

Filamentous bacteria inhabiting the sheaths of marine *Thioploca* spp. on the Chilean continental shelf

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Introduction

Nitrate-respiring, ammonia-producing, sulfur-oxidizing mats of *Thioploca* spp. constitute the dominant microbial community on eastern South Pacific continental shelf sediments (Fossing *et al.*, 1995; Jørgensen & Gallardo, 1999; Otte *et al.*, 1999). Co-occurring with *Thioploca* within the same habitat, a new complex community of filamentous bacteria has been described recently, but their species composition and functions still remain unknown; they are termed 'macro-bacteria' (Gallardo & Espinoza, 2007a, b) and differ in size (*c.* 1–10 µm cell diameter, and up to several millimeters filament length) from the considerably larger mat-forming *Thioploca* spp. (*c.* 10–45 µm cell diameter, and up to several centimeters filament length). The *Thioploca* mat is characterized by a highly active sulfur cycle with high rates of sulfate reduction and sulfur reoxidation (Thamdrup & Canfield, 1996; Ferdelman *et al.*, 1997; Schubert *et al.*, 2000), indicating very active populations of sulfate-reducing bacteria growing within or associated with the mats. The microbial associations within the *Thioploca* mat are com-

Abstract

A new component of the benthic *Thioploca* mat microbial ecosystem on the Chilean continental shelf was detected by epifluorescence microscopy: filamentous, bacterial endobionts of 4–5-µm filament diameter and length sometimes exceeding 1 mm. These filaments were identified as growing within *Thioploca* sheaths located between the sediment surface and *c.* 5 cm depth. Their location coincided with maximal biomass and biovolume of *Thioploca* filaments in surficial sediments, and with maximal abundance and activity of sulfate-reducing bacterial populations near the sediment/water interface. FISH and environmental characteristics support the working hypothesis that these endobiont populations are members of the filamentous, sulfate-reducing bacterial genus *Desulfonema*. Found at several sampling stations over a decade-long interval (1994–2006), these populations appear to be a stable component of the Chilean *Thioploca* mat ecosystem.

posed of heterotrophic bacteria, some of which utilize sulfate as a terminal electron acceptor. The polysaccharide sheaths of *Thioploca* spp. are colonized by filamentous sulfate-reducing bacteria of the genus *Desulfonema* (Fukui *et al.*, 1999) in addition to many other uncultured bacteria. For example, some *Thioploca* sheath endobionts hybridize with 16S rRNA gene probes originally designed for *Alpha-proteobacteria* (Muyzer & Ramsing, 1995). 16S rRNA gene sequence analyses of freshwater *Thioploca* sheaths from Lake Biwa, Japan, revealed a sheath-associated community consisting of members of the *Bacteroidetes*, the uncultured OP8 division, the *Acidobacteria* and *Betaproteobacteria* (Kojima *et al.*, 2006). Cloning and sequencing of dissimilatory sulfite reductase genes (*dsrA*) from freshwater *Thioploca* sheaths revealed phylotypes related to the deltaproteobacterial genera *Desulfobacter*, *Desulfonema*, *Desulfococcus*, *Desulfosarcina*, *Desulfomonile*, and *Desulforhopalus* (Kojima *et al.*, 2006).

This study extends these observations on *Thioploca*-associated microbial populations by reporting the detection, distribution, and microbial and biogeochemical habitat characteristics of a frequently observed filamentous

endobiont associated with the sheaths and bundles of *Thioploca araucae* and *Thioploca chilae* on the Chilean continental shelf near Concepción.

Materials and methods

In January and February 1997 and in January 2006, benthic *Thioploca* spp. mats and sediment cores were sampled by a multicorer and a box corer with RV *Kay-Kay* at Stations 7 and 18 (c.f. Thamdrup & Canfield, 1996; Schulz *et al.*, 2000) on the Chilean Continental shelf near Concepción. Sediment cores from Station 18, c. 20 km offshore northwest of the Bay of Concepción (88 m depth; 36°30'8"S, 73°07'6"W), and from nearshore Station 7 located at the entrance to the Bay of Concepción (37 m depth; 36°36'5"S, 73°00'6"W) were collected and analyzed (Fig. 1a–c). While aboard the ship, the sediment cores were maintained at near *in situ* temperature (10–12 °C). Upon returning to shore, the cores were subsampled: subcores of 2.5 cm diameter and 20–30 cm length were extruded from the core liner and placed on a dissection plate. Here, 1-cm slides of sediment and *Thioploca* bundles (multiple filaments within a sheath) were sliced off using sterile surgical blades and sterile spatulas. *Thioploca* bundles were removed from sediment slices with forceps and toothpicks, rinsed in filter-sterilized seawater, and fixed in 1 × phosphate-buffered saline (PBS) with 4% paraformaldehyde at 10 °C for 1 day, and then transferred to a 1 × PBS:ethanol mixture (1:1 volume) until hybridization 1–3 days later (Teske *et al.*, 1999). Intact *Thioploca* bundles were found from the sediment surface to a depth of c. 15–20 cm. The upper 10 cm of the sediment yielded more *Thioploca* bundles than the deeper layers similar to previous observations (Schulz *et al.*, 1996, 2000).

FISH

FISH hybridization of *Thioploca* samples was performed as described previously (Teske *et al.*, 1995). In brief, short 0.5–1-cm segments were cut from larger *Thioploca* bundles with surgical blades. The pieces were placed into wells of Teflon-coated 6-well slides, which allowed hybridizations of three *Thioploca* samples – one from the sediment surface, one from an intermediate depth, and one from a deep sediment layer – each in duplicate (Table 1). Pieces of the same *Thioploca* bundle or, if not available, *Thioploca* bundles from the same sediment sample were used in parallel hybridizations to check for consistent hybridization results. The *Thioploca* samples were dehydrated in a three-step ethanol series (50%, 80%, and 100%) and hybridized with fluorescein- and Cy3-labeled 16S rRNA gene-targeted FISH probes (see the summary of the probes and individual experiments in the legend of Table 1) at 37 °C and 30% formamide concentration for 2–3 h following a previously described hybridization protocol (Teske *et al.*, 1999). A large

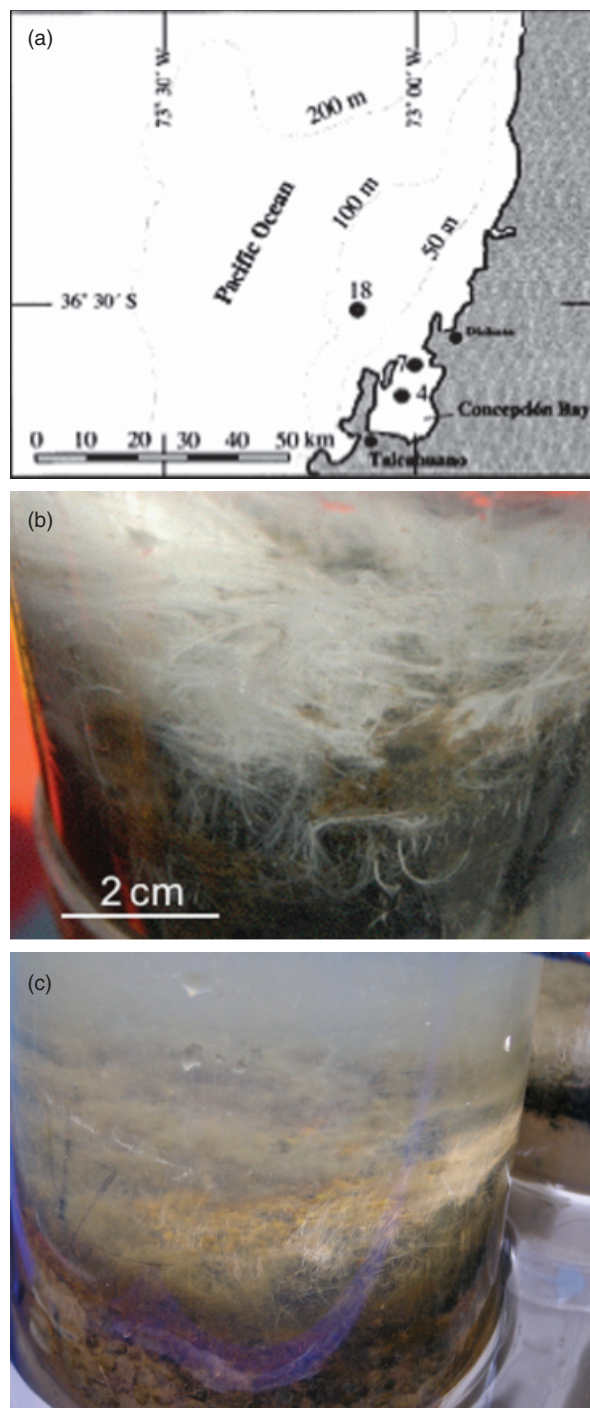


Fig. 1. (a) Map of sampling sites at Station 18 on the inner shelf and Station 7 within the Bay of Concepción. (b) A dense lawn of *Thioploca* filaments covering a sediment core recovered from Station 7. (c) A typical sediment core from Station 18 with surficial *Thioploca* mats, its appearance dominated by olive-brown organic phytodetritus.

(50 mL) Falcon tube was fitted with a small paper towel wetted in hybridization buffer, and served as a moisturized incubation chamber for the hybridization slide while

Table 1. FISH detection of filamentous bacteria within and on *Thioploca* sheaths

	Exp. 1 <i>T. chileae</i> Station 18, January 2006 Co385/ln385	Exp. 2 <i>T. chileae</i> Station 18, January 2006 Co385/ln385	Exp. 3 <i>T. chileae</i> Station 18, January 2006 DSS658/alf19	Exp. 4 <i>T. chileae</i> Station 18, January 2006 Co385/ln385	Exp. 5 <i>T. araucae</i> Station 7 January 2006 Co385/ln385	Exp. 6 <i>T. araucae</i> Station 7 January/February 1997 Co385/CFB319	Sheath endobiont/ <i>Thioploca</i> length January/February 1997
0–1 cm				++	++	++++++	3.3
1–2 cm	++	+ –	+ –				
2–3 cm				++	++	++++++	0.3
4–5 cm	++	--	--		--	+- - - - -	< 0.3
8–9 cm	--	--	--				
> 9 cm				++			

Summary of FISH experiments using two simultaneously applied FISH probes (listed in the top row) with filamentous endobionts of Chilean *Thioploca* spp. Experiment nos 1–5 were performed as simultaneous analyses of two small *Thioploca* segments (c. 3–4 mm long) cut from different *Thioploca* bundles, using the same hybridization slide. Detection of filamentous epibionts on both *Thioploca* segments is indicated by two positive values (++); one positive and one negative specimen is indicated by + – values; absence of epibionts from both samples is indicated by two negative values (– –). The data and + – in column 6 are based on hybridizations of six *Thioploca* segments. For Nos 1, 2, 4, and 5, the fluorescein-labeled probe CoSRB385 (5'-CGGCGTTGCTGCTCAGG-3') was used in combination with Cy3-labeled competitive probe lnSRB385 (5'-CGGCGTGCCTGCTCAGG-3'; Rabus *et al.*, 1996). The hybridization in column 3 was performed with fluorescein-labeled probe DSS658 targeting the *Desulfosarcinales* (5'-TCCACTTCCCTCTCCAT-3') (Manz *et al.*, 1998) and Cy3-labeled probe alf19b (5'-CGTTCGYTCTGAGCCAG-3'). In column 4, the positive hybridization result at 9–10 cm depth originated from endobionts on a single, very thick *Thioploca* bundle that was pulled from the 9–10 cm depth layer, and cut into two pieces for parallel hybridization. FISH experiment no. 6 was performed with rhodamine-labeled 385CoSRB in combination with fluorescein-labeled probe CFB319a (5'-TGGTCCGTGTCTCAGTAC-3'; Manz *et al.*, 1996). For this dataset no. 6, the length ratio of 385CoSRB-stained endobiont and of underlying *Thioploca* bundle was determined by epifluorescence microscopy, using $\times 40$ magnification and a 50- μm counting grid.

floating in a 37 °C water bath. A 5-min counterstaining step with 4',6'-diamino-2-phenylindole (DAPI) was performed after the hybridization was concluded with two washing steps with washing buffer and water. FISH hybridization and DAPI stain images were viewed and photographed using a Zeiss Axiophot 2 epifluorescence microscope at the Austral Microbial Diversity Course, Dichato, in January 2006, using exposure times of 1–2 s, $\times 20$ magnification, and AXIOVISION 4.4 image acquisition software. Because confocal microscopy was not available, the images were focused on focal planes that yielded the best overall picture of the filamentous endobionts. For Fig. 3 and Supporting Information, Fig. S1, composites of two images were prepared.

Most probable number (MPN) counts

Cultivable sulfate-reducing bacteria in *Thioploca* sediment were quantified by triplicate MPN dilution as described previously (Teske *et al.*, 1996), using 20 mM lactate and 10 mM acetate as substrates in selective anaerobic media for sulfate reducers (Widdel & Bak, 1992). *Thioploca* mat samples and sediment from 0 to 1, 2 to 3, 6 to 7, and 16 to 17 cm depths of Station 7 were homogenized for 5 min using a hand-help potter homogenizer, before dilution and inoculation into MPN vials.

Results

Filamentous endobionts associated with the *Thioploca* sheaths hybridized positively with probe CoSRB385 de-

signed for members of the completely oxidizing sulfate-reducing deltaproteobacterial family *Desulfobacteraceae*, whose members oxidize a wide substrate spectrum, including acetate to CO₂, in competitive hybridization with the nearly identical (single mismatch) probe lnSRB385 for the incompletely oxidizing families *Desulfobulbaceae* and *Desulfovibrionaceae*, whose oxidation pathways stop at acetate and thus do not complete the remineralization of organics to CO₂ (Rabus *et al.*, 1996; Figs 2 and 3). The filamentous endobionts did not hybridize with probe lnSRB385 during competitive hybridization with the lnSRB/CoSRB385 probe combination. Filamentous endobionts were also observed with probe DSS658 (Manz *et al.*, 1998) targeting the *Desulfosarcinales* lineage within the *Desulfobacteraceae* (Fig. 4); the hybridization experiment with this probe aimed at detecting a wide range of members of the *Desulfosarcinales*, the dominant group of deltaproteobacterial sulfate reducers in marine sediments (Ravenschlag *et al.*, 2000; Klepac *et al.*, 2004). The filamentous endobionts did not yield a hybridization signal with 16S rRNA gene probes for archaea, *Beta*- and *Gammaproteobacteria*, and *Alphaproteobacteria* (with probes and procedures as described previously; Teske *et al.*, 1995) and *Cytophaga*–*Flavobacter*–*Bacteroides* phylum (Manz *et al.*, 1996) (data not shown).

In most cases, the filamentous bacteria were observed within the *Thioploca* sheaths, following the length axis of the sheath and the lengthwise orientation of the *Thioploca* filaments within the sheath; therefore, they are referred to as endobionts. Within their overall lengthwise orientation,

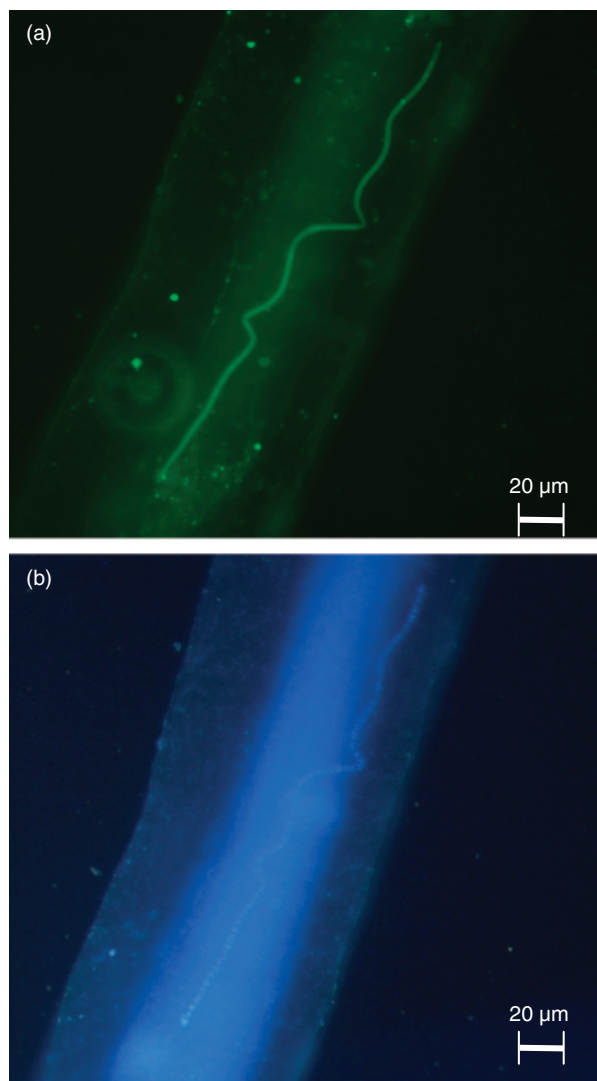


Fig. 2. (a) FISH of a *Thioploca* bundle with a filamentous sheath endobiont bacterium of c. 360 µm length, using competitive 16S rRNA gene probe combination fluorescein-labeled 385CoSRB (green) and rhodamine-labeled 385InSRB (red; no visible hybridization signal, not shown). (b) The same filament counterstained with DAPI, and viewed under UV excitation. The sample is from Station 18 at 4–5 cm sediment depth. The scale bar corresponds to 20 µm.

the filaments can turn around and bend back on themselves in single or even multiple loops (Fig. 3). In a few cases, filaments were also found on the surface of the *Thioploca* sheaths, forming loosely attached loops and coils that may represent preparation artifacts.

The filamentous endobionts were observed in the upper sediment layers, down to 4–5-cm sediment depth, but disappeared almost entirely below 5 cm depth, with the exception of a single *Thioploca* bundle (Table 1). The hybridized endobionts have a filament diameter of c. 4–5 µm (Figs 2–4). Viewed under UV excitation, the filaments

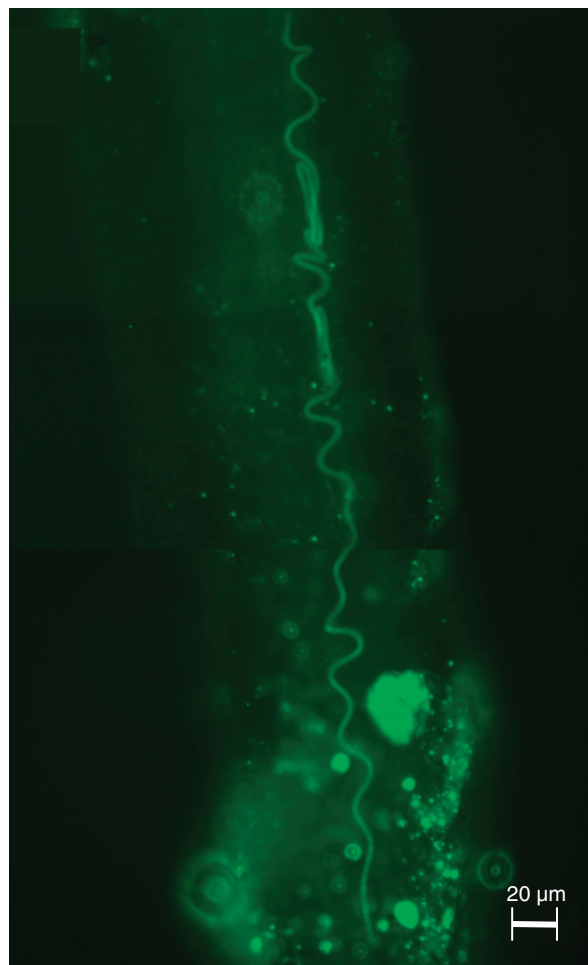


Fig. 3. FISH of a *Thioploca* bundle with a filamentous sheath endobiont bacterium of c. 1 mm length, using competitive 16S rRNA gene probe combination fluorescein-labeled 385CoSRB (green) and rhodamine-labeled 385InSRB (red; no visible hybridization signal, not shown). The figure is a composite of two adjacent image frames. The sample is from Station 18 at 4–5 cm sediment depth. The scale bar corresponds to 20 µm.

showed bright DAPI-stained segments that probably correspond to individual cells, c. 4 µm in width and 6–8 µm in length (Fig. 2b). The filaments can reach considerable lengths of 1 mm and more (Figs 3, 4, and S1). The 0.36 mm length of the DAPI stained and hybridized filament in Fig. 2a and b matches the length range of shorter observed filaments (examples measured 0.22, 0.45, 0.70, and 0.75 mm, not shown). The observed filament lengths are probably conservative estimates, because length may be affected by cutting or any mechanical disturbance of *Thioploca* bundles before and during sample preparation, FISH, and DAPI staining.

The endobionts occur in the upper few centimeter of the *Thioploca* mats (upper 4–5 cm) and appear to grow within the sheaths of active *Thioploca* bundles; they were never observed within the blackish sheaths of decaying *Thioploca*

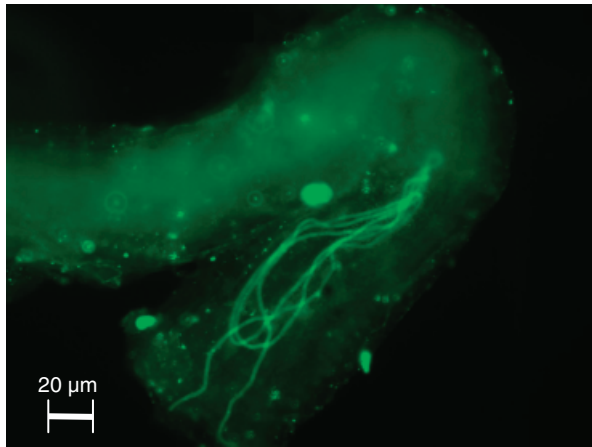


Fig. 4. FISH of a *Thioploca* bundle with a filamentous sheath endobiont bacterium of > 1 mm length, using fluorescein-labeled 16S rRNA gene probe DSS658 (green) for members of the *Desulfosarcinales*. The sample is from Station 18 at 1–2 cm sediment depth. The scale bar corresponds to 20 μm .

bundles (Gallardo, 1992). The length ratio of integrated endobiont filaments and underlying *Thioploca* sheath was determined for a sample set from Station 7; the length ratio increased toward the sediment surface by at least an order of magnitude (Table 1, right column). These upper sediment layers were also characterized by a high abundance of cultivable sulfate-reducing bacteria, determined at Station 7 by MPN counts, which, to the best of our knowledge, are the first available MPN data for the Chilean *Thioploca* mats (Table 2). The MPN counts indicated the highest concentrations, 4.6×10^6 cells mL^{-1} , in the upper 0.5 cm of the sediment for both lactate and acetate as substrates. These numbers are probably underestimates, because growth was detected in the maximum dilutions tested. At 2–3 and 6–7 cm, the MPN counts amounted to 0.9 and 2.4×10^6 cells mL^{-1} for lactate and acetate. These are among the highest MPN counts of sulfate-reducing bacteria recorded for marine sediments. At 16–17 cm sediment depth, the MPN counts had decreased to 0.02 and 0.009×10^6 cells mL^{-1} on lactate and acetate, < 0.5% of the near-surface MPN counts (Table 2). The MPN counts represent population densities for single-celled sulfate reducers; filamentous sulfate reducers of the genus *Desulfonema* were not found in the MPN tubes, in contrast to previous MPN counts in hypersaline cyanobacterial mats with abundant *Desulfonema* spp. (Teske *et al.*, 1998). In combination with multiple sulfate reduction rate measurements at Station 7 in 1994 and 2006, the integrated MPN counts (lactate plus acetate) allowed the calculation of sulfate reduction rates per cell, in the range of 30–200 fmol sulfate reduced per cell and day within the upper *Thioploca* mat sediments (Table 2). These cell-specific rates are overestimates, probably by several orders of magnitude, due to

their dependence on cultivation-based MPN counts, which underestimate sulfate-reducing cell densities compared with molecular quantifications (Sahm *et al.*, 1999) or pure culture experiments with known cell densities (Jørgensen, 1978).

Discussion

Repeated FISH surveys of *Thioploca* spp. sampled in 1994 (Fukui *et al.*, 1999), 1997, and 2006 (this study) have detected highly conspicuous, filamentous bacterial endobionts in *Thioploca* sheaths. During this interval, *Thioploca* spp. and the associated endobionts survive El Niño years (1998) characterized by oxygenated bottom water, significantly reduced *Thioploca* populations, and reduced sulfate-reducing activity in the bottom sediment (Schubert *et al.*, 2000). Thus, the endobionts represent consistently recurring, resilient members of the *Thioploca* mat ecosystem. Their benthic environment is characterized by peak sulfate reduction rates, high numbers of cultivable sulfate-reducing bacteria, and cell-specific sulfate reduction rates that are in the range characteristic for highly organic-rich microbial mats and marine sediments.

FISH results constrain the identity of the filamentous endobionts in several ways, and, while not definitive, support the working hypothesis that the filaments are novel members of the filamentous, sulfate-reducing genus *Desulfonema*. The observed hybridization pattern with competitive probes CoSRB385 (positive) and InSRB385 (negative) is characteristic for members of the *Desulfobacteriaceae* within the *Deltaproteobacteria* (Rabus *et al.*, 1996). As a caveat, the probe combination CoSRB385 and InSRB385 also matches various gram-positive bacteria and *Deltaproteobacteria* that do not reduce sulfate, such as *Geobacteraceae*, *Myxobacteraceae*, and the genus *Nitrospina*; thus, the probes are selective, but not specific for deltaproteobacterial sulfate reducers (Teske *et al.*, 1996). Positive hybridization with the more specific probe DSS658 (Fig. 4) indicates that filamentous endobionts are members of the *Desulfobacteriales*, a monophyletic, deltaproteobacterial lineage within the *Desulfobacteraceae* that includes the cultured genera *Desulfosarcina*, *Desulfococcus*, *Desulfofaba*, *Desulfofrigus*, *Desulfatibacillum*, the species *Desulfobacterium cetonicum*, and numerous environmental phylotypes from marine sediments and rhizospheres, where they constitute the dominant deltaproteobacterial population (Ravenschlag *et al.*, 2000; Klepac *et al.*, 2004). The only known filamentous members of the *Desulfobacteraceae* and the *Desulfobacteriales* are cultured species of the genus *Desulfonema* (*Desulfonema magnum*, *Desulfonema limicola*, and *Desulfonema ishimotoni*). *Desulfonema* is not a monophyletic genus, and the 16S rRNA gene sequences of different *Desulfonema* species form divergent lineages within the *Desulfobacteriales*; therefore,

Table 2. Summary of abundance and activity of sulfate-reducing bacteria (SRB) and *Thioploca* spp. at Station 7

	(A)	(B)	(C)	(D)	(E)	(F)
Sediment layer (cm)	<i>Thioploca</i> biomass (mg mL ⁻¹)	SRB mL ⁻¹ MPN with Lactate	SRB mL ⁻¹ MPN with acetate	Sulfate reduction rates (nmol mL ⁻¹ day ⁻¹)	Sulfate reduction rates (nmol mL ⁻¹ day ⁻¹)	Sulfate reduced per cell (fmol cell ⁻¹ day ⁻¹)
0–1	4.36	≥ 4 600 000	≥ 4 600 000	1050*	721	≤ 78.4–114.13
1–2	3.82	ND	ND	1100	357	
2–3	2.95	900 000	2 400 000	650	145	43.9–197.0
3–4	2.43	ND	ND	450	134	
4–5	1.40	ND	ND	350	107	
5–6	0.80	ND	ND	350	62	
6–7	0.28	900 000	2 400 000	NDL	71	21.5
7–8	0.12	ND	ND	NDL	53	
8–9	0.09	ND	ND	200	55	
9–10	0.20	ND	ND	200	48	
10–11	0.11	ND	ND	NDL	43	
11–12	0.04	ND	ND	NDL	43	
12–13	0.04	ND	ND	NDL	42	
13–14	0.04	ND	ND	NDL	42	
14–15	0.05	ND	ND	NDL	42	
15–16	0	ND	ND	NDL	42	
16–17	0	20 000	9000	NDL	39	1345

Datasets from: (A) Station 7, January 2006. The biomass data for 0–9 cm depth are averages from four cores; the data for 9–17 cm depth are from a single core (L. Holmkvist, E. Arning, K. Küster *et al.*, unpublished data). (B and C) Station 7, January 1997. MPN quantification of sulfate-reducing bacteria (SRB) with lactate and acetate as substrates. ND, not determined (this study). (D) Station 7, March 1994. The rate marked with an asterisk is the average of two separately measured rates for the 0–0.5 and 0.5–1 cm sediment layers. NDL, near detection limit based on published data plot (Thamdrup & Canfield, 1996). (E) Station 7, March 1994 (Ferdelman *et al.*, 1997). Sulfate reduction rates measured in January and February 1997 (B. Strotmann, unpublished data) and January 2006 (L. Holmkvist, E. Arning, K. Küster *et al.*, unpublished data) have yielded similar average rates and depth profiles. Profiles change toward lower sulfate reduction rates near the sediment surface in El Niño years, such as 1998 (Schubert *et al.*, 2000). (F) Cell-specific sulfate reduction rates calculated from sulfate reduction rates in column 4 and 5, divided by the sum of MPN (acetate) plus MPN (lactate). These and similar MPN-based cell-specific sulfate reduction rates from hypersaline cyanobacterial mats (64–1490 fmol cell⁻¹ day⁻¹; Teske *et al.*, 1998), are overestimates by at least two orders of magnitude, due to systematic underestimates of sulfate-reducing cell numbers by cultivation-dependent MPN quantification compared with molecular methods. Cell-specific sulfate reduction rates for pure cultures are in the range of 0.2–50 fmol cell⁻¹ day⁻¹ (Jørgensen, 1978), and cell-specific rates based on quantitative 16S rRNA gene blotting are between 0.01 and 0.09 fmol cell⁻¹ day⁻¹ (Sahm *et al.*, 1999).

single *Desulfonema*-targeted FISH probes do not allow zero-mismatch detection of all *Desulfonema* species (Fukui *et al.*, 1999). Further, the three cultured *Desulfonema* species show single mismatches to probe DSS658. A more detailed, species-specific identification of the endobionts will require 16S rRNA gene sequences derived from individual endobiont filaments, as a basis for the development of species- and strain-specific FISH probes. However, the working hypothesis that these filamentous endobionts represent novel species of the sulfate-reducing genus *Desulfonema* within the *Desulfobacteriales* is consistent with biogeochemical habitat characteristics, observed occurrence patterns, and morphological evidence.

Habitat preference and activity of sulfate-reducing bacteria

The MPN counts showed a strong preference of sulfate-reducing bacteria for the top layer of the *Thioploca* mat and sediment, consistent with the observed vertical occurrence pattern of the filamentous endobionts (Table 2). At Station

7, the MPN counts for sulfate-reducing bacteria increase toward their maximum at the surface of the *Thioploca* mats and sediment (Table 2), coinciding with the maximal biovolume of *Thioploca* per milliliter sediment (Schulz *et al.*, 1996), maximal *Thioploca* biomass, and with maximal sulfate reduction rates of *c.* 700–1500 nmol mL⁻¹ day⁻¹ (Thamdrup & Canfield, 1996; Ferdelman *et al.*, 1997; L. Holmkvist, E. Arning, K. Küster *et al.*, unpublished data). The MPN-based cell-specific sulfate reduction rates in the *Thioploca* mat sediments (Table 2) were similar to MPN-based cell-specific sulfate reduction rates within hypersaline cyanobacterial mats, characterized by a highly active sulfur cycle (Teske *et al.*, 1998). Thus, the sulfate-reducing bacterial population and activity maxima coincide with the biomass maxima of sulfur-oxidizing *Thioploca* spp. near the sediment surface, indicative of a highly active microbial sulfur cycling.

Consistent morphology

The filament diameter of 4–5 µm and filament length of up to 1 mm or longer falls into the range of cultured species of

the genus *Desulfonema*, *D. magnum*, *D. limicola*, and *D. ishimotonii* (Widdel *et al.*, 1983; Fukui *et al.*, 1999), and is congruent with the largest size class of *Desulfonema* filaments (*c.* 4–5 μm diameter) that were detected on the surface of *Thioploca* sheaths by FISH hybridization with *Desulfonema*-targeted probe DNMA657 (Fukui *et al.*, 1999). This probe matches the 16S rRNA gene sequences of *D. magnum* and *D. limicola*, and has a single mismatch with *D. ishimotonii* (Fukui *et al.*, 1999).

Habitat preference of *Desulfonema*

The genus *Desulfonema* is a characteristic community component of sulfur-oxidizing as well as phototrophic microbial mats: *Desulfonema*-related *dsrAB* gene clones were consistently obtained from freshwater *Thioploca* sheaths sampled in Germany and Japan (Kojima *et al.*, 2006). Further, molecular and cultivation surveys demonstrated that members of the genus *Desulfonema* constitute dominant sulfate-reducing bacterial populations in hypersaline cyanobacterial mats, and grow in strongly stratified abundance patterns with occurrence maxima in the uppermost mat layers (*c.* 2–3 mm) at the oxycline (Teske *et al.*, 1998; Minz *et al.*, 1999a, b).

Implications for microbial ecosystem structure and functioning

The initial discovery of *Thioploca*–*Desulfonema* associations (Fukui *et al.*, 1999) suggested a spatially close sulfur-cycling system, where the sulfide produced by *Desulfonema* is rapidly reoxidized as an electron and energy source by *Thioploca* spp. The low sulfide concentrations (often below 10 μM) in active *Thioploca* sediments would be consistent with this effective mode of sulfur cycling (Hüttel *et al.*, 1996; Zopfi *et al.*, 2008). The key environmental control factor that determines the depth stratification of the endobiont filaments remains to be identified. Because sulfate-reducing bacteria are quite tolerant to high sulfide concentrations, and *in situ* sulfide concentrations remain low within the upper 10–20 cm of the sediment, sulfide removal by *Thioploca* spp. is probably not the sole control factor. Other potential control parameters are the availability and quality of organic carbon substrates within the sediment or *Thioploca* sheaths, or the availability of reduced nitrogen species provided by *Thioploca* spp. Interestingly, all currently known *Desulfonema* species have been cultured with ammonia as the principal nitrogen source in sulfate-reducer medium (Widdel & Pfennig, 1981).

The endobionts show a widespread phenomenon in the *Thioploca* mats in which different filamentous bacteria share the same sheath. *Thioploca* species with different filament diameters frequently share a common sheath; these mixed sheaths account for *c.* 20% of randomly picked *Thioploca*

sheaths (Schulz *et al.*, 1996). The smaller species, *T. chileae*, (filament diameter 12–22 μm) occurs quite frequently (17% of all sheaths examined) in sheaths dominated by the larger species *T. araucae* with a filament diameter between 28 and 42 μm ; the reverse pattern, *T. araucae* colonizing of sheaths dominated by *T. chileae*, is less common (3%) (Schulz *et al.*, 1996). These field observations suggest that filamentous bacteria with a smaller diameter can colonize the sheaths of larger filaments more easily than vice versa. Because of their smaller filament diameter of *c.* 4–5 μm , endobionts apparently have no difficulty colonizing sheaths dominated by *T. chileae* (predominant at Station 18) and *T. araucae* (predominant at Station 7). Light microscopy observations revealed filamentous bacteria with diameters of around 5 μm (range 1–8 μm) in large numbers within *Thioploca* sheaths of both species at Stations 7 and 19 (Schulz *et al.*, 1996); specific members of this intra-sheath filament community could belong to the endobiont population reported in this study. Similar colonization pathways could apply to other nonfilamentous bacteria that have been detected in the *Thioploca* sheaths (Muyzer & Ramsing, 1995; Kojima *et al.*, 2006).

The *Thioploca* endobionts fall into the size range of recently described, complex communities of free-living, morphologically diverse, filamentous macrobacteria from the surficial sediments and *Thioploca* mats of the eastern South Pacific continental shelf. The morphologies are varied, with diameters ranging from < 1 to < 10 μm , and lengths from around 10 μm to usually several hundreds and at times several thousands of micrometers (Gallardo & Espinoza, 2007a, b). Some overlap between these free-living filamentous populations and the *Thioploca*-associated filamentous endobionts is clearly possible.

These findings highlight the unexplored complexity of sulfur-cycling filamentous microbial communities in *Thioploca* mats and their sediments. The communities of (1) free-living, filamentous sulfur-cycling macrobacteria, (2) mat-forming megabacteria, such as large *Beggiatoa* and *Thioploca* spp., (3) filamentous endobionts growing on and within the sheath of *Thioploca* spp. that are most likely members of the sulfate-reducing genus *Desulfonema*, and (4) free-living sulfate-reducing and sulfur-oxidizing bacteria (e.g. Brinkhoff *et al.*, 1999) within the surficial sediments represent a complex, sulfur-oxidizing and sulfate-reducing microbial ecosystem that catalyzes the highly dynamic sulfur cycle in numerous locations along the eastern South Pacific region, and requires more detailed analysis by coordinated cultivation-based and molecular approaches. The specific identification of selected free-living and endobiotic populations and their metabolic activities will require species- or strain-specific FISH hybridization based on 16S rRNA gene sequencing and probe design, uptake tests with radiolabeled substrates and microautoradiography of specific filaments,

or enrichment and cultivation of individual strains for a closer examination.

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References

- Brinkhoff T, Muyzer G, Wirsén CO & Kuever J (1999) *Thiomicrospira chilensis* sp. nov., a mesophilic obligately chemolithoautotrophic sulfur-oxidizing bacterium isolated from a *Thioploca* mat. *Int J Syst Bacteriol* **49**: 875–879.
- Ferdelman TG, Lee C, Pantoja S, Harder J, Bebout BM & Fossing H (1997) Sulfate reduction and methanogenesis in a *Thioploca*-dominated sediment off the coast of Chile. *Geochim Cosmochim Acta* **61**: 3065–3079.
- Fossing H, Gallardo VA, Jørgensen BB *et al.* (1995) Concentration and transport of nitrate by the mat-forming sulphur bacterium *Thioploca*. *Nature* **374**: 713–715.
- Fukui M, Teske A, Assmus B, Muyzer G & Widdel F (1999) Physiology, phylogenetic relationships, and ecology of filamentous sulfate-reducing bacteria (Genus *Desulfonema*). *Arch Microbiol* **172**: 193–203.
- Gallardo VA (1992) On the presence of metal stained organic material in *Thioploca* shelf bottoms off Bay of Concepcion, Chile. *Gayana Oceanol* **1**: 27–33.
- Gallardo VA & Espinoza C (2007a) New communities of large filamentous sulfur bacteria in the Eastern South Pacific. *Int Microbiol* **10**: 97–102.
- Gallardo VA & Espinoza C (2007b) Large multicellular filamentous bacteria under the oxygen minimum zone of the eastern South Pacific: a forgotten biosphere. Instruments, Methods, and Missions for Astrobiology X. *Proc SPIE* **6694**: H1–H11.
- Hüttel M, Forster S, Klöser S & Fossing H (1996) Vertical migration in the sediment-dwelling sulfur bacteria *Thioploca* spp. in overcoming diffusion limitations. *Appl Environ Microbiol* **62**: 1863–1872.
- Jørgensen BB (1978) A comparison of methods for the quantification of bacterial sulfate reduction in coastal marine sediments. III. Estimation from chemical and bacteriological field data. *Geomicrobiol J* **1**: 49–64.
- Jørgensen BB & Gallardo VA (1999) *Thioploca* spp.: filamentous sulfur bacteria with nitrate vacuoles. *FEMS Microbiol Ecol* **28**: 301–313.
- Klepac V, Bahr M, Crump BC, Teske AP, Hobbie JE & Polz MF (2004) High overall diversity and dominance of microdiverse relationships in salt marsh sulphate-reducing bacteria. *Environ Microbiol* **6**: 386–398.
- Kojima H, Koizumi Y & Fukui M (2006) Community structure of Bacteria associated with sheaths of freshwater and brackish *Thioploca* species. *Microb Ecol* **52**: 765–773.
- Manz W, Amann R, Ludwig W, Vancanneyt M & Schleifer KH (1996) Application of a suite of 16S rRNA specific oligonucleotides designed to investigate bacteria of the phylum *Cytophaga-Flavobacter-Bacteroides* in the natural environment. *Microbiology* **142**: 1097–1106.
- Manz W, Eisenbrecher T, Neu TR & Szewzyk U (1998) Abundance and spatial distribution of gram-negative sulfate-reducing bacteria in activated sludge investigated by in-situ probing with specific 16S rRNA targeted oligonucleotides. *FEMS Microbiol Ecol* **25**: 43–61.
- Minz DJ, Flax J, Green SJ, Muyzer G, Cohen Y, Wagner M, Rittmann BE & Stahl DA (1999a) Diversity of sulfate-reducing bacteria in oxic and anoxic regions of a microbial mat characterized by comparative analysis of dissimilatory sulfite reductase genes. *Appl Environ Microbiol* **65**: 4666–4671.
- Minz DJ, Fishbain S, Green SJ, Muyzer G, Cohen Y, Rittmann BE & Stahl DA (1999b) Unexpected population distribution in a microbial mat community: sulfate-reducing bacteria localized to the highly oxic chemocline in contrast to a eukaryotic preference for anoxia. *Appl Environ Microbiol* **65**: 4659–4665.
- Muyzer G & Ramsing NB (1995) Molecular methods to study the organization of microbial communities. *Water Sci Technol* **32**: 1–9.
- Otte S, Kuenen JG, Nielsen LP, Pearl HW, Zopf J, Schulz HN, Teske A, Strothmann B, Gallardo VA & Jørgensen BB (1999) Nitrogen, carbon, and sulphur metabolism in natural *Thioploca* samples. *Appl Environ Microbiol* **65**: 3148–3157.
- Rabus R, Fukui M, Wilkes H & Widdel F (1996) Degradative capacities and 16S rRNA-targeted whole-cell hybridization of sulfate-reducing bacteria in an anaerobic enrichment culture utilizing alkylbenzenes from crude oil. *Appl Environ Microbiol* **62**: 3605–3613.
- Ravenschlag K, Sahn K, Knoblauch C, Jørgensen BB & Amann R (2000) Community structure, cellular rRNA content, and activity of sulfate-reducing bacteria in marine arctic sediments. *Appl Environ Microbiol* **66**: 3592–3602.
- Sahn K, MacGregor BJ, Jørgensen BB & Stahl DA (1999) Sulphate reduction and vertical distribution of sulphate-reducing bacteria quantified by rRNA slot-blot hybridization in a coastal marine sediment. *Environ Microbiol* **1**: 65–74.
- Schubert CJ, Ferdelman TG & Strotmann B (2000) Organic matter composition and sulfate reduction rates in sediments off Chile. *Org Geochem* **31**: 351–361.
- Schulz HN, Jørgensen BB, Fossing HA & Ramsing NB (1996) Community structure of filamentous, sheath-building sulfur

- bacteria, *Thioploca* spp., off the coast of Chile. *Appl Environ Microbiol* **62**: 1855–1862.
- Schulz HN, Strotmann B, Gallardo VA & Jørgensen BB (2000) Population study of the filamentous sulfur bacteria *Thioploca* spp. off the Bay of Concepción, Chile. *Marine Ecol-Prog Ser* **200**: 117–126.
- Teske A, Ramsing NB, Küver J & Fossing H (1995) Phylogeny of *Thioploca* and related filamentous sulfide-oxidizing bacteria. *Syst Appl Microbiol* **18**: 517–526.
- Teske A, Wawer C, Muyzer G & Ramsing NB (1996) Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager Fjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments. *Appl Environ Microbiol* **62**: 1405–1415.
- Teske A, Ramsing NB, Habicht K, Fukui M, Küver J, Jørgensen BB & Cohen Y (1998) Sulfate-reducing bacteria and their activities in cyanobacterial mats of Solar Lake, Sinai (Egypt). *Appl Environ Microbiol* **64**: 2943–2951.
- Teske A, Sogin ML, Nielsen LP & Jannasch HW (1999) Phylogenetic position of a large marine *Beggiatoa*. *Syst Appl Microbiol* **22**: 39–44.
- Thamdrup B & Canfield DE (1996) Pathways of carbon oxidation in continental margin sediments off central Chile. *Limnol Oceanogr* **41**: 1629–1650.
- Widdel F & Bak BF (1992) Gram-negative mesophilic sulfate-reducing bacteria. *The Prokaryotes*, 2nd edn (Balows A, Trüper HG, Dworkin M, Harder W & Schleifer KH, eds), pp. 3352–3378. Springer Verlag, New York.
- Widdel F & Pfennig N (1981) Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. I. Isolation of new sulfate-reducing bacteria enriched with acetate from saline environments. Description of *Desulfobacter postgatei* gen. nov., sp. nov. *Arch Microbiol* **129**: 395–400.
- Widdel F, Kohring GW & Meyer F (1983) Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. *Arch Microbiol* **134**: 286–294.
- Zopfi J, Böttcher ME & Jørgensen BB (2008) Biogeochemistry of sulfur and iron in *Thioploca*-colonized surface sediments in the upwelling area off Central Chile. *Geochim Cosmochim Acta* **72**: 827–843.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. FISH of *Thioploca* bundle with a filamentous sheath endobiont bacterium of c. 1.7 mm length, using competitive 16S rRNA gene probe combination fluorescein-labeled 385CoSRB (green) and rhodamine-labeled 385InSRB (red; no visible hybridization signal, not shown).

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