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Sulfur respiration in a marine chemolithoautotrophic *Beggiatoa* strain

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The chemolithoautotrophic strain *Beggiatoa* sp. 35Flor shows an unusual migration behavior when cultivated in a gradient medium under high sulfide fluxes. As common for *Beggiatoa* spp., the filaments form a mat at the oxygen–sulfide interface. However, upon prolonged incubation, a subpopulation migrates actively downward into the anoxic and sulfidic section of the medium, where the filaments become gradually depleted in their sulfur and polyhydroxyalkanoates (PHA) inclusions. This depletion is correlated with the production of hydrogen sulfide. The sulfur- and PHA-depleted filaments return to the oxygen–sulfide interface, where they switch back to depositing sulfur and PHA by aerobic sulfide oxidation. Based on these observations we conclude that internally stored elemental sulfur is respired at the expense of stored PHA under anoxic conditions. Until now, nitrate has always been assumed to be the alternative electron acceptor in chemolithoautotrophic *Beggiatoa* spp. under anoxic conditions. As the medium and the filaments were free of oxidized nitrogen compounds we can exclude this metabolism. Furthermore, sulfur respiration with PHA under anoxic conditions has so far only been described for heterotrophic *Beggiatoa* spp., but our medium did not contain accessible organic carbon. Hence the PHA inclusions must originate from atmospheric CO_2 fixed by the filaments while at the oxygen–sulfide interface. We propose that the directed migration of filaments into the anoxic section of an oxygen–sulfide gradient system is used as a last resort to preserve cell integrity, which would otherwise be compromised by excessive sulfur deposition occurring in the presence of oxygen and high sulfide fluxes. The regulating mechanism of this migration is still unknown.

Keywords: *Beggiatoa*, sulfur reduction, gradient cultivation, microelectrodes, migration

INTRODUCTION

The genus *Beggiatoa* comprises large, filamentous bacteria that inhabit diverse sulfidic environments, such as sediments (Winogradsky, 1887; Jørgensen, 1977; Nelson and Castenholz, 1982; McHatton et al., 1996), springs (Winogradsky, 1887; Macalady et al., 2006), and activated sludge (Farquhar and Boyle, 1971). The motile filaments typically aggregate in a narrow overlapping zone of opposed oxygen and sulfide diffusion gradients where they form a sharply demarcated mat (Faust and Wolfe, 1961; Nelson and Jannasch, 1983; Nelson et al., 1986). Within this mat, *Beggiatoa* spp. oxidize sulfide with oxygen, depleting both compounds (Nelson et al., 1986). This process is accompanied by deposition of elemental sulfur inside the filaments.

Several filamentous and non-filamentous members of the *Beggiatoaceae* (Salman et al., 2011) are capable of anaerobic sulfide oxidation with nitrate as electron acceptor (Fossing et al., 1995; McHatton et al., 1996; Schulz et al., 1999). Dissimilatory nitrate reduction enables these organisms to colonize anoxic environments such as deeper layers in sediments, microbial mats, or gradient cultures (Sweerts et al., 1990; Mussmann et al., 2003; Sayama et al., 2005; Kamp et al., 2006; Hinck et al., 2007; Jørgensen et al., 2010). Nitrate-based sulfide oxidation seems to have been of great importance for some members of the *Beggiatoaceae*, as suggested by their ability to highly concentrate nitrate from the ambient water and store it in intracellular vacuoles (Fossing et al., 1995; McHatton et al., 1996; Schulz et al., 1999; Sayama, 2001; Mussmann et al., 2003; Kalanetra et al., 2004, 2005; Hinck et al., 2007). For example, internal nitrate concentrations of 4–44 mM were found in narrow, hypersaline *Beggiatoa* spp. cultivated at an external nitrate concentration of 50 μM (McHatton et al., 1996), whereas up to 100–800 mM of nitrate were reported for marine *Thiomargarita* spp. cells from an environment with ambient nitrate concentrations of 5–28 μM (Schulz et al., 1999). However, also non-vacuolated strains were shown to use externally provided nitrate as a terminal electron acceptor (Sweerts et al., 1990; Kamp et al., 2006).

We cultivated the chemolithoautotrophic, marine strain *Beggiatoa* sp. 35Flor in an agar-stabilized oxygen–sulfide gradient medium. Upon prolonged incubation in the presence of medium to high sulfide fluxes, we observed an unusual migration behavior, where a subpopulation of filaments moved downward from the oxygen–sulfide interface. These filaments were able to survive although sulfide concentrations were high and terminal electron acceptors that are known to be used by *Beggiatoa* spp., i.e., oxygen and nitrate, were not detectable in the medium or the filaments. In this study we investigated the possibility of an alternative

metabolism of *Beggiatoa* sp. 35Flor under anoxic, nitrate-free, and sulfidic conditions, and discuss its possible ecological significance and link to the peculiar migration behavior.

MATERIALS AND METHODS

STRAIN AND CULTIVATION

The strain *Beggiatoa* sp. 35Flor was enriched from a black band disease of scleractinian corals from the coast of Florida. The filaments are about 6 μm wide (Kamp et al., 2008), and the cells contain a central vacuole filled with polyphosphate (Brock and Schulz-Vogt, 2011). The strain can so far only be cultivated in the presence of *Pseudovibrio* sp. FO-BEG1, which was isolated in pure culture from the very same enrichment (Schwedt, unpublished). Different attempts of obtaining a pure culture of *Beggiatoa* sp. 35Flor failed so far, indicating that there is an important interaction between these strains. However, the nature of this interaction is currently not resolved and might not be specific. The clonal *Beggiatoa* culture used in this study was eventually obtained by inoculating gradient media with a single, washed filament (Schulz-Vogt, unpublished).

Cultivation was performed in tubes with an agar-based mineral gradient medium designed for chemolithoautotrophic growth of *Beggiatoa* spp. (Nelson et al., 1982; Nelson and Jannasch, 1983) using artificial seawater (Kamp et al., 2008). The medium was composed of a sulfidic bottom agar plug (1.5% w/v agar) covered with a sulfide-free, semisolid top agar layer (0.25% w/v agar) of ∼5 cm height. Both agar layers were prepared by mixing separately autoclaved salt and agar solutions. The salt solution comprised 100 mL artificial seawater (470.57 mM NaCl, 24.6 mM $MgCl_2{\cdot}6H_2O$, 16.6 mM $MgSO_4{\cdot}7H_2O$, 4.5 mM $CaCl_2{\cdot}2H_2O$ and 13.7 mM KCl; 27.5 g NaCl, 5 g $MgCl_2{\cdot}6H_2O$, 4.1 g $MgSO_4{\cdot}7H_2O$, 0.66 g $CaCl_2{\cdot}2H_2O$, and 1.02 g KCl in 1 L distilled water), 2.9 g NaCl, and 1 drop of 1 mol L^{-1} KOH in case of the bottom agar medium, or 240 mL artificial seawater and 4.32 g NaCl in case of the top agar medium. The agar solution contained 80 mL distilled water and 2.7 g double-washed agar (bottom agar medium), or 96 mL distilled water and 0.9 g double-washed agar (top agar medium). The top agar medium further received sterile mineral solution (3.2 mM K_2HPO_4, 139.5 μM Na_2MoO_4, 3.9 mM $Na_2S_2O_5$, and 107.3 μM $FeCl_3{\cdot}6H_2O$; 555 mg K_2HPO_4, 28.72 mg Na_2MoO_4, 750 mg $Na_2S_2O_5$, 29 mg $FeCl_3{\cdot}6H_2O$ in 1 L distilled water), trace element, and vitamin solutions as specified in Kamp et al. (2008) as well as 0.72 mL of a sterile 1 mol L^{-1} $NaHCO_3$ solution. The bottom agar medium was supplemented with 0.7–3.6 mL sterile 1 mol L^{-1} Na_2S (4–20 mM final concentration), depending on the experiment. The medium was prepared free of nitrate, nitrite, and nitric oxide, as verified by measurements with an NO_X analyzer (CLD 66, Eco Physics, Rösrath, Germany). Gas exchange with the atmosphere was possible, and opposing gradients of oxygen and sulfide were allowed to form for 1 to 2 days before inoculation. The cultures were inoculated about 1 cm below the air–agar interface using 100 μL of filament suspension from an established mat. The cultures, from which the inoculum was taken, were prepared as described above and grown under low sulfide flux conditions for 6–10 days. The inoculum contained *Beggiatoa* sp. 35Flor filaments and *Pseudovibrio* sp. FO-BEG1 cells and was free of oxidized nitrogen species as confirmed with an NO_X analyzer. All incubations were performed at room temperature in the dark.

Migration behavior of the *Beggiatoa* sp. 35Flor filaments was investigated in culture tubes with variable fluxes of sulfide from the bottom agar. During these incubations, the distribution of filaments in the same tube was determined simultaneously with vertical profiles of H_2S and pH. Filaments from parallel culture tubes were subsampled and used for microscopic determination of their sulfur and PHA inclusions. Additional parallel tubes were used for the measurement of oxygen and sulfide fluxes, the proportion of broken filaments and the internal sulfur content.

TRANSFER EXPERIMENT WITH SULFUR-FREE FILAMENTS

To verify that sulfur-free filaments from the anoxic subpopulation of an aged culture (cultivated under high sulfide flux) were alive, able to migrate back to the oxygen–sulfide interface and re-establish their sulfide-oxidizing metabolism, they were transferred into the anoxic section of a fresh gradient medium (low sulfide flux conditions). All cultivation media were prepared in plexiglass tubes (2 × 12 cm in size) with lateral holes (Brock and Schulz-Vogt, 2011). Fresh medium for inoculation with sulfur-free filaments was pre-incubated with *Pseudovibrio* sp. FO-BEG1. This was done to ensure a sufficient cell density of the accompanying bacterium irrespective of the inoculum as it seems to be required for growth of *Beggiatoa* sp. 35Flor but its abundance is negligible in the anoxic part of the gradient medium. Subsequently, sulfur-free filaments were removed laterally from the aged culture and injected laterally into the fresh medium at a depth of about 1 cm below the oxygen–sulfide interface. The media were inspected visually for development of a mat.

MICROSENSOR MEASUREMENTS

Microsensors for O_2 (OX-10 standard), H_2S (H_2S-10), and pH (pH-10) were purchased from Unisense A/S (Aarhus, Denmark). The external reference for the pH electrode was manufactured and connected in-house. Calibration of the H_2S sensor was performed in anoxic, acidified artificial seawater (pH < 2) to which anoxic Na_2S stock solution was added stepwise. The exact sulfide concentration of the stock solution was determined by iodometric titration. Total sulfide (S_{tot}) profiles were calculated from measured H_2S and pH profiles using the equation $S_{tot} = H_2S \times [1 + K_1/H_3O^+]$, with $pK_1 = 6.569$ at 21°C and 39‰ salinity (Millero et al., 1988). The oxygen sensor was two-point calibrated in a calibration chamber filled with artificial seawater. Signal readings were taken in water saturated with N_2 and ambient air. Oxygen concentrations at the respective salinity and temperature were calculated according to Weiss (1970). The pH electrode was calibrated using buffer solutions of pH 4.01, pH 7.00, and pH 9.21 (Mettler-Toledo, Giessen, Germany). All sensors were calibrated immediately before the measurement. In case of long time series measurements the sensor calibration was checked afterward and a possible drift was corrected for. Sulfide fluxes were calculated using Fick's first law of diffusion ($J = -D\,\partial c/\partial x$). The diffusion coefficient D for HS^- was corrected for temperature (21°C) according to Jørgensen and Revsbech (1983), resulting in a value of 1.56×10^{-9} m^2 s^{-1}.

Vertical profiling in 250 μm steps was performed with sensors mounted on a motorized linear positioner (VT-80, Pollux motor, Micos, Eschbach, Germany) controlled by a computer using a software tool for automated microsensor measurements (μ-Profiler, L. Polerecky, http://www.microsen-wiki.net). The sensors were aligned by manually adjusting their tips to the air–agar interface using a dissecting microscope (Stemi 2000-C, Zeiss, Jena, Germany).

FILAMENT IMAGING

The distribution of sulfur-containing *Beggiatoa* sp. 35Flor filaments in gradient cultures was monitored using time-lapse photography. An amber light-emitting diode (LXHL-NM98, Luxeon, Philips, San Jose, CA, USA) was positioned below the culture tube and switched on for 1 s when an image was taken with a cooled CCD camera (Sensicam, PCO, Kelheim, Germany). Illumination and image acquisition in 10 min intervals were controlled by a custom-written computer program (Look@Molli, B. Grunwald, http://www.microsen-wiki.net).

Intensities in the recorded images were horizontally averaged over an area with visible filaments (∼5 mm wide, ∼2 cm high), and the resulting vertical profiles were assembled into a 2D map with the *x*-axis representing incubation time and the *y*-axis corresponding to depth. Since the average image intensity was proportional to the density of sulfur globules, which were present exclusively inside filaments, vertical movement of sulfur-rich filaments was detected as a change in the shape of the vertical intensity profile. In contrast, an increase and decrease in the profile intensity that was not accompanied with the change in the profile shape indicated accumulation and depletion of sulfur inside the filaments, respectively. Because this method relied on light scattering from sulfur inclusions, it did not allow visualization of sulfur-free filaments.

STAINING OF INTERNAL PHA

Staining with Nile Red was used to visualize PHA inclusions in the filaments. A subsample of 90 μL from a culture tube was incubated for 5 min with 10 μL of a Nile Red (Sigma-Aldrich, Steinheim, Germany) staining solution (25 mg L^{-1} in dimethyl sulfoxide). The filament suspension was transferred onto a poly-L-lysine (Sigma-Aldrich) coated microscope slide for immobilization of the filaments. Fluorescence of Nile Red was excited with a laser at 546 nm and emission was recorded above 590 nm (filter set 15, Zeiss, Jena, Germany) using an epifluorescence microscope (Axiophot equipped with AxioCam MRm, Zeiss, Jena, Germany).

IDENTIFICATION OF INTERNAL SULFUR

Under a bright-field microscope, internal sulfur globules in *Beggiatoa* spp. usually appear as highly refractive, round inclusion bodies inside the filaments. We used high performance liquid chromatography (HPLC) to confirm that the globules observed in the studied strain *Beggiatoa* sp. 35Flor were indeed composed of sulfur. A suspension of filaments was fixed with 3.2% (v/v) formaldehyde for 2 h at room temperature. Two 1 mL subsamples were mixed with 2 mL artificial seawater or HPLC-grade methanol (Applichem, Darmstadt, Germany), and shaken over night in glass vials. Filaments from both treatments were examined microscopically the next day. The methanol extract was centrifuged at 13.000 rpm (Centrifuge 5417R, Eppendorf, Hamburg, Germany) for 5 min to remove agar and cell debris. The supernatant was filtered (Acrodisc syringe filter 4472, Pall Life Science, NY, USA) and subsequently measured by HPLC (Kamyshny et al., 2009), using elemental sulfur standards as reference.

MONITORING OF *BEGGIATOA* sp. 35FLOR CELL INTEGRITY

The proportion of damaged filaments in cultures grown for 7 and 13 days under low and high sulfide flux conditions was quantified by visual inspection using a microscope. Samples of the mat at the oxygen–sulfide interface were taken from three parallel tubes per sulfide flux treatment, and the proportion was calculated from about 150–200 filaments counted per each sample. The significance of differences between treatments (high vs. low sulfide flux) and points of time were evaluated with a *t*-test, using log-transformed percentages of damaged filaments to ensure variance homogeneity between the compared data sets.

RESULTS

MIGRATION OF *BEGGIATOA* sp. 35FLOR IN GRADIENT CULTURES

Beggiatoa sp. 35Flor filaments aggregated and formed a dense mat at the oxygen–sulfide interface within the gradient medium (**Figure 2A** and Movie S1 in Supplementary Material). In cultures with medium to high sulfide fluxes (**Table 1**) a subpopulation of filaments began a downward migration to the anoxic zone about 3–4 days after establishment of the mat. For medium sulfide fluxes, this migration resulted in a layer with homogenous filament density extending up to 2–3 mm below the mat (**Figure 1**). In contrast, for high sulfide fluxes, the migrating filaments were not homogeneously distributed but progressively aggregated in a region distinctly separated from the mat at the oxygen–sulfide interface (**Figures 1** and **2B**). Because the aggregation of filaments in the anoxic part increased the chance of detecting metabolic products all further experiments were conducted with cultures growing under a high sulfide flux.

Migration of filaments in cultures with a high sulfide flux followed a general pattern illustrated in **Figure 2E**. During the initial 3–4 days of incubation, the mat at the oxygen–sulfide interface gradually formed. After about 6–7 days the sulfur-globule density in the mat decreased moderately, followed by a more pronounced decrease after 8–9 days. These decreases were correlated with two pronounced events of downward migration at days 5–6 and 7–8, respectively (arrows 1 and 2 in **Figure 2E**). After reaching a depth of

Table 1 | Diffusive sulfide fluxes in gradient cultures from this study.

Na_2S (mmol L^{-1}) in bottom agar	Time (days)	Flux (mmol m^{-2} day^{-1})[a]
4 (low flux)	7	4.7 ± 1.2
	13	6.8 ± 0.3
10 (medium flux)	7	14.1 ± 1.9
	13	14.7 ± 1.7
16 (high flux)	7	27.3 ± 5.1
	13	17.1 ± 3.5

[a]*The flux values are given as average ± SD of parallel measurements in five tubes.*

around 10 mm, the migrating filaments formed a layer of increased filament density. These filaments slowly disappeared from view due to a gradual loss of their internal sulfur granules. The disappearance of filaments was accompanied by a parallel increase in the sulfur-globule density in the mat at the oxygen–sulfide interface (arrow 3 in **Figure 2E**), suggesting that the sulfur-depleted filaments returned to this zone and switched back to sulfide oxidation, thereby depositing sulfur. This was confirmed by transfer experiments, which showed that sulfur-depleted filaments transferred from the anoxic subpopulation of an aged culture into the anoxic section of a fresh gradient medium formed, within 12 days, a new mat of sulfur-containing filaments at the oxygen–sulfide interface.

SULFIDE PRODUCTION BY FILAMENTS IN THE ANOXIC SECTION

Throughout the incubation, sulfide oxidation in the mat at the oxygen–sulfide interface was confirmed by pronounced acidification and steep gradients of total sulfide (**Figures 2C,D**). A small but detectable peak in the H_2S profile was observed at a depth of ~10 mm when the anoxic subpopulation was present (**Figure 2D**). As pH varied only smoothly with depth in this region, the H_2S peak was not caused by pH variation but indicated a true production of sulfide at and around this depth. This production was strongly spatially and temporally correlated with the presence of the anoxic subpopulation (**Figure 2F**), suggesting that it was linked to the metabolic activity of the filaments from this subpopulation.

CELL INTEGRITY, SULFUR AND PHA CONTENT OF SINGLE FILAMENTS

Beggiatoa sp. 35Flor filaments accumulated elemental sulfur and PHA during growth at the oxygen–sulfide interface. Sulfur inclusions were visible as dark, highly refractive globules in bright-field micrographs (**Figures 3A,B**). These globules disappeared when filaments were treated with methanol, and the corresponding extracts featured a single pronounced peak in the HPLC chromatogram at the retention time of 3.738 min ± 0.007 ($n = 27$), which matched the sulfur standard peak at 3.728 min ± 0.006 ($n = 9$). PHA inclusions appeared as strongly fluorescent globules in images of Nile Red-stained samples (**Figure 3E**). With increasing sulfide flux the amount of internal sulfur strongly increased (compare **Figures 3A,B**), whereas PHA inclusions were equally abundant in all treatments (data not shown). When grown under high sulfide flux, most filaments from the mat at the oxygen–sulfide interface were densely filled with sulfur and PHA inclusions (**Figures 3B,E**). In contrast, filaments from the anoxic subpopulation were heterogeneous with respect to their inclusion density; while some were densely filled with sulfur and PHA, others lacked both (**Figures 3D,F**).

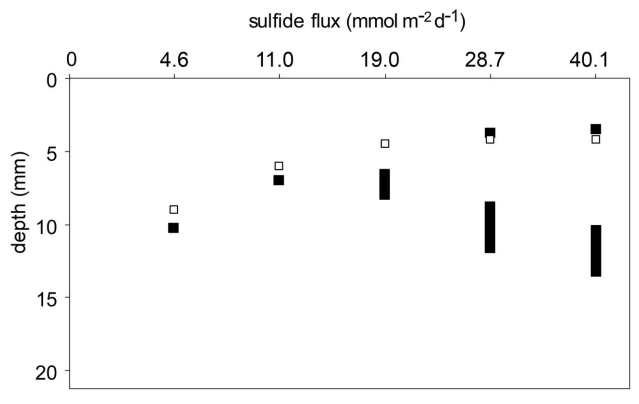

FIGURE 1 | Distribution of *Beggiatoa* sp. 35Flor filaments over depth in gradient cultures with different sulfide fluxes after 6 (open symbols) and 12 (closed symbols) days of cultivation. The flux values were estimated from measurements after 7 days (**Table 1**).

The proportion of damaged filaments (**Figure 3C**) from the mat at the oxygen–sulfide interface increased with sulfide flux. In cultures growing for 1 week under low sulfide flux, most filaments were intact, with only 0.9% ± 1.0 ($n = 3$) filaments damaged, whereas this proportion was significantly higher (13.2% ± 3.3, $n = 3$, $p = 0.011$) in cultures grown at high sulfide flux. The proportion of damaged filaments also increased with time: after 2 weeks of growth, this increase was significant for cultures with high sulfide flux (50.1% ± 7.2, $n = 3$, $p = 0.007$) but not in cultures with low sulfide flux (2.0% ± 1.5, $n = 3$, $p = 0.429$).

DISCUSSION

Sulfide production by members of the genus *Beggiatoa* is known from chemoheterotrophic strains that were cultivated in liquid medium and artificially exposed to short-term anoxic conditions (Schmidt et al., 1987). Based on those experiments it was hypothesized that sulfur respiration may provide *Beggiatoa* spp. in gradient systems with energy for return from the anoxic zone to the oxygen–sulfide interface under changing environmental conditions. In this study, we cultivated the chemolithoautotropically growing strain *Beggiatoa* sp. 35Flor in an oxygen–sulfide gradient medium, and observed a directed migration of the filaments from the oxygen–sulfide interface into the anoxic and sulfidic zone where they reduced internal sulfur to sulfide. This suggested an alternative or additional function of sulfur respiration in *Beggiatoa* filaments.

We propose that the observed behavior is a "last resort" survival strategy of *Beggiatoa* sp. 35Flor at prolonged incubation under high sulfide fluxes. Under this condition the filaments become densely filled with sulfur and were often observed to burst. By moving to the anoxic zone of the gradient system, the filaments can prevent further deposition of sulfur through aerobic sulfide oxidation and even reduce the amount of storage compounds by sulfur respiration with PHA. Sulfur-depleted filaments can eventually migrate back to the oxygen–sulfide interface, where they resume aerobic sulfide oxidation and accumulate new sulfur globules. An involvement of the accompanying *Pseudovibrio* sp. strain in the observed migration and metabolism is unlikely due to its negligible abundance in the region of the anoxic subpopulation (Schwedt, unpublished).

SULFUR RESPIRATION FOR REGULATION OF THE AMOUNT OF STORED SULFUR

The alternation between sulfide oxidation and sulfur reduction in spatially separated environments seems to allow *Beggiatoa* sp. 35Flor to control the amount of stored sulfur beyond the scope of enzymatic regulation. Sulfide is oxidized aerobically by

Beggiatoa spp. in a two-step process via internally stored sulfur ($2H_2S + O_2 \rightarrow 2S^0 + 2H_2O$) further to sulfate ($2S^0 + 3O_2 + 2H_2O \rightarrow 2SO_4^{2-} + 4H^+$). The regulation of these reactions is unknown in *Beggiatoa* spp., but the presence of internal sulfur globules demonstrates that the two reactions are not always well balanced. Principally, a balanced sulfur content can be achieved by either down-regulating sulfide oxidation or up-regulating sulfur oxidation. Possibly, sulfide oxidation is controlled kinetically and cannot be regulated by the cell, because both O_2 and H_2S freely diffuse into the cytoplasm. This is supported by observations on other closely related filamentous and non-filamentous large sulfur bacteria, which both immediately increase their respiration rate upon addition of sulfide to the medium (Schulz and de Beer, 2002; Høgslund et al., 2009). Moreover, Fenchel and Bernard (1995) reported for marine *Beggiatoa* spp. that the sulfide flux into the mat did not drop after the oxygen flux was decreased, indicating that the ratio of sulfide oxidation to sulfur oxidation shifted to favor sulfide. Therefore, up-regulation of sulfur oxidation seems the more likely mechanism to balance the internal sulfur content. However, at high sulfide fluxes the frequently observed bursting of *Beggiatoa* sp. 35Flor filaments that were densely filled with sulfur globules indicates that

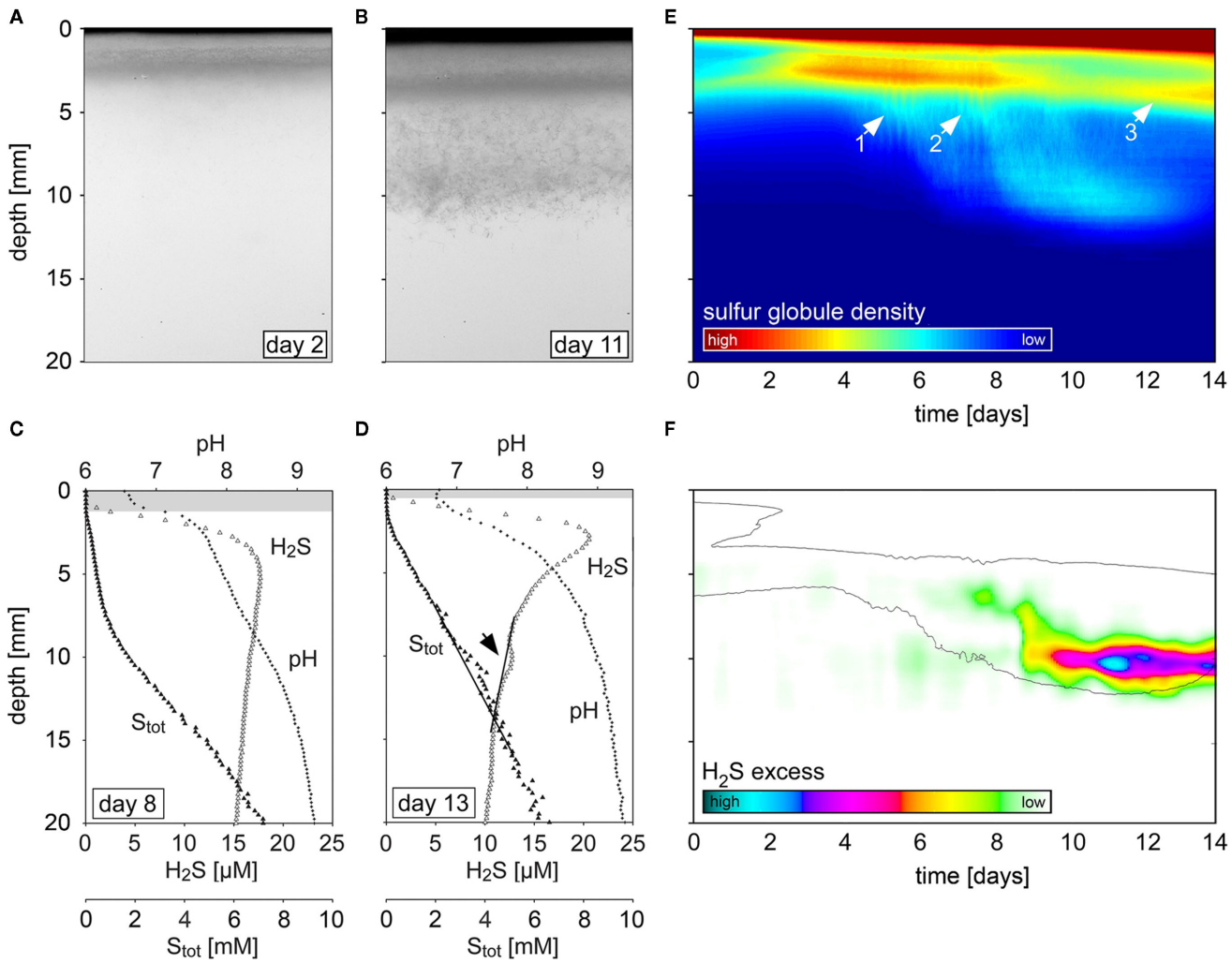

FIGURE 2 | Relationship between the migration of *Beggiatoa* sp. 35Flor filaments and the dynamics of O_2, pH, H_2S, and S_{tot} (total sulfide) in gradient cultures with high sulfide flux. (A + B) Images of culture tubes showing the filament distribution after 2 and 11 days. **(C + D)** Examples of pH, H_2S, and total sulfide profiles in 8- and 13-day-old gradient cultures. Shaded areas mark the oxic zone. **(E)** Average sulfur-globule density as a function of time and depth, showing the dynamics of the filament distribution and their sulfur content. Arrows 1 and 2 indicate the onset of major downward migration events, arrow 3 indicates the onset of an increase in the filament density in the mat at the oxygen–sulfide interface. Although the timing of these events varied amongst experimental runs, the general pattern was reproducible. A time-lapse movie of migrating filaments, from which the sulfur-globule density plot was calculated, is provided as a supplementary material (Movie S1 in Supplementary Material). **(F)** H_2S excess as a function of time and depth, calculated by subtracting the measured H_2S profile from the background trend. The trend was derived from the H_2S concentrations measured above and below the peak [line indicated by arrow in **(D)**]. Contour lines of the sulfur-globule density from **(E)** are overlaid. Data shown in **(A,B,E,F)** are from the same culture tube, profiles in **(C,D)** are from a parallel culture tube.

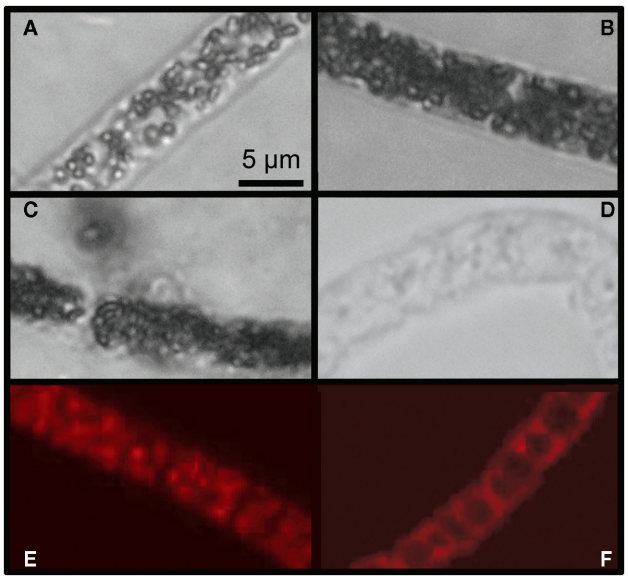

FIGURE 3 | Bright-field and fluorescence micrographs illustrating the typical appearance of *Beggiatoa* sp. 35Flor filaments cultivated under different conditions. (A+B) Filaments from the mat at the oxygen–sulfide interface of 6-days-old cultures growing under low **(A)** and high **(B)** sulfide flux conditions. **(C+D)** Filaments from cultures grown under high sulfide flux conditions, collected from the mat at the oxygen–sulfide interface after 27 days **(C)** and from the anoxic subpopulation after 12 days **(D)**. **(E+F)** Nile Red-stained filaments from a 14-day-old culture, collected from the mat at the oxygen–sulfide interface **(E)** and from the anoxic subpopulation **(F)**. Bright fluorescence in **(E)** originates from PHA inclusions, whereas lower fluorescence in **(F)** is due to staining of the cell membrane lipids.

further up-regulation of sulfur oxidation did not occur, e.g., due to enzymatic rate limitation.

As an alternative to enzymatic regulation, the filaments may leave the overlapping zone of oxygen and sulfide in order to starve themselves of external electron donor or acceptor, thereby interrupting sulfur deposition. A negative chemotactic response to oxygen (Møller et al., 1985) presumably prevented the filaments from moving upward into the oxic section of the gradient system. Instead, they migrated downward into the anoxic and sulfidic section, where sulfide could no longer be oxidized to sulfur due to the lack of an electron acceptor. It is surprising that these filaments moved into the sulfidic zone, because elevated sulfide concentrations have previously been reported to be toxic for *Beggiatoa* spp. (Winogradsky, 1887; Keil, 1912; Nelson et al., 1986). However, all earlier studies were done under oxic conditions. Our study indicates that *Beggiatoa* sp. 35Flor can tolerate higher sulfide concentrations under anoxic conditions, whereas under oxic conditions high sulfide concentrations can cause cell death indirectly by inducing excessive sulfur accumulation.

METABOLISM OF *BEGGIATOA* IN THE ANOXIC ZONE OF GRADIENT SYSTEMS

The depletion of sulfur and polyhydroxyalkanoate inclusions together with the production of sulfide suggests that *Beggiatoa* sp. 35Flor reduced internal sulfur by oxidizing stored carbon in the anoxic part of the gradient system. It is not known which type of PHA was synthesized by *Beggiatoa* sp. 35Flor, but for the most frequent PHA, poly(3-hydroxybutyrate) (PHB), the reaction $[C_4O_2H_6]_n + n{\cdot}9S^0 + n{\cdot}6H_2O \rightarrow n{\cdot}4CO_2 + n{\cdot}9H_2S$, which is pH-neutral, is in agreement with the observed pH profiles. Oxidation of stored sulfur was most probably not performed by filaments of the anoxic subpopulation, as oxygen and nitrate, which are the electron acceptors known to be used by members of the *Beggiatoaceae*, were not present. This is supported by the fact that we did not observe a decrease in pH at the corresponding depth interval in the gradient medium, which would be a sign of sulfuric acid production through oxidation of sulfur with oxygen ($2S + 3O_2 + 2H_2O \rightarrow 2SO_4^{2-} + 4H^+$) or nitrate ($5S + 6NO_3^- + 2H_2O \rightarrow 5SO_4^{2-} + 3N_2 + 4H^+$ or $4S + 3NO_3^- + 7H_2O \rightarrow 4SO_4^{2-} + 3NH_4^+ + 2H^+$). Likewise, sulfur disproportionation would produce sulfuric acid ($4S + 4H_2O \rightarrow 3H_2S + SO_4^{2-} + 2H^+$). The filaments of the anoxic subpopulation seem to gain energy chemoorganotrophically from oxidation of PHA with sulfur. However, no accessible source of fixed carbon is present in the medium, so that the PHA must have been previously synthesized through CO_2 fixation during chemolithotrophic growth on oxygen and sulfide at the oxygen–sulfide interface. Generation of PHA through excess CO_2 fixation was not described for *Beggiatoa* spp. so far, but is known from other bacteria (Schlegel et al., 1961). By reducing stored sulfur with a carbon reserve compound created previously through costly CO_2 fixation, the filaments did not exploit environmental resources in the anoxic environment. Instead, this process might be used by *Beggiatoa* sp. 35Flor as the only possibility to empty storage space under high sulfide fluxes.

The presence of filamentous *Beggiatoaceae* in the anoxic section of oxygen–sulfide gradient systems has so far been shown in multiple laboratory and field studies (Sweerts et al., 1990; Mussmann et al., 2003; Kamp et al., 2006; Hinck et al., 2007; Preisler et al., 2007; Jørgensen et al., 2010). However, in these systems nitrate was present either externally or internally and could have been used for oxidizing reduced sulfur compounds in the anoxic zone of the sediment. Nitrate respiration could, however, be excluded in our experiments as NO_X compounds were absent from medium and filaments.

THE ROLE OF SULFUR REDUCTION BY *BEGGIATOA* spp. IN THE ENVIRONMENT

The migration behavior and sulfur reduction by *Beggiatoa* filaments described in our study may occur and play the same role also in natural habitats. This is supported by the fact that the sulfide fluxes in our cultures (**Table 1**) were well within the range of fluxes previously measured in different natural *Beggiatoa* mats (**Table 2**), and that a strong heterogeneity in internal sulfur content of *Beggiatoa* filaments was also observed for filaments collected from natural mats (Sassen et al., 1993; Bernard and Fenchel, 1995). We suggest that, in natural habitats, filaments respond to high sulfide fluxes either by moving laterally to an adjacent region with a lower flux or, if this is not possible, by migrating vertically into the sulfidic and anoxic sediment section below, where they respire sulfur (**Figure 4**). However, the conditions at which

Table 2 | Diffusive sulfide fluxes in natural *Beggiatoa* spp. mats.

Sediment from	Measured	Flux (mmol m^{-2} day^{-1})	Reference
Lagoon	*ex situ*	38	Jørgensen and Revsbech (1983)
Arctic lagoon	*ex situ*	34	Jørgensen et al. (2010)
Coast	*ex situ*	4.3 ± 2	Preisler et al. (2007)
Harbor[a]	*ex situ*	ca. 12–100	Fenchel and Bernard (1995)
Deep sea mud volcano	*ex situ*	13 ± 4	de Beer et al. (2006)
Deep sea mud volcano	*in situ*	19 ± 3	de Beer et al. (2006)
Deep sea mud volcano	*in situ*	11.6	Lichtschlag et al. (2010)
Deep sea mud volcano	*in situ*	40	Grünke et al. (2011)

All fluxes were calculated based on sulfide profiles obtained with microsensors (silver–silver or Clark type electrodes). When possible, values are given as average ± SD of parallel measurements.

[a] *Minimum and maximum values were estimated from a graph presented in the cited study.*

these phenomena occur will depend on the possible maximum oxidation rates of sulfide and ultimately sulfur, which likely define the tolerance of different *Beggiatoa* species toward high sulfide fluxes.

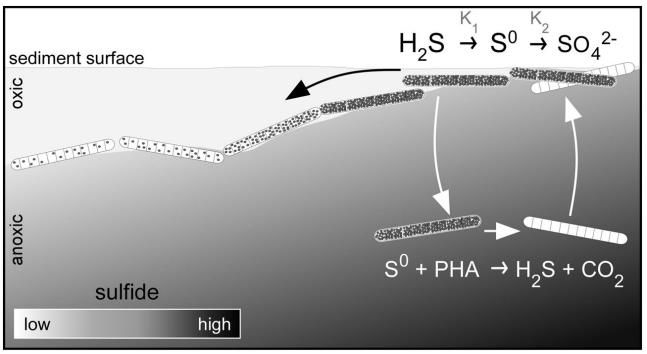

FIGURE 4 | Proposed function of sulfur reduction as a survival strategy of *Beggiatoa* spp. under high sulfide fluxes. In locations with high sulfide fluxes (right side) filaments become excessively filled with sulfur (black dots inside the filaments), because the oxidation rates of sulfide to sulfur (K_1) and sulfur to sulfate (K_2) are not well balanced ($K_1 > K_2$). To prevent bursting, the filaments could move into a region with a lower sulfide flux (black arrow) where these two reactions may proceed in a balanced way. If this is not possible, filaments could leave the oxygen–sulfide interface and move down into the anoxic region to reduce their internal sulfur deposits and thus prevent bursting (white arrow). They do so by using internally stored PHA as an electron donor to reduce S^0 to H_2S. After emptying storage space, the filaments return to the oxygen–sulfide interface, and continue with aerobic sulfide oxidation.

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SUPPLEMENTARY MATERIAL

The Movie S1 for this article can be found online at http://www.frontiersin.org/Microbial_Physiology_and_Metabolism/10.3389/fmicb.2011.00276/abstract

Movie S1 | Time-lapse video of *Beggiatoa* sp. 35Flor filaments cultivated under low and high sulfide flux conditions. In presence of a low sulfide flux the filaments stay in a confined layer whereas pronounced downward migration is evident in cultures with a high sulfide flux.

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