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SHORT COMMUNICATION

Identification of the bacterial symbiont *Entotheonella* sp. in the mesohyl of the marine sponge *Discodermia* sp.

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The lithistid sponge *Discodermia dissoluta* (family Theonellidae), is found in deep-waters throughout the Caribbean sea and is the source of discodermolide, a natural product with potential anticancer properties, and other secondary metabolites. As with other sponges, large numbers of microbes are harbored in the sponge mesohyl. The microbial population of the sponge mesohyl shows an abundance of large filamentous microbes. Fractionation of the dissociated sponge allowed enrichment of this microbe, which was then identified by analysis of the 16S rRNA genes. Its identity was confirmed through the use of fluorescent *in situ* hybridization. These studies have allowed the identification of this eubacterial microbe as belonging to the genus *Entotheonella*.
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Introduction

Sponges contribute significantly to the total fauna of sessile marine organisms worldwide and dominate the benthic community of some Caribbean and other tropical waters (Hentschel *et al.*, 2002). Even though sponges feed on microorganisms, large numbers of bacteria are also known to be harbored within the extracellular mesohyl matrix of many sponges, living in symbiosis with their host (Imhoff and Stöhr, 2003). This microbial community has been shown to be distinct from that of marine plankton or marine sediments and therefore is specific to the sponge, rather than to its environment (Hentschel *et al.*, 2006). Even though a comprehensive overview of microbial diversity from marine invertebrates can be achieved through various culture-dependent and culture-independent techniques (Fieseler *et al.*, 2004; Lesser *et al.*, 2004), a combination of microscopic methods such as transmission electron microscopy (TEM) and sequence-specific fluorescence *in situ* hybridization (FISH) may enable the precise localization of particular microbes within the invertebrate host.

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In this study, we identify a filamentous microorganism in the lithistid sponge *Discodermia* sp. (family Theonellidae) with 16S rRNA gene analysis and localize it to the sponge mesohyl using TEM and FISH probing.

Materials and methods

Chemicals were purchased from Sigma (St Louis, MO, USA), unless otherwise stated. Primers and probes were purchased from MWG Biotech (High Point, NC, USA).

Samples of *Discodermia* sp. were collected at depths between 480 and 500 ft (146–152 m) around Grand Bahama Island (Bahamas), using the Research Vessels Seward Johnson I and Seward Johnson II and the Johnson-Sea-Link I submersible in October 2000 and March 2001.

Observations by TEM were made on 2×3 mm fragments of sponge tissue, fixed using a modified 'low osmium pre-fixative' technique as described previously (Eisenman and Alfert, 1981). In one series of samples, ruthenium red was added to each solution in the proportion of 50 mg per 100 ml (Luft, 1971a, b) to enhance contrast of the cell coat. The samples were post-fixed for 3 h in 1% osmium tetroxide in 0.2 M sodium cacodylate and 0.3 M NaCl, dehydrated through a graded ethanol series, and embedded in ERL 4206 (Spurr, 1969).

The procedure for the separation of symbiotic bacteria from sponge cells was carried out as previously described (Bewley *et al.*, 1996). The separation yielded a pellet (1P), highly enriched in filamentous bacteria, while still containing a small number of unicellular bacteria and sponge cells, and a supernatant (1S) containing a mixture of sponge cells and filamentous and unicellular bacteria.

1P samples were placed in a sterile mortar chilled with liquid nitrogen, in which the pellet was ground to a fine powder. The ground pellet was then transferred into a clean microcentrifuge tube, to which 500 µl of GTC buffer (4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.5% SDS, pH 7.0) was added. DNA was extracted using a standard phenyl–chloroform–isoamylalcohol (24:1:1) protocol. The pellets were allowed to air dry before being resuspended in 50 µl of deionized H_2O.

Two sets of universal bacterial primers (Table 1) were used to amplify ~750 and ~1300 bp of the 16S small subunit rRNA gene, using published protocols. Cleaned PCR reactions were shipped overnight to Northwoods DNA, Inc. (Solway, MN, USA), for sequencing. Edited sequences were queried using NCBI BLAST v 2.2 (Altschul *et al.*, 1997).

FISH with Cy3 monolabeled (5′) rRNA-targeted probes was carried out using probes covering most bacteria (EUB388, Amann *et al.*, 1990; EUBII, EUBIII, Daims *et al.*, 1999) and a probe for most δ-proteobacteria (DELTA495suite, Loy *et al.*, 2002). An *Entotheonella* sp. specific probe, designed using the ARB probe design tool and a control probe with one mismatch, was monolabeled (5′) with FAM to enable dual hybridization with DELTA495suite. All probes are listed in Table 1. Protocols were performed as described previously, using a 1:10 (w/v) suspension of sponge cells fixed in 96% (v/v) ethanol (Brück *et al.*, 2007). Hybridization was carried out at 46 °C for both probes while washing was at 48 °C. Bacteria were evaluated using an Olympus IX51 microscope (Center Valley, PA, USA) with an epifluorescence attachment (Olympus U-RFL-T) and Cy3 and FAM filter sets. Image analysis was performed using an Olympus DP70 camera system and Cell^F imaging software.

All phylogenetic reconstruction for sequences obtained from bacterial culture was performed using ARB (Ludwig *et al.*, 2004). *De novo* trees were constructed using the ARB neighbor joining distance matrix with Felsenstein correction and termini (.-=0) and position variance (123456789.-=0) filters. New sequences generated in this study are available in GenBank (DQ519075, DQ519076, EU159414).

Results

Transmission electron microscopy on thin sections of sponge tissue, confirmed the presence of the filamentous organism in the sponge mesohyl in addition to a large number of other unicellular microorganisms (Figures 1a and b). The organism was absent in choanocyte chambers and other areas of the sponge. After cell separation and DNA extraction, 16S rRNA-specific primers showed amplification of ~750 and ~900 bp bands in fractions containing the filamentous organism. The final edited sequences of 501, 602 and 875 bp were 96, 97 and 93%, similar to uncultured *Entotheonella* sp. (AY897125, AY897120) from a *Discodermia* sp. metagenomic library previously described in literature (Schirmer *et al.*, 2005).

FISH was performed using EUB338, EUB338II, EUB338III, DELTA495a, ENP1442 and ENP1442c. A control sample was also prepared without probes. EUB338 hybridized well with the filamentous structures contained in the enriched fraction and showed similar signal strength when compared to an *Escherichia coli* standard mixed with the

Table 1 Amplification primers and hybridization (FISH) probes

Primer or probe	*Sequence*[a]	*Positions*[b]	*% Formamide*	*Target*	*Reference*
Probes					
EUB338	GCT GCC TCC CGT AGG AGT	338–355	35	(Eu)bacterial 16S rDNA	Amann *et al.* (1990)
EUB338II	GCA GCC ACC CGT AGG TGT	338–355	50	Planctomycetales	Daims *et al.* (1999)
EUB338III	GCT GCC ACC CGT AGG TGT	338–355	50	Verruncomicrobiales	Daims *et al.* (1999)
DEL TA495suite	A(**A/G**)T TAG CCG G(**C/T**)G CTT CCT	495–512	35	δ-Proteobacteria	Loy *et al.* (2002)
ENP1442	TCA CCC CAA TCA CCC CGC	1442–1459	30	*Entotheonella sp.*	This study
ENP1442c	TCA CCC CGA TCA CCC CGC	1442–1459	30	Mismatch Probe to ENP1442	This study
Primers					
519F	CAG C**(C/A)**G CCG CGG TAA T**(A/T)**C	519–537	N/A	(Eu)bacterial 16S rDNA	Lane *et al.* (1985)
1406R	ACG GGC GGT GTG T**(A/G)**C	1406–1420	N/A	(Eu)bacterial 16S rDNA	Lane *et al.* (1985)
Eco9F	GAG TTT GAT CCT GGC TCA	9	N/A	(Eu)bacterial 16S rDNA	Sfanos *et al.* (2005)
Lop27rc	GAC TAC CAG GGT ATC TAA TC	804	N/A	(Eu)bacterial 16S rDNA	Sfanos *et al.* (2005)

[a]The nucleotides in boldface type are degenerate nucleotides.
[b]*E. coli* numbering.

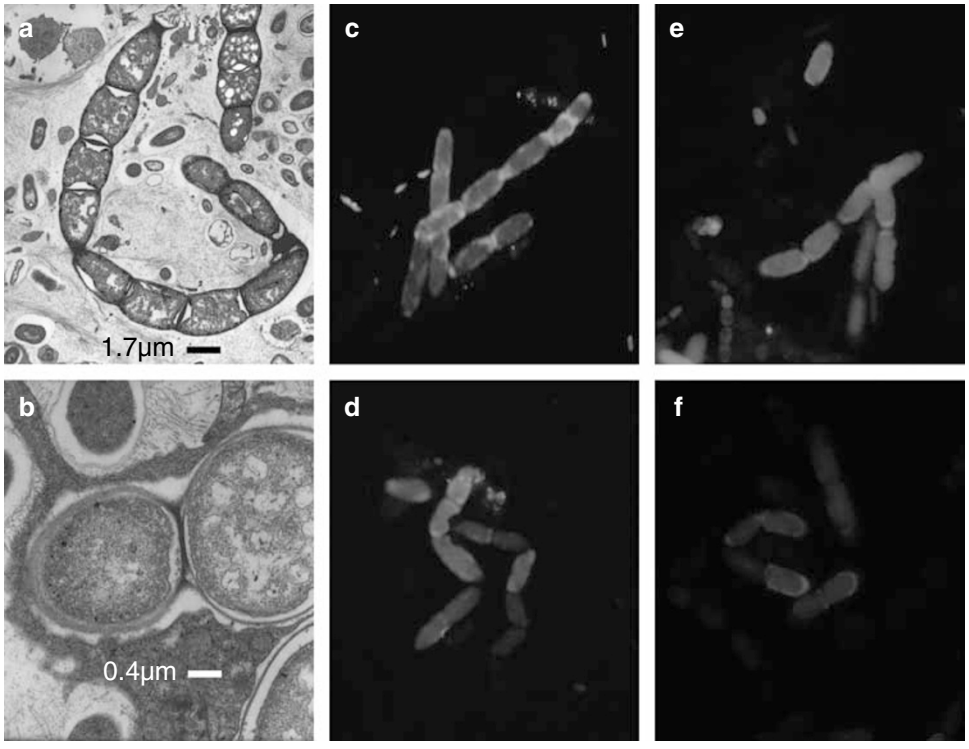

Figure 1 (**a**) Transmission electron microscopy of filamentous structures in the mesohyl of *Discodermia* sp. fixed in the presence of ruthenium red and treated with hydrofluoric acid prior to embedding (bar = 1.7 μm); (**b**) TEM of sponge material showing fine structures fixed without ruthenium red and treated with hydrofluoric acid after embedding (bar = 0.4 μm); (**c**) FISH image (× 1000 magnification) of *Discodermia* sp. sample enriched for filamentous structures and spiked with *E. coli* as fluorescence control (EUB338); (**d**) FISH image (× 1000 magnification) of *Discodermia* sp. sample enriched for filamentous structures and hybridized with DELTA495suite. Figures (**c**) and (**d**) were taken using identical gain and exposure settings, (**e**) FISH image (× 1000 magnification) *Discodermia* sp. sample enriched for filamentous structures and hybridized with ENP 1442; (**f**) FISH image (× 1000 magnification) *Discodermia* sp. sample enriched for filamentous structures and hybridized with a control probe ENP 1442c containing 1 mismatch to ENP1442.

hybridization mixes. EUB338II and EUB338III hybridized only weakly. A similar level of fluorescence was observed when no probe was used, suggesting that the signal was due to autofluorescence. When the enriched samples were hybridized to the probes targeting *Entotheonella* sp. and total δ-proteobacteria (ENP1442, DELTA495suite), strong hybridization was observed and the filamentous structures were illuminated by the probes showing that the sequence originated within those organisms (Figure 1d). The mismatch probe illuminated weakly at identical gain levels, suggesting autofluorescence. Dual hybridization with DELTA495suite and the *Entotheonella* sp. specific probe (ENP1442), resulted in organisms emitting a yellow signal in composite images (not shown).

Discussion

In this study, we identified the filamentous microorganism in the lithistid sponge *Discodermia* sp. as *Entotheonella* sp. through 16S rRNA gene analysis and FISH probing. The genus *Entotheonella* was first proposed by Schmidt *et al*. (2000) as a genus in the δ-subdivision of Proteobacteria, '*Candidatus Entotheonella palauensis*' isolated from the Palauan marine sponge *Theonella swinhoei*. Another study, examining metagenomic libraries from total sponge tissue of *D. dissoluta* and enriched bacterial symbionts showed an abundance of *Entotheonella* sp. related sequences (Schirmer *et al*., 2005). However, due to the nature of the later study, no direct confirmation or no assumptions based on their location within *D. dissuluta* were made. We concluded that *Entotheonella* sp. is an abundant member of the symbiotic microbial community of the sponge mesohyl of *Discodermia* sp. As in *T. swinhoei*, where *E. palauensis* is thought to produce theopalauamide (Schmidt *et al*., 2000), all *Entotheonella* sp. found in this study were specific to the sponge mesohyl. TEM further showed that *Entotheonella* is not present in other areas of the sponge such as the choanocyte chambers where other unicellular microorganisms are present. This indicates a direct microbe–host association between *Discodermia* sp. and *Entotheonella* sp. instead of being a result of indiscriminate filter feeding (Figure 2).

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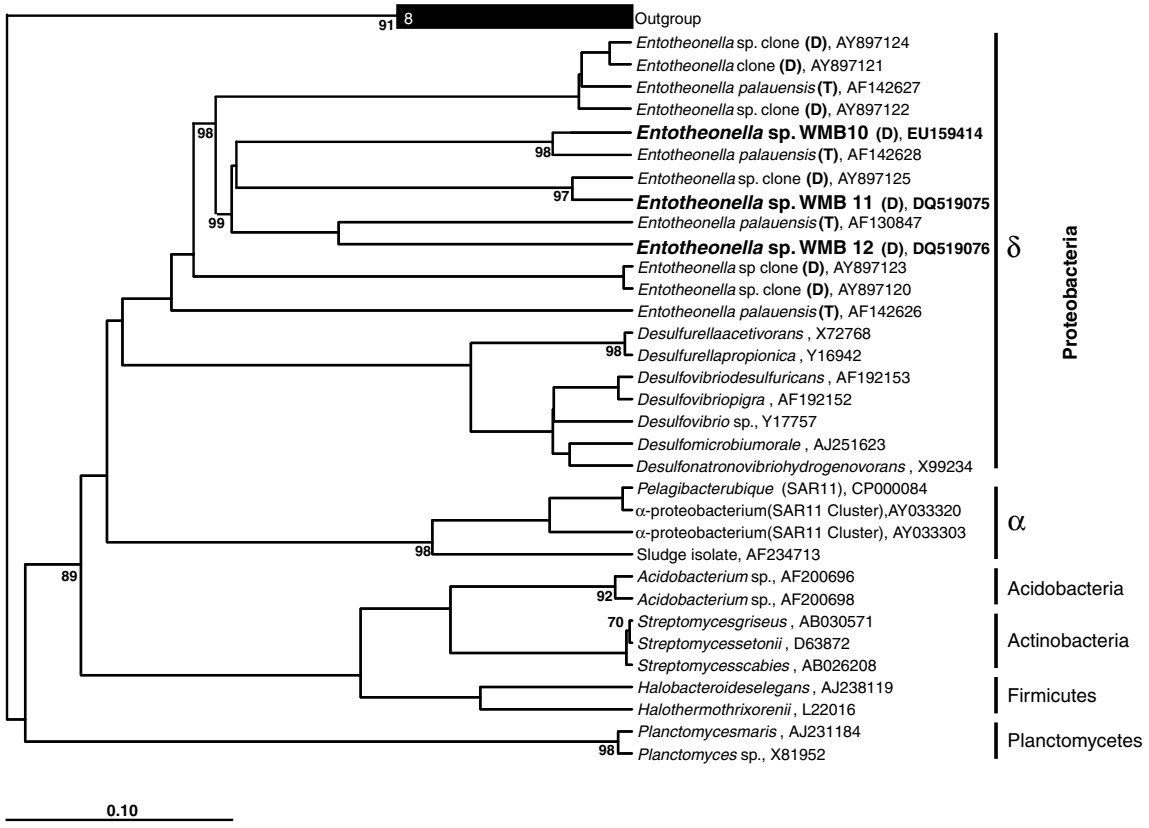

Figure 2 Distance-based neighbor-joining phylogeny of 16S rRNA sequences, including putative *Entotheonella* sequences (WMB10, WMB11, WMB12). Sponge sources for *Entotheonella* sp. are given behind sequence names: (D) *Discodermia* sp., (T) *T. swinhoei*. Reference sequences are derived from ARB and are written with their corresponding accession numbers. Bootstrap percentages after 1000 replications for the most significantly supported clades are shown in bold below the nodes.

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