The Journal of Microbiology (2010) Vol. 48, No. 4, pp. 411-418
DOI 10.1007/s12275-010-0017-x

New Phylogenetic Lineages of the *Spirochaetes* Phylum Associated with *Clathrina* Species (Porifera)[§]

Sven C. Neulinger[1,2], Rüdiger Stöhr[1,2], Vera Thiel[2], Rolf Schmaljohann[2], and Johannes F. Imhoff[1,2]*

[1]*Kieler Wirkstoff-Zentrum KiWiZ (Center for Marine Natural Products), Am Kiel-Kanal 44, D-24106 Kiel, Germany*
[2]*Leibniz Institute of Marine Sciences (IFM-GEOMAR), Duesternbrooker Weg 20, D-24105 Kiel, Germany*

(Received January 12, 2010 / Accepted March 25, 2010)

Though spirochetes have been repeatedly found in marine sponges and other invertebrates, little attention has been paid to the specificity of this association. This study demonstrates that different geno- and morphotypes of spirochetes can reside within the same sponge individual and develop in considerable numbers. Specimens of the calcareous sponge *Clathrina clathrus* collected from the Adriatic Sea off Rovinj (Croatia) were found to harbor spirochete-like bacteria, which were characterized by scanning electron microscopy (SEM), 16S rRNA gene analysis, and catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH). Two novel spirochete sequence types related to the *Brachyspiraceae* could be retrieved. By use of specifically designed CARD-FISH probes, the *C. clathrus*-associated sequences could be assigned to a linear and a helical spirochete morphotype. Both were located within the sponge mesohyl and resembled the spirochete-like cells identified by SEM. In addition, from a *Clathrina* sp., most likely *C. coriacea*, that originated from Indonesian coastal waters, four different spirochete type sequences were recovered. Two of these also affiliated with the *Brachyspiraceae*, the other two were found associated with the *Spirochaetaceae*, one with the genera *Borrelia* and *Cristispira*.

***Keywords*:** *Spirochaetes*, *C. clathrus*, sponge-associated bacteria, CARD-FISH, 16S rRNA gene analysis

Bacteria have been regularly found in marine sponges by microscopic observations, and it has long been suspected that they may have a specific function for their host. Detailed electron microscopic observations (Boury-Esnault *et al.*, 1984; Vacelet *et al.*, 1989) demonstrated the impressive abundance of bacteria in many marine sponges. The application of molecular genetic methods revealed bacterial signatures specifically associated with many marine sponges (Hentschel *et al.*, 2002, 2003, 2006; Imhoff and Stöhr, 2003; Wang, 2006; Taylor *et al.*, 2007; Thiel *et al.*, 2007a, 2007b). This emphasizes the view that such bacterial communities can—at least partially—be considered as sponge-specific.

Bacteria of the class *Spirochaetes* are long known for inhabiting invertebrates (Noguchi, 1921), some being infectious, such as representatives of the genera *Borrelia* and *Treponema*. In recent molecular genetic studies, spirochetes have regularly been identified in sponges, though no information is available on the specificity of this association (Hentschel *et al.*, 2002; Schirmer *et al.*, 2005; Hill *et al.*, 2006; Taylor *et al.*, 2007; Isaacs *et al.*, 2009).

The cosmopolitan sponge *Clathrina clathrus* is found in undisturbed areas of the upper littoral with good water quality, under umbrageous overhangs and in caves. The species belongs to the group of calcareous sponges (class Calcarea), with spicules of calcium carbonate supporting the animal's structure. Its habitus is tubular with internal cisterns lined by a layer of choanodermal cells. The anastomosing tubes are of neon yellow color and range between 0.5 and 3 mm in diameter.

In initial surveys with *C. clathrus* collected off Rovinj (Croatia) in 2002, 2005, and 2006, distinct spirochete-like bacteria were detected by scanning electron microscopy (SEM). These findings inspired the characterization of spirochetes associated with free-living Adriatic *C. clathrus* by 16S rRNA gene analysis and catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) presented in this paper. In addition, also spirochetes associated with an aquarium-reared *Clathrina* sp. from Sulawesi (Indonesia) were investigated using 16S rRNA gene analysis.

Materials and Methods

Sampling and fixation

C. clathrus was collected by scuba diving from the Adriatic Sea off Rovinj (Croatia) in the years 2002, 2005, and 2006 (Supplementary data Table 1). The sampling locations are located on a stretch of 17 km with distances of 3 and 14 km between each other (closest distance by water). The samples were transferred into autoclaving bags filled with 2-5 L of seawater from the collection site and kept at temperature during transport to the Ruđer Bošković Institute in Rovinj. At the institute, the sponge tissue was rinsed several times with sterile-filtered seawater and divided into subsamples. Sponge subsamples were preserved in 1% glutaraldehyde in seawater for SEM and in 50% glycerol for DNA extraction. For CARD-FISH, the sponge subsample was fixed in sterile-filtered seawater with 3.7% formaldehyde for 2 h. It was then transferred into an ethanol series of 25%, 30%, 50% (v/v) in sterile-filtered seawater for 20 min each before final transfer into 50% (v/v) ethanol in sterile-filtered seawater. All

* For correspondence. E-mail: jimhoff@ifm-geomar.de; Tel: +49-431-600-4450, -4433; Fax: +49-431-600-4452
§ Supplemental material for this article may be found at http://www.springer.com/content/120956

subsamples were transported to IFM-GEOMAR at -20°C and stored at this temperature until further processing.

Additionally, a *Clathrina* sp. from Sulawesi, Indonesia, reared in a seawater aquarium at the Center for Marine Natural Products (KiWiZ) in Kiel for 1.5 years, was sampled and frozen at -20°C for DNA extraction and 16S rRNA analysis. As opposed to *C. clathrus*, this specimen was white, tightly interwoven, and featured oscules, resembling the habitus of *C. coriacea*.

Scanning electron microscopy

Water in sponge samples was replaced by a graded ethanol series and subsequent critical-point drying. After mounting, samples were sputtered with Au/Pd and observed with a Zeiss DSM 940 scanning electron microscope.

DNA extraction

From all sponge samples, DNA was extracted using the PowerSoil™ DNA Isolation kit (Mo Bio, USA). About half a cubic centimeter of sponge material was employed. To minimize DNA shearing, the 'alternative lysis method' proposed by the manufacturer (heating to 70°C instead of vortexing) was applied with 4 times elongated incubation times as compared to the manufacturer's protocol. DNA was eluted in 100 µl elution buffer.

DNA amplification for cloning

Three parallel PCRs were conducted, each with a total reaction volume of 25 µl containing 6 µl of DNA extract as template. PCR was conducted with 1 U Phusion™ High-Fidelity DNA Polymerase (Finnzymes, Finland) according to manufacturer's instructions. The universal forward primer 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) (Grabowski, 2002) and the spirochete-specific reverse primer C90 (5′-GTTACGACTTCACCCTCCT-3′) (Dewhirst *et al.*, 2000) (0.05 µM each) were used. PCR conditions were 3 min at 98°C; 35 cycles of: 10 sec at 98°C, 30 sec at 54°C, 45 sec at 72°C; 1 terminal elongation step of 5 min at 72°C. PCR products of the three parallels were pooled and purified by excision from a 1% agarose gel in Tris-Acetate-EDTA buffer and subsequent extraction with the NucleoSpin® Extract II kit (Macherey-Nagel, Germany). Elution from spin columns was done in 30 µl elution buffer.

Cloning

Terminal deoxyadenosine addition was carried out in 100 µl of 1×

Table 1. 16S rRNA gene sequences obtained from Adriatic *C. clathrus* and Indonesian *Clathrina* sp.

Clone	Source	Length (nt)	Closest relatives[a]	Accession no. of next relative	Similarity (%)	Phylogenetic affiliation	Reference of closest relative
Spiro_A1	Adriatic *C. clathrus*	1409	clone LG048, microbial mat	AY605166	78.8	*Spirochaetes*	Guerrero *et al*. (unpublished)
			Spirochaeta alkalica strain Z-7491	X93927	77.3	*Spirochaetaceae*, *Spirochaeta*	Zhilina *et al.* (1996)
			Brachyspira pilosicoli strain ATCC 51139 = Smarlab 2300042[b]	AY155458	75.1	*Brachyspiraceae*, *Brachyspira*	Berger *et al*. (unpublished)
Spiro_A2	Adriatic *C. clathrus*	1415	clone GHI14, marine bivalve crystalline style	EU857763	84.3	*Spirochaetes*	Wichels *et al*. (unpublished)
			Borrelia sinica strain CMN3 = JCM 10505	AB022101	76.5	*Spirochaetaceae*, *Borrelia*	Masuzawa *et al.* (2001)
			Brachyspira pilosicoli strain ATCC 51139 = Smarlab 2300042[b]	AY155458	77.8	*Brachyspiraceae*, *Brachyspira*	Berger *et al*. (unpublished)
Spiro_I1	Indonesian *Clathrina* sp.	780	clone GHIV10, marine bivalve crystalline style	EU857749	85.6	*Spirochaetaceae*	Wichels *et al*. (unpublished)
			Spirochaeta alkalica strain Z-7491	X93927	84.0	*Spirochaetaceae*, *Spirochaeta*	Zhilina *et al.* (1996)
Spiro_I2	Indonesian *Clathrina* sp.	750	isolate SRODG093, bivalve *Saccostrea glomerata*	FM995182	93.4	*Spirochaetaceae*	Green and Barnes (unpublished)
			Borrelia turcica strain IST7 = JCM 11958	AB111849	84.1	*Spirochaetaceae*, *Borrelia*	Guner *et al.* (2004)
Spiro_I3	Indonesian *Clathrina* sp.	803	clone FW104-184, groundwater well sediment	EF693554	84.1	*Spirochaetes*	Cardenas *et al.* (2008)
			Treponema medium subsp. *bovis* strain T19	EF061249	76.9	*Spirochaetaceae*, *Treponema*	Evans *et al.* (2008)
			Brachyspira pilosicoli strain ATCC 51139 = Smarlab 2300042[b]	AY155458	75.6	*Brachyspiraceae*, *Brachyspira*	Berger *et al*. (unpublished)
Spiro_I4	Indonesian *Clathrina* sp.	780	clone KM3c_6F_FF, deep-sea sediment	FJ197494	91.8	*Spirochaetes*	Kouridaki *et al*. (unpublished)
			Treponema porcinum strain 14V28	AY518274	77.8	*Spirochaetaceae*, *Treponema*	Nordhoff *et al.* (2005)
			Brachyspira pilosicoli strain ATCC 51139 = Smarlab 2300042[b]	AY155458	76.4	*Brachyspiraceae*, *Brachyspira*	Berger *et al*. (unpublished)

[a] Closest uncultivated relative and type strain assigned by BLAST.
[b] Representative of *Brachyspiraceae* as the most closely related group assigned by phylogenetic calculations.

ThermoPol buffer (New England Biolabs, Germany) with 4 U of *Taq* DNA Polymerase (New England Biolabs) and 200 μM dATP (Roche, Germany) for 30 min at 72°C. The products were purified with the NucleoSpin® Extract II kit and eluted in 20 μl elution buffer. Cloning was carried out using the StrataClone PCR Cloning kit (Agilent Technologies, USA) according to manufacturer's instructions. Clone forming units were separated into 96-well plates filled with nuclease-free water. Cells were disintegrated by heating to 95°C for 10 min. 16S rRNA gene inserts were re-amplified with the primer pair T3 (5′-AATTAACCCTCACTAAAGGG-3′) and T7 (5′-TAATAC GACTCACTATAGGG-3′). The total reaction volume was 25 μl containing 6 μl of *E. coli* cell suspension, 1 U of *Taq* DNA Polymerase, 0.1 μM of each primer, 50 μM of each dNTP, and 1× ThermoPol buffer. PCR conditions were 5 min at 95°C; 35 cycles of: 10 sec at 95°C, 30 sec at 50°C, 1 min 30 sec at 72°C; 1 terminal elongation step of 5 min at 72°C. Aliquots of 5 μl were taken from each PCR product and tested for their correct length (about 1,600 base pairs, including the T3 and T7 priming sites) by agarose gel electrophoresis.

Sequencing

Purification of PCR products and sequencing procedure were as described previously Neulinger *et al*. (2008). Nearly complete sequences were obtained by sequencing with primers T3 and T7, followed by assembling of the two overlapping partial sequences with the module SeqMan of the Lasergene v8 software package (DNAStar, USA).

Phylogenetic analysis

Sequence data were visually checked for quality issues. The Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) (Altschul *et al.*, 1990) was used for classification of the bacterial sequences and determination of closest relatives. Data of sequences from this study, their closest relatives, and sequences of sponge-associated spirochetes from other studies with their closest relatives were imported into the SILVA SSU reference database release 98 (http://www.arb-silva.de) (Pruesse *et al.*, 2007) using ARB v07.12.06org (Ludwig *et al.*, 2004) and aligned according to 16S rRNA secondary structure information. Sequences not already present in the SILVA database were obtained from the EMBL nucleotide sequence database (http://www.ebi.ac.uk/embl) (Kulikova *et al.*, 2004). A positional mask (Neulinger *et al.*, 2008) was applied that only allowed for unambiguously alignable sequence positions in all following phylogenetic assays. Aligned sequences were incorporated into the phylogenetic 'backbone' tree of the SILVA database consisting of over 310,000 bacterial sequences, using the 'Parsimony interactive' option of ARB. Sequences were grouped into operational taxonomic units (OTUs) if the proportion of identical sequence positions shared between any two of them was ≥98.7%, which corresponds approximately to affiliation with the same species (Stackebrandt and Ebers, 2006). The longest sequence of each OTU was assigned reference sequence for that OTU. Maximum likelihood calculations with 100 bootstrap replicates and maximum parsimony calculations with 1,000 bootstrap replicates were conducted using the online version of PHYML v3.0 (http://www.atgc-montpellier.fr/phyml/) (Guindon and Gascuel, 2003) and PHYLIP DNAPARS v3.6a3 (Felsenstein, 1989) implemented in ARB, respectively. For phylogenetic calculations, a subset of 21 sequences ≥1,000 nucleotides (nt) was selected, and the corresponding subset of the ARB 'backbone' tree was used as starting tree in PHYML sustaining topology and branch lengths. The most appropriate model of nucleotide substitution for the maximum likelihood calculation was determined with the program ModelGenerator v0.85 (Keane *et al.*, 2006). Sequences <1,000 nt were subsequently added by use of the ARB parsimony method without changing the tree topologies (cf. Thiel *et al.*, 2007a, 2007b).

Nucleotide sequence accession numbers

Reference clone sequences of OTUs from Adriatic *C. clathrus* and aquarium-reared Indonesian *Clathrina* sp. samples obtained in this study were deposited in the EMBL nucleotide sequence database (Kulikova *et al*., 2004) under accession numbers FN424156 to FN424161.

Probe design

Target sites for CARD-FISH probes specific for the spirochetes found in this study were determined with the ARB probe design module. Suitable probe sequences were refined to optimize probe affinity according to Yilmaz and coworkers (Yilmaz and Noguera, 2004; Yilmaz *et al.*, 2006). Horseradish peroxidase (HRP)-labeled oligonucleotide probes (Supplementary data Table 2) were purchased from biomers.net (Germany) and prepared as described previously (Neulinger *et al.*, 2009). Probe specificity was verified with probeCheck (Loy *et al.*, 2008).

Thin sectioning and fluorescence *in situ* hybridization

Thin sections (8 μm) were produced and processed by CARD-FISH basically as described previously (Neulinger *et al*., 2009), only with hybridization times elongated to 24 h. Simultaneous marking of bacteria on the same thin section with the two sequence-specific probes was accomplished as follows: After the first signal amplification with fluorescein tyramide, HRP was inactivated with H_2O_2 as described previously (Neulinger *et al*., 2009). Then a second hybridization and signal amplification with Cy5 tyramide was carried out. Air-dried thin sections were mounted as described previously (Neulinger *et al*., 2009).

Fluorescence microscopy and image processing

Thin sections were viewed on a Zeiss Axiophot epifluorescence microscope, equipped with filter sets for fluorescence detection of fluorescein, Cy3, and Cy5, and a 100× Plan-NEOFLUAR objective. Image stacks were taken with a digital still camera by manually advancing the focus layer through the Z-axis of the whole thin section in steps of ~0.5 μm. Deconvolved composite images were produced from these stacks with the software Helicon Focus v4.77 (Helicon Soft Ltd.). Images of adjacent object regions were stitched together to a single panorama image with the same program. Overlay images for the simultaneous display of fluorescence signals of different dyes were produced with Photoshop® CS4 (Adobe). Color enhancement was employed equally to all image parts. Color hues were altered to make the images color blindness-proof. Cell numbers of spirochete-like cells within the sponge were estimated on the basis of numbers of cells marked on microphotographs. The thickness of section was used to calculate numbers per volume from the image area.

Results

Scanning electron microscopy (SEM)

Among various bacterial morphotypes, SEM images taken from Adriatic *C. clathrus* sampled in 2002, 2005, and 2006 (Figs. 1A, B, and C) all showed linear spirochete-like morphotypes with a cell diameter of 0.3 μm, coil wavelength of 0.3 μm, coil amplitude of 0.5 μm, and a conspicuous perimetric crest. In the sample from 2002 (Fig. 1B), also a putative helical spirochete-like morphotype with a diameter of

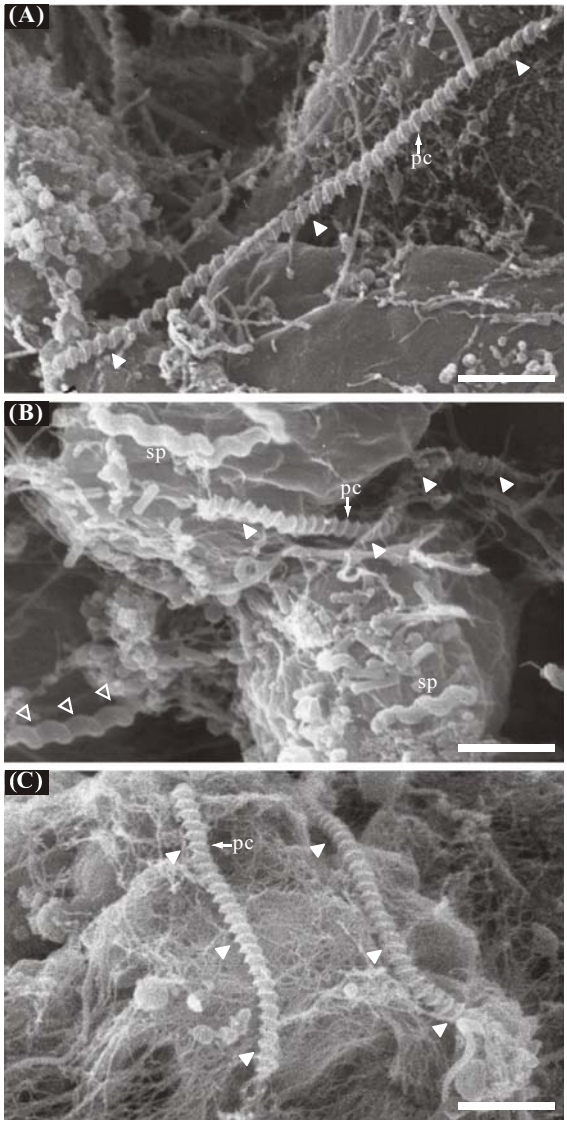

Fig. 1. Scanning electron micrographs showing spirochete-like and other bacterial cells within the mesohyl of Adriatic *C. clathrus* collected in 2002 (A), 2005 (B), and 2006 (C). Filled, upward-pointing arrowheads (A, B, C) mark single linear spirochete-like morphotypes; outlined, downward-pointing arrowheads (B) mark a single putative helical spirochete-like morphotype. pc (with arrow), perimetric crest of the linar spirochete-like morphotype; sp, spirillum. Scale bars, 2 μm.

0.4 μm, 0.8 μm coil wavelength and 0.6 μm coil amplitude was discovered.

Phylogenetic analysis

Using PCR primers specific for the *Spirochaetes*, a total of 75 16S rRNA gene sequences were obtained from Adriatic *C. clathrus* (length from 621 to 1,415 nucleotides, with 70 sequences >1,000 nt). They clustered into two distinct operational taxonomic units (OTUs) (containing 46 and 29 sequences, respectively), with an in-group similarity of ≥99.8% and a similarity of ≤80.5% between the groups. Sequences Spiro_A1 and Spiro_A2 were selected as references for these two OTUs, sharing only 74.4% of their sequence positions.

From Indonesian *Clathrina* sp., five 16S rRNA gene sequences (length from 750 to 803 nt) were retrieved, clustering into 4 OTUs of 71.1%-86.9% sequence similarity. These sequences revealed only low similarities (70.7%-86.7%) to those from the Adriatic *C. clathrus*. Similarity values of all sequences retrieved in this study are summarized in Supplementary data Table 3.

The phylogenetic tree shown in Fig. 2 represents the recognized spirochete families, *Leptospiraceae*, *Brachyspiraceae*, and *Spirochaetaceae*, with the *Leptospiraceae* forming an outgroup to the other spirochete sequences (supported by high bootstrap values according to both calculation methods). In contrast to the spirochetes found in *Clathrina*, all hitherto known sponge-associated spirochete sequences group within distinct and separate lineages of the *Spirochaetaceae* (marked by shaded boxes in Fig. 2) (Hentschel *et al.*, 2002; Schirmer *et al.*, 2005; Hill *et al.*, 2006; Taylor *et al.*, 2007; Isaacs *et al.*, 2009; Holmes and Blanch, unpublished; Lee *et al*., unpublished; Xu *et al*., unpublished). Only two spirochete sequences from the Indonesian *Clathrina* sp. (OTUs Spiro_I1 and Spiro_I2) cluster with the *Spirochaetaceae*. However, both are only distantly related to the so far known clusters of *Spirochaetaceae* from other sponges. The Spiro_I2 sequence is distantly associated to the genera *Borrelia* and *Cristispira*.

All other spirochete OTUs were associated with the *Brachyspiraceae* represented by *Brachyspira pilosicoli* as the only cultivated representative together with their uncultivated relatives. They form sister groups to these known representtatives, one of which harbors the OTUs Spiro_A2 and Spiro_I3 and Spiro_I4, the other is represented solely by clones of Spiro_A1 (Fig. 2). Similarities between *B. pilosicoli* and clones Spiro_A1 and Spiro_A2 from the Adriatic *C. clathrus* were in the same range as similarities between these two clone sequences, pointing out the large distance of both clone sequences from the reference organism. The closest known relatives of spirochetes from Adriatic *Clathrina clathrus* and Indonesian *Clathrina* sp. are given in Table 1.

In general, the similarities of all OTUs to each other and to sequences of closest cultivated and uncultivated relatives were far below the threshold of 98.7% proposed for species discrimination (Stackebrandt and Ebers, 2006) and approx. 95% for distinction of genera (Bosshard *et al.*, 2003) and therefore new species and genera or even families of spirochetes may occur in *Clathrina* spp.

Catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH)

CARD-FISH with the broadband bacterial probe set EUB338 I to III revealed a dense and morphologically diverse consortium of bacterial cells in *C. clathrus*, namely on the outer perimeter of the sponge tubes (Fig. 3A). Two spirochete-like cell types were visible within the mesohyl (Fig. 3A), a linear (Fig. 3B), and a helical one (Fig. 3C).

Double hybridization of a *C. clathrus* thin section with probes SPIRO657 (specifically targeting clone Spiro_A1-type 16S rRNA) and SPIRO1009 (specifically targeting clone Spiro_A2-type 16S rRNA) resulted in a distinct fluorescence of only the target spirochete-like cells (Fig. 3D). The linear (Fig. 3E) and helical (Fig. 3F) morphotypes were clearly distinguishable by fluorescein and Cy5 tyramide signal

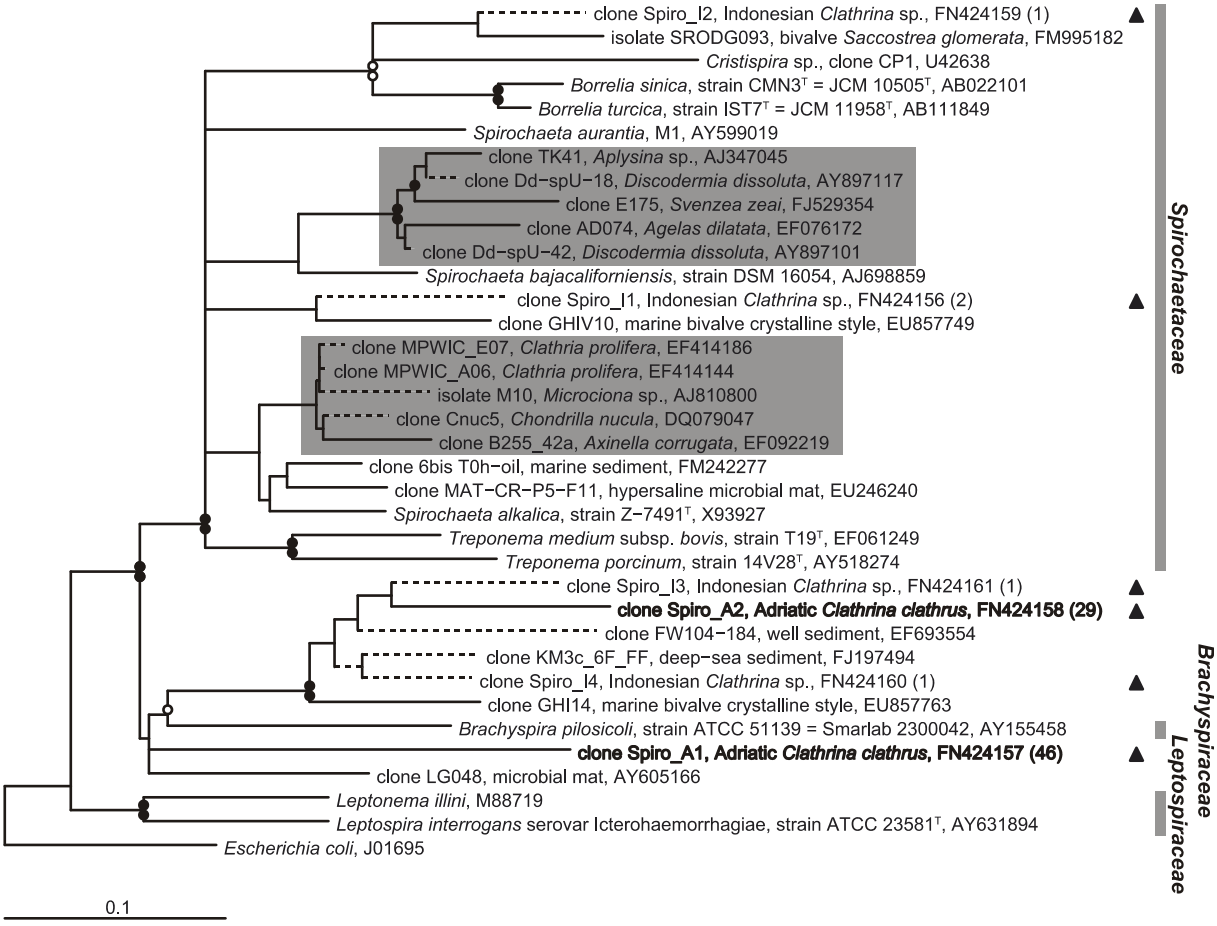

Fig. 2. Consensus tree from maximum likelihood (ML) (based on the generalized time-reversible model of nucleotide substitution with gamma distribution) and maximum parsimony (MP) calculations with OTUs from this study (indicated by filled triangles), their closest uncultured and type-strain relatives (top BLAST hits), and sequences of sponge-associated spirochetes from other studies with their closest relatives. The latter are denoted by shaded boxes. OTUs from Adriatic *C. clathrus* are in bold type. For each sequence, its name, source (if available), EMBL accession number, and the number of similar clones with a 98.7% identity cutoff (sequences from this study) are given. Bootstrap support of respective clusters is indicated by filled (≥90%) and open (≥75%) circles (cf. Taylor *et al*., 2007), determined from 100 (ML, upper) and 1,000 (MP, lower) resamples. The sequence of *E. coli* (J01695) was used to root the tree. All ML and MP trees and bootstrap analyses are based on sequences >1,000 nucleotides (nt). Sequences <1,000 nt were subsequently added to the ML tree by use of the ARB parsimony method without changing the topology and are indicated by dashed branches (cf. Thiel *et al.*, 2007a, 2007b). Scale bar, 0.1 nucleotide substitutions per alignment position.

amplification, respectively. As each probe showed at least one central mismatch to all other known bacterial sequences and multiple mismatches to the respective other spirochete target sequence, probe specificity was maintained even at low-stringency conditions (20% formamide in the hybridization buffer). Hybridization with the control probe NON338 did not result in unspecific staining of spirochete-like cells or other bacterial structures (data not shown).

Both morphotypes co-occurred in the mesohyl in considerable numbers on the order of 10^6 cells/cm^3, and without noticeable spatial separation or difference in abundance (Fig. 3D). The spirochetes were intact and of different lengths, indicating active growth of these cells within the sponge. In addition, the bright staining by CARD-FISH points to a high ribosome content and thus to metabolic activity at the time of sampling.

Discussion

Spirochete sequences retrieved from *Clathrina* spp. during this study formed distinct new lineages within the *Spirochaetes* phylum and did not conform with previously identified clusters of spirochetes found in sponges and other marine invertebrates. Phylogenetic calculations with ML and MP unequivocally classified most of the clones as sister groups to *Brachyspiraceae* (Fig. 2). The family *Brachyspiraceae* currently consists only of the genus *Brachyspira* with *B. pilosicoli* as cultured representative. While the representative clones Spiro_A2 from Adriatic *C. clathrus* and Spiro_I3 and Spiro_I4 from Indonesian *Clathrina* sp. are members of one lineage, clone Spiro_A1 from Adriatic *C. clathrus* forms a separate lineage at the root of the former. As similarities of both lineages to the known *B. pilosicoli* are far below the 95% threshold for distinction of genera (Bosshard *et al*., 2003), they

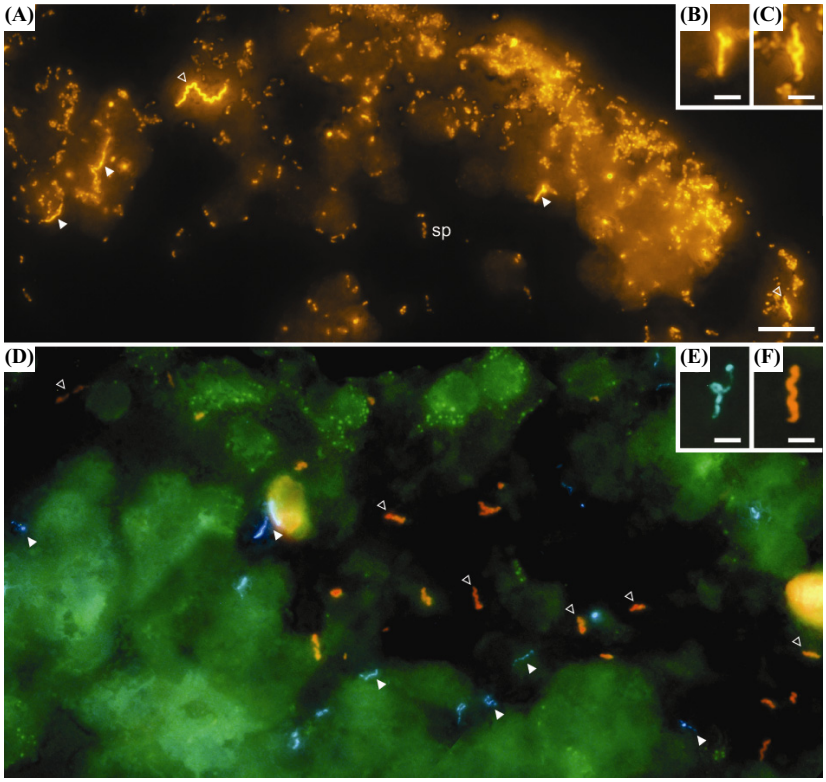

Fig. 3. Epifluorescence microphotographs of bacteria on thin sections of *C. clathrus*. (A) Overview of a cross-sectioned sponge tube with hybridization signals of probes EUB338 I to III with Cy3 tyramide signal amplification (TSA; orange). (B and C) Enlarged microphotographs of linear (B) and helical (C) cells visible within the sponge mesohyl. (D) Double hybridization with probes SPIRO657 (+fluorescein TSA; cyan) and SPIRO1009 (+Cy5 TSA; red). (E and F) Detailed micrographs of linear (E) and helical (F) bacterial cells hybridized with probes SPIRO657 and SPIRO1009, respectively. Filled, upward-pointing arrowheads mark linear cells; outlined, downward-pointing arrowheads mark helical cells. sp, spirillum. Scale bars=10 μm (A, D), and 2 μm (B, C, E, F).

may represent new genera or even new families related to the *Brachyspiraceae*.

Reasons for the distinct discovery of new spirochete lineages during this study may be the high abundance of the spirochetes within one of the sponges (*C. clathrus* from the Adriatic Sea) but also the use of the spirochete-specific reverse primer C90 (Dewhirst *et al*., 2000). Four different OTUs retrieved from an aquarium-reared Indonesian *Clathrina* sp. could be detected using this primer and depict the presence of a likely diverse number of spirochete representatives (Fig. 2). Many studies intended to cover the whole sponge-associated bacterial community by universal primers bear the risk that whole bacterial groups remain underrepresented or even undetected. Specific primers, while narrowing the scope of the analysis, ensure that the group of interest—in this case the *Spirochaetes*—is covered with high efficiency. The picture that emerges from the present study points to a basically high diversity within the *Spirochaetes* phylum and in addition a much broader spectrum of spirochetes appears to be present in sponges (represented by the investigated *Clathrina* spp.) and possibly other marine invertebrates, than so far recognized.

Only highly specific CARD-FISH probes designed for this study allowed the unambiguous *in situ* identification of both spirochete morphotypes in *C. clathrus*. With SEM only the linear morphotype was unequivocally identified, whereas cells of the helical morphotype could easily be taken for spirilla (Fig. 1B). In fact, from initial SEM surveys it did not become clear that *C. clathrus* actually harbored two types of spirochetes. Even with CARD-FISH, it is difficult to discriminate between the two spirochete types based on morphology alone, given that the linear morphotype appears also twisted (Figs. 1B and E), although not as evenly coiled as the helical one (Figs. 1C and F). The more rigid form of the linear morphotype with SEM (Fig. 1) is most probably an artifact of sample preparation. This demonstrates that the use of highly specific CARD-FISH probes along with SEM is highly advisable for *in situ* detection and identification of bacteria in complex systems such as sponges.

From comparison of Figs. 1 and 3, it appears that the linear spirochete morphotype depicted by SEM is longer than most of the respective specimens illustrated by CARD-FISH. While this holds true for the pictures shown, it must be noted that the goal of imaging in the present study was to find well-defined specimens (namely with SEM) rather than to conduct a systematic search for different cell lengths. The apparent discrepancy in cell lengths between SEM and CARD-FISH images is thus not necessarily systematic. Moreover, it has been known for long that "the length of spirochetes is dependent upon the stage of growth and the environmental

conditions, and that length should be used as a criterion for classification only if the organisms are grown under strictly controlled conditions" (Canale-Parola *et al.*, 1968).

In the case of the linear spirochete morphotype from Adriatic *C. clathrus* the association can be regarded as temporally and spatially stable, as proven by comparison of samples from different years and locations by two methods (SEM and CARD-FISH, Figs. 1 and 3). In the case of the Indonesian *Clathrina* sp. specimen the situation is less clear. This specimen had been aquarium-reared for 1.5 years and the artificial environment could possibly have influenced its bacterial community composition. Few studies deal with the effects of aquarium keeping on sponge-associated bacteria. In *Aplysina aerophoba*, bacterial community composition appears to be very resilient upon environmental changes (Friedrich *et al.*, 2001; Thoms *et al.*, 2003; Gerçe *et al.*, 2009). On the other hand, the diversity of bacteria associated with *Mycale laxissima* (Mohamed *et al.*, 2008a) and *Ircinia strobilina* (Mohamed *et al.*, 2008b) increased in aquaculture, while that of *Clathria prolifera* decreased (Isaacs *et al*., 2009). Namely spirochetes associated with *C. prolifera* in its natural habitat could not be detected in the cultured sponge (Isaacs *et al*., 2009). Obviously, the effects of aquaculture on sponge-associated bacteria strongly depend on the observed sponge species and maybe the culture conditions. Therefore it remains unclear whether the spirochetes in the aquarium-reared Indonesian *Clathrina* sp. specimen were part of the sponge's natural bacterial community or acquired during culture.

In any case, however, these data present first evidence that representatives of two or more spirochete genera or even families can simultaneously reside on the same sponge host, whether in the wild or under culture, and that a distinct "set" of *Spirochaetes* is present in different sponges.

Sponge-associated 16S rRNA gene sequences of spirochetes from other studies form two monophyletic clusters marked by shaded boxes in Fig. 2. The upper cluster constitutes a sponge-specific group sensu Hentschel *et al*. (2002) and was recognized by Taylor *et al*. (2007): It comprises at least three sequences that (i) have been recovered from different sponge species and/or from different geographic locations, (ii) are more closely related to each other than to any other sequence from non-sponge sources, and (iii) cluster together independently of the treeing method. The lower marked cluster in Fig. 2 fulfills conditions (i) and (ii) of the above definition, but since it consists only of short sequences that were added to the tree ex post, condition (iii) cannot be tested. Members of neither cluster were detected here. The present study demonstrates that additional clusters of spirochetes may specifically be associated with sponges and representatives thereof have been identified in *Clathrina* spp. from Indonesia and from the Adriatic Sea.

One of the fundamental principles in ecology states that two species cannot occupy the same ecological niche. Since the two *C. clathrus*-associated spirochetes lack spatial separation within the mesohyl (Fig. 3D), their ecological niches must be defined by physiological differences. Ecology and physiology of sponge-derived spirochetes have not been addressed so far by any pertinent study. The large phylogenetic distances of the newly discovered *Clathrina*-associated spirochetes make it difficult to reasonably infer physiological properties from comparison with closest known relatives. Thus, at present all deliberations on the nature of sponge-spirochete symbiosis are speculative.

Remarkably, three of the novel spirochete OTUs discovered in this study had relatives recovered from marine bivalves, two of them demonstrably from the mollusks' crystalline style. This structure is a noncellular, gelatinous, cellulase-containing rod. Its purpose lies in extracellular digestion, which is achieved by both pestle-like grinding of food particles and the release of enzymes (Margulis and Hinkle, 1992). The best-known (but not the only) spirochete genus colonizing this habitat is *Cristispira* (Margulis and Hinkle, 1992). It is feasible that *Cristispira* takes advantage from the enzymatic activity of the style. A similar pattern may apply to *Clathrina*-associated spirochetes, at least to the species represented by clone Spiro_I2 from Indonesian *Clathrina* sp., which is most closely related to *Cristispira* (Fig. 2). However, sponge mesohyl-associated spirochetes might utilize extracellular enzymes provided by concomitant bacteria as well as by the sponge itself.

Acknowledgements

We thank F. Brümmer, T. Staufenberger, K. Nagel, and the staff of the Ruđer Bošković Institute in Rovinj, Croatia, for their assistance with sponge sampling.

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