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Isolation and purification of Australian isolates of the toxic cyanobacterium *Microcystis aeruginosa* Kütz.

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Abstract

Isolation and laboratory culture of *Microcystis aeruginosa* Kütz. using a growth medium (MLA medium) suitable for both non-axenic and axenic cultures is described. Seventeen established strains of *M. aeruginosa* were subjected to one or more of three purification methods: centrifugation cleaning, sulphide gradient selection, and antibiotic treatment (Imipenem®). While each method purified only about half of the strains attempted, the selective application of each method, based on the morphological characteristics of the strains, succeeded in purifying 12 of the 17 strains. Three of the 5 strains not purified were contaminated with a sulphide-tolerant, Imipenem-resistant spirochaete, *Spirochaeta* cf. *aurantia*, which could not be detected on normal, broad spectrum bacterial test media. The presence of this bacterial species was detected only by phase contrast and DAPI (4,6-diamidino-2-phenylindole) stained fluorescence microscopy.

Introduction

One of the most common causes of hepatotoxicity and odour problems in Australian inland waterways and lakes are blooms of the planktonic, gas-vacuolate cyanobacterial genus *Microcystis*. Blooms of *Microcystis* species, which form buoyant colonies of small (2–8 μm diameter), coccoid cells embedded in a gelatinous matrix (Baker, 1992), can produce a range of potent cyclic heptapeptide hepatotoxins called microcystins (Carmichael, 1992).

The environmental and genetic factors controlling the production of microcystins are poorly understood, therefore the establishment of laboratory cultures is critical for controlled laboratory studies. During a study to investigate these factors we established a collection of *M. aeruginosa* strains from Australian blooms. The established clonal, uni-algal cultures were sufficient for most culture studies; however, some genetic approaches, particularly methods using Polymerase Chain Reaction (PCR) techniques, required bacteria-free (axenic) cultures. We therefore sought to establish axenic strains.

There are many methods for reducing bacterial contaminants and obtaining axenic cultures of cyanobacteria (Rippka et al., 1988; Castenholz, 1988). Standard plating methods used for bacterial cultures have proved largely unsuccessful with *Microcystis* species due to the tight association of contaminating bacteria with the gelatinous material surrounding colonies (Rippka et al., 1988). Of the range of published methods, two have been generally successful with *Microcystis* strains; sulphide gradient selection (Parker, 1982) and repeated disaggregation and centrifugation (Shirai et al., 1990). Sulphide selection is a technically simple method relying on increased tolerance of some cyanobacteria to sulphides. However, it is not clear how applicable this method is to a broad range of strains with varying sulphide tolerances. The centrifugation method relies on the buoyancy of gas-vacuolate *Microcystis* cells, and cannot be applied to non-vacuolate strains. The extent to which cells can be disaggregated may affect the success of the process, and some strains appear to be resistant to purification by this method (Shirai et al., 1990).

Another approach is the addition of broad spectrum antibacterial compounds to reduce bacterial contami-

nants, such as sodium hypochlorite (Fogg, 1942), or phenol or detergents (Carmichael & Gorham, 1974). Treatment of cultures with broad-spectrum antibiotics has also been generally successful for reducing bacterial contamination in algal cultures (e.g. Hoshaw & Rosowski, 1973; Guillard, 1973). Earlier cyanobacterial workers used penicillin (e.g. Rippka, 1981) and cycloserine (Vaara et al., 1979); however, increasing widespread resistance to antibiotics in the environment (e.g. Boon, 1992) appears to have reduced the effectiveness of many antibiotics used for purification. More recently, a comparatively new broad spectrum antibiotic, Imipenem, has been used to purify a broad variety of cyanobacterial isolates (Ferris & Hirsch, 1991).

The broad applicability of the available methods for purifying *Microcystis* strains which may vary in their phenotypic characteristics, such as gas-vacuolation and sulphide tolerance, is currently unknown. In this study we compare the efficiency of modified sulphide gradient and disaggregation/centrifugation methods, and the antibiotic treatment method of Ferris & Hirsch (1991) for obtaining axenic cultures of 17 strains of *Microcystis aeruginosa*.

Material and methods

Growth media

The growth medium used for isolation and purification (MLA, see Table 1) is substantially modified from ASM-1 medium (Gorham et al., 1964). The base ASM-1 nutrients and trace metals were used, but Na_2HPO4 and $MgCl_2$ were omitted and the K_2HPO_4 concentration was doubled. The trace metal salts were changed by substituting $CuSO_4 \times 5H_2O$ and $ZnSO_4 \times 7H_2O$ for $CuCl_2$ and $ZnCl_2$ (which were available in our laboratory). The $MnCl_2 \times 4H_2O$ concentration was reduced from 7.0 μM to 2.0 μM, and molybdenum as $Na_2MoO_4 \times 2H_2O$ (essential co-factor for nitrate reductase [Rippka, 1988]), and selenium as H_2SeO_3 (essential trace metal for some species [Castenholz, 1988]) were added. Sodium hydrogen carbonate was added to improve buffering capacity and provide a carbon source, and vitamins B_{12}, biotin and thiamine which are important for some species, (Rippka, 1988) were also added.

Liquid medium was prepared in one of two ways (1) For isolation and maintenance of stock cultures, the medium was prepared by filter-sterilisation. A 40 times concentrate of nutrients/salts solution was prepared in Milli-Q (Millipore, $\geq$18 MΩ resistance) water and filter-sterilised through a 0.22 μm filter (Dynagard, Microgon, CA, USA) into an autoclave-sterilised, screw-capped, glass bottle and stored at 4 °C until use. Sterile concentrated nutrient/salts were then added aseptically to autoclave-sterilised Milli-Q water at 25 ml l^{-1} of medium. Autoclave-sterilised $NaHCO_3$, $CaCl_2$ and filter-sterilised vitamins were then added and the final medium dispensed into sterilised flasks or petri dishes. (2) The medium for purification and maintenance of axenic cultures was essentially the same but non-sterile stock solutions were added to Milli-Q water and the pH adjusted to 7.8–8.0 with 1.0 M HCl. The non-sterile medium was then dispensed into Erlenmeyer flasks plugged with Steristoppers (Heinz-Herenz, Germany) covered with foil dust-caps, and autoclaved for 15 minutes at 121 °.

Table 1. Composition of MLA medium (mg l^{-1} of final media, pH = 7.8–8.0).

FSA constituents	Final Concentation (mg l^{-1})
Salts/nutrients	
$MgSO_4.7H_2O$	49.10
$NaNO_3$	170.00
K_2HPO_4	34.80
H_3BO_3	2.40
Trace metal mix [a b]	
Na_2EDTA	4.56
$FeCl_3.6H_2O$	1.58
$NaHCO_3$	1.20
$MnCl_2.4H_2O$	0.36
$CuSO_4.5H_2O$	0.01
$ZnSO_4.7H_2O$	0.022
$CoCl_2.6H_2O$	0.01
$Na_2MoO_4.2H_2O$	0.006
Additional nutrients/trace metals	
H_2SeO_3	0.0012
$CaCl_2.2H_2O$	29.40
$NaHCO_3$	16.80
Na_2SO_3	12.60
Vitamins [c]	
Thiamine HCl	0.10
Biotin	5×10^{-4}
Cyanocobalamin (B_{12})	5×10^{-4}

[a] Na_2EDTA dissolved before adding other ingredients.
[b] Stored at 4°C as a concentrated (1000 ×) combined stock solution.
[c] Prepared by filter sterilisation and stored at 4°C as a concentrated (1000 ×) stock solution.

Table 2. Test media and incubation conditions used for verifying purity.

Test media	Preparation and incubation
ANB aerobic broth	FSA medium plus 0.1% nutrient broth (Accumedia) incubated for 28 days at 22°C. Inoculated 1 ml into 5 ml.
ANB anaerobic broth	As above but incubated for 28 days at 22°C under anaerobic conditions (Gaspak BBL anaerobic system). Inoculated 1 ml into 5 ml.
TGB broth	Thioglycollate Broth (Accumedia) incubated for 10 days at 22°C. Inoculated 0.5 ml into 5 ml.
GCA agar	FSA agar (1%) plus 0.2% glucose and 0.02% Casamino acids (Difco) incubated for 10 days at 22°C. 1 ml spread-plate.
PYE agar	1.5% agar plus 0.2% Bacteriological Peptone (Accumedia) and 0.2% Yeast Extract (Accumedia) incubated for 10 days at 22°C. 1 ml spread-plate.
PCA agar	Standard Methods Agar (Accumedia) incubated at 22°C and 37°C for 10 days. 1 ml spread-plate.

The solid FSA growth medium was modified by increasing the $NaHCO_3$ concentration tenfold to 168 mg l^{-1} to counteract the acidity of agar and agarose and to further improve the buffering capacity, and the addition of 0.1 mM (0.0126 g l^{-1}) Na_2SO_3 as a growth promoter for purified cultures (Parker, 1982). Solidified FSA medium was prepared by mixing equal volumes of double-strength salts/nutrients solution with 2.0% (1.0% final conc.) Difco Bacto agar or 0.8% (0.4% final conc.) agarose (Molecular Biology Grade, Promega Corp., WI, USA.), which had been autoclaved separately. The nutrients/salts and agar or agarose were cooled to 45 °C and mixed together aseptically, as recommended by Allen (1968). Autoclave-sterilised $NaHCO_3$, $CaCl_2$ and Na_2SO_3, and filter-sterilised vitamins were added and the complete medium was poured into sterilised (γ-irradiated) 90-mm diameter polystyrene petri dishes.

Isolation and culture

Cultures isolated during this study were collected from *Microcystis aeruginosa* blooms, placed in a sterile McCartney bottle, and sent to the laboratory overnight. Samples were initially diluted (approx. 1:1) with sterile MLA medium with the addition of filter-sterilised cycloheximide (100 μg ml^{-1}) and nystatin (100 μg ml^{-1}) to prevent the growth of eukaryotic and fungal contaminants. Non-axenic, uni-algal stock cultures were established from the diluted samples by either or both of the following two methods: (1) Non-clonal stock cultures were obtained by inoculating small volumes of the sample directly into 50 ml conical flasks containing 40 ml of MLA medium. (2) Clonal isolates were obtained by resuspending floating colonies in Milli-Q water to disaggregate the cells. Subsamples of the suspension (0.1 ml) were spread-plated with a glass 'hockey stick' onto 1% MLA agar or 0.4% agarose MLA medium. These plates were sealed with parafilm, inverted, and incubated for 3–4 weeks at 30 °C ± 1.0 °C.

Purification methods

Not all purification methods were applied to all strains, due to their phenotypic characteristics. Only gas-vacuolate strains were used for the centrifugation method. The antibiotic method could be applied to both vacuolate and non-vacuolate strains; however it could not be applied to some strains due to the unavailability of Imipenem powder from the supplier.

Cultures and plates for purification experiments were incubated at one or both of the following temperature/light conditions: (1) 20 °C, 30 μmol photon m^{-2} s^{-1} white fluorescent light (30 μmol photon m^{-2} s^{-1}) with a 18:6 light:dark (L:D) cycle (referred to as 20 °C incubation), or (2) 30 °C, 30 μmol photon m^{-2} s^{-1} and 18:6 L:D cycle (referred to as 30 °C incubation). Individual colonies were then isolated with supporting agar by a platinum micro-spade (a flattened platinum needle) and transferred to sterile flasks containing 40 ml of MLA medium. All cultures were then subsequently maintained at 20 °C ± 1.0 °C under cool

white fluorescent light (5–20 μmol photon m^{-2} s^{-1}) with a 18:6 L:D cycle.

Centrifuge method. Approximately 1 ml of late-logarithmic-phase culture was disaggregated by dilution with 20 ml of Milli-Q water (Parker, 1982). About half of the disaggregated cells were then added to a sterile 10 ml centrifuge tube and cleaned according to the two-step method of Shirai *et al.* (1991). The top ml of supernatant, including the buoyant *Microcystis* cells, was withdrawn with a Pasteur pipette and resuspended in 9 ml of sterile Milli-Q water. Five serial dilutions were prepared with sterile Milli-Q water and the 1/10, 1/100 and 1/1000 dilutions were inoculated by spread-plating on to MLA agar or agarose. The plates were sealed with parafilm and incubated in an inverted position at 30 °C. They were examined after 7 days and every 2 days thereafter for signs of growth and bacteria-free colonies. Presumptive 'clean' colonies were isolated with supporting agar and aseptically transferred to liquid autoclaved MLA medium in 25 ml flasks or fresh MLA agar plates, and incubated at 20 °C.

Sulphide selection gradients. Sulphide selection plates were prepared either according to the methods of Parker (1982) or modified as follows. Approximately one ml of late-logarithmic-phase culture was disaggregated by dilution with 20 ml sterile Milli-Q water and agitated with a vortex mixer. Three serial dilutions of the cell suspension were prepared in sterile Milli-Q and the 1/10 and 1/100 dilutions spread-plated onto the surface of three pre-poured, dried, MLA agar or agarose 90 mm diameter plates. Plates were left to dry for 10–15 minutes and then 1, 2 or 3 drops of 1.3 M Na_2S (BDH Chemicals) were dropped onto one side of the plates above pre-marked spots on the underside. The plates were covered and left to absorb the Na_2S for 2–4 hours, then inverted, sealed with parafilm, and incubated at 30 °C. After 2–4 weeks of incubation, dense, green colonies growing in the zone free of bacterial colonies were transferred by micro-spade to fresh MLA agarose plates, or directly into autoclaved MLA medium, and incubated at 20 °C. The colonies transferred to solid medium were incubated for a further 2–4 weeks and subsequently halved and transferred to autoclaved MLA medium.

Antibiotic treatment. Purification was carried out using the broad spectrum β-lactam antibiotic Imipenem (Merck, Sharp and Dohme, Sydney, Australia) according to Ferris and Hirsch (1991), modified as described below. *Microcystis* cultures (20 ml) were grown to mid-logarithmic phase as described previously and 400 μl sterile nutrient solution (2.5% sucrose, 0.5% yeast extract, 0.5% Peptone G) and 400 μl of sterile Imipenem stock (0.5% w/v) was added to give a final antibiotic concentration of 100 μg ml^{-1}. The flask was wrapped in a double layer of aluminium foil and incubated at 20 °C for 24 to 48 h with continuous shaking at 180 rpm. After incubation the cells were harvested by centrifugation at 9000 g and washed twice with sterile MLA medium. Serial dilutions were prepared (1/10, 1/100, 1/1000) in sterile Milli-Q water and spread-plated on to MLA agar. The plates were then sealed with parafilm, incubated for 2–4 weeks at 30 °C and regularly examined for bacteria-free colonies under a dissecting microscope and an inverted microscope equipped with phase-contrast optics. Clean colonies were transferred with a microspade to flasks of liquid MLA medium and incubated at 20 °C.

Bacterial numbers in cultures before and after 24 and 48 h of Imipenem treatment were assessed in three trials using strain MATE-01. One ml subsamples of culture were serially diluted to 10^{-6} dilution in 9 ml sterile Milli-Q water. All dilutions were spread-plated onto Standard Methods Agar (Accumedia) and incubated at 22 °C for 48 h. The number of colony-forming units (CFU) on two suitable dilutions were counted and averaged. The counts from each of the three replicates were averaged to obtain CFU ml^{-1}.

Verification of purity

Established presumptive axenic clones were inoculated into 25 ml flasks with foil covered Steristoppers and grown at 20 °C until clearly senescent (yellow/white cells, cloudy medium). The cultures were then inoculated into several test media (see Table 2).

After incubation, the liquid test media were examined visually for turbidity. Subsamples were stained with DAPI (Coleman, 1980; Porter & Feig, 1980) and examined directly for bacteria at × 440 and × 1250 magnification under a Zeiss Axioplan microscope by phase contrast and epifluorescence illumination (Zeiss filter set 02). Solid test media were examined visually and microscopically, under a Wild M7 stereomicroscope, for evidence of macroscopic and microscopic colonies of bacteria. Plates were also examined for spreading and gliding bacteria by phase contrast examination at × 400 under a Leitz Labovert inverted micro-

scope. If there was no evidence of bacteria in any of the test media, cultures were judged to be axenic.

Chemicals

The Imipenem antibiotic was obtained as a dry pure powder donated by Merck Sharp and Dohme, Sydney, Australia. Aqueous stock solutions of Imipenem (0.5% w/v) were prepared in Milli-Q water, filter-sterilised and used immediately or frozen at −70 °C until use. Stock solutions of cycloheximide (1.0% w/v, aqueous solution) and Nystatin (1.0% w/v prepared in *N,N*-dimethlyformamide) were filter-sterilised and stored at 4 °C and −20 °C, respectively, until use. Sodium sulphide crystals were stored in a parafilm-sealed bottle at 4 °C until use. Aqueous stock solutions (1.3 M) for sulphide gradient agar plates were prepared by dissolving crystals into boiled Milli-Q water in a fume hood. One ml aliquots were quickly placed into 1.5 ml capped polyethylene centrifuge tubes and frozen at −20 °C until use. The fluorescent stain, DAPI, supplied by Sigma Chemicals, was prepared as an aqueous stock solution (approx 0.1 μg ml^{-1}1), filter-sterilised and stored at −20 °C until use. All other chemicals used in culture media were of at least AR grade or equivalent.

Results

A total of 17 strains of *Microcystis aeruginosa* (9 macroscopic colonial and gas-vacuolate, 7 non gas-vacuolate and non-colonial, 1 gas-vacuolate and non-colonial) were used for the axenic isolation experiments (Table 3). Of the 17 strains, 12 could be purified by one or more of the methods detailed (Table 4).

The centrifuge cleaning method was successful with vacuolate strains easily disaggregated in Milli-Q or distilled water. After one cycle of centrifuge cleaning, 4 of the 7 strains used were successfully purified, with 75–95% of presumptively bacteria-free colonies later confirmed to be axenic (Table 4). The technique was not successful with MAMB-02 which could not be disaggregated, nor with MAMB-03 or MAGL-02, which were contaminated with clear, rapidly spreading bacterial colonies that rapidly spread throughout the plate before axenic *Microcystis* colonies could be isolated. Axenic isolates purified by this method generally retained their colonial morphology and gas-vacuoles rather than dissociate into microscopic colonies or single cells.

The sulphide gradient method was applied to *Microcystis* strains that could be substantially disaggregated in Milli-Q water. Successful gradients usually exhibited a ring of *M. aeruginosa* colonies growing within the selection zone. Colonies within this zone appeared to be bacteria-free; the size of the selection zone varied considerably depending on the strain used. Sulphide gradients purified 3 of the 9 strains used; 87–100% of presumptive axenic colonies picked from the selection zone were subsequently found to be bacteria-free. For 3 strains (MABA-01, MAPI-01 and MATE-01), presumptive axenic colonies were isolated and subsequently appeared to be uncontaminated on all test media (see Table 4). However, examination by phase contrast and DAPI/fluorescence-staining microscopy showed the presence of a spirochaete, tentatively identified as *Spirochaeta* cf. *aurantia*. These cultures were subsequently subjected to the antibiotic purification procedure without success. All cultures purified using this procedure lost their colonial morphology and gas-vesicles.

To compare our spread-plate method with the pour-plate method of Parker (1982), two strains (MAGL-02 and MAMB-03) were subjected to sulphide gradient purification with pour-plate gradients. In both cases pour-plates were unsuccessful. For MAGL-02 the selection zone was smaller than the spread plate (<5 mm) and it was difficult to remove colonies embedded in the agarose; none could be removed without contact with other bacterial colonies.

The Imipenem antibiotic purification method was applied to 6 strains, ranging from unicellular, non-gas-vacuolate, through to colonial, gas-vacuolate strains (see Table 4). After 10 to 14 days' incubation, presumptive bacteria-free colonies were identified using a stereomicroscope. Contaminant bacterial colonies were usually clear, spreading types, necessitating careful examination of plates by phase-contrast microscopy to identify colonies for isolation. Four of the 6 strains were successfully purified; 67–100% of isolates of each strain were found to be axenic. The remaining two strains, MAPI-01 and MATE-01 which were contaminated with the spirochaete, could not be purified by this method. Strains purified using Imipenem retained a similar morphology to the parent culture, and retained gas-vesicles, if present.

Imipenem treatment substantially reduced the number of bacterial CFU in a culture of strain MATE-01. The initial bacterial numbers were found to be 5.10×10^6 (SD = 2.65×10^5) CFU ml^{-1}. After 24 hours of treatment the bacterial count had been

Table 3. Isolation site and physical characteristics of the cultures used for the axenic culture experiments.

Strain	Location isolated	Gas vacuoles	Macroscopic colonies	Disaggregation in Milli-Q
MAGO-01	Gobba Lagoon, Wagga Wagga, NSW	-	-	+
MALB-01	Lake Burley-Griffin, ACT	+	+	+
MASY-01	Malpas Reservoir, NSW	-	-	+
MAMB-01	Mt Bold Reservoir, SA	-	-	+
MAMB-02	Mt Bold Reservoir, SA	+	+	-
MAMB-03	Mt Bold Reservoir, SA	+	+	-
MAPI-01	Water supply, Palm Is. Qld	-	-	+
MAPI-02	Water supply, Palm Is. Qld	-	-	+
MASH-01	Goulburn River, Shepparton, VIC	+	+	+
MATE-01	Lake Centenary, Temora, NSW	-	-	+
MAGR-01	Raw Water Supply, Griffith, NSW	+	+	+
MASM-01	St Marys sewage lagoon, TAS	+	+	+
MABA-01	Farm dam, Barambola, NSW	+	+	+
MAWB-01	Werribee sewage lagoon, VIC	+	+	+
MAGL-02	Gippsland Lakes, VIC	+	+	+
MATA-01	Tallaroop Reservoir, VIC	+/-	-	+
NIVA CYA-43	*a*	-	-	+

a Data unknown.

reduced by 2.5 orders of magnitude to 1.05×10^4 (SD = 7.02×10^2) CFU ml^{-1}. Further incubation for a total of 48 h reduced the bacterial count another 2 orders of magnitude to 150 CFU ml^{-1} (SD = 96.0).

Discussion

Purity verification

Several methodological problems were encountered when verifying culture purity. While plating or inoculation of old cultures onto the test media was a convenient and rapid way of assessing contamination, examination of old, presumptively axenic cultures by DAPI staining and fluorescence microscopy was a more reliable test. However, some components of lysed *Microcystis* cells, notably cyanophycin granules (structured granules) (Simon, 1987), fluoresce in much the same way as bacteria and are easily confused with small coccoid bacteria. Phase contrast reveals the refractile appearance of structured granules, which can used to discriminate them from the non-refractile bacterial cells.

The finding that three apparently bacteria-free strains, purified by the sulphide gradient method, were contaminated by spirochaete bacteria illustrates the importance of microscopic examination as the critical test of axenicity. The spirochaete cells were difficult to visualise with phase contrast because of their narrow diameter (0.2–0.3 μm) and are often intimately associated with *Microcystis* colonies. They were, however, easily visualised by DAPI staining and fluorescence microscopy. The broad-spectrum test media used did not support the growth of the spirochaete contaminant found in this study. Therefore it is important to either include spirochaete growth media in axenic-testing regimes, or examine presumed axenic cultures by phase contrast and fluorescent stain microscopy to verify the purity.

Purification methods

The modified two-step centrifuge method presented here was successful with 4 of the 7 gas-vacuolate strains tested, failing with the strains which could not be completely disaggregated before centrifugation. It is also much less time-consuming (2–4 weeks) than the repeated cycle processes of Shirai *et al.* (1989; 1990). Their method was also unsuccessful with some *Microcystis* strains, however the repetitive, two-step centrifuge method did eventually purify the resistant strains. It remains possible that repeated centrifugation and re-isolation could improve the success rate, however, each cycle is time consuming (4–6 weeks) and difficulties with strains that cannot be disaggregated are likely due to the tight association of bacteria

Table 4. Summary of axenic culture attempts and success rate.

Method	Strain	Isolates made	Successful[a] growth	Axenic isolates	Success[b] (%)
Centrifuge/spread[c]	MAGL-02	0	-	-	-
	MAGR-01	24	21	20	95
	MALB-01	8	8	6	75
	MAMB-02	0	-	-	-
	MAMB-03	0	-	-	-
	MASH-01	24	22	21	95
	MAWB-01	15	14	11	79
Sulphide/spread	MABA-01	4	2	0	0[d]
	MAGL-02	6	5	5	100
	MAMB-03	0	-	-	-
	MAPI-01	5	5	0	0[d]
	MAPI-02	0	-	-	-
	MASM-01	21	15	13	87
	MATA-01	5	4	4	100
	MATE-01	4	4	0	0[d]
	NIVA CYA-43	0	-	-	-
Sulphide/pour	MAGL-02	0	-	-	-
	MAMB-03	0	-	-	-
Imipenem/spread	MAGO-01	7	5	5	100
	MAMB-01	6	4	4	100
	MAMB-02	12	3	2	67
	MAPI-01	10	10	0	0[d]
	MASY-01	8	7	7	100
	MATE-01	7	5	0	0[d]

Note: In cases where no isolates were made there were either no 'clean colonies' or was no apparent growth.

[a] Successful growth refers to isolates that were subsequently viable and could be tested for axenic status.

[b] Percentage of isolates showing successful growth that were subsequently found to be axenic.

[c] Only strains that were gas-vacuolate and could be disaggregated in Mill-Q water could be used for this method.

[d] These cultures were contaminated with a spirochaete that could not be removed by any of the methods trialed.

with the colonial matrix. One serious disadvantage of centrifuge methods are that they are not suitable for cultures that have become non-gas-vacuolate, a common effect of long-term laboratory culture. However, newly established *M. aeruginosa* isolates usually retain buoyancy for several months or more (unpublished observations) and centrifugation could, therefore, be applied to recently acquired strains that can be disaggregated in Milli-Q water.

The sulphide gradient method described here, which purified 3 of 8 strains used, is easily applied to both gas-vacuolate and non-vacuolate strains. The method is substantially quicker to undertake using the spread-plate methods with pre-prepared solid media than the pour plate method of Parker (1982). One strain that could not be purified by Parker's (1982) method was subsequently purified from a spread plate gradient suggesting it is also a more reliable purification method. Selection of bacteria-free colonies is very reliable (87–100% of colonies selected were axenic) in cases where a clear selection zone is created. However, high sulphide tolerance does not appear to be a universal feature of *Microcystis* strains. Some strains exhibited low sulphide tolerance, or were less toler-

ant of sulphide than the contaminating bacteria, and sulphide selection failed. It is also of some concern that strains isolated from sulphide gradients lost both gas-vacuolation and colonial morphology despite the non-axenic parent retaining these characters, implying a direct physiological effect on the cells or strong selection for cells without these characters expressed.

The success of Imipenem for purifying *Microcystis* strains was encouraging (4 of 6 cultures purified), however, two cultures contaminated by a spirochaete bacterium could not be purified despite 48 h treatment with Imipenem. This may be due to resistance to Imipenem or, as spirochaetes are highly motile in solid media (particularly softer agar and agarose; Canale-Parola, 1981), any remaining viable cells may recontaminate *Microcystis* colonies on solid media before they can be isolated. The reduction in bacterial numbers by Imipenem incubation (2.5 orders of magnitude in 24 h) is consistent with Ferris & Hirsch's (1991) data. However, we found that 48 h incubation reduced bacterial contamination considerably more (4.5 orders of magnitude), with little apparent loss of cyanobacterial viability.

Despite the success of Imipenem, there are several major drawbacks with the purification method. Firstly, the use of antibiotics to purify cyanobacterial cultures concerns a number of workers due to possible selection pressures imposed during isolation, leading to the isolation of abberant phenotypes or antibiotic resistant strains (B. A. Whitton, personal communication). However, this does not invalidate the method for some studies which investigate particular characters such as toxin production. The researcher can, in most cases, determine the phenotypic character of interest (i.e. test for toxin production) after purification.

Secondly, the presence of an apparently Impipenem-resistant spirochaete can lead to failure of the purification. Spirochaetes of the genus *Spirochaeta* are either obligately or facultatively anaerobic and are commonly isolated from freshwater environments and anoxic, sulphide-rich, lake and river sediments (Canale-Parole, 1981). They are, therefore, likely to be a common contaminant of cyanobacterial cultures from aquatic habitats. They are also presumably tolerant to high sulphide concentrations, explaining their presence even after sulphide-gradient purification.

Thirdly, Imipenem is unstable (Merck, Sharpe and Dohme, personal communication) and is not yet widely available. Later attempts to purify additional *M. aeruginosa* strains with Imipenem met with poor results, despite storage at −70 °C. Fresh supplies of pure Imipenem proved difficult to obtain from the manufacturing company and the method could not be used to purify additional strains. Another carbapenem-based antibiotic, Meropenem (ICI Pharmaceuticals), has recently been released for clinical trials indicating a similar broad spectrum of activity (Mudaliar *et al.*, 1994). It is a considerably more stable compound, and may be a useful alternative to Imipenem. The authors could not trial this antibiotic as it was not commercially available from ICI Pharmaceuticals.

Purification of cyanobacterial strains, particularly *Microcystis* species, is time consuming. All three improved purification methods presented here produced axenic *Microcystis aeruginosa* cultures and are relatively rapid and simple to use. However, only about half of the strains we used could be purified by each method. By using the three methods, all but 5 of 17 strains were purified. The three methods could not all be applied to all strains due to the different physical characteristics of the strains and, in this study, problems obtaining Imipenem. The common factor in the success of all the methods is that a researcher must be able to identify the bacteria-free colonies, which requires practice and experience. Additionally, problems created by the diversity of growth characteristics and environmental tolerances means that the success of any one method of purification is by no means guaranteed with all strains. However, researchers prepared to use a variety of techniques, such as those described here, should succeed in isolating axenic cultures.

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References

Allen MM (1968) Simple conditions for growth of unicellular blue-green algae on plates. J. Phycol. 4: 1–4.

Baker PD (1992) Identification of common noxious Cyanobacteria. Part II – Chroococcales and Oscillatoriales. Urban Water Research Association of Australia Research Report 46.

Boon PI (1992) Antibiotic resistance of aquatic bacteria and its implications for limnological research. Aust. J. mar. freshwater Res. 43: 847–859.

Canale-Parola (1981) Free-living anaerobic and facultatively anaerobic Spirochaetes: The genus *Spirochaeta*. In Starr MP, Stolp H, Trüper HG, Balows A, Schlegel HG (eds), The Prokaryotes, Vol. 1. A Handbook on Habits, Isolation and Identification of Bacteria. Springer-Verlag, Berlin, Germany: 538–547.

Carmichael WW (1992) Cyanobacteria secondary metabolites – the cyanotoxins. J. appl. Bact. 72: 445–459.

Castenholz RW (1988) Culturing methods for cyanobacteria. Meth. Enzymol. 167: 68–92.

Ferris MJ, Hirsch CF (1991) Method for isolation and purification of cyanobacteria. Appl. environ. Microbiol. 57: 1448–1452.

Fogg GE (1942) Studies on nitrogen fixation by blue-green algae. 1. Nitrogen fixation by *Anabaena cylindrica* Lemm. J. exp. Biol. 19: 78–87.

Gorham PR, McLachlan J, Hammer UT, Kim WK (1964) Isolation and culture of toxic strains of *Anabaena flos-aquae* (Lyngb.) de Bréb. Verh. int. Ver. Limnol. 15: 796–804.

Guillard RRL (1973) Methods for microflagellates and nanoplankton. In Stein JR (ed) Handbook of Phycological Methods: Culture methods and growth measurements. Cambridge University Press, London, UK: 69–86.

Hoshaw RW, Rosowski JR (1973) Methods for microscopic algae. In Stein JR (ed.) Handbook of Phycological Methods: Culture methods and growth measurements. Cambridge University Press, London, UK: 53–68.

Mudaliar U, Furyk N, Wilson V, Infanti J, Mayall B (1994) Comparative in-vitro antibacterial activity of Meropenem. In Abstracts of Australian Society for Microbiology Annual Scientific Meeting, Melbourne, 1994, Abstract P02.32, Aust. Microbiol. 15(4): 132.

Parker DL (1982) Improved procedures for the cloning and purification of *Microcystis* cultures (Cyanophyta). J. Phycol. 18: 471–477.

Porter KG, Feig YS (1980) The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. 25: 943–948.

Rippka R (1988) Isolation and purification of cyanobacteria. Meth. Enzymol. 167: 3–27.

Rippka R, Waterbury JB, Stanier RY (1981) Isolation and purification of cyanobacteria: Some general principles. In Starr MP, Stolp H, Trüper HG, Balows A, Schlegel HG (eds), The Prokaryotes, Vol. 1. A Handbook on Habits, Isolation and Identification of Bacteria. Springer-Verlag, Berlin, Germany: 212–220.

Simon RD (1987) Inclusion bodies in the cyanobacteria: cyanophycin, polyphosphate, polyhedral bodies. In Fay P, Van Baalen C (eds), The Cyanobacteria. Elsevier Science Publishers B.V., Amsterdam, Netherlands: 199–226.

Shirai M, Matumaru K, Ohotake A, Takamura Y, Aida T, Nakano M (1989) Development of a solid medium for growth and isolation of axenic *Microcystis* strains (Cyanobacteria). Appl. environ. Microbiol. 55: 2569–2571.

Shirai M, Ohtake A, Takaai S, Matsumoto S, Sakamoto T, Sato A, Aida T, Harada K, Shimada T, Suzuki M, Nakano M (1990) Toxicity and toxins of natural blooms and isolated strains of *Microcystis* spp. (Cyanobacteria) and improved procedure for purification of cultures. Appl. environ. Microbiol. 57: 1241–1245.

Vaara T, Vaara M, Niemela S (1979). Two improved methods for obtaining axenic cultures of cyanobacteria. Appl. environ. Microbiol. 38: 1011–1014.