Method for Isolation and Purification of Cyanobacteria

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A method employing nutrient saturated glass fiber filters allowed the isolation of the same numbers of cyanobacteria from freshwater as were obtained with medium solidified with agar, while providing a 2- to 15-fold reduction in the number of accompanying heterotrophic bacteria. Imipenem, a broad-spectrum β-lactam antibiotic which inhibits peptidoglycan biosynthesis, was superior to some other β-lactam antibiotics for reducing the numbers of heterotrophic bacterial contaminants associated with freshly isolated cyanobacteria to a level which facilitated the production of axenic cyanobacterial cultures.

Despite the existence of morphologically diverse cyanobacteria in a wide variety of terrestrial and aquatic habitats, work with these bacteria has been restricted to a relatively few representatives. This seems to be partly the result of difficulties encountered in both the isolation and the subsequent purification of these bacteria. It has been suggested that the techniques normally used to isolate cyanobacteria may severely limit the number of cyanobacterial species which can be readily cultured (9). Agar, which is routinely used as a solidifying agent in bacteriological media, is known to contain impurities (1, 7, 15), and some of these are suspected to be responsible for the repeated observation that agar is inhibitory to the growth of some cyanobacteria (1). Different approaches have been used in an attempt to lower or eliminate the growth-inhibitory effects of agar. These have included the use of low agar concentrations (3, 24), agar-washing procedures (1, 15), the separate sterilization of agar and nutrient solutions (3), and the substitution of agarose or other alternative solidifying agents (24, 26). Perhaps an even more frustrating aspect of dealing with cyanobacteria is the often arduous and time-consuming work that is involved in attempting to produce axenic cultures contrasted with the low rate of success. Various approaches have been taken to try to develop more-efficient methods to purify contaminated cyanobacteria. These have included mechanical separations of the cyanobacteria and bacterial contaminants by micromanipulation (6), differential filtration (13, 19), and repeated transfer of cells (2, 25, 27). Other approaches have involved the use of an agent which is judged to be relatively harmless to the cyanobacteria but is toxic to the bacterial contaminants. Agents used have included phenol (8, 18), sodium hypochlorite (11), detergents (18), sodium sulfide (20), UV or gamma irradiation (5, 12, 14), elevated temperature (4, 29), and antibiotics (21, 22, 27). As part of our studies to explore the cyanobacteria as a possible source of new and useful pharmaceutical compounds (23), we attempted to develop methods which would be more effective for the isolation and purification of cyanobacteria. As part of our approach, we endeavored to eliminate the use of agar or any other organic solidifying agents in our isolation media and we attempted to find an effective means of generating axenic cultures of cyanobacteria. We report here that glass fiber filters can serve as a suitable substitute for organic solidifying agents and that imipenem, a

relatively new, broad-spectrum β-lactam antibiotic that inhibits bacterial peptidoglycan biosynthesis (16, 17), is more effective than some other β-lactam antibiotics at reducing the number of heterotrophic bacterial contaminants present with freshly isolated cultures of cyanobacteria, aiding in the production of axenic cultures.

(A preliminary account of this work has been published [10a].)

MATERIALS AND METHODS

Media and incubation conditions. BG-13 medium consisted of (per liter) $NaNO_3$ (1.5 g), $NaHCO_3$ (1.7 g), K_2HPO_4 (31 mg), $MgSO_4 \cdot 7H_2O$ (75 mg), $CaCl_2 \cdot H_2O$ (36 mg), Na_2CO_3 (20 mg), citric acid (6 mg), ferric ammonium citrate (6 mg), disodium magnesium EDTA (1 mg), H_3BO_3 (2.86 mg), $Mn_2Cl_2 \cdot 4H_2O$ (1.81 mg), $ZnSO_4 \cdot 7H_2O$ (220 μg), $Na_2MoO_4 \cdot 2H_2O$ (390 μg), $CuSO_4 \cdot 5H_2O$ (80 μg), and $CoCl_2 \cdot 6H_2O$ (40 μg). The pH of BG-13 medium was 7.5 to 7.6 when it was incubated under an atmosphere of 5% (vol/vol) CO_2 in air. BG-12 medium consisted of BG-13 medium minus the sodium bicarbonate and containing 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) buffer (1.2 g/liter), pH 7.5. For some experiments, BG-12 and BG-13 media were supplemented with cycloheximide and nystatin, each at a concentration of 100 μg/ml or with low concentrations of organic nutrients as indicated in the text. The water used to prepare BG-12 and BG-13 media was purified to a resistance of 18 MΩ with a Milli-Q water system (Millipore). All agar media were prepared with 1.5% (wt/vol) Noble agar (Difco). All media were sterilized by autoclaving for 20 min at 15 lb/in². For BG-12 and BG-13 agar media, double-strength agar and double-strength nutrient solution were prepared, sterilized separately, and then combined according to the method of Allen (3). BG-13 glass fiber plates were prepared by packing five 90-mm-diameter glass fiber filters (Whatman 934-AH) into the bottom of a Pyrex glass petri plate (100 by 20 mm), covering the plate, and then sterilizing the plate by heating at 260°C for 30 min. After cooling, the filters were saturated with 20 ml of sterile BG-13 medium. Inoculated plates were incubated at 25°C under an atmosphere of 5% (vol/vol) CO_2 in air. The plates were illuminated with 40-W cool-white fluorescent lamps at an irradiance of 3 to 5 klux. Broth cultures were shaken at 180 to 200 rpm under the same temperature, atmosphere, and illumination conditions as described for the plates.

Enumeration of cyanobacteria and heterotrophic bacteria.

Freshwater and sediment samples were collected from various areas in eastern Pennsylvania and northern New Jersey. A sample was shaken to suspend sediment, and then triplicate 10-μl aliquots were removed and diluted into 100 ml of sterile distilled water which was vacuum filtered through a sterile 47-mm-diameter polycarbonate membrane filter (0.4-μm pore diameter; Nuclepore). The filters were aseptically transferred, inoculum side up, onto plates of BG-12 or BG-13 medium containing nystatin and cycloheximide. The plates were incubated for 14 days, after which the cyanobacterial colonies growing on the surface of the membrane filters were counted with the aid of a dissecting microscope (×10 to ×50 magnification). The mean CFU per filter was calculated, and the variation between means was analyzed by Student's t test (95% confidence interval).

The number of heterotrophic bacteria present on the surface of a membrane filter then was determined by placing each filter into 10 ml of sterile dilution buffer (HEPES, 1.2 g/liter; $NaNO_3$, 1.5 g/liter; pH 7.5) and dispersing the cells by treatment for 3 min in an ultrasonic cleaning bath (model B-220; Branson). Serial dilutions of the resulting cell suspensions were prepared and plated in duplicate on BG-12 medium supplemented with glucose (1.0 g/liter), nutrient broth (Difco; 0.8 g/liter), and cycloheximide. After incubation in the dark at 25°C under ambient atmosphere for 1 week, the bacterial colonies present on the plates were counted with a colony counter (American Optical). The mean CFU per filter was calculated, and the variation between means was analyzed by Student's t test (95% confidence interval). The number of heterotrophic bacterial contaminants present in broth cultures of cyanobacteria was determined similarly by plating serial dilutions of the broth culture in duplicate on BG-12 medium which contained nystatin and cycloheximide and was supplemented with glucose, yeast extract (Difco), and Bacto-Peptone (Difco), each at 100 mg/liter.

Purification of cyanobacteria with antibiotics. Contaminated cyanobacterial colonies growing on the surface of membrane filters overlaying glass fiber medium were picked and transferred into 20 ml of sterile BG-12 medium in a 50-ml Delong flask. The mixed culture was incubated for 3 to 4 weeks under the conditions described previously in order to obtain sufficient cyanobacterial biomass, and then 400 μl of a sterile nutrient solution (SNS) consisting of 2.5% (wt/vol) sucrose, 0.5% (wt/vol) Difco yeast extract, and 0.5% (wt/vol) Difco Bacto-Peptone was added along with 400 μl of sterile antibiotic solution (see below) to give a final antibiotic concentration of 100 μg/ml. The culture then was incubated for 18 to 24 h in the dark at 180 to 200 rpm under an atmosphere of 5% (vol/vol) CO_2 in air. After incubation, the cyanobacteria were harvested by centrifugation at 17,000 × g for 15 min at 25°C. The cells were washed twice by centrifugation with volumes of sterile BG-12 medium equal to the original culture volume and finally suspended in 1/10 of the original volume. It was usually necessary to gently disperse the cyanobacterial cells with a sterile tissue homogenizer in order to produce a cell suspension which could be easily pipetted and plated onto BG-12 agar containing nystatin and cycloheximide. The plates were incubated for 2 to 4 weeks and observed at weekly intervals for the growth of cyanobacteria. With a dissecting microscope (×10 to ×50 magnification), purified colonies or filaments of cyanobacteria were picked and transferred to plates of BG-12 agar. The purity of isolates was confirmed by phase-contrast microscopy (×500 to ×1,250 magnification) and by inoculating cyanobacterial growth into BG-12 broth supplemented with

0.01% (wt/vol) (each) glucose, yeast extract (Difco), and Bacto-Peptone (Difco) and into GNB broth medium, which consisted of 1.0% (wt/vol), glucose and 0.8% (wt/vol) nutrient broth (Difco). Broth cultures were incubated at 25°C with shaking at 180 to 200 rpm under ambient atmosphere and statically under anaerobic conditions in an anaerobic jar (Gas-Pak; BBL). If no growth of heterotrophic bacteria was observed under any of the conditions after incubation for 1 month, the cultures were judged axenic.

Growth characteristics and morphology observed by phase-contrast microscopy were used to identify cyanobacteria according to established criteria (10).

Chemicals. Aqueous stock solutions (0.5% [wt/vol]) of cefoxitin, ampicillin, penicillin G, imipenem, and cycloheximide (1.25% [wt/vol]) were prepared fresh, sterilized by filtration, and added to cooled molten agar or broth media. Stock solutions of nystatin (10% [wt/vol]) were prepared in N,N-dimethylformamide. Penicillin G (potassium salt) was purchased from Calbiochem, ampicillin (sodium salt) was purchased from Sigma, and cycloheximide and nystatin were purchased from U.S. Biochemicals. Cefoxitin (sodium salt) and imipenem were supplied by Merck Sharp & Dohme Research Laboratories. All other chemicals were of the highest purity available.

RESULTS

Comparison of cyanobacterial and bacterial growth on agar and glass fiber media. An experiment was done to determine whether there was any difference in the numbers of cyanobacteria and accompanying heterotrophic bacteria recovered from aquatic samples when inoculated membrane filters were incubated on agar or glass fiber medium. Each sample was plated in triplicate on BG-13 agar and BG-13-saturated glass fiber medium. After 14 days of incubation, the mean number of cyanobacterial CFU present on each membrane filter was determined. Statistical analysis of the results presented in Table 1 indicates that the mean number of cyanobacterial colonies recovered from the same water sample was the same on glass fiber medium and on agar. However, statistical analysis indicated a difference between the mean number of heterotrophic bacterial CFU present on the agar medium and the number on the glass fiber medium. As shown in Table 2, the mean number of heterotrophic bacterial CFU occurring on filters incubated on the glass fiber medium was 4 to more than 15 times lower than that observed on the agar medium. In a similar experiment, the inoculated filters were incubated in the dark to eliminate any

TABLE 1. Comparison of agar and glass fiber medium for the isolation of cyanobacteria

Water sample	No. of cyanobacterial CFU/membrane filter (mean ± SD)[a] on:	
	Agar medium	Glass fiber medium
M41	18.3 ± 5.8	26.3 ± 2.3
M44	1.30 ± 1.5	3.70 ± 2.1
M51	0.33 ± 0.58	0.33 ± 0.58
M52	12.0 ± 4.4	27.0 ± 13.5

[a] By using polycarbonate membrane filters as described in the text, each water sample was plated in triplicate on both BG-13 agar and BG-13-saturated glass fiber filters and incubated. Then for each water sample, the mean number of cyanobacterial colonies which formed on each medium was determined.

TABLE 2. Comparison of the number of contaminant bacteria present on membrane filters incubated on agar and glass fiber media

Water sample	10^6 bacterial CFU/filter (mean ± SD)[a] on:	
	Agar medium	Glass fiber medium
M41	290 ± 94.8	65.8 ± 49.4
M44	293 ± 96.8	58.0 ± 61.6
M51	820 ± 138.0	48.6 ± 37.8
M52	291 ± 41.0	37.2 ± 33.5

[a] By using polycarbonate membrane filters as described in the text, each water sample was plated in triplicate on both BG-13 agar and BG-13-saturated glass fiber filters and incubated. Then for each water sample, the mean number of contaminant bacteria present on each medium was determined.

TABLE 3. Comparison of the efficacy of various antibiotics to reduce the number of contaminant bacteria present in a cyanobacterial culture

Antibiotic (100 μg/ml)	10^8 Contaminant CFU/ml of culture (mean ± SD)	
	Before antibiotic treatment	After antibiotic treatment
Imipenem	2.9 ± 0.25	0.029 ± 0.0014
Cefoxitin	2.2 ± 0.18	0.22 ± 0.029
Ampicillin	2.0 ± 0.11	9.7 ± 0.071
Penicillin G	3.1 ± 0.028	28.0 ± 8.4
None (control)	3.3 ± 0.18	37.0 ± 11.7

effects of growing cyanobacteria, and it was observed that 1.9 to 19.4 times fewer heterotrophic bacteria were detected on the glass fiber medium (data not shown).

Comparison of different β-lactam antibiotics to reduce the number of contaminants. Some efforts have been made to use β-lactam antibiotics which inhibit peptidoglycan synthesis to eliminate the contaminant heterotrophic bacteria associated with freshly isolated cyanobacteria (21, 22). Most recent approaches (22) have been based on the rationale that if the mixed culture is placed under conditions (usually in the dark with elevated levels of organic nutrients) in which growth of the cyanobacteria is restricted while that of the heterotrophic contaminants is stimulated, then the antibiotic will kill those bacteria which are rapidly dividing and spare those which do not divide or which divide very slowly. While the rationale seems reasonable, this approach seems to have suffered from its inability to significantly reduce the numbers of contaminant bacteria present, apparently as a result of the narrow antibacterial spectrum of the β-lactam antibiotics which were used. Since imipenem (Fig. 1), a β-lactam antibiotic which inhibits peptidoglycan biosynthesis and offers a very broad antibacterial spectrum (16, 17), had not been tested, an experiment was done to compare it with other β-lactam antibiotics, some of which had been previously used (21, 22). Five equivalent cultures of a contaminated Oscillatoria sp. (MJF-1) were supplemented with SNS, and four of the cultures each received a different antibiotic. Immediately after the additions were made, the number of contaminant bacteria present in each of the cultures was determined. All of the cultures were then incubated in the dark for 18 h, after which the number of bacteria present in each culture was determined again. The results are presented in Table 3 and show that following treatment with 100 μg of imipenem per ml, the number of

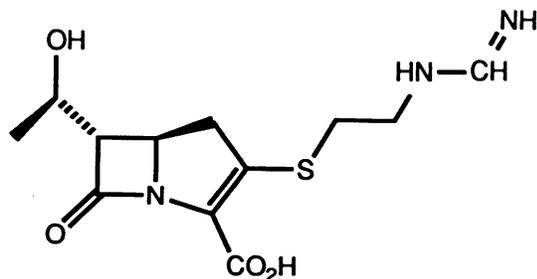FIG. 1. Structural configuration of imipenem.

contaminant bacteria was 1.0% of the initial number. Of the other antibiotics tested, ampicillin and penicillin G were observed to be ineffective at reducing the initial number of bacteria, while cefoxitin, a semisynthetic derivative of cephamycin C (28), was less effective than imipenem. This was reflected in the observation that after plating, whereas viable cyanobacteria were obtained from each of the antibiotic-treated cultures, only the imipenem-treated culture produced axenic cyanobacterial growth. The results of similar experiments with other contaminated cyanobacterial isolates (data not shown) also showed imipenem to be superior to the other β-lactam antibiotics at reducing the number of bacterial contaminants to a level which allowed the isolation of axenic cyanobacteria.

The effect of concentration and length of incubation on the ability of imipenem to reduce the numbers of contaminant bacteria is shown in Fig. 2. Equivalent contaminated cultures of Oscillatoria sp. (MJF-1) were supplemented with SNS and treated with different concentrations of imipenem. The number of contaminant bacteria in each culture was then monitored over a period of 32 h. After 8 h of incubation, the number of bacteria in each imipenem-treated culture had decreased almost 100-fold and continued to decrease with longer incubation such that after 32 h, the number of contaminant bacteria in each imipenem-treated culture had declined by almost 3 orders of magnitude. In contrast, the number of contaminant bacteria in a control culture which did not receive imipenem was found to be almost 10 times higher after 32 h.

DISCUSSION

The substitution of glass fiber filters for the agar normally used in solid media and the use of imipenem to reduce the numbers of bacterial contaminants associated with cyanobacterial isolates seem to be useful additions to the array of methods used to isolate and purify cyanobacteria. The major advantage provided by glass fiber filters as a substitute for agar appears to be a reduction in the number of accompanying contaminant bacteria. The greater number of contaminant heterotrophic bacteria observed on agar medium presumably is due to increased growth of these bacteria supported by organic nutrients which are known to be present in agar (7). With media prepared with glass fiber filters, it might be expected that such nutrients would not be present or would be present at much lower concentrations. The observation in these experiments that as many cyanobacteria are isolated with glass fiber medium as with agar medium suggests that the use of glass fiber filters in place of agar does not inhibit the growth of cyanobacterial isolates. When the morphology of the cyanobacteria isolated from an
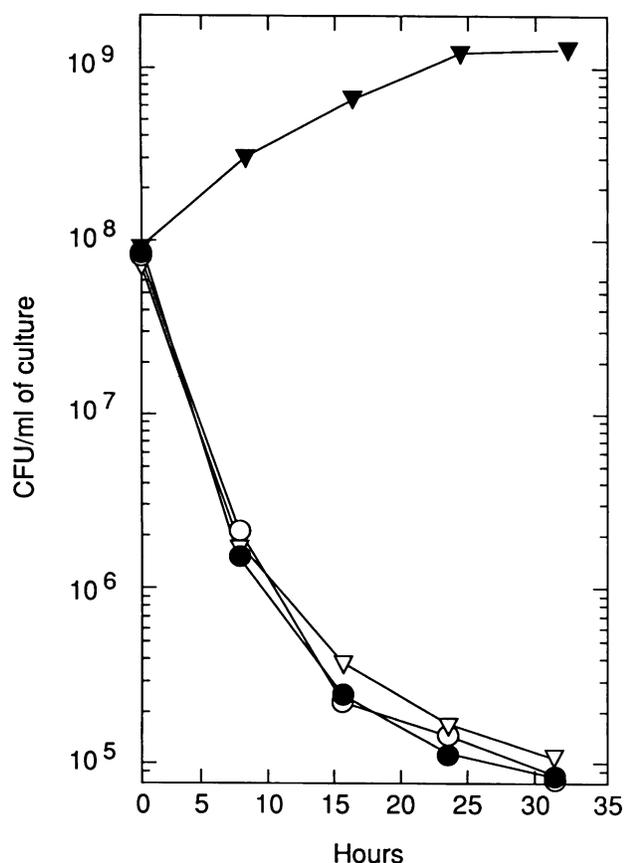
FIG. 2. Effect of incubation time and different concentrations of imipenem on the number of contaminant bacteria present in a cyanobacterial culture. ▼, no imipenem; ▽, 10 μg/ml; ●, 50 μg/ml; ○, 100 μg/ml.

individual sample with agar medium was compared with that of the isolates from glass fiber medium, no great difference was seen, suggesting that the use of glass fiber medium does not influence the selection of cyanobacterial isolates. Also, almost all of the cultures of filamentous or unicellular cyanobacteria which were isolated with glass fiber medium were found to grow on agar medium with no apparent toxic effects. However, while it was not observed in these studies, occasional axenic isolates of cyanobacteria, particularly unicellular forms, which do not exhibit growth on agar-containing media have been obtained with the glass fiber medium (unpublished data). Thus, it is anticipated that the use of glass fiber filters may offer a way to isolate some cyanobacteria which could not be isolated on media solidified with agar.

The greater capacity of imipenem to reduce the numbers of contaminant bacteria and the ability of cyanobacteria to tolerate incubation with it in the dark appear to make imipenem superior to other β-lactam antibiotics which have been used in an attempt to generate axenic cultures of cyanobacteria. The very broad antibacterial spectrum of imipenem may allow it to be more generally useful in eliminating a wide range of different heterotrophic bacteria which may be encountered in efforts to produce axenic cyanobacteria. Our own experience has shown that imipenem is effective in producing axenic cultures of morphologically diverse cyanobacteria which were isolated from various freshwater, marine, and terrestrial environments. Using the procedures described, over the past 5 years we have been able to produce more than 500 axenic cultures of cyanobacteria. Currently we are able to successfully obtain axenic cultures about 80% of the time with one treatment of imipenem; however, our experience has shown that the most critical step in the entire process is identifying and picking axenic colonies or filaments, which still requires considerable skill and patience.

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