

Isolation of planctomycetes from *Aplysina* sponges

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ABSTRACT: There is mounting molecular evidence that bacteria belonging to the phylum Planctomycetes are abundant in marine sponges including members of the genus *Aplysina*. In an attempt to culture planctomycete bacteria from *Aplysina* sponges, 116 bacterial strains were isolated on selective oligotrophic media. Screening of the strain collection by fluorescence *in situ* hybridization with the planctomycete-specific probe Pla46 yielded 3 positive candidates. Nearly complete sequencing of the respective 16S rRNA genes revealed that the isolates were affiliated with 2 distinct clusters of the genus *Pirellula*: 1 isolate was obtained from a Mediterranean sponge, 1 from a Caribbean sponge and a third from Caribbean seawater. To our knowledge this is the first report of cultured Planctomycetes from marine sponges. The isolates grew slowly on oligotrophic media and failed to grow on nutrient-rich media. *Pirellula* sp. Strain 797 was pink-pigmented while the other 2 isolates, 16 and 81, were non-pigmented. Transmission electron microscopy revealed a pear- or droplet-shaped cell morphology that is characteristic of the genus *Pirellula*. The application of strain-specific oligonucleotide probes to sponge tissue cryosections showed that the isolates contribute only a minor fraction to the total microbial community that is associated with *Aplysina* spp. sponges.

KEY WORDS: *Pirellula* · Planctomycete · *Aplysina* · Sponge · Porifera · Sponge-associated microorganism · 16S rDNA

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INTRODUCTION

The planctomycetes form a phylum within the domain *Bacteria* that is characterized by a peptidoglycan-free cell wall, cell compartmentalization due to the presence of a membrane-bound nucleoid (Lindsay et al. 1997, 2001), a short 5S rRNA sequence, as well as a budding mode of reproduction (for review see Fuerst 1995). Currently, there are 4 known genera, the *Planctomyces*, *Gemmata*, *Isosphaera* and *Pirellula*. They were originally discovered as freshwater planktonic organisms and have subsequently been found in a variety of marine, hypersaline, freshwater and terrestrial environments (Schlesner 1986, Ward et al. 1995, Wang et al. 2002). With the aid of selective cultivation media and the application of molecular microbial ecology tools, their natural abundances are becoming

increasingly known (Liesack & Stackebrandt 1992, Neef et al. 1998, Derakshani et al. 2001). However, because the typical marine media (e.g. ZoBell's medium) are inhibitory to the growth of planctomycetes, few have been cultivated from the marine environment. Accordingly, the development of improved methods for the cultivation of these and other oligotrophic bacteria is still a challenging task.

There is mounting evidence that planctomycete bacteria occur within marine sponges. Fuerst et al. (1998, 1999) described 6 distinct morphotypes in various tropical sponges. All of these bacteria shared the presence of 'membrane-bound nuclear bodies', a feature that is so far unique to the planctomycete division. In contrast to classical planctomycetes, the sponge-associated bacteria appear to lack polar differentiation (lack of a polar cap). Because the cell walls of these sponge-

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associated bacteria resemble an S-layer-type membrane, an affiliation with the *Archaea* has been proposed (Fuerst et al. 1999). Bacteria with nucleoid-like structures have also been identified in the mesohyl matrix of Mediterranean verongid sponges (Vacelet 1975, Friedrich 1998, Friedrich et al. 1999). Moreover, *in situ* hybridization with the planctomycete-specific probe Pla46 produced abundant fluorescent signals in *Aplysina aerophoba* (Friedrich et al. 2001) and the Australian sponge *Rhopaloides odorabile* (Webster et al. 2001), suggesting that planctomycetes may be a numerically important component of the sponge microbiota. The aim of this study was to cultivate planctomycetes from *Aplysina* spp. sponges, to determine their phylogenetic affiliation by 16S rRNA genes sequencing and to verify their presence in sponge tissues by the application of strain-specific oligonucleotide probes.

MATERIALS AND METHODS

Sponge collection. *Aplysina aerophoba* sponges (class Demospongiae, order Verongida, family Aplysinidae) were collected by SCUBA diving at depths of 5 to 15 m offshore of Banyuls-sur-Mer, France (GPS: 42° 29' N, 03° 08' E) in May 2000. The sponges *A. fistularis*, *A. insularis* and *A. archeri* were collected by SCUBA diving during a research expedition to the Bahamas with the RV 'Seward Johnson', of the Harbor Branch Oceanographic Institution (HBOI), USA, in August 2000. Samples were collected offshore of Little San Salvador island (GPS: 24° 32.68' N, 75° 55.73' W). Seawater and sediment samples were taken from the immediate vicinity of the sponges as controls to see whether putative planctomycete isolates would be sponge-specific. The samples were processed immediately after collection using ship-board laboratory facilities. The remaining sponge samples were frozen for future fluorescence *in situ* hybridization (FISH) studies.

Microbial isolation and cultivation. Sponge tissues were excised from the center of individual sponge samples using an alcohol-sterilized cork borer, and the ends were removed with a scalpel. Tissues were rinsed 3× in sterile seawater, minced with a razor blade, and homogenized with a Dounce homogenizer. Sponge tissues were also plated prior to homogenization using a sterile glass rod and plate spinner. Homogenates of sponge tissues were diluted with sterile seawater from 10⁻¹ to 10⁻⁵ and 3 × 100 µl were plated on the following media (l⁻¹): M13 (peptone 0.25 g, yeast extract 0.25 g, glucose 0.25 g, agar 18 g, distilled water 750 ml, artificial seawater 250 ml) and M30a (Solution 1: Hunter's basal salts 20 ml, 0.1 M Tris HCl pH 7.5 50 ml, agar 18 g, artificial seawater 250 ml, distilled water 630 ml;

Solution 2: *N*-acetylglucosamine 2.0 g, sodium phosphate 0.1 g, vitamin solution 5 ml, distilled water 45 ml) (Schlesner 1994), oligotrophic agar (Santavy et al. 1990) and seawater agar (agar 15 g, artificial seawater 1000 ml) (Jensen et al. 1996). Sediment samples were supplemented with sterile seawater and vortexed prior to plating. Seawater samples were plated without further dilution immediately after collection. All media were supplemented with 50 µg ml⁻¹ cycloheximide to inhibit fungal growth and 100 µg ml⁻¹ ampicillin to select for planctomycetes over bacteria that possess peptidoglycan as a cell wall component. Plates were inspected at weekly intervals for up to several months. Following repeated single-colony isolation on ampicillin-containing media, a total of 116 strains were obtained in pure culture and deposited in a strain collection.

Fluorescence *in situ* hybridization (FISH) In order to screen the strain collection for the presence of planctomycetes, FISH was performed using Planctomycete-specific Probe Pla46 (Table 1). The universal probe mix EUB338, EUB338-II and EUB338-III targeting the domain *Eubacteria* served as a control that sufficient microbial biomass had adhered to the microscope slides (Daims et al. 1999). Bacterial isolates were fixed overnight in 4 % paraformaldehyde-PBS (phosphate-buffered saline) at 4°C. Following centrifugation at 10 000 rpm (12 000 × *g*) (Biofuge Heraeus), the cells were washed 2× with PBS. We applied 25 µl of the cell suspension to microscope slides with grooves, and heat-fixed at 37°C. The slides were dehydrated in an aqueous ethanol series (50, 80 and 96 %) for 3 min each, dried, and stored at -20°C prior to use. Oligonucleotide probes were applied to slides (1 µl) at a final concentration of 3 ng of labelled probe µl⁻¹ hybridization buffer (MWG, Biotech). Hybridization of sponge tissue cyrosections was performed as described previously by Friedrich et al. (2001). Each probe was applied to at least 5 different tissue sections of 1 sponge. More than 5 different *Aplysina aerophoba* and *A. fistularis* specimens were investigated. *In situ* hybridization and microscopical visualization were performed as described elsewhere (Manz et al. 1992, Friedrich et al. 2001).

Cloning, sequencing and phylogenetic analysis. DNA extraction, PCR amplification, cloning and sequencing of the 16S rRNA genes were performed following the methods of Hentschel et al. (2001). For PCR amplification, the universal primers 27f (5'-GAG-TTTGATCCTGGCTCA'3) and 1385r (5'-CGGTGTGT-(A/G)CAAGGCC'3) according to *Escherichia coli* 16S rDNA numbering were used (Lane 1991). Nearly complete 16S rRNA gene sequences were aligned using the ABI Prism Autoassembler Version 2.1 software (Perkin Elmer) and compared to known sequences in

Table 1. Oligonucleotide probes used for *in situ* hybridization. FA: formamide concentration of hybridization buffer

Probe	Sequence 5' → 3'	% FA	Specificity	16S rRNA position in <i>Escherichia coli</i>	Source
Domain-specific probe mix					
EUB338	GCTGCCTCCCGTAGGAGT	35	Domain <i>Eubacteria</i>	338–355	Amann et al. (1995)
EUB338-II	GCAGCCACCCGTAGGTGT				Daims et al. (1999)
EUB338-III	GCTGCCACCCGTAGGTGT				Daims et al. (1999)
Phylum-specific probe					
Pla46	GACTTGCATGCCTAATCC	30	Phylum <i>Planctomycetes</i>	46–63	Neef et al. (1998)
Order-specific probe					
Pla8863	GCCTTGCGACCATACTTCCC	35	Order <i>Planctomycetales</i>	886–904	Neef et al. (1998)
Competitor	GCCTTGCGACCGTACTCCC				
Strain-specific probes					
Pir197	CTCGAGCGGAATCTCACCAT	30	Strain <i>Pirellula</i> sp. 797	197–216	K. H. Schlesner (pers. comm.)
Pir81	GTCCCCTTAGAAGCAAGC	30	Strain <i>Pirellula</i> sp. 81	81–98	Present study

the NCBI (National Center for Biotechnology Information) GenBank database using the basic local alignment search tool (BLAST) algorithm (Altschul et al. 1990). The sequences of the isolates were aligned with the closest neighbors using CLUSTAL W (Thompson et al. 1994). Phylogenetic trees were constructed using the Kitsch algorithm (Fitsch-Margoliash and least-squares method with evolutionary clock) from the PHYLIP program (Felsenstein 1993). *Chlamydia psittaci* was used as an outgroup. CLUSTAL W and PHYLIP were accessed through the website of the Computing Center of Pasteur Institute (see: <http://bioweb.pasteur.fr>). The sequences were deposited in the NCBI GenBank under Accession Nos. AF453518 to AF453520.

Design of strain-specific FISH probe. A strain-specific probe for the sponge-derived *Pirellula* sp. Strain 81 was designed during the ARB workshop (September 2001) using the ARB software package (O. Stunk & W. Ludwig (available at <http://www.mikro.biologie.tu-muenchen.de>). The hybridization stringency was adjusted such that *Pirellula* sp. 81 yielded bright signals whereas non-target organisms that contained 1 to a few mismatches in the 16S rRNA target region (*P. marina*, *Pirellula* sp. 16, *Pirellula* sp. 797) did not.

Transmission electron microscopy. Following cultivation of the isolates in liquid medium, bacterial biomass was fixed in 50 mM cacodylate/2.5 % glutaraldehyde in artificial seawater (ASW) for 24 h. Following centrifugation (1800 × *g* for 10 min) and washing with PBS, the samples were postfixed in 2 % osmium tetroxide/3 × PBS for 24 h. They were dehydrated in an ethanol series (50, 70, 80, 90, 3 × 100 %, each for 1 h) and subsequently incubated in propylene oxide (3 × 1 h). Following overnight incubation in 1:1 (v/v) propy-

lene oxide/glycidether 100 (Epon 812; Roth) and 2 additional 3 h incubation steps in glycidether, the resin was polymerized at 60°C for 3 d. The samples were sectioned with an ultramicrotome (OM U3, C. Reichert) and contrasted with 1 % uranyl acetate and lead citrate. Sections were examined with a Zeiss EM 10 electron microscope operating at 80 kV. Transmission electron microscopy (TEM) on sponge tissues was performed as described previously (Friedrich et al. 2001).

RESULTS AND DISCUSSION

Screening of the strain collection using the planctomycete-specific probe Pla46 revealed 3 positive candidates. Isolate 797 was obtained from the Mediterranean sponge *Aplysina aerophoba*, Isolate 81 from the Caribbean sponge *A. fistularis* and Isolate 16 was recovered from Caribbean seawater. The fluorescence signal of individual cells of all strains appeared in a ring-shaped manner that is consistent with the presence of a highly condensed nucleoid, as is characteristic of the genus *Pirellula* (Neef et al. 1998). As expected, the positive control *P. marina* produced a similar ring-shaped fluorescence, whereas the negative control *Escherichia coli* produced no signal. These findings demonstrate that planctomycetes can be cultivated from marine sponges.

Nearly complete 16S rRNA gene-sequencing revealed that all isolates of this study belonged to the genus *Pirellula* (Fig. 1). The Mediterranean sponge isolate, 797, is most closely related to *Pirellula* sp. Schlesner 1 from the Kiel Bight (>99.1 % sequence similarity) and to Planctomycete Strain 608 from Hawaii (98.4 % sequence similarity). Caribbean Sponge Isolate 81 is most closely related to a group of

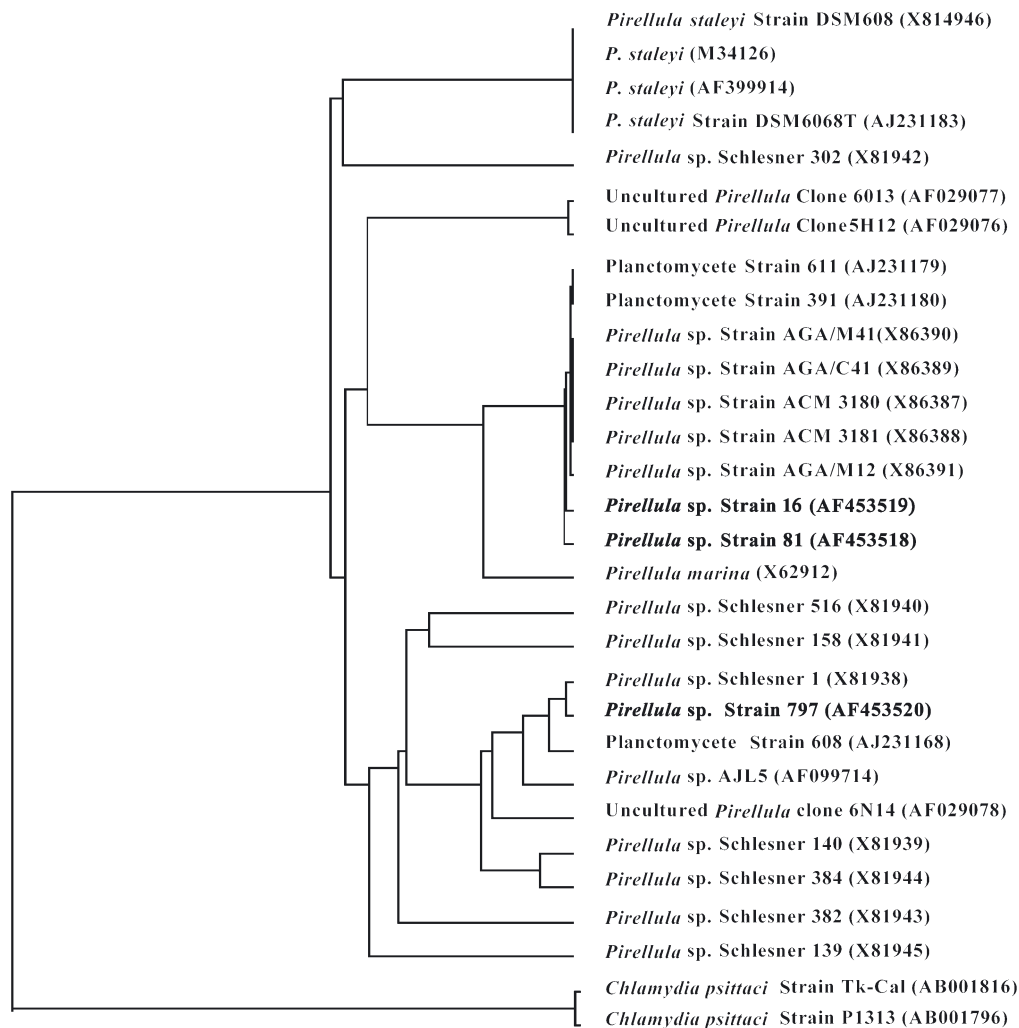


Fig. 1. Phylogenetic tree derived using Fitch-Margoliash and least-squares method with an evolutionary clock from DNADIST of PHYLIP. Database accession numbers are shown in parentheses. Strains in bold face: isolates obtained during present study

non-pigmented *Pirellula* sp. strains isolated from giant tiger prawn postlarvae (>99.0% sequence similarity), to Planctomycete Strains 391 and 611 (>98.9% sequence similarity) and to the Caribbean seawater isolate 16 (98.1% sequence similarity). Mediterranean Isolate 797 and Caribbean Isolates 16 and 81 fall within 2 distinct phylogenetic clusters within the genus *Pirellula*. The generally accepted phylogenetic definition of a species states that strains must show >97% 16S rRNA sequence similarity (Stackebrandt & Goebel 1994). Interestingly, the sequence similarities of both Mediterranean (Isolate 797) and Caribbean (Isolate 16, 81) isolates to the most closely related previously described *Pirellula* sp., *P. marina*, are quite low (89.1% and 92.4 and 93.1%, respectively), supporting previous suggestions that they may constitute new *Pirellula* species (Fuerst et al. 1997).

The affiliation of the sponge isolates to the genus *Pirellula* is supported by their colony appearance and by morphological characteristics documented in this study and reported elsewhere (Staley et al. 1992, Fuerst 1995, Fuerst et al. 1997). *Pirellula* sp. Isolate 797 grew slowly on M30a medium (approximately 1.0 mm colony diameter after 3 wk) with *N*-acetylglucosamine as the sole carbon and nitrogen source. This observation suggests that this strain is capable of chitin degradation, as has been previously reported for Planctomycetes (Schlesner 1994, Fuerst 1995). The colonies were pink-pigmented, opaque, circular, glistening and pulvinate, with an entire edge. In contrast, Isolates 16 and 81 grew slowly only on M13 medium (approximately 0.7 mm colony diameter after 3 wk). These colonies were non-pigmented, opaque, circular, glistening and flat, with an entire edge. The fact that

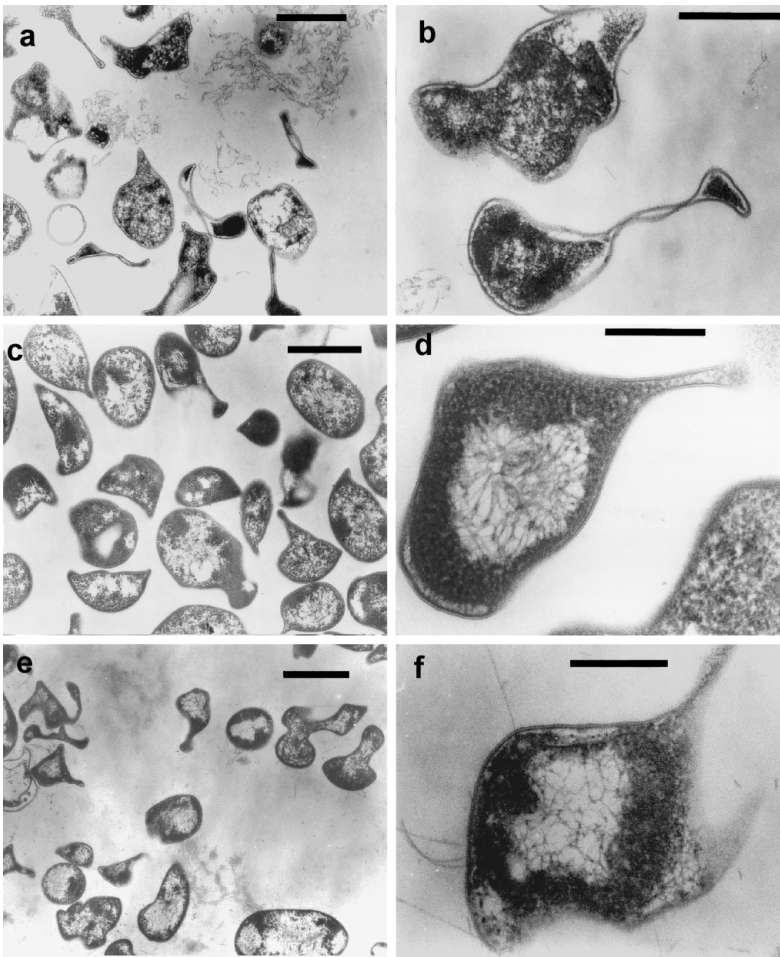


Fig. 2. *Pirellula marina* (a,b) and *Pirellula* sp. Strains 81 (c,d) and 16 (e,f). Transmission electron microscopy. Scale bars = 1 μm (a,c,e), 0.5 μm (b) and 0.25 μm (d,f)

P. marina (positive control) (Fig. 2a,b); $1.20 \pm 0.28 \mu\text{m}$ (length) \times $0.72 \pm 0.29 \mu\text{m}$ (width) ($n = 12$) for *Pirellula* sp. 81 (Fig. 2c,d); $0.99 \pm 0.33 \mu\text{m}$ (length) \times $0.59 \pm 0.16 \mu\text{m}$ (width) ($n = 16$) for *Pirellula* sp. 16 (Fig. 2e,f). *Pirellula* sp. Isolate 16 showed fibrillar appendages (Fig. 2f). The condensed nucleoid contained thin filaments that were easily discernible in the enlargements in Fig. 2d,f). The characteristic, stain-accumulating crateriform structures were only visible on the cell surface of negatively stained cells and could therefore not be identified.

In agreement with previous studies (Friedrich et al. 2001), hybridization of *Aplysina aerophoba* tissues with Planctomycete-specific Probe Pla46 resulted in abundant fluorescent signals (Fig. 3a). Hybridization of *A. fistularis* tissues with the probe Pla46 resulted in consistent, but somewhat lower, abundances (Fig. 3b). Similar results were obtained with Planctomycetales-specific

Isolate 797 exhibited different phenotypic characteristics than the other 2 isolates (16 and 81) corroborates the phylogenetic identification of 2 discrete clusters within the genus *Pirellula*. The fact that closely related strains were recovered from a sponge sample and the surrounding seawater suggests that at least *Pirellula* sp. Strain 81 is not specifically associated with *Aplysina fistularis*.

TEM observations revealed the ovoid, pear- or droplet-shaped cell morphology that is characteristic of the genus *Pirellula* (Fig. 2). The size of the cells was: $1.12 \pm 0.25 \mu\text{m}$ (length) \times $0.56 \pm 0.27 \mu\text{m}$ (width) ($n = 12$) for

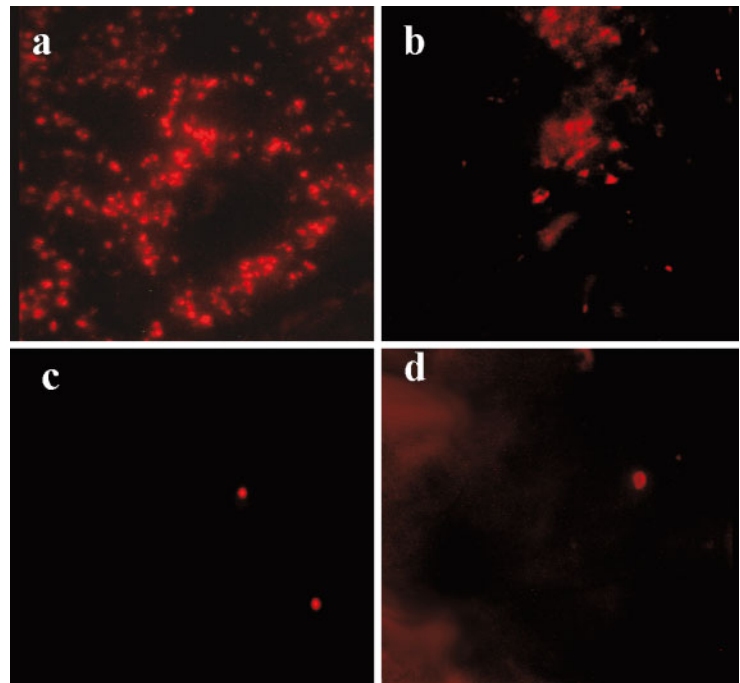


Fig. 3. *Aplysina aerophoba* (a,c) and *A. fistularis* (b,d). Fluorescence *in situ* hybridization of tissue cryosections with (a,b) Planctomycete-specific Probe Pla46, (c) Strain-specific Probe Pir197, and (d) Strain-specific Probe Pir81

Probe Pla886c, albeit some unspecific background binding was noted (data not shown). In order to assess whether *Pirellula* sp. Strains 797 and 81 constitute a significant fraction of the sponge-associated bacterial community, strain-specific FISH probes were applied (Table 1). However, hybridization of *A. aerophoba* tissue cryosections with Probe Pir197 resulted in the detection of only a few fluorescent cells (Fig. 3c). Similarly, only a few cells were detected following hybridization of *A. fistularis* tissue cryosections with the newly designed Probe Pir81 (Fig. 3d). Here, the fluorescent signal resembled the ring-shaped structure indicative of cell compartmentalization. The application of Probe Pir197 to *A. fistularis* and of Probe Pir81 to *A. aerophoba* did not produce fluorescent signals (data not shown).

In summary, the application of FISH using strain-specific probes revealed that the isolated *Pirellula* sp. Strains 797 and 81 did not match the abundance of cells detectable with the Planctomycete-specific Probe Pla46. Apparently, the standard culture methods applied in this study are not adequate for the majority of planctomycete bacteria that can be visualized by FISH in the *Aplysina* sponge species examined. The data of this study are consistent with the observation that 16S rDNA library construction from sponges does not yield planctomycete sequences (Webster et al. 2001, Hentschel et al. 2002, 2003). The discrepancies between culturable and nonculturable approaches to natural microbiological communities are a common phenomenon in microbial ecology (Amann et al. 1995) and demonstrate a pressing need for the development of new, innovative methods for culturing marine bacteria (for novel methods see Zengler et al. 2002). Finally, the phylogenetic identity of the elusive fraction of sponge-associated microorganisms that shows highly unusual cell compartmentalization in the form of nucleoid-containing bodies remains to be elucidated.

Acknowledgements. We gratefully acknowledge the marine operations personnel at the Laboratoire Arago (Banyuls-sur-Mer, France) and the captain and crew of the RV 'Seaward Johnson' (HBOI, Florida) for their expert help during sponge collection. We thank D. Gade (MPI für Marine Mikrobiologie, Bremen) and K. H. Schlesner (Institut für Mikrobiologie, Universität Kiel) for the isolation of *Pirellula* sp. Strain 797 from *Aplysina aerophoba* and for providing us with FISH Probe Pir197. We also thank J. R. Pawlik (University of North Carolina, Wilmington) for excellent cruise organization, C. A. Kauffman for technical assistance on board ship, G. P. Concepcion (Marine Science Institute, University of the Philippines) for bioassay facilities, and W. L. Barraquio (Institute of Biology, University of the Philippines) for assistance with photography of the isolates. This work was generously supported by grants to U.H. (SFB567 and bmb+f grant 'Biotec-Marin') and to S.P.-E. (DAAD research fellowship).

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Editorial responsibility: Gerhard Herndl,
Den Burg, Texel, The Netherlands

Submitted: March 10, 2003; Accepted: July 16, 2003
Proofs received from author(s): October 8, 2003