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Establishment of an Axenic Culture of Microcystin-Producing *Microcystis aeruginosa* Isolated from a Korean Reservoir

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In order to establish an axenic (bacteria-free) culture of *Microcystis aeruginosa* NIER 10039 isolated from a Korean reservoir, the culture was subjected to sequential treatment, including ultrasonication, washing, and addition of antibiotics. Three broad-spectrum antibiotics, namely, kanamycin, ampicillin, and imipenem, were applied separately in that order. Axenicity of the culture was confirmed by cultivation on bacterial media and observation under epifluorescence and scanning electron microscopes. We are the first to establish an axenic culture of a *Microcystis* strain isolated from Korean reservoirs and can be used in physiological and molecular studies to control toxic *Microcystis* blooms.

Keywords: Axenic culture, *Microcystis*, Korean reservoir, sequential treatment, antibiotics

Microcystin-producing cyanobacteria commonly appear in aquatic ecosystems around the world and can threaten the health of humans and animals [4]. *Microcystis* is one of the best studied cyanobacterial genera because it is representative of microcystin-producing cyanobacteria and commonly appears in aquatic ecosystems around the world [4]. Further, these organisms are one of the major bloom-forming cyanobacteria found in Korean freshwaters along with *Anabaena* [1, 15].

Many kinds of bacteria adhere to the surfaces of cyanobacteria in large numbers. In order to avoid interference by these bacteria in physiological and molecular studies on the target cyanobacteria, an axenic (bacteria-free) culture of cyanobacteria is needed [5, 9].

Over thirty *Microcystis* strains isolated from several eutrophic lakes in U.S.A., Europe, Australia, China, and Japan have been induced as axenic cultures by centrifugation cleaning, cultivation on solid medium, selection using sulfide gradients, and antibiotic treatments [3, 8, 10, 14, 22]. So far, no axenic culture of *Microcystis* strains isolated from Korean reservoirs has been obtained.

The objective of this study was to obtain axenic culture of *Microcystis* strains isolated from a Korean eutrophic reservoir by subjecting the culture to sequential treatment, including ultrasonication, washing, and the addition of antibiotics.

Methods, Results and Discussion

In order to establish an axenic culture, we used *Microcystis aeruginosa* NIER 10039 courteously provided from the National Institute of Environmental Research (NIER) in Korea. This strain was isolated from the Daechung reservoir, a representative one in Korea used as a source of drinking water supply [12]. The culture was maintained in CB medium in an incubation room at 25°C under fluorescent light at ~40 μE/m^2/s on a 10:14-h dark/light cycle [19].

In order to eliminate heterotrophic bacteria from a cyanobacterial culture, the culture was subjected to sequential treatment, including ultrasonication, washing, and addition of antibiotics. The cyanobacterial culture was diluted with the same volume of CB medium, and treated with an ultrasonic cleaner (150W, WUC-D06H; Daehan Scientific, Korea) for 5 min. It was vigorously vortexed for 30 s, and then filtered through a 1.0-μm pore-sized membrane filter (mixed cellulose ester; Advantec, Japan). The filter was washed thrice with sterilized sodium-phosphate buffer (0.1 M, pH 7.0). Cyanobacteria on the filter paper were then suspended by vortexing for 2 min in a cap tube containing fresh CB medium.

After treatment, the culture was stained with 4', 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich Co., U.S.A.) [16] and observed under an epifluorescence microscope

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(Nikon Eclipse 80i; Tokyo, Japan). Ultrasonication was effective in dispersing *Microcystis* colonies. Filtration with a 1-μm pore-sized membrane filter followed by filter-washing enhanced the removal efficiency of bacterial cells in culture because the size of cultured *Microcystis* cells is bigger than that of bacteria. However, even after these treatments, bacterial cells were not completely removed.

Antibiotics are widely used to selectively remove bacteria associated with cyanobacteria [2, 5, 9]. In this study, three broad-spectrum antibiotics, namely, kanamycin (Bioshop Canada Inc., Canada), ampicillin (Biosesang Inc., Korea), and imipenem (Sigma-Aldrich Co.), were used. Kanamycin is an aminoglycoside antibiotic, which prevents mRNA translation by affecting the 30S ribosomal subunit, and ampicillin and imipenem, which are β-lactam antibiotics, inhibit peptidoglycan biosynthesis. The antibiotic concentrations to be used were determined on the basis of the minimal lethal concentration of the agents, which was determined according to non-inhibition surrounding the test-disk-added antibiotics using a modified disk diffusion method [13].

When each antibiotic was added to the cyanobacterial culture to examine the susceptibility of bacteria to the antibiotic, bacterial growth was observed on growth media; this result indicates that the axenic culture was not established. Although it has been reported that imipenem is the most effective antibiotic for obtaining an axenic culture [2, 6], it could not completely inhibit bacterial growth when used alone. When a cocktail of the three antibiotics was used, it produced severe discoloration and death of *Microcystis* cells.

Antibiotics were added sequentially in order to obtain the axenic culture of filamentous cyanobacteria *Phormidium animalis* and *Arthrospira platensis* [5, 21]. Cyanobacterial cells present on the filter paper subjected to ultrasonication and washing as described above were resuspended in CB medium containing kanamycin (final concentration, 50 μg/ml). After overnight incubation at 25°C, the culture was filtered through a 1.0-μm pore-sized membrane filter to remove kanamycin from the solution. The filter was transferred onto fresh medium after vigorous vortexing for 2 min, and then ampicillin was added (final concentration, 100 μg/ml). The steps of filtration and washing were repeated after overnight cultivation, and then imipenem was added (final concentration, 50 μg/ml). On the next day, imipenem was removed by filtration. The cyanobacterial cells on the filter were resuspended in fresh CB medium and cultured at 25°C for 2 weeks. Axenicity was examined by using three methods: cultivation of bacteria on the medium, epifluorescence examination, and scanning electron microscope (SEM) examination.

In order to check for bacterial growth, the culture was spreaded on three different kinds of media, which are commonly used to culture bacteria: nutrient agar (NA) medium, 1/10 NA medium, and R2A medium [5]. The plates were kept in a dark incubator at 25°C for 2 weeks, and bacterial growth was examined. Because bacteria grow faster than cyanobacteria at the same temperature in the dark, the bacterial colony will be detected on the plates if the contaminants are still in the culture [9]. For epifluorescence microscope examination, the culture was stained with DAPI and observed under an epifluorescence microscope [16]. The samples were treated in a routine manner to prepare specimens for SEM; the samples were subjected to fixation and dehydration, and were observed under a SEM (Hitachi S4300N; Hitachi, Japan).

The culture that was sequentially treated with three antibiotics and spread on three different kinds of bacterial culture media showed no bacterial growth. Furthermore, no bacteria were observed under an epifluorescence microscope and SEM as shown in Fig. 1; this result indicates that an axenic culture of *Microcystis aeruginosa* NIER 10039 was finally obtained. Other sequential combinations of antibiotics

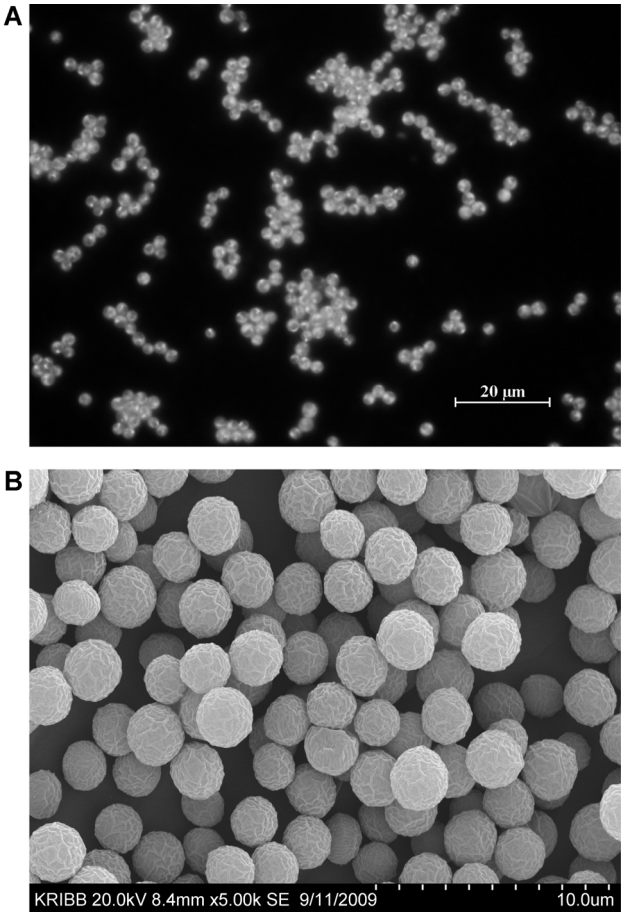

Fig. 1. Epifluorescence microphotograph showing a DAPI-stained axenic culture of *Microcystis aeruginosa* NIER 10039 (**A**) and a scanning electron micrograph (**B**) of axenic *Microcystis aeruginosa* NIER 10039 after ultrasonication, washing, and antibiotic treatment.

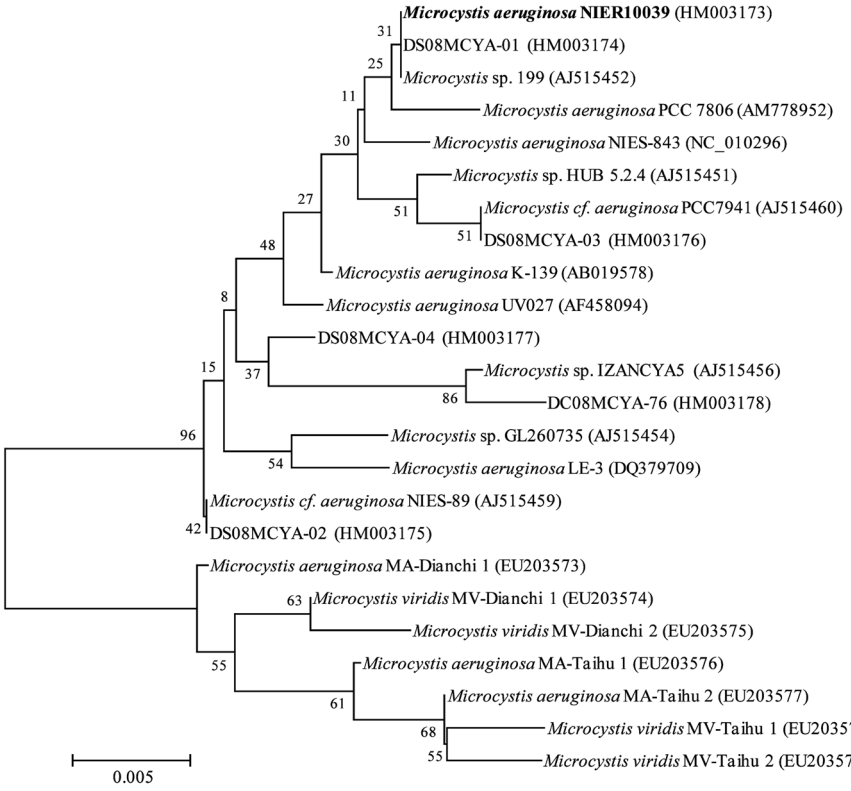

Fig. 2. Phylogenetic tree based on the partial *mcyA* sequence of *Microcystis aeruginosa* NIER 10039 and those taken from the GenBank database.
The tree was constructed by using the neighbor-joining method, and local bootstrap probabilities are indicated at the nodes.

could not remove bacterial contaminants completely. It had been previously reported that the order in which antibiotics are used is important for the establishment of an axenic culture [21]; however, there is no scientific explanation for this. This strategy was also effective for obtaining an axenic culture of *Mychonastes* sp. in our laboratory (data not shown).

Part of the *mcyA* gene in NIER 10039, which codes for a key enzyme in the production of microcystins [7, 17, 18], was amplified using mcyA-Cd1F/mcyA-Cd1R polymerase chain reaction (PCR) primers [7], and the PCR product was sequenced by Solgent (Korea). This sequence and previously reported ones were aligned using the ClustalW multiple alignment function in the BioEdit program, and phylogenetic trees were constructed with MEGA4 using the neighbor-joining tree algorithm [20]. The GenBank accession numbers of the sequences reported here are HM003173 to HM003178.

The sequence of the targeted region of the *mcyA* gene in NIER 10039 (GenBank Accession No. HM003173) was identical to that of *Microcystis* sp. 199 isolated from Rusutjärvi Lake in Finland [7] and that of a dominant clone, DC08MCYA-01 (HM003174), which occupied 35% and 65% of the clonal library constructed from Daechung and Chungju Reservoirs, respectively, in 2008 (Fig. 2) [11]. These results indicated that the established axenic

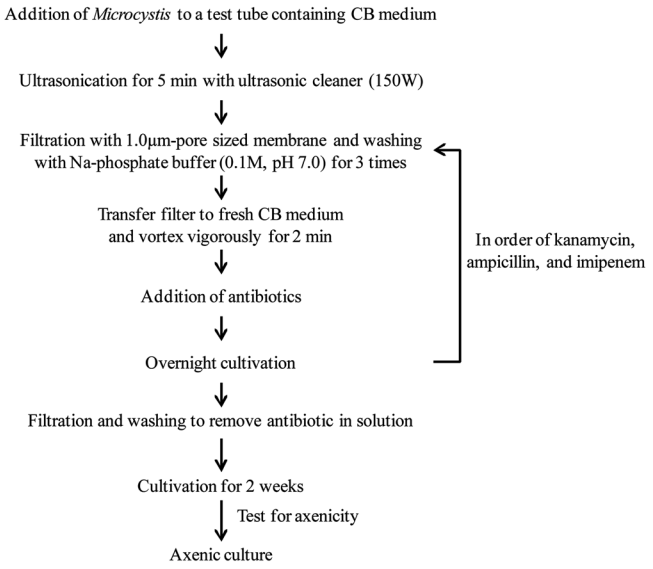

Fig. 3. Purification procedure for obtaining an axenic culture of *Microcystis aeruginosa* NIER 10039.

strain is one of the dominant *Microcystis* strains that produce microcystins in Korean reservoirs.

In conclusion, we are the first to successfully obtain an axenic culture of a *Microcystis* strain isolated from a Korean reservoir using the strategy shown in Fig. 3. This strain is expected to be useful in research on physiological and molecular approaches to control harmful algal blooms originated by *Microcystis* strains in Korean reservoirs.

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