Comparison of primer sets for the study of Planctomycetes communities in lentic freshwater ecosystems

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Comparison of primer sets for the study of *Planctomycetes* communities in lentic freshwater ecosystems

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Summary

In search of a primer set that could be used to study *Planctomycetes* dynamics in lakes and especially via fingerprinting methods, e.g. denaturing gradient gel electrophoresis (DGGE), three existing specific primer sets, developed for marine and soil systems, have been tested on water samples from four freshwater ecosystems. The first primer set (PLA46F/PLA886R) allowed PCR amplification of *Planctomycetes* sequences in only one of the four ecosystems, whereas the second primer set (PLA40F/P518R) amplified *Planctomycetes* sequences in all the studied ecosystems but with a low specificity, since sequences belonging to *Verrucomicrobiales* and *Chlamydiales* clades were also amplified. Finally, the third primer set (PLA352F/PLA920R) allowed amplification of *Planctomycetes* sequences in the four ecosystems with a very high specificity. It amplified all known *Planctomycetes* genera and yielded the highest Operational Taxonomic Unit (OTU) richness and diversity estimates. *In silico* analyses supported these results. Further experiments comparing PLA352F/PLA920R to PLA46F/P1390R (a primer set generating a longer PCR fragment, also used to study *Planctomycetes*) yielded very similar results. Our findings suggest that the primer set PLA352F/PLA920R provides good estimates of *Planctomycetes* richness and diversity compared with other, and can thus be used to study *Planctomycetes* dynamics in lentic freshwater ecosystems.

Introduction

The order of *Planctomycetales* belongs to a separate phylum in the domain bacteria on the basis of their 16S rRNA gene sequences (Schlesner and Stackebrandt, 1986; Woese, 1987). It is now known that these bacteria are ubiquitous and display several specificities in terms of cell structure (e.g. cell compartmentalization), genetics and physiology (Fuerst, 1995; Lindsay *et al*., 2001). These bacteria have been isolated from soils, marine and freshwaters, hot springs, wastewater treatment plants and invertebrate animals (e.g. Schlesner, 1986; 1994; Giovannoni *et al*., 1987; Bornemann and Triplett, 1997; Fuerst *et al*., 1997; Axelrood *et al*., 2002; Pimentel-Elardo *et al*., 2003; Gade *et al*., 2004; Köhler *et al*., 2008). Since the discovery of the implication of *Planctomycetes* in the anaerobic ammonia oxidation (anammox) process (Strous *et al*., 1999; Kuypers *et al*., 2003), the number of studies dealing with these bacteria has considerably increased over years. However these studies generally concern marine ecosystems, soils, wastewater treatment plants and springs (Strous *et al*., 1999; Schmid *et al*., 2001; Chouari *et al*., 2003; Elshahed *et al*., 2007; Rich *et al*., 2008; Shu and Jiao, 2008; Woebken *et al*., 2008) and only a few studies have been carried out on these bacteria in lentic freshwater ecosystems. In the latter, *Planctomycetes* have been found to be involved in anammox (Schubert *et al*., 2006) and in change in the quality of dissolved organic matter (Tadonléké, 2007), two key processes in the functioning of aquatic systems. However while the first cited study focused on particular *Plancmotycetes*, the second was based on fluorescence *in situ* hybridization, with no information on *Planctomycetes* composition.

In order to increase our knowledge on the structural and functional diversity of the *Planctomycetes* communities in freshwater ecosystems, we need to have a set of primers allowing specific amplification of this group and an evaluation of its diversity. We wanted that this primer set generates a small size fragment, which can be used in a fingerprinting approach such as denaturing gradient gel electrophoresis (DGGE), although the latter is not restricted to short fragments. In this goal, we tested three existing primer sets developed in marine and soil systems PLA40F/P518R (Derakshani *et al*., 2001),PLA46/PLA886R (Pyneart *et al*., 2003) and PLA352F/PLA920R (Mühling *et al*., 2008), and described as *Planctomycetes*-specific (Table 1). Our study was performed on water samples from four contrasting freshwater ecosystems (two French peri-alpine lakes, Annecy and Bourget, and two reservoirs in Burkina Faso, Bagre and Pouytenga) and was complemented by *in silico* analyses, which compared the theoretical performance of these primers in the context of the current Ribosomal Database Project (RDP) database. Finally, we compared the results provided by the best primer set with those obtained with a primer set (PLA46F/P1390R) generating a longer fragment, and used by other authors to study *Planctomycetes* (Chouari *et al*., 2003).

Table 1. *Planctomycetes*-specific 16S rRNA gene PCR primer sets used in this study and conditions of each PCR reaction.

				PCR conditions							
				Denaturation		Annealing		Elongation			
Primers	**Target group**	**Sequences (5′–3′)**	**No. of cycles**	**Temperature (°C)**	**Time (min)**	**Temperature (°C)**	**Time (min)**	**Temperature (°C)**	**Time (min)**	**References**
PLA40F	*Planctomycetales*	CGG CRT GGA TTA GGC ATG	30	94	0,5	60	1	72	2	Boon *et al.* (2000); Derakshani *et al.* (2001)
P518R	*Eubacteria*	ATT ACC GCG GCT GCT GG								
PLA46F	*Planctomycetales*	GGA TTA GGC ATG CAA GTC	30	95	1	56	1	72	2	Pyneart *et al.* (2003)
PLA886R	*Planctomycetales*	GCC TTG CGA CCA TAC TCC C								
PLA352F	*Planctomycetales*	GGC TGC AGT CGA GRA TCT	30	96	1	58	1	74	1	Mühling *et al.* (2008)
PLA920R	*Planctomycetales*	TGT GTG AGC CCC CGT CAA								
PLA46F	*Planctomycetales*	GGA TTA GGC	30	94	1	57	1	72	2	Chouari *et al.* (2003)

1. For DNA extraction and PCR amplification, 250 ml of water sample from each lake was first filtered through a 2-µm-pore-size polycarbonate membrane filter (Nuclepore) to eliminate larger eukaryotes. The bacterioplankton remaining in the filtrate was then collected, through a gentle filtration, on a 0.2 -µm-pore-size polycarbonate membrane filters (Nucleopore). Nucleic acid extraction was carried out as described in Dorigo and colleagues (2006). PCR amplifications were performed with the PTC-100™ Thermal Cycler (MJ Research) using three sets of primers (PLA46F/PLA886R, Pyneart *et al.*, 2003; PLA40F/P518R, Derakshani *et al.*, 2001; and PLA352F/PLA920R, Mühling *et al.*, 2008). To compare the two sets of primers PLA352F/PLA920R and PLA46F/P1390R, PCR amplifications have been performed only on water samples from the Lake Bourget (referred to as 'Bourget 2'). For each primer set, a range of annealing temperatures was tested to determine empirically the temperature that resulted in the most specific PCR product.

Table 1. *Planctomycetes*-specific 16S rRNA gene PCR primer sets used in this study and conditions of each PCR reaction.

Primers	Target group	Sequences (5′–3′)	PCR conditions						References
			No. of cycles	Denaturation		Annealing		Elongation		
				Temperature (°C)	Time (min)	Temperature (°C)	Time (min)	Temperature (°C)	Time (min)	
P1390R	*Eubacteria*	ATG CAA GTC GAC GGG CGG TGT GTA CAA								

Results and discussion

Efficacy and ability of each primer set to amplify *Planctomycetes* sequences

The *in silico* analyses (theoretical approach) using the RDP database showed that the forward and the reverse of the primer set PLA352F/PLA920R matched with a higher number of *Planctomycetes* sequences (60% and 52% respectively), and also exhibited lower percentages of match outside this bacterial group than the forward and the reverse from the two others primer sets (Table 2). It is, however, important to note that in spite of a good specificity of primers obtained by theoretical analysis, experimental analyses are necessary and can reveal a high degree of non-specificity (Morales and Holben, 2009).

Table 2. *Planctomycetes*-specific 16S rRNA gene PCR primers used in this study and their specificity revealed by the *in silico* analysis.

Primer	Target group	Sequence (5′→3′)	*Escherichia coli* position	Identical matches within the target group	Percentage matches within the target group compared with all *Planctomycetes* sequences[a]	Percentage matches within the target group compared with total hits
PLA46F	Planctomycetales	GGA TTA GGC ATG CAA GTC	46–63	2838	30	77
PLA886R	Planctomycetales	GCC TTG CGA CCA TAC TCC C	886–904	4574	48	95
PLA40F	Planctomycetales	CGG CRT GGA TTA GGC ATG	40–57	2446	26	86
P518R	Eubacteria	ATT ACC GCG GCT GCT GG	518–534	1784	18	0.24
PLA352F	Planctomycetales	GGC TGC AGT CGA GRA TCT	350–367	5684	60	91
PLA920R	Planctomycetales	TGT GTG AGC CCC CGT CAA	920–937	4973	52	99

- **a.**
  A total of 9488 *Planctomycetes* sequences were available in the RDP database.
- The theoretical performance of each PCR primer was tested (*in silico* analyses) using the 'Probe Match' function within the Ribosomal Database Project (RDP) (Cole *et al*., 2005).

Very contrasting results were found in the PCR efficiency and specificity using the first three primer sets tested. The primer set PLA46F/PLA886R allowed amplification of *Planctomycetes* sequences (100% of sequences obtained belonged to *Planctomycetales* order) but only in Lake Bourget. No amplification was obtained with this primer set for samples from the three other ecosystems (Table 3). This primer set has previously been used to estimate the *Planctomycetes* diversity in marine environments and in digesters of wastewater treatment plants (Pyneart *et al*., 2003; Shu and Jiao, 2008). Previous studies indicated that a low specificity of the primers and a poor efficacy of the PCR reaction can yield poor amplification (Becker *et al*., 2000), as found with this primer set in our study. Moreover, recent studies (Bru *et al*., 2008; Wu *et al*., 2009) have shown that single mismatch in the regions where primers bind onto the template strands can also greatly affect the PCR efficacy. This set of primers might thus be able to detect and amplify only a restricted number of *Planctomycetes* sequences among those present in freshwater ecosystems. In a recent review, Amann and Fuchs (2008) reported that the coverage of the probe PLA46 was 44% (559 of 1271) of the *Planctomycetes* 16S rRNA sequences in the SILVA database. Our *in silico* analysis in the context of a larger database (RDP) indicates a lower coverage (30%) for the PLA46F as a primer for this bacterial group (Table 2).

Table 3. Analysis of the 16S rDNA diversity (Chao1 estimator, total number of sequences, number of *Planctomycetes* sequences and OTUs and percentage of *Planctomycetes* sequences) for each primer set and lake.

Primers	Lakes	Total number of sequences	Number of *Planctomycetes*		Chao1 estimates (±SD)
			Sequences	OTUs	
PLA46F/PLA886R	Annecy	ND	ND	ND	ND
	Bourget 1	94	94	10	10.5 (1.29)
	Bagre	ND	ND	ND	ND
	Pouytenga	ND	ND	ND	ND
PLA40F/P518R	Annecy	26	5	5	ND
	Bourget 1	72	48	7	12 (7.04)
	Bagre	70	47	5	5 (0.01)
	Pouytenga	57	55	2	2 (0)
PLA352F/PLA920R	Annecy	86	85	9	9 (0.25)
	Bourget 1	94	93	9	12 (4.18)
	Bourget 2	90	89	5	6 (2.19)
	Bagre	88	84	21	26 (4.21)
	Pouytenga	88	86	5	5 (0)
PLA46F/P1390R	Bourget 2	78	76	6	7 (2.22)

1. After amplification, fragments were cloned in the pGEM-T system II vector (Promega) and then transformed into JM109-competent cell (Promega) according to the manufacturer's instructions. Ninety-six positive clones (white colonies) obtained from each clone library from each lake and each primer set were randomly selected, verified by PCR using the commercial primers SP6/T7 and finally sequenced (GATC Biotech). Sequences were then edited, aligned with Genedoc (Nicholas and Nicholas, 1997) and finally checked for chimera using Bellerophon (Huber *et al*., 2004) and the Ribosomal Database Project (RDP, Cole *et al*., 2005). Operational Taxonomic Units (OTUs) were defined on the basis of a ≥ 98% sequence identity. The Chaos-1 and abundance-based coverage estimators of species richness were calculated using the software 'EstimateS' (http://viceroy.eeb.uconn.edu/estimates). ND, not determined.

The second primer set, PLA40F/P518R, allowed to amplify *Planctomycetes* sequences in the four lakes but the specificity of the amplification varied considerably (Table 3). The proportion of sequences belonging to *Planctomycetes* ranged from a value as low as 13% in Lake Annecy to 96% in the reservoir Pouytenga, intermediate values being found in Lake Bourget (51%) and in the reservoir Bagre (67%). This primer set amplified members of other bacterial orders, which are phylogenetically close to *Planctomycetes*, namely *Verrucomicrobiales* and *Parachlamydiales* (13% and 9% of the total of sequences respectively) in Lake Annecy, and C*hlamydiales* (6% of all sequences) in the reservoir Bagre. This lack of *Planctomycetes* specificity has previously been reported for the primer PLA40F in soils and rice roots (Derakshani *et al*., 2001). These authors pointed out that PLA40F revealed no mismatch for almost all *Planctomycetes* and only a very few mismatches for members of the *Verrucomicrobia* and some *Chlamydia.* Our results suggest that the primer set PLA40F/P518R might not be suitable for study of freshwater planktonic *Planctomycetes*, especially in the framework of fingerprinting approach on these communities.

The third primer set tested (PLA352F/PLA920R) provided the best results in term of specificity and efficiency (Table 3). Indeed, a positive PCR amplification was obtained in all sampling sites and almost all the sequences (> 97%) belonged to the *Planctomycetes* phylum. The remaining sequences (< 3%) belonged to a group of 'unclassified bacteria'.

Operational Taxonomic Unit (OTU) richness found in the *Planctomycetes* communities using the three sets of primers

The number of *Planctomycetes* Operational Taxonomic Units (OTUs) identified in the samples from the four ecosystems with the different primer sets did not differ significantly from the values of the Chao1 estimator (Sign test, $P = 0.25$, Table 3), showing that we obtained a sufficient number of sequences to evaluate the *Planctomycetes* OTU richness in our samples. This finding was confirmed by the rarefaction curves which were asymptotic in most cases (not shown). Finally it appeared for the same primer set that there were large variations in the richness index between the samples from the four ecosystems (Table 3). Compared with the two others, the primer set PLA352F/PLA920R allowed to obtain higher numbers of OTUs and higher values of the Chao1 index in almost all cases (Table 3). This suggests that the primer set PLA352F/PLA920R was able to amplify a larger set of *Planctomycetes* sequences than the two others, and that the latter underestimated the richness of the *Planctomycetes* community. These results were supported by the *in silico* analyses, which revealed only a few sequences detected by PLA352F/PLA920R outside the group of *Planctomycetes* in the context of RDP (Table 2). As pointed out in previous works (Lueders and Friedrich, 2003), our findings confirmed that it is necessary to perform a careful evaluation of the PCR primers used to assess the diversity in microbial communities. Hence, the primer set PLA352F/PLA920R seemed to provide the best compromise considering the ability to obtain a positive PCR amplification, the specificity of the amplification and the ability to detect a large number of OTUs within the *Planctomycetes* communities.

Planctomycetes diversity obtained using PLA352F/PLA920R versus PLA46F/P1390R

Based on the above results, further experiments were performed to compare results obtained with PLA352F/PLA920R with those found with the primer set PLA46F/P1390R (Chouari *et al*., 2003). PLA352F/PLA920R generates a short enough PCR fragments (568 bp) to be used in DGGE (Mühling *et al*., 2008), one of the most used fingerprinting approach. PLA46F/P1390R allows amplification of a 1344 bp fragment, and has been used to study *Planctomycetes* communities in waste water treatment plants (Chouari *et al*., 2003). The comparison was performed on a sample from Lake Bourget (Bourget 2). Results showed that the two primer sets presented a high specificity to *Planctomycetes*, as 99% and 98% of sequences obtained with PLA352F/PLA920R and PLA46F/P1390R, respectively, belonged to *Planctomycetales*. The number of OTUs (5 and 6) and the composition of the *Planctomycetes* community amplified by these two primer sets were very similar (Table 3). In addition, these two primer sets yielded similar values for the Chao1 (Table 3). This indicated that, despite the smaller size of PCR fragments generated by PLA352F/PLA920R, the latter did not underestimate *Planctomycetes* diversity, in our samples, compared with PLA46F/P1390R.

Phylogenetic analysis of the sequences obtained with the primers PLA352F/PLA920R

Based on the above results, a phylogenetic analysis was performed with data obtained with PLA352F/PLA920R. To date, seven cultured genera of *Planctomycetes* (*Planctomyces*, *Gemmata*, *Isosphaera*, *Pirellula*, *Schlesneria, Singulisphaera* and *Zavarzinella*) have been described (Staley *et al*., 1992; Kulichevskaya *et al*., 2007; 2008; 2009) but several additional *Candidatus* genera (*Brocadia*, *Kuenenia, Scalindua, Annamoxoglobus* and *Jettenia*) playing a key role in the anammox process (Strous *et al*., 1999; Kuypers *et al*., 2003; Kartal *et al*., 2007; Schmid *et al*., 2007; Quan *et al*., 2008) have recently been proposed. Among the 437 *Planctomycetes* sequences obtained with the primer set PLA352F/PLA920R in our four sampling sites, 39 OTUs displaying a ≥ 98% sequence identity were identified. Thirty-eight of these OTUs were distributed in four clusters containing all *Planctomycetes* genera [cluster I (*Pirellula/Blastopirellula*), cluster II (*Planctomyces/Schlesneria*), cluster III (*Isosphaera/Singulisphaera*) and cluster IV (*Gemmata/Zavarzinella*)]. This indicated that the primers PLA352F/PLA920R were able to amplify members of all the *Planctomycetes* genera currently described (Fig. 1). Among these 38 OTUs, 20 (~53%) belonged to cluster I, which comprises *Pirellula/Blastopirellula* genera. A high diversity of the *Pirellula* genus and its dominance within *Planctomycetes* communities have been reported by previous studies in other freshwater environments, namely wastewater treatment plants and river biofilms (Chouari *et al*., 2003; Brümmer *et al*., 2004). Our results, together with those from these previous studies, suggest that members of this genus are probably well adapted to freshwater ecosystems and might play a particular role in these environments.

One OTU (OTU 28), amplified in the reservoir Bagre, showed a high degree of similarity with sequences belonging to anammox *Planctomycetes* (Fig. 1). This suggests that the primer set PLA352F/PLA920R is potentially able to detect and amplify freshwater *Planctomycetes* involved in anaerobic ammonium oxidizing processes. This may be of interest even though obtaining of a better representation of anammox organisms when they are present might require primer specific to these organisms (Schmid *et al*., 2005). The existence of anoxic

microzones in detrital aggregates (Shanks and Reeder, 1993) might be a factor allowing the presence of anammox bacteria in oxygenated water columns, as found in this study.
All the sequences sharing ≥ 98% sequence identity with sequences from GenBank were closely related to environmental sequences and not to cultivated organisms. In addition, 46% of the OTUs displayed sequence similarity < 98% with sequences from GenBank® database (Table S1), which confirmed that the *Planctomycetes* from freshwater ecosystems are still poorly known. As previously found for dominant phyla in freshwater communities (Humbert *et al*., 2009), the fact that best blast hits were all obtained with sequences from freshwater habitats (lakes, rivers and reservoirs) seems to support the conclusions of Brümmer and colleagues (2004) suggesting that *Planctomycetes* species from freshwaters are different from those from marine environments.
In conclusion, we propose that at the current state of knowledge, the primer set PLA352F/PLA920R could be used to study pelagic *Planctomycetes* in freshwater ecosystems specifically via fingerprinting methods.

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