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Rapid establishment of clonal isolates of freshwater autotrophic picoplankton by single-cell and single-colony sorting

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Abstract

We describe single-cell and single-colony sorting protocols which allowed for rapid establishment of a diverse culture collection of clonal autotrophic picoplankton (APP) isolates originating from oligotrophic and oligo-mesotrophic subalpine lakes. Overall sort recoveries, expressed as the percentage of sorted microwells exhibiting APP growth, ranged from 5% to 17% depending on the type of APP, but the growth success varied greatly (from 0% to 68%) depending on the origin of the sorted sample. We applied two direct sequencing and two denaturing gradient gel electrophoresis (DGGE) protocols to identify and characterize the genetic purity of 21 of our picocyanobacteria cultures, namely, direct sequencing of the 16S rRNA gene and *cpcBA*-IGS region, and DGGE analyses involving a 194-bp fragment of the internal transcribed spacer (ITS) and a ca. 500-bp fragment of the phycocyanin (PC) operon (*cpcBA*-IGS, novel protocol described herein). Of those 21 picocyanobacteria cultures obtained by single-cell/single-colony sorting and subsequently characterized genetically/screened for genetic purity, only one culture was composed of multiple picocyanobacterial strains.

Keywords: Cell culture; Eukaryotic autotrophic picoplankton; Flow cytometry; Phycocyanin; Phycoerythrin; *Synechococcus*

1. Introduction

Autotrophic picoplankton (APP) are microscopic (0.7 to 2.0 μm) cyanobacterial or eukaryotic planktonic phototrophs. They are of global biogeochemical and ecological importance in freshwater and marine ecosystems, are used as model organisms for investigating the genetic prerequisites and evolution of phototrophy, and are likely to be increasingly exploited and studied for industrial purposes (Apt and Behrens, 1999; Raven, 1998; Stockner et al., 2000; Weisse, 1993). Despite the interest in APP, our understanding of their biodiversity, physiology, ecology and industrial potential is significantly hampered by the considerable time, effort and unintended selectivity associated with classical isolation techniques such as plating (Brand, 1986; Ernst, 1991, Ernst et al., 2003; Rippka et al., 2000). Ernst (1991), for example, noted that standard plating techniques favored the isolation of phycocyanin (PC)-rich picocyanobacteria, which comprise a small fraction of the picocyanobacteria inhabiting subalpine lakes (Weisse, 1988, 1993). Phycoerythrin (PE)-rich picocyanobacteria took a period of up to 40 weeks before plated colonies were sufficiently large for transfer into liquid culture (Ernst,

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1991). Finally, many picocyanobacteria do not form isolated colonies on agar or agarose amended with culture medium BG11, and transfer between solid and liquid culture remains problematic (Ernst et al., 2003; Rippka et al., 2000).

The goal of this study was to develop a protocol for improved flow sorting of freshwater APP which, in combination with flow-cytometric and molecular screening, should reduce the time needed to establish a culture collection and enhance the likelihood that the cultures were clonal. Previous reports of flow sorting as a means of establishing APP cultures are few and anecdotal. Yentsch et al. (1983) reported the isolation of a marine *Synechococcus* strain, but did not asseses its genetic purity and gave no details as to the number of inoculum cells. Moore et al. (1998) sorted bright and dim *Prochlorococcus* cells co-occurring in freshly collected marine samples. They succeeded in establishing cultures of each type from the sorted cells and showed that each retained its unique flow cytometric and genetic signatures. Since, however, cultures were initiated by sorting many hundreds of cells into a given culture vessel (Lisa Moore, personal communication), such cultures may not be clonal. Finally, Reckermann (2000) used flow sorting to obtain *Synechococcus* cultures from a water sample taken from a coastal environment, but did not evaluate their genetic purity nor assess the likelihood that the isolates were obtained from single cells.

Since freshwater APP, particularly picocyanobacteria, form 'microcolonies' in addition to occurring as solitary cells (Crosbie et al., 2003b; Stockner et al., 2000), we established picocyanobacterial cultures by sorting either single cells or single colonies (i.e. single-cell cloning or single-colony 'cloning'). The picocyanobacterial microcolonies sorted in this study consisted predominantly of an assemblage of tightly packed, PE-rich coccoid, ovoid or rod-shaped picocyanobacteria, occurring in both natural samples and enrichment cultures, and which are amenable to flow-cytometric sorting (Crosbie et al., 2003b). When viewed using an epifluorescence microscope, cells from the same microcolony always exhibited identical morphology, suggesting that microcolonies had formed by clonal cell division. Nonetheless, the possibility that some microcolonies included 'foreign' picocyanobacterial cells (e.g. as the result of aggregation processes) emphasized the need to assess the genetic purity of cultures obtained by sorting single colonies.

2. Materials and methods

2.1. Enrichment cultures and natural sample

Water samples from the surface 20-m water column of five subalpine lakes (Mondsee, Irrsee, Hallstättersee, Attersee and Traunsee—all located in Upper Austria) were collected by an integrating sampler (Schröder, 1969) (Schröder sampler from Züllig, CH-9424 Rheineck, Switzerland; volume collected = 750 ml), transferred immediately to thermostable containers (temperature of water samples ranged between 3 and 15 °C), and transported in darkness to the laboratory. Within 5 h of sampling, 200-ml subsamples were amended with 50 ml of culture media (BG11, Stanier et al., 1971) and gravity-filtered at 15 °C and under low light (10 μmol quanta m^{-2} s^{-1} white light, continuously supplied by 'cool-white' fluorescent tubes) using sterile Sartorius filtration units fitted with 2-μm (nominal pore size) Millipore type TTTP filters. For each of a total of seven enrichment cultures (see Table 2B), gravity filtration and the addition of culture media were repeated on a weekly basis and, 3–4 weeks after collection of the original water samples, harvested for single-cell/single-colony sorting. In addition, single-cell/single-colony sorting was also conducted immediately upon collection (March 2, 2001) of a sample from Lake Mondsee. This sample was also collected from the surface 20-m water column using a Schröder sampler.

2.2. Flow-sorting, epifluorescence microscopy and culture in microplate wells and flasks

We used a FACSVantage SE™ equipped with CloneCyt Plus™ software (v. 3.1, BD Systems, San Jose, CA, USA) and an INNOVA™ Enterprise™ II-621 ion laser (Coherent, Santa Clara, CA, USA) providing 170 ± 10 mW 488-nm output (UV-blocker engaged). For single-cell/single-colony sorting, the sorter was operated in 'Counter' mode with drop-drive attenuation switched on, drop-drive frequency set to 17,400 Hz, dead time set to 7.5 μs, and sheath

fluid (0.1% NaCl PBS, autoclaved) and sample differential pressures set to 9 PSI and 8 relative units, respectively. To maximize the flow-cytometric discrimination of the APP populations, we used a 100-μm (orifice diameter) sort nozzle, a broad bandpass red filter (695 ± 40 nm) and a custom-made overlay added to the obscuration bar of the forward scatter collection lens. The jets produced by smaller nozzles were more difficult to align and resulted in pure laser noise when overlays were not added to the forward scatter obscuration bar, while adding overlays reduced cell scattering and fluorescence to an extent that individual APP populations could not be readily distinguished from each other and from the noise. Contamination was minimized by flushing the fluidics with 70% ethanol for ca. 30 min, followed by Milli-Q water (for up to 1 h).

Monoalgal, non-axenic cultures of PE-rich (BO 8807, BO 8809) and PC-rich (BO 8801) picocyanobacteria (Ernst et al., 1992), and three eukaryotic autotrophic picoplankton (EAP) taxa (*Choricystis* sp. strain Pi98/1A1, *Chlorella* cf. *minuttissima* strain CCAP 211-52 and strain KR 96/1; courtesy of D. Hepperle) (Hepperle and Schlegel, 2002) were used to aid setting up of the flow cytometers for sorting/analyzing of APP from enrichment cultures and natural samples (all reference cultures were grown under the same or similar conditions as used for growth of enrichment cultures). Finally, instrument settings and gate boundaries for single-cell/single-colony sorting of APP populations were refined by bulk sorting from enrichment cultures/natural sample into microwells containing 2 ml of Milli-Q, and then observing the sorted (10 000–50 000 cells per defined gate) and fixed (paraformaldehyde, 0.2% final concentration) cells by epifluorescence microscopy (Zeiss filter sets #01, 05 and 14).

Sort gates were classified as targeting PE-rich picocyanobacteria, PC-rich picocyanobacteria or EAP. Fig. 1 shows an example of gates used to sort APP populations from enrichment cultures. By comparing the a priori (i.e. sort-gate) classification of APP cells with the identity of APP cells found growing in microwells, we determined the number of misclassifications that resulted in growth of the 'wrong' cell type—e.g. a microwell exhibiting growth of PE-rich picocyanobacteria when a PC-rich cell was supposedly sorted.

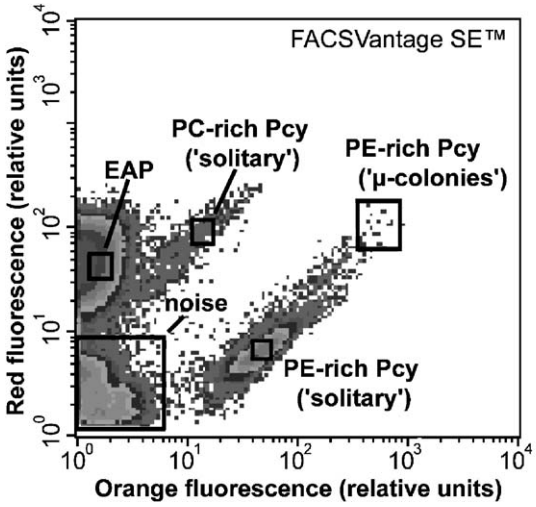

Fig. 1. Example of gates used for single-cell and single-colony sorting of APP populations sourced from enrichment cultures originating from subalpine lakes. Pcy = picocyanobacteria, PE = phycoerythrin, PC = phycocyanin, EAP = eukaryotic autotrophic picoplankton. Note that the cytogram shows the flow-cytometric signature of an undiluted enrichment culture. Prior to single-cell/single-colony sorting, enrichment cultures were diluted with BG11 to achieve total event rates of $<200\ s^{-1}$.

Just prior to single-cell/single-colony sorting, enrichment cultures were diluted with culture media to achieve total event rates of <200 events s^{-1} and single APP cells or microcolonies sorted by depositing a 4-drop sort package (i.e. sort envelope set to 3 drops, but 0.5 drops was added to each end of the sort envelope due to the use of 'Counter' mode) into tissue-culture microplate (24-well format, Iwaki, 3820-024) wells containing 2 ml of culture media. High sort purities were expected given the conditions under which APP were sorted (Leary, 2000; Lindmo et al., 1990)—a total event rate of $<200\ s^{-1}$, target event rates of between 10 and 100 s^{-1}, and a cycle time of ca. 10 μs (i.e. an average spacing of 89 or more drops per event).

To control for the possibility that multiple target cells had been deposited into a given well, we restricted single-cell sorting (but not single-colony sorting) to microplate rows 'A' and 'C', thoroughly mixed their contents under sterile conditions using a pipette, and then transferred half their contents into half-filled wells of rows 'B' and 'D' (i.e. half of A1 well into B1 well, half of C1 into D1, etc.).

Transferred contents were replaced with equal amounts of culture media and each 'sorted microplate' fitted with its plastic lid and placed to culture conditions (15 °C, 30 μmol quanta m^{-2} s^{-1} white light, continuously supplied by 'cool-white' fluorescent tubes).

Four to six weeks following their inoculation by single-cell or single-colony sorting, 250 μl from each microplate well was harvested under sterile conditions and processed by a three-color FACSCalibur™ flow cytometer (BD Systems) for evidence of APP growth. The operation of the FACSCalibur was as follows. Using WorklistManager™ software (v. 3.1f, BD Systems), data acquisition was automatically delayed for a period of 15 s prior to the analysis of individual samples to allow the autoloader to gently shake the samples (5 s), to flush the sample interrogation cuvette with new sample and to allow the flow rate to stabilize (10 s). Flow rate was determined by an adaptation of the gravimetric protocol of Olson et al. (1993). Specifically, the flow rate was given by the regression slope of analyte weight change versus time, plotting the results from 6 FALCON™ tubes filled with standard analyte (3 ml of integrated sample added to 100 μl of bead working stock; FACSFlow™ sheath fluid (BD Systems)), analyzed for periods of between 0.5 and 12 min using standard instrument settings. Semi-automatic classification of APP populations was performed on CellQuest™ (v. 3.1f) data files using Attractors™ (v.3.0.0) software (BD Systems) (see Crosbie et al., 2003b). Total sample event rates were always 5- to 10-fold below event rates at which coincidence may become significant (ca. 500 events s^{-1}). A count of at least 5-fold greater than that observed for sheath-fluid controls was nominally taken as evidence of APP growth.

A small subset of the microplate wells showing evidence of APP growth was transferred aseptically to flasks containing 50 ml of culture media and the flasks placed to culture conditions. Flask contents were examined periodically by flow cytometry and by a Zeiss Axioplan microscope equipped with a charge-coupled device (CCD) camera (Hitachi, model KP-M1E/K). Picocyanobacteria were differentiated from EAP based upon their epifluorescent characteristics under green and blue excitation (MacIsaac and Stockner, 1993; Weisse, 1988). PE-rich picocyanobacteria were characterized by their orange-red fluorescence under green and their yellow-orange fluorescence under blue excitation. PC-rich picocyanobacteria and EAP appeared purple-red or red at both green and blue excitation. They were differentiated from each other based on the presence or absence of a chloroplast.

2.3. Characterization of picocyanobacterial isolates by direct sequencing and DGGE

We applied two direct sequencing and two denaturing gradient gel electrophoresis (DGGE) protocols to identify and characterize the genetic purity of our picocyanobacterial isolates, namely, direct sequencing of the 16S rRNA gene and *cpcBA*-IGS region, and DGGE analyses involving a 194-bp fragment of the internal transcribed spacer (ITS) (adapted from Becker et al., 2002) and a ca. 500-bp fragment of the phycocyanin operon (*cpcBA*-IGS protocol described herein). The primers used in this study, and their target regions, are summarized in Table 1.

DNA used in each of the direct sequencing or DGGE protocols was extracted from 10 to 50 ml of culture material using the DNA extraction protocol of Tillett and Nielan (2000), or the FastDNA kit and FastPrep instrument (Bio 101), and its concentration and purity determined by measuring the absorption ratio A_{260}/A_{280} and/or electrophoresis through 1% agarose in TBE buffer (45 mM Tris–borate, 1 mM EDTA). All PCRs were performed in 25-μl volumes using a MWG-Biotech^{Primus} thermocycler, and products visualized by electrophoresis through 1.5% (w/v) agarose in TBE buffer and photographed under UV transillumination after staining with ethidium bromide (EB).

All DGGE analyses were performed using a Bio-Rad Dcode™ universal mutation detection system. For sequencing of DGGE bands, prominent bands in EB- or SYBR Gold-stained DGGE gels were excised using a sterile scalpel and DNA eluted overnight at 4 °C in 30 μl of sterile Milli-Q H_20. One to two microliters of the solution was amplified using the same primers, reaction mixture and thermocycling conditions used to generate PCR products for a given DGGE screen (see below). The resulting PCR products were pooled,

Table 1
PCR primers used in this study and their target sites

Primer	Sequence (5′ → 3′)	Target site	Reference
16S5′F	AGAGTTTGATCCTGGCTCAG	5′end of 16S rRNA gene	Scheldman et al., 1999
B23S5′R[a]	CTTCGCCTCTGTGTGCCTAGGT	5′end of 23S rRNA gene	Lepere et al., 2000
cpcBF(UFP)[a]	TAGTGTAAAACGACGGCCAGTTGYYTK CGCGACATGGA	3′ flanking region of *cpcB*	Robertson et al., 2001
cpcAR(URP)[a]	TAGCAGGAAACAGCTATGACGTGGTGT ARGGGAAYTT	5′ flanking region of *cpcA*	Robertson et al., 2001
PITSANF	CGTAACAAGGTAGCCGTAC	3′end of 16S rRNA gene	Becker et al., 2000
PITSEND[b]	CTCTGTGTGCCAAGGTATC	5′end of 23S rRNA gene	Becker et al., 2000
PITSGCANF	GTGATGTCTGAGTAATTTATTCTCAGGC	ITS fragment, forward primer	Becker et al., 2002
PITSGC	GCCGCGCCCGCCGGCCGCCCGCGCGCCC GCCGCCGCGCCCGCCGCCGCCCGGCGGA ATTATAAATATAGGAGCTCTCGCCGCAAC	ITS fragment, reverse primer	Becker et al., 2002
cpcBF(DGGE)[†]	CGCCCGCCGCGCCCCGCGCCCGCCCCGC CGCCCCCGCCCGTGYYTKCGCGACATGGA	3′ flanking region of *cpcB*	Robertson et al., 2001[†]

[a] Cyanobacteria-biased.
[b] Biased for picophytoplankton clade (sensu Urbach et al., 1988).
[†] Modified for DGGE by the addition of a GC clamp to cpcBF primer of Robertson et al. (2001).

purified using the QIAquick PCR Purificaton kit (Qiagen), sequenced unidirectionally (VBC Genomics), and the sequence data subsequently compared to that obtained by direct sequencing and to sequences held in GenBank, using CLUSTALX (Thompson et al., 1997) and BLAST (Altschul et al., 1997), respectively.

(i) *Direct sequencing*. 16S rRNA gene and *cpcBA*-IGS sequences were amplified using the primer pairs 16S5′F/B23S5′R (Lepere et al., 2000; Scheldman et al., 1999) and cpcBF(UFP)/cpcAR(URP) (Robertson et al., 2001), respectively (Table 1). PCRs were performed in multiples, products pooled, purified using the QIAquick PCR Purificaton kit (Qiagen) and then sequenced (VBC Genomics) bidirectionally. Further details can be found in Crosbie et al. (2003a).

(ii) *DGGE and sequencing of excised bands*. ITS fragments were amplified and analyzed by DGGE using a protocol adapted from Becker et al. (2002). Briefly, ITS fragments were amplified in two successive PCRs, conducted as a nested PCR. The first PCR used primer pairs PITSANF and PITSEND (Table 1), and final PCR reactant concentrations and PCR cycling parameters as follows: 0.3 mM each deoxynucleoside triphosphate, 0.2 μM each primer, 10 to 100 ng of template DNA, 2.5 mM $MgCl_2$ and 0.625 U of Taq (Qiagen) polymerase. Cycling parameters: 95 °C for 3 min, followed by 30 cycles of 60 °C for 2.5 min and 94 °C for 40 s. This was followed by a final elongation step of 70 °C for 5 min. One to two microliters of the first assay mixture was used as template for the second PCR, which used primers PITSGCANF and PITSGC (Table 1), and final PCR reactant concentrations and PCR cycling parameters as follows: 0.3 mM each deoxynucleoside triphosphate, 0.2 μM each primer, ca. 1 to 2 μl of the first PCR product, 2.5 mM $MgCl_2$ and 0.625 U of Taq (Qiagen) polymerase. Cycling parameters: 95 °C for 3 min, followed by 35 cycles of 65 °C for 1.5 min and 94 °C for 30 s. This was followed by a final elongation step of 70 °C for 5 min. For DGGE, ca. 600 ng of PCR product was electrophoresed through a 10%–40% denaturing gradient for 4 h at 200 V.

cpcBA-IGS fragments used for DGGE were amplified using the primers cpcBF(DGGE) and cpcAR (URP) (Table 1). Final PCR reactant concentrations and PCR cycling parameters were as follows: 0.3 mM each deoxynucleoside triphosphate, 0.3 μM each primer, ca. 50 to 100 ng of template DNA, 2.5 mM $MgCl_2$, 0.5 U of Taq (Qiagen) polymerase. Cycling parameters: 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. This was followed by a final elongation step of 72 °C for 7 min. For DGGE, 100 to 400 ng of PCR product was electrophoresed through a 40%–70% denaturing gradient for 16 h at 100 V.

3. Results

3.1. APP growth in microwells

APP growth was observed in 15% of microplate wells putatively inoculated with single APP cells (Table 2A). Overall recoveries ranged from 5% (from 288 wells), for EAP-preclassified cells to 17% (from 96 wells) for cells preclassified as PC-rich picocyanobacteria (Table 2A), but the growth success varied greatly (from 0% to 68%) depending on the origin of the sorted sample (Table 2B). Misclassifications resulting in growth of the 'wrong' cell type were rare (1–5%) (Table 2A).

In support of the assumption that the majority of cultures obtained from single-cell sorting were initiated from single APP cells, APP growth in *both* of paired sort-control wells was observed only six times (of the fifty-three 24-well microplates that were sorted in the 'split-wells' format, APP growth was observed in 71 wells).

3.2. Direct sequencing and DGGE

Twenty-one picocyanobacterial cultures obtained by single-cell/single-colony sorting were characterized genetically and screened for their genetic purity. Many of our new isolates are similar to PE-rich isolates from other subalpine lakes, falling into two groups (B&H) (Fig. 2). Additionally, we have proposed the new group 'I', which contains four PC-rich isolates from L. Mondsee and an Arctic tundra pond (Crosbie et al., 2003a; Vincent et al., 2000) (cluster designations and their nomenclature are based on the groups proposed originally by Robertson et al. (2001), with modifications according to Ernst et al. (2003) and Crosbie et al. (2003a)).

Recent studies on freshwater picocyanobacteria have found greater sequence heterogeneity in *cpcBA*-IGS and ITS loci in comparison to that present in their 16S rRNA genes (Becker et al., 2002; Crosbie et al., 2003a; Ernst et al., 2003; Robertson et al., 2001). Accordingly, we were able to discriminate closely related isolates with either the ITS DGGE or *cpcBA*-IGS DGGE protocol (Fig. 3), but failed to do so (N.D. Crosbie, unpublished data) when using a protocol which targets a ca. 600-bp fragment of the 16S rRNA gene [i.e. the protocol of Nübel et al. (1997), modified as described by Garcia-Pichel et al. (2002)].

DGGE examination of PCR products amplified with the PITSGCANF and PITSGC primers (Table 1) often revealed more than one band from a single isolate starting template (Fig. 3A), but the banding pattern exhibited a high degree of similarity between templates originating from putatively identical or closely related picocyanobacteria strains (compare Fig. 2 with Fig. 3A). One explanation for the multiple bands is that PCR with the PITSGCANF and PITSGC primers produced both homoduplex and heteroduplex molecules which could be separated by DGGE. This explanation is supported by two observations. First, sequences which appeared to

Table 2A
Percentage of *all* sorted wells showing phycoerythrin-rich picocyanobacteria (PE), phycocyanin-rich picocyanobacteria (PC) or eukaryotic autotrophic picoplankton (EAP) growth for a priori sort gate classfications PE, PC and EAP

Pre-sort classification	Number of sorted wells	Post-sort APP classification		
		%PE	%PC	%EAP
PE	1193	14	0	1
PC	96	1	17	0
EAP	288	0	0	5

The data were pooled from eight sorting 'experiments' (i.e., see Table 2B, below).

Table 2B
Percentage of sorted wells showing PE, PC or EAP growth for seven enrichment cultures and one (†) natural sample (collected in the same manner as described for the enrichment cultures—see Section 2)

Origin	Collection date[a]	Post-sort APP classification		
		%PE	%PC	%EAP
Mondsee	2/5/01	68(72)	NA	NA
Mondsee†	3/2/01	44(119)	NA	NA
Mondsee	3/5/01	8(48)	NA	NA
Irrsee	5/6/01	0(96)	NA	3(96)
Hallstättersee	6/6/01	10(282)	NA	NA
Mondsee	6/15/01	6(288)	17(96)	10(96)
Attersee	8/14/01	4(192)	NA	NA
Traunsee	8/23/01	5(96)	NA	0(96)

Number of wells sorted per APP group given in parentheses.
[a] Month/day/year.

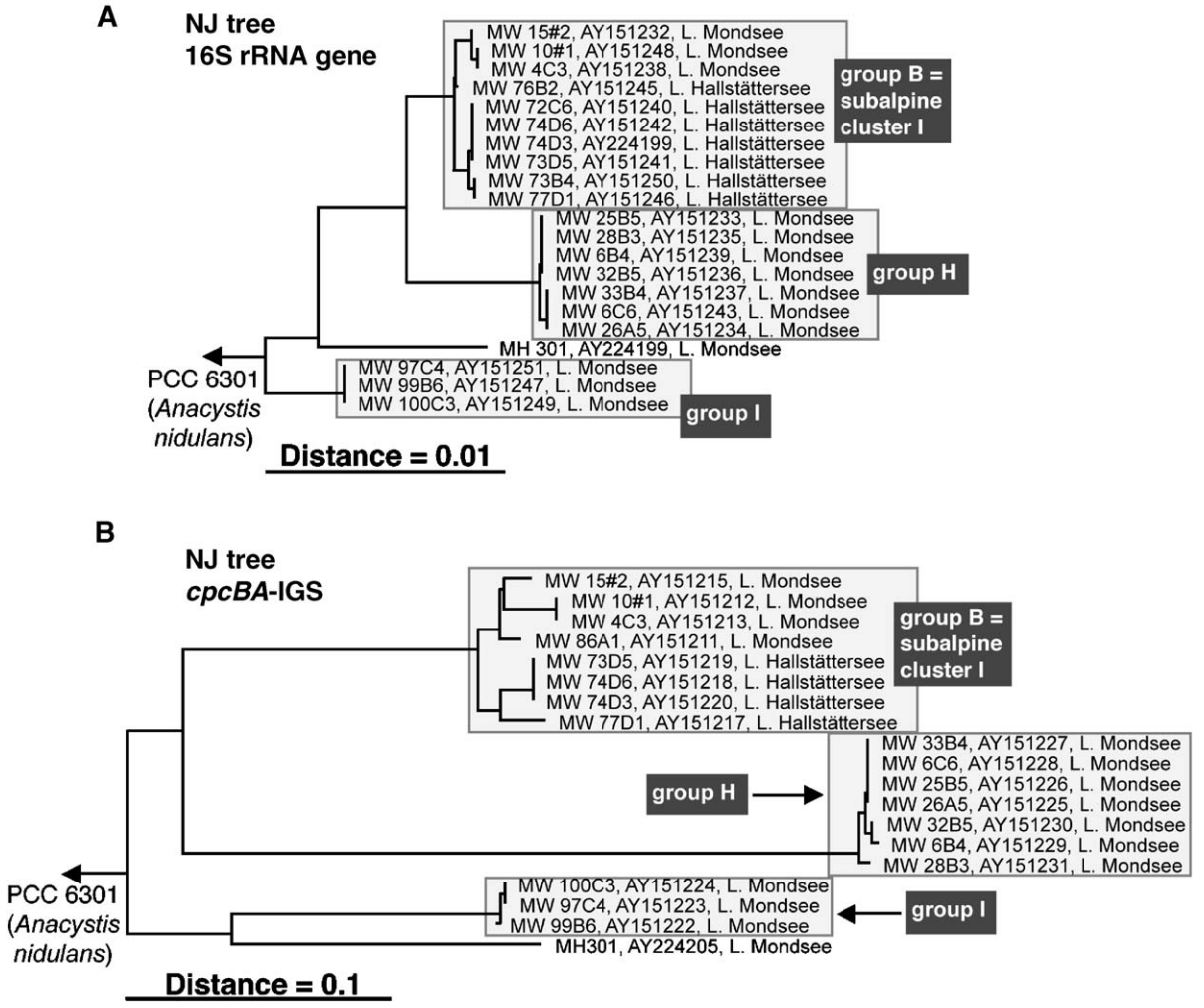

Fig. 2. Neighbour-joining (NJ) trees illustrating the phylogenetic relationship of isolates initiated by single-cell/single-colony sorting. (A) 16S rRNA gene (1383 nt positions) sequences, and (B) *cpcBA*-IGS (362 nt positions) sequences. These trees were constructed by removing sequences not obtained from isolates initiated by single-cell/single-colony sorting (except for MH 301, which was obtained by dilution culture) from trees constructed from more comprehensive datasets (Crosbie et al., 2003a). Terminal branches show isolate name, GenBank accession number, and the lake from which the isolate was obtained. Cluster designations and their nomenclature are based on groups originally proposed by Robertson et al. (2001), with modifications according to Ernst et al. (2003) and Crosbie et al. (2003a). The outgroup was *Synechococcus* PCC 6301 (formerly *Anacystis nidulans*).

originate from heteroduplex molecules—as suggested by BLAST searches and attempts to align the sequences with those obtained through direct sequencing—were excised from locations higher in the gradient. This has been previously observed by Ferris and Ward (1997), and is probably due to weakening of the hydrogen bonding between the double strands at base pair mismatches. Second, multiple prominent bands were not observed in DGGE analyses of the *cpcBA*-IGS fragments (Fig. 3B), with the exception of a single culture which showed two prominent bands (result not shown). Excision and subsequent sequencing of both bands showed that the culture contained two picocyanobac-

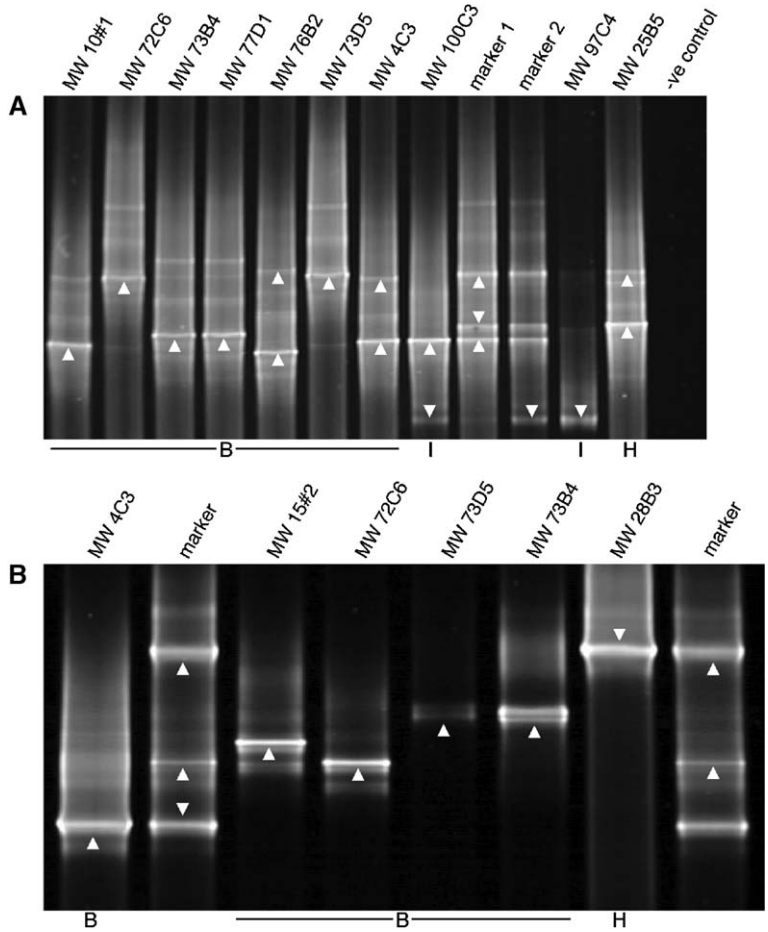

Fig. 3. DGGE screening of *Synechococcus*-type picocyanobacteria isolated from subalpine lakes by single-cell/single-colony sorting. The phylogenetic group (see Fig. 2) to which each strain belongs is given beneath each gel image. Arrows show bands which were excised and subsequently sequenced uni-directionally; for details, see Section 2. (A) Migration behaviour of ITS fragments amplified from eight PE-rich and two PC-rich isolates and electrophoresed through a 10% to 40% denaturing gradient for 4 h at 200 V. Marker 1 contained approximately equal concentrations of DNA amplified from MW 4C3, MW 25B5, MW 72C6 and MW 100C3. Marker 2 contained the same mixture except that approximately twice the amount of MW 100C3 DNA was added. (B) Migration behaviour of *cpcBA*-IGS fragments amplified from six PE-rich isolates and electrophoresed through a 40% to 70% denaturing gradient for 16 h at 100 V. The marker contained approximately equal concentrations of MW 4C3, MW 72C6, MW 73D5 and MW 28B3.

teria strains, identical or at least closely related to isolates MH 301 and MW 33B4.

4. Discussion

We were able to rapidly establish a diverse culture collection of clonal freshwater picocyanobacterial strains by single-cell and single-colony sorting in combination with flow-cytometric and molecular screening protocols. In the case of PE-rich picocyanobacteria (red strains), the time to establish 20 clonal cultures was reduced from up to 40 weeks to 3–4 weeks. There appeared to be little selectivity for PE-rich or PC-rich picocyanobacteria, although further testing is required since PC-rich picocyanobacteria were available for sorting from only a single enrichment culture (from which three PC-rich isolates were obtained). Apparently, EAP were also successfully isolated (Tables 2A and 2B) despite the use of a

culture medium (BG11) which is optimized for cyanobacterial growth (Stanier et al., 1971).

Sort recoveries, expressed as the percentage of sorted microwells exhibiting APP growth, were highly variable. Since sorting 'experiments' were conducted under standardized conditions, on eight separate occasions, either different picocyanobacterial strains had very different sort-sensitivities and/or (an) other factor(s) was/were at play. One possibility concerns the heterotrophic bacteria that are co-sorted with the APP and the role they may play in establishing cultures. Although an axenic culture is often desired, the variety of biotic interactions that can exist among species may be critically important to the health and survival of APP (Brand, 1986). Some bacteria, for example, are known to sequester inhibitory compounds, to which the few APP cells may be especially vulnerable (Brand, 1986). During single-cell/single-colony sorting, the wide 'safety margin' (i.e. 4-drop sort envelope, 'Counter' mode) may have promoted the seeding of microwells with accompanying heterotrophic bacteria that benefit APP growth under culture conditions.

The success of our approach probably rests with several factors, the relative importance of which was not determined, since our goal was only to develop and test sorting and screening protocols which we assumed would enable a large reduction in the time to establish clonal APP cultures—focussing on the PE-rich picocyanobacteria which dominate the lakes from which isolation of APP was desired. Factors, however, necessary or likely to have been important in achieving our goal, include (i) the use of enrichment cultures to promote the growth of APP prior to sorting including, we assume, that of metabolically inactive or rare populations. Nonetheless, it was possible to obtain cultures by sorting directly from a natural sample (Table 2B); (ii) repeated filtration of enrichment cultures to prevent overgrowth by larger phytoplankton; (iii) the use of a low-salinity sheath fluid to minimize the potential for osmotic stress and the introduction of impurities (e.g. metals) into microplate wells (Pinkel and Stovel, 1985; Sieracki et al., in press); (iv) sorting from enrichment cultures/natural sample that contained APP sourced from the entire euphotic zone (i.e. by collecting lake water with an integrating Schröder sampler) from several lakes and during different seasons (Winter, Spring and Summer); (v) inoculating microwells with both single cells and single colonies since freshwater picocyanobacteria exhibit a diversity of growth forms; and (vi) the ability to efficiently screen cultures by flow cytometric and molecular methods.

In spite of these caveats, our approach significantly widened the known diversity of freshwater picocyanobacteria, and the phylogenetic analyses suggest that our isolates may be considered representative for large, deep lakes (Crosbie et al., 2003a).

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