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Testing of primers for the study of cyanobacterial molecular diversity by DGGE

C. Boutte*, S. Grubisic, P. Balthasart, A. Wilmotte

Center for Protein Engineering, Institute of Chemistry B6, University of Liège, B-4000, Liège, Belgium

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Abstract

Denaturing Gradient Gel electrophoresis (DGGE) is a PCR-based technique which is widely used in the study of microbial communities. Here, the use of the three specific 16S rRNA cyanobacterial specific primers CYA359F, CYA781R(a) and CYA781R(b) on the assessment of the molecular diversity of cyanobacterial communities is examined. Assignments of the reverse primers CYA781R(a) and CYA781R(b) with cyanobacterial strain sequences showed that the former preferentially targets filamentous cyanobacteria whereas the latter targets unicellular cyanobacteria. The influence of the GC clamp position on the forward or on reverse primer and the use of the two reverse primers separately or in equimolar mixture were investigated. Three environmental samples were subjected to amplification with 6 combinations of primers. The 6 banding patterns as well as the sequences of the bands extracted were analysed and compared. In addition, to assess the effect of the position of the GC clamp, the melting profiles of the sequences of *Aphanizomenon flos-aquae* PMC9707 and *Synechococcus* sp. MH305 were determined, with the GC clamp in the 3′ or 5′ position. Results showed that the use of two separate amplifications allowed a more complete study of the molecular diversity of the cyanobacterial community investigated. Furthermore, similar richness and identical phylogenetic assignments of extracted bands were obtained irrespective of the positioning of the GC clamp.

Keywords: 16S ribosomal RNA; Denaturating gradient gel electrophoresis; Cyanobacteria; Molecular diversity

1. Introduction

16S rRNA PCR-DGGE (Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis) is one of the most frequently used technique to assess the genetic diversity of microbial communities (Muyzer, 1999; Ercolini, 2004). This method allows the separation of small DNA fragments (maximum size of 1000 bp) of the same length but of different sequence according to their melting properties (Nollau and Wagener, 1997). Indeed, fragments with only one single base substitution can be separated with this technique (Myers et al., 1985). Typically, so as to prevent a complete denaturation of the double-stranded fragments, a GC clamp is added to the 5′ end of the forward primer (Myers et al., 1985; Sheffield et al., 1989; Casamayor et al., 2000; Sekiguchi et al., 2001; Lyautey et al., 2005). However, the effect of the position of this GC clamp on the forward or reverse primer on the quality of the patterns obtained has not previously been investigated.

The numbers, positions and intensities of the DGGE bands obtained can be used to determine the diversity of natural samples (Muyzer, 1999; Garcia-Pichel et al., 2003; Lyautey et al., 2005). Several biases which may

* Corresponding author. Tel.: +32 4 366 33 10; fax: +32 4 366 33 64.
E-mail address: c.boutte@student.ulg.ac.be (C. Boutte).

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cause pitfalls in the interpretation of the DGGE patterns have been reported, like heteroduplex formation, amplification errors, the presence of multiple copies of 16S rRNA operons (Speksnijder et al., 2001; Wintzingerode et al., 1997), co-migration of different DNA fragments (Sekiguchi et al., 2001) and the presence of several melting domains in a DNA fragment (Kisand and Wikner, 2003). Nevertheless, some of these problems can be solved by the excision of the bands followed by reamplification and sequencing, as the identity of the organisms can be ascertained if the DNA fragment analysed is polymorphic.

Nübel et al. (1997) have designed the cyanobacterial primers CYA359F (forward), CYA781R(a) and CYA781R(b) (reverse) for specific amplification of a 379 bp 16S rRNA gene sequence. CYA781R(a) and CYA781R(b) differ by two polymorphic bases situated at positions 7 and 23 (5′ to 3′). These primers have the advantage of giving a PCR product which corresponds to variable regions V3 and V4, and contains significant information for phylogenetic assignments (Yu and Morrison, 2004). Indeed, they have been used unmodified (e.g. Casamayor et al., 2000; Garcia-Pichel et al., 2001; Abed et al., 2002; Geiß et al., 2004) or slightly adapted (Zwart et al., 2005) for numerous DGGE studies investigating cyanobacterial diversity in environmental samples.

In the present study, we aim to determine (1) the variation in the banding profiles caused by the position of the GC clamp on the forward or reverse primer, and (2) the combination of the primers designed by Nübel et al. (1997) which allows an optimum investigation of the diversity of the cyanobacterial community. To achieve this, we analysed and compared the DGGE fingerprints obtained with the six pairs of primers CYA359FGC-CYA781R(a), CYA359FGC-CYA781R(b), CYA359FGC-CYA781R(a+b), CYA359F-CYA781RGC(a), CYA359F-CYA781RGC(b), CYA359F-CYA781RGC(a+b), where GC means the GC clamp. In addition, as many as possible DGGE bands were sequenced to assess the diversity of the sequences obtained.

2. Materials and methods

2.1. In silico match of the reverse primers CYA781R(a) and CYA781R(b)

Using the Probe Search tool of the software package ARB (Ludwig et al., 2004) with an alignment of 1124 cyanobacterial strain sequences, the theoretical matches of the reverse primers CYA781R(a) and CYA781R(b) were investigated.

2.2. Sampling and DNA extraction

Environmental samples were collected at the Esch-sur-Sûre reservoir in North Luxembourg. The typical genera detected microscopically in this site during spring and summer were *Anabaena*, *Aphanizomenon* and *Planktothrix*. In August, populations of *Limnothrix* and *Snowella* have been reported. *Woronichinia* have been detected in autumn whereas no cyanobacteria have been observed during winter (Willame et al., MS in prep.).

Samples were collected by filtration of 500 ml water on 0.2 μm-pore-size filters (Supor, Pall Life Science, USA). The filters were stored in 2 ml lysis buffer (40 mM EDTA, 400 mM NaCl, 0.75 M sucrose, 50 mM Tris HCl pH 8.3) (Giovannoni et al., 1990) and immediately frozen at −20 °C. Three environmental samples were collected for the comparison of the primer pairs, 0ES27 (04/07/2000), 0ES32 (08/08/2000) and 0ES39 (26/09/2000). DNA was extracted from the filters as described by Wilmotte et al. (2002) with modifications as follows. For the enzymatic lysis step, a volume of 30 μl of proteinase K (20 mg ml^{-1}, MBI Fermentas, Lithuania) was used. Two milliliters of hot phenol (Sigma, USA)/chloroform/isoamylalcohol (Merck, Germany) (25:24:1) was added and incubated for 10 min at 56 °C. During the precipitation step, nucleic acids were precipitated from the supernatant (divided in several Eppendorf tubes) by addition of two volumes of ethanol and incubated for two hours at −20 °C. The tubes were centrifuged for 20 min at 16,000 g and after extraction, DNA was subjected to a purification step using the Prep-A-Gene DNA Purification Kit (Biorad, USA).

2.3. PCR reactions

As a direct amplification of the DNA with the Nübel primers gave a weak signal (data not shown), a semi-nested PCR reaction was performed so as to increase the sensitivity of the DGGE analysis. The 16S rRNA fragments were first amplified using the primers CYA359F and 23S30R (Taton et al., 2003). The second PCR reaction was performed with CYA359F and CYA781R(a) and/or (b), with the GC clamp on the forward or reverse primer. All the primers used are listed in Table 1 and were synthetized and purified by Gel PAGE (Polyacrylamide Gel Electrophoresis) at Eurogentec (Belgium). For the first PCR, 0.5 μl of the isolated DNA was added to 49.5 μl of the amplification mixture, giving rise to final concentrations of 1 X Super Taq Plus buffer, 1 mg ml^{-1} BSA (bovine serum albumin), 200 μM dNTP mix, 0.5 μM of each of the forward and reverse primers and

Table 1
Sequences of the PCR primers used (*: 16S *E. coli* position, #: 23S *E. coli* position)

Primer names	Position 3′ end	Sequence (5′-3′)	References
CYA359F	359*	ggggaattttccgcaatggg	Nübel et al., 1997
CYA359FGC	359*	cgcccgccgcgccccgcgcccgtcccgccgccccccgcccgggggaattttccgcaatggg	Nübel et al., 1997
CYA784R	784*	gactac**a**ggggtatctaatccc	derived from Nübel et al., 1997
CYA781R(a)	781*	gactac**t**ggggtatctaatccc**a**tt	Nübel et al., 1997
CYA781RGC(a)	781*	cgcccgccgcgccccgcgcccgtcccgccgccccccgcccgactac**t**ggggtatctaatccc**a**tt	Nübel et al., 1997
CYA781R(b)	781*	gactac**a**ggggtatctaatccc**ttt**	Nübel et al., 1997
CYA781RGC(b)	781*	cgcccgccgcgccccgcgcccgtcccgccgccccccgcccgactac**a**ggggtatctaatccc**ttt**	Nübel et al., 1997
23S30R	30#	cttcgcctctgtgtgcctaggt	Taton et al., 2003

The polymorphic bases for the primers CYA781R(a) and CYA781R (b) are in bold.

0.8 U/μl of Super Taq Plus (HT Technology, UK) in a final volume of 50 μl. The amplification was performed using an Icycler (Bio-Rad, USA). The PCR program included 1 cycle of initial denaturation at 94 °C for 5 min, followed by 10 cycles with a denaturing step of 45 s at 94 °C, an annealing step of 45 s at 54 °C, and an elongation step of 2 min at 68 °C. The final elongation was done during 7 min at 68 °C. 0.5 μl of the first PCR product was then added to 49.5 μl of the second PCR master mix (same composition as above, except for the primers used). This was performed with a combination of the forward primer CYA359F and the reverse primers CYA781R(a) and (b), separately or mixed. The GC clamp was positioned on the forward or reverse primers. We used the same PCR reagents as in the first reaction and the cycling conditions used where as described by Nübel et al. (1997), with the exception of the elongation temperature, specific of the Super Taq Plus (68 °C instead of 72 °C). Reference marker prepared by PCR amplifications of DNA fragments of *Cylindrospermum* PCC7417, *Calothrix* 328, *Aphanizomenon* 202a and *Nostoc* 152 with the primers CYA359F and CYA781RGC(a) and with CYA359F-CYA781RGC(b) amplifications of DNA fragments from *Microcystis* 130 and *Synechococcus* GL150636 was added in three lanes of each DGGE gel.

2.4. DGGE

The products of the second PCR reaction were analysed by DGGE. This was performed using a DCode System (Bio-Rad Laboratories, USA) with a 6% acrylamide gel (acrylamide/bis 37.5/1) (w/v) and a denaturating gradient from 45% to 55%. The 100% denaturating solution was composed of 7 M urea and 40% formamide (v/v). The samples were prepared by adding 4 μl of loading dye solution (0.09% bromophenol blue, 0.09% xylen cyanol, 60% glycerol, 60 mM EDTA) to 20 μl of the PCR product. The electrophoresis conditions were chosen on the basis of a perpendicular gel and a time-travel experiment (data not shown). DGGE were performed at 60 °C during 999 min at a constant voltage of 45 V. The gel was stained with 2 μl Gelstar dye (BioWhittaker Molecular Applications, USA) mixed with 15 ml of water (milli-Q). Bands were visualised under UV light and the central part of these was excised with a scalpel washed with pure ethanol. The excised acrylamide gel portion was stored in an Eppendorf tube containing 100 μl of sterilized water at room temperature for 1 hour and then the tubes were stored at −20 °C. 0.5 to 1 μl of supernatant was used for the reamplification reaction with CYA359F-CYA784R. The PCR products were sequenced commercially (Genome Express, France) using the CYA784R primer. The sequences of approximatively 360 bp were submitted to a BLAST analysis, and the results are shown in Table 3. The 36 sequences obtained have been deposited in Genbank database under the accession numbers AY646773 to AY646808.

2.5. Analysis of the banding patterns

Scanning of the gel was performed with a Fluor-S Max MultiImager (Bio-Rad, USA). The bands were localised using the manufacturer's software (Quantity One Software Package) on the basis of the peaks present in the densitometric curves calculated for each lane. The number of OTUs (Operational Taxonomic Units) was defined as the number of DGGE bands (Diez et al., 2001). The banding profiles were analysed using the GelCompar II Software 2.5 (Applied Maths, Belgium) after alignment with the reference marker.

2.6. Determination of melting profiles

The theoretical effect of the presence of the GC clamp on the forward or reverse primer was analysed using the Poland algorithm with the MELT94 software following the author's instructions (Lerman and Silverstein, 1987). This software performs the calculation of the melting profile of a known sequence, and allows the

addition of a GC clamp of 29 bp in 5′ or 3′ position. The 378–781 positions (based on the *E. coli* sequence) of the 16S rRNA of *Aphanizomenon flos-aquae* PMC9707 and *Synechococcus* sp. MH305 were used as template, to which CYA359F, CYA781R(a) or (b) primers and a GC clamp were added at the 3′ or 5′ end. These sequences correspond to the most frequent BLAST affiliations found among our sequences (six for *Aphanizomenon* and three for *Synechococcus*).

3. Results and discussion

3.1. In silico match of the reverse primers CYA781R(a) and CYA781R(b)

Of 397 heterocystous cyanobacterial sequences, 95.2% matched with the primer CYA781R(a) including 73% of the sequences of the genus *Chroococcidiopsis*, this being a sister group of the heterocystous cyanobacteria (Fewer et al., 2002) (Table 2). Concerning the non-heterocystous filamentous strains, 69.3% of the sequences matched with CYA781RGC(a). Of 381 sequences from unicellular strains, 92.6% matched with the primer CYA781R(b), 5.0% matched with CYA781R(a) and 2.4% did not match. Of the 46 sequences which did not match with either CYA781R(a) or (b), 11 sequences contain ambiguities, 3 contain gaps, and 32 are due to mismatches. A list of all the sequences used and their matches is available at http://www.cip.ulg.ac.be/appendix.htm. Thus, because the polymorphism at position 23 is situated in the region critical for the specificity of annealing during PCR, the reverse primer (a) amplifies preferentially the filamentous cyanobacteria, whereas the reverse primer (b) targets mainly the unicellular cyanobacteria.

3.2. Comparison of data when the GC clamp is on the forward or reverse primer

For the three environmental samples 0ES27, 0ES32 and 0ES39, different DGGE patterns were obtained for the six primer combinations used, CYA359FGC-CYA781R(a), CYA359FGC-CYA781R(b) CYA359FGC-CYA781R(a+b), CYA359F-CYA781RGC(a), CYA359F-

Table 2
Matches for CYA781R(a) and CYA781R(b) primers with 1124 16S rRNA sequences of cyanobacterial strains examined using the Probe Search tool of the software package ARB (Ludwig et al., 2004)

Heterocystous	CYA 781R(a)	CYA 781R(b)	No match	Filamentous non-heterocystous	CYA 781R(a)	CYA 781R(b)	No match	Unicellular *sensu stricto*	CYA 781R(a)	CYA 781R(b)	No match
Anabaena sp	82	0	0	*Arthrospira sp*	4	1	0	*Acaryochloris*	0	3	0
Anabaenopsis sp	4	0	0	*Geitlerinema sp*	6	1	0	*Aphanocapsa sp*	0	1	1
Aphanizomenon sp	31	0	2	*Halospirulina sp*	4	0	0	*Aphanothece sp*	0	2	0
Capsosira sp	1	0	0	*Leptolyngbya sp*	0	20	3	*Chamaesiphon sp*	0	1	1
Calothrix sp	8	0	2	*Limnothrix sp*	2	3	0	*Cyanobium sp*	0	4	0
Chlorogloeopsis sp	3	0	0	*Lyngbya sp*	9	0	3	*Cyanothece sp*	0	8	0
Chroococcidopsis sp	16	3	3	*Microcoleus sp*	45	0	7	*Dactylococcopsis sp*	0	1	0
Coleodesmium sp	1	0	2	*Oscillatoria sp*	24	36	6	*Dermocarpa sp*	0	13	0
Cylindrospermopsis sp	51	0	0	*Phormidium sp*	19	19	2	*Dermocarpella sp*	0	2	0
Cyanospira sp	0	0	1	*Planktothricoides sp*	6	0	0	*Euhalothece sp*	0	4	0
Cylindrospermum sp	4	0	0	*Planktothrix sp*	85	1	0	*Gloeocapsa sp*	0	2	0
Fischerella sp	9	0	0	*Plectonema sp*	0	2	0	*Gloeothece sp*	0	4	0
Gloeobacter sp	3	0	0	*Pseudanabaena sp*	10	1	0	*Halothece sp*	0	1	0
Hapalosiphon sp	4	0	0	*Spirulina sp*	6	1	0	*Merismopedia sp*	0	1	0
Mastigocladopsis sp	1	0	0	*Symploca sp*	5	0	0	*Microcystis sp*	0	70	3
Mastigocladus sp	2	0	0	*Trichodesmium sp*	8	0	0	*Myxosarcina sp*	0	2	0
Nodularia sp	41	0	0	*Tychonema sp*	7	0	0	*Pleurocapsa sp*	0	6	0
Nostoc sp	94	0	4	Total	240	85	21	*Prochlorococcus sp*	0	26	0
Nostochopsis sp	2	0	0	Percentage	69.3	24.6	6.1	*Prochloron sp*	0	2	0
Scytonema sp	4	0	2					*Prochlorothrix sp*	0	5	0
Spirestris sp	3	0	0					*Staniera sp*	0	3	0
Stigonema sp	2	0	0					*Synechococcus sp*	18	184	3
Symphyonema sp	2	0	0					*Synechocystis sp*	1	6	1
Symphyonemopsis sp	1	0	0					*Xenococcus sp*	0	2	0
Tolypothrix sp	3	0	0					Total	19	353	9
Westiellopsis sp	6	0	0					Percentage	5.0	92.6	2.4
Total	378	3	16								
Percentage	95.2	0.8	4.0								

The list of all sequences used for each genus and their matches are given at http://www.cip.ulg.ac.be/appendix.htm.

CYA781RGC(b) and CYA359F-CYA781RGC(a+b). The numbers of bands obtained for each primer pair varied (Fig. 1) and the average number of bands determined for the patterns obtained with CYA359F-CYA781R(a), CYA359F-CYA781R(b) and CYA359F-CYA781R(a+b) were similar whether the GC clamp was on the forward or reverse primer (6.6 and 5.0; 8.0 and 9.3; 7.3 and 6.0 respectively, Fig. 1). Concerning the reamplification of the DGGE bands, a sequence could not be obtained for all band excised. Moreover, identical sequences were obtained from different bands in the same lane. This is in agreement with the observation of Nikolausz et al. (2005) that dominant amplicons could be distributed at different positions in the same pattern. If several domains have similar melting properties, stochastic effects might cause one to denaturate before the other in a fraction of the amplicon population and could also explain the presence of different bands with the same sequence in one lane.

BLAST analyses of the sequences obtained with the GC clamp on the forward and reverse primers gave the same closest relatives for samples 0ES27 (*A. flos-aquae* PMC9707 and PCC7905 and *Synechococcus* MH305), 0ES32 (*A. flos-aquae* PMC9707, *Synechocystis* PCC6803 and *Synechococcus* MH301) and 0ES39 (*Limnothrix redekii* CCAP1443/1 and *Synechocystis* PCC6803) (Table 3).

The differences in the banding profiles obtained with the GC clamp on the forward or reverse primer can be explained by the melting properties of the amplicons. The melting profiles obtained for the same sequence with the GC clamp on the forward or reverse primer were different whereas there was no effect with the primers CYA781R(a) and (b) even though these differ from each other by two nucleotides (Fig. 2). The modification of the melting properties could be explained by the GC content of the forward and reverse primers, 55% and 44%, respectively. Thus the reverse primers had a lower melting temperature and this can be observed in Fig. 2 where the melting temperature of the DNA segment at the 3′ end is lower than that at the 5′ end, irrespective of the presence of the GC clamp. When the GC clamp is situated at the 5′ end, the lower melting temperature of the reverse primer CYA781R has a more pronounced

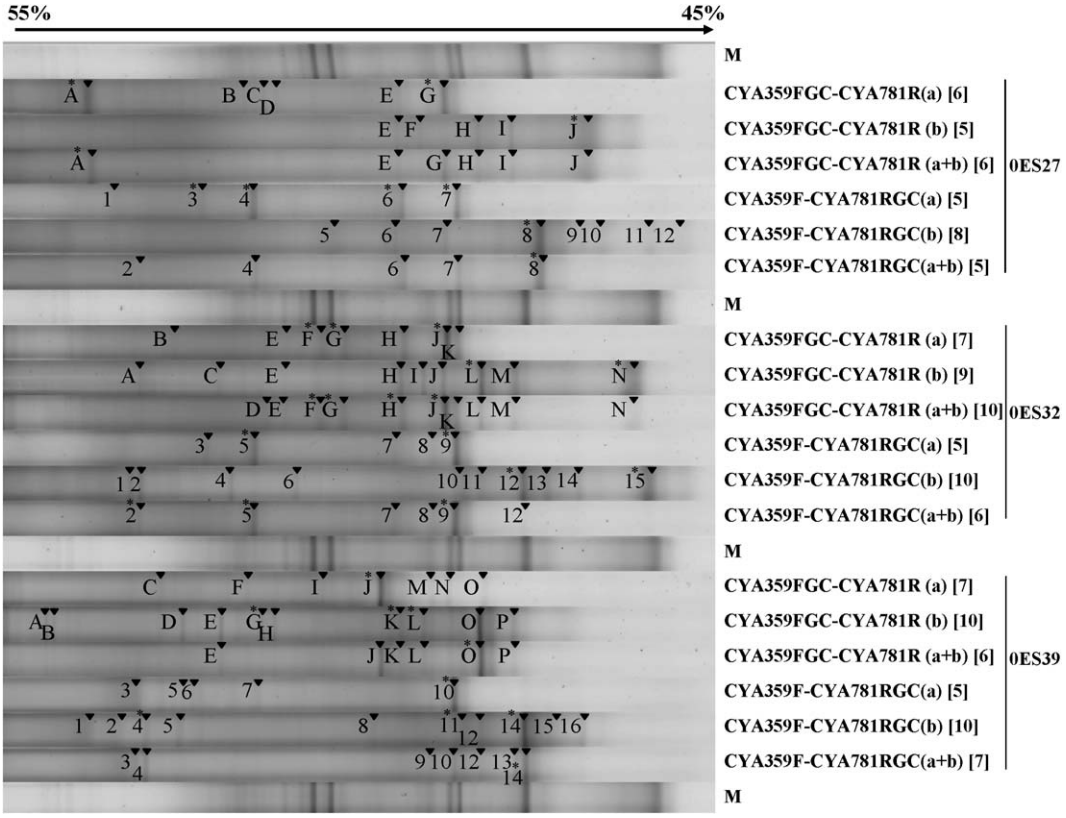

Fig. 1. DGGE patterns and designations of the bands obtained with the 6 primer pairs for samples 0ES27, 0ES32 and 0ES39. Visible bands are indicated by an arrow and bands at the same positions were given the same designation. A star indicates the bands for which a sequence was obtained. The numbers between brackets indicate the number of bands obtained for the lane.

Table 3
Results of the BLAST analysis of the sequences obtained from the sequenced DGGE

Sample	Primer pair	Band designation	Accession number	Most similar sequence	BLAST Similarity (%)
0ES27	CYA359FGC-CYA781R(a)	G	AY646774	*Aphanizomenon flos-aquae* PMC9707 (AJ293130)	100
	CYA359FGC-CYA781R(a)	A	AY646775	*Aphanizomenon flos-aquae* PCC7905 (AY038035)	99
	CYA359FGC-CYA781R(b)	J	AY646776	*Synechococcus* sp. MH305 (AY224198)	99
	CYA359FGC-CYA781R(a+b)	A	AY646777	*Aphanizomenon flos-aquae* PCC7905	99
	CYA359F-CYA781RGC(a)	3	AY646778	*Aphanizomenon flos-aquae* PMC9707	100
		4	AY646780		
		7	AY646781		
	CYA359F-CYA781RGC(a)	6	AY646779	*Aphanizomenon flos-aquae* PCC7905	99
	CYA359F-CYA781RGC(b)	8	AY646782	*Synechococcus* sp. MH305	99
	CYA359F-CYA781RGC(a+b)	8	AY646783	*Synechococcus* sp. MH305	99
0ES32	CYA359FGC-CYA781R(a)	F	AY646784	*Aphanizomenon flos-aquae* PMC9707	100
		G	AY646785		
		J	AY646786		
	CYA359FGC-CYA781R(b)	N	AY646787	*Synechococcus* sp. MH301 (AY224199)	97
	CYA359FGC-CYA781R(b)	L	AY646788	*Synechocystis* sp. PCC6803 (AY224195)	94
	CYA359FGC-CYA781R(a+b)	F	AY646789	*Aphanizomenon flos-aquae* PMC9707	100
		G	AY646790		
		H	AY646791		
		J	AY646792		
	CYA359F-CYA781RGC(a)	5	AY646793	*Aphanizomenon flos-aquae* PMC9707	100
		9	AY646794		
	CYA359F-CYA781RGC(b)	15	AY646795	*Synechococcus* sp. MH301	97
	CYA359F-CYA781RGC(b)	12	AY646796	*Synechocystis* sp. PCC6803	94
	CYA359F-CYA781RGC(a+b)	2	AY646797	*Aphanizomenon flos-aquae* PMC9707	100
		5	AY646798		
		9	AY646799		
0ES39	CYA359FGC-CYA781R(a)	J	AY646800	*Limnothrix redekei* CCAP 1443/1 (AJ580007)	99
	CYA359FGC-CYA781R(b)	G	AY646801	*Synechocystis* sp. PCC6803	94
		K	AY646802		
		L	AY646803		
	CYA359FGC-CYA781R(a+b)	O	AY646804	*Synechocystis* sp. PCC6803	94
	CYA359F-CYA781RGC(a)	10	AY646805	*Limnothrix redekei* CCAP 1443/1	99
	CYA359F-CYA781RGC(b)	4	AY646806	*Synechocystis* sp. PCC6803	94
		11	AY646807		
		14	AY646808		
	CYA359F-CYA781RGC(a+b)	14	AY646773	*Synechocystis* sp. PCC6803	94

effect, and the double-stranded DNA will melt at a lower temperature. This can also explain why the patterns produced with the GC clamp on the forward primer gave bands situated closer to the top of the DGGE gel.

Moreover, several melting domains are observed for the two sequences, irrespective of the position of the GC clamp. Ideal melting profiles are flat, and the presence of several melting domains typically results in extended (fuzzy) bands in the migration direction (Kisand and Wikner, 2003). Such fuzzy bands can be observed for the bands with sequences closely related to *A. flos-aquae* PMC9707 and *Synechococcus* sp. MH305 (0ES27: bands G, J, 4, 7, 8; 0ES32: bands H, J, 5, 9 in Fig. 1). Furthermore, the polymorphisms in high melting domains might be undetected, because the dissociation of low melting domains, which occurs earlier, strongly slows down the migration of the fragments (Fischer and Lerman, 1983; Wu et al., 1998).

3.3. Comparison of the use of the reverse primers CYA781R(a) and CYA781R(b) used separately or together

The lowest average number of bands observed for the DGGE patterns was obtained when using CYA781R(a) alone (5.8: Fig. 1). The primer pair CYA359F-CYA781R(b) gave the highest average number of bands, irrespective of the GC clamp position (8.6) whereas the mixture of reverse primers gave an intermediate score (6.6). The diversity of bands obtained was lower when both reverse primers were used together than the sum of the bands obtained separately with the primers

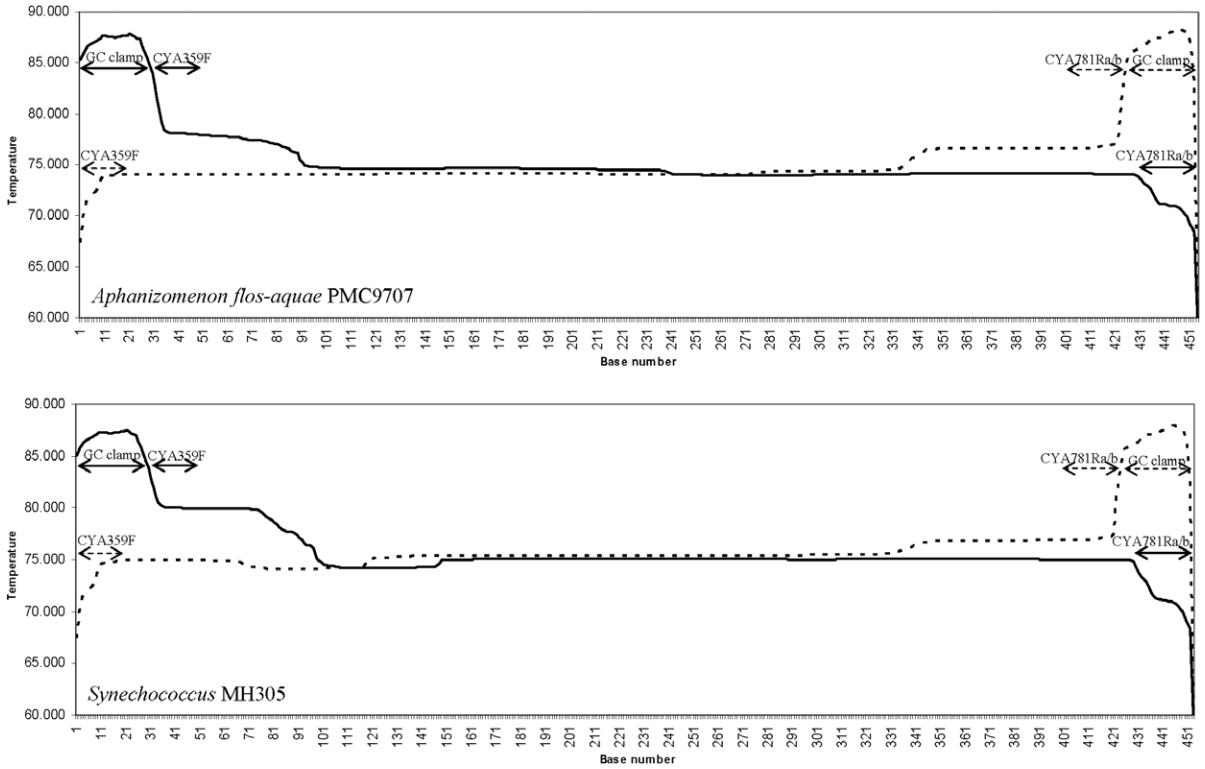

Fig. 2. Melting profiles obtained with the primer pair CYA359FGC-CYA781R(a/b), (solid line) and CYA359F-CYA781RGC(a/b) (dotted line) for sequence positions 378–781 of the 16S rRNA sequence of *Aphanizomenon flos-aquae* PMC9707 and for *Synechococcus* MH305. CYA781R(a/b) indicates CYA781R(a) or CYA781R(b) as identical profiles that were produced with primers CYA359F-CYA781R(a) and CYA359F-CYA781R(b), with the GC clamp on the forward or reverse primer. The zones corresponding to the primers and the GC clamp are indicated by an arrow.

(a) and (b). This result is supported by the DGGE patterns (Fig. 1).

In sample 0ES27, with the GC clamp on the forward primer, 10 bands (A to J) were produced in total for the separate primers instead of 6 with the combined primers (A, E, G, H, I, J, Fig. 1). With CYA359F-CYA781RGC(a+b), five bands were present in the DGGE pattern (2, 4, 6, 7, 8) whereas 11 bands were detected with the separate reverse primers.

Concerning sample 0ES32, 13 bands were obtained with the GC clamp on the forward primer with separate reverse primers (bands A, B, C, E, F, G, H, I, J, K, L, M, N) while only 10 bands could be observed when the reverse primers were mixed. With the GC clamp on the reverse primer, 15 bands in total were obtained for the separate reverse primers (1 to 15) and 6 bands (2, 5, 7, 8, 9, 12) when a combination of both primers were used.

For 0ES39 and CYA359FGC, 16 bands were thus recovered in total (A to P). With the mixture of the reverse primers, 6 bands were visible. When the GC clamp was on the reverse primer, 7 bands were visible in the pattern obtained with the mixture of the reverse primers (3, 4, 9, 10, 12, 13, 14) and 14 when the number of bands in the separate patterns were summed.

For each of the samples analysed, with the GC clamp on the forward primer, only one type of sequence was recovered with the mixture of the reverse primers. The same result was obtained with the GC clamp on the reverse primer. The sequences are related to *Aphanizomenon flos-aquae* PCC7905 (with the GC clamp on the forward primer) or to *Synechococcus* MH305 (with the GC clamp on the reverse primer) for 0ES27, to *Aphanizomenon flos-aquae* PMC9707 for 0ES32, and to *Synechocystis* PCC6803 for 0ES39.

With the separate PCR amplifications and irrespective of the position of the GC clamp, a higher number of genera was detected than with the mixture of primers according to the sequences obtained. Two (related to *L. redekei* CCAP 1443/1 and *Synechocystis* PCC6803 for 0ES39) or three (related to *A. flos-aquae* PMC9707, *A. flos-aquae* PCC7905 and *Synechococcus* MH305 for 0ES27 and to *A. flos-aquae* PMC9707, *Synechococcus* MH301 and *Synechocystis* PCC6803 for 0ES32) sequences were obtained. These

results are congruent with the theoretical match of the reverse primers.

It is believed that, when used together, the reverse primers compete to template hybridization between them, and the genetic fingerprint obtained is thus less complete.

By making separate amplifications with the reverse primers (a) and (b), a more complete picture of the real diversity of the environmental sample can be obtained. If we consider sample 0ES32, four and three sequences related to the filamentous cyanobacterium *A. flos-aquae* were obtained with CYA359FGC-CYA781R(a+b) and CYA359F-CYA781RGC(a+b), respectively. This taxa probably dominated the environmental sample. Only when primer (b) was used alone could the unicellular populations (related to *Synechococcus* sp. MH301 and *Synechocystis* sp. PCC6803) be detected. Thus, the use of separate primers is recommended, especially when performing straightforward excisions and reamplifications of bands, where it is not always possible to obtain sequences of all bands.

4. Conclusion

DGGE was originally designed to detect point mutations in human genomic DNA (Sheffield et al., 1989). In medical research, detection of mutations by DGGE can be optimized by careful analysis of the melting profiles and adaptations of the primers. This is not possible when using this method for community analysis. In addition, for cyanobacteria, the primers described by Nübel et al. (1997) are the only specific primers available. The results of our study show that the banding patterns obtained with the GC clamp on the forward or on the reverse primer varies and this is due to the influence of the GC clamp position on the melting profile. Nevertheless, the number of bands observed are very similar irrespective of the position of the GC clamp. Moreover, except when using reverse primers mixed with sample 0ES27, the same band sequences were obtained with the GC clamp on the forward or reverse primer.

In addition, we advise to use the reverse primers CYA781R(a) and (b) separately. It gives a more complete view of the cyanobacterial community composition. The primers (a) and (b) target filamentous and unicellular cyanobacteria, respectively, and their separate use allows the two different types of populations to be revealed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mimet.2005.09.017.

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