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Effects of low-shear modeled microgravity on a microbial community filtered through a 0.2-μm filter and its potential application in screening for novel microorganisms

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The effects of low-shear modeled microgravity (LSMMG) on a microbial community filtered through a 0.2-μm filter were investigated, and the potential application of LSMMG in the screening of microorganisms was evaluated. Pond water was passed through a 0.2-μm filter and the filtrate inoculated into two kinds of media (Schneider's insect medium, and ten-times-diluted Schneider's insect [0.1-Sch] medium). The cultures were incubated under LSMMG and normal-gravity and the microbial cell growth rates compared. Cell growth rates, final cell concentrations, and substrate consumption rates were higher in the LSMMG culture than in the normal-gravity culture. The microbial communities obtained under the various culture conditions were subjected to denaturing gradient gel electrophoresis (DGGE), revealing three different groups of microorganisms: (i) microorganisms whose growth rates were increased by LSMMG; (ii) microorganisms whose growth rates were suppressed or inhibited by LSMMG; and (iii) microorganisms whose growth rates were not affected by LSMMG. Sequence analysis of the microorganisms whose growth rates were increased by LSMMG showed that some had high similarity with unculturable microorganisms. When these microorganisms that displayed similarity with unculturable microorganisms were cultivated on agar plates, some of the DGGE bands present in the LSMMG culture were also present. We show that it is possible to isolate and cultivate uncultured microorganisms by using combinations of LSMMG, normal-gravity, and agar plate culturing techniques.

[**Key words:** Low-shear modeled microgravity culture; Screening method; Denaturing gradient gel electrophoresis; Rotating wall vessel; 0.2-μm-passable-microorganisms]

In the 19th century, both Robert Koch and Louis Pasteur developed techniques for isolating microorganisms and growing them in pure cultures. Since then, many microorganisms have been isolated, maintained in pure cultures, and utilized in the production of useful metabolites such as antibiotics and amino acids. The establishment of pure cultures of microorganisms has contributed to the development of the pharmaceutical, food, and chemical industries. However, it is becoming increasingly difficult to isolate and maintain pure cultures of new microorganisms, which is significantly limiting progress in industrial microbiology, developed on the basis of pure culture systems. Using independent molecular techniques, it has been estimated that more than 99% of microorganisms existing in nature cannot be isolated and maintained in pure cultures (1–4). To overcome this problem, it has become necessary to develop novel microbial cell culture systems for the isolation of microorganisms. In the present study, we explored the potential of using low-shear modeled microgravity (LSMMG) culture to isolate novel groups of microorganisms. It has been reported that LSMMG affects the physiological activities of various microbial cells in culture. Nickerson et al. (5) reported that *Salmonella enterica* serovar Typhimurium cultured under LSMMG are more virulent and can be recovered in higher numbers from murine spleen and liver following oral infection compared with organisms grown under normal-gravity. Furthermore, when compared with identical growth conditions at normal-gravity, Salmonellae cultured under LSMMG display increased resistance to environmental stresses (acid, thermal, and osmotic), increased ability to survive within macrophages, and altered protein levels (6,7). Other reports have shown effects of LSMMG on resistance to radiation and phage induction (8), secondary metabolite production (9–11), length of lag phase (12–15), and bio-film formation (16,17).

Although LSMMG culture of many microorganisms has been reported, no reports have detailed whether the sensitivities of microorganisms to LSMMG vary. It is not yet clear whether all microorganisms respond in the same way to LSMMG. Furthermore, most studies on LSMMG cultures employed monocultures; the cultivation of microbial communities under LSMMG and the application of LSMMG culture for the screening of microorganisms have not been reported.

Filtration of liquids through 0.2-μm filters is also a common method for the removal of microorganisms from heat-sensitive solutions. Filtration using a 0.2-μm filter is frequently referred to as 'sterile filtration', reflecting the general belief that all living

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organisms are excluded from the filtrate. However, it has been demonstrated that some bacterial species can pass through 0.2-μm filters and many kinds of novel bacteria were isolated from 0.2-μm-filtrated samples by using the acclimatization method (18,19).

In the present study, the effects of LSMMG on the growth of 0.2-μm-passable-microorganisms community in pond water filtrate and its potential application in screening for novel microorganisms were investigated.

MATERIALS AND METHODS

High-aspect-ratio rotating wall vessel bioreactor used for LSMMG culture Cells were cultivated under LSMMG by means of a high-aspect-ratio rotating wall vessel (HARV) bioreactor (Synthecon, Inc., Houston, Tex., USA [Schwarz, R. P. and Wolf, D. A., U.S. Patent 4988623, 1991]). The bioreactor keeps the cells in suspension as they continually 'fall' through the medium under 1 ×*g* conditions. The HARV bioreactor produces an environment in which the gravitational vectors are randomized over the surfaces of the cells. This randomization emulates a state of constant free-fall, creating a sustained LSMMG environment. The HARV design allows constant rotation normal to the gravitational field, which results in environmental vectors. This system was designed to simulate in the laboratory the effects of weightlessness or microgravity on cells (20).

Prevention of media evaporation through humidification Cultivation in the HARV bioreactor results in media evaporation, which leads to a reduction in culture volume and consequent bubble formation. This affects the gravity experienced by the cells, and long-term cultivation of cells under LSMMG is difficult because of this bubble formation (21). To prevent medium evaporation, a special incubator (the ultrasonic humidifier control system FT-10N-14 [Ucan Co. Ltd., Tokyo, Japan] was attached to the incubator CN-25 B [width = 265 mm, depth = 260 mm, height = 360 mm, chamber capacity = 25 L, Mitsubishi Electric Engineering Co. Ltd., Tokyo, Japan]), that could be accurately maintained at 95–98% relative humidity with temperature control was constructed (the relative humidity and temperature were homogenous inside the chamber). The HARV bioreactor (reactor volume and culture volume = 10 mL) was set inside the incubator, and the culture volume inside the vessel was monitored during the LSMMG cultivation.

Cultivation of microbial community under LSMMG Two different media were used for cultivating the microbial community: (i) Schneider's insect medium (pH 6.7) (Sigma, MO, USA) (22). This medium is used in our laboratory for the isolation of microorganisms. (ii) Ten-times-diluted Schneider's insect [0.1-Sch] medium (pH 6.7).

Sample collection A water sample from a pond within the premises of the University of Tsukuba, Japan, was used as the model environmental microbial community. The sample was filtered through a membrane filter (pore size: 0.2 μm; Millipore, Billerica, MA, USA), and the filtrate (40 mL) was centrifuged at 10,000 ×*g* for 30 min. The supernatant was discarded and the remaining cell pellet resuspended in 10 mL of one of the media and cultivated in the HARV bioreactor (reactor volume and culture volume = 10 mL) under LSMMG or normal-gravity. In the microgravity orientation, the axis of rotation of the HARV was perpendicular to the direction of the gravity force vector, and in the normal-gravity orientation, the axis of rotation was parallel to the direction of the gravity force vector. The cells were incubated at 37°C, and the rotation speed of the HARV bioreactor was 50 rpm. The concentration of the microbial community was monitored by measuring unit optical density at 400 nm ($U.O.D._{400}$), and a glucose C kit II (Wako Pure Chemical Industries) was used to measure the glucose concentration in the medium. All the experiments were performed in triplicates. The results were expressed as mean values and there were less than 6% deviation in the results.

Analysis of the microbial community A FastDNA kit (Bio 101, Vista, CA, USA) was used to extract the DNA from the cultivated cells, and the extract was subjected to denaturing gradient gel electrophoresis (DGGE) according to the method described by Muyzer et al. (23) and Iwamoto et al. (24). Total DNA was extracted from the cells. The V3 region of the bacterial 16S rDNA fragment was amplified using the PCR primers 357F-GC (5′-CGCCCGCCGCGC GCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3′) and 518R (5′-GTATTACCGCGGCTGCTGG-3′). Each amplification reaction mixture (20 μL) consisted of 0.5 U *Ex Taq* DNA polymerase (Takara Shuzo, Kyoto, Japan), 1 μL of total DNA solution, 2 μL of 10× PCR buffer, 0.25 μM of each primer, and a mixture containing 100 μM deoxynucleoside triphosphate. A touchdown program (23,25) was implemented as follows: after the initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 1 min and 72°C for 1 min were performed, and then the reaction mixture was kept at 72°C for 7 min. During the reaction cycle, the annealing temperature was decreased by 1°C steps from 65°C to 56°C every second cycle in the first 20 cycles. The annealing temperature was 55°C for the last 10 cycles. The amplicons were purified with a QIAquick PCR purification kit (Qiagen, Crawley, UK), and DNA concentration was determined by measuring the OD_{260} of the purified amplicon solutions. For the DGGE, 250 ng of the purified amplicons was used, and it was performed using the D-code system (Bio-Rad Laboratories, Hercules, CA, USA). Acrylamide (8%) gels were prepared and electrophoresed with 0.5× Tris–acetate–EDTA (TAE) buffer (1× TAE buffer consisted of 0.04 M Tris base; 0.02 M sodium acetate; and 10 mM EDTA, pH 7.4). The DGGE gel contained 20–70% gradients of urea and formamide in the direction of electrophoresis as denaturants. The 100% denaturant consisted of 40% (v/v) formamide and 7 M urea. DGGE was performed at a constant voltage of 35 V at 60°C for 24 h. The gel was stained with SYBR Gold nucleic acid gel stain (Molecular Probes, Eugene, OR, USA) and photographed using a UV transilluminator. The DGGE band profile was analyzed using an image-analyzing system (Image master; Amersham Pharmacia Biotech, Uppsala, Sweden), and the densities and migration distances of the bands were calculated.

DNA was extracted from the gel pieces by incubation at 4°C for 3 days, and then 0.5 μL of the supernatant was used as the template DNA in a touchdown PCR performed with the PCR primers GC-2 (5′-GAAGTCATCATGACCGTTCTGG-CACGGGGGGCCTA-3′) (25) and 518R. The amplification mixture and conditions of the touchdown PCR were the same as those used for the amplification of DNA for DGGE as described above. The resulting amplicons were electrophoresed again on a DGGE gel to verify the position of the original band. Subsequently, the amplicons were purified using a QIAquick PCR purification kit (Qiagen) and then cloned by using the pGEM-T Easy Vector System (Promega, Madison, WI, USA). Ligation products were transferred into competent cells of *Escherichia coli* DH5-α. White colonies were randomly picked and screened directly for inserts by colony PCR using primers for the vector (primers T7 and SP6). Plasmid DNA was prepared from the clones using a QIAprep Spin Miniprep Kit (Qiagen). Plasmid DNA was then sequenced according to the direction of insertion by using a CEQ 2000 XL DNA sequencer (Beckman, Fullerton, CA, USA). All of the sequences were analyzed using the SeqMan software (DNAstar, Madison, WI, USA) and compared with the sequences of reference organisms by performing a BLAST search (26,27) at the website of the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/).

Nucleotide sequence accession number The nucleotide sequences of partial 16S rRNA genes have been deposited in the DDBJ nucleotide sequences databank under the following accession numbers: UM-CS2, AB667795; UM-CS3, AB667796; UM-MS1, AB667797; UM-M0.1S2, AB667798; UM-M0.1S4, AB667799; UM-M0.1S5, AB667800.

Plate wash (PW)-PCR-DGGE analysis The PW-PCR-DGGE analysis (4) was performed as follows; all of the bacterial colonies that grew on the agar plates were suspended in sterilized distilled water and placed into a 1.5-mL microtube. The suspensions were collected by centrifuging at 12,000 ×*g* for 10 min. The total DNAs of the bacterial colonies were extracted using a FastDNA kit (Bio 101) and analyzed using the PCR-DGGE method as described above.

RESULTS AND DISCUSSION

Without humidity control, about 4% of the culture broth (Schneider's insect medium) evaporated within 12 h of cultivation, and this led to bubble formation (Fig. 1). By operating the HARV bioreactor inside the specially constructed incubator and keeping the relative humidity between 95% and 99%, culture broth evaporation was prevented and the LSMMG condition was maintained for

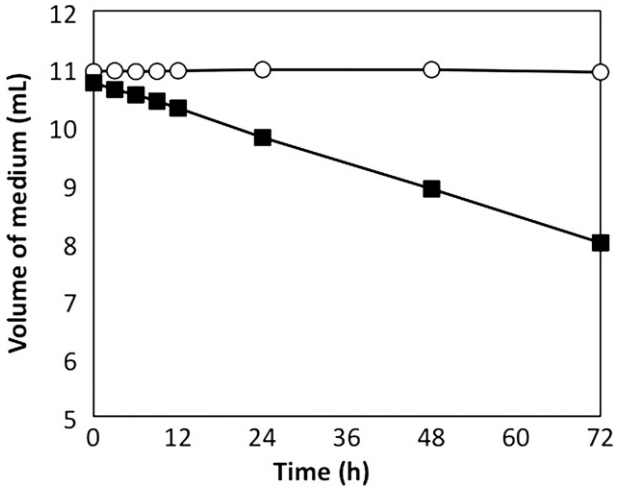

FIG. 1. Effect of humidification on Schneider's insect medium volume in the HARV bioreactor. Symbols: open circles, relative humidity kept between 95% and 99%; closed squares, no humidity control.