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ORIGINAL ARTICLE

Novel observations of *Thiobacterium*, a sulfur-storing Gammaproteobacterium producing gelatinous mats

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The genus *Thiobacterium* includes uncultivated rod-shaped microbes containing several spherical grains of elemental sulfur and forming conspicuous gelatinous mats. Owing to the fragility of mats and cells, their 16S ribosomal RNA genes have not been phylogenetically classified. This study examined the occurrence of *Thiobacterium* mats in three different sulfidic marine habitats: a submerged whale bone, deep-water seafloor and a submarine cave. All three mats contained massive amounts of *Thiobacterium* cells and were highly enriched in sulfur. Microsensor measurements and other biogeochemistry data suggest chemoautotrophic growth of *Thiobacterium*. Sulfide and oxygen microprofiles confirmed the dependence of *Thiobacterium* on hydrogen sulfide as energy source. Fluorescence *in situ* hybridization indicated that *Thiobacterium* spp. belong to the Gammaproteobacteria, a class that harbors many mat-forming sulfide-oxidizing bacteria. Further phylogenetic characterization of the mats led to the discovery of an unexpected microbial diversity associated with *Thiobacterium*.

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Introduction

The genus *Thiobacterium* was first described by Molisch in 1912 (Molisch, 1912). In the years after this discovery, the conspicuous sulfur-storing, non-motile rods embedded in a gelatinous matrix were found in other marine and continental locations worldwide. They occur in thermal and sulfur springs (Devidé, 1952; Lackey and Lackey, 1961; Lackey *et al.*, 1965; Vouk *et al.*, 1967; Anagnostidis, 1968; Scheminzky *et al.*, 1972; Fjerdingstad, 1979), but also in sulfidic marine and brackish waters (Molisch, 1912; Lackey *et al.*, 1965; Seki and Naganuma, 1989). So far two different morphologies of the gelatinous mats have been described: a spherical or bladder-like shape (Molisch, 1912; Devidé, 1952; Vouk *et al.*, 1967; Anagnostidis, 1968; Scheminzky *et al.*, 1972; Seki and Naganuma, 1989) and a dendroid shape (Lackey and Lackey, 1961; Lackey *et al.*, 1965). First experiments on the chemical and elemental composition of the gelatinous material led to the assumption that it consists of an allophane–sulfur–hydrogel (Vouk *et al.*, 1967; allophane is an amorphous hydrous aluminum silicate). In contrast to the varying shapes and sizes of the gelatinous material, the morphology of the rod-shaped microbes was very consistent in most studies. An experiment targeting the physiology of *Thiobacterium* suggested that under aerobic conditions these organisms may express a eurythermally mesophilic and slightly halophilic behavior (Seki and Naganuma, 1989). Most importantly, the enrichment study indicated that *Thiobacterium* cells are themselves forming the gelatinous masses, most likely to retain a favorable spatial position in their habitat with access to the sulfide and oxygen sources.

In spite of all past observations, knowledge on the genus *Thiobacterium* is still rather poor, because none of its members have been cultivated. By morphological analogy and ecological context, it was associated with the family Thiotrichaceae of the Gammaproteobacteria in Bergey's manual of systematic bacteriology (Kuenen, 2005) and the Encyclopedia of Life (http://www.eol.org/pages/97513). Its classification as one genus *Thiobacterium* is based on consistent morphological observations of rod-shaped cells with chain-like inclusions of up to

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20 spherical sulfur granules (Lackey and Lackey, 1961), embedded in gelatinous matter (Kuenen, 2005). However, no 16S ribosomal RNA (rRNA) gene sequence has yet been attributed to a *Thiobacterium* sp., and the taxonomic positioning of this genus within the group of sulfur bacteria is uncertain. Furthermore, the energy sources, niche-selection and ecological role of *Thiobacterium* remain unknown. The main problems in the investigation of these mat-forming bacteria are their rarity and the fragility of mats, colonies and cells (Molisch, 1912; Lackey and Lackey, 1961; Vouk *et al.*, 1967; Seki and Naganuma, 1989).

This study investigated *Thiobacterium* mats from three sulfidic marine habitats, including a minke whale bone collected offshore Sweden, deep-sea sediments from the Storegga Seeps off Norway and a shallow-water cave in Greece. To broaden our knowledge on the genus *Thiobacterium* and its ecological role, (i) the geochemical gradients within the gelatinous masses were analyzed, (ii) the cells were microscopically and phylogenetically characterized, and (iii) the overall microbial community composition associated with the gelatinous mats was examined.

Materials and methods

Site description and sample collection
All sampling locations and prevailing environmental conditions are described in Table 1. Whale bones were recovered from the carcass of a female minke whale that was previously implanted at 125 m depth in Kosterfjord, Sweden (58°53.1′N, 11°6.4′E; Glover *et al.*, 2005; Dahlgren *et al.*, 2006). Since their recovery, the bones had been maintained at 7–8 °C in aquaria flushed with filtered seawater (Glover *et al.*, 2005). Sampling of a small spherical *Thiobacterium* mat was achieved by pipetting.

Deep-sea *Thiobacterium* mats were obtained from the Storegga area off Norway during the 'VICKING' expedition with the RV *Pourquoi Pas?* and the ROV *Victor 6000* (IFREMER) in June–July 2006 from a small seep structure covered with a whitish mat (Dive 275-05; core CT-02; 64°45.27′N, 4°58.87′E). Aboard the ship, the *Thiobacterium*-containing core was immediately transferred to a cold room. Sampling occurred directly after the dive through mechanical disruption with a pipette.

The gelatinous mats of the shallow-water cave ('Blue Pot Cave'; 39°10.66′N, 20°12.54′E) off Paxos (Greece) were first discovered by Paul Bowbeer (Oasi-Sub-Diving) and Dr Thomas Beer. Initial samples of the spheres, native and fixed in 4% formaldehyde, reached the MPI Bremen in September 2006. A second sampling in Paxos took place in August 2007. The partially sun-lit cave is located at approximately 23 m depth. Whole gelatinous spheres and cave water were sampled into sterile tubes and transported in a cool chamber to the on-site laboratory. Subsampling of the gelatinous mats was carried out either by preserving whole spheres for biogeochemical analyses, or by dissecting single spheres with a sterile scalpel for microscopic, phylogenetic and fluorescence *in situ* hybridization (FISH) analyses.

Microscopy
Subsamples of the same gelatinous mats sampled for DNA extraction were analyzed directly after sampling by bright field and phase contrast microscopy. Visualization of subsamples stained with different fluorochromes was achieved by using a Zeiss LSM 510 and the appropriate software (Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

Staining
Subsamples of the gelatinous mats were preserved in either 2% or 4% formaldehyde/seawater at room temperature, 4 °C or −20 °C. When applying the protein-targeting fluorochrome SYPRO Red (Molecular Probes, Invitrogen Corporation, Karlsruhe, Germany), staining was conducted at room temperature for at least 45 min (4 h maximum). The dye was diluted beforehand in artificial seawater (salinity 34‰) to a 5 × concentrated solution. Slides were directly subjected to microscopy. The DNA-targeting fluorescent stain 4′,6-diamidino-2-phenylindole (DAPI) was also applied to fixed material of the gelatinous spheres. Subsamples of the structures were placed onto individual spots of Teflon-coated slides and were dried at 46 °C for 1 h. Staining was conducted for 7 min at room temperature with 15 µl of a 2.5 µg ml^{-1} DAPI solution. To remove excess DAPI, the preparations were shortly rinsed with distilled water, followed by rinsing in 96% ethanol and subsequent drying at room temperature. Preparations were finally mounted in a 2:3 mix of Vecta Shield (Vector Laboratories Inc., Burlingame, CA, USA) and Citifluor (Agar Scientific Ltd, Essex, UK), stored at −20 °C and subjected to microscopy the following day.

Microsensor measurements
High-resolution geochemical gradients were measured on a *Thiobacterium* mat during the 'VICKING' expedition in 2006 (IFREMER) with a laboratory set up. Microsensors for pH, O_2 and H_2S were used, and sensors were calibrated as previously described (Revsbech and Ward, 1983; Jeroschewski *et al.*, 1996; de Beer *et al.*, 1997). The total sulfide ($H_2S + HS^- + S^{2-}$) was calculated from the H_2S concentrations and the local pH using equilibrium constants. Microsensors were mounted on a motor-driven micromanipulator and data acquisition was performed using a DAQ-PAD 6015 (National Instruments Corporation, Austin, TX, USA) and a computer. Relative to the microsensor tips, the surface of

Table 1 Physicochemical characteristics of habitats in which *Thiobacterium* spp.-resembling microbes and gelatinous mats occurred

Location[a]	*Habitat*	*H_2S*[b]	*pH*	*Temp.*	*Salinity*	*Depth*[c]	*Reference*
Trieste (Italy)	Marine water	Assumed	—	—	—	1 m	Molisch (1912)
Dubrovnik (Croatia)	Sulfur spring[d]	Detected	—	—	—	1 m	Devidé (1952)
Venice (Florida)	Sulfur spring[d]	~5 μM	7.2	29 °C	17‰	1 m	Lackey and Lackey (1961); Lackey *et al.* (1965)
Titusville (Florida)	Polluted water[d]	—	—	12.5 °C	17‰	Shallow	Lackey and Lackey (1961); Lackey *et al.* (1965)
Cedar Keys (Florida)	Tide pools[d]	—	—	—	17‰	1 m	Lackey *et al.* (1965)
Bad Gastein (Austria)	Thermal spring	~15–23 μM	8.7–9.3	43.5–45.5 °C	—	0 m	Vouk *et al.* (1967)
Amélie-Les-Bains (France)	Thermal spring	~103 μM[e]	9.1[e]	22 °C; 45 °C	—	0 m	Scheminzky *et al.* (1972)
Greece	Hot springs	Detected	—	—	—	0 m	Anagnostidis (1968)
North Fiji Basin[f]	Hydrothermal vent	—	7.5	—	27.8‰	2671 m	Seki and Naganuma (1989)
Tjärnö (Sweden)	Whale bone	Detected	—	7–8 °C[g]	34.3–34.7‰[g]	aq	This study
Storegga Seeps (Norway)	Marine sediment	Detected	7.8–7.9	−1 °C	34.1–35.5‰	745 m	This study
Paxos (Greece)	Marine cave	b.d.[h]	8.0–8.2	15–20 °C	39‰	23 m	This study

[a]Includes observations on *Thiobacterium* that could clearly be attributed to the genus under investigation. Publications providing only scarce morphological information (Miyoshi, 1897; Caldwell and Caldwell, 1974; Gugliandolo and Maugeri, 1993; Yang *et al.*, 2008), or describing microbes that appear morphologically deviant from *Thiobacterium* (Miyoshi, 1897; Naganuma *et al.*, 1990; Hedoin *et al.*, 1996; Mosso *et al.*, 2002) are not listed.
[b]Hydrogen sulfide was occasionally detected only qualitatively, or its presence was mentioned without presenting data on measured concentrations. Hydrogen sulfide concentrations were estimated for Venice (Florida) from 0.162 p.p.m. H_2S (Lackey and Lackey, 1961), for Bad Gastein (Austria) from 0.5–0.8 mg kg^{-1} H_2S (Vouk *et al.*, 1967) and for Amélie-Les-Bains (France) from 3.5 mg l^{-1} H_2S (Scheminzky *et al.*, 1972).
[c]If no explicit value for depth was given, this is now indicated by a substitute value of 1 m. A value of 0 m resulted from the observation of gelatinous structures directly on rock surface overflown by water from thermal springs (Vouk *et al.*, 1967; Scheminzky *et al.*, 1972), or where structures were found on the water surface (Anagnostidis, 1968). The minke whale bones from Tjärnö were kept in an aquarium, which is indicated by aq.
[d]Mixture of seawater/freshwater.
[e]Values determined for a nearby source.
[f]One *Thiobacterium* sp. was isolated from water as single rods, and formation of the gelatinous matrix was observed during a laboratory life cycle experiment. Salinity and pH were determined by Seki and Naganuma (1989) for the seawater used in enrichment cultures (collected from the Strait of Georgia, Canada).
[g]Values obtained from Glover *et al.* (2005).
[h]Hydrogen sulfide was detected qualitatively, but measurements yielded values below detection limit (b.d.).

the sediment or of the gelatinous sphere was determined with the help of a dissection microscope. During the analyses, the core was kept in an aquarium with water cooled to approximately 1 °C to simulate *in situ* temperature conditions. A gentle jet stream, pointing at the water surface of the core, stirred the overlying water and assured the development of a distinct diffusive boundary layer. Flux calculations were performed as in Lichtschlag *et al.* (2010).

Composition of the gelatinous matrix

Three spheres from the cave mats were separated from sample water and cleaned from attached sediment particles as efficiently as possible. They were then combined and stored in a sterile polypropylene tube at −20 °C until further processing. Wet weight of the spheres was determined as 13.35 g. After freeze-drying for 4 days, total dry weight was determined as 0.61 g.

Three replicate subsamples of the dried material, with initial weights between 24 and 25 mg, were used for measuring total carbon, nitrogen and sulfur content of the material with a Thermo Fisher Scientific FlashEA 1112 (Thermo Fisher Scientific Inc., Waltham, MA, USA). No acidification was performed before the measurements. In addition, inorganic carbon was measured with a CM5012 CO_2 Coulometer (UIC Inc., Joliet, IL, USA) for three separate replicate subsamples with initial weights between 21 and 22 mg. Finally, organic carbon, organic nitrogen and organic/elemental sulfur content were calculated accordingly. All values were corrected to a normalized dry weight, which excluded the total sea salt content of approximately 39‰.

Analyses of carbon and nitrogen isotopic composition were conducted with two replicate subsamples of 26 and 27 mg of the freeze-dried material, using a Thermo Fisher Scientific MS DELTA^{plus} XP gas isotope ratio mass spectrometer (Thermo Fisher Scientific Inc.). Isotopic ratios were corrected against air-N_2 and the international Vienna Pee Dee Belemnite (VPDB) standard for obtaining $\delta^{15}N$ or $\delta^{13}C$, respectively. No acidification was performed before the measurements.

Fluorescence in situ *hybridization*

Subsamples of the cave mats chosen for FISH analyses were fixed in 1 ml of 4% formaldehyde/seawater for 1 h at room temperature. They were then directly transferred to Whatman Nuclepore

Track-Etch Membranes (PC MB; 25 mm; 0.2 μm; Whatman, Schleicher and Schuell, Maidstone, UK), the latter being captured in an appropriate filtration apparatus and supported by Whatman ME25 membrane filters (mixed cellulose ester; 25 mm; 0.45 μm; Whatman). After removal of excess fixative, the filters were dried for 2 min at 50 °C on a heating plate and were finally stored separately at −20 °C in sterile Petri dishes.

FISH was performed as described earlier (Snaidr *et al*., 1997). Mono-labeled probes (Biomers, Ulm, Germany) and hybridization conditions are listed in Table 2. All filter sections were counterstained with 10 μl of a 1 or 2.5 μg ml^{-1} DAPI solution before rinsing with distilled water and air-drying. Finally, the filters were mounted in separate spots of Teflon-covered slides with a 2:3 mix of Vecta Shield (Vector Laboratories Inc.) and Citifluor (Agar Scientific Ltd) and were stored at −20 °C before microscopy.

Construction of 16S rRNA gene libraries and sequencing

Subsamples for phylogenetic analyses were stored at −20 °C, preserved in either PCR-grade water (Sigma-Aldrich Biochemie GmbH, Hamburg, Germany), 1 × TE buffer (Promega Corporation, Madison, WI, USA) or without any fixative. Three different approaches for obtaining total community DNA were applied,

Table 2 Oligonucleotide probes and hybridization conditions used for FISH analyses in this study

Probe	*Probe specificity*	*Probe sequence (5′-3′)*	*Target site within 16S/23S rRNA gene*[a]	*FA*[b] *(%)*	T_h[c]/T_w[d] *(°C)*	*Positive hybridization with* Thiobacterium *cells*	*Reference*
EUB338(I-III)	Most bacteria	Equimolar mixture of three probes	16S, 338–355	35	46/48	Yes	Daims *et al.* (1999)
EUB338-I	Most bacteria	GCTGCCTCCCGTAGGAGT	16S, 338–355	35	46/48		Amann *et al.* (1990)
EUB338-II	Planctomycetales	GCAGCCACCCGTAGGTGT	16S, 338–355	35	46/48		Daims *et al.* (1999)
EUB338-III	Verrucomicrobiales	GCTGCCACCCGTAGGTGT	16S, 338–355	35	46/48		Daims *et al.* (1999)
NON338	Negative control	ACTCCTACGGGAGGCAGC	16S, 338–355	10	46/48	No	Wallner *et al.* (1993)
GAM42a[e]	Gammaproteobacteria	GCCTTCCCACATCGTTT	23S, 1027–1043	35	46/48	Yes	Manz *et al.* (1992)
BET42a[e]	Betaproteobacteria	GCCTTCCCACTTCGTTT	23S, 1027–1043	35	46/48	No	Manz *et al.* (1992)
GAM660	Free-living or endosymbiotic sulfur-oxidizing bacteria in the Gammaproteobacteria	TCCACTTCCCTCTAC	16S, 660–674	35	46/48	No	Ravenschlag *et al.* (2001)
Gm705	Methanotrophs in the Gammaproteobacteria except *Methylocaldum*	CTGGTGTTCCTTCAGATC	16S, 705–722	10	46/48	No	Gulledge *et al.* (2001)
Mγ669	*Methylobacter* spp. and *Methylomonas* spp.	GCTACACCTGAAATTCCACTC	16S, 669–690	10	46/48	No	Eller *et al.* (2001)
LMU	*Leucothrix mucor*	CCCCTCTCCCAAACTCTA	16S, 652–669	10	46/48	No	Wagner *et al.* (1994)
G123T[e]	*Thiothrix* spp.	CCTTCCGATCTCTATGCA	16S, 697–714	10	46/48	No	Kanagawa *et al.* (2000)
829-Thioploca	Marine *Thioploca* spp. and *Beggiatoa* sp.	GGATTAATTTCCCCCAACATC	16S, 829–849	10	46/48	No	Teske *et al.* (1995)
Blim575	Marine *Beggiatoa* spp.	CTAGCCGCCTACATACGC	16S, 575–592	10	46/48	No	Mußmann *et al.* (2003)
Blim193	Marine *Beggiatoa* spp.	AAAAGACGCCCTCTTTCC	16S, 193–210	10	46/48	No	Mußmann *et al.* (2003)
VSO673	Unattached, vacuolate, sulfur-oxidizing spp.	CGCTTCCCTCTACTGTAC	16S, 656–673	10	46/48	No	Kalanetra *et al.* (2004)
WPF464	Attached, vacuolate, filamentous spp.	AGCTTTAAGTTTTTCTTCCC	16S, 445–464	10	46/48	No	Kalanetra *et al.* (2004)
Thm465	*Thiomargarita* spp. Amon mud volcano	GTCAAGACTCTAGGGTAT	16S, 465–482	10	46/48	No	Girnth *et al.* (submitted)
Thm482[f]	*Thiomargarita* spp.	CTTCTTCTATTGCTGATG	16S, 482–499	10	46/48	No	This study
Biwa829	Freshwater *Thioploca* spp.	AGGTATACCCTTCCAACGTC	16S, 829–849	10	46/48	No	Kojima *et al.* (2003)
ThioBar894[f]	*Thiobacillus baregensis*	TGAGTTTCAACTTCCGGC	16S, 894–911	10	46/48	No	This study
ThioBar272[f]	*Thiobacillus baregensis*	CTACTGTATCGTCGCCTT	16S, 272–289	10	46/48	No	This study
THIO1	*Acidithiobacillus* spp.	GCGCTTTCTGGGGTCTGC	16S, 1275–1292	10	46/48	No	González-Toril *et al.* (2003)
Thio820	*Acidithiobacillus* spp.	ACCAAACATCTAGTATTCATCG	16S, 816–837	10	46/48	No	Peccia *et al.* (2000)
LaSp60	Tubeworm endosymbionts, uncult. Gammaproteobacteria	CCATCGTTACCGTTCGAC	16S, 60–77	10	46/48	No	Duperron *et al.* (2009)
RifTO147	*Rif/Tev/Oas* symbiont	GATTTCTCCGAGTTGTCC	16S, 147–164	10	46/48	No	Nussbaumer *et al.* (2006)
RifTO445	*Rif/Tev/Oas* symbiont	TCCTCAGGCTTTTCTTCC	16S, 445–462	10	46/48	No	Nussbaumer *et al.* (2006)
Ohaa1-65	*O. haakonmosbiensis* symbiont	AGCTCTTGCTGTTACCGT	16S, 65–82	10	46/48	No	Lösekann *et al.* (2008)

Table 2 (Continued)

Probe	*Probe specificity*	*Probe sequence (5′-3′)*	*Target site within 16S/23S rRNA gene*[a]	*FA*[b] *(%)*	T_h[c]/T_w[d] *(°C)*	*Positive hybridization with* Thiobacterium *cells*	*Reference*
Ohaa1-77	*O. haakonmosbiensis* symbiont	GCCAAGAGCAAGCTCTTG	16S, 77–94	10	46/48	No	Lösekann *et al.* (2008)
Ohaa2-60	*O. haakonmosbiensis* symbiont	GCATCGTTACCGTTCGAC	16S, 60–77	10	46/48	No	Lösekann *et al.* (2008)
Ohaa2-77	*O. haakonmosbiensis* symbiont	CCTGCTAGCAAGCTAGCA	16S, 77–94	10	46/48	No	Lösekann *et al.* (2008)

Abbreviations: FISH, fluorescence *in situ* hybridization; rRNA, ribosomal RNA.
[a]*Escherichia coli* positions.
[b]Formamide concentration in the hybridization buffer.
[c]Hybridization temperature.
[d]Washing temperature.
[e]Mixed in a 1:1 ratio with unlabeled competitor oligonucleotides, to assure specific hybridization (Manz *et al.*, 1992; Amann and Fuchs, 2008).
[f]These probes represent newly developed oligonucleotides which have not been tested in corresponding positive control experiments because of inavailability of the respective organisms/16S rRNA gene sequence.
Probe specificity is given for the original design. In addition to the listed probes, nine other, but so far unpublished probes, were used: two designed for a hypersaline *Beggiatoa* sp., one designed for sulfur-oxidizing endosymbionts, one designed for *Thiomargarita* spp.[f] and five designed for smaller subgroups within the Gammaproteobacteria. None of them positively hybridized with the target organisms, why publication of the probe sequences was reserved for the original developers. Specific gammaproteobacterial probes were used at lower stringency than published, that is, 10% formamide, to create favorable hybridization conditions. For probe Gm705 specificity had been achieved by the original publishers at $T_d = 51\,^{\circ}C$ (midpoint dissociation temperature).

including (i) freezing and thawing, (ii) an extraction method developed for marine microorganisms embedded in exopolysaccharides (E1; Narváez-Zapata *et al.*, 2005) and (iii) the UltraClean Soil DNA Isolation Kit (E2; MO BIO Laboratories Inc., Carlsbad, CA, USA).

Universal bacterial primers GM3F (5′-AGAGTTTG ATCMTGGC-3′; Muyzer *et al.*, 1995) and GM4R (5′-T ACCTTGTTACGACTT-3′; Muyzer *et al.*, 1995) were used to amplify a nearly complete region of the 16S rRNA gene. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), ligated into pGEM-T Easy (Promega Corporation) and transformed into One Shot TOP10 Chemically Competent *Escherichia coli* (Invitrogen Corporation). After plasmid preparation (Montage Plasmid $Miniprep_{HTS}$ Kit; Millipore GmbH, Schwalbach, Germany), inserts were subjected to *Taq* cycle sequencing with an ABI Prism 3130x Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing was either conducted with the M13R vector primer, or by using the bacterial primers 907R (5′-CCGTCAATTCCTT TRAGTTT-3′; Muyzer *et al.*, 1995) and primer2 (5′-ATTACCGCGGCTGCTGG-3′; Muyzer *et al.*, 1993). Both sequencing approaches yielded good-quality fragments of the 16S rRNA gene with lengths between 400 and 700 bp.

Sequence analyses
Partial sequences derived from 907R and primer2 were assembled with Sequencher 4.6 software (Gene Codes Corporation, Ann Arbor, MI, USA) and manually checked. In addition, all libraries were screened for putative chimeras with the Mallard program at a cut-off of 99.9% (Ashelford *et al.*, 2006). Anomalous sequences were further investigated by using Pintail (Ashelford *et al.*, 2005), with nearest neighbors of the sequences obtained by using the SILVA-based SINA Webaligner (Pruesse *et al.*, 2007). Sequences with genuine chimeric signals were excluded from further analyses.

To search for a sequence common to either all libraries or all sample sites, gammaproteobacterial sequences, as previously determined with the RDP Classifier (Wang *et al.*, 2007), and their nearest neighbors (obtained by using the SILVA-based SINA Webaligner and ARB database) were used for tree reconstruction in ARB (Ludwig *et al.*, 2004) by applying maximum likelihood (RAxML).

Nucleotide sequence accession numbers
All sequences have been submitted to the EMBL database under accession no. FN597289 to FN597418 (Gammaproteobacteria) and FN662907 to FN663128 (all other sequences).

Results

Visual description of the gelatinous mats
At all three sampling sites the gelatinous mats were spherically shaped masses. A second, less dominant, layer-like mat type was found only in the shallow-water cave. The environmental settings prevailing at these three sites and in previously described habitats are summarized in Table 1.

On the whale bone a yellowish–white gelatinous sphere was observed (Figure 1a). Its diameter was approximately 4 mm. The sphere had evolved within 1 to 2 days in an area that was scraped free

of bacterial mats of *Beggiatoa* and *Arcobacter* (as indicated by microscopy). During sampling the entire gelatinous sphere was removed and in the next few days nothing alike grew back on the bone.

The mats on top of the deep-sea methane seep sediment had an approximate diameter of 1.5 cm (Figure 1b). Under bright light conditions the gelatinous spheres seemed to be covered by a thin white layer with some thicker white filaments. The white color of the gelatinous spheres is an indication for the presence of elemental sulfur, and microscopic analyses later revealed a high density of light-reflecting granules typical for the sulfur inclusions of giant sulfide-oxidizing bacteria (Teske and Nelson, 2006). The white filaments were also observed surrounding the gelatinous masses on top of the sediment and were morphologically attributed to bacteria of the genus *Beggiatoa*, mat-forming giant sulfide oxidizers known to occur on highly reduced sulfidic sediments (Ahmad *et al.*, 1999; Mills *et al.*, 2004; de Beer *et al.*, 2006). The underlying sediment was characterized by a beige-brown top layer above a dark gray to blackish horizon.

The largest accumulation of gelatinous mats in this study was found in a shallow-water cave off Paxos in Greece (Figure 1c; Supplementary Figure 1; Supplementary Results and Discussion). The cave walls were almost completely covered by gelatinous mats over an estimated area of 5 to 7.5 m^2, with a sharp boundary below the top of the cave (Dr Thomas Beer, personal communication). Two morphological types of mats could be observed without a particular zonation in the cave (Figure 1c). The dominating type consisted of spherical structures showing diameters between less than 1 cm and more than 10 cm, the second type was characterized by a rather thin layering of the gelatinous material. Both spherical- and layer-like structures of the gelatinous mats appeared whitish similar to the deep-sea spheres. The presence of elemental sulfur and white filamentous bacteria was verified by microscopy. The mats were present in summer 2006 and 2007, but had vanished in January 2007 when they were replaced by a dense filamentous mat on the cave walls (Paul Bowbeer, personal communication).

Microscopic description of Thiobacterium *microbes*

Microscopic examination of the gelatinous spheres from all three sites revealed the presence of high numbers of rod-shaped, sometimes slightly curved chains of sulfur granules. The *Thiobacterium* spp. embedded in the gelatinous material found on the whale bone had an approximate size of 3–8 μm × 0.9–2 μm (Figure 2a), those from the deep-sea ranged from 3–11 μm × 0.7–2 μm (Figure 2b), and the ones from the shallow-water cave were from 2–9 μm × 0.3–2 μm in size (Figure 2c). The number of sulfur granules per cell varied between 3 and 5 (whale bone), 3 and 11 (deep-sea sediment) as well as 3 and 10 (cave). In addition, single and pairs of sulfur granules were

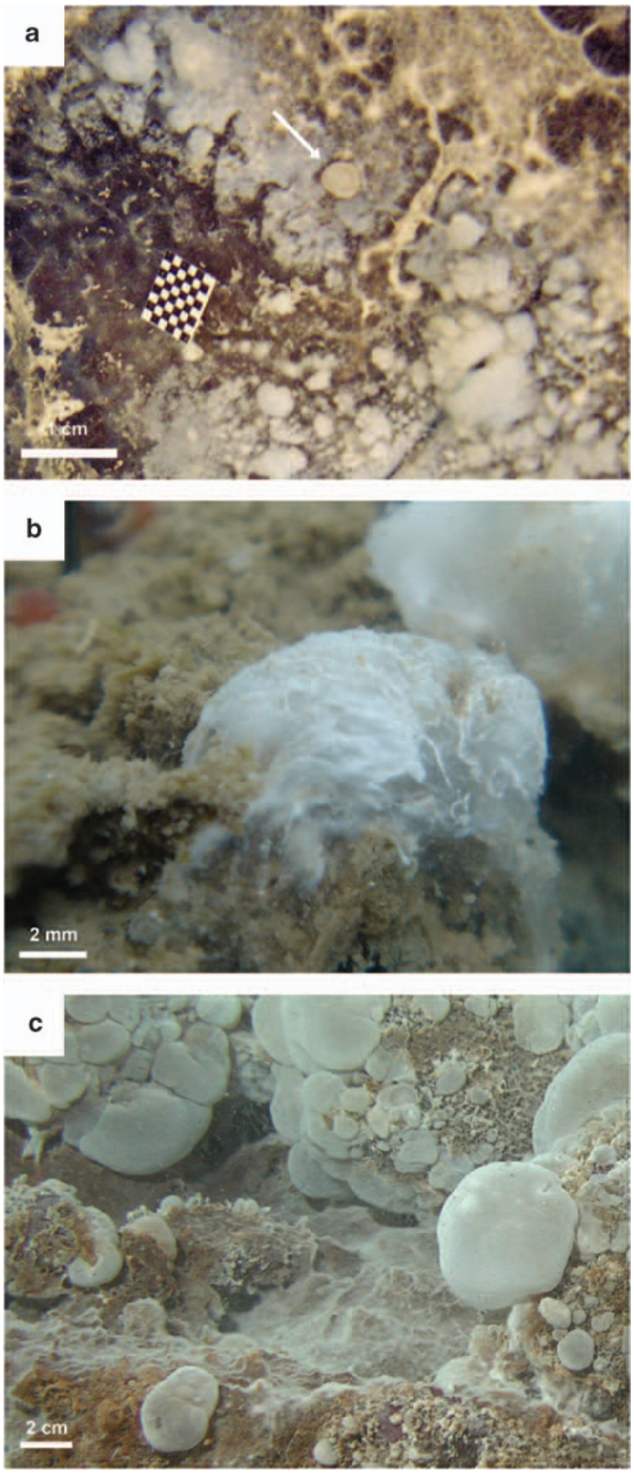

Figure 1 *Thiobacterium* gelatinous mats analyzed in this study. (**a**) Gelatinous sphere with an estimated diameter of 4 mm, found on top of a minke whale bone kept in an aquarium. In addition, the bone was covered by dense mats of thiotrophic bacteria (*Beggiatoa* and *Arcobacter*). (**b**) Gelatinous sphere found on top of a sediment core collected at the Storegga Seeps. (**c**) Assemblage of spherically shaped and layer-like gelatinous masses coating the walls of a shallow-water cave off Paxos in Greece.

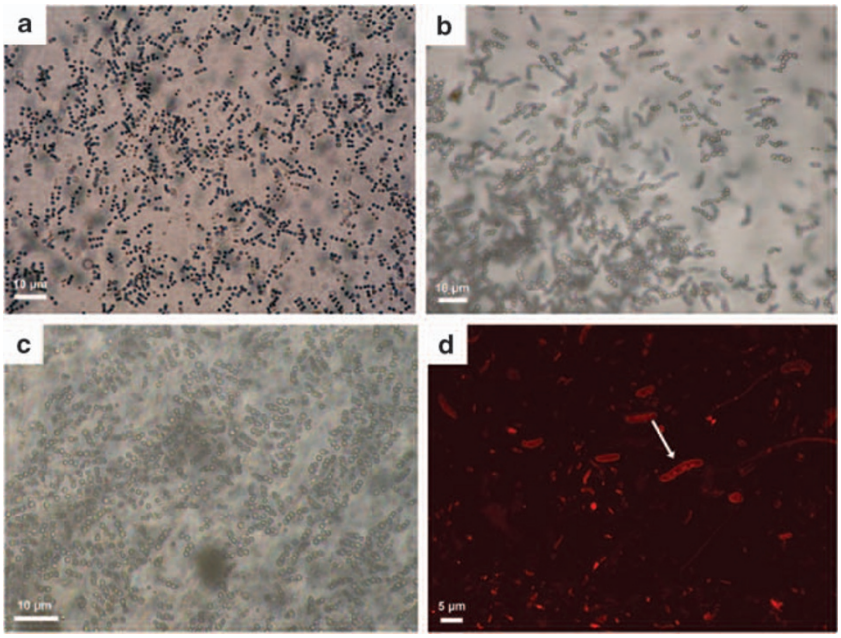

Figure 2 Microscopic observations on *Thiobacterium* cells embedded in the gelatinous spheres. (**a**) Whale bone sample (bright field), (**b**) deep-sea sample (phase contrast) and (**c**) shallow-water cave sample (phase contrast). In all samples, chains of elemental sulfur granules were visible. (**d**) True cell outlines around the granules were not visible by phase contrast and bright field microscopy, but by staining with the protein-targeting fluorochrome SYPRO Red using epifluorescence microscopy (staining was conducted with fixed material recovered from the cave in 2006).

observed, but it was not clear whether those belonged to living cells or remnants of cell decay. Except for the *Thiobacterium* cells from the whale bone, we found that even within one cell the size of the sulfur inclusions could vary by up to a factor of 5. By phase contrast microscopy cell outlines around the granules could not be observed, but became visible after staining fixed material with the protein-targeting fluorochrome SYPRO Red (Figure 2d). We observed a loss of the internal sulfur deposits in ethanol fixation, because ethanol dissolves elemental sulfur. However, even with formaldehyde/seawater fixation, the sulfur granules vanished with time. At all three sites, immediately after sampling, the *Thiobacterium* cells embedded in the gelatinous mats appeared to be non-motile. However, microscopic examination of gelatinous material obtained from the underside of a red leaf-like structure (cave) revealed a few rod-shaped cells showing directed or tumbling movement outside of the matrix.

In addition to *Thiobacterium*, microscopic analyses of the gelatinous spheres from all three sample sites revealed the presence of many other organisms embedded in and associated with the gelatinous mats. This unexpectedly high microbial diversity is described separately in the Supplementary Results and Discussion and in Supplementary Figure 2.

Microsensor measurements

Microsensors were used to measure high-resolution profiles of oxygen and sulfide concentration, as well as pH, (i) through a gelatinous deep-sea sphere (diameter approximately 1.5 cm) and the first 1.5 cm of the underlying sediment (Figure 3a) and (ii) through the top 4 cm of sediment immediately next to the sphere (Figure 3b). Oxygen penetrated the gelatinous matrix to a depth of approximately 3 to 4 mm. Sulfide from the sediment diffused into the underside of the structure, but no local overlap of oxygen and sulfide was observed in the sphere. The pH profiles showed several minima and maxima, but generally decreased in the sphere, possibly as a consequence of sulfide oxidation. In the underlying sediment, no oxygen could be detected and sulfide concentrations reached a maximum of 3.2 mM at a depth of 1.5 cm below the sphere. The sulfide and oxygen fluxes into the sphere were 5 and 11 mmol m^{-2} d^{-1}, respectively, matching the stoichiometry of sulfide oxidation with oxygen as electron acceptor. Next to the sphere, oxygen penetrated approximately 2 mm into the sediment. Sulfide was not detectable until 2.7 cm below seafloor and then increased linearly with depth. At 2 cm below seafloor, the pH reached a minimum of 7.6, below which it increased with depth. The oxygen uptake of the sediment next to the sphere was comparable to that in the sphere with 10 mmol m^{-2} d^{-1}.

Elemental and isotopic composition of the matrix

The dried matter comprised 5.48% (w/w) organic carbon, 1.06% (w/w) nitrogen and 3.62% (w/w) organic and elemental sulfur. The molar C:N:S ratio

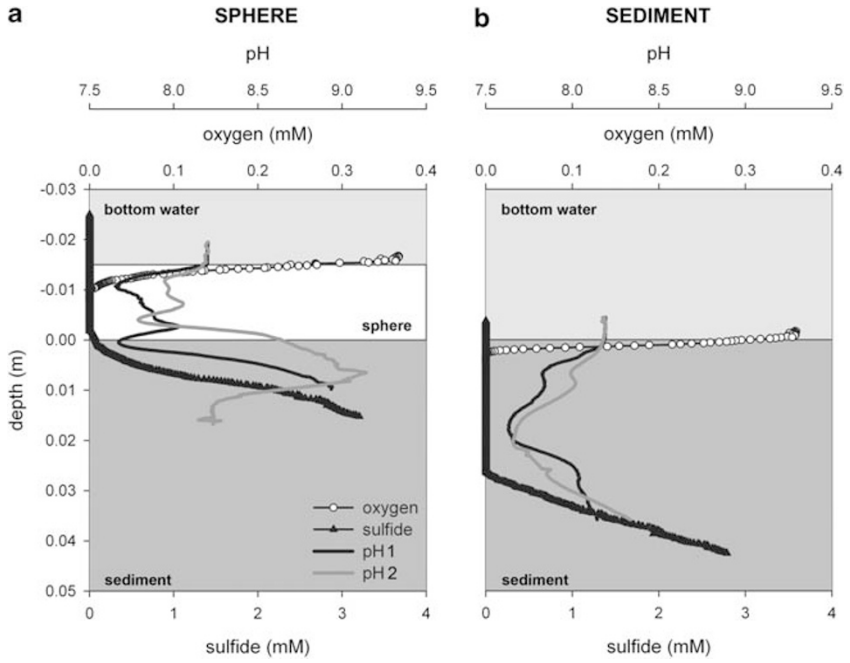

Figure 3 Oxygen, sulfide and pH microprofiles measured (**a**) through a gelatinous sphere with a diameter of approximately 1.5 cm and (**b**) within reference sediment next to the sphere (recovered from deep-water seafloor at the Storegga Seeps). Single, *ex situ* measured profiles are shown. Replicate pH profiles 1 (black) and 2 (gray) were retrieved from (**a**) the same gelatinous sphere and (**b**) the adjacent sediment. The microprofiles clearly show that the gelatinous spheres had formed in an area in which hydrogen sulfide reached the sediment surface. Sulfide and oxygen did not overlap within the sphere, raising the question of how electron transport is mediated within the sphere during sulfide oxidation.

of 6:1:1.5 reflects the enrichment of the cells and matrix with elemental sulfur. The $\delta^{13}C$ measured for two replicates of the freeze-dried cave material resulted in values of −16.1 and −18.3‰. The $\delta^{15}N$ was determined for the same replicates as −6.0 and −6.2‰. More details on the composition of the matrix are given in the Supplementary Results and Discussion.

Ribosomal RNA gene analyses

Bacterial 16S rRNA gene libraries were constructed with subsamples of the gelatinous spheres from all three sampling sites. Gamma-, Delta- and Epsilonproteobacteria dominated the mat samples from all three sites. Other abundant classes of bacterial 16S rRNA genes included the Alphaproteobacteria, as well as Flavobacteria, Sphingobacteria, Planctomycetacia and Clostridia (Supplementary Table 1; Supplementary Results and Discussion). In spite of the observed abundance of *Thiobacterium* cells in all samples, it was not possible to identify common phylotypes dominating the clone libraries of all three sites (all bacterial libraries per site combined). A substantial diversity of 16S rRNA gene sequences belonging to the Gammaproteobacteria was retrieved from all three mats (Figure 4), including many sequences of sulfur-oxidizing bacteria, such as different types of *Beggiatoa*, sulfide-oxidizing symbionts of tubeworms, and bacteria from hot springs, hydrothermal vents, cold seeps and lava. Subsequent FISH analyses with the GAM42a probe on fixed subsamples of the cave spheres revealed that the *Thiobacterium* cells belong to the Gammaproteobacteria (Figure 5). Unfortunately, further attempts to confine phylogenetic relatedness using more specific oligonucleotide probes for FISH were not successful (Table 2). A probe including all sulfide-oxidizing and sulfur-storing members of the Thiotrichaceae has not been developed so far. Specific gammaproteobacterial probes, if not otherwise indicated, were used at lower stringency than published, that is, 10% formamide, to create favorable hybridization conditions. However, none of the established FISH probes specific for certain subgroups within the Gammaproteobacteria resulted in positive hybridization with *Thiobacterium* spp.-resembling cells, including those for the Thiotrichaceae-associated genera *Beggiatoa*, *Thioploca*, *Thiomargarita*, *Thiothrix* and *Leucothrix*, as well as probes for other organisms involved in the microbial cycling of sulfur like *Acidithiobacillus,* or for endosymbionts of seep and vent fauna. Further approaches to identify a candidate 16S rRNA gene sequence for *Thiobacterium* by single cell sorting were also not successful (data not shown) and detailed sequence analyses of *Thiobacterium* cells will require special attention in future experiments.

Discussion

Thiobacterium classification

Since its first description by Molisch in 1912 (Molisch, 1912), no representative of this genus has been

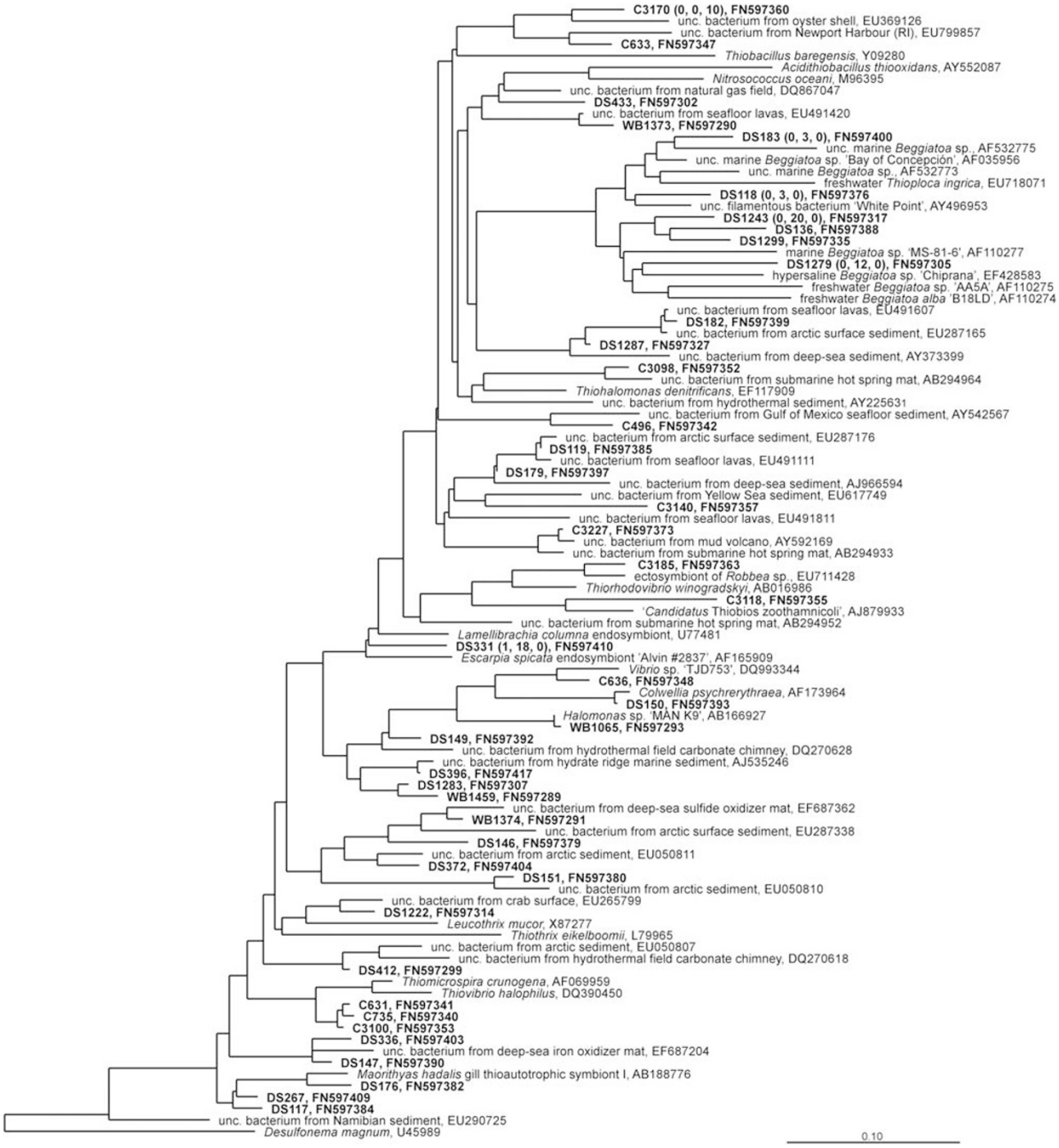

Figure 4 Maximum likelihood tree showing gammaproteobacterial 16S rRNA gene sequences obtained in this study (bold type; WB = whale bone mat, DS = deep-sea sediment mat, C = cave mat) to reference sequences within the Gammaproteobacteria. Only selected sequences are shown. For large clusters of sequences obtained in this study, the number of sequences associated with the given representative is indicated in parentheses, following the order WB, DS and C. Initial calculations were conducted with nearly full-length sequences (*Escherichia coli* positions 99–1331). Partial sequences (bold type) were added subsequently to the reconstructed tree by applying parsimony criteria. Deltaproteobacterial sequences were used as outgroup.

cultivated or genomically classified. The visual observation of internally stored sulfur granules and the biogeochemical analyses discussed below suggest that *Thiobacterium* is closely related to other known sulfide-oxidizing and sulfur-storing members of the Thiotrichaceae. Positive hybridization of *Thiobacterium* cells with the probe GAM42a (76% group coverage; Amann and Fuchs, 2008) confirmed their phylogenetic affiliation with the class of Gammaproteobacteria (Figure 5), as previously suggested based on morphological analogies (Kuenen, 2005). However, no common gammaproteobacterial phylotypes (Figure 4)

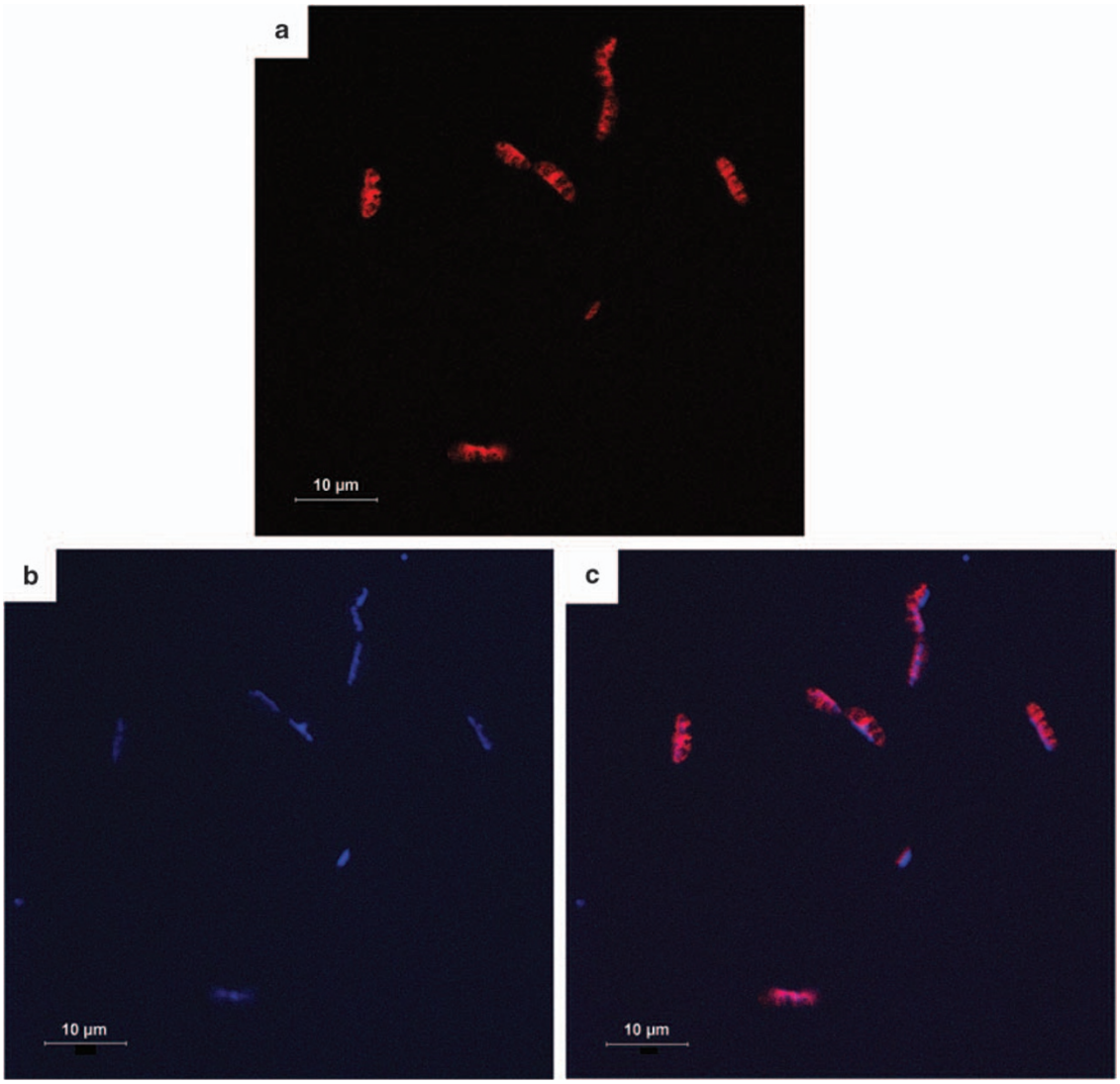

Figure 5 FISH images of *Thiobacterium* cells (cave samples 2007). (**a**) Positive signal after staining with the Gammaproteobacteria-targeting probe GAM42a (unlabeled competitor BET42a). (**b**) Corresponding DAPI stain. (**c**) Overlay of (**a, b**). In addition, staining of the *Thiobacterium* cells with the probe BET42a (unlabeled competitor GAM42a) did not result in any positive signal. Autofluorescence of the cells was found to be negligible.

dominating the clone libraries of all three sites could be identified. Reasons might include that one of the universal primers does not have full access to its target site on the *Thiobacterium* spp.-16S rRNA gene, or that the amplification of this gene is underrepresented compared with that of other bacteria with the applied PCR conditions, a problem also known from the giant sulfide oxidizer *Thiomargarita namibiensis* (Schulz, 2006). However, considering the broad range of environmental conditions from which it was described, the genus *Thiobacterium* could be composed of rather different phylotypes, as it is known for the genus *Beggiatoa* (Figure 4; Ahmad *et al*., 2006; Teske and Nelson, 2006).

Niches of Thiobacterium

Environmental conditions under which *Thiobacterium* mats have been observed include pH values between 7.2 and 9.3, temperatures ranging from −1 °C to 45.5 °C and water depths between 0 and 2700 m (Table 1). Low $\delta^{15}N$ values of −6.0 and −6.2‰ measured for the cave mats in this study suggest autotrophic growth of the associated organisms (see also Supplementary Results and Discussion). The main environmental factor selecting for *Thiobacterium* seems to be the availability of the electron donor hydrogen sulfide. Their ability to store massive amounts of sulfur, and their preference for sulfidic habitats suggests that *Thiobacterium* could be a sulfide oxidizer, belonging at least functionally into the same group as other mat-forming members of the Thiotrichaceae such as *Beggiatoa*, *Thiomargarita*, *Thioploca* and *Thiothrix*. With microsensor measurements it could be shown that the gelatinous spheres had exclusively formed in an area in which hydrogen sulfide reached the sediment surface and that this hydrogen sulfide was

depleted within the sphere (Figure 3a). The oxidation of sulfide occurs in several steps, from sulfide to sulfur, and from sulfur to sulfate. Sulfide oxidation can be driven by oxygen, nitrate and Mn- and Fe-oxides. The microsensor profiles (Figure 3a) indicate that the matrix had consumed stoichiometric amounts of oxygen and sulfide (2:1 in aerobic sulfide oxidation), but sulfide and oxygen did not overlap. A pronounced gap between oxygen and sulfide has previously also been observed for *Thioploca* and *Beggiatoa* spp. that are capable of bridging this gap by means of their gliding motility and the use of internally stored nitrate as additional electron acceptor under anoxic conditions (Fossing *et al.*, 1995; Huettel *et al.*, 1996; Mußmann *et al.*, 2003; Sayama *et al.*, 2005; Lichtschlag *et al.*, 2010). However, *Thiobacterium* are neither known to express a gliding behavior within the gelatinous matrix, nor to possess an internal vacuole for nitrate storage. The nature of a potential intermediate electron shuttle therefore remains unknown. The existence of a microoxic and microsulfidic environment within the matrix can be ruled out, as at undetectably low levels of reactants, the diffusive transport will be too low for significant metabolic activity. Also metal cycling, as in the suboxic zone, can be excluded as no solid phase transport seems possible in these gel-like structures. The potential involvement of electrical currents in the oxidation of sulfide remains a possibility (Nielsen *et al.*, 2008).

It is unknown how and when *Thiobacterium* can outcompete other sulfide-oxidizing bacteria for energy and space. On the whale bone, it grew after *Beggiatoa* and *Arcobacter* mats had been physically removed, indicating that it may only be able to compete with others after specific disturbances. When it grew, *Beggiatoa* was always observed, either growing thinly on the gelatinous matrix or more densely next to it, indicating that it may compete with *Thiobacterium* for the same energy source and space.

Conclusion and further questions

Morphological and ecological characteristics of *Thiobacterium* cells and mats suggest that this genus is closely related to other sulfide-oxidizing and sulfur-storing bacteria of the family Thiotrichaceae. Most interestingly, we found a stoichiometrical oxidation of sulfide to sulfate with oxygen without them overlapping. Motile microbes resembling the morphology of *Thiobacterium* have been reported few times (Lackey and Lackey, 1961; Lackey *et al.*, 1965; this study), but so far were never truly accounted to this genus. Molisch described similar motile organisms separately as *Bacillus thiogenus* (Molisch, 1912), hence it remains an important question whether they can move inside their gels. It also remains hypothetical which factors trigger the attachment of free cells of *Thiobacterium* in a certain habitat and whether mat formation may undergo a succession in which other pioneer microbes may be needed. As often reported, the gelatinous masses of *Thiobacterium* were found to be attached to a substratum, for example, the algae *Lyngbya* (Lackey and Lackey, 1961), rock surface (Vouk *et al.*, 1967), sediment (this study) or a whale bone (this study), suggesting that the production of a gel may not only be used for competing against other microbes, but also functions as an anchor in areas with favorable environmental conditions (Vouk *et al.*, 1967; Seki and Naganuma, 1989). Further it is not clear what role quorum sensing, density-dependent cell–cell communication, may have in the formation, maturation and final disintegration of the gelatinous mats. In this respect, future studies need to assess (i) the mechanisms triggering the appearance of *Thiobacterium* in conspicuous gelatinous mats, (ii) the access to electron donor and acceptor by the cells embedded in the thick matrix, (iii) the 16S rRNA gene-based taxonomic affiliation of *Thiobacterium* spp. and (iv) whether and what role other microbes may have in the development of the *Thiobacterium* mats in natural environments.

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