Monitoring a widespread bacterial group: *in situ* detection of planctomycetes with 16S rRNA-targeted probes

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The group of planctomycetes represents a separate line of descent within the domain *Bacteria*. Two phylum-specific 16S rRNA-targeted oligonucleotide probes for planctomycetes have been designed, optimized for *in situ* hybridization and used in different habitats to detect members of the group *in situ*. The probes, named PLA46 and PLA886, are targeting all or nearly all members of the planctomycete line of descent. Planctomycetes could be detected in almost all samples examined, e.g. a brackish water lagoon, activated sludge, and other wastewater habitats. *In situ* probing revealed quite uniform morphology and spatial arrangement of the detected cells but profound differences in abundance ranging from less than 0.1% to several percentage of the total cells. Single coccoid cells with diameters between 1 and 2.5 µm were dominating in most samples with the exception of the lagoon, in which rosettes of pear-shaped cells were abundant. The planctomycetes showed generally no hybridization signals with the bacterial probe EUB338, which is in accordance with base changes in their 16S rRNA sequences. A discrete ultrastructure of planctomycete cells was suggested by double staining with rRNA-targeted probes and the DNA-binding dye 4',6-diamidino-2-phenylindole (DAPI). The probe-conferred fluorescence was distributed in a ring-shaped manner around a central DAPI spot. The two probes developed extend the existing set of group-specific rRNA-targeted probes and help to elucidate the basic composition of bacterial communities in a first step of differential analysis. *In situ* hybridization of environmental samples indicated widespread presence of planctomycetes in different ecosystems.

Keywords: planctomycetes, rRNA-targeted probes, fluorescence *in situ* hybridization (FISH), *in situ* identification

INTRODUCTION

Planctomycetes are aerobic, Gram-negative, chemoheterotrophic bacteria with mostly spherical, ovoid or pear-shaped cell morphology. They share some unique

characters that distinguish them from other bacteria, most importantly the lack of peptidoglycan in their cell walls and the budding type of reproduction. Some members have typical acellular appendages. Main ecological features of the group have been recently reviewed (Fuerst, 1995). Pure cultures have been isolated from diverse aquatic habitats such as freshwater lakes, brackish water and sea water as well as from a hot spring (Schmidt, 1978; Schlesner, 1986, 1994; Giovannoni *et al.*, 1987). So far, four genera, *Planctomyces*, *Pirellula*, *Gemmata* and *Isosphaera*, are validly described (Staley *et al.*, 1992; Holt *et al.*, 1994). A new

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Abbreviations: BOD₅, biological oxygen demand within 5 d incubation; DAPI, 4',6-diamidino-2-phenylindole; FLUOS, 5(6)-carboxy-fluorescein-*N*-hydroxysuccinimide ester; TAMRA, 5(6)-carboxy-tetramethylrhodamine-*N*-hydroxysuccinimide ester; WWTP, wastewater treatment plant.

order, *Planctomycetales*, was proposed (Schlesner & Stackebrandt, 1986) that encompasses all four genera.

Analysis of rRNA molecules demonstrated that this order comprises a separate line of descent within the domain *Bacteria* clearly separated from the other phyla (Stackebrandt *et al.*, 1984; Woese, 1987). Their 16S rRNA sequences possess unique signatures. Other idiosyncrasies are shorter 5S rRNA molecules in comparison to other bacterial lineages (Bomar *et al.*, 1988) and unlinked *rrn* operon structures detected in two members (Liesack & Stackebrandt, 1989; Ward-Rainey *et al.*, 1996). The four genera constitute distinct phylogenetic lineages within the phylum (Bomar *et al.*, 1988; Staley *et al.*, 1992). In a recent study on the phylogenetic position of 22 environmental isolates by 16S rDNA sequence analysis, considerable diversity within the order *Planctomycetales* was shown. In fact, none of the newly studied strains was related to any previously described species at species level (Ward *et al.*, 1995).

The members of the four genera possess typical cell shapes. On this basis they could be differentiated (Holt *et al.*, 1994). Further differences exist regarding the type of cell aggregation, e.g. rosette formation for *Planctomyces* and *Pirellula* or as filaments of coccoid cells for *Isosphaera pallida*, and type of motility, such as gliding as described for *I. pallida* versus via flagellation for members of the other three genera.

Up to a few years ago planctomycetes had been supposed to be restricted to aquatic environments, since all known pure cultures have been isolated from habitats like freshwater or marine samples. In fact, even in 1992 in *The Prokaryotes* the group has been described as solely aquatic (Staley *et al.*, 1992). Therefore, it was surprising

when the first evidence for occurrence of planctomycetes in terrestrial habitats came in a study on 16S rDNA clone sequences from Australian soils (Liesack & Stackebrandt, 1992). Later, further studies reporting the presence of planctomycete 16S rDNA/rRNA sequences in different habitats, such as in marine snow from offshore seawater (DeLong *et al.*, 1993), sludge from a sequencing batch reactor (Bond *et al.*, 1995), marine sediments (Gray & Herwig, 1996), silt-loam soils (Borneman *et al.*, 1996; Lee *et al.*, 1996) and in postlarvae from prawns (Fuerst *et al.*, 1997), indicated that they are widely distributed. However, cloning approaches yield only sequence data without the possibility of acquiring additional information on the organisms. Up to now, isolation of a pure culture from any non-aquatic habitat has not been reported.

It was the goal of this study to develop rRNA-targeted oligonucleotide probes for the evaluation of the natural abundance of planctomycetes by *in situ* hybridization. Here we report the development, optimization and *in situ* application of two probes on the phylum level to address the question of how far distributed this group of organisms is in environmental habitats.

METHODS

Organisms and culture conditions. The following reference strains were used in this study: *Gemmata* sp. SCH633, *Isosphaera* sp. SCH394, *Pirellula marina* DSM 3645^T, *Pirellula* sp. SCH1, *Pirellula* sp. SCH140 [all obtained from the Institut für Allgemeine Mikrobiologie (IFAM) strain collection], *Planctomyces limnophilus* ATCC 43296^T, *Brevibacterium linens* DSM 20425^T, *Cellulomonas turbata* LMG 4072^T, *Chryseobacterium gleum* LMG 8333, *Comamonas testos-*

Table 1. Sampling locations

Brackish water lagoon	Size	Chemical characteristics
Lake Albufera, south of Valencia, Spain	Surface, 2.2 km ² ; medium depth, 0.75 m	Salinity, 0.2 %; pH, 8–10; hypereutrophic
Treatment plant location for activated sludge samples	Population equivalents	Type of wastewater and plant processing
Airolo, Ticino, Switzerland	About 10000	Municipal; one biological stage
Freising*	110000	Municipal, dairy and brewery sewages; one stage
Grüneck*	100000	Mainly municipal; one stage
Haag*	3600	Municipal; one stage
München-Dietersheim, Germany	1000000	Mainly municipal sewage; two stages
München-Großlappen, Germany	1000000	Mainly municipal; two stages
Valencia-Pinedo, Spain	650000	Mainly municipal; one stage
Other samples from WWTPs	Origin of sample	Type of processing
Sand filter, München-Dietersheim	Sampling depth, 1.0 m	1.5-m-thick filter bed of 2.5 mm quartz grains; removal of suspended solids
Influent and effluent of Valencia-Pinedo		

* These plants are located in the county of Upper Bavaria close to München.

Table 2. Oligonucleotide probes

Name	OPD designation*	Sequence from 5' to 3'	Position†	Stringency‡	Reference
PLA46	S-P-Planc-0046-a-A-18	GACTTGCATGCCTAATCC	46–63	30	This study
PLA886	S-P-Planc-0886-a-A-19	GCCTTGCGACCATACTCCC	886–904	35§	This study
EUB338	S-D-Bact-0338-a-A-18	GCTGCCTCCCGTAGGAGT	338–355	0	Amann <i>et al.</i> (1990)
EUB008	S-D-Bact-0008-a-A-19	TGAGCCAGGATCAAACCTCT	8–26	0	Hicks <i>et al.</i> (1992)

* Standardized probe name according to proposal of Alm *et al.* (1996); OPD, oligonucleotide probe database.

† Target site in 16S rRNA according to *E. coli* numbering (Brosius *et al.*, 1981).

‡ Amount of formamide (% , v/v) in hybridization buffer.

§ Hybridization together with equimolar amount of competitor oligonucleotide cPLA886, 5' GCCTTGCGACCGTACTCCC 3'.

teroni LMG 1800^T and *Escherichia coli* DSM 30083^T (ATCC, American Type Culture Collection, Rockville, MD, USA; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; LMG, Laboratorium voor Mikrobiologie, Universiteit Ghent, Belgium). Planctomycete strains were grown on the following solid media (18 g agar l⁻¹) as described by Schlesner (1994): PYGV for SCH394, M31 supplemented with peptone and yeast extract (0.25 g l⁻¹ of each) for ATCC 43296^T and SCH633 and M13a for DSM 3645^T, SCH1 and SCH140. All other strains were cultivated as outlined in the respective catalogues of strains.

Cell fixation. Fixation with 4% (w/v) paraformaldehyde and subsequent treatment was performed according to Amann *et al.* (1990). Cell material was collected either directly from solid media or from exponentially growing liquid cultures. It was resuspended in 200 µl PBS (130 mM NaCl, 10 mM sodium phosphate buffer, pH 7.4) and 600 µl fixative was added.

Samples. Characteristics of the investigated habitats are given in Table 1. Surface water was sampled from the brackish coastal lagoon (Aznar *et al.*, 1991) as well as from inflow and outflow channels of one wastewater treatment plant (WWTP), mixed liquor from activated sludge basins and solid material from the sand filter bed. Liquid and solid samples were fixed immediately after sampling with paraformaldehyde solution and further processed as described by Wagner *et al.* (1993) and Neef *et al.* (1996), respectively.

Oligonucleotide probes. Probe sequences and target sites for the probes used in this study are displayed in Table 2. Oligonucleotides synthesized with a 5'-C₆-aminolinker were purchased from MWG-Biotech. Labelling with the fluorochromes 5(6)-carboxy-fluorescein-*N*-hydroxysuccinimide ester (FLUOS; Boehringer Mannheim) and 5(6)-carboxy-tetramethylrhodamine-*N*-hydroxysuccinimide ester (TAMRA; Molecular Probes) and subsequent purification of the oligonucleotide-dye conjugates were performed as described by Amann *et al.* (1990).

Hybridizations. Hybridizations were done on gelatin-coated glass slides with six wells for independent positioning of samples. Aliquots of fixed reference cells and environmental samples were spotted on single wells, air-dried and dehydrated by passing them through an ethanol series (50, 80 and 96%, v/v) for 3 min each. Hybridizations were performed in isotonicity equilibrated humid chambers for 1.5 h at 46 °C. Hybridization buffer (10 µl) containing 0.9 M NaCl, 20 mM Tris/HCl, pH 8.0, 0.01% SDS, the specific amount of formamide (Table 2) and 5 ng FLUOS- or TAMRA-labelled probe µl⁻¹ were applied on each well of the slide. Washing was

achieved by immediate rinsing with 1 ml prewarmed (48 °C) washing buffer followed by a 20 min immersion in washing buffer at 48 °C. The washing buffer contained 20 mM Tris/HCl, pH 8.0, 0.01% SDS and, depending on the hybridization stringency, between 0.056 M and 0.9 M NaCl. Subsequently, slides were rinsed with deionized water. After air-drying, slides were optionally counterstained with 4',6-diamidino-2-phenylindole (DAPI) (see below) and embedded in Citifluor.

DAPI staining. Counterstaining with the DNA-binding dye DAPI (Boehringer Mannheim) was performed following the washing step of the hybridization protocol. Single wells on the slide were covered with 30 µl DAPI solution (1 µg ml⁻¹ in PBS) and incubated at room temperature for 10 min in the dark. After rinsing with water and air-drying the slide could be mounted in Citifluor.

Microscopy and documentation. Examination was done with a Zeiss Axioplan microscope (Carl Zeiss) equipped with Zeiss filter sets no. 09 and 15 and Chroma filter sets HQ-Cy3 and HQ-FITC (Chroma) for probe-conferred and Zeiss filter set no. 01 for DAPI-conferred fluorescence. Photomicrographs were taken with a Kodak Ektachrome P1600 colour reversal film and a Kodak TMAX 400 black-and-white film. Exposure times were 5–15 s and 0.5–1 s for colour micrographs of probe-conferred and DAPI epifluorescence, respectively, and 60 s for the black-and-white micrographs.

Signal quantification. Fluorescence conferred by probes PLA46-TAMRA and PLA886-TAMRA to reference cells was quantified using a CCD camera (CF 15/2; Kappa) attached to the epifluorescence microscope and the fg-cell image analysis program (Captec), as described by Trebesius *et al.* (1994). Between 50 and 250 cells were analysed for each data point. Values of probe-conferred fluorescence intensities of each cell were divided by cell area to determine intensity values independent of cell size. Mean values of signal intensities were normalized relative to the signal of the probe EUB008-FLUOS to correct for the different contents of ribosomes in the reference strains. Sigmoidal fitting of melting curves was carried out using the graphics software Origin (version 3.5; Microcal Software).

RESULTS AND DISCUSSION

Design of 16S rRNA-targeted probes

Probe design was initially performed on the basis of about 3500 complete and partial 16S rRNA sequences

(a) PLA46

Probe sequence	3' - CCTAATCCGTACGTTTCAG - 5'
Target sequence	5' - GGAUUAGGCAUGCAAGUC - 3'
<i>Gemmata</i> sp. strain SCH633	
<i>Chlamydia</i> spp.	...g.....
Sequences from clone libraries	...A..A.....
<i>Spirochaeta thermophila</i>	.UC.....
<i>Brevinema andersonii</i>	.UU.....
<i>Chryseobacterium gleum</i>	...g..C...A.....u
<i>Pyrobaculum islandicum</i>	..C.A..C.....g....
Majority of the spirochaetes	.UC...A.....

(b) PLA886

Probe sequence	3' - CCCTCATACCAGCGTTCCG - 5'
Target sequence	5' - GGGAGUAUGGUCGCAAGGC - 3'
<i>Gemmata</i> sp. strain SCH633	
<i>Brevibacterium linens</i>	C.....
<i>Holophaga foetida</i>	C.....
<i>Buchnera aphidicola</i>	C.....
<i>Desulfovibrio longus</i>	C.....
<i>Nitrospina gracilis</i>	C.....
<i>Blattabacterium</i> spp.	C.....
Most actinomycetes	C..C.....
<i>Bacillus</i> spp.	C..C.....
<i>Thermus</i> spp.	C..C.....
<i>Vibrio cholerae</i>	C.....A.
<i>Xanthomonas</i> spp.	C.....A.
<i>Escherichia coli</i>	C.....Au
<i>Comamonas testosteroni</i>	C.....Au
<i>Chlamydia</i> spp.	A.....C..C.....G

Fig. 1. Difference alignments for probes PLA46 (a) and PLA886 (b). 16S rRNA sequences at the target sites of the probes are displayed for representative reference organisms. Upper-case letters indicate nucleotide changes leading to strong mismatches; lower-case letters indicate changes leading to weak mismatches. In (a) the sequences from clone libraries include some clone sequences from Australian soils and a sequencing batch reactor (Liesack & Stackebrandt, 1992; Bond *et al.*, 1995). Eukaryotic organisms with an identical target site for probe PLA886 (b) in their 18S rRNA sequences are plants, fungi and diverse protists.

present in the TUM rRNA database (Dr Ludwig, Technical University Munich) in July 1995. The set of data contained at this time 17 sequences of members of the order *Planctomycetales*. For probe design the respective tool from the ARB software package (O. Strunk & W. Ludwig, Technical University Munich, Germany; URL: <http://www.biol.chemie.tu-muenchen.de/pub/ARB>) was used. Two target regions for oligonucleotide probes were found to be highly diagnostic for the members of the *Planctomycetales* covering nucleotide positions 46–63 (*E. coli* numbering; Brosius *et al.*, 1981) and 886–904, respectively. The probes were named PLA46 and PLA886. The former region around helices 4 and 5 has been used as target site for identification of planctomycetes already by Liesack & Stackebrandt (1992). Another probe specific for planctomycetes and some other bacteria targeting a further region of the 16S rRNA has additionally been reported (Fuerst *et al.*, 1997). Meanwhile, the number of available 16S rRNA sequences for the *Planctomycetales* has increased markedly to 75 full or partial sequences (TUM rRNA database, August 1997). However, applying recent sequence checks both probes are still targeting all planctomycete sequences except four partial sequences from 16S rDNA clones (Bond *et al.*, 1995) for PLA46 and one nearly complete sequence from an isolate (Ward *et al.*, 1995) for PLA886. However, both probes recognize

no sequences from any other prokaryotes (recent probe check against Ribosomal Database Project data release 7.0, July 1998; Maidak *et al.*, 1997), therefore confirming the uniqueness of both target regions for the planctomycetes. The results of the alignment checks are displayed in Fig. 1. Surprisingly, inclusion of 18S rRNA sequences in the probe checks showed that some groups of eukaryotes possess the identical target region for probe PLA886. Plants, fungi and some protists like *Acanthamoeba* spp., *Cryptosporidium* spp. or *Toxoplasma* spp. belong to these groups, which, however, do not form a monophyletic cluster within the domain *Eucarya*.

Optimization of hybridization conditions

The aim of probe development was single cell identification by *in situ* hybridization in environmental samples. Therefore, specificity of both probes was tested directly by whole-cell hybridization with fluorochrome-labelled probes and representative reference organisms. All six tested planctomycetes, which represent the four lineages and include four pigmented strains, showed no considerable autofluorescence and yielded strong hybridization signals with both oligonucleotide probes. *Gemmata* sp. SCH633 displayed the most uniform signals.

Fig. 2. Probe-binding profiles of probes PLA46 and PLA886. The relative strength of hybridization was determined at increasing concentrations of formamide by quantification of intensities of the fluorescence signals. Graphs show results for probe PLA46-TAMRA (a) against *Gemmata* sp. strain SCH633 and probe PLA886-TAMRA (b) against *Gemmata* sp. strain SCH633 (■) and *B. linens* DSM 20425T (●). Hybridizations with PLA886 were done with an equimolar amount of competitor cPLA886. The mean fluorescence intensity of probe-positive cells after hybridization at 0% formamide was defined as 100%. Single data points mark arithmetic means $\pm$ SD.

Therefore, optimal hybridization stringencies were evaluated with this strain in formamide series by quantification of fluorescence intensities (Fig. 2).

Probe PLA46 yielded strong signals up to 30% (v/v) formamide followed by a significant decline at 35% (v/v) formamide. *Chryseobacterium gleum* LMG 8333 with four base changes within the target strand was the reference strain tested showing the highest sequence similarity (see Fig. 1a). Cells of this organism displayed no signals even without formamide in the hybridization buffer. Considering strength and position of the mismatches in other non-target organisms which were not available for specificity testing, such as, e.g. spirochaetes, a formamide concentration of 30% (v/v) was chosen as optimal stringency for PLA46. Members of the genus *Chlamydia* contain only one weakly discriminating base change at position 5 of the target region (position 50 in *E. coli* numbering). It is probable that this mismatch could not be discriminated at the applied stringency of 30% (v/v) formamide. Therefore, it is

assumed that *Chlamydia* spp. could also be detected with probe PLA46. For probe PLA886 preliminary experiments indicated that an unlabelled competitor oligonucleotide, cPLA886, always has to be used to effectively prevent hybridization of PLA886 to some actinomycetes and other outgroup bacteria (for details see Fig. 1b). PLA886 has only one central C-A mismatch with such non-target sequences, which is insufficient for full discrimination without a competitor able to capture the 16S rRNAs of these organisms. At a formamide concentration of 35% (v/v) and an equimolar amount of cPLA886 signals of the non-target reference *B. linens* were decreased to the low level of autofluorescence, while *Gemmata* sp. SCH633 still yielded strong signals. Therefore, 35% (v/v) formamide was set as optimal hybridization stringency for PLA886.

At the optimized hybridization conditions all six reference strains showed strong signals with both PLA probes, whereas all tested organisms from other bacterial phyla displayed no fluorescence signals.

In situ detection in different habitats

Fixed samples originating from different habitats were probed with PLA46 and PLA886. The results displayed in Table 3 show that members of the *Planctomycetales* could be found in nearly all examined samples. Estimated fractions, relative to DAPI counts, ranged from the presence of only single cells to significant numbers of approximately 5% of all cells. In all examined samples the planctomycetes were mainly attached to particulate matter or cell clusters. Activated sludge was examined extensively as an example of a highly diverse ecosystem. Two successive hybridizations with PLA886 and PLA46 labelled with different fluorochromes allowed the determination of the degree of accordance between the cells detected with each of the two probes. The fraction of double-stained cells was usually above 95% (Table 3 and Fig. 3a).

Planctomycetes in activated sludge occurred mainly as single spherical or ovoid cocci with diameters between 1.0 and 2.5 μ m. Cells were located mostly within or at the edge of sludge flocs and in particular attached to filaments (see Fig. 3a), but seemed to be randomly distributed in the sample material. This distribution pattern could be observed in all 27 activated sludge samples analysed. The relative quantities of planctomycetes differed significantly between sewage treatment plants. A marked difference in abundance could be observed even between the two stages of the high-capacity municipal plant München-Großlappen. In the first stage with a load value of approximately 200 mg BOD₅ l⁻¹ always less than 0.1% of DAPI-positive cells reacted with the PLA probes. However, in the second stage with a load of 20 mg BOD₅ l⁻¹ approximately 2–5% of all cells were probe-positive. Relative enrichment of planctomycetes in the second stage is striking and suggests a possible role of this group in the degradation of more recalcitrant compounds.

Table 3. Microscopic analysis of environmental samples for the presence of planctomycetes by probing with PLA46 and PLA886

Sample*	Microscopic appearance of the detected planctomycete population	Abundance†	Accordance‡
Lake Albufera			
Lagoon and outflow channel to the sea (7)	Rosettes of ovoid cells, single cocci; budding cells observable	++/+++§	++
Activated sludge			
Großlappen, stage I (13)	Single cocci, sparse groups of cocci located within flocs	+	++
Großlappen, stage II (4)	Cocci of different size, sometimes in groups, filaments of ovoid cells	+++	+++
Dietersheim, stage I (3)	Cocci, single or in groups, few rods	++	+
Dietersheim, stage II	Single cocci	++	+
Valencia-Pinedo (2)	Mainly single cocci, sometimes in groups	++	++
Airolo	Cocci, mainly single	++	+
Grüneck	Single cocci	++	+
Freising	Large cocci, single or in groups	+++	+
Haag	Small cocci	+++	+
Other samples from WWTPs			
Sand filter, Dietersheim	Cocci, thin filaments	++	++
Influent, Valencia-Pinedo	Single cocci	+	+
Effluent, Valencia-Pinedo	—	—	—

* See Table 1; numbers in parentheses indicate the number of samples examined from the respective habitat.

† Estimated fraction of PLA-positive cells relative to total cells visualized by DAPI staining. +++, 0.5–5%; ++, 0.1–0.5%; +, <0.1%; —, no positive cells detectable.

‡ Fraction of PLA46 and PLA886 double-stained cells relative to all detected planctomycete cells. +++, Only single cells not double-stained; ++, >99%; +, >95%.

§ Different fractions of positive cells in the different samples examined.

Formation of rosettes as observed in the brackish lagoon Lake Albufera (Fig. 3b) has been described for members of the genera *Planctomyces* and *Pirellula*. The pear-shaped cells displayed bright signals. The rosettes comprised around one-half of all planctomycetes in the lagoon samples. Some of the cells seemed to be in the state of budding. The samples contained significant numbers of phototrophic organisms displaying remarkable autofluorescence, especially in the red spectrum range. Clear identification with TAMRA-labelled probes was possible only using the HQ-Cy3 filter set, which enabled the differentiation of orange-yellowish hybridization signals and reddish background fluorescence, whereas the Zeiss filter set no. 15 was not appropriate for this differentiation based on colour.

PLA probes visualized cocci and thin filaments in biofilms from the sand filter of a WWTP. The examined sample originated from a layer within the filter bed where oxygen-free conditions prevail nearly all the time. Besides the discovery of planctomycete 16S rDNA clone sequences in an anaerobic digester (Godon *et al.*, 1997) this is to our knowledge the first report on observation of planctomycetes in an anoxic habitat. Meanwhile,

probe PLA46 has also been used in a silty clay soil sample where planctomycetes were found to comprise a significant part of the bacterial population (Zarda *et al.*, 1997).

Lack of detectability of planctomycetes with the bacterial probe EUB338

An interesting observation in this study was that all cells detected with the PLA probes showed only a very low to no hybridization signal with probe EUB338 (Fig. 3c). The lack of detectability with this frequently used bacterial probe is in accordance with available sequence information. All known planctomycete 16S rRNA sequences show at least one base change within the target region of probe EUB338. Three of the four lineages (the *Planctomyces*, *Pirellula* and *Gemmata* lineages) even possess three base changes, two of which lead to strong mismatches. It is therefore by no means surprising that reference strains of planctomycetes as well as environmental populations are hardly detected with probe EUB338. The lack of hybridization with EUB338 can even be regarded as a third probe-based proof, besides

Fig. 3. Detection of planctomycetes in environmental samples. For (a) and (b), identical fields were viewed by phase-contrast (left) and epifluorescence (right) microscopy. (a) Hybridization of activated sludge from the WWTP Valencia-Pinedo with FLUOS-labelled probe PLA886 and TAMRA-labelled probe PLA46. The yellow colour in the double exposure indicates simultaneous binding of the green and the red-emitting probes. (b) Hybridization of a sample from the Albufera lagoon with TAMRA-labelled probe PLA46. Signals were detected with the HQ-Cy3 filter set. Typical rosette morphotypes are marked by arrowheads. (c) Lack of detectability of planctomycetes with bacterial probe EUB338. Hybridization of activated sludge with FLUOS-labelled probes PLA46 and PLA886 and TAMRA-labelled probe EUB338. Double exposure with fluorescein- and rhodamine-specific filter sets demonstrates that planctomycetes do not bind the bacterial probe. (d) Intracellular ultrastructure of activated sludge. Hybridization of activated sludge with TAMRA-labelled probe PLA46 and counterstaining with DAPI. Double exposure with DAPI- and rhodamine-specific filter sets shows the concentration of DNA within the hybridized cells (arrowheads). All photomicrographs have been taken at $\times 1000$ magnification. Bar, 20 μm .

Fig. 4. Ring-shaped appearance of probe-conferred fluorescence in planctomycetes. Hybridization of an artificial mixture of cells of *Gemmata* sp. SCH633 and *B. linens* DSM 20425^T with FLUOS-labelled probe PLA886. Phase-contrast (left) and epifluorescence (right) micrographs show identical microscopic fields. Bar, 20 μ m.

positive reaction with PLA46 and PLA886, for affiliation of the detected cells to the *Planctomycetales*. The lack of reaction with this most commonly used bacterial probe means that planctomycetes have not been recognized in probe-based *in situ* investigations yet. Consequently, the use of the PLA probes increases the fraction of bacteria which can be detected and classified by *in situ* hybridization with rRNA-targeted probes in environmental samples. For a bulk soil sample Zarda *et al.* (1997) have already shown that by combined use of EUB338 and PLA46 the total number of detectable bacteria could be increased from 40 to 47 %.

Intracellular distribution of fluorescence signals in planctomycetes

A second unusual observation was that for some of the planctomycete cells probe-conferred fluorescence was not distributed evenly across the whole cell body but was ring-shaped with a dark area in the centre of the cell, which seems to be free of ribosomes. Fig. 4 displays this character for cells of *Gemmata* sp. SCH633. In all examined environmental samples some of the planctomycetes showed such ring-shaped hybridization signals. Concomitantly with the structuring of the probe-conferred signal a complementary distribution of DAPI-conferred fluorescence was found. DAPI visualized the DNA, which was strongly concentrated at one spot in the middle or the edge of the cell body. Together DAPI

and the probe-bound fluorochrome cover the whole cell body (Fig. 3d). Since reference cells show the same pattern of distribution of DNA and rRNA fluorescence this is regarded as a feature typical for planctomycetes. The observation could be nicely explained by the concentration of nuclear material in a distinct part of the cell, referred to as the nucleoid, which has been reported for pure cultures of *Gemmata obscuriglobus* (Franzmann & Skerman, 1984), *I. pallida* (Staley *et al.*, 1992) and recently two *Pirellula* species (Lindsay *et al.*, 1997).

Concluding remarks

The two newly developed probes for planctomycetes extend the existing set of group-specific rRNA-targeted oligonucleotides on the phylum to subclass level for a rapid *in situ* classification of individual bacterial cells (Amann *et al.*, 1995). Combined application of both PLA probes ensured reliable *in situ* identification in environmental samples by double staining of phylum members. *In situ* analysis demonstrated the presence of planctomycetes in a variety of habitats and revealed marked differences in abundance despite widely similar morphotypes of the detected cells. The probes allow the examination of the abundance of planctomycetes in habitats from which they have already been isolated or shown to be present by PCR-based methods. Furthermore, they enable one to check identity of cells which have been solely observed by phase-contrast microscopy and affiliated by morphology but not successfully isolated such as diverse *Planctomyces* species (Staley *et*

al., 1992). Probing may therefore assist isolation of these fastidious organisms and could be helpful to close the gap between the molecular information obtained in recent years and the existing knowledge about the organisms themselves.

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