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Two Improved Methods for Obtaining Axenic Cultures of Cyanobacteria

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Scoring the agar plate before incubation under unidirectional light led to a rapid separation of gliding filamentous cyanobacteria from their contaminating bacteria. Twenty strains were purified by the method. Additionally, 13 axenic cyanobacterial strains were isolated from pour plates made after treatment of cyanobacterial cultures in tryptone-yeast extract-glucose broth with cycloserine in darkness to select for obligate photoautotrophs.

The difficulty often encountered in the isolation of axenic cultures of cyanobacteria is well reflected by the great variety of purification methods used. The methods include ordinary plating procedures, e.g., numerous restreakings on mineral medium (1, 8, 15, 16, 19), techniques making use of gliding movement and phototaxis of motile cyanobacteria (1-4, 8), treatments of cyanobacterial cultures with toxic chemicals (7, 11), heat (20), ultraviolet light (11) or gamma irradiation (12), and mechanical separations such as micromanipulation (3), filtration (9), and equilibrium centrifugation (14). In this report we describe two purification methods which have proved in our hands to be rapid and efficient.

We isolated 33 different cyanobacterial strains, most of them from Finnish lakes, ponds, ditches, and soil and from the Gulf of Finland. The strains were identified according to Rippka et al. (13). Three nonaxenic strains were obtained from other laboratories. The strains were grown in BG-11 medium (15). Enrichment cultures were supplemented with cycloheximide (Sigma Chemical Co., St. Louis, Mo.) at a concentration of 50 mg/liter to eliminate contaminating eucaryotes (21). For preparation of solid media, BG-11 medium was supplemented with 1% (wt/vol) separately sterilized agar (Difco Laboratories, Detroit, Mich.), and, when establishing clones from single axenic cells, with 10 U of catalase (Sigma) per ml (6, 17). The incubation took place at 26°C under continuous illumination of 500 to 900 lx from cool white fluorescent tubes (Airam, Helsinki, Finland).

The purity of cyanobacterial cultures was verified first by the absence of bacterial growth on TYG agar and in TYG broth (tryptone [Difco], 5.0 g; yeast extract [Difco], 2.5 g; glucose, 1.0 g per liter) after incubation of several weeks at 26°C. The final proof of purity was a careful microscopic examination (1,125×, phase-contrast illumination) of aged cyanobacterial cultures (15) and of cultures incubated for 3 days in darkness on BG-11 medium supplemented with 0.5% (wt/vol) glucose and 0.02% (wt/vol) Casamino Acids.

Twenty-two cyanobacterial strains glided actively on the agar. They belonged to the following genera: *Pseudoanabaena* (11 strains), *Oscillatoria* (7 strains), *Lyngbya-Phormidium-Plectonema* group (3 strains), and *Anabaena* (1 strain).

Burton and Lee (6) have developed a rapid method for purifying *Beggiatoa* strains, gliding non-photosynthetic bacteria. They scored the agar surface with parallel lines along which the *Beggiatoa* filaments glided directly away from the inoculum site and liberated themselves from their contaminants.

We purified the gliding cyanobacterial strains by making use of their phototactic movement on scored agar. *Oscillatoria* strains were purified on BG-11 agar supplemented with 0.1% (wt/vol) $Na_2S \times 9H_2O$ (pH approximately 10), which stimulated their gliding. Other strains were purified on the ordinary BG-11 agar. The surface of usually 3-day-old agar plates was scored with parallel lines with a flamed rough glass triangle. Small pieces of young cyanobacterial vegetation were transferred to the center of scored agar plates. The plates were incubated at 26°C under unidirectional light, the scores parallel with incident light (50 to 100 lx). After overnight incubation, the plates were examined microscopically (125×, bright-field illumination). The cyanobacterial filaments glided much further from the inoculum site on scored agar than on unscored agar (Table 1) because they glided directly along the scores towards the incident light, whereas on unscored agar they tended to change their direction on the way (Fig. 1). The direct gliding led to a rapid separation of cyano-

bacterial filaments from their adhering contaminants. The axenic filaments were picked on an agar block with a sterile injection needle and subcultured on fresh agar plates. Some strains did not tolerate this manipulation, and the axenic filaments had to be isolated otherwise. Instead of cutting off the agar blocks around the filaments, the entire contaminated area of the scored agar was cut off and replaced with fresh agar.

TABLE 1. *Maximal gliding distances from the inoculum site of some cyanobacterial strains on scored and unscored agar plates*[a]

Strain	Type	Maximal gliding distances (mm)[b] on:	
		Unscored agar	Scored agar
HB1	*Pseudoanabaena*	4	18
GH1	*Pseudoanabaena*	8	23
II	LPP group[c]	4	16
AN	*Anabaena*	1	9
LII	*Oscillatoria*	2[d]	33[d]
G9L	*Oscillatoria*	6[d]	43[d]

[a] Incubation under unidirectional light (50 to 100 lx) at 26°C overnight.

[b] The figures are averages of values from five separate plates.

[c] *Lyngbya-Phormidium-Plectonema* group.

[d] On BG-11 agar supplemented with 0.1% (wt/vol) $Na_2S \times 9H_2O$.

Axenic cultures were established from 20 of 22 cyanobacterial strains already after the first attempt. All of them grew on BG-11 agar, although two *Lyngbya-Phormidium-Plectonema* strains grew much better on BG-11 agar supplemented with 0.05% (wt/vol) tryptone, 0.025% (wt/vol) yeast extract, and 0.01% (wt/vol) glucose; one *Pseudoanabaena* strain grew better on BG-11 agar supplemented with 0.01% (wt/vol) yeast extract; and one *Oscillatoria* strain grew better in BG-11 liquid medium.

The *Anabaena* strain and one *Oscillatoria* strain did not grow after purification. They grew on neither BG-11 agar, liquid medium, nor BG-11 agar supplemented with any of the above-mentioned supplements. There are several notes that some cyanobacterial strains have not grown as pure culture (16, 18; V. B. D. Skerman, personal communication). It is interesting that both the strains were of planctic origin.

The 14 non-actively gliding cyanobacterial strains were grouped into the following genera: *Synechococcus* (8 strains), *Synechocystis* (2 strains), *Nostoc* (1 strain), *Oscillatoria* (1

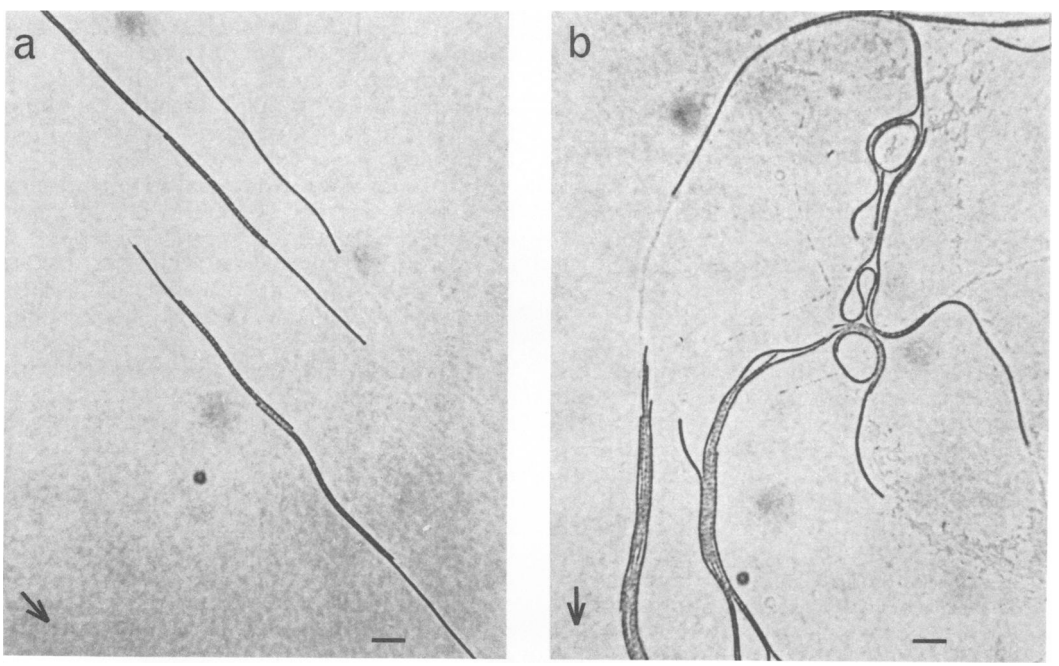

FIG. 1. *Gliding of cyanobacterial filaments (Pseudoanabaena strain O) towards the incident light on scored (a) and unscored (b) agar. In (b) bacterial contaminants are also seen. Pictures are taken from the peripheral zone of cyanobacterial vegetation. The incubation was as described in the legend to Table 1. Bar, 15 μm. The arrow indicates the direction of the incident light.*

TABLE 2. *Viable counts of contaminating bacteria[a] and cyanobacteria[b] before and after the cycloserine enrichment[c] of some cyanobacterial cultures*

Strain	Type	Counts per ml			
		Before cycloserine enrichment		After cycloserine enrichment	
		Bacteria	Cyanobacteria	Bacteria	Cyanobacteria
CB3	*Synechocystis*	2.4×10^7	4.2×10^6	1.9×10^5	3.2×10^6
CTR	*Synechococcus*	1.2×10^8	7.7×10^6	1.0×10^6	2.3×10^6
HR5	*Oscillatoria*	6.8×10^6	7.7×10^4	2.3×10^5	7.0×10^4

[a] The viable count on TYG agar after incubation for 1 week at 26°C.

[b] The viable count in BG-11 pour plates after incubation for 3 weeks at 26°C in the light.

[c] The cycloserine enrichment was made in TYG broth with cycloserine concentration of 1 mg/ml. Incubation was at 26°C for 24 h in darkness.

strain), *Pseudoanabaena* (1 strain), and the *Lyngbya-Phormidium-Plectonema* group (1 strain). None of them achieved any visible growth when incubated in TYG broth for 1 week in darkness. In purification of these strains, we applied the general principles of the classic antibiotic enrichment method, originally developed for isolation of auxotrophic bacterial mutants (10). We dark incubated the cyanobacterial culture in TYG broth with an antibiotic which selectively killed growing bacteria but left the resting cyanobacteria viable. Basically, this method is related to the phenol treatment of the cyanobacterial culture in darkness, used successfully by Carmichael and Gorham (7) in the purification of several cyanobacterial strains.

We found cycloserine to be the drug of choice in the antibiotic enrichment. All of the 52 contaminant strains isolated from the cyanobacterial cultures were sensitive to the cycloserine concentration of at least 2 mg/ml, as determined as minimum inhibitory concentration in TYG broth after incubation at 26°C for 48 h. On the other hand, 20 contaminant strains were resistant to ampicillin, 22 were resistant to cephalosporins, 23 were resistant to carbenicillin, and 48 were resistant to penicillin.

The enrichment procedure was as follows. Young cyanobacterial culture was first starved for 48 h in darkness. The culture was diluted (1:10) with TYG broth and incubated for some hours in darkness to let contaminating bacteria start growing. Cycloserine (Sigma) was added to the culture in most cases at a concentration of 1 mg/ml, and the culture was further dark incubated at 26°C for 24 h, after which appropriate dilutions were pour plated into BG-11 agar. After incubation for 2 to 3 weeks at 26°C in the light, the plates were examined and the axenic cyanobacterial colonies were subcultured.

Table 2 shows the quantitative results of the antibiotic enrichment of some cyanobacterial cultures. The amount of contaminating bacteria was generally decreased approximately 100-fold (as determined on TYG agar after incubation for 1 week at 26°C), whereas the amount of cyanobacteria was decreased by much less.

Twelve of fourteen cyanobacterial strains, some of them very mucous, were easily purified at the first attempt. In BG-11 pour plates made after the enrichment there were always at least some axenic cyanobacterial colonies to be found. Additionally, one *Synechococcus* strain was also purified by this method, but it formed single axenic colonies only on BG-11 agar supplemented with 0.01% (wt/vol) yeast extract. One *Synechococcus* strain did not survive the enrichment at all.

When the antibiotic enrichment was performed in the light all the cyanobacterial strains were killed. The relative cycloserine resistance of cyanobacteria in darkness was thus due to their inability to grow in darkness in TYG broth. The antibiotic enrichment method is certainly not suitable for purification of those cyanobacteria which are facultatively capable of rapid chemoheterotrophic growth.

Cycloserine was the only antibiotic effective against all the contaminants of our cyanobacterial cultures, and thus it seems to be the drug of choice in the antibiotic enrichment method. Other choices or combinations of cell wall synthesis-inhibiting antibiotics should be tried if any contaminant proved to be cycloserine resistant.

Recently, Rippka and Coursin purified two cyanobacterial strains with antibiotic enrichment using ampicillin as the selective antibiotic (R. Rippka, personal communication).

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