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# Enrichment and identification of large filamentous sulfur bacteria related to *Beggiatoa* species from brackishwater ecosystems of Tamil Nadu along the southeast coast of India

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ABSTRACT

*Beggiatoa* species are filamentous sulfide-oxidizing bacteria belonging to the family *Beggiatoaceae* that contains several largest bacteria known today. These large sulfur bacteria occur in diverse ecosystems and play an important role in the global sulfur, nitrogen and phosphorus cycle. In this study, sediment samples from brackishwater shrimp culture ponds and other brackishwater ecosystems from Tamil Nadu, southeast coast of India, were enriched for *Beggiatoa* species. Extracted hay medium supplemented with catalase was used and were incubated for two weeks at 28 °C. Out of seven set-ups, four yielded positive growth of filamentous sulfide-oxidizing bacteria. The filaments were several millimeters long, ranged in width between 2 and 15 μm and exhibited typical gliding motility. The 16S rRNA gene of four single filaments representing the four positive enrichments was subjected to PCR-DGGE followed by sequencing. All four filaments were affiliated to the *Beggiatoaceae*, but showed less than 89% identity with the *Beggiatoa* type strain *Beggiatoa alba* and less than 93% identity with any other sequence of the family. One of the four filaments revealed a nearly full-length 16S rDNA sequence (1411 bp) and it formed a monophyletic cluster with two of the partial DGGE-16S rRNA gene sequences (99–100% identity) within the *Beggiatoa* species cluster. These organisms could possibly represent a novel genus within the family *Beggiatoaceae*. The fourth partial sequence affiliated with less than 93% sequence identity to the genera *Parabeggiatoa*, *Thioploca* and *Thiopilula*, and was likewise strongly delineated from any sequence published in the family.



## Introduction

Aquaculture is an important economic activity in India and other Southeast Asian countries. Aquaculture involves the use of artificial feeds rich in nitrogen, sulfur and organic compounds that alter the natural carbon, nitrogen and sulfur cycles. Microorganisms such as bacteria, fungi and protozoa carry out active decomposition of left over feed and metabolites, forming inorganic compounds such as ammonia ($NH_3$), hydrogen sulfide ($H_2S$) and carbon dioxide ($CO_2$) via mineralization [19]. In sediments, sulfate and sulfide are constantly recycled by oxidation and reduction steps, which are predominantly carried out by two main groups of prokaryotes – sulfate reducers and sulfide oxidizers [10]. Sulfur-reducing bacteria, such as *Desulfobacter* spp., *Desulfovibrio* spp. and *Desulfuromonas* spp., use sulfate as an electron acceptor, and are thus the primary producers of large quantities of $H_2S$. While this reduction pathway is strictly anaerobic, the oxidation of reduced sulfur compounds can be aerobic or anaerobic. *Archaea*, such as *Sulfolobales* spp., and many bacteria, including *Rhodobacter* spp., *Rhodospirillum* spp., *Thiobacillus* spp., *Paracoccus* spp., *Thiolkalivibrio* spp., *Thiomicrospira* spp., *Thiovulum* spp., *Macromonas* spp. and *Sulfurimonas* spp., are known to be responsible for sulfur oxidation [30]. Filamentous sulfur bacteria, such as the genera *Beggiatoa* and *Thioploca*, are some of the largest and most conspicuous bacteria in nature that are known to oxidize reduced forms of sulfur [28]. They grow at the oxic/anoxic interface and are thus usually found as white mats at the surface or within the top few centimeters of sulfide-rich sediments [22]. These bacteria convert sulfide into elemental sulfur and store it as sulfur granules. These organisms are of considerable importance as they link the local carbon, sulfur and nitrogen cycles [13]. Marine *Beggiatoa* species have been shown to oxidize hydrogen sulfide at rates that are several thousand times higher than the rates of spontaneous chemical oxidation [14,23].

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Reports on the occurrence of such sulfide-oxidizing bacteria in aquaculture ponds are scarce, except for a few reports based on conventional MPN data [1,8]. Hence, this study was undertaken to understand the occurrence and distribution of sulfide-oxidizing bacteria in shrimp culture ponds and other brackishwater ecosystems with special reference to filamentous sulfur bacteria such as *Beggiatoa* species. In the present study, filamentous sulfide-oxidizing bacteria were enriched from aquaculture ecosystems located along the southeast coast of India. The enriched filaments were identified using PCR-DGGE and 16S rRNA gene sequence analysis.

## Materials and methods

### *Sampling*

Sediment samples were collected from shrimp culture ponds with a duration of culture (DOC) of over 60 days and from various brackishwater ecosystems located along the southeast coast of India (Fig. 1 and Table 1). Sediments were sampled using sterile PVC core samplers, collected in sterile containers, transported on ice to the laboratory and processed within 24 h. The latitude and longitude of the sampling sites were recorded using a GPS device (Explorist 210, Canada).

### *Enrichment and isolation*

Enrichment was carried out in extracted hay medium, as described by Cataldi [6]. Briefly, hay was extracted by boiling in tap water for about 30 min and the water was decanted. Repeated boiling and decanting was carried out at least five times with cold tap water rinses between each boiling/decanting step. The extracted hay was left in water overnight, and then decanted and dried at room temperature for two days. Approximately 1 g of dried hay was added to 100 mL of artificial seawater [16] in a 250 mL Erlenmeyer flask and autoclaved. Filter-sterilized catalase was added to the medium at a final concentration of 35 U/mL [33] and then subsequently inoculated with 1–2 g of sediment sample. After 1–2 weeks incubation at 28 °C in the dark, the enrichments were observed for the presence of whitish thread-like mats and tufts in the medium, and these were examined microscopically for the presence of typical *Beggiatoa* filaments.

The tufts from enrichment cultures were washed twice with sterile 0.01% sodium azide solution [33] prepared in artificial seawater, followed by two washes and a 5-min soak in filtered artificial seawater containing catalase (35 U/mL). Washed filaments were inoculated onto five different media (Table 2) for isolation.

### *Microscopy*

Microscopic examination of sediment samples and enrichment cultures was carried out using a Zeiss Axiostar II microscope (Carl Zeiss, Germany) fitted with a digital camera (Jenoptik, Germany), and images were captured using ProgRes (Capture pro 2.1) software. Washed filaments were subjected to scanning electron microscopy after fixing the filaments as described by de Albuquerque et al. [7]. After fixation, the filaments were coated with gold–palladium using an auto fine sputter coater and imaged with a Jeol JSM 6360LV scanning electron microscope (Tokyo, Japan) operating at 15 kV.

### *DNA extraction from single filaments*

Filaments were washed as described above, placed on the medium (with 1% agar) and allowed to glide on the surface [33]. After 6 h incubation at room temperature (30 ± 2 °C), the cultures

**Table 1**
Occurrence of filamentous sulfide-oxidizing bacteria in brackishwater ecosystems based on direct microscopy and by enrichment.

| Sampling site | Latitude/longitud&alinity (ppt) | | Presence of filaments revealed by direct microscopy of samples | Growth of filaments in extracted hay medium | Oxygen preference as deduced from location of filaments in semi-solid media | Shape and arrangement | Diameter (μm) | Motility | Sulfur inclusions | Isolate code | GenBank accession numbers |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Kattur shrimp pond | 13°12.467′N 80°19.136′E | 45 | + | + | Microaerophilic | Single filaments or tufts | 8–10 | Gliding | + | CSO3 | JN674458 |
| Coum River (Barmouth) – two samples collected with a one year interval | 13°04.105′N 80°17.304′E | 34 | + | + | Microaerophilic | Single filaments or tufts | 6–8 | Gliding | + | CSO2 | JN588607 |
| | 13°04.115′N 80°17.324′E | 35 | + | + | Microaerophilic | Single filaments or tufts | 13–15 | Gliding | + | CSO4 | JN674459 |
| Muttukadu shrimp pond | 12°48.533′N 80°14.778′E | 27 | + | + | Microaerophilic | Single filaments or tufts | 5–6 | Gliding | + | CSO1 | HM598303 |
| Kovalam Creek | 12°46.699′N 80°14.978′E | 30 | – | – | – | – | – | – | – | – | – |
| Buckingham Canal (Mamallapuram) | 12°37.937′N 80°11.929′E | 32 | – | – | – | – | – | – | – | – | – |
| Marakkanam paddy field | 12°11.704′N 79°56.685′E | ND | + | + | – | Single filaments | 2–3 | Gliding | – | NA | – |

+, presence; –, absence; NA, not assigned; ND, no data.

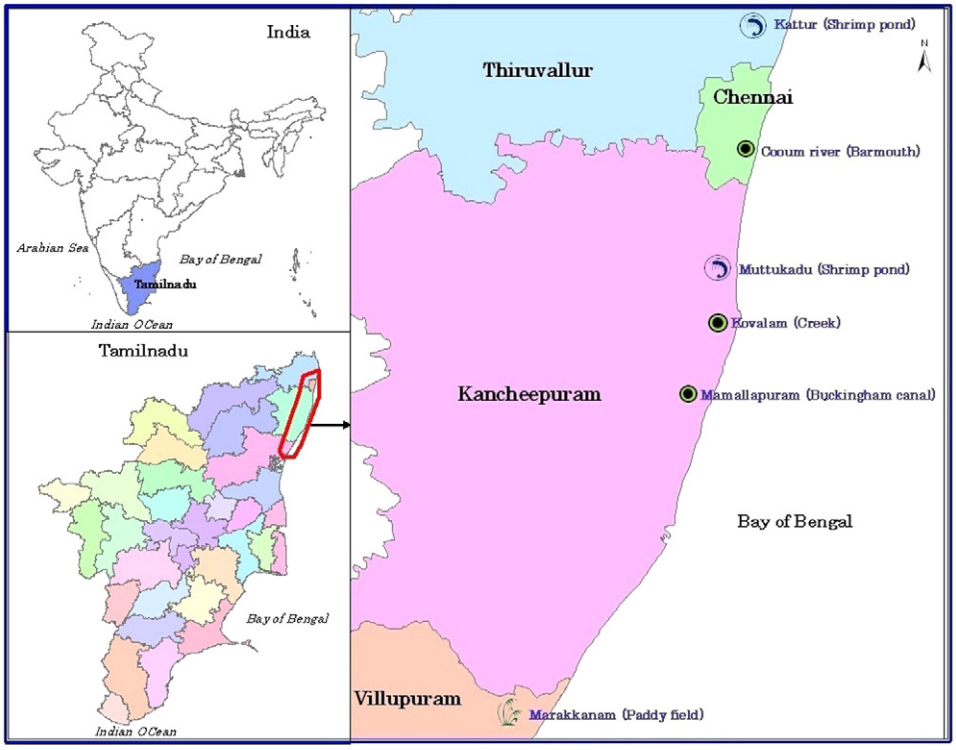


**Fig. 1.** Sites sampled in Tamil Nadu, southeast coast of India, for enrichment and recovery of filamentous sulfur-oxidizing bacteria.

were observed with a stereozoom microscope (Olympus, Japan). Single filaments that glided away from the source of inoculation and that were thus considered to contain no or few contaminants were picked using sterile 10 μL pipette tips and transferred to sterile microcentrifuge tubes. The DNA was extracted using a DNA express kit (Himedia, Mumbai, India) according to the manufacturer's instructions.

### *PCR-DGGE analysis and sequencing*

The extracted DNA was used as a template to amplify the variable V3–V5 region of the 16S rRNA gene using the universal primers 357f-GC and 907rM, as described by Muyzer et al. [20] (Table 3). The total reaction mixture (50 μL) contained 20 pM of each of the primers, 25–50 ng of template DNA, 1× Taq DNA polymerase buffer, 2 U of Taq DNA polymerase (New England Biolabs, Ipswich, USA), 0.2 mM of dNTPs, and 1.5 mM $MgCl_2$. Amplification was carried out using an iCycler (BioRad, Hercules, USA) with initial denaturation at 95 °C for 4 min and 10 touchdown cycles of denaturation at 95 °C for 30 s, annealing at 65 °C for 30 s (with the annealing temperature decreasing by 1 °C in each cycle), extension at 72 °C for 1 min, followed by 20 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and a final extension at 72 °C for 5 min. Amplified products were electrophoresed on a 1.5% agarose gel in 0.5× TBE buffer at 50 V for 45 min, stained with ethidium bromide and visualized using a Gel Documentation system (BioRad, Hercules, USA).

**Table 2**
Growth of filamentous sulfide-oxidizing bacteria in various media.

| Medium used | Survival/other observations | Reference |
|---|---|---|
| BP medium (Pringsheim medium) | 2–3 days | [33] |
| MP medium (modified Pringsheim medium) | 4–5 days | [33] |
| Yeast extract medium | 1 day/heterotrophic contamination | [5] |
| Diluted beef extract medium | 1 day/heterotrophic contamination | [2] |
| Improved isolation medium using calcium alginate | 1 day/heterotrophic contamination, motility of the filaments enhanced | [5] |
| Sulfide-gradient medium | 4–5 weeks | [21] |

The PCR products (~100 ng) were subjected to denaturing gradient gel electrophoresis (DGGE) in a 6% polyacrylamide gel (acrylamide:bis-acrylamide, 37.5:1.0) with a denaturant gradient of 30–60% (with 100% denaturant consisting of 7 M urea plus 40% formamide). DGGE was carried out as described by Muyzer et al. [20] using a DCode apparatus (BioRad, Hercules, USA). Electrophoresis was carried out in 1× TAE buffer at 100 V for 17 h at a constant temperature of 60 °C. The gel was stained for 20 min with SYBR gold nucleic acid stain (1:10,000×) in 1× TAE buffer and visualized using a Gel Documentation system. All bands were excised, eluted, reamplified and checked for purity and migration patterns in DGGE, as described by Ponnusamy et al. [25]. Reamplified products showing single bands were amplified using primers 357f (Non-GC clamped) and 907rM (Table 3). The amplicons were then purified using HiYield PCR Clean-up kit (RBC, Taiwan) and sequenced using an automated DNA sequencer (1st Base, Malaysia).

For the DNA sample that yielded a single band in DGGE profiling (CSO1), the universal primers fD1 and rP2 [38] (Table 3) were used to amplify the nearly full-length sequence of the 16S rRNA gene. The amplification program consisted of initial denaturation at 94 °C for 3 min followed by 30 cycles of 94 °C for 10 s, 56 °C for 1 min, 72 °C for 30 s and a final extension at 72 °C for 5 min. The amplicons were checked, purified and sequenced as described above.

### *Phylogenetic analysis*

The sequences were initially checked for anomalies using Pintail [3]. Phylogenetic trees based on 16S rRNA gene sequences were constructed using the ARB software package [18] and the Silva database release 102 as Ref. [26]. Initially, six trees were calculated

**Table 3**
16S rRNA primers used in this study.

| Primer | Sequence (5′ to 3′) | Reference |
|---|---|---|
| 357f | CCTACGGGAGGCAGCAG | [20] |
| 357f-GC | GCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGG GGGG CCTACGGGAGGCAGCAG | [20] |
| 907rM | CGTCAATTCMTTTGAGTTT | [20] |
| fD1 | GAGTTTGATCCTGGCTCA | [38] |
| rP2 | ACGGCTACCTTGTTACGACTT | [38] |

using neighbor-joining and maximum likelihood algorithms with 0%, 30% and 50% conservatory filters. A total of 285 full-length 16S rRNA gene sequences and nucleotides at positions 252–1463 were considered for tree construction. The three partial sequences (CSO2, CSO3 and CSO4) were added to each tree using parsimony criteria without allowing changes in the general tree topologies. Bootstrap calculation of 1000 runs was performed with maximum likelihood and 0% conservation. Fig. 5 shows an excerpt of the sequences used for calculation, giving a clear overview for the affiliation of the filaments investigated here within the *Beggiatoaceae*. Based on the 0% maximum likelihood tree, multifurcations were introduced at nodes that were both not supported by all treeing conditions [24] and that showed bootstrap values lower than 60%. All nodes retained were supported by all calculation conditions and showed high bootstrap values, as indicated in Fig. 5. Sequence identities were also calculated in ARB.

### *Nucleotide sequence accession numbers*

The partial 16S rRNA gene sequences of the four single filaments CSO1–CSO4 and other co-existing bacteria were deposited in the GenBank/EMBL/DDBJ databases under the accession numbers HM598303, JN588607, JN674458, JN674459, JN804558, JN804559, JN804560 and JN804561.

## Results

### *Enrichment and characterization of filamentous sulfide-oxidizing bacteria*

A total of seven samples from six different sources were enriched for *Beggiatoa*. Visible tufts (macroscopic star shaped colonies) of filamentous sulfide-oxidizing bacteria appeared in five enrichments within two to three weeks of incubation (Fig. 2). Microscopic examination revealed that the filaments consisted of disc-shaped cells that contained sulfur inclusions (Table 1), and these filaments showed gliding motility, which is a typical feature of *Beggiatoa* species. Addition of catalase stimulated the growth of these filaments, revealing a prominent increase in the visibility of tufts in four enrichments, except for enrichment F. The filaments aggregated to tufts on hay and on the surface of the glass. The tufts dispersed when transferred to liquid BP medium without hay, and single filaments exhibited gliding motility on semi-solid BP (1% agar) medium. The filaments in all five enrichments were several millimeters long, whereas the width of the filaments varied from 2 to 15 μm. CSO3 and CSO4 had wider filaments (8–15 μm), whereas CSO1 and CSO2 had thinner filaments (5–8 μm) (Table 1). The filaments from enrichment F were the smallest of the five (2 μm) (Table 1 and Fig. 3). After transferring, the filaments glided towards the subsurface of the soft BP (0.2% agar) medium. On semi-solid (1% agar) BP medium most of the filaments formed rotating coiled structures on the surface with a typical *Beggiatoa* etch, while some glided irregularly on the agar surface. After the addition of pyridine to the filaments, release of sulfur granules and the formation of external sulfur crystals were observed. The scanning electron micrographs revealed the septation in the filaments and the slime that was binding them together (Supplementary Fig. S1).

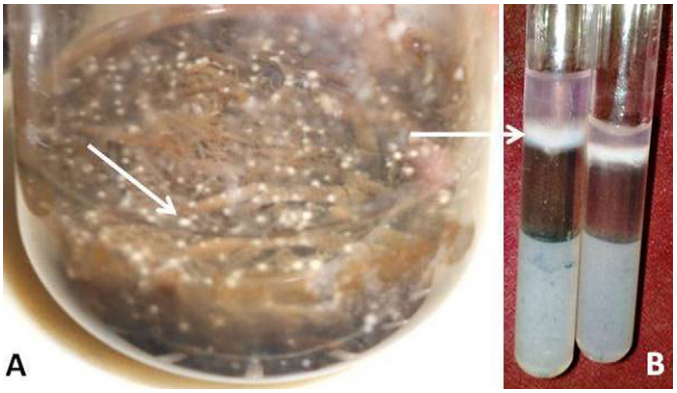


**Fig. 2.** (A) Whitish thread-like tufts indicative of growth of *Beggiatoa* spp. in extracted hay medium. (B) Filaments forming a distinct white mat in sulfide-gradient medium.

Enrichments of filamentous sulfide-oxidizing bacteria recovered from sampling locations A, $B_1$, $B_2$ and C were designated as CSO3, CSO2, CSO4 and CSO1, respectively. The filaments of enrichment CSO1 grew actively on repeated subcultures in extracted hay and sulfide-gradient media, while they failed to survive in BP medium, MP medium or yeast extract medium (Table 2).

### *DGGE and sequencing*

DGGE analysis of 16S rRNA gene PCR products of single filaments retrieved from the four enrichment cultures (CSO1, CSO2, CSO3 and CSO4) showed a specific banding pattern for each of the four filaments. Reproducible DGGE patterns could be obtained from the same DNA extract across multiple PCRs and the gradient range 30–60% was found suitable for identification of such *Beggiatoa*-like enrichments. Enrichment C was sampled four times for single filaments that were similarly processed for PCR-DGGE-based identification, and all four 16S sequences retrieved from the four filaments of enrichment C were 100% identical, indicating that the enrichment could have contained only a small phylogenetic diversity of sulfur bacteria. PCR-DGGE was carried out only once for other enrichments (A, $B_1$ and $B_2$). Enrichment F yielded poor growth and the shortage of intact filaments hampered the analysis by PCR-DGGE. Each of the four enrichments yielded one prominent band along with 2–3 faint bands (Fig. 4). Bands 1 and 6 (Fig. 4) could not be reamplified after repeated attempts and were thus discarded. All other bands could be reamplified and were sequenced. The sequence of the predominant bands 3, 4, 8 and 10 in the four samples (Fig. 4) showed an identity of up to 92% with sequences of other filamentous sulfide-oxidizing bacteria, such as *Beggiatoa* spp. or *Parabeggiatoa* spp., whereas other bands (2, 5, 7, and 9; Fig. 4) showed affiliation to distantly related genera (*Sulfurimonas*, *Idiomarina*, *Halobacillus* and *Castellaniella*, respectively). The purified filament of CSO1 yielded only a single band on the DGGE gel

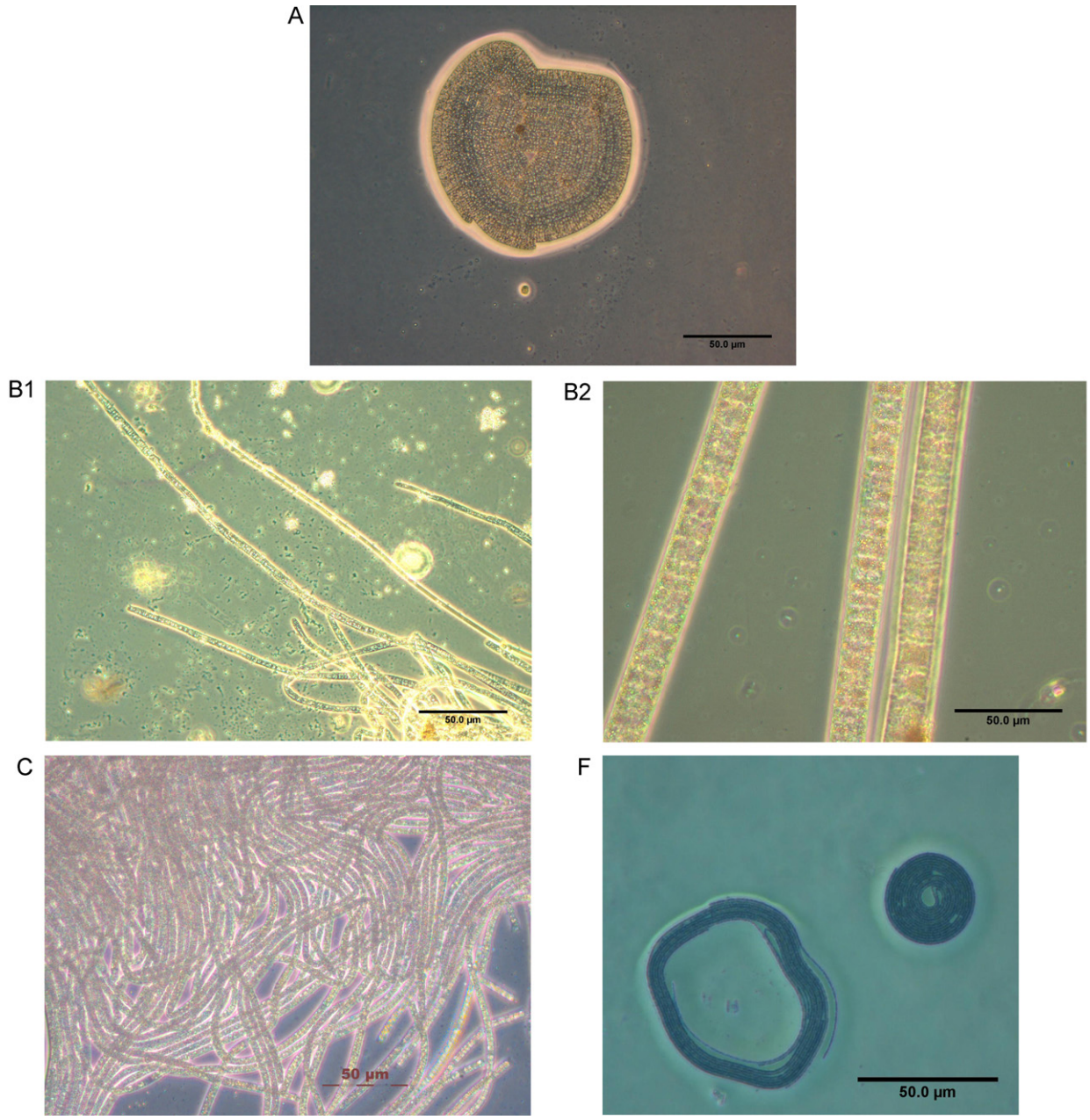


**Fig. 3.** Phase contrast micrographs of five different enrichment cultures of *Beggiatoa* spp. (A) CSO3 from Kattur shrimp pond, ($B_1$ and $B_2$) CSO2 and CSO4 from Cooum River, (C) CSO1 from Muttukadu shrimp pond, and (F) enrichment from Marakkanam paddy field.

(band 10; Fig. 4) and a nearly full-length 16S rRNA gene sequence could be retrieved from this DNA sample.

*Phylogenetic analysis*

16S rDNA sequence analysis of DGGE bands and one full-length gene sequence revealed that all four filaments were affiliated to the *Beggiatoaceae* family. The filament from enrichment CSO1 yielded a 16S rRNA gene sequence of 1411 nucleotides and showed highest sequence identity with *Beggiatoa* sp. MS-81-6 and *Beggiatoa* sp. 35Flor (accession numbers AF110277 and FR717278, respectively, from marine habitats) and only 88% sequence identity with the *Beggiatoa* type strain *Beggiatoa alba* (accession number L40994, from a freshwater habitat). The dominant DGGE bands of the filaments picked from enrichments CSO2 and CSO4 (579 and 545 nucleotides long, respectively) were 100% identical in their overlapping nucleotide composition and formed a monophyletic group with the CSO1 sequence (99% identity; Fig. 5). This cluster grouped with a branch containing other *Beggiatoa* spp. (99% bootstrap confidence) isolated or enriched from marine or hypersaline habitats. Accordingly, sequences CSO2 and CSO4 also showed highest sequence identities with the strains *Beggiatoa* sp. MS-81-6 and *Beggiatoa* sp. 35Flor (91%), and only 88% with *B. alba*. The filament retrieved from enrichment CSO3 yielded a prominent DGGE band of 546 nucleotides, which fell out of the *Beggiatoa* cluster and appeared to be separated from any sequence belonging to the *Beggiatoaceae* (Fig. 5). Owing to the shortness of the CSO3 sequence and the distant relationship to any other published sequence, the classification of this filament was complicated. Highest sequence identities were 93% to *Thiopilula* sp. (accession numbers FR690971, FR690972 and FR690973), 92% to *Parabeggiatoa* sp. (accession numbers FJ875196, FJ875197, FJ875198 and FJ875199) and again only

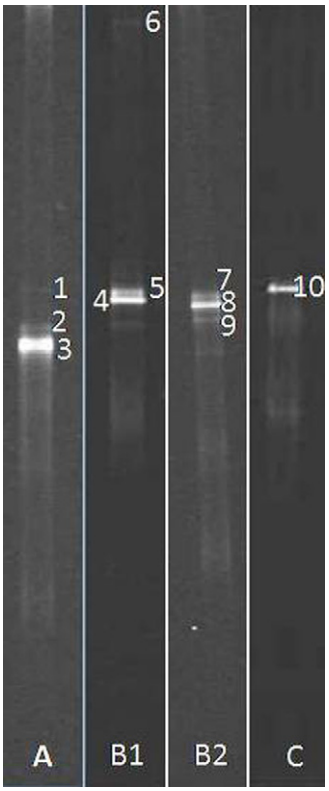


**Fig. 4.** PCR-DGGE profiling of 16S rRNA genes of CSO3, CSO2, CSO4 and CSO1 filaments.

86% to *B. alba*. Interestingly, in the reconstructed tree (Fig. 5), the CSO3 sequence formed a monophyletic group (100% bootstrap confidence) with partial DGGE sequences (706 nucleotides) retrieved from 10 μm wide filaments from Tokyo Bay [17] (TokyoA, 91% identity and TokyoC 92% identity), indicating that filament CSO3 may indeed be affiliated to other filamentous sulfide-oxidizing bacteria.

## Discussion

Out of the seven samples, filamentous sulfur bacteria were enriched successfully from two samples originating from shrimp ponds and three other brackishwater sediments with a discernible sulfide smell. In samples without a sulfide smell, such filaments were neither observed during direct microscopic examination nor grew within two to three weeks of incubation on extracted hay medium. Generally, extracted hay medium yielded the highest number of positive enrichments of sulfide-oxidizing filaments after two to three weeks incubation, and filaments appeared predominantly as distinct white cottony tufts. However, none of the filaments analyzed in this study were closely related to the *Beggiatoa* type strain *B. alba*. All four sequences separated from the *B. alba* cluster with less than 88% sequence identity and to any other sequence in the *Beggiatoaceae* family by less than 93%. Accordingly, the filamentous sulfide-oxidizing bacteria enriched in this study shared prominent morphological features of described *Beggiatoa* spp., but should be taxonomically separated from this genus (organisms of the same genus must share a 16S rDNA sequence identity of 95–98% [37,39]). Nevertheless, the ecological behavior of the filaments analyzed here was very comparable to described *Beggiatoa* spp. and other large filamentous sulfur bacteria of the family. The formation of intracellular sulfur granules by oxidation of reduced sulfur sources was reported to be one of the basic, defining features of the genus *Beggiatoa* [35]. The filaments enriched here occurred in sulfidic sediments and most filaments contained sulfur inclusions. After enrichment, filaments in four out of five enrichment cultures (A, $B_1$, $B_2$ and C) contained sulfur inclusions, indicating that they actively carried out sulfide oxidation. The extraction of sulfur granules with solvents, such as pyridine, and their refractile appearance when intact cells were viewed with a light microscope were used to confirm the presence of intracellular sulfur granules [31]. When grown in gradient medium, it was observed that the filaments accumulated at the oxic/anoxic interface by gliding, as has been described for marine lithotrophic *Beggiatoa* spp. [4,21]. Thus, it can be assumed that these brackishwater filaments were lithotrophic and may play a significant role

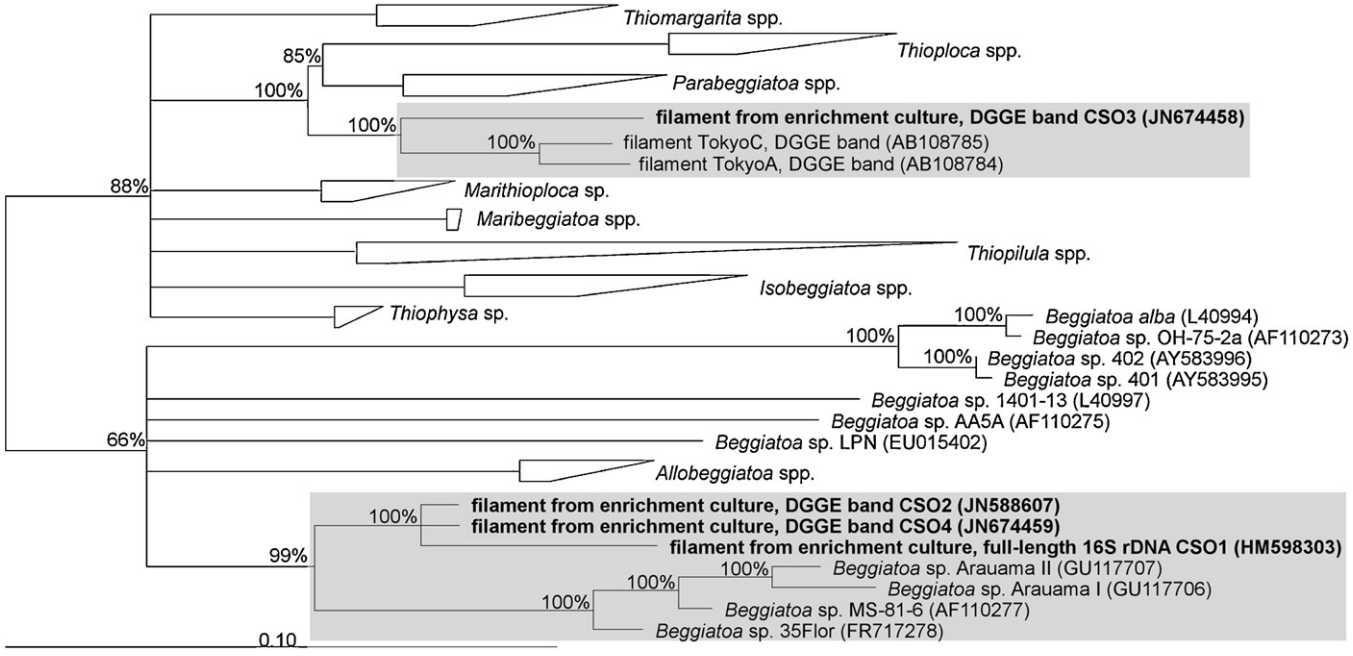


**Fig. 5.** Phylogeny of PCR-DGGE derived sequences of single filaments enriched from brackishwater ecosystems of Tamil Nadu, India. The gray boxes indicate the two clades containing the sequences of the filaments studied and the closest related sequences previously published. The classification of taxa in the family *Beggiatoaceae* is based on Salman et al. [27].

in the sulfide oxidation pathway in their natural habitats, where their metabolic activity could thus shape the local ecosystem. The heterotrophic growth capabilities of the *Beggiatoa*-like filaments were also tested, but none of the cultures exhibited heterotrophic growth. Even though the inoculum of CSO1 was obtained from an organically rich ecosystem, filaments failed to survive in the media containing only organic compounds, such as acetate, malate or lactate, as an electron source. Also, the lysis of the filaments within 24 h incubation on these media and predominant growth of contaminating heterotrophic bacteria suggested that the physiology of the filaments could be of a lithotrophic nature. Gliding of the filaments towards the subsurface region in BP and sulfide-gradient medium (0.2% agar) may possibly indicate that they were microaerophilic, and coiling of the filaments observed on BP medium (1.0% agar) may be a response to oxidative stress.

Interestingly, when using the sulfide-gradient medium for enrichment, the filaments were much more contaminated with other bacteria, and the growth yield was comparatively reduced and not as distinct as that observed on extracted hay medium. This may be due to the possible role of complex polymers, such as cellulose residues, present in hay that may better support sulfate reduction near the surface of hay, providing the hydrogen sulfide necessary to enrich the sulfur bacteria [35].

Considering the catalase negative nature of *Beggiatoa* spp. [15,33], external addition of catalase was used here for enrichment and it did in fact stimulate growth of filaments. Furthermore, filaments grew well in enrichments, which also contained other bacteria, rather than appearing as isolated tufts on agar media, such as BP or MP. An explanation for this observation could be that the co-existing bacteria could help in alleviating the effect of hydrogen peroxides that form in the oxic zone of the enrichment. The occurrence of *Halomonas* spp. and *Sulfurimonas* spp. in these enrichments that have been previously reported to carry out denitrification [12] and sulfur oxidation [34], respectively, points towards the possible interconnected metabolic processes taking place in these microenvironments.

The PCR-DGGE method was chosen for this study since it served the dual purpose of identifying the target organisms in the enrichment and simultaneously revealing the purity of the extracted DNA. Although DNA was extracted from washed filaments that were allowed to glide on an agar surface, the DGGE profile showed several other bands suggesting that several other bacteria were co-extracted. The length of the filament and its outer mucus sheath very likely offers an ideal surface for other bacteria to adhere. Thus, the purification of *Beggiatoa*-like filaments free from other bacteria is challenging. The PCR-DGGE technique used in this study, involving universal bacterial primers, enabled retrieval of partial 16S rRNA gene information from the filaments and their putative contaminants, whereas those amplicons originating from contaminating bacteria could be successfully separated. The band pattern could thus be used initially to analyze the purity of the picked filament. In a next step, the nearly full-length 16S rRNA gene sequence could be obtained from an uncontaminated filament DNA extraction, as shown for filament CSO1. Partial sequences (~550 bp) derived from PCR-DGGE were still useful for approximate affiliation within the family of large sulfur bacteria. While Kojima and Fukui [17] employed a nested PCR method for identification, the PCR-DGGE method [20] used here also yielded information on the bacteria associated with *Beggiatoa* enrichments.

Reclassification of the *Beggiatoaceae* family has been proposed recently [27] based on 16S rDNA sequence data. One large cluster within the family still contains several sequences classified as *Beggiatoa* spp., although these sequences share only low sequence similarity [27]. Several studies have attempted to obtain 16S rDNA sequence data for sulfur bacteria from various ecosystems, such as a minimum oxygen zone of the tropical South Pacific, biofilms, corals and hydrothermal vents, however, some of these studies indicated that obtaining phylogenetic data from this group of organisms was challenging [9,11,29,32]. In the present study, attempts made to retrieve *Beggiatoa* 16S rDNA sequences using a *Beggiatoa*-specific amplification protocol [36] were unsuccessful. Hence, the use of PCR-DGGE was used for retrieving the 16S rDNA sequences from the enriched filaments. Recently, a high number of 16S rRNA gene sequences have been published from non-filamentous large sulfur bacteria, however, full-length 16S rRNA gene sequences retrieved from filamentous sulfur bacteria, such as *Beggiatoa* spp., remain scarce.

In conclusion, this study demonstrates for the first time the occurrence of sulfide-oxidizing filaments of the family *Beggiatoaceae* in shrimp culture ponds and other brackishwater ecosystems on the Indian sub-continent. Phylogenetic analysis suggests that the enrichments include filaments that share morphological features with described *Beggiatoa* species, but which delineate phylogenetically at the genus level from *B. alba*. Further sequencing will be necessary to resolve the affiliation of filaments enriched in this study within the family *Beggiatoaceae*. The fact that only one enrichment culture (CSO3) could be successfully maintained from this study highlights the absence of a suitable media for enriching the diverse sulfur bacteria occurring in brackishwater ecosystems. More research is required to understand the ecological significance of filamentous sulfur bacteria, but this study certainly demonstrates the significance of brackishwater ecosystems as study sites for the investigation of the distribution and diversity of large sulfur bacteria and their role in the sulfur cycle.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2012.05.006.

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