Transient development of filamentous *Thiothrix* species in a marine sulfide oxidizing, denitrifying fluidized bed reactor

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Abstract

In this study, microscopic and molecular microbial analyses were integrated to characterize rapidly developing white filamentous tufts in a fluidized bed reactor used for nitrate removal from a marine recirculating fish culture system. Formation and rapid elongation of the tufts (often exceeding 50 mm day $^{-1}$) was strongly correlated to transient elevated sulfide concentrations ($> 50\,\mu M$) in the reactor. The dominant bacterial constituents of these tufts were filamentous gram-negative bacteria with densely packed intracellular sulfur granules. Using 16S rRNA gene analysis and fluorescence *in situ* hybridization it was found that these filamentous bacteria represented a novel *Thiothrix* phylotype closely related (97% sequence identity) to a previously identified *Thiothrix* strain endogenous to the marine crustacean *Urothoe poseidonis*. In addition to filamentous morphotypes, rosette-shaped morphotypes of *Thiothrix* were also detectable within the tufts.

Introduction

Sulfidic environments, such as marine sediments (Mussmann *et al.*, 2003), hydrothermal vents (Ruby *et al.*, 1981), sulfur-rich streams (Engel *et al.*, 2003; Rudolph *et al.*, 2004) and wastewater reactors (Kanagawa *et al.*, 2000; Nielsen *et al.*, 2000), are often characterized by dense white tufts or mats. The major constituents of these complex structures are filamentous sulfide oxidizing bacteria, such as certain *Epsilonproteobacteria* (Engel *et al.*, 2003; Rudolph *et al.*, 2004), and *Gammaproteobacteria*, such as *Beggiatoa* (Larkin & Strohl, 1983; Martinez *et al.*, 1997), *Thioploca* (Larkin & Strohl, 1983; Kojima *et al.*, 2003) and *Thiothrix* (McGlannan & Makemson, 1990; Martinez *et al.*, 1997; Moissl *et al.*, 2002; Ferrera *et al.*, 2004).

The filamentous, colorless sulfur-oxidizing *Thiothrix* genus are metabolically versatile with various species capable of autotrophic, mixotrophic and heterotrophic growth (Larkin & Strohl, 1983; Nielsen *et al.*, 2000). *Thiothrix* species are generally considered to be obligate aerobes, although some strains are metabolically active under denitrifying conditions (Nielsen *et al.*, 2000). Formation of intracellular sulfur granules in the presence of sulfide (Larkin & Strohl, 1983) and the formation of rosettes and gonodial holdfast (Larkin & Nelson, 1987) are defining features of this genus. *Thiothrix* species have been previously identified in bioreactors treating sulfide effluents where they are most likely involved in oxygen-mediated sulfide oxidation (Ferrera *et al.*, 2004; Cytryn *et al.*, 2005). In bioreactors and in wastewater treatment plants they often contribute to the problem of sludge bulking (Nielsen *et al.*, 2000; Kanagawa *et al.*, 2000).

On occasions, filamentous tuft formations have also been detected in an environmentally sustainable marine fish culture system. Water in this system is purified via an aerobic trickling filter for nitrification and a parallel anaerobic loop consisting of a digestion basin (DB) and a fluidized bed reactor (FBR), where nitrate and organic carbon are gasified (Gelfand *et al.*, 2003; Cytryn *et al.*, 2003). Anaerobic conditions within the sludge layer of the DB typically stimulate sulfate reduction resulting in sulfide production to concentrations that in some parts of the digester may exceed 3 mM (Cytryn *et al.*, 2003). A significant fraction of this sulfide is reoxidized within less reduced zones in the upper aqueous part of the DB, and further oxidized in the FBR. Under normal operating conditions,

sand particles in the FBR are coated with a thin brownish-colored biofilm (average floc diameter of 1.2 mm). However, on several independent occasions during more than 4 years of system operation, elevated sulfide concentrations in the FBR were associated with the development of dense white filamentous tufts that completely coated the FBR flocs (average diameter of 3.0 mm). During these episodes, filamentous tufts also developed on the walls of the FBR column, in connective tubing and within the settling basin at the FBR outlet. Sulfide amendment (50–100 μM) also stimulated development of filamentous tufts in batch experiments, where filaments formation was observed on the surface of FBR flocs within 2–3 h after amendment (Cytryn *et al*., 2005). Microsensor analysis of these filamentous flocs demonstrated high rates of sulfide oxidation under oxic as well as anoxic conditions in the presence of nitrate (Cytryn *et al*., 2005).

The aim of the present work was to investigate the morphology, phylogeny and chemical composition of filamentous tufts in the above-described marine biofiltration system using a combination of microscopic techniques and molecular analyses, including 16S rRNA gene analysis and fluorescence *in situ* hybridization (FISH).

Materials and methods

System description

The overall structure and performance of the intensive recirculating mariculture system studied here has been previously described in detail (Cytryn *et al*., 2003; Gelfand *et al*., 2003). Briefly, water from the upper part of a double-drain fish basin (2.3 m^3), stocked with Gilthead Seabream (*Sparus aurata*) to a final density of 20–50 $kg\,m^{-3}$, was pump-recirculated at a rate of one to two fish basin volumes per hour over a trickling filter. Simultaneously, water from the bottom center of the fish basin flowed into the DB (working volume of 0.4–0.5 m^3) at a rate of 0.1–0.25 fish basin volumes per hour. Water from the upper layers of the DB outlet was pumped (at rates of 5–7 $L\,min^{-1}$) into the cylindrical FBR through a vertical pipe that extended from the top center to approximately 3 cm above the base of the FBR. The FBR (working volume of 6.26 L) was filled with sand (average diameter 0.7 mm), which served as carrier material for the biofilm. Up-flow from the inlet pipe caused the flocs (biofilm-attached sand grains) to float within the column. Water from the FBR outlet at the top of the cylinder was drained back to the fish basin after passing through a settler for removal of particulate matter, which was funneled back into the DB. Artificial seawater (ASW; salinity 20 ppt) used in the system was prepared by addition of sea salt (Red Sea Pharm Ltd., Eilat, Israel) to tap water. The system was supplemented daily with 500–1000 g of feed (Matmor Ltd., Ashdod, Israel) comprised of 45% fat and 19% protein.

Sampling

Filamentous tufts developed on FBR flocs on several independent occasions during more than 4 years of system operation. In this study, morphological and physiology analyses focused primarily on samples from two periods (December 2001 and May 2004). These samples represented two separate fish growth cycles. In between these cycles, the system was thoroughly cleaned; sludge was removed and system water and FBR sand were replaced. Filament-coated flocs were sampled through faucets situated at set intervals on the FBR column. Alternatively, tufts that coated the settler at the FBR outlet were collected using sterile tweezers. For DNA extraction, approximately 0.05 g of tufts were detached with a sterile scalpel, placed into sterile 2 mL screw-capped DNA extraction tubes and frozen at $-20\,^{\circ}C$ until DNA extraction was performed.

Microscopic analyses

Morphology of individual white filaments and gram staining was visualized via phase contrast microscopy using an Olympus BX40 microscope (Olympus, Tokyo, Japan). Filamentous tufts were examined in a Joel JSM-5410LV scanning electron microscope (Tokyo, Japan). During this procedure, energy dispersive microanalysis (EDS) of selected filaments was carried out using an Oxford link ISIS series No. 300 analyzer (Oxford Instruments, Bucks, UK).

DNA extraction, PCR and denaturing gradient gel electrophoresis (DGGE)

DNA was extracted using a previously described, modified bead-beating method (Oved *et al*., 2001). PCR amplification was performed using the extracted DNA as a template with the general bacterial primer pair 11F and 1392 R (Table 1). In addition, *Thiothrix* phylotypes were identified using a general bacterial forward primer (341F), with an added GC clamp at its 5′ end, and a *Thiothrix*-specific reverse primer (Thio695R) (Table 1). The Thio695R primer was found to be specific for all *Thiothrix* sequences currently present in the GenBank database. Each 50 μL reaction for both PCR reactions contained the following components: 1.5 U Taq DNA polymerase (Red Taq; Sigma, St Louis, MO), Taq buffer containing a final magnesium concentration of 2.5 mM, dNTPs (20 nmol each), 12.5 μg bovine serum albumin, 25 pmol of each primer and 1.2 μL DNA template. For the 11F-1392R primer set, the PCR program was carried out with an initial denaturation step of 95 °C for 60 s followed by 33 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and elongation at 72 °C for 80 s.

Table 1. Probes and primers used in this study

Probe/primer*	Sequence	Target site†	Target organism (s)	Formamide (%)	References
11F	GTTTGATCCTGGCTCAG	16S (11–28)	Domain bacteria	—	Kane *et al*. (1993)
1392R	ACGGGCGGTGTGTRC	16S (1392–1407)	Domain bacteria	—	Lane (1991)
341F‡	CCTACGGGAGGCAGCAG	16S (341–358)	Domain bacteria	—	Muyzer *et al*. (1998)
Gam42a	GCCTTCCCACATCGT TT	23S (1027–1043)	*Gammaproteobacteria*	35	Manz *et al*. (1992)
Bet42a§	GCCTTCCCACTTCGTTT	23S (1027–1043)	*Betaproteobacteria*	35	Manz *et al*. (1992)
G123T	CCT TCCGATCTCTACGCA	16S (697–714)	*Thiothrix*	40	Kanagawa *et al*. (2000)
Thio695R	CTTCCGATCTCTATGCAT	16S (695–713)	*Thiothrix*	—	This study
G1B	TGTGTTCGAGTTCCTTGC	16S (1029–1046)	*Thiothrix eikelboom* type 021N grp I	30	Kanagawa *et al*. (2000)
G2M	GCACCACCGACCCCT TAG	16S (842–859)	*Thiothrix eikelboom* type 021N grp II	35	Kanagawa *et al*. (2000)
G3M	CTCAGGGAT TCCTGCCAT	16S (996–1013)	*Thiothrix eikelboom* type 021N grp IIII	30	Kanagawa *et al*. (2000)

*Primers indicated with F and R suffixes.

†rRNA position according to *Escherichia coli* numbering.

‡DGGE primer attached to 40 bp GC-clamp – 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3′.

§Unlabeled competitor oligonucleotide applied together with probe Gam42a to prevent nonspecific hybridization with β-subclass proteobacteria.

DGGE, denaturing gradient gel electrophoresis.

Cycling was completed with a final elongation step at 72 °C for 2 min. For the 341F-Thio695R primer set, the PCR program was identical, but elongation took place for 30 s. The presence and size of PCR amplicons were determined by agarose gel electrophoresis (2%) and staining with ethidium bromide.

PCR for DGGE was performed using the *Thiothrix* genus-specific primer set 341F and Thio695R (Table 1), both directly on DNA extracted from tufts and on selected colonies from general 16S rRNA gene clone libraries, in order to screen for unique *Thiothrix* phylotypes. DGGE was performed using a D-gene system (BioRad, Hercules, CA), as previously described (Muyzer *et al*., 1998). In this analysis, a 1 mm thick 6% (weight in volume) polyacrylamide gel with a 35–55% denaturing gradient was run for 3.5 h in 1 × TAE buffer at a constant voltage of 250 V. Following electrophoresis, the gels were stained with GelStar nucleic acid stain (Bio Whittaker Molecular Applications Inc., Rockland, ME) and photographed on a UV transillumination table (302 nm) with a Kodak KDS digital camera (Kodak Co., New Haven, CT). Selected DGGE bands were carefully excised on a Dark Reader Transilluminator (Clare Chemical Research Inc., Dolores, CO) and purified as described previously (Muyzer *et al*., 1998).

Cloning and sequence analyses

The pGEM-T Easy vector system (Promega, Madison, WI) was used to clone 11F-1392R PCR products amplified from the December 2001 and May 2004 samples. Ligation and transformation reactions were performed according to the protocol described by the manufacturer. A total of 20 colonies were screened with the *Thiothrix*-specific DGGE primers. Plasmids from the three successfully amplified colonies were purified with the Jet Quick Miniprep Plasmid Purification System (Genomed GmbH, Löhne, Germany) according to the protocol provided by the manufacturer.

Purified plasmids were sequenced using the Applied Biosystems (Foster City, CA) PRISM Dye Terminator Cycle Sequencing Ready Reaction kit with Ampli Taq DNA polymerase and the T7 primer as suggested by the manufacturer. The sequencing products were analyzed with an Applied Biosystems 377 DNA sequencer.

Phylogenetic analysis

The almost complete 16S rRNA gene sequences (11F-1392R) detected in this study were incorporated into a prealigned database of 16S rRNA gene sequences using the aligning tool in the ARB phylogenetic program package (Ludwig *et al*., 2004). Neighbor joining, maximum likelihood and parsimony trees were then constructed with previously described reference sequences using the Felsenstein correction method, and a consensus tree was assembled based on the merged topologies of the three trees (Ludwig *et al*., 2004).

FISH

Slurries of filamentous tufts were fixed with fresh 4% paraformaldehyde solution for 1–2 h, washed twice with phosphate-buffered saline (PBS) and stored in PBS-ethanol (1 : 1) at −20 °C until further processing (Amann *et al*., 1990; Manz *et al*., 1992). Filamentous tufts were either directly used for hybridization analysis or presectioned using a Jung frigocut microcryotome (Leica Microsystems, Nussloch, Germany). After freezing and removal of the plastic material, radial sections with a thickness of 20 µm were prepared at −18 °C, immobilized on gelatin-coated microscope slides, and dehydrated in an ethanol series (Schramm *et al*., 1996). *In situ* hybridizations of cells in the

biofilm were performed with fluorescently labeled, rRNA-targeted oligonucleotide probes according to earlier descriptions (Schramm *et al.*, 1996). The probes used in this study and their specific hybridization conditions are listed in Table 1. Probes labeled with the sulfoindocyanine dyes Cy3 and Cy5 were obtained from either Biometra (Göttingen, Germany) or from Interactiva Biotechnologie (Ulm, Germany). Following hybridization, filamentous tufts were stained with 1 mg L^{-1} 4′, 6′-diamidino-2-phenylindole (DAPI) solution for 10 min in order to estimate the total bacterial amount within the tufts. Samples were analyzed by standard epifluorescence microscopy on a Zeiss Axioplan II microscope and by confocal laser scanning microscope on a Zeiss LSM 510 microscope (Carl Zeiss, Jena, Germany).

Sequence accession numbers

The almost complete 16S rRNA *Thiothrix* gene sequence detected in this study was deposited in Genbank under accession number DQ067608.

Results

Observations on occurrence of white filamentous tufts

Filamentous tufts were detected in the system on several independent occasions during operation of the system between January 2000 and June 2004. In the present study, samples from December 2001 and May 2004, characterized by significant formation of these tufts, were analyzed. Their development was directly correlated to elevated sulfide concentrations in the DB (Fig. 1), which was attributed to increased organic load in this component. Generally, tuft development occurred when FBR inlet concentrations exceeded 50 μM. Under these conditions, tuft elongation was extremely rapid, often exceeding 50 mm day^{-1} (not shown). Once the DB reached equilibrium, sulfide production in the DB and, hence, FBR inlet sulfide concentrations decreased. This resulted in the disappearance of the filamentous tufts, generally within 1–5 weeks.

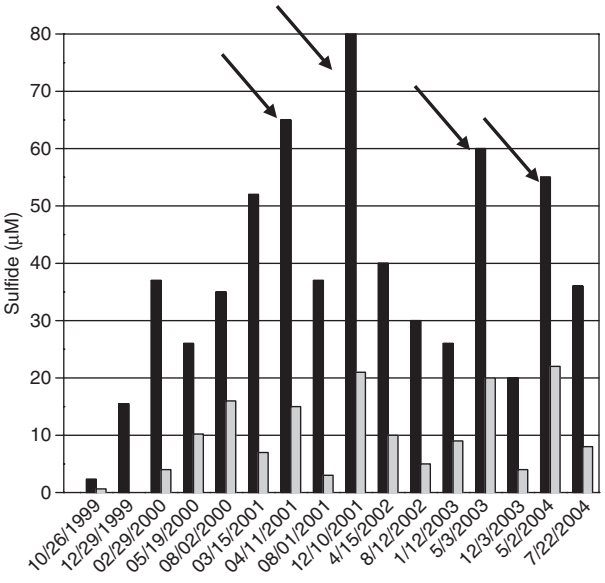

Fig. 1. Inlet (dark gray) and outlet (light gray) sulfide concentrations in the fluidized bed reactor over 4 years of sampling. Arrows indicate periods where filamentous tufts developed.

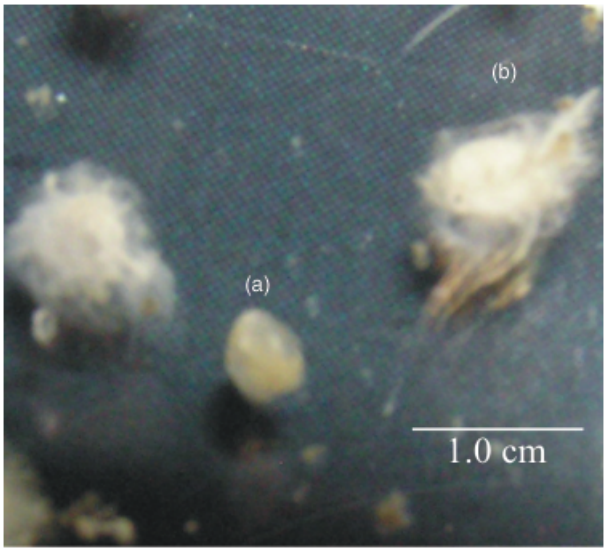

Fig. 2. Photograph showing morphology of plain type (a) and white filament coated (b) fluidized bed reactor flocs.

Microscopic analysis

At different sampling times, the FBR was characterized by occurrence of different floc morphologies (Cytryn *et al.*, 2005). Generally, under normal sulfide concentrations (< 30 μM), brown biofilm-coated flocs were present. However, increased sulfide concentrations (> 50 μM) resulted in the development of white filament-coated flocs (Fig. 2). Microscopic analyses of tufts from the white flocs (from both the FBR flocs and the settler) showed that they were dominated by a gram-negative filamentous bacterium that contained densely packed intracellular sulfur granules (Figs 3a and b). Scanning electron microscopy showed segmentation within the filaments (Fig. 3c) and average length and width of single filament segments were approximately 1.5 and 1 μm, respectively. EDS performed directly on randomly selected filaments showed that they were composed of approximately 10% sulfur, consistent with sulfur granules detected in microscopic analysis.

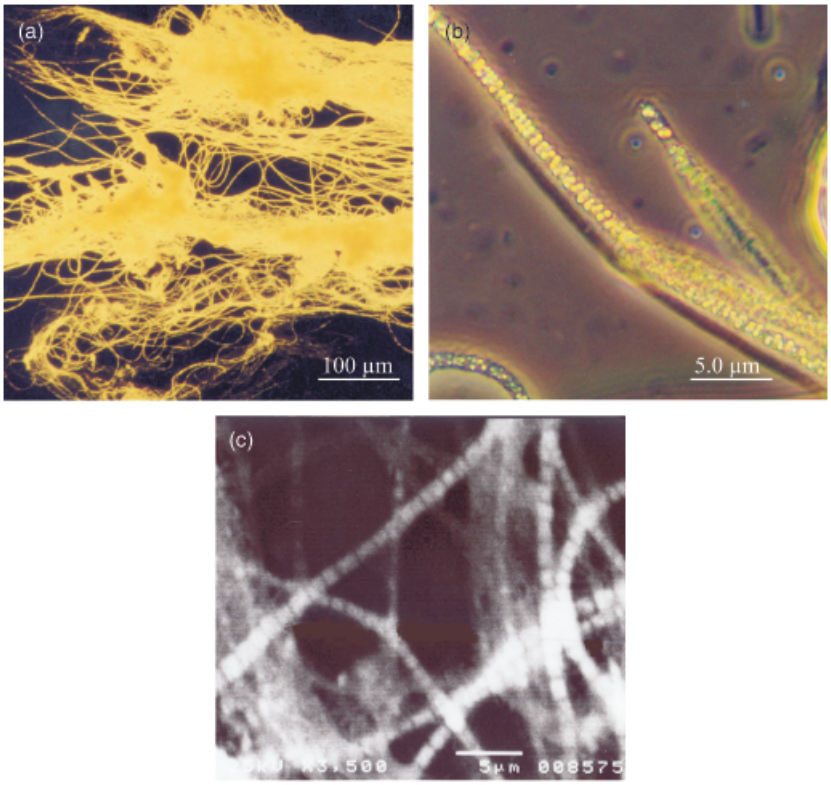

Fig. 3. Low magnification (a), high magnification (b) and scanning electron (c) microscopic analyses of filamentous tufts detached from fluidized bed reactor flocs.

FISH analysis

4′, 6′-diamidino-2-phenylindole staining of the filamentous tufts revealed several bacterial morphotypes (Fig. 4a). One filamentous bacterial morphotype accounted for 50–90% of the total DAPI-stained tuft biomass. These filaments strongly hybridized with the *Thiothrix* genus-specific probe G123T (Figs 4b and c) but not with probes that target previously described *Thiothrix* subgroups (G1B, G2M and G3M; Table 1). In addition to the prevailing filamentous morphotypes, several rosette-like morphotypes (Fig. 4d) also hybridized with both *Gammaproteobacteria* and *Thiothrix* genus-specific probes (Table 1).

Phylogenetic analysis

Denaturing gradient gel electrophoresis analysis of filamentous tufts using the *Thiothrix*-specific primer set Thio695FGC-907R revealed an identical, dominant phylotype in all of the FBR samples analyzed. The almost complete 16S rRNA gene sequence of this *Thiothrix* phylotype was identified by screening clones from 11F-1392R amplified libraries with the *Thiothrix*-specific DGGE primer set described above. Phylogenetic analysis (Fig. 5) showed that this phylotype clustered (97% sequence identity) with an epibiotic filamentous *Thiothrix* strain associated with a marine amphipod crustacean (Gillan & Dubilier, 2004).

Discussion

Elevated sulfide concentrations in the FBR often resulted in rapid developing macroscopic tufts within the FBR column, tubing, pump and settler at the FBR outlet. After cleaning, substantial filaments redevelopment inside these components was visible within 24 h, demonstrating the rapid expansion of these tufts under favorable growth conditions. These filamentous matrices often caused substantial sludge bulking and flow disruption, which had detrimental mechanical/hydrodynamic effects on the FBR functional capacity. Conversely, the sulfide oxidizing capacity of these filaments is crucial for regulating sulfide concentrations in the system, and thus their development safeguards the fish from toxic effects of sulfide carryover from the anaerobic loop during periods of elevated sulfide concentrations (Cytryn *et al.*, 2005).

Microscopy, FISH and 16S rRNA gene analyses demonstrated that filamentous tufts were principally comprised of a singular *Thiothrix* phylotype related to a novel uncultured *Thiothrix* strain endogenous to the marine crustacean *Urothoe poseidonis* (Fig. 5) (Gillan & Dubilier, 2004). Interestingly, sequence analysis indicated that the *Thiothrix* phylotype identified in the FBR tufts shared higher similarity to this marine phylotype than to previously described *Thiothrix* species previously identified in freshwater sulfidic springs (Moissl *et al.*, 2002) or wastewater treatment systems

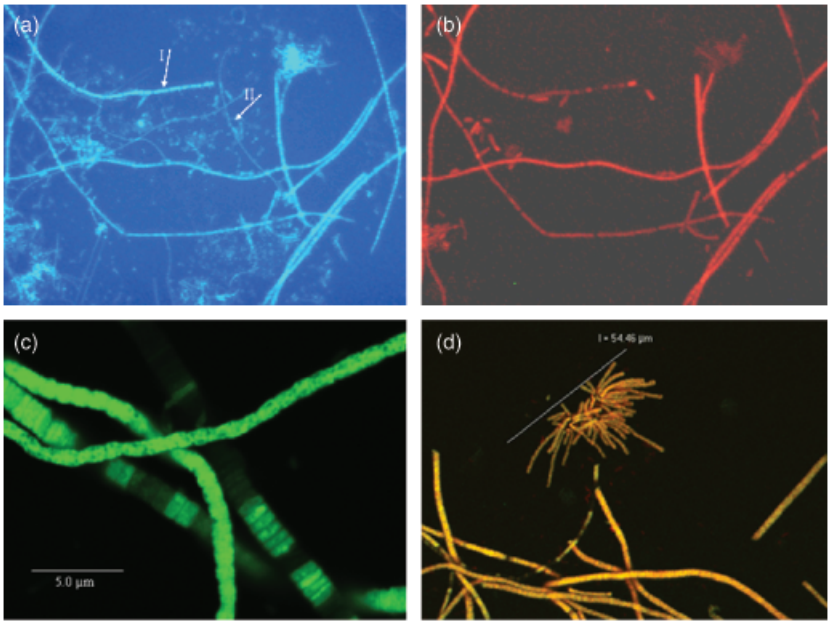

Fig. 4. Microscopic images (epifluorescence and laser scanning microscope) after staining of filamentous tuft sections with fluorescence *in situ* hybridization (FISH). (a) 4′, 6-diamino-2-phenyllidone (DAPI) stain showing the *Thiothrix* filamentous morphotype (I) and an additional filamentous morphotype (II) that did not hybridize with either *Gammaproteobacteria*- or *Thiothrix* genus-specific probes. (b) Image after FISH of the same sample using the CY3-labled *Thiothrix*-genus specific probe G123T. (c) Filament segments after FISH with the CY3-labled *Thiothrix* genus-specific probe G123T. (d) Rosette formation from filamentous tuft sections. Micrograph shows dual hybridization with the CY3-labled *Thiothrix* genus-specific probe G123T and probe Gam42a for members of the *Gamma*-subclass of *Proteobacteria*.

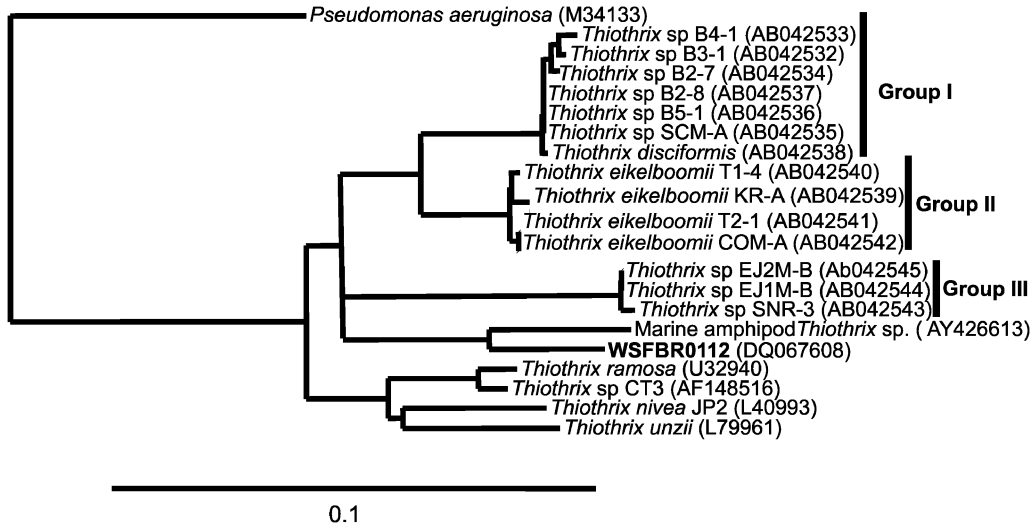

Fig. 5. Phylogenetic tree based on almost complete 16S rRNA gene sequences of the dominant *Thiothrix* phylotype from the fluidized bed reactor (FBR) filamentous tufts (WSFBR0112) in relation to previously described *Thiothrix* strains. The topology of the tree is based on the consensus of trees of > 1000 informative positions generated using the Neighbor Joining, Parsimony and Maximum Likelihood methods (ARB phylogenetic package). Scale bar = 10% estimated difference in nucleotide sequence position.

(Kanagawa *et al.*, 2000; Nielsen *et al.*, 2000; Ferrera *et al.*, 2004). This may suggest a marine-based origin of these strains, and similar strains may also exist in other sulfidic marine environments.

Previous 16S rRNA gene analyses have identified a number of other bacterial phylotypes within these filamentous tufts including *Roseobacter*, *Hydrogenobacter* and *Bacteroidetes* strains (Cytryn *et al.*, 2005; E. Cytryn, unpublished). Presently, it is not clear whether or not a specific association exists between these strains and the *Thiothrix* filaments.

High flow rates and sheering forces in the FBR make surface attachment a crucial characteristic for bacterial survival in these environments. *Thiothrix* species are known to produce polar adhesive structures (holdfasts), leading to cell asymmetry and cell attachment in a distinct orientation (Larkin & Nelson, 1987). Frequently, the cells attach to inanimate surfaces and to one another by polar production

of fimbriae and EPS holdfasts and form rosette-like aggregates (Larkin & Nelson, 1987; Williams *et al.*, 1987). Similar rosette-like formations were also observed within the filamentous tufts examined in this study (Fig. 4d).

Thiothrix growth is generally limited to microaerophilic or aerobic conditions (Larkin & Strohl, 1983). In the FBR, oxygen concentrations are relatively low ($< 50\,\mu M$) and oxygen is generally completely depleted within the top 100 μm of the FBR floc biofilm (Cytryn *et al.*, 2005). Owing to their adhesive capacity, *Thiothrix* species may be able to position themselves within sulfide/oxygen-boundary layers and outward extension via filament formation may enable them to access zones of higher oxygen concentrations, potentially enhancing their growth capacity.

In the course of this research we determined that a single, intracellular sulfur accumulating, *Thiothrix* phylotype was the dominant bacterial component of the filamentous tufts in the FBR. The rapid development of this phylotype following insurgences of sulfide ($> 50\,\mu M$) and the comparatively swift depletion following return to steady-state conditions were indicative of a k-type growth strategy (Andrews & Harris, 1986). Development of this phylotype in the mariculture system appears to be dependent on elevated sulfide concentrations, high-flow environments and microaerophilic or aeaerobic conditions. Future efforts will focus on isolating this phylotype in order to enhance understanding of its physiological characteristics and metabolic capacities.

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