A PCR technique based on the Hip1 interspersed repetitive sequence distinguishes cyanobacterial species and strains

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The use of primers based on the Hip1 sequence as a typing technique for cyanobacteria has been investigated. The discovery of short repetitive sequence structures in bacterial DNA during the last decade has led to the development of PCR-based methods for typing, i.e. distinguishing and identifying, bacterial species and strains. An octameric palindromic sequence known as Hip1 has been shown to be present in the chromosomal DNA of many species of cyanobacteria as a highly repetitious interspersed sequence. PCR primers were constructed that extended the Hip1 sequence at the 3' end by two bases. Five of the 16 possible extended primers were tested. Each of the five primers produced a different set of products when used to prime PCR from cyanobacterial genomic DNA. Each primer produced a distinct set of products for each of the 15 cyanobacterial species tested. The ability of Hip1-based PCR to resolve taxonomic differences was assessed by analysis of independent isolates of *Anabaena flos-aquae* and *Nostoc ellipsosporum* obtained from the CCAP (Culture Collection of Algae and Protozoa, IFE, Cumbria, UK). A PCR-based RFLP analysis of products amplified from the 23S–16S rDNA intergenic region was used to characterize the isolates and to compare with the Hip1 typing data. The RFLP and Hip1 typing yielded similar results and both techniques were able to distinguish different strains. On the basis of these results it is suggested that the Hip1 PCR technique may assist in distinguishing cyanobacterial species and strains.

Keywords: cyanobacteria, typing, identification, Hip1, repetitive sequence primed PCR

INTRODUCTION

The ability to distinguish species and strains of bacteria is essential for environmental and epidemiological studies. In recent years molecular genetic techniques have been developed to supplement or supplant conventional methods that are based on techniques such as immunological (serotyping) and bacteriophage-susceptibility characteristics (Tenover *et al.*, 1995). The newer molecular genetic techniques all seek to detect differences in DNA sequence structure present either in the chromosomal DNA of a species or in a contained plasmid. One common approach assesses differences, often in the presence of restriction endonuclease sites, that are present in a small segment of the genome such

as a particular gene. Methods based on the intergenic regions within the rRNA cistrons (rDNA sequencing and ribotyping) have been particularly popular (Stull *et al.*, 1988; Vila *et al.*, 1996). Another, well-regarded technique assesses RFLPs generated by restriction of chromosomal DNA with rare-cutting restriction endonucleases and the use of pulsed-field gel electrophoresis to separate the fragments (Arbeit *et al.*, 1990; Frey *et al.*, 1996; Le-Bourgeois *et al.*, 1993).

A third method employs the PCR reaction to show differences between species by analysing the size of the DNA products amplified from a genomic DNA template by a variety of primers. In higher organisms (Welsh & McClelland, 1990), sets of random primers have been used to generate randomly amplified polymorphic DNA (RAPD)-PCR products, which produce banding patterns, when separated on agarose gels, that are characteristic of species and even individual organisms.

Abbreviations: ITS, internal transcribed spacer; RAPD, randomly amplified polymorphic DNA; STRR, short tandemly repetitive repeat.

The minimum size of a primer which is capable of specific interaction with a DNA sequence (10–12 bp) and the much smaller size of the bacterial genome as compared to that of higher organisms limit the general application of RAPD techniques to bacteria, although several RAPD-PCR techniques for distinguishing bacteria have been described (Lipman *et al.*, 1996; Mahenthiralingam *et al.*, 1996; Schmidt *et al.*, 1991). Similar techniques have been developed that are based on primers designed to hybridize with repeated sequence structures present in bacterial DNA. The primers permit amplification of the DNA sequences between those adjacent repeated sequences which are present in a suitable orientation and distance apart. A number of different examples of the technique have been published, including ERIC (Enterobacterial Repetitive Interspersed Consensus) (Hulton *et al.*, 1991) and REP (Repetitive Extragenic Palindrome) (Versalovic *et al.*, 1991; Giesendorf *et al.*, 1993; Georgiou *et al.*, 1995).

Classically the distinguishing of different species of cyanobacteria has relied essentially upon identifying morphological and developmental characteristics by light microscopy (Rippka, 1988; Castenholz *et al.*, 1992). Considerable expertise is required to identify species since both morphological and developmental characteristics can vary with the growth conditions (Evans *et al.*, 1976; Doers & Parker, 1988). In some instances it is not too difficult to identify cyanobacterial isolates to the genus level, particularly where morphological characteristics are significantly different from most other genera, e.g. *Calothrix*. However, for many genera, including *Oscillatoria*, *Lyngbya* and *Phormidium*, it is often difficult for the non-expert to be confident of their diagnosis. The problems of identification increase further at the species level and little is known about subspecies variation, i.e. strains.

Although they would be useful in distinguishing cyanobacterial isolates and in maintaining laboratory and collection cultures, only a few molecular genetic techniques for typing cyanobacteria have been developed. The cloning and sequencing of rRNA cistrons has most frequently been used to identify cyanobacteria in environmental samples and to investigate phylogeny (Britschgi & Giovannoni, 1991; Ferris *et al.*, 1996; Fuhrman *et al.*, 1993; Nelissen *et al.*, 1994; Neilan, 1996; Schmidt *et al.*, 1991). Lotti *et al.* (1996) used the restriction endonucleases *Bfa*I and *Hpa*I to create unique RFLP banding patterns of chromosomal DNA to distinguish symbiotic *Nostoc* isolates. Neilan *et al.* (1995) found RFLPs, in PCR-amplified products of the phycocyanin gene, that distinguished between toxic *Microcystis* and *Anabaena* strains. Neilan (1996) reported the use of a RAPD technique to distinguish *Anabaena* and *Microcystis* isolates responsible for producing nuisance blooms in freshwater.

Filamentous heterocystous cyanobacteria contain STRR (short tandemly repetitive repeat) sequences (Mazel *et al.*, 1990) and LTRR (long tandemly repetitive repeat) sequences, that have been used as Southern blot RFLP

hybridization probes (Rouhiainen *et al.*, 1995) and PCR typing (Rasmussen & Svenning, 1998) to distinguish cyanobacteria. Another interspersed repeated sequence known to be common to many, but not all, cyanobacterial species is an 8 bp, highly iterated palindromic sequence known as Hip1 (Gupta *et al.*, 1993). Robinson *et al.* (1995) have shown that the use of Hip1 as a primer in PCR amplification from genomic DNA generated product banding patterns that were characteristic of species. However, large numbers of products are generated with some species, producing complex banding patterns. We demonstrate here that DNA amplification from cyanobacterial DNA templates using primers based on the Hip1 sequence, but extended by an additional two bases at the 3' end, yields reproducible banding patterns that distinguish different species and isolates (strains) of cyanobacteria.

METHODS

Cyanobacteria and culture conditions. A range of cyanobacteria (Table 1), maintained in the CCAP collection (<http://www.ife.ac.uk/ccap/>) and varying in morphology from simple unicells to forms with branched filaments, heterocysts and akinetes, were examined in this study. Species cultured in JM medium or BG11 medium (Tompkins *et al.*, 1995) were grown under 'environmental' conditions (15 °C, a 12:12 h light:dark cycle, irradiance 25 µmol m⁻² s⁻¹). Species cultured in AA/4 medium (Allen & Arnon, 1955) were grown at 29 °C under a 24 h light regime (30 µmol m⁻² s⁻¹). Isolates used to assess the resolution of subspecific differences are listed in Table 2.

Exponential or early stationary phase cultures (10 ml) were harvested by centrifugation (3500 r.p.m., 10 min) and washed twice in the appropriate sterile medium and/or twice in sterile TE buffer (10 mM Tris/HCl, pH 7.2, 1.0 mM EDTA), with a final resuspension in 750 µl TLES buffer (50 mM Tris/HCl, pH 9.5, 150 mM LiCl, 5 mM EDTA, 5% SDS).

DNA isolation. The resuspended material was added to a screw-capped Eppendorf tube filled with glass microbeads (acid washed, 425–600 nm, Sigma) to the 500 µl mark. The tubes were attached by screw clamps to a Griffin flask shaker and shaken at maximum revolutions (approximately 300 r.p.m.) for 7 min to break open cells. The release of phycoerythrin and phycocyanin pigments from some species allowed cell breakage to be monitored by eye. Cell debris was removed by centrifugation in a microfuge (13 200 r.p.m., 8 min). The supernatant was removed to a fresh tube containing an equal volume (500 µl) of phenol/chloroform/isoamyl alcohol (25:24:1, by vol.; Sigma) and mechanically mixed for 1 min followed by centrifugation (microfuge, 13 200 r.p.m., 15 min) and removal of aqueous supernatant to a fresh tube. The phenol/chloroform/isoamyl alcohol extraction was repeated. A full 15 min centrifugation for both extractions decreased protein and polysaccharide contamination of the final product.

A one-tenth volume (50 µl) of 5 M potassium acetate (3 M potassium acetate in 2 M acetic acid) was added to the supernatant. The sample was placed on ice for 10 min, during which time a turbid suspension appeared in some samples. This suspension was pelleted by centrifugation (microfuge, 13 200 r.p.m., 15 min) and the supernatant retained. Two volumes (1 ml) of absolute ethanol were added and the sample

Table 1. Test species of cyanobacteria

Species	Isolate identification	Medium
<i>Anabaena cylindrica</i>	CCAP 1403/2a	JM
<i>Oscillatoria amoena</i>	CCAP 1459/39	JM
<i>Calothrix</i> sp.	CCAP 1429/1	BG11
<i>Nostoc flagelliforme</i>	CCAP 1553/33	BG11
<i>Anabaenopsis circularis</i>	CCAP 1402/1	BG11
<i>Fischerella muscicola</i>	CCAP 1427/1	BG11
<i>Symploca muscorum</i>	CCAP 1478/1	BG11
<i>Synechocystis</i> sp.	CCAP 1480/4	BG11
<i>Nostoc muscorum</i>	CCAP 1453/20	BG11
<i>Anabaena</i> sp.	CCAP 1446/1C	BG11
<i>Anabaena flos-aquae</i>	CCAP 1403/13A, /13B, /13D to /13H	BG11
<i>Nostoc ellipsosporum</i>	CCAP 1453/2, /11, /15 to /19	BG11
<i>Anabaena</i>	ATCC 27892	AA/4
<i>Anabaena</i>	PCC 7120	AA/4
<i>Nostoc</i> Mac R1	P. Bisen (Barkatullah University, India)	AA/4

Table 2. Isolates used to assess resolution of subspecific differences

See the CCAP on-line database [<http://www.ife.ac.uk/ccap/>] for further information on these strains.

Species	Isolate identification	Origin, year of isolation and other characteristics
<i>Anabaena</i> sp.*	CCAP 1403/13A	Sewage oxidation pond, Mississippi, USA; 1964
<i>Anabaena flos-aquae</i>	CCAP 1403/13B	Windermere, Cumbria, England; 1964
<i>Anabaena flos-aquae</i>	CCAP 1403/13C	Blelham Tarn, Cumbria, England; 1972
<i>Anabaena flos-aquae</i>	CCAP 1403/13D	Wales; 1976; mutant, no gas vacuoles
<i>Anabaena flos-aquae</i>	CCAP 1403/13E	Wales; 1976; mutant, helical filaments
<i>Anabaena flos-aquae</i>	CCAP 1403/13F	Windermere, Cumbria, England; 1976; reisolation of CCAP 1403/13B
<i>Anabaena flos-aquae</i>	CCAP 1403/13G	Windermere, Cumbria, England; 1976; reisolation of CCAP 1403/13B
<i>Anabaena flos-aquae</i>	CCAP 1403/13H	Windermere, Cumbria, England; 1964
<i>Anabaena flos-aquae</i> †	CCAP 1446/1C	Unknown
<i>Nostoc ellipsosporum</i>	CCAP 1453/2	Freshwater ditch, Utrecht, Netherlands
<i>Nostoc ellipsosporum</i>	CCAP 1453/11	Fresh water, Sweden; 1950
<i>Nostoc ellipsosporum</i>	CCAP 1453/15	Fresh water; USA
<i>Nostoc ellipsosporum</i>	CCAP 1453/16	Fresh water; 1966
<i>Nostoc ellipsosporum</i>	CCAP 1453/17	Fresh water; 1967
<i>Nostoc ellipsosporum</i>	CCAP 1453/18	Fresh water, Wisconsin, USA; 1968
<i>Nostoc ellipsosporum</i>	CCAP 1453/19	Fresh water

* Formerly designated *A. flos-aquae*.

† Formerly designated *A. inaequalis*.

placed at -20°C for at least 20 min. DNA, heavily contaminated by polysaccharide in samples derived from some species, was recovered by centrifugation (microfuge, 13 200 r.p.m., 8 min) and washed with 70% ethanol prior to drying of the sample in an airflow cabinet at room temperature and resuspension in TE buffer containing DNase-free RNase A ($25\text{ }\mu\text{g ml}^{-1}$; Sigma). DNA recovered by this technique

varied from 0.2 to $1.2\text{ }\mu\text{g}$ per 10 ml of culture. DNA preparations were stored at -20°C and further diluted in TE buffer prior to use in PCR.

DNA concentrations were estimated directly from ethidium bromide fluorescence in agarose gel images against standard quantities of λ bacteriophage DNA, either by using a Pharmacia gel documentation system and associated software

or from scanned images of gel photographs analysed using NIH Image software, version 1.59, on a Macintosh computer. NIH Image software is available by ftp transfer protocols from Info-mac archives (e.g. ftp://src.doc.ic.ac.uk/packages/info-mac/) that are mirrored worldwide.

PCR conditions. PCR reactions were either run as 50 µl volumes in 300 µl thin-walled PCR tubes (Perkin Elmer) or 25 µl volumes in 96-tube plates (skirted Thermo-Fast 96) sealed with Thermo-Seal (heat-sealing foil) using a Combi Thermo-Sealer (Advanced Biotechnologies). Reactions normally contained approximately 20–30 pg DNA template (samples shown in Fig. 3 contained up to 3 ng DNA template), one primer (0.6 µM), deoxynucleoside triphosphate mixture (40 nM each), 1.0 unit *Taq* DNA polymerase (Gibco-BRL) or other heat-stable DNA polymerase, one-tenth volume of the appropriate 10× buffer supplied by the manufacturer, supplemented where appropriate to give a final MgCl₂ concentration of 2.0 mM, in a total volume of 50 µl, or half these amounts in 25 µl volumes.

Reactions were cycled on an Ericomp DeltaCycler II thermal cycler using a temperature profile of one cycle of 95 °C, 5 min; 30 cycles of 95 °C, 30 s; 30 °C, 30 s; 72 °C, 60 s; one cycle of 72 °C, 5 min.

There are 16 possible variants of primers based on the Hip1 sequence GCGATCGC and containing two additional nucleotides at the 3' end. Four of the 16 possible primers were chosen arbitrarily and used for this study: HipCA (GCGATCGCCA), HipAT (GCGATCGCAT), HipTG (GCGATCGCTG) and HipGC (GCGATCGCGC). The fifth primer used in this study, HipTC (GCGATCGCTC), corresponds to a sequence that is found upstream of *nifH* genes and other genes transcribed during heterocyst development in *Anabaena* species (Beesley *et al.*, 1994). PCR products were separated by agarose gel electrophoresis in TBE buffer according to standard protocols (Sambrook *et al.*, 1989) by loading 2–10 µl of the reaction mixture mixed with loading buffer onto an agarose gel containing 1.5% (w/v) agarose (NBL Gene Sciences) and 1.5% (w/v) NuSieve3:1 (FMC Bioproducts). In some instances (Fig. 3) a deliberate overloading of the gel is shown in order that minor products (light bands) are visible. Typically a loading of 2–3 µl of reaction mixture was sufficient to show banding patterns suitable for typing. All the extended Hip1 PCR profiles shown were repeated at least five times.

The method of Lu *et al.* (1997) was used to provide an alternative means of characterizing the *Anabaena flos-aquae* and *Nostoc ellipsosporum* isolates. This technique employs PCR to amplify products from the 16S–23S rDNA using the primer pairs R1/R18, R14/R18 or R17/R18, as described by Wilmotte *et al.* (1993). R1/R18-primed PCR amplifies the 16S rDNA, the internal transcribed spacer (ITS) region, tDNA^{le}, or tDNA^{le} and tDNA^{ala}, and the 5' end of 23S rDNA. R14/R18-primed PCR amplifies the same rDNA region, but starting from the 3' end of 16S rDNA. R17/R18-primed PCR amplifies part of the ITS region, starting at tDNA^{le}. Products formed in R17/R18-primed PCR were analysed for restriction polymorphisms using the restriction endonucleases *Hinf*I, *Dde*I and *Alu*I, identified as discriminating cyanobacterial species by Lu *et al.* (1997). The rDNA PCR and RFLP analysis was repeated three times.

The results presented were scanned from the photographic images obtained from a Pharmacia gel documentation system (Pharmacia Biotech) and the digitized images were inverted, corrected for brightness and contrast, and labelled using Adobe Photoshop version 2.5 prior to printing. The size of

PCR products was determined using software associated with the gel documentation system and a 123 bp ladder DNA standard (Gibco-BRL).

RESULTS

Optimization of the extended Hip1 PCR

The template DNA preparation procedure used for extended Hip1 typing yields relatively small quantities of impure DNA, but was adopted for the attributes of simplicity, rapidity and general application to diverse cyanobacterial species. To assess whether this crude method allowed reproducible PCR, cultures of *Anabaena* ATCC 27892 were grown and extracted separately. No significant difference was observed in products obtained by extended Hip1 PCR from these templates (Fig. 1a). Extended Hip1 PCR was achieved directly from cultures concentrated 10-fold, resuspended in TE buffer and 3 µl used in place of template DNA. Rasmussen & Svenning (1998) achieved PCR using an STRR primer from cyanobacterial cells (filaments). However, in this study both the productivity and the reproducibility of the Hip1 PCR reaction, particularly in formation of larger PCR products, was decreased when whole-cell suspensions were employed instead of a DNA template. This may be a consequence of the different PCR conditions employed.

Concentration of template. The concentration of DNA template that was required for extended Hip1 PCR was determined empirically. Most DNA preparations adequately supported the PCR reaction at a concentration of 20 pg per 50 µl reaction (Fig. 1b), but others, notably those from species in which substantial polysaccharide contamination was apparent, required up to 3 ng per 50 µl reaction to achieve good productivity, presumably due to some inhibition of the PCR reaction. However 3 ng quantities are not excessive. Various quantities of cyanobacterial templates have been employed in PCR reactions reported in the literature; for example, 100 ng (Lu *et al.*, 1997), 50 ng (Nelissen *et al.*, 1994; Rasmussen & Svenning, 1998), 10 ng (Bolch *et al.*, 1996) and 5 ng (Wilmotte *et al.*, 1994) of template DNA per reaction.

Reaction with contaminant bacteria. To assess the effect of bacterial contamination, DNA preparations of purified freshwater coliform and streptococcal isolates were used in extended Hip1 PCR and were shown to form products (Fig. 1c).

Concentration of primer. Primer concentration was determined empirically. Concentrations between 100 and 600 nM did not affect the number of bands obtained, but productivity of the reaction was improved at the higher primer concentration. Primer concentrations above 600 nM and enzyme activities above 2 units per 50 µl reaction increased nonspecific background relative to discrete products, the two factors being interdependent (data not shown).

Concentration of Mg²⁺. The MgCl₂ concentration of PCR reactions directly affects product formation through the

Fig. 1. (a) Assessment of variation due to template preparation. DNA prepared separately from different cultures of *Anabaena* ATCC 27892 was purified and used as template in a PCR reaction employing HipCA primer. Four examples are shown. (b) Assessment of DNA template concentration. An *Anabaena* ATCC 27892 DNA template was serially diluted 10-fold and 3 µl used as template in a PCR reaction employing HipTG primer under standard conditions. Lanes: 1, 123 bp ladder; 2, 3 ng template DNA; 3, 300 pg template DNA; 4, 30 pg template DNA; 5, 3.0 pg template DNA. (c) Hip primer products from freshwater bacterial isolates. A PCR reaction employing DNA templates prepared from coliform (lanes 2 and 3) and streptococcal (lanes 4 to 7) freshwater bacterial isolates and HipTG primer was performed under standard conditions. Lane 1, 123 bp ladder. (d) Effect of varying Mg^{2+} concentration. A PCR reaction employing an *A. cylindrica* DNA template and HipCA primer was performed under standard conditions. Lanes: 1, 123 bp ladder; 2, no Mg^{2+} ; 3, 0.5 mM Mg^{2+} ; 4, 1.0 mM Mg^{2+} ; 5, 1.5 mM Mg^{2+} ; 6, 2.0 mM Mg^{2+} ; 7, 2.5 mM Mg^{2+} ; 8, 3.0 mM Mg^{2+} . (e) Effect of using different DNA polymerase preparations. A PCR reaction employing an *A. circularis* DNA template and HipTG primer was performed under standard conditions. The DNA polymerase preparations used were: lane 1, *Taq* polymerase, HT Biotechnologies, UK; lane 2, *Taq* polymerase, Applied Biotechnologies, UK; lane 3, *Taq* polymerase, Perkin Elmer, UK; lane 4, *Taq* polymerase, Gibco-BRL; lane 5, *Tbr* polymerase, Northumbria Biochemicals, UK; lane 6, 123 bp ladder. (f) Effect of annealing temperature. PCR reactions employing a *Nostoc* Mac DNA template and HipGC primer were performed under standard conditions, but at different annealing temperatures. Lanes: 1, 30 °C; 2, 32 °C; 3, 34 °C; 4, 36 °C; 5, 38 °C; 6, 40 °C; 7, 50 °C; 8, 60 °C.

stringency of primer-template interaction. A minimum of 1.5 mM $MgCl_2$ was required to obtain products, but no significant difference was observed in products obtained from reactions containing between 2.0 and 3.0 mM $MgCl_2$ (Fig. 1d).

Number of cycles. Although 30 PCR reaction cycles were used in this work, 20 cycles were sufficient to yield ample product with most templates (data not shown). A minimum of 30 s denaturation was sufficient, but reduction of the extension step below 30 s decreased productivity of longer products, particularly with some template preparations. Use of a stepped programme in which the time taken to raise the annealing temperature, 30 °C, to the extension temperature, 72 °C, was delayed, in order to encourage extension and stabilization of the short Hip1 primers, yielded products that did not differ in number or quantity from those obtained with the standard profile (data not shown).

Source of polymerase and PCR machine. Variation in PCR typing techniques due to the use of different supplies of

Taq DNA polymerase has been reported (Meunier & Grimont, 1993). The products formed in extended Hip1-primed PCR from cyanobacterial genomic DNA templates did not vary significantly when any of four different *Taq* polymerase preparations, obtained from different manufacturers, or *Tbr* polymerase was used to catalyse the reactions, even when the reactions were constructed with the different manufacturers' buffers and $MgCl_2$ solutions (Fig. 1e). However, it was necessary to compare equivalent enzyme activities per PCR reaction and/or equivalent loading of PCR products on agarose gels. Comparison of different quantities of products may be complicated by low-band-intensity products, which, *in extremis*, are visible at high loading, but not at low loading. In this limited study, comparison of banding patterns of different intensities could not give rise to confusion between species. Even so, recognition and compensation for low yield in the PCR reaction is recommended. Extended Hip1 PCR yielded the same products when cycling was performed with three different PCR machines (Deltacycler II system, Ericomp;

Fig. 2. Comparison of the PCR products formed in reactions primed with different extended Hip1 primers. Template DNA isolated from *Anabaena* PCC 7120 (a) and *A. flos-aquae* CCAP 1403/13B (b).

DNA thermal cycler, Perkin Elmer; GeneAmp 2400 PCR system, Perkin Elmer). Nor was variation encountered in the use of PCR tubes or sealed 96-tube plates or due to the position of a tube in a heating block (data not shown).

Annealing temperature. The use of annealing temperatures in the PCR cycle between 30 °C and 40 °C did not significantly alter the products obtained with extended Hip1 PCR except that a few products increased or decreased in yield (Fig. 1f). At higher temperatures (50–60 °C) some products were decreased in intensity/productivity, while other products, which formed low-intensity bands on agarose gel electrophoresis when obtained from low-annealing-temperature PCR (30 °C), increased in productivity and appeared as high-intensity bands on agarose gel electrophoresis.

Comparison of the five primers

Each of the five extended Hip1 primers reproducibly yielded a distinct set of products when used individually to prime PCR from the same cyanobacterial genomic DNA template (Fig. 2). Each of the five extended Hip1 primers reproducibly yielded a distinct set of products from each of 15 cyanobacterial species tested. Examples of HipTG-, HipGC- and HipCA-primed PCR reactions

Fig. 3. Comparison of the PCR products obtained in extended Hip1 PCR from ten different cyanobacterial DNA templates using HipTG (a), HipGC (b) and HipCA (c).

with ten different cyanobacterial species are shown in Fig. 3.

Resolution of strains within species

To assess the resolution of subspecies differences afforded by extended Hip1 primer typing, two separate sets of isolates of cyanobacterial species, which have been maintained in the CCAP, were used. These sets of isolates were taken as likely to represent different strains of cyanobacterial species. One set, of eight isolates, originally classified as *Anabaena flos-aquae*, originated

Fig. 4. Comparison of the R14/R18-primed rDNA PCR products obtained for the *A. flos-aquae* (a) and *N. ellipsosporum* (b) isolates.

predominantly from UK sources. Isolate CCAP 1403/13A was originally recorded as *A. flos-aquae*, but has since been reclassified as *Anabaena* sp. It was included in the experiment as a sample that was likely to be distinct. The other set, of seven isolates classified as *Nostoc ellipsosporum*, originated from a variety of European and North American sources (Table 2).

The rDNA analysis of the *A. flos-aquae* isolates showed that isolates CCAP 1403/13A to /13H produced similar PCR products and restriction products except that isolate 1403/13D showed an additional product in R14/R18-primed PCR (Fig. 4). Isolate 1446/1C was distinct from the other *A. flos-aquae* isolates (Figs 4 and 5). Hip1 typing confirmed the differences between isolates identified by rDNA analysis. The CCAP 1403/13B, /13E, /13F, /13G and /13H isolates yielded indistinguishable products by rDNA analysis and in all five extended Hip1-primed PCR reactions. Isolate CCAP 1446/1C was clearly distinguished by both rDNA- and Hip1-primed PCR. Examples of HipCA- and HipAT-primed PCR products are shown in Fig. 7. However, although distinguished by an additional product in R14/R18-primed PCR (Fig. 4), CCAP 1403/13D was

Fig. 5. Comparison of the R17/R18-primed rDNA PCR products obtained for the *A. flos-aquae* (a) and *N. ellipsosporum* (b) isolates.

not distinguished by Hip1-primed PCR (Fig. 7). In contrast, isolate CCAP 1403/13A was clearly distinguished from the majority of CCAP 1403 isolates by Hip1 PCR (Fig. 7), but was distinguished by rDNA analysis only by slightly smaller R17/R18 major PCR product and restriction fragments (Figs 5 and 6).

The rDNA PCR analysis of the seven *N. ellipsosporum* isolates showed that they were diverse (Figs 4 and 5), in either the size of the PCR products or restriction fragments formed. Extended Hip1-primed PCR also yielded diverse products, confirming the differences between species identified by rDNA analysis; examples of HipCA- and HipAT-primed PCR products are shown in Fig. 8. Major and minor PCR products were observed for both the R14/R18-primed products and the R17/R18-primed products of both sets of isolates (Figs 4 and 5).

DISCUSSION

The discovery of the Hip1 sequence and its presence in many, though not all, cyanobacteria (Robinson *et al.*, 1995) allowed an assessment of a cyanobacterial typing technique which, akin to the repetitive-sequence-

Fig. 6. RFLP analysis of the R17/R18-primed rDNA PCR products obtained for the *A. flos-aquae* isolates using *DdeI* (a), *AluI* (b) and *HinfI* (c).

Fig. 7. Extended Hip1-primed PCR products obtained for the *A. flos-aquae* isolates using HipCA primer (a) and HipAT primer (b).

orientated PCR techniques used with bacteria (e.g. Versalovic *et al.*, 1991; Vila *et al.*, 1996) and cyanobacteria (Rasmussen & Svenning, 1998), is based on the

Fig. 8. Extended Hip1-primed PCR products obtained for the *N. ellipsosporum* isolates using HipCA primer (a) and HipAT primer (b).

amplification of DNA between adjacent repeated Hip1 sequences in the chromosomal DNA of cyanobacteria. However, the frequency of Hip1 repeats in the DNA of some cyanobacteria is high. For example, Robinson *et al.* (1995) estimated from analysis of database sequence that the Hip1 sequence occurred on average every 320 bp in the chromosomal DNA of *Synechococcus* PCC 6301. Consequently the number of discrete products obtained in a Hip1-primed PCR reaction is very large and not conducive to distinguishing species by a simple comparison of agarose gel electrophoresis patterns. To decrease the number of PCR products obtained and therefore provide a clearer and more distinctive banding pattern, primers were constructed in which two additional bases were added to the 3' end of the Hip1 sequence.

DNA polymerases extend the 3' end of primers. Since the complementarity of the 3' end of the primer with the template dictates efficient extension and PCR product formation, extending the 3' end of the palindromic Hip1 sequence by two nucleotides should restrict efficient priming to those genomic Hip1 sequences in which the flanking sequences complement the two nucleotide extension. This strategy was successful, as shown by the different PCR products obtained from a cyanobacterial genomic DNA template with each of the 5' extended Hip1 primers that were tested (Fig. 2).

The prime objective of the work was to assess the ability of extended Hip1-primed PCR to clearly distinguish

species and strains of cyanobacteria. In the three examples shown, ten species of cyanobacteria drawn from a variety of genera were tested and yielded clearly distinct extended Hip1-primed PCR products (Fig. 3). The extended Hip1 primers generated PCR products and agarose gel electrophoresis banding patterns that clearly distinguished all ten species of cyanobacteria. These included the three *Nostoc* and two *Anabaena* species that present examples of closely related species occupying the same genera.

One characteristic of the banding patterns on agarose gel electrophoresis obtained from extended Hip1 PCR products is the presence of bands of widely different intensities resulting from the different quantities of products formed (Fig. 3). This variation in productivity was reproducible and was not affected by *Taq* polymerase activity, Mg^{2+} concentration or primer concentration. Such variation in the intensity of bands (i.e. productivity of individual products) is a characteristic of RAPD and repetitive-sequence PCR as shown by published data (e.g. for bacteria, Meunier & Grimont, 1993; Gillings & Holley, 1997; Mahenthiralingam *et al.*, 1996; and cyanobacteria, Rasmussen & Svenning, 1998). Experiments showed that some high-intensity products declined in productivity as the PCR annealing temperature was increased, indicating that they resulted from inexact priming from template sites that were not completely complementary to the primer (Fig. 1f). Consequently inexact priming does not provide a simple explanation for the formation of low-intensity products. Other low-intensity products increased in productivity as the annealing temperature increased. This would be consistent with the hypothesis that intramolecular template secondary structure occludes some priming sites, reducing productivity of some Hip1-primed products. Since most typing reactions employ annealing temperatures well below 65 °C, at which template secondary structure is minimized, occlusion of primer sites by secondary structure may explain the variation in PCR product intensity which is common to PCR typing techniques.

To explore the resolution afforded by extended Hip1 PCR, two sets of isolates present in the CCAP collection (Tables 1 and 2) were analysed. The Hip1 analysis compared favourably with the rDNA analysis in discriminating similar and distinct strains. Both techniques found the *A. flos-aquae* strains to be similar and the *N. ellipsoforum* strains to be more diverse. However, the two techniques differ in their discrimination of isolates CCAP 1403/13A and /13D. Clearly there exists an element of chance as to whether a chosen technique will distinguish particular strains and it would be unwise to rely upon the results of a single technique as evidence that two isolates are identical.

The products obtained from R1/R18-primed (data not shown) and R14/R18-primed (Fig. 4) PCR contained minor products as reported by Lu *et al.* (1997). These minor products were ascribed by Lu *et al.* (1997) to heterogeneity for heterocyst differentiation or the formation of heteroduplexes containing conserved 3' and 5'

ends, but highly variable ITS sequences. Minor products were also present in R17/R18-primed rDNA PCR (Fig. 5). Such minor products were not reported by Lu *et al.* (1997). In addition to the explanations suggested by Lu *et al.* (1997) for minor products, they may also be attributed to a template at low concentration, e.g. a contaminating bacterial DNA – although the R18 primer discriminates against bacterial rDNA amplification (Lu *et al.*, 1997; Nelissen *et al.*, 1996) – or rare cyanobacterial rDNA cistrons that retain the R17 primer site within the tDNA^{11e} gene, but are of a different structure within the 3' end of the ITS region. Alternatively they may represent poor amplification of products primed from sites outside the rRNA cistrons. RFLP analysis of R17/R18-primed PCR products was complicated by the presence of the minor products, but the restriction fragments obtained for the *A. flos-aquae* isolates were similar to those described by Lu *et al.* (1997) for the *A. flos-aquae* isolate included in their survey.

Extended Hip1 PCR appears from this initial study to be robust, contain less inherent variability than reported for other PCR typing systems (Meunier & Grimont, 1993) and be applicable to a wide range of cyanobacterial species and strains. Hip1 PCR produces products with bacterial templates (Fig. 1c). While fewer than those normally found with cyanobacterial templates, this necessitates the purification of cyanobacterial isolates prior to PCR typing. Use of different enzyme activities per PCR reaction and/or comparison of different loads on agarose gels were identified as potential sources of confusion, particularly where less intense bands (low-productivity products) are included. As with other enzyme-based assays, a consistent and uniform construction of the assay and analysis of products is required, with recognition of and compensation for poor amplification of products.

The CCAP database contains information describing the *A. flos-aquae* and *N. ellipsoforum* isolates used in this study (compiled in Table 2). The six related strains of *A. flos-aquae* all originated from UK sources (Lake Windermere and Wales) over a period of 12 years. The two distinct strains are probably of American origin. Note that CCAP 1446/1C was originally designated *Anabaena inaequalis* and later reclassified as *A. flos-aquae*. CCAP 1403/13A, originally designated *A. flos-aquae*, has been reclassified as *Anabaena* sp. (Tompkins *et al.*, 1995). The *N. ellipsoforum* isolates, which appear to be more diverse than the *A. flos-aquae* group, have a more varied geographical origin. The number of isolates tested is small, but would suggest that strain variation is an aspect of major geographical location and that ecotypes within a region are more closely related.

Five of the 16 possible Hip1 extended primers have been shown to produce different PCR products (Figs 2 and 3) for each species of cyanobacterium studied and to distinguish cyanobacterial strains (Figs 7 and 8). One advantage of Hip1 typing is the degree of resolution that is afforded by possible use of all 16 extended Hip1

primers. Even so, as with other PCR-based techniques, the need to assume that size of product is an indicator of identical products, and the relatively low information content of the data, detract from the use of extended Hip1 PCR for phylogenetic studies. Extended Hip1 PCR typing may be of use in distinguishing cyanobacterial strains and species where a large number of samples are involved, e.g. the enumeration of purified isolates from environmental samples, in the maintenance of stock cultures or as a preliminary assay to distinguish between multiple strains prior to rDNA sequence analysis.

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