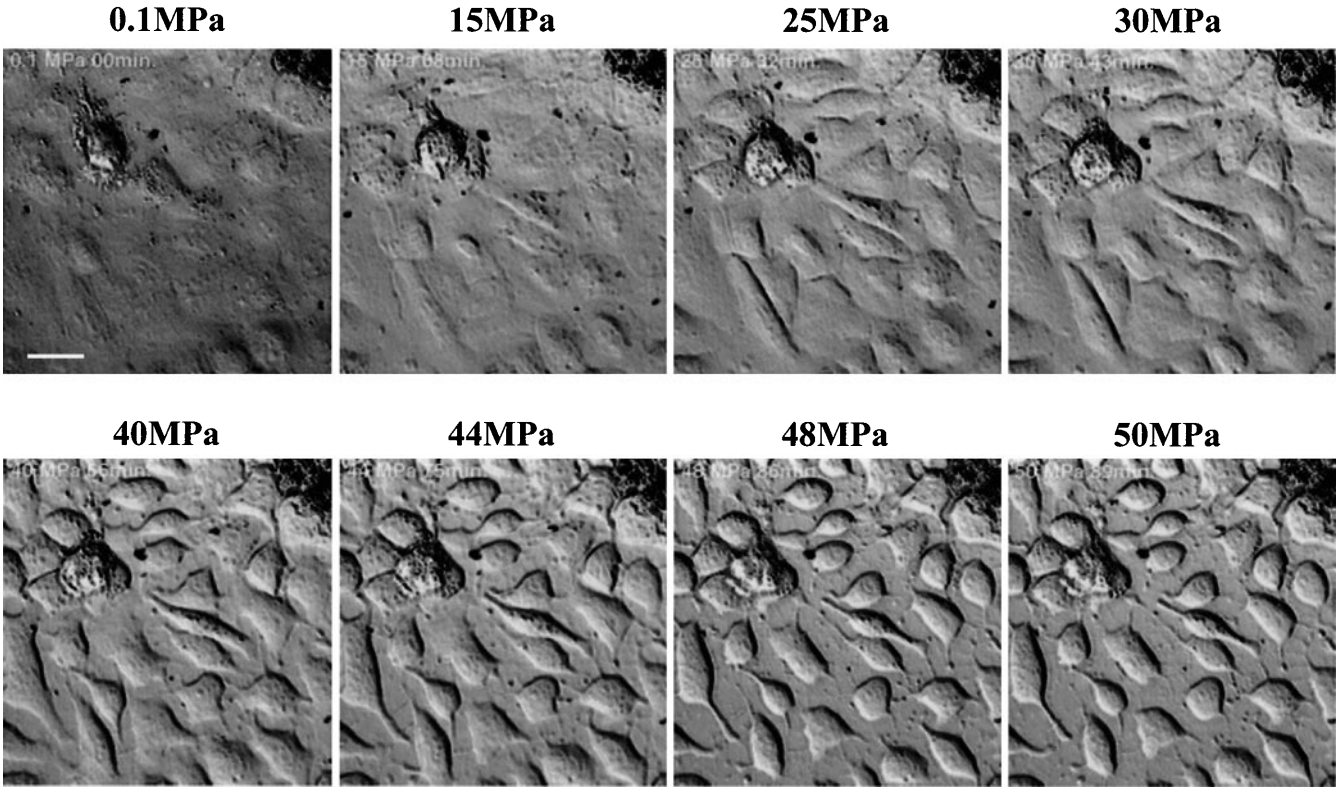


Fig. 6. Time series of differential interference contrast images of HeLa cells under pressure. The *white bar* indicates 20 µm. Compression and medium perfusion rates were 0.56 MPa/min and 0.8 ml/min, respectively

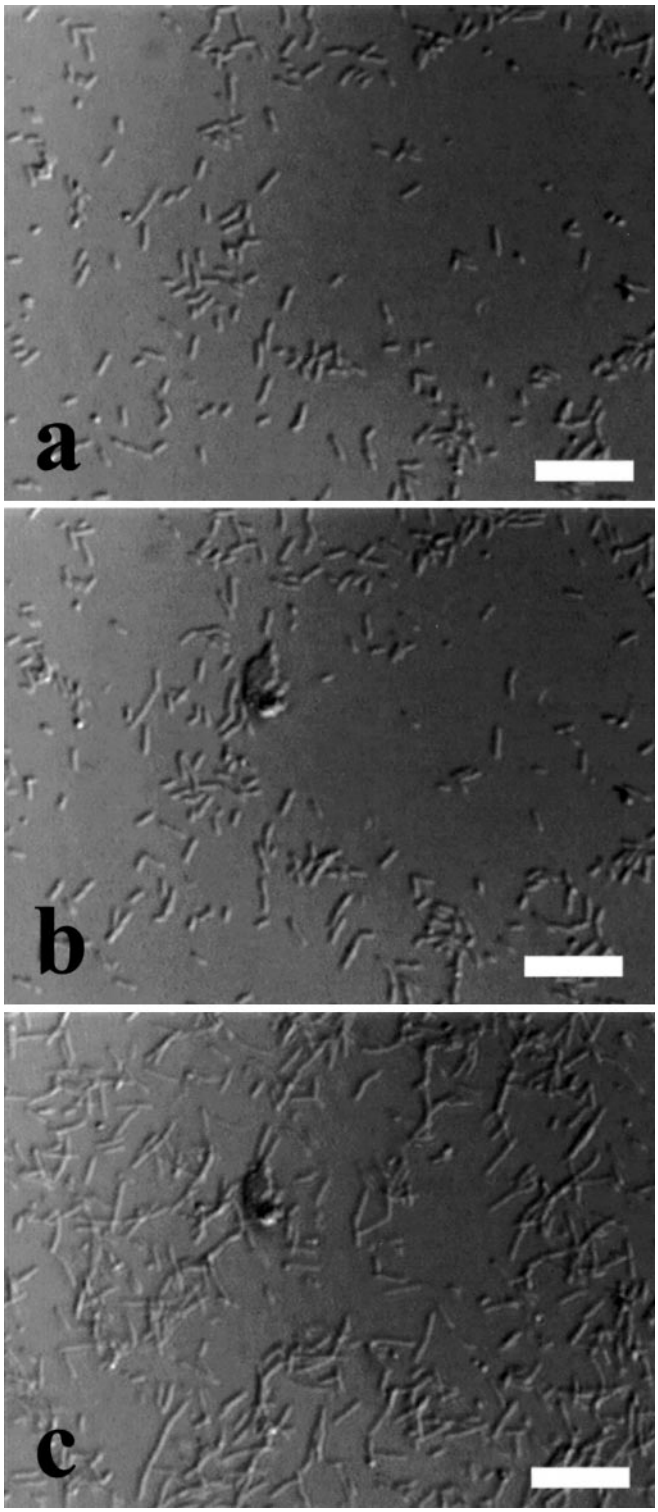


Fig. 7a–c. Pressure-induced elongation of *Escherichia coli* cells. The *white bars* indicate 10 μm. Compression and medium perfusion rates were 2.25 MPa/min and 0.8 ml/min, respectively. **a** 0 min, 0.1 MPa; **b** 20 min, 45 MPa; **c** 10 h, 45 MPa

the cells were disrupted at 50 MPa pressure after 17 h of cultivation. Since 30 MPa or greater hydrostatic pressure reversibly disrupts the organization of the cytoskeleton (Landau 1960, 1961; Bourns et al. 1988; Crenshaw et al. 1996), it appears that such high pressures may alter the thermodynamics of the assembly of cytoskeletal proteins, forcing these proteins to depolymerize.

In cell division of *E. coli*, the first event is polymerization of FtsZ into the Z ring at the future division sites. The other proteins, FtsA, FtsQ, FtsL, FtsI, FtsN, ZipA, and FtsK, localize to the division sites (Margolin 1999; Weiss et al. 1999). The pressure-induced elongation of *E. coli* cell would be induced by results in malfunction of the cell division proteins. Further research should be conducted to clarify the pressure-induced elongation using green fluorescent protein-labeled cell-division proteins.

In conclusion, the novel chamber system described here allows modulation of the cellular environment (chemical milieu, temperature, and pressure) to stimulate, cultivate, and observe terrestrial and abyssal cells.

Acknowledgments We thank Yoichi Ishikawa and Mitsuaki Iwase of ABLE Co., Ltd. for technical assistance and helpful advice.

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