Development of a Solid Medium for Growth and Isolation of Axenic *Microcystis* Strains (Cyanobacteria)

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Solid media on a base of B-12 or CB medium with agarose or agarose of low melting temperature were developed for the cultivation of *Microcystis* species. The media with 0.4% gel showed the highest number of CFU, and increasing the gel concentration resulted in a reduction of the number of CFU. There was no difference in the numbers of CFU between pour and spread plates made of the solid media. By using the solid media, 31 clones of *Microcystis* species were isolated from natural blooms in Lake Kasumigaura, and 5 axenic strains (1 of *M. wesenbergii* and 4 of *M. aeruginosa*) were established from the clones.

In most instances, *Microcystis* species, colony-forming cyanobacteria (blue-green algae), have caused massive surface blooms in eutrophic water (5, 13), and some of the strains and blooms have been found to produce peptide toxins (2, 3). The culturing of cyanobacteria on agar media has been routinely used for maintenance or purification of certain species, but it has long been known that *Microcystis* species cannot grow properly in agar medium (12). Allen and Gorham (1) reported that although some agent(s) in agar inhibited the growth of *Microcystis* species as well as other blue-green algae, the washed agar in ASM-1 medium supported the growth of these algae. To prevent the formation of a growth inhibitor(s) in agar medium, it was suggested that the agar and the minimal medium should be autoclaved separately and then mixed together after cooling (12). Since *Microcystis* organisms form mucilaginous colonies and some bacteria are usually tightly associated with the colonies, isolation of axenic strains from the colonies is very difficult (4, 13). The efforts to obtain axenic cyanobacteria were carried out by radiation with UV light, phenol treatment, addition of antibiotics to culture medium, and physical separation (6). It is essential to obtain bacteria-free cultures of *Microcystis* species for physiological, genetic, and taxonomic studies. In this paper, we deal with the development of solid media suitable for the growth of *Microcystis* species and report a practical method for the isolation of axenic strains from natural blooms.

MATERIALS AND METHODS

Strains and blooms. Natural blooms that consisted of 95% or more *Microcystis* species were collected from Lake Kasumigaura in Ibaraki prefecture, Japan. The strains isolated from the blooms were identified by following the description by Komárek (9). *Microcystis wesenbergii* K-3A (8), an axenic strain isolated from Lake Kasumigaura, was used as the test strain for the development of solid media.

Media. B-12 (11) and CB (14) media were used as liquid media and the base for solid media. B-12 medium has the following composition (in milligrams per liter of deionized distilled water): $NaNO_3$, 100; K_2HPO_4, 10; $MgSO_4 \cdot 7H_2O$,

75; $CaCl_2 \cdot 2H_2O$, 40; Na_2CO_3, 20; ferric citrate, 6 (autoclaved separately); disodium EDTA $\cdot$ $2H_2O$, 1; and vitamin B_{12}, 0.1. CB medium has the following composition (in milligrams per liter of deionized distilled water): $Ca(NO_3)_2 \cdot 4H_2O$, 150; KNO_3, 100; $MgSO_4 \cdot 7H_2O$, 40; β-disodium glycerophosphate, 50; bicine, 500; biotin, 0.0001; vitamin B_{12}, 0.0001; and thiamine hydrochloride, 0.01, with 3 ml of PIV metals. PIV metals consisted of the following (in milligrams per 100 ml of deionized distilled water): $FeCl_3 \cdot 6H_2O$, 19.6; $MnCl_2 \cdot 4H_2O$, 3.6; $ZnSO_4 \cdot 7H_2O$, 2.2; $CoCl_2 \cdot 6H_2O$, 0.4; $Na_2MoO_4 \cdot 2H_2O$, 0.25; and disodium EDTA $\cdot$ $2H_2O$, 100. The pH of both the B_{12} and CB media was adjusted to 9.0. Agar (chemical grade 1), Agar-noble (low-ash agar), agarose, and Agarose low gel temperature (low-melting-temperature agarose) were purchased from Daiichi Chemical Co. (Tokyo, Japan), Difco Laboratories (Detroit, Mich.), Marine Colloids, Inc., (Rockland, Maine), and Bio-Rad Laboratories (Richmond, Va.), respectively. Solid media were prepared on a base of B-12 or CB medium with these gels and then sterilized at 121°C for 15 min.

Culture. The cells were cultured in liquid medium (5 ml of medium per test tube [17.5 by 130 mm]) under 2,000-lx illumination at 30°C for 7 to 10 days. The cultures were diluted appropriately, pipetted onto sterile petri dishes, mixed with molten but cool solid medium (0.1 ml of diluted solution per 10 ml of medium) and then allowed to harden (pour plates). Alternatively, the diluted cultures (0.1 ml) were placed on the surface of the hardened, solid medium in the petri dishes and spread over the medium with a bent glass rod (spread plates). These plates were sealed with Parafilm and incubated under 2,000-lx illumination in an inverted position at 30°C. The mean and standard error of the colony numbers in a group of five plates were calculated.

Test for purity of *Microcystis* species. Bacterial contamination was tested by phase-contrast microscopy and cultivation. For the latter procedure, the cultured cells were heavily inoculated into the following three media (1 ml of culture per 10 ml of medium): diluted nutrient agar (0.5 g of meat extract, 1 g of peptone, 0.5 g of NaCl [pH 7.0] per liter), thioglycolate (TGC) medium (Nissui Seiyaku Co., Tokyo, Japan), and potato dextrose agar (Nissui Seiyaku Co.) and cultured at 30°C for 3 weeks. A group of five cultures was used for each test.

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TABLE 1. Effect of agarose concentration on colony formation

Medium	10^3 CFU/ml[a] at % solidified agents				
	0.4	0.5	0.6	0.8	1.0
Agarose	61.3 ± 3.5	45.7 ± 2.4	21.0 ± 5.6	18.0 ± 1.5	5.3 ± 1.8
LT agarose[b]	55.7 ± 3.9	51.4 ± 2.5	45.7 ± 10.5	24.7 ± 2.0	23.7 ± 1.2

[a] Mean ± standard error ($n = 5$).
[b] LT agarose, Low-melting-temperature agarose.

RESULTS AND DISCUSSION

Development of solid media. Strain K-3A was cultured in B-12 medium for 7 to 10 days. To break up the colonies in culture and to separate them into single cells, the culture was vigorously shaken by a vortex mixer for 2 min and serially diluted 10-fold in sterilized, deionized, distilled water. Each dilution was vigorously stirred for 30 to 60 s. Aliquots (0.1 ml) of the 100-fold dilution were poured into the melted B-12 agar medium (10 ml) that contained various concentrations (0.4 to 1%) of agar, agar-noble, agarose, or low-melting-temperature agarose and were kept at 40°C in a water bath. Then, the agar media were solidified in dishes at room temperature and cultured. Two weeks later, colonies had grown in the media with agarose or low-melting-temperature agarose (Table 1 and Fig. 1) but not in those with agar or agar-noble. As an exception, several colonies formed in 0.4% agar- or agar-noble-solidified B-12 medium when the original cells were inoculated without stirring and dilution. The highest number of CFU was observed in the medium with 0.4% gel. When the concentration of the gel was increased, there was a reduction in the number of CFU in the medium (Table 1). Agars that consist of agarose and agaropectin are contaminated with various impurities (10), and some agars contain water-soluble lytic agents against cyanobacteria (1). The lytic agents (inhibitors) in agars can be removed by extensive washing (4). Probably some of the impurities or inhibitors against cyanobacteria still persist after the process to produce gels, and the use of the low-purification gels or use of a higher concentration of the partially purified gels may inhibit the growth of cyanobacteria.

There was no difference in the growth rates of B-12 and CB agarose media in terms of CFU numbers (data not shown). The colonies could be visualized on day 5 after initiation of culture, and the number reached maximum on days 10 to 14. The *Microcystis* cells grown in 0.4% agarose medium formed mucilaginous colonies. The color of the colonies was green at first but changed to white 3 to 4 weeks after the initiation of culture. The cells in the white colonies lost their ability to make colonies in a subsequent culture.

The efficiency of colony formation in the pour plates was quite similar to that in the spread plates, but the colony formation was greatly influenced by the kind of solution used for cell dilution (Table 2). The numbers of colonies in the cultures in which the cells had been prepared by the dilution with distilled water were apparently higher than in those prepared by the dilution with CB medium (15 mosM) or 0.05% saline (20 mosM). Salt in the solution may hinder the segregation of the cells in colonies.

Isolation of axenic strains. Figure 2 shows the isolation procedure for unicellular and axenic strains from natural blooms. Collected blooms were allowed to stand in a separator funnel. The lower aqueous layer was discarded. The obtained buoyant cells in the top layer were suspended in sterilized water. This procedure was repeated five times to wash the cells. Then, the washed cells were vigorously stirred with a vortex mixer for 1 to 2 min and then serially diluted 10-fold in sterilized water. To break the blooms into single cells and to separate the *Microcystis* cells from other cyanobacteria and bacteria, an appropriate dilution was again vigorously stirred for 30 to 60 s before plating, poured into CB medium with 0.4% agarose, allowed to solidify, and incubated at 30°C for 7 to 10 days (first culture). When the colony formation was observed, the *Microcystis* cells in the colonies were transferred by a sterilized toothpick to fresh agarose medium and incubated again (second culture). Long-term culture in the first culture should be avoided because
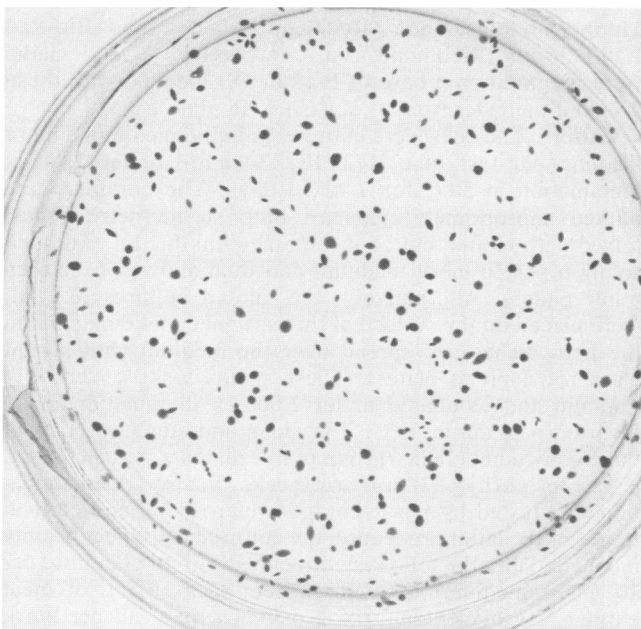

FIG. 1. Colonies of *M. wesenbergii* K-3A on an agarose plate. An aliquot (0.1 ml) of a 100-fold dilution of culture was plated on 0.5% agarose in B-12 medium with the pour plate technique, and the plate was incubated for 14 days.

TABLE 2. Influence of dilution and plating technique on number of CFU

Diluted solution	10^3 CFU/ml[a]	
	Pour plate	Spread plate
H_2O	53.2 ± 1.3[b]	54.5 ± 4.6
CB medium	35.2 ± 2.5	NT[c]
0.05% NaCl	32.9 ± 1.9	NT

[a] Mean ± standard error ($n = 5$).
[b] Significantly different from CFU in CB medium and 0.05% NaCl medium ($P < 0.01$) as determined by the t test.
[c] NT, Not tested.

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Blooms
    │
    │wash several times
    │
    ┌─────────────────────────────────┐
    │stir with vortex mixer           │
    ↓plate out on agarose medium      │
Colonies on first culture plate      │
    │inoculate                        │
    │                                 │
Colonies on second culture plate     │
    │inoculate                        │
Liquid culture (5ml/tube)            │
    │test for purity                  │
    │(microscopy)                     │
    ↑                                 │
    └─────────────────────────────────┘
Liquid culture (100ml/flask)         │
    │test for purity                  │
    │(heavy inoculation)              │
    ↓─────────────────────────────────┘

Axenic strains
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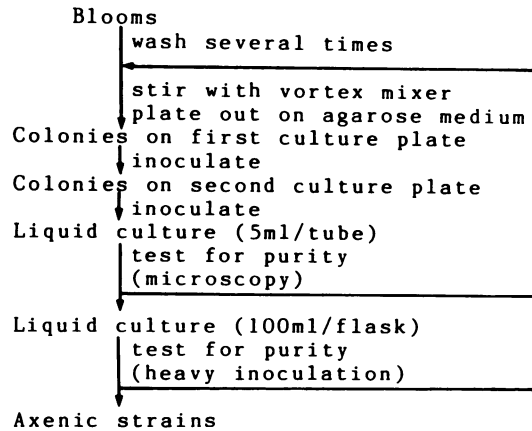
FIG. 2. Isolation procedure for axenic *Microcystis* strains.

the possibility for contamination by bacteria and fungi increases. The cells in fully propagated colonies in the second culture were inoculated into CB medium (5 ml per tube) and cultured for 1 to 2 weeks. Then, the cultures were examined by a phase-contrast microscope to determine whether any contamination had occurred. If contamination was observed, the culture was returned to the first step of the isolation procedure, which was repeatedly performed until the culture was verified to be free of contamination. Gentle sonication and stirring with glass beads were not effective for the isolation of *Microcystis* species, because these procedures decreased the CFU of *Microcystis* species and were not effective for removing contaminating bacteria. The cells from the cultures that were confirmed to be contamination-free by microscopic examination were inoculated into 100 ml of CB medium in a 200-ml Erlenmeyer flask and then cultured. The resulting cultures were heavily inoculated into the test media to detect the presence of any contamination. When contaminating microbes were observed, the whole procedure was repeated.

The bloom used here for *Microcystis* isolation was observed microscopically to consist of large numbers of *M. aeruginosa* and *M. wesenbergii* and of small numbers of *M. viridis* and *Anabaena* species. As the result of the procedures described above, 31 clones of *Microcystis* (30 of *M. aeruginosa* and 1 of *M. wesenbergii*) were obtained, and these cells were in the form of single cells. We failed to isolate *M. viridis* and *Anabaena* species, but this does not mean that our procedure is inadequate to isolate them, since these two species can grow in our newly developed medium (unpublished observation). The clone of *M. wesenbergii* was purified by one cycle of the isolation procedure. Four axenic strains of *M. aeruginosa* were isolated by three cycles of the procedure. Ten clones of *M. aeruginosa* were bacteria-free in microscopic examination but were found to be contaminated when tested by heavy inoculation. The other 16 clones of *M. aeruginosa* were still nonaxenic in microscopic observation after three cycles of the isolation procedure, although the number of contaminating bacteria significantly decreased when compared with those in the first cultures.

After our intensive effort to get the axenic strains, some clones still contained bacteria. It might be possible that some species of cyanobacteria cannot exist without bacterial ectosymbionts.

The procedures for isolation and purification of cyanobacteria have not been standardized yet. The procedure described here is simple and useful for establishment of clones and axenic strains of *Microcystis* species. It is known that the addition of phenol to algal cultures reduced the number of contaminating bacteria (4), and UV irradiation is useful for isolating axenic strains (7). The combination of our procedure with these methods may be expected to better eliminate bacteria that attach strongly to or are embedded in the mucilage surrounding the *Microcystis* colonies.

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