Electron donors and acceptors for members of the family *Beggiatoaceae*

Dissertation

zur Erlangung des Doktorgrades

der Naturwissenschaften

- Dr. rer. nat. -

dem Fachbereich Biologie/Chemie

der Universität Bremen

vorgelegt von

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Bremen, November 2013

Die vorliegende Doktorarbeit wurde in der Zeit von Februar 2009 bis November 2013 am Max-Planck-Institut für marine Mikrobiologie in Bremen angefertigt.

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Tag des Promotionskolloquiums: 16.12.2013

To Finn

Summary

The family *Beggiatoaceae* comprises large, colorless sulfur bacteria, which are best known for their chemolithotrophic metabolism, in particular the oxidation of reduced sulfur compounds with oxygen or nitrate. This thesis contributes to a more comprehensive understanding of the physiology and ecology of these organisms with several studies on different aspects of their dissimilatory metabolism. Even though the importance of inorganic sulfur substrates as electron donors for the *Beggiatoaceae* has long been recognized, it was not possible to derive a general model of sulfur compound oxidation in this family, owing to the fact that most of its members can currently not be cultured. Such a model has now been developed by integrating information from six *Beggiatoaceae* draft genomes with available literature data (**Section 2**). This model proposes common metabolic pathways of sulfur compound oxidation and evaluates whether the involved enzymes are likely to be of ancestral origin for the family.

In **Section 3** the sulfur metabolism of the *Beggiatoaceae* is explored from a different perspective. Besides oxidizing stored elemental sulfur further to sulfate, members of this family can use sulfur as a terminal electron acceptor under anoxic conditions. So far, sulfur respiration in the *Beggiatoaceae* has only been discussed in the context of energy acquisition, but the here presented data suggest that this reaction could also be employed to dispose of stored sulfur when sulfide is oxidized at high rates. If strongly sulfidic conditions and high sulfide oxidation rates persist, sulfur can accumulate in such an excessive manner that the cell integrity can eventually not be maintained.

Reduced sulfur compounds are surely the most important electron donors for chemolithoautotrophically growing *Beggiatoaceae*, but the traditional focus on this topic has left other possible inorganic electron donors largely unexplored. Hence, a major part of this thesis is dedicated to investigating the capacity of *Beggiatoaceae*

to use molecular hydrogen as an electron donor. Physiological experiments have shown that a chemolithoautotrophic *Beggiatoa* strain oxidizes hydrogen at high rates and under various conditions, indicating that hydrogen could play an important role in the metabolism of the *Beggiatoaceae* (**Section 4.1**). The physiological studies on hydrogen oxidation have been complemented by screening all available *Beggiatoaceae* draft genomes and several cultured members of the family for hydrogenase-encoding genes (**Section 4.2**). [NiFe]-hydrogenase genes from four phylogentically and functionally distinct clades have been identified repeatedly, illustrating that the capacity for hydrogen oxidation in the *Beggiatoaceae* is likely both, widespread and versatile. The possible influence of hydrogen oxidation on the metabolic plasticity of the *Beggiatoaceae* is discussed and environmental settings are pointed out, in which hydrogen oxidation could be important for members of the family.

In recent years, it became evident that molecular hydrogen can indeed be an important electron donor for sulfur bacteria of very different phylogenetic origin and lifestyle. The general discussion of this thesis therefore presents a comparison of how much energy members of the family *Beggiatoaceae*—and sulfur bacteria in general—could gain from the oxidation of reduced sulfur compounds and molecular hydrogen (**Section 5**). This comparison includes both, thermodynamic and biochemical considerations.

Zusammenfassung

Die Familie *Beggiatoaceae* umfasst große, farblose Schwefelbakterien, die für ihren chemolithotrophen Stoffwechsel, im speziellen die Oxidation reduzierter Schwefelverbindungen mit Sauerstoff und Nitrat, bekannt sind. Die vorliegende Arbeit trägt mit verschiedenen Studien zum dissimilatorischen Stoffwechsel zu einem tiefergehenden Verständnis der Physiologie und Ökologie dieser Organismen bei. Obwohl anorganische Schwefelverbindungen seit jeher als wichtige Elektronendonatoren für diese Familie angesehen werden, war es bis jetzt nicht möglich ein allgemeines Modell für die Oxidation von Schwefelverbindungen innerhalb der Familie aufzustellen, da die meisten ihrer Mitglieder zur Zeit nicht kultivierbar sind. Ein solches Modell wurde jedoch jetzt entwickelt, indem Informationen aus sechs teilsequenzierten Genomen aus der Familie mit verfügbaren Literaturdaten zusammengefasst wurden (**Abschnitt 2**). Im Rahmen dieses Modells werden gemeinsame Stoffwechselwege der Schwefeloxidation vorgeschlagen und die Ursprünglichkeit der betreffenden Enzyme für die Familie wird abgeschätzt.

In **Abschnitt 3** wird der Schwefelstoffwechsel der Familie *Beggiatoaceae* aus einer anderen Perspektive betrachtet. Neben der Oxidation zu Sulfat kann gespeicherter Elementarschwefel von Mitgliedern dieser Familie auch als terminaler Elektronenakzeptor verwendet werden. Bis jetzt wurde Schwefeloxidation in den *Beggiatoaceae* nur aus Sicht des Energiegewinns betrachtet, aber die hier präsentierten Daten weisen darauf hin, dass diese Reaktion auch bei hohen Sulfid-Oxidationsraten genutzt werden könnte um gespeicherten Schwefel zu entsorgen. Für den Fall dass stark sulfidische Bedingungen und hohe Sulfid-Oxidationsraten über längere Zeit anhalten, kann Schwefel so ausgiebig eingelagert werden, dass schlussendlich die Zellintegrität nicht mehr aufrecht erhalten werden kann.

Reduzierte Schwefelverbindingen sind mit Sicherheit die wichtigsten Elektronendonatoren für chemolithoautotroph wachsende *Beggiatoaceae*, aber durch die langanhaltende Fokussierung auf dieses Thema wurden andere mögliche anorganische Elektronendonatoren kaum untersucht. Daher beschäftigt sich ein Hauptteil dieser Arbeit damit, die Fähigkeit der *Beggiatoaceae* molekularen Wasserstoff zu oxidieren, zu untersuchen. Physiologische Experimente haben gezeigt, dass ein chemolithoautotropher *Beggiatoa*-Stamm Wasserstoff mit hohen Raten und unter verschiedensten Bedingungen oxidiert, so dass Wasserstoff im Metabolismus der Familie eine wichtige Rolle spielen könnte (**Abschnitt 4.1**). Die physiologischen Studien zur Wasserstoffoxidation sind um eine Überprüfung der verfügbaren teilsequenzierten *Beggiatoaceae*-Genome und verschiedener kultivierter Mitglieder der Familie auf Hydrogenase-Gene ergänzt worden (**Abschnitt 4.2**). Die wiederholte Identifizierung von [NiFe]-Hydrogenasen aus vier phylogenetisch und funktionell verschiedenen Gruppen weist darauf hin, dass die Fähigkeit zur Wasserstoffoxidation in der Familie *Beggiatoaceae* wahrscheinlich sowohl weit verbreitet als auch vielseitig ist. Der möglich Einfluss von Wasserstoffoxidation auf die metabolische Anpassungsfähigkeit der *Beggiatoaceae* wird diskutiert, genauso wie natürliche Umgebungen, in denen Wasserstoffoxidation eine wichtige Funktion für Mitglieder der Familie erfüllen kann.

In den letzten Jahren wurde vermehrt deutlich, dass molekularer Wasserstoff in der Tat ein wichtiger Elektronendonator für Schwefelbakterien von unterschiedlichstem phylogenetischen Ursprung und Lebensstil sein kann. Die Gesamtdiskussion dieser Arbeit (**Abschnitt 5**) widmet sich daher der Frage wie viel Energie Mitglieder der Familie *Beggiatoaceae* — und Schwefelbakterien im Allgemeinen — mit der Oxidation reduzierter Schwefelverbindungen gewinnen können. Dieser Vergleich berücksichtigt sowohl thermodynamische als auch biochemische Überlegungen.

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List of Abbreviations

(very common abbreviations are not listed)

APAT	APS:phosphate adenylyltransferase
APS	adenosine-5'-phosphosulfate
Ca.	*Candidatus*
cAMP	cyclic adenosine monophosphate
DMS	dimethyl sulfide
DMSO	dimethyl sulfoxide
DNRA	dissimilatory nitrate reduction to ammonium
DSR	dissimilatory sulfite reductase
FCSD	flavocytochrome *c*-sulfide dehydrogenase
LSU	large subunit
PHA	polyhydroxyalkanoate
PHB	poly(3-hydroxybutyrate)
Qmo	quinone-interacting membrane-bound oxidoreductase
RAxML	Randomized Axelerated Maximum Likelihood
rDSR	reverse dissimilatory sulfite reductase
SQR	sulfide:quinone oxidoreductase
SSU	small subunit
S_{tot}	total sulfide (H_2S + HS^- + S_2^-)

Chapter 1

Introduction

1.1 The family Beggiatoaceae

1.1.1 Members of the family Beggiatoaceae belong to the physiological group of sulfur bacteria

In his seminal papers from 1887, Sergei Winogradsky introduced the concept of chemolithotrophy when he reported for the first time on organisms gaining energy exclusively from the oxidation of inorganic compounds. He focussed his description on filamentous bacteria of the genus *Beggiatoa*, which were known to appear conspicuously whitish due to highly refractive inclusions of elemental sulfur (Cramer, 1870; Cohn, 1875). Winogradsky was able to show that the sulfur appeared as an intermediate in the oxidation of hydrogen sulfide to sulfate. Further and most importantly, he demonstrated that sulfide was essential for growth of the investigated *Beggiatoa* spp., while organic compounds were apparently not used. Consequently, he concluded that reduced sulfur compounds are the only substances fueling respiration in these bacteria and thereby function equivalent to organics in other organisms. In order to estimate how widespread the remarkable properties he had observed in *Beggiatoa* spp. are, Winogradsky continued by investigating other prokaryotic and eukaryotic organisms for their tolerance towards sulfide, their sulfide requirement, and the internal deposition of sulfur globules during

sulfide oxidation. Only few bacteria proved to be similar to *Beggiatoa* in their peculiar relation to reduced sulfur compounds, including *Monas okenii* (Ehrenberg, 1838; renamed as *Chromatium okenii* by Petry, 1852, and listed in the Approved Lists of Bacterial Names by Skerman et al., 1980), *Monas vinosa* (Ehrenberg, 1838; recently renamed as *Allochromatium vinosum* by Imhoff et al., 1998) and *Clathrocystis roseopersicina* (Cohn, 1875; renamed as *Lamprocystis roseopersicina* and listed in the Approved Lists of Bacterial Names by Skerman et al., 1980), which all are members of the family *Chromatiaceae*. Winogradsky introduced the collective term *Schwefelbacterien* (sulfur bacteria), for this—as he vaguely said—

> *höchst merkwürdige physiologische Gruppe wegen der eigentümlichen Rolle, welche der Schwefel in ihren Lebensprocessen spielt.*[1]

Later in his essays (1887), he defined sulfur bacteria as organisms, which (i) oxidize hydrogen sulfide, (ii) deposit globules of amorphous sulfur intracellularly, (iii) oxidize stored sulfur further to sulfate, (iv) require reduced sulfur compounds for growth, and (v) prosper under conditions, which do not support growth of heterotrophs. With this wording, he recognized that sulfur bacteria are a collection of species unified by common physiological properties rather than being defined by a close evolutionary relationship. Nevertheless, his latter definition is very strict and usually not applied, when the term sulfur bacteria is used. Other authors used different definitions and these can be remarkably divergent. Some use the term for all prokaryotes, which oxidize or reduce sulfur compounds for dissimilatory purposes, while others limit its use to those prokaryotes, which deposit globules of elemental sulfur intracellularly (see Fjerdingstad, 1979). While there is no accepted, universal definition of the term sulfur bacteria, it is today commonly used for photolithotrophic and chemolithotrophic prokaryotes, which oxidize sulfide for dissimilatory purposes. **Figure 1.1** gives an overview over taxa including representatives to which this definition applies. This thesis focusses on sulfur bacteria of the family *Beggiatoaceae* and their chemolithotrophic metabolism, but other sulfur bacteria will be referred to in several instances to point out traits, which appear to be common to or typical for the entire group.

[1] English translation of the German original text (Winogradsky, 1887): "most strange physiological group, due to the peculiar role sulfur plays in their life processes."

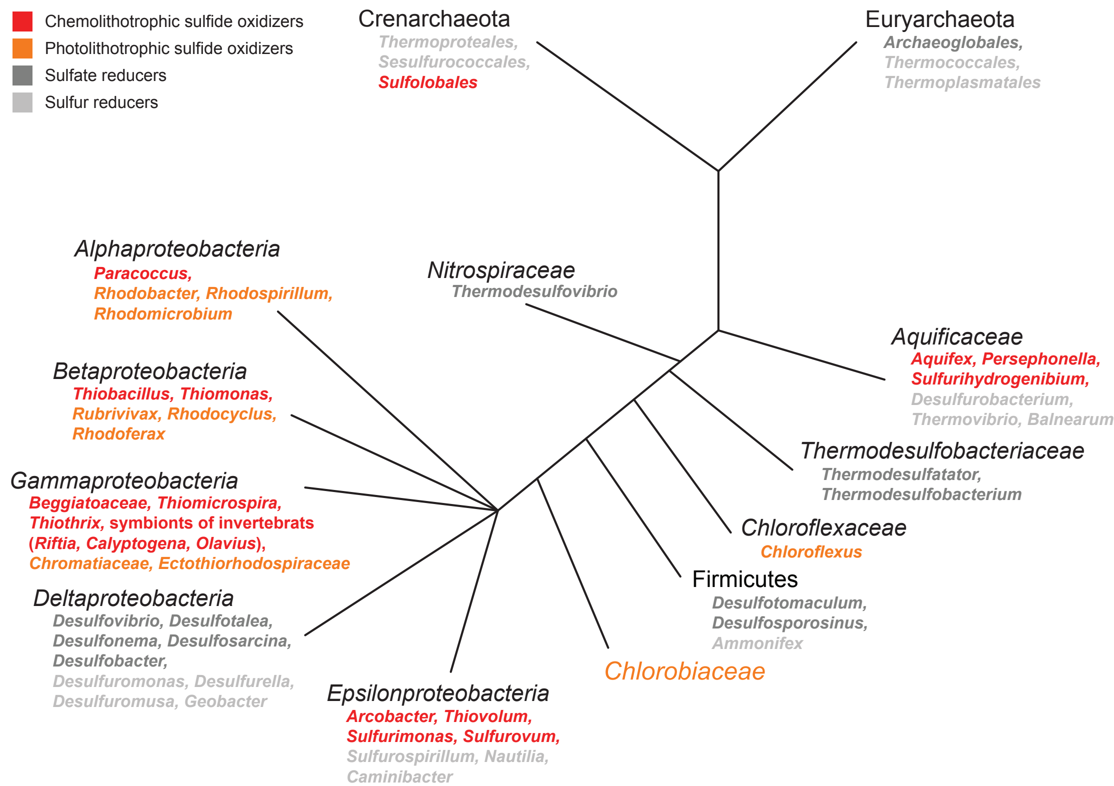

Figure 1.1 | Phylogenetic relationship of prokaryotes oxidizing or reducing inorganic sulfur compounds for dissimilatory purposes. Taxa including sulfur bacteria, i.e. chemolithotrophic and photolithotrophic prokaryotes, which oxidize sulfide and other reduced sulfur compounds for energy gain, are highlighted in red and orange. Prokaryotes, which use sulfate or sulfur as respiratory electron acceptors are shown in dark and light grey. This listing is not comprehensive and the branch lengths of the phylogenetic tree are not drawn to scale. Adapted from Sievert et al. (2007).

Living on sulfide, members of the family *Beggiatoaceae* are typical inhabitants of sulfidic environments such as organic-rich freshwater sediments (e.g. Winogradsky, 1887; Keil, 1912; Strohl and Larkin, 1978; Mezzino et al., 1984; Sweerts et al., 1990), coastal eutrophic zones (e.g. Jørgensen, 1977; Sayama, 2001), productive upwelling regions (e.g. Gallardo, 1977; Schulz et al., 1999), sites of hydrothermal venting and seeping (e.g. Prince et al., 1988; Jannasch et al., 1989; de Beer et al., 2006; Kalanetra et al., 2004), productive cyanobacterial mats in lakes and lagoons (e.g. Garcia-Pichel et al., 1994; Hinck et al., 2007, 2011), and activated sludge (Farquhar and Boyle, 1971).

1.1.2 Morphological and phylogenetic diversity

The family *Beggiatoaceae* is known for the impressive morphological diversity of its members (Salman et al., 2011, 2013). Filaments made up of disc-shaped or cylindrical cells can occur singly (e.g. Winogradsky, 1887; **Figure 1.2 A**), as bundles surrounded by a common mucous sheath (e.g. Lauterborn, 1907, **Figure 1.2 B**), and in rosetta-like structures, which may be attached to solid surfaces (e.g. Kalanetra et al., 2004; **Figure 1.2 C**). The diameter of such filamentous *Beggiatoaceae* covers a wide range, reportedly from < 1 μm up to about 200 μm (Winogradsky, 1888; Larkin and Henk, 1996). An even larger morphological diversity with respect to cell shape, arrangement, and size exists among the non-filamentous *Beggiatoaceae*. These are known to occur as unicellular, spherical cells (e.g. Kalanetra et al., 2005; **Figure 1.2 D**), as spherical or cylindrical cells stringed in mucus-coated chains (e.g. Schulz et al., 1999; **Figure 1.2 E** and **F**), and as circumspherical cells arranged in regular (e.g. Kalanetra et al., 2005; **Figure 1.2 G**) or irregular clusters (e.g. Salman et al., 2011; **Figure 1.2 H**) of various sizes. Aggregations of spherical cells were further found embedded in thick mucus clumps (**Figure 1.2 I**), enclosed in an envelope (**Figure 1.2 J**), and residing in empty diatom frustules (all Salman et al., 2011; **Figure 1.2 K**). In addition, elongated and attached cells in different states of budding were described (Bailey et al., 2011; **Figure 1.2 L**) as well as single, seemingly budding cells (Salman et al., 2011; **Figure 1.2 M**). Featuring cell diameters between 9 and 750 μm (Schulz et al., 1999; Salman et al., 2011), the non-filamentous *Beggiatoaceae* include the largest known bacterial cells.

Even though the presence of such distinct morphological traits suggests a classification scheme based on morphology, this would not correctly reflect the phylogenetic relationships within the family as inferred from 16S rRNA gene analyses (Salman et al., 2011). Many morphological traits such as the occurrence as single cells, the formation of chains, filaments, and bundles of filaments, the attachment to surfaces, and a dimorphic life cycle with budding cells are polyphyletic, i.e. occur in phylogenetically distinct lineages of the family (Salman et al., 2013). On the other hand, multiple—up to seven—distinct morphotypes grouped without an obvious

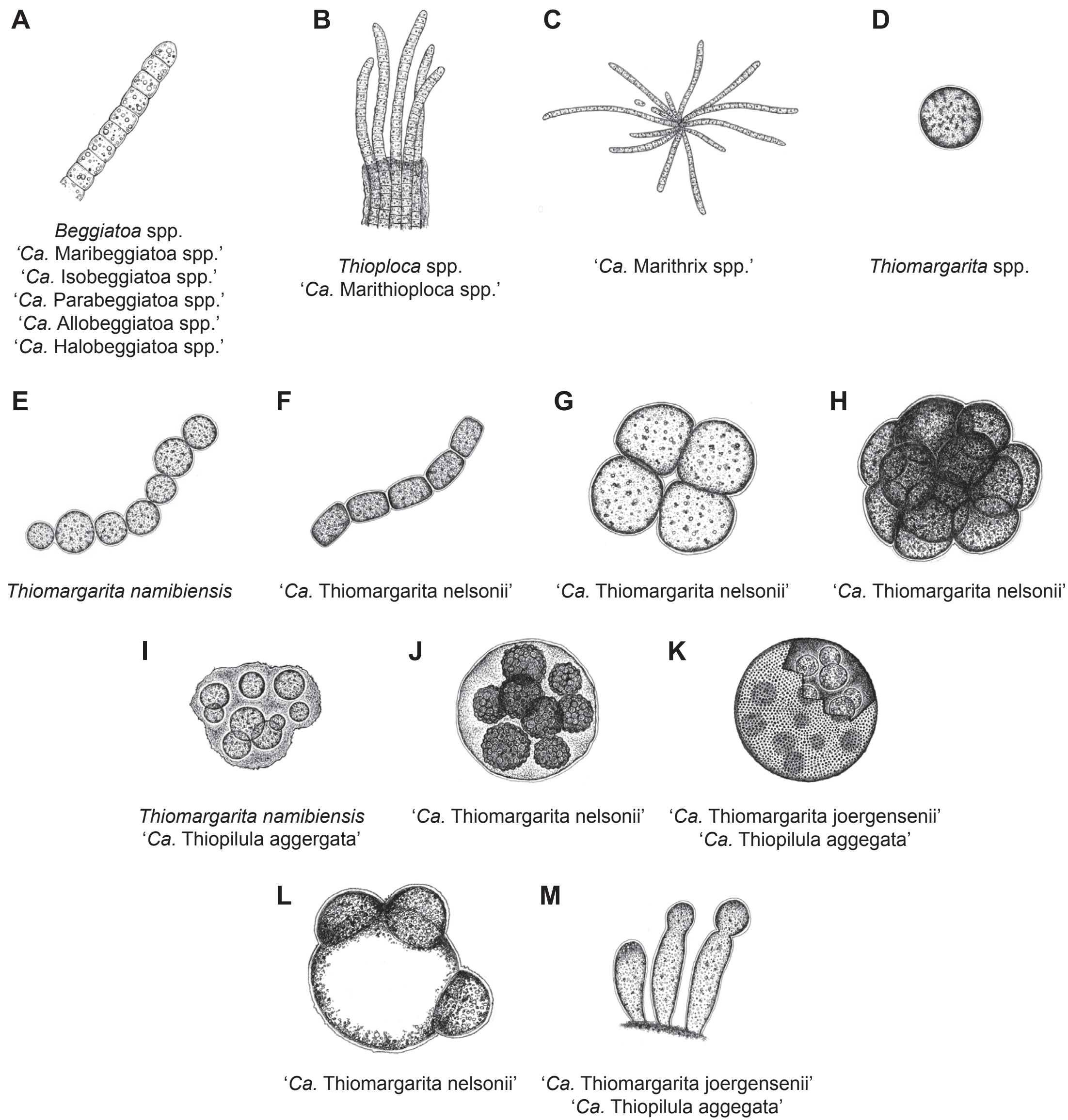

Figure 1.2 | Morphological diversity within the family *Beggiatoaceae*. Illustrations of the currently known *Beggiatoaceae* morphotypes are shown together with the reported phylogenetic affiliations. Adapted from Salman et al. (2013).

phylogenetic separation in several clusters, which are monophyletic according to 16S rRNA gene trees (Salman et al., 2011, 2013). Currently, the family *Beggiatoaceae* comprises twelve genera, among which nine have a *Candidatus* state, and several presently unnamed phylogenetic clusters (Salman et al., 2013). To the great disadvantage of a comprehensive physiological characterization of the family *Beggiatoaceae*, most of these genera and clusters have no cultured representatives. Physiological data have also been obtained from presently unculturable members of the family in incubation experiments with environmental samples (e.g. Otte et al., 1999; Schulz and de Beer, 2002; Høgslund et al., 2010), but such experiments are only suited for particular questions. Likewise, no genetic system is available for any representative of the family *Beggiatoaceae*, ruling out the testing of hypotheses on enzyme functions with deletion mutants.

From an ecophysiological point of view the presence of different morphotypes within the family *Beggiatoaceae* is interesting, because these imply different life strategies. The filamentous, non-attached representatives are typically motile by gliding and can reach speeds of about 6–7 μm s^{-1} (Dunker et al., 2010). Thereby, these morphotypes are able to respond quickly to changes in biogeochemical gradients and track the movement of the oxygen-sulfide interface or bridge spatially separated reservoirs of oxidant and reductant (see below). Being able to control their exposure to oxygen and sulfide by fast movement, these strains are usually less tolerant to elevated concentrations of either substance than their sedentary counterparts (Salman et al., 2013). In contrast, sessile morphotypes or morphotypes with a limited motility have to rely on environmental factors bringing about an alternating exposure to sulfide on the one and oxygen or nitrate on the other hand. This may be realized by resuspension of sulfidic host sediment in the oxic and nitrate-rich water column, turbulent flow of sulfide- and oxygen-rich water at hydrothermal vents (Kalanetra et al., 2004; Kalanetra and Nelson, 2010), occasional overflow with sulfidic brine (Girnth et al., 2011), or the movement of host macrofauna between oxic water and sulfidic sediment (Bailey et al., 2011). Due to their different life strategies, distinct morphotypes usually prevail in a given habitat, but to some extent co-occurrences have been reported (e.g. Schulz et al., 1999; Kalanetra et al., 2005; Bailey et al., 2011; Salman et al., 2011).

1.1.3 Physiology

Sulfur metabolism

All studied members of the family *Beggiatoaceae* are capable of oxidizing sulfide to elemental sulfur, which they deposit intracellularly in form of small globules or droplets (Cramer, 1870; Cohn, 1875; Winogradsky, 1887; Keil, 1912). The exact chemical nature of such bacterial sulfur inclusions is heavily debated (e.g. Prange et al., 1999; Pasteris et al., 2001; Pickering et al., 2001; Prange et al., 2002; George et al., 2008) but in *Beggiatoaceae* it appears that cyclooctasulfur is the storage form while inorganic polysulfides are the activated, metabolizable form (Berg et al., 2013). In this family, sulfur globules reside in the periplasm and are surrounded by a complex, likely proteinaceous envelope, which is thought to be of structural function (Strohl et al., 1981b, 1982; Schmidt et al., 1986). Most *Beggiatoaceae* oxidize stored sulfur further to sulfate, but two freshwater *Beggiatoa* strains have been reported to lack this ability (Schmidt et al., 1987). Alternatively, stored sulfur can be reduced with organic compounds or molecular hydrogen under anoxic conditions to supply the cells with energy for maintenance and movement (Nelson and Castenholz, 1981b; Schmidt et al., 1987). Larkin et al. (1994) found hollow pyrite tubes of the size of *Beggiatoa* filaments in the sediment beneath *Beggiatoa* mats and suggested that these tubes may have been formed when the filaments produced sulfide under anoxic conditions. Thus, stored sulfur represents a reserve of both, electron donor and acceptor, which *Beggiatoaceae* can deploy flexibly to meet their current needs. A more detailed introduction into the biochemistry and enzymology of sulfur compound oxidation in sulfur bacteria is presented in **Section 1.3**.

Oxygen requirement and mat formation

Most members of the family *Beggiatoaceae* use oxygen as an electron acceptor for sulfide oxidation (e.g. Keil, 1912; Nelson et al., 1986b; Schulz and de Beer, 2002; Høgslund et al., 2009). Because oxygen reacts also abiotically with sulfide, these substrates co-occur *in situ* almost exclusively in a narrow overlapping zone between

opposed gradients. Importantly, this overlapping zone offers low concentrations and a high supply with both substrates at the same time. Therefore, it is an ideal habitat for most *Beggiatoaceae*, which require both compounds but are sensitive to higher concentrations of either.

Motile, filamentous members of the family exhibit negative chemotactic responses to elevated concentrations of oxygen and sulfide, which allow them to track the oxygen-sulfide interface (determined thresholds for '*Ca.* Marithioploca' are $> 10\ \mu$M oxygen and $> 150\ \mu$M sulfide; Hüttel et al., 1996). The filaments typically aggregate at this interface, where they consume the upward-diffusing sulfide and the downward-diffusing oxygen. Thereby, they reduce the overlapping zone of oxygen and sulfide to a minimum, steepen the biogeochemical gradients even more, and lower the concentrations in their immediate vicinity (**Figure 1.3 B**). Concentrations of up to only 10 μM oxygen and 150 μM sulfide were measured in a natural *Beggiatoa* mat (Jørgensen and Revsbech, 1983) and even lower concentrations of up to 7.5 μM oxygen and 10 μM sulfide were recorded in *Beggiatoa* mats grown in artificial oxygen-sulfide gradient medium (Nelson et al., 1986a). Keil (1912) kept *Beggiatoa* spp. in liquid culture and reported that growth occurred only between 1.3–2.6% oxygen (optimum 1.5%) and 0.08–0.22% sulfide (optimum 0.11%) in the gas phase. Because the tolerated concentrations of oxygen and sulfide are so low and the range supporting growth is so narrow, cultures of sulfide-oxidizing *Beggiatoaceae* are best maintained in artificial gradient media, which allow for steep gradients, i.e. low concentrations of and a sufficient supply with oxidant and reductant. (**Figure 1.3 A**; e.g. Nelson et al., 1982; Nelson and Jannasch, 1983; Nelson et al., 1986a,b). In these cultures, the filaments can form a mat at a position, where oxygen and sulfide fluxes optimally support growth, but do not raise concentrations to a toxic level (**Figure 1.3 C**).

Nitrogen metabolism and vacuolation

Besides using oxygen as a terminal electron acceptor, many members of the family *Beggiatoaceae* can respire with nitrate under anoxic conditions (e.g. Sweerts et al., 1990; McHatton et al., 1996; Kamp et al., 2006). Most, but not all of these

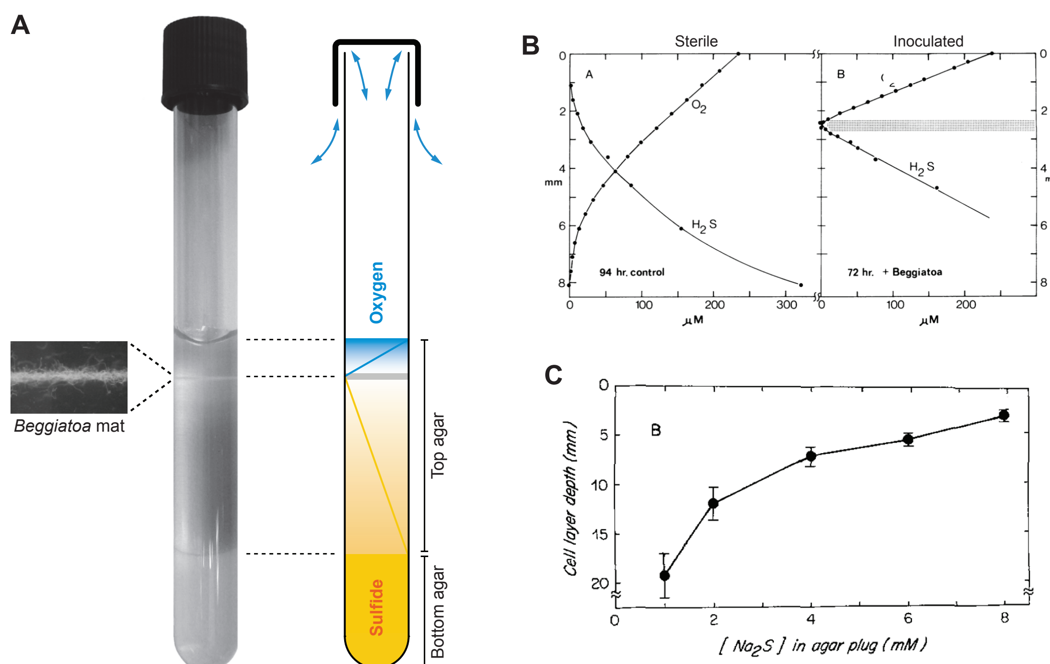

Figure 1.3 | Cultivation of filamentous *Beggiatoaceae* in oxygen-sulfide gradient medium. (**A**) Image and schematic drawing of a cultivation tube with oxygen-sulfide gradient medium prepared according to Nelson et al. (1982). The solid and sulfidic bottom agar is covered with a semisolid, initially sulfide-free top agar. The semisolid consistency of the top agar allows for gliding movement of the filaments. Sulfide from the bottom agar diffuses up into the top agar, where it eventually meets with downward-diffusion atmospheric oxygen. A *Beggiatoa* mat grows at the interface of the oxic and sulfidic zones. (**B**) Profiles of oxygen and sulfide in sterile and inoculated oxygen-sulfide gradient media. The overlap of oxygen and sulfide extends over about 7 mm in the sterile medium, in which both substances react only abiotically. The gradients are much steeper in the inoculated medium and meet only in the 0.5 mm thick *Beggiatoa* layer (shaded area). From Nelson et al. (1986a). (**C**) Depth of *Beggiatoa* mats in oxygen-sulfide gradient media in relation to the bottom agar sulfide concentration. The higher the concentration of sulfide in the bottom agar is, the closer the mat is positioned to the air-agar interface. From Nelson and Jannasch (1983).

nitrate-respiring representatives are able to accumulate large amounts of nitrate intracellularly and thereby become independent of an external supply with terminal oxidants. Assuming an initial intracellular nitrate concentration of 300 mM, Schulz et al. (1999) calculated that a *Thiomargarita* cell of 180 μm diameter could live for the enormous period of 40–50 days on stored nitrate. The independence from external sources of oxidant (and reductant) is of utmost importance for sessile members of the family *Beggiatoaceae*, because they will only infrequently be exposed to the respective substances. However, also motile filaments will benefit

from such internal reserves, because these typically live in steep gradients, which may change suddenly and thereby cut off filaments temporarily from the supply with oxidant or reductant.

Usually, nitrate appears to be stored in intracellular vacuoles and a recent study showed that nitrate respiration in '*Ca.* Allobeggitoa spp.' generates a proton motive force over the vacuolar membrane, which drives ATP production (Beutler et al., 2012). However, nitrate accumulation has also been reported for members of the family, in which no vacuoles were evident (Kojima et al., 2003, 2007; Høgslund et al., 2010) and not all vacuolated members seem to use this intracellular compartment for nitrate storage (Kalanetra et al., 2004, 2005; Brock et al., 2012). Compilations of studies reporting on (i) the presence of vacuoles and (ii) nitrate accumulation in members of the *Beggiatoaceae* are presented in **Tables S1.2** and **S1.3** (pages 60–62). The presence or absence of vacuoles seems to correlate with cell diameter rather than phylogeny (Brock et al., 2012) as vacuoles are typically found in cells with diameters above 5–6 μm (**Table S1.2**). In these larger cells, vacuoles are thought to be necessary for counteracting diffusion limitation by restricting the metabolically active cytoplasm to a peripheral layer of only 0.5–2 μm thickness (Larkin and Henk, 1989; Schulz et al., 1999; Schulz and Jørgensen, 2001). Similar to vacuolation, nitrate respiration is likely not restricted to certain genera. Most genera of the family include members, for which nitrate enrichment has been demonstrated and these are most probably also capable of nitrate respiration.

Two different pathways exist for the dissimilatory reduction of nitrate, being denitrification (reduction to N_2) and the dissimilatory nitrate reduction to ammonium (DNRA). Importantly, denitrification and DNRA have opposite effects on the budget of fixed nitrogen, because denitrification removes biologically available nitrogen from ecosystems, counteracting eutrophication, whereas nitrogen species are recycled as ammonium by DNRA, promoting eutrophication. Studies on the pathway of dissimilatory nitrate reduction in members of the family *Beggiatoaceae* provided contrasting results with some studies supporting denitrification (e.g. Sweerts et al., 1990; Beutler et al., 2012) and others arguing for DNRA (e.g. Otte et al., 1999; Høgslund et al., 2009). Hence, it is currently not clear, which of the two pathways

is realized in members of the family *Beggiatoaceae* and it is even possible that different strains employ different pathways. In addition to nitrate respiration, various strains of marine and freshwater *Beggiatoaceae* influence the cycling of nitrogen by fixing N_2 (Nelson et al., 1982; Polman and Larkin, 1988).

Carbon metabolism and modes of nutrition

Comprehensive descriptions of the nutritional modes of *Beggiatoaceae* have been challenging ever since. The essential issue is that *Beggiatoaceae* in general can use organic compounds as sources of both, energy and carbon. Thus, members of the family can grow lithotrophically or organotrophically (gain energy from the oxidation of inorganic or organic substrates) and autotrophically or heterotrophically (derive carbon from CO_2 or organics). Because it is often difficult to differentiate clearly between these nutritional modes and even more complicated to describe the phenotypic plasticity of a strain exhaustively, the term 'mixotrophy' was introduced for a nutritional mode, in which "concurrent use is made of organic and inorganic sources of carbon or energy, or both" (Kelly, 1971). Even though embracing the reported physiological versatility of many *Beggiatoaceae*, the imprecision of this term adds a lot to the confusion.

When Winogradsky (1887) first described the genus *Beggiatoa* physiologically, he proposed that these bacteria were growing either lithoautotrophically or lithoheterotrophically. i.e. oxidized inorganic sulfur substrates for energy gain while using carbon dioxide or organic substances for the production of biomass. Keil (1912) confirmed Winogradsky's suggestion of chemolithoautotrophy in pure cultures of *Beggiatoa* by showing that oxygen, hydrogen sulfide, and carbon dioxide but not organics were essential for growth. Later, other authors were only able to cultivate *Beggiatoa* strains heterotrophically (e.g. Faust and Wolfe, 1961; Scotten and Stokes, 1962; Pringsheim, 1964; Strohl and Larkin, 1978; Nelson and Castenholz, 1981a) and the early findings of autotrophy in *Beggiatoa* were questioned (see Nelson and Jannasch, 1983). Autotrophy in *Beggiatoa* was only demonstrated unequivocally by Nelson and Jannasch (1983), who showed by $^{14}CO_2$ incorporation that $\geq$ 89% of the protein carbon in *Beggiatoa* sp. MS-81-6 originate from

carbon dioxide. As *Beggiatoa* sp. MS-81-6 is also capable of entirely heterotrophic growth, the strain is considered to be a facultative chemolithoautotroph. Hagen and Nelson (1996) reported on an obligately chemolithoautotrophic strain, *Beggiatoa* sp. MS-81-1c, but noted that also this strain could produce up to about 22% of its cell carbon from acetate. Further, the strain *Beggiatoa alba* B18LD has been shown to oxidize sulfide for energy gain and fix carbon dioxide while not being able to grow in the absence of organic carbon compounds (Güde et al., 1981; Strohl et al., 1981a). Altogether, many strains of the family *Beggiatoaceae* seem to be capable of adapting their nutritional modes to the given conditions, some to a greater, some to a lesser extent. Hence, extensive studies would be required to describe the litho-, organo-, auto-, and heterotrophic potentials of a given strain comprehensively.

If members of the *Beggiatoaceae* consume organic substrates, these are typically organic acids such as acetate, pyruvate, lactate, succinate, fumarate, and malate (Faust and Wolfe, 1961; Pringsheim, 1964; Burton and Morita, 1964; Nelson and Castenholz, 1981a; Mezzino et al., 1984). The utilization of alcohols such as methanol and ethanol has been reported (Nelson and Castenholz, 1981a; Mezzino et al., 1984; Jewell et al., 2008) and amino acids were used in some cases (Pringsheim, 1964) while sugars were not metabolized (Scotten and Stokes, 1962; Nelson and Castenholz, 1981a). Several *Beggiatoaceae* are further known to deposit carbon reserves within the cytoplasm (Strohl et al., 1982), which may be composed of polyhydroxyalkanoates (Strohl and Larkin, 1978) or glycogen (Schulz and Schulz, 2005). In *Beggiatoa alba* B18LD, polyhydroxyalkanoates were shown to account for up to 56% of the cellular dry weight (Güde et al., 1981; Strohl et al., 1981a).

1.2 Sulfur cycling

Sulfur can assume a variety of stable valence states ranging from –2 (e.g. sulfide) to +6 (sulfate). This variable valence, together with the tendency of sulfur atoms to catenate and their ability to form covalent bonds with carbon atoms, gives rise to numerous organic and inorganic sulfur compounds and an accordingly complex

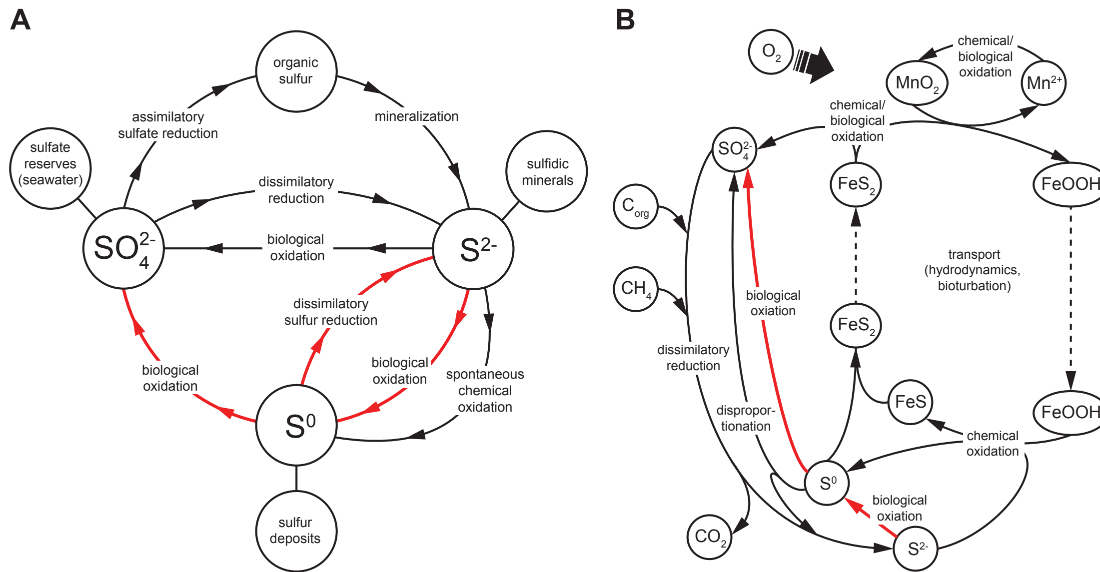

Figure 1.4 | **Sulfur cycling**. Members of the family *Beggiatoaceae* are involved in the reactions highlighted in red. (**A**) Conceptual illustration of sulfur cycling in the biosphere. Adapted from Robertsen and Kuenen (1992). (**B**) Schematic illustration of sulfur cycling in sediments. Adapted from Jørgensen and Kasten (2006).

sulfur cycle. This section will not provide a comprehensive overview of sulfur cycling in nature but will instead focus on the dissimilatory part of the biological sulfur cycle and emphasize the reactions occurring in sediments, which are of particular interest with respect to the family *Beggiatoaceae* (**Figure 1.4**).

The sulfur cycle of sediments is fueled by the input of organic matter. Many different physiological groups of microorganisms such as aerobes, denitrifiers, sulfate reducers, and fermenters take part in the degradation of this organic matter and produce sulfide when mineralizing organosulfur compounds. In quantitative terms, sulfate respiration is, however, a much more important process for the production of sulfide in sediments. Using sulfate as the terminal electron acceptor, sulfate-reducing bacteria couple the degradation of organic matter, biomass as well as hydrocarbons, to the production of sulfide. In marine sediments, sulfate-reducing bacteria contribute significantly to the degradation of organic matter (Jørgensen, 1982), owing to the high concentration of sulfate in seawater (29 mM; Jørgensen and Kasten, 2006). However, sulfate respiration is also observable in the sediments

Table 1.1 | Several sulfur compounds involved in the biogeochemical sulfur cycle. Normal environmental concentrations of oxygen, nitrate, metal oxides, Fe^{2+}, and Mn^{2+} as well as circumneutral pH are considered for chemical oxidation or reduction. From Canfield et al. (2005).

Formula	Name	Oxidation level	Dissimilatory reduction	Assimilatory reduction	Chemotrophic oxidation	Phototrophic oxidation	Dispropor-tionation	Chemical oxidation	Chemical reduction
SO_4^{2-}	Sulfate	+6	+	+	–	–	–	–	–
SO_3^{2-}	Sulfite	+4	+	+	+	+	+	+	+
$S_3O_6^{2-}$	Trithionate	+3.33	+	?	+	+	?	?	+
$S_4O_6^{2-}$	Tetrathionate	+2.5	+	?	+	?	+	?	+
$S_2O_3^{2-}$	Thiosulfate	-1, +5	+	+	+	+	+	+	–
S^0	Elemental sulfur	0	+	+	+	+	+	+	+
H_2S	Hydrogen sulfide	–2	–	–	+	+	–	+	–
FeS_2	Pyrite Marcasite	–1	–	–	+	–	–	+	(+)

of freshwater bodies, which typically feature much lower sulfate concentrations (ca. 0.1 mM; Jørgensen and Kasten, 2006). In these systems, sulfate reduction is possible because of high sulfate turnover rates and efficient bacterial sulfate uptake systems (Roden and Tuttle, 1993, and references therein). Other partially oxidized sulfur species such as sulfite, thiosulfate, tetrathionate, and elemental sulfur can likewise serve as respiratory electron acceptors. Members of the family *Beggiatoaceae* were shown to reduce sulfur to sulfide under anoxic conditions, but this process is likely more relevant in physiological terms than with respect to the biogeochemical sulfur cycle. Sulfide can eventually be oxidized by a variety of biotic and abiotic pathways and members of the *Beggiatoaceae* play an important role in the biotic reactions. While the direct re-oxidation of sulfide to sulfate would close the sulfur cycle, most sulfide is in fact oxidized to sulfur compounds of intermediate redox states. These sulfur compounds can then be oxidized, reduced, and disproportionated (**Table 1.1**), making the sulfur cycle a complex system with a number of shunts and subcycles (Jørgensen and Kasten, 2006).

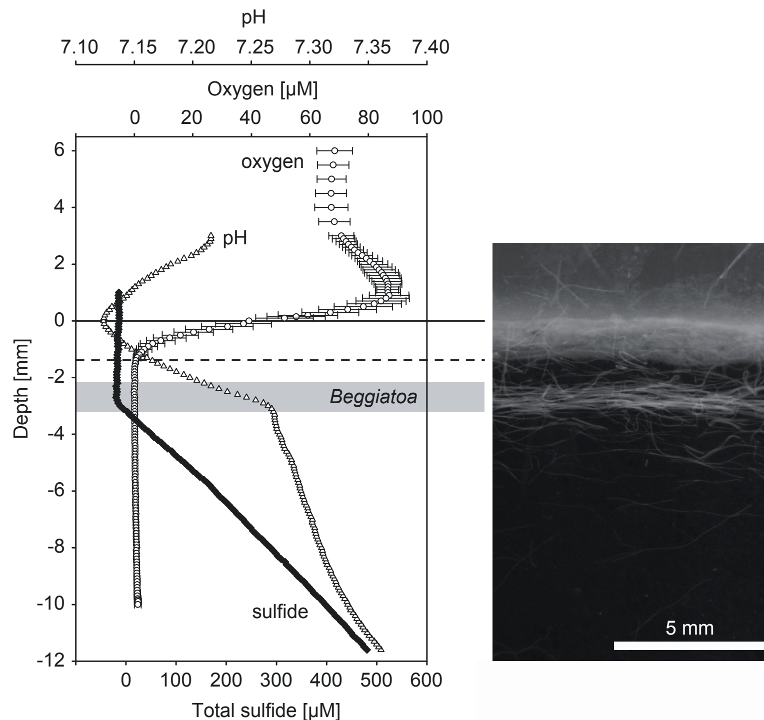

Figure 1.5 | Creation of a suboxic zone by filamentous *Beggiatoaceae*. Microsensor profiles were measured in a sediment core from Åarhus Harbor. The oxygen profile represents the average (± standard deviation) of three replicate measurements. An image of the profiled core is shown on the right side (to scale). The sediment surface is indicated by a solid line; a dashed line denotes the lower boundary of the oxic, iron and manganese oxide-rich layer (bright sediment in the image). The layer with the *Beggiatoa* mat, in which the upward-diffusing sulfide is consumed, is shaded in grey. Kreutzmann (unpublished data).

In sediments, populations of phototrophic sulfur bacteria usually develop when sulfide and light meet while chemotrophic sulfur bacteria often prosper when sulfide comes into contact with oxygen- or nitrate-rich water. Dense populations of chemotrophic sulfur bacteria, usually referred to as mats, are often dominated by members of the family *Beggiatoaceae* (e.g. Gallardo, 1977; Jannasch et al., 1989; Schulz et al., 1999; Kalanetra et al., 2004). Estimates of the extent to which these populations contribute to the bulk sulfide oxidation in their host sediments cover a wide range, from about 3% to >50% (Jørgensen, 1977; Fossing et al., 1995; Ferdelman et al., 1997; Brüchert et al., 2003; Mußmann et al., 2003; Preisler et al., 2007). Within the bounds of dense *Beggiatoaceae* mats—*in situ* as well *in vitro*—biological sulfide oxidation is, however, estimated to be up to a thousand times faster than the abiotic oxidation by iron and manganese (oxyhydr)oxides so that

nearly all of the sulfide removal will be biological (Jørgensen and Revsbech, 1983; Nelson et al., 1986a). Hence, sulfur bacteria may not typically be responsible for most of the sulfide removal in their host sediments, but dense sulfur bacterial populations can act as effective sulfide filters between the sediment and the water column by scavenging the upward-diffusing sulfide, which was not removed or precipitated by abiotic reactions within the sediment. As long as the sulfide flux is low enough for the sulfur bacteria to cope with, they will thus prevent the diffusion of this toxic and reducing substance into the water column and promote the stability on an oxic water body. If the sulfide flux exceeds a certain threshold—which is different for each strain—most *Beggiatoaceae* populations die.

Many members of the family *Beggiatoaceae* can respire with nitrate (**Section 1.1.3**) and therefore are able consume sulfide deeper within the sediment before its diffusion into oxic, iron and manganese (oxyhydr)oxide-rich layers. The oxidation of sulfide with nitrate allows these *Beggiatoaceae* to grow to dense populations despite a high abiotic sulfide oxidation potential of the sediment. Concurrently, it leads to the generation of a suboxic zone (e.g. Sweerts et al., 1990; Mußmann et al., 2003; Kamp et al., 2006; Hinck et al., 2007; **Figure 1.5**), in which neither oxygen nor sulfide is present. In addition to using nitrate as an electron acceptor for sulfide oxidation, many *Beggiatoaceae* can store nitrate abundantly in internal vacuoles (**Section 1.1.3**). This enhances not only their ability to compete with the abiotic oxidation of sulfide in the sediment. Likewise, it confers an advantage in the competition with other nitrate-respiring sulfide oxidizers, because nitrate-storing *Beggiatoaceae* can consume sulfide before it diffuses into nitrate-rich sediment horizons.

1.3 Enzymes of sulfur compound oxidation

United in physiology, sulfur bacteria share several biochemical pathways for sulfur compound oxidation, which, in turn, depend on the presence of several typical enzymes. This section gives an overview over the common sulfur compound oxidation pathways and the associated enzymes shared by many sulfur bacteria (summarized in **Figure 1.6**).

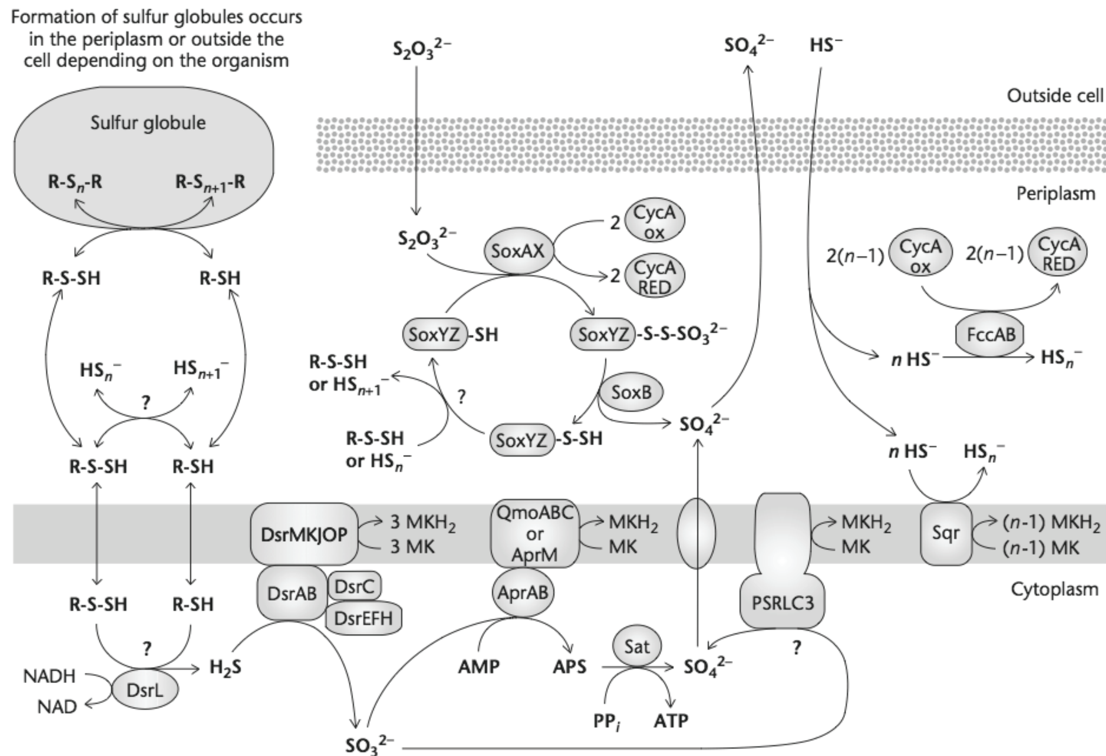

Figure 1.6 | Compiliation of enzymes involved in the oxidative sulfur metabolism. PSRLC3 is a polysulfide reductase-like protein, CycA is cytochrome *c*, and R stands for an organic residue. A periplasmic sulfite:ferricytochrome *c* oxidoreductase of the SorAB type, a SoxCD sulfur dehydrogenase, and enzymes of the tetrathionate intermediate pathway are not shown. From Dahl et al. (2008a).

1.3.1 Oxidation of sulfide to elemental sulfur

Two enzymes are considered to catalyze the dissimilatory oxidation of sulfide to elemental sulfur in photo- and chemolithotrophic sulfur bacteria. **Sulfide:quinone oxidoreductase** (SQR; EC 1.8.5.4; Shahak et al., 1992; Arieli et al., 1994; Schütz et al., 1997, 1998; Nübel et al., 2000; Shibata and Kobayashi, 2001; Marcia et al., 2009) is a monomeric or homomultimeric flavoprotein, which couples sulfide oxidation to quinone reduction. **Flavocytochrome *c*-sulfide dehydrogenase** (FCSD; hydrogen sulfide:flavocytochrome *c* oxidoreductase; EC 1.8.2.3; Fukumori and Yamanaka, 1979; Visser et al., 1997; Kostanjevecki et al., 2000), in contrast, reduces soluble *c*-type cytochromes. FCSDs are generally described to be composed of a flavoprotein and a cytochrome *c* subunit, but also a monomeric flavopro-

tein with FCSD activity has recently been identified (Quentmeier et al., 2004). Phylogenetically, SQRs and flavoprotein (sub)units of FCSDs represent distinct subfamilies of the disulfide-oxidoreductase flavoproteins, which are more closely related to each other than to any other member of this family (Theissen et al., 2003; Gregersen et al., 2011). As periplasmic enzymes or membrane-bound enzymes with a periplasmically oriented active site, SQR and FCSD are both taking up sulfide from the periplasm. However, the mechanisms for membrane-targeting and translocation are not well understood for all representatives and appear to be rather heterogeneous (Dolata et al., 1993; Visser et al., 1997; Schütz et al., 1999; Shahak and Hauska, 2008; Marcia et al., 2009; Gregersen et al., 2011).

SQR or FCSD are known to occur in many phylogenetically dissimilar sulfur bacteria and a simultaneous presence of both was confirmed in several instances (Visser et al., 1997; Kostanjevecki et al., 2000; Theissen et al., 2003; Mußmann et al., 2007; Gregersen et al., 2011). While the requirement for SQR during sulfide oxidation was clearly established *in vivo* with deletion mutants (Schütz et al., 1999; Chan et al., 2009) or by heterologous expression in *sqr*-deficient bacteria (Shibata and Kobayashi, 2001), sulfide oxidation by FCSD was only shown *in vitro* with enzymes of different species (Kusai and Yamanaka, 1973; Fukumori and Yamanaka, 1979; Visser et al., 1997; Quentmeier et al., 2004). Additional experimental support for the *in vivo* involvement of FCSD in sulfide oxidation, however, comes from the upregulation of FCSD transcription in the presence of sulfide (Kostanjevecki et al., 2000). Nevertheless, SQR is generally assumed to be the main sulfide-oxidizing enzyme in sulfur bacteria, even though it remains elusive under which conditions the two enzymes function and thus may complement each other.

1.3.2 Oxidation of elemental sulfur to sulfite

Genes of the *dsr* cluster (*dsrABCEFHLMKJOPN*) are the only ones known to be essential for the oxidation of elemental sulfur in sulfur bacteria (Pott and Dahl, 1998; Dahl et al., 2005; Lübbe et al., 2006; Sander et al., 2006; Cort et al., 2008; Dahl et al., 2008b; Grimm et al., 2008; Loy et al., 2009). The first two genes of this cluster encode the subunits of the **reverse dissimilatory sulfite reduc-**

tase (rDSR; hydrogen-sulfide:acceptor oxidoreductase; EC 1.8.99.1), the enzyme catalyzing the oxidation of sulfide to sulfite. Homologous, but phylogenetically distinct DsrAB proteins constitute the dissimilatory sulfite reductase of sulfate-reducing prokaryotes (Hipp et al., 1997; Meyer and Kuever, 2007; Loy et al., 2009), which catalyzes the reduction of sulfite to sulfide with trithionate and thiosulfate appearing as byproducts (Lee and Peck Jr., 1971; Jones and Skyring, 1975; Crane et al., 1997).

A detailed model for sulfur oxidation via the rDSR pathway was derived from intensive genetic and biochemical studies in the purple sulfur bacterium *Allochromatium vinosum* (reviewed by e.g. Grimm et al., 2008; Dahl et al., 2008a). Remarkably, the model envisages sulfur oxidation to start with a reductive activation, followed by transport of sulfur into the cytoplasm with an organic perthiol serving as a carrier (Pott and Dahl, 1998; Dahl et al., 2005). Relocation of the sulfur substrate into the cytoplasm is important, since none of the cytoplasmic or membrane-bound Dsr proteins can act directly on the periplasmic sulfur globules. It appears that sulfide—as a toxic and volatile compound—is not set free in the cytoplasm but is channeled directly to the rDSR, which eventually catalyzes the oxidation of sulfide to sulfite.

The remaining proteins encoded in the core *dsr* operon of sulfur oxidizers (Sander et al., 2006; Grimm et al., 2008) are either not directly involved in sulfide oxidation or their function is still a matter of debate. DsrL, an NADH:acceptor oxidoreductase and putative disulfide reductase, was suggested to catalyze the reductive release of sulfide from the perthiolic carrier molecule in the cytoplasm (Dahl et al., 2005). DsrC and the three subunits of the DsrEFH complex are similar, small proteins which together may be involved in sulfur and electron transfer from or to the rDSR (Dahl et al., 2005; Cort et al., 2008; Dahl et al., 2008b). DsrC plays a central role in the current model of the rDSR pathway as the substrate donor for the rDSR and is assumed to cycle between thiolic, persulfidic and disulfidic states (Cort et al., 2008; Grein et al., 2010a). Unlike DsrC, the DsrEFH complex appears to be restricted to prokaryotes that employ the DSR pathway in the oxidative direction (Sander et al., 2006; Grimm et al., 2008) and was, in addition, shown to be

essential for this process (Dahl et al., 2008b). Different functions were proposed for the transmembrane complex DsrMKJOP. While initially believed to participate in the transfer of electrons from the rDSR to the photosynthetic reaction center via quinone pool (Dahl et al., 2005), the complex was later suggested to operate in reverse and transfer electrons from a periplasmic, unspecified sulfur substrate into the cytoplasm (Grein et al., 2010a,b). These electrons were proposed to reduce the DsrC disulfide, which is thought to be formed during the catalytic cycle, and regenerate its sulfide-accepting, dithiolic form (Grein et al., 2010a). Biochemical data argue for the latter hypothesis (Grein et al., 2010a), so that the fate of electrons from the oxidation of sulfur to sulfite is currently unclear. DsrN is similar to cobyrinic acid a, c-diamide synthase and seems to be important for maturation of the siro(haeme)amide prosthetic group of the rDSR. Even though DsrN is not absolutely required for sulfur oxidation, the process is heavily impaired in a $\Delta dsrN$ deletion mutant (Lübbe et al., 2006).

1.3.3 Oxidation of sulfite to sulfate

Once produced, sulfite can be oxidized to sulfate via two alternative, energy-conserving pathways. The direct oxidation of sulfite is catalyzed by molybdenum-containing **sulfite dehydrogenases** (sulfite:ferricytochrome-c oxidoreductase; EC 1.8.2.1; Charles and Suzuki, 1966; Kappler and Dahl, 2001; Kappler, 2008, 2011) of the sulfite oxidase enzyme family, which feed electrons into the respiratory chain via cytochrome *c*. The enzymes, for which sulfite dehydrogenase activity was initially described (Lu and Kelly, 1984; Kurek, 1985; Quentmeier et al., 2000), belong to the thiosulfate-oxidizing multienzyme complex (**Section 1.3.4**) and thus are nowadays considered to function as sulfur dehydrogenases during thiosulfate oxidation rather than oxidizing sulfite (Friedrich et al., 2001, 2005b). Several other complex-independent enzymes of the sulfite oxidase family were later shown to exhibit sulfite dehydrogenase activity (Kappler et al., 2000; de Jong et al., 2000; Myers and Kelly, 2005; D'Errico et al., 2006; Di Salle et al., 2006; Denger et al., 2008; Wilson and Kappler, 2009). These are thought to catalyze the oxidation of various organic and inorganic sulfur compounds, but as the vast majority of

prokaryotic sulfite oxidase-like enzymes is presently not characterized, their actual metabolic function is mostly unknown (Kappler, 2008, 2011). The best characterized directly sulfite-oxidizing enzyme is the SorAB sulfite:cytochrome *c* oxidoreductase of *Thiobacillus novellus*, which is a periplasmic protein (Kappler et al., 2000).

Indirect, AMP-dependent sulfite oxidation involves the transient formation of adenosine-5'-phosphosulfate (APS) and conserves energy via both, substrate-level and oxidative phosphorylation (Peck, 1960; Michaels et al., 1970; Kappler and Dahl, 2001; Ghosh and Dam, 2009). The pathway starts with a flavin-containing, reverse-acting **APS reductase** (AMP,sulfite:acceptor oxidoreductase; EC 1.8.99.2), which catalyzes the oxidative formation of APS from sulfite and AMP (Peck, 1960, 1961a,b; Fritz et al., 2000). The physiological electron acceptor of the APS reductase is not known, but electron exchange with the quinone pool is discussed for APS reductases of sulfate reducers and the related lineage II APS reductases (Meyer and Kuever, 2007) of sulfur oxidizers, in particular. Subunits of a quinone-interacting membrane-bound oxidoreductase (Qmo) complex are typically encoded in the genomes of these organisms, in the case of sulfate reducers and *Chlorobiaceae* often adjacent to APS reductase subunits (Meyer and Kuever, 2007). The subunits of this QmoABC complex are homologous to the HdrA (both, QmoA and QmoB) and HdrEC (fusion protein QmoC) subunits of the soluble and membrane-bound heterodisulfide reductases (Pires et al., 2003). Different lines of evidence suggest that the QmoABC complex acts a quinol:APS reductase oxidoreductase in sulfate reducers and *Chlorobiaceae* (Pires et al., 2003; Zane et al., 2010; Rodriguez et al., 2011; Ramos et al., 2012), even though direct electron exchange between both enzymes could so far not be demonstrated in biochemical assays (Pires et al., 2003; Ramos et al., 2012). However, it has to be noted that a transmembrane quinone-interacting subunit (i.e. a QmoC or HdrE homolog) is typically not encoded in the genetic vicinity of the QmoAB subunits in the genomes of *Beta-* and *Gammaproteobacteria* (Meyer and Kuever, 2007). Accordingly, it is unclear how the QmoAB subunits of these organisms and, ultimately, their APS reductases are coupled to the electron transport chain.

APS can subsequently be cleaved by either of two enzymes, which both couple the release of sulfate to substrate-level phosphorylation. **ATP sulfurylase** (ATP:sulfate adeylyltransferase; EC 2.7.7.4; Renosto et al., 1991) exchanges AMP-bound sulfate with free pyrophosphate, yielding one ATP per oxidized sulfite. **APS:phosphate adenylyltransferase** (APAT, formerly named ADP sulfurylase; EC 2.7.7.5) uses orthophosphate as a substitute for sulfate, releasing ADP. Together with **adenylate kinase** (ATP:AMP phosphotransferase; EC 2.7.4.3), which occurs ubiquitously and transfers orthophosphate residues between adenine nucleotides (2 ADP $\rightleftharpoons$ ATP + AMP), the latter enzyme yields ½ ATP per oxidized sulfite. ADP sulfurylase was so far mostly detected in strains that simultaneously carried also the more energy-efficient ATP sulfurylase and affinities of both enzymes for APS indicated that lysis with pyrophosphate is generally preferred (Peck, 1960; Dahl and Trüper, 1989; Brüser et al., 2000). Regarding the *in vivo* function of ADP sulfurylase, Brüser et al. (2000) hypothesized that the enzyme may operate analogous to a safety valve and remove APS efficiently under pyrophosphate limitation in order to prevent the accumulation of toxic sulfite.

Overall, the direct oxidation of sulfite seems to be far more common in sulfur bacteria and most strains that are capable of indirect oxidation via the APS pathway carry the respective enzymes only in addition to a sulfite dehydrogenase (Kappler and Dahl, 2001; Kappler, 2011). Studies in *Allochromatium vinosum*, a purple sulfur bacterium, which encodes both pathways, showed not only that the direct pathway accounts for most of the sulfite oxidation observed (69–100%, depending on the irradiance; Sánchez et al., 2001) but also that APS reductase is entirely dispensable (Dahl, 1996). This apparent preference of the direct oxidation pathway is somewhat surprising, as indirect oxidation enables additional energy conservation via substrate-level phosphorylation. However, the regulation and (concerted) function of both sulfite oxidation pathways are not well understood.

1.3.4 Oxidation of thiosulfate

Prokaryotic thiosulfate oxidation can proceed via at least three pathways, the SOX pathway (Friedrich et al., 2001, 2005b, 2008), the SOX/DSR pathway (also termed

'branched thiosulfate oxidation pathway'; Hensen et al., 2006; Grimm et al., 2008), and the tetrathionate intermediate pathway (Ghosh and Dam, 2009). Biochemically, these pathways differ in the intermediates formed during the oxidation of thiosulfate to sulfate. While the SOX pathway is devoid of free intermediates, the transient deposition of elemental sulfur is obligate for the SOX/DSR pathway and tetrathionate is formed when thiosulfate is oxidized via the tetrathionate intermediate pathway.

SOX enzymes were first isolated by Lu and Kelly (1983) and the pathway was later studied in depth by the group of C. G. Friedrich. Seven polypeptides, which form four distinct periplasmic proteins, constitute the thiosulfate oxidizing multienzyme complex of the canonical SOX pathway (reviewed by Friedrich et al., 2001, 2005b, 2008). Thiosulfate oxidation via this enzyme complex is thought to operate as follows: The protein **SoxYZ** acts as a sulfur substrate carrier and as such interacts with all other proteins of the complex (Quentmeier and Friedrich, 2001). The reaction cycle is initiated by **SoxAX**, which couples thiosulfate oxidatively and covalently to a conserved cysteine residue of SoxYZ (Bamford et al., 2002). The manganese-containing protein **SoxB** then catalyzes the hydrolytic cleavage of a sulfate residue (originating from the former sulfone sulfur atom) from the SoxYZ-bound substrate. The remaining sulfane sulfur is oxidized to the level sulfone by the molybdenum-containing **sulfur dehydrogenase SoxCD** (Quentmeier et al., 2000). SoxB then cleaves the second sulfone sulfur from SoxYZ, releasing a second sulfate molecule. The electrons liberated during thiosulfate oxidation via the SOX system are transferred to cytochrome *c* (Friedrich et al., 2001). Subsets of these Sox proteins may also catalyze the oxidation of other sulfur substrates such as sulfide, sulfur, and sulfite (Kappler and Maher, 2013).

The SOX/DSR pathway is a modification of the canonical SOX pathway, which is characterized by absence of a SoxCD sulfur dehydrogenase (Hensen et al., 2006; reviewed by Grimm et al., 2008; Kappler and Maher, 2013). As a consequence, SoxYZ-bound sulfane sulfur cannot be oxidized in organisms featuring the SOX/DSR pathway. Instead, this sulfane sulfur is thought to remain bound to the carrier protein and form a covalent bond with the sulfane sulfur of the next

thiosulfate molecule, which is added by SoxAX. According to this model, the sulfane sulfur atoms of thiosulfate would catenate on SoxYZ and at some point be transferred to sulfur globules for storage (Kappler and Maher, 2013). However, the transfer mechanism and the involved enzymes are currently unknown. Sulfur, which is transiently deposited in organisms using the SOX/DSR pathway, is thought to be oxidized to sulfate via the rDSR pathway (**Section 1.3.2**). In contrast to organisms, which employ the canonical SOX pathway, the *sox* genes of organisms using the SOX/DSR pathway are usually not present in a single cluster but are encoded in several separate loci (Kappler and Maher, 2013).

Acidophilic prokaryotes seem to preferentially employ the tetrathionate intermediate pathway for thiosulfate oxidation but also neutrophiles have been reported to oxidize thiosulfate accordingly (Ghosh and Dam, 2009). The function of the tetrathionate intermediate pathway is currently not well understood on both, the enzymatic and the genetic level. The literature data are conflicting with respect to the subcellular location of the involved enzymes and the interacting electron carriers (reviewed by Ghosh and Dam, 2009). Hence, it appears that several distinct versions of the tetrathionate intermediate pathway exist and a detailed discussion will be out of the scope of this introduction. However, the general sequence of reactions is agreed on and starts with the oxidation of thiosulfate to tetrathionate, which is catalyzed by a **thiosulfate dehydrogenase**. Tetrathionate is subsequently oxidized to sulfite by a **tetrathionate hydrolase** and sulfite is oxidized to sulfate by a **sulfite dehydrogenase** (Ghosh and Dam, 2009).

1.3.5 Production of reducing equivalents

Organisms growing autotrophically need to provide reducing equivalents in addition to ATP for CO_2 fixation and other assimilatory purposes. Since the redox potentials of sulfur compounds involved in the oxidative sulfur metabolism are generally more positive than the redox potential of the $NAD(P)^+/NAD(P)H$ couple, a direct reduction of $NAD(P)^+$ is not possible. Under these circumstances electrons are lifted in the reverse direction through the more redox-negative section of the electron transport chain to a reverse-acting **NADH-dehydrogenase**

(NADH:ubiquinone oxidoreductase; EC 1.6.5.3). Chemolithotrophic and phototrophic (type-II reaction center) sulfur bacteria power the reverse electron transport with energy from the the proton motive force (Griesbeck et al., 2000) and thus need to invest additional energy in CO_2 fixation.

1.4 Molecular hydrogen in the biosphere

A main topic of this thesis is the use of molecular hydrogen by members of the family *Beggiatoaceae*. With the exception of a single study, which demonstrated that a heterotrophic *Beggiatoa* strain was able to oxidize hydrogen under short-term anoxia (Schmidt et al., 1987), H_2 has never been discussed in the context of *Beggiatoaceae* metabolism and ecophysiology. In order to estimate the ecological significance of hydrogen oxidation for members of the family *Beggiatoaceae*, this section presents an overview over the turnover of hydrogen in the environment.

Even though important in terms of atmospheric chemistry, the tropospheric H_2 budget tells only little about hydrogen cycling in the biosphere. This is due to the fact that biospheric hydrogen production and consumption are generally tightly coupled, meaning that large amounts of H_2 are turned over in the biosphere without affecting the troposphere. In the biosphere, the main hydrogen evolving reactions are fermentative processes and nitrogen fixation (**Figure 1.7**; Aragno and Schlegel, 1992). Further biotic H_2 evolving processes such as anaerobic CO oxidation (Kerby et al., 1995), phosphite oxidation (Yang and Metcalf, 2004), and redox-balancing in course of photosynthesis (Appel et al., 2000; Cournac et al., 2003) exist, but these are thought to be of minor importance. In addition to biotic processes, geological hydrogen sources can be of local importance for the biosphere (Aragno, 1992). Significant amounts of molecular hydrogen are regularly contained within the fluids and gases emitted at terrestrial and submarine sites of geothermal activity (Welhan and Craig, 1979; Lilley et al., 1982; Aragno, 1992; Petersen et al., 2011). Correspondingly, hydrogen-oxidizing prokaryotes, both free-living and symbiotic, have been be identified at such sites (e.g. Aragno, 1992; Petersen et al., 2011).

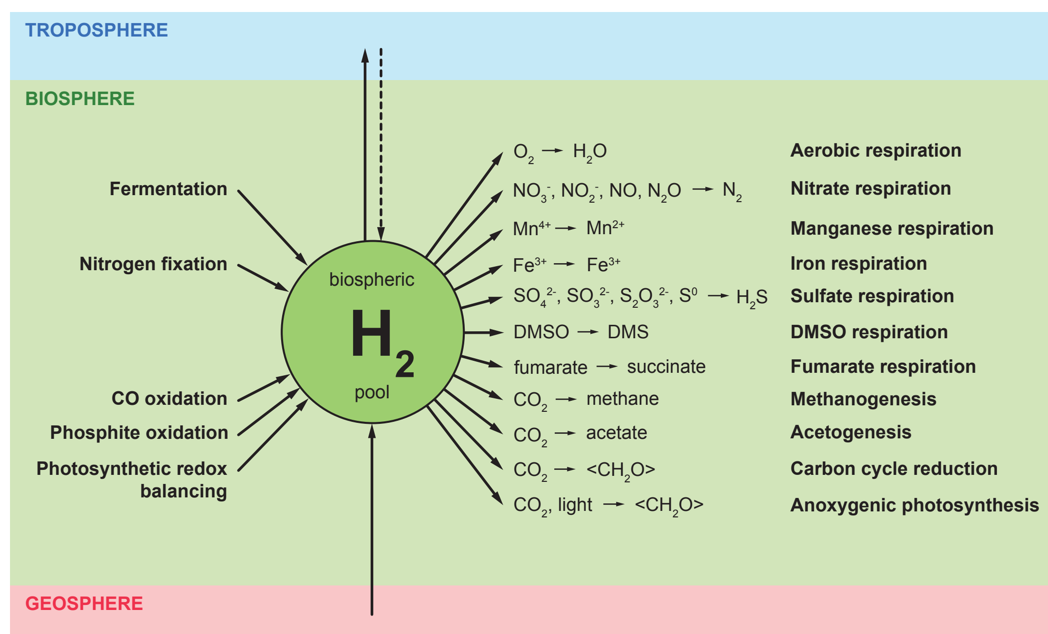

Figure 1.7 | Scheme illustrating hydrogen transfer within the biosphere. Sources and sinks for biospheric H_2 are shown. The dashed arrow represents uptake of tropospheric hydrogen by soils, a process that is putatively catalyzed by extracellular, soil particle-bound hydrogenases. $<CH_2O>$ represents organic matter. Adapted from Aragno and Schlegel (1992) according to the text; H_2-fueled dimethyl sulfoxide (DMSO) and manganese respiration were reported by e.g. Laurinavichene et al. (2002) and Lovley et al. (1989).

Fermentative reactions are responsible for a large fraction of organic matter decomposition under anaerobic conditions, may it be in sediments, the intestines of macroorganisms, or other habitats. Devoid of a (functional) electron transport chain, fermenters re-oxidize their metabolic electron carriers by direct transfer of excess electrons to organic substrates or protons, in the latter case producing H_2. However, most fermentative reactions are inhibited thermodynamically already at very low concentrations of H_2. These reactions are only possible because the fermenting organisms live in close—syntrophic—association with H_2 consumers such as methanogens, homoacetogens, and sulfate reducers (Aragno and Schlegel, 1992; Schwartz and Friedrich, 2006). If syntrophic organisms keep the H_2 concentration beyond the critical threshold, which in turn depends on the type of fermentation (Schwartz and Friedrich, 2006), fermenters can produce large amounts of molecular hydrogen over time (Hoehler et al., 2002). Hydrogen evolution by the nitrogen-fixing enzyme nitrogenase is, in contrast, not inhibited by H_2 (Rivera-Ortiz and

Burris, 1975) so that free-living and symbiotic diazotrophs can give rise to high environmental hydrogen concentrations. The frequent export of nitrogenase-evolved H_2 to the troposphere illustrates that hydrogen production by nitrogen-fixing microorganisms must in fact regularly exceed the biospheric uptake capacity (Hoehler et al., 2002).

Owing to the high redox potential of the H^+/H_2 couple (E_0' = -414 mV; Thauer et al., 1977) molecular hydrogen can be oxidized with nearly all biologically important electron acceptors (**Figure 1.7**). Microorganisms, which are capable of hydrogen uptake via one or several of these processes, are ubiquitous and highly active with respect to H_2 consumption. Accordingly, molecular hydrogen is a usually low-concentrated (< 70 nM) and short-lived compound under most environmental conditions (Hoehler et al., 1998, 2002).

The concentration of any metabolizable compound at any place in the environment is controlled by its local production and consumption rates as well as its diffusive and advective transport from or to this place. Transport processes will surely play an important role for H_2 from geothermal sources, but low concentrations and high turnover rates will usually preclude a significant transport of biologically produced H_2. Thus, with the exception of H_2 from geothermal sources, environmental hydrogen concentrations will either be controlled by production or consumption. Depending on the mode of control, hydrogen concentrations can differ pronouncedly (Hoehler et al., 2002). Organic-rich and anoxic sediments are typical examples of consumption controlled environments (Hoehler et al., 1998, 2002). These feature very low and steady H_2 levels, which usually correspond to the lowest concentration that thermodynamically allows H_2 uptake under the given conditions (Hoehler et al., 1998). Thus, a concentration of 0.031 ± 0.005 nM H_2 was measured in incubation experiments with nitrate as the terminal electron acceptor, while higher concentrations were recorded when reactions characterized by a smaller change in free energy dominated (i.e. 133 ± 15 nM in case of acetogenesis; Hoehler et al., 1998). Accordingly, hydrogen concentrations typically increase with depth in organic-rich sediments, corresponding to the decrease in the redox potential of the terminal electron acceptors used (Hoehler et al., 2002).

In contrast to sediments dominated by fermentation, hydrogen concentrations are production-controlled in phototrophic microbial mats (Hoehler et al., 2002). These environments are characterized by highly dynamic biogeochemical conditions, which change pronouncedly in the course of the diel cycle (e.g. Dillon et al., 2009). During the day, photosynthetically active cyanobacteria produce organic matter and oxygen (Canfield and Des Marais, 1993; Des Marais, 1995). At night, photosynthesis is interrupted and oxygen production comes to a halt. With ceasing oxygen production, the sulfide front rises up to the surface of the mat, which eventually becomes anoxic and sulfidic (Canfield and Des Marais, 1993; Des Marais, 1995; Dillon et al., 2009). Now, the accumulated biomass can fuel hydrogen-evolving and oxygen-sensitive processes such as fermentation and nitrogen fixation in the upper section of the mat (Hoehler et al., 2001; Omoregie et al., 2004). Hydrogen consumers cannot keep up with the enormous nightly increase in the hydrogen production rate, supposedly owing to the highly dynamic conditions (Hoehler et al., 2002). Accordingly, H_2 concentrations in such dynamic, production-controlled systems can transiently be much higher than in steady-state, consumption-controlled environments (Hoehler et al., 2001, 2002). Moreover, in photosynthetic microbial mats, the relative spatial distribution of H_2 is inverted with respect to the terminal electron acceptors used. Cyanobacterial fermentation and nitrogen fixation flood the uppermost layer of the mat with hydrogen, where more redox-positive acceptors are used than in deeper layers. Thus, in production-controlled systems, molecular hydrogen can be available to microorganisms, which usually cannot consume significant amounts of H_2 in consumption-controlled systems for thermodynamic or spatial reasons.

1.5 Enzymes of hydrogen metabolism

1.5.1 Hydrogenases

As different as hydrogen-metabolizing organisms may be in terms of physiology and phylogeny, they all share the ability to express hydrogenases (Schwartz and Friedrich, 2006). These enzymes catalyze the simplest of all redox half reactions,

the formation and dissociation of molecular hydrogen ($2\,H^+ + 2\,e^- \rightleftharpoons H_2$). *In vitro*, most hydrogenases support both, forward and backward reactions, while usually functioning as designated H_2-uptake or H_2-evolving enzymes *in vivo* (Vignais et al., 2001). Physiologically, hydrogenases perform two major tasks, being the contribution to the transmembrane proton motive force and the (re)establishment of cellular redox homeostasis (Vignais and Colbeau, 2004; Vignais, 2008). Hydrogenases are widely distributed within the bacterial and archaeal domains, but were likewise found in eukaryotes (Vignais and Billoud, 2007). In the latter, hydrogenases occur mainly in organelles of endosymbiontic origin, i.e. hydrogenosomes and chloroplasts (Horner et al., 2002). Prokaryotes, which have the ability to metabolize H_2, often encode several hydrogenases of different degrees of similarity (Vignais and Billoud, 2007). A considerable amount of work has been invested to elucidate and differentiate the *in vivo* functions of these enzymes, so that a wealth of biochemical, genetic, structural, and phylogenetic information on hydrogenases has been gathered.

One of the most elementary findings in hydrogenase research was that the ability to metabolize H_2 seems to have arisen repeatedly throughout evolutionary history. Three phylogenetically unrelated classes of hydrogenases were identified, which can be distinguished by their idiosyncratic proteinaceous cores and the presence of distinct metal cofactors (Vignais and Billoud, 2007). [NiFe]-hydrogenases (Volbeda et al., 1995) and [FeFe]-hydrogenases (Peters et al., 1998) feature characteristic binuclear metal centers at their active sites. In contrast, [Fe]-hydrogenases, which were initially termed 'metal-free hydrogenases' contain only a mononuclear, redox-inactive iron (Shima et al., 2008). Hydrogenases of these three classes are not equally distributed within the three domains of life. [NiFe]-hydrogenases seem to occur exclusively in *Bacteria* and *Archaea*, while [FeFe]-hydrogenases appear to be restricted to *Bacteria* and *Eucarya*. Within the *Bacteria*, [NiFe]-hydrogenases are widespread while [FeFe]-hydrogenases were identified mainly in representatives of the *Firmicutes* and *Deltaproteobacteria*. Functionally, [NiFe]-hydrogenases often catalyze hydrogen oxidation, while enzymes of the [FeFe]-hydrogenases are usually involved in H_2 evolution (Vignais and Billoud, 2007). [Fe]-hydrogenases are rare in comparison. So far, enzymes of this class were only identified in

methanogens (Vignais and Billoud, 2007), in which they apparently substitute a [NiFe]-hydrogenase under nickel-limitation and catalyze the reduction of methenyl-tetrahydromethanopterin (Afting et al., 1998). [FeFe]- and [Fe]-hydrogenases are not relevant for this thesis and hence will not be considered in detail.

1.5.2 [NiFe]-hydrogenases

Hydrogenases of the [NiFe]-class differ profoundly in size, the kind and number of their structural subunits as well as the type of redox partners they interact with. Nevertheless, all share a similar heterodimeric core that is composed of a large, catalytic and a small, electron-transferring subunit. Phylogenetic reconstructions showed that these subunits co-evolved (Vignais et al., 2001) and 3D structures demonstrated an intimate interaction via a large contact area (Volbeda et al., 1995).

The [NiFe]-hydrogenase large subunit (LSU) has an approximate size of ca. 60 kDa and carries the active site with the binuclear [NiFe] center. This center is coordinated by four cysteine residues or three cysteines and a selenocysteine ([NiFeSe]-hydrogenases) as well as three to five inorganic and diatomic ligands such as CN^- and CO (Vignais and Billoud, 2007). One CO and two CN^- ligands are thought to be minimally required as ligands of the iron atom, so that the molecular formula of the basic [NiFe] cofactor is $NiFe(CN)_2CO$ (Pierik et al., 1999). The coordinating (seleno)cysteines are arranged in two pairs, which are encoded in two conserved regions situated at the N- and C-termini of the LSU sequence (Vignais et al., 2001). The small subunit (SSU) has an approximate size of ca. 30 kDa and is usually equipped with three [FeS] clusters that constitute an electron relay from or to the active site (Vignais et al., 2001; Vignais and Billoud, 2007). The iron-sulfur cluster, which is proximal to the active site, is critical for hydrogenase function (Vignais et al., 2001) and its coordination can profoundly influence the biochemical properties of the hydrogenase (Goris et al., 2011). The exchange of molecular hydrogen with the cytoplasm or periplasm is thought to happen via several hydrophobic channels, which connect the active site to the surface of the protein (Montet et al., 1997). Protons probably access or leave the active site via a relay

of four histidines and one glutamate (Volbeda et al., 1995). Additional subunits and accessory proteins are often encoded together with the [NiFe]-hydrogenase core subunits in the genomes of H_2-metabolizing organisms (Vignais et al., 2001).

1.5.3 Phylogenetic clusters of [NiFe]-hydrogenases correspond to functional categories

The class of [NiFe]-hydrogenases is divided in four major phylogenetic groups and several subgroups (compare **Figure 1.8**) that correspond notably well to differences in subunit composition, biochemistry, and metabolic function (Wu and Mandrand, 1993; Vignais et al., 2001; Vignais and Billoud, 2007; Vignais, 2008; Pandelia et al., 2012).

Group 1 hydrogenases, the so-called H_2-uptake hydrogenases, are extracytoplasmic enzymes, which couple H_2 oxidation to the generation of a proton motive force (Vignais et al., 2001). Several oxidants of considerably different redox potentials such as oxygen, nitrate, dimethyl sulfoxide, fumarate, sulfur, sulfate, and CO_2 have been shown to serve as terminal electron acceptors in this process (Vignais et al., 2001; Laurinavichene and Tsygankov, 2001; Laurinavichene et al., 2007). Soluble H_2-uptake hydrogenases are known from sulfate reducers, but most enzymes of this group are membrane-bound and transfer electrons via a membrane-integral cytochrome *b* subunit to the quinone pool (Vignais et al., 2001; Vignais, 2008). The cytochrome subunit and a hydrophobic, C-terminal segment of the SSU anchor Group 1 hydrogenases in the cytoplasmic membrane (Vignais et al., 2001). A recent phylogenetic study (Pandelia et al., 2012) identified several subgroups of H_2 uptake hydrogenases with distinct compositional and biochemical characteristics, which are likely to function in different metabolic contexts. Among these are the oxygen tolerant 6C-hydrogenases, which catalyze aerobic H_2 oxidation (Goris et al., 2011), HybA-hydrogenases, which preferentially couple to terminal electron acceptors of lower redox potential (Laurinavichene and Tsygankov, 2001), and Isp-hydrogenases, which require a HdrDE-like and putatively disulfide-reducing complex for *in vivo* activity (Palágyi-Mészáros et al., 2009).

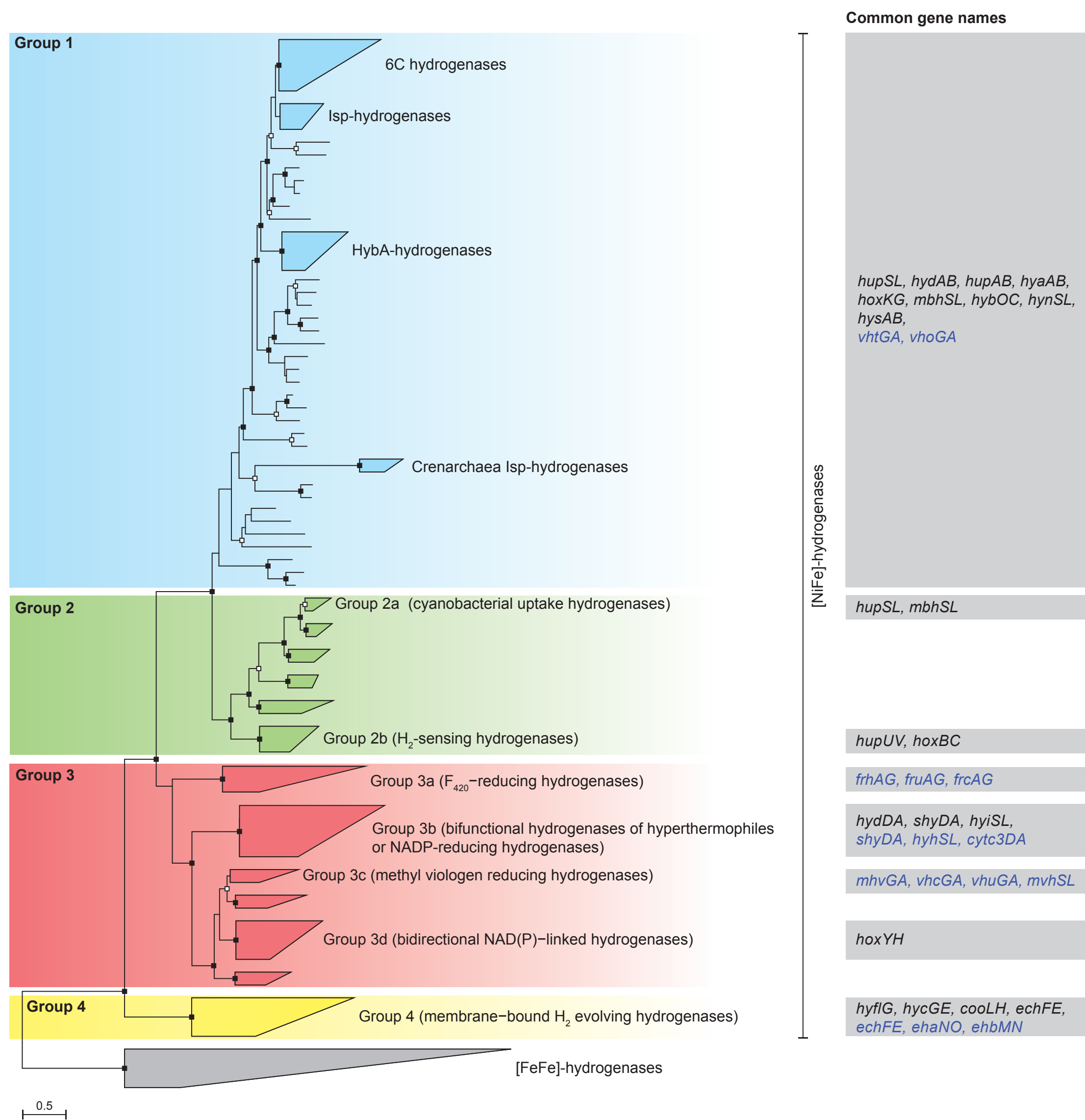

Figure 1.8 | Phylogenetic tree of [NiFe] hydrogenases large subunits. The tree was calculated based on a MAFFT alignment (version 7; Katoh and Standley, 2013) of 2038 sequences using the rapid bootstrap analysis of the RAxML algorithm (Stamatakis et al., 2008). Only representative sequences are shown and accepted clusters are named according to Vignais et al. (2001), Vignais and Billoud (2007), and Pandelia et al. (2012). Further research may assign unnamed clusters to established groups or identify these as distinct types. Bootstrap values were calculated from ten replicate trees. Plain branching, open boxes (□), and filled (■) boxes denote nodes with bootstrap values of ≤ 50%, 51–75%, and 76–100%, respectively. Names commonly used for genes encoding [NiFe]-hydrogenase small and large subunits of the respective clades (Vignais and Billoud, 2007) are given on the right side. Names of bacterial genes shown in black and names of archaeal genes written in blue.

Group 2 comprises heterodimeric and cytoplasmic [NiFe]-hydrogenases, the SSU of which is devoid of an N-terminal membrane-targeting motif (Vignais et al., 2001). The group splits in several clusters (**Figure 1.8**), among which are the cyanobacterial uptake hydrogenases (Group 2a) and the H_2-sensing hydrogenases (Group 2b). Cyanobacterial uptake hydrogenases are ubiquitous in diazotrophic cyanobacteria (Vignais et al., 2001) and have been shown to recycle H_2 produced during nitrogen fixation (Happe et al., 2000). According to their function in H_2 uptake, they are thought to be bound to the cytoplasmic face of the membrane (Vignais et al., 2001). H_2-sensing hydrogenases are involved in the regulation of uptake hydrogenase expression in presence of H_2 (Vignais et al., 2005; Friedrich et al., 2005a). Even though these enzymes catalyze H_2 oxidation, their activity is very low and they do not conserve energy (Vignais et al., 2001).

Most Group 3 hydrogenases are cytoplasmic, heteromultimeric, and physiologcally reversible enzymes which interact with soluble cofactors (Vignais et al., 2001; Vignais and Billoud, 2007; Vignais, 2008). Several phylogenetic subgroups were identified, which correspond to the physiological or artificial electron carrier being oxidized or reduced (Vignais et al., 2001; Vignais and Billoud, 2007). F_{420}-reducing hydrogenases (Group 3a) are trimeric enzymes present in methanogenic archaea. They reduce the cytoplasmic electron carrier 8-hydroxy-5-deazaflavin (coenzyme F_{420}; Jacobson et al., 1982; Jin et al., 1983), which transfers H^- anions analogous to NAD(P)H (DiMarco et al., 1990). Group 3b comprises heterotetrameric enzymes with hydrogenase and sulfur reductase activities, which were termed 'sulfhydrogenases' upon their identification in hyperthermophilic prokaryotes (Ma et al., 1993). Due to uncertainties concerning their *in vivo* function, the group was renamed 'bifunctional hydrogenases of hyperthermophiles' (Vignais et al., 2001), but homologs were later detected in mesophiles as well (Ng et al., 2000; Vignais and Billoud, 2007). Group 3b hydrogenases catalyze redox reactions involving $NAD(P)^+/NAD(P)H$, S^0/H_2S and H^+/H_2 and it was suggested that they dispose of excess electrons originating from fermentation or generate NADPH for biosynthetic purposes (Ma et al., 1993, 1994, 2000; Kanai et al., 2011). Accordingly, another name, 'NADP-reducing hydrogenases', has been suggested for Group 3b enzymes (Vignais and Billoud, 2007). Methyl viologen-reducing hydrogenases or

F_{420}-non-reducing hydrogenases (Group 3c) are membrane-bound and part of the H_2:heterodisulfide oxidoreductase complex in several methanogenic archaea (Setzke et al., 1994; Stojanowic et al., 2003). This complex couples hydrogen oxidation to the reduction of the coenzyme-M/coenzmye-B disulfide via the electron carrier methanophenazine (Abken et al., 1998; Brodersen et al., 1999). Bidirectional NAD(P)-linked hydrogenases (Group 3d) are soluble or loosely membrane-associated enzymes of four to six subunits. All Group 3d hydrogenases seem to share two dimeric modules, namely a hydrogenase and an NADH-dehydrogenase (diaphorase) module (Vignais and Billoud, 2007). They function reversibly and reduce $NAD(P)^+$ with H_2 or H^+ with NAD(P)H depending on the redox status of the cell. In cyanobacteria, Group 3d hydrogenases were shown to produce H_2 during dark fermentation and at dark/light shifts, when the light-dependent reactions are generating low-potential electrons while the dark reactions are not sufficiently active to consume these (Appel et al., 2000; Cournac et al., 2003).

Group 4 comprises energy-conserving, membrane-associated H_2 evolving hydrogenases of at least six subunits, which seem to couple the oxidation of carbonyl-groups to H_2 evolution while conserving energy in a transmembrane proton gradient (Vignais et al., 2001; Vignais and Billoud, 2007; Vignais, 2008). Among the Group 4 enzymes is (i) the hydrogenase 3 of *E. coli*, which is part of the formate-hydrogen lyase complex (Böhm et al., 1990; Sawers, 2005), (ii) the CooLH hydrogenase of *Rhodospirillum rubrum*, which—in cooperation with the bacterium's CO dehydrogenase—catalyzes the oxidation of CO to CO_2 and H_2 (Fox et al., 1996a,b), and (iii) the Ech hydrogenase of *Methanosarcina barkeri* thought to be involved in the oxidation of acetate (Künkel et al., 1998; Meuer et al., 1999).

1.5.4 Nitrogenase is an hydrogen-evolving protein

A notable exception to the otherwise consistent involvement of hydrogenases in H^+/H_2 redox reactions is the evolution of molecular hydrogen as a by-product of nitrogen fixation via the enzyme nitrogenase (Bulen and LeComte, 1966). Indeed, hydrogen production is an intrinsic property of nitrogenase so that H_2 is evolved

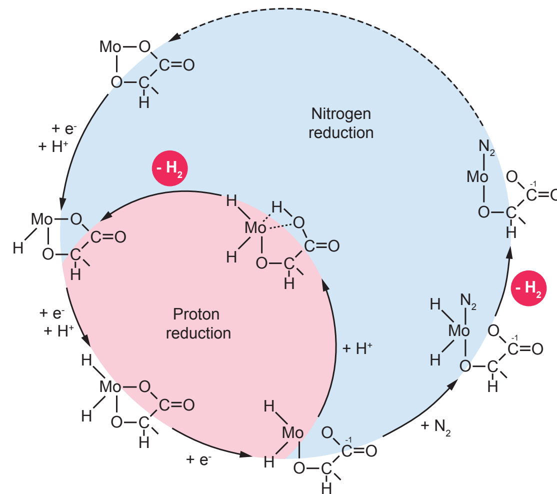

Figure 1.9 | Proposed scheme of hydrogen evolution by nitrogenase. The scheme comprises both, evolution of H_2 from proton reduction in the absence of nitrogen fixation and H_2 evolution coupled to the binding of N_2. Reactions of N_2 reduction (dashed arrow) are not shown. Adapted from Burgess and Lowe (1996).

whenever N_2 is fixed (Hadfield and Bulen, 1969; Simpson and Burris, 1984; Burgess and Lowe, 1996). Several diazotrophic organisms therefore express uptake hydrogenases under nitrogen-fixing conditions to recycle at least a part of this otherwise lost reducing power (Brito et al., 1997; Axelsson et al., 1999; Elsen et al., 2000; Happe et al., 2000). By increasing the respiration rate, hydrogen recycling may help to protect the highly oxygen-sensitive nitrogenase from O_2 in aerobic diazotrophs (Aragno and Schlegel, 1992).

The partial pressure of N_2 was found to influence hydrogen evolution reciprocally, but H_2 production cannot be suppressed completely. In absence of N_2, the production of H_2 is maximal with all the reducing power available to the enzyme being channeled into proton reduction, while only about 40% of the reducing power are used for this purpose at normal atmospheric pressure of N_2 (Rivera-Ortiz and Burris, 1975). At even higher N_2 pressures, the share of reducing power used for proton reduction is not much lower; 25–30% still end up in H_2 at an N_2 pressure of 50 atm (Simpson and Burris, 1984). Thus, the ratio of evolved hydrogen to fixed nitrogen depends on the experimental conditions (Burgess and Lowe, 1996), but at least one molecule H_2 appears to be produced per molecule N_2 being reduced

($N_2 + (6 + 2n)H^+ + (6 + 2n)e^- + p(6 + 2n)ATP \longrightarrow 2\,NH_3 + nH_2 + p(6 + 2n)ADP + p(6 + 2n)P_i$; with $n = 1$ and $p = 1$; Rees et al., 2005). However, the experimentally determined H_2:N_2 ratio is often higher so that relatively more H_2 is produced and relatively more ATP is hydrolyzed ($n > 1$ and $p > 1$; Rees et al., 2005). The so far most likely explanation for hydrogen evolution by nitrogenase, which is consistent with a minimal H_2:N_2 ratio of 1:1, was put forward by Lowe and Thorneley (1984), who suggested that nitrogen binds to the nitrogenase by displacement of molecular hydrogen (**Figure 1.9**). Further hypotheses for H_2 evolution by nitrogenases were reviewed by Burgess and Lowe (1996) but are out of the scope of this introduction.

1.6 Objectives

With this thesis I seek to expand and deepen the knowledge on the dissimilatory metabolism and the ecophysiology of the family *Beggiatoaceae*. Since the first genus of this family was described by Trevisan in 1842, the physiology of these conspicuous bacteria has been studied continuously and the oxidative sulfur metabolism has always been a major focus of this research. Yet, a broad-scale comparative study on this topic has never been conducted, most probably because data were difficult to obtain for the large number of currently unculturable members of the family. However, the availability of six draft genomes together with the large body of physiological and biochemical data from other strains does now allow for such a comparative analysis (**Section 2**). With this study I aimed at identifying pathways of sulfur compound oxidation, which have most likely been present in the last common ancestor of the family, pathways, which constitute the metabolic core of the family's extant members, as well as pathways, which confer metabolic distinctiveness to the different strains of the family.

While studying the marine chemolithoautotrophic strain *Beggiatoa* sp. 35Flor, we observed that a share of filaments migrated into the anoxic and sulfidic section of the oxygen-sulfide gradient medium, when the sulfide flux in the culture exceeded a certain threshold. Commonly, nitrate is thought to be used as an alternative

electron acceptor by *Beggiatoaceae* under anoxic conditions, but neither was nitrate present in these cultures nor did the downward migration seem to confer any advantage to the filaments under the given conditions. Therefore, we tried to find a rationale for this apparently pointless behavior and wanted to determine how *Beggiatoa* sp. 35Flor filaments gain energy under anoxic conditions (**Section 3**).

Given that many members of the family *Beggiatoaceae* are capable of lithotrophic growth, it is surprising that almost no attempts were undertaken to identify whether inorganic substrates other than reduced sulfur compounds can support growth of these bacteria. Molecular hydrogen, the likely most favorable inorganic electron donor, is used as a growth substrate by various other sulfur bacteria and a single study provided indications, that hydrogen might also be oxidized by a heterotrophic *Beggiatoa* strain (Schmidt et al., 1987). In order to promote a more comprehensive understanding of hydrogen metabolism in the family *Beggiatoaceae*, we studied this topic from different perspectives. First, we sought to identify under which conditions hydrogen is oxidized by the strain *Beggiatoa* sp. 35Flor, whether the strain couples hydrogen oxidation to carbon dioxide fixation, whether hydrogen can serve as an exclusive electron donor for this strain, and in which habitats hydrogen oxidation could in general be important for members of the family *Beggiatoaceae* (**Section 4.1**). In addition, we studied the presence and diversity of hydrogenase genes in different members of *Beggiatoaceae* to estimate how widespread hydrogen oxidation is within the family and how it may be integrated into the metabolism of these sulfur bacteria (**Section 4.2**).

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Supplemental material

Table S1.2 | Vacuolation in members of the family *Beggiatoaceae*.

Organism[a]	Source	Diameter [μm]	Vacuole	Evidence[g]	P/BV[b] [mg cm^{-3}]	Reference
bundled trichomes (*Thioploca ingrica*)	Lake Baikal (low-temp. hydrothermal vents)	2–5	+	LM[c]	N/A	Zemskaya et al. (2001, 2009)
bundled trichomes ('*Ca.* Marithioploca spp.')	Chilean continental shelf	12–20; 30–43	+	LM, EM	N/A	Maier and Gallardo (1984)
bundled trichomes ('*Ca.* Marithioploca spp.')	Chilean continental shelf	15–20; 30-40	+	LM	N/A	Fossing et al. (1995)
single filaments ('*Ca.* Marithioploca spp.')	Bay of Concepción	35–40	+	LM	N/A	Teske et al. (1999)
single filaments ('*Ca.* Maribeggiatoa vulgaris')	Monterey Canyon (Clam Field Seep)	65–85	+	P/BV	24	McHatton et al. (1996)
single filaments ('*Ca.* Maribeggiatoa vulgaris')	Carmel Canyon	20–76	+	P/BV	8.9 ± 1.1	Kalanetra et al. (2004)
single filaments ('*Ca.* Allobeggiatoa salina')	Lake Chiprana (8% salinity)	6–8	+	MDY-46	N/A	Hinck et al. (2007, 2011)
single filaments ('*Ca.* Allobeggiatoa salina')	Lake Chiprana (8% salinity)	6–8	+	FITC	N/A	Hinck et al. (2011)
single filaments ('*Ca.* Allobeggiatoa salina')	Lake Chiprana (8% salinity)	N/A	+	Newport Green	N/A	Hinck et al. (2011)
single filaments ('*Ca.* Allobeggiatoa salina')	Solar saltern, Ibiza (6% salinity)	6–8	+	DCFDA	N/A	Hinck et al. (2011)
single filaments ('*Ca.* Allobeggiatoa salina')	Solar saltern, Ibiza (15% salinity)	9	+	DCFDA	N/A	Hinck et al. (2011)
single filaments ('*Ca.* Allobeggiatoa salina')	Inactive saltern, Formentera (15% salinity)	N/A	+	CMFDA	N/A	Hinck et al. (2011)
single filaments ('*Ca.* Allobeggiatoa salina')	Guerrero Negro, Mexico (8% salinity)	9–10	+	CMFDA	N/A	Hinck et al. (2011)
single filaments ('*Ca.* Para- or Isobeggiatoa spp.')	Limfjorden	9–12	+	LM	N/A	Mußmann et al. (2003)
single filaments ('*Ca.* Isobeggiatoa spp.')	Jade Bight (intertidal mud flat)	9–11	+	LM	N/A	Mußmann et al. (2003)
single filaments ('*Ca.* Isobeggiatoa spp.')	Tokyo Bay	30	+	LM	N/A	Kojima and Fukui (2003)
single filaments ('*Ca.* Isobeggiatoa spp.')	Svalbard/ Smeerenburgfjorden	5–50	+	LM	N/A	Jørgensen et al. (2010)
single filaments (*Beggiatoa* sp. 35Flor)	Florida (diseased coral)	6	+	DAPI[e]	N/A	Brock and Schulz-Vogt (2011)
single filaments (named *Beggiatoa mirabilis*)	Kiel	ca. 14.6–45[f]	+[d]	Neutral Red	N/A	von Zastrow (1953)
single filaments	Svalbard/ Ymerbukta	5–12	+	LM	N/A	Jørgensen et al. (2010)
single filaments	Guaymas Basin (hydrothermal vents)	88–140	+	EM, P/BV	9.5	Nelson et al. (1989); McHatton et al. (1996)
single filaments	Tokyo Bay (hypertrophic coastal sediment)	ca. 9	+	LM	N/A	Sayama (2001)
single filaments	Gulf of Mexico (hydrocarbon seeps)	10–200	+	EM	N/A	Larkin and Henk (1996)
single filaments	Araruama & Itaipu Lagoons	6.5–34	+[d]	FITC	N/A	de Albuquerque et al. (2010)
spherical cells (*Thiomargarita* spp.)	Namibian continental shelf	100–300	+	P/BV, FITC	4.5	Schulz et al. (1999)
spherical cells (*Thiomargarita* spp.)	Gulf of Mexico (hydrocarbon seeps)	180–375	+	P/BV, FITC	21.5 ± 1.4	Kalanetra et al. (2005)
spherical cells (*Thiomargarita* spp.)	Amon Mud Volcano	24–65	+	FITC	N/A	Girnth et al. (2011)
spherical cells	N/A	9	+	N/A	N/A	Salman et al. (2011)
vacuolate attached filaments ('*Ca.* Marithrix spp.')	White Point (hydrothermal vents)	4–112	+	LM, P/BV, FITC	7.6 ± 0.8	Kalanetra et al. (2004)
vacuolate attached filaments ('*Ca.* Marithrix' spp.')	Juan de Fuca Ridge (hydrothermal vents)	9–96	+	P/BV, FITC	13.4 ± 1.5	Kalanetra and Nelson (2010)
vacuolated attached filaments	Warm Mineral Springs, Florida	13	+	LM,EM	N/A	Larkin and Henk (1989)

(Continued on next page.)

Table S1.2 (Continued from previous page.)

Organism[a]	Source	Diameter [μm]	Vacuole	Evidence[g]	P/BV [mg cm^{-3}]	Reference
bundled trichomes (*Thioploca ingrica*)	Lake Biwa	3–5.6	–	EM	N/A	Kojima et al. (2003)
bundled trichomes (*Thioploca ingrica*)	Hjarbæk fjord	2.3–4	–	N/A	N/A	Høgslund et al. (2010)
single filaments (*Beggiatoa* sp. MS-81-6)	Great Sippiwisset Salt Marsh	4	–	EM, P/BV	121 ± 17	Nelson et al. (1982); McHatton et al. (1996); Ahmad et al. (2006)
single filaments (*Beggiatoa* sp. MS-81-1c)	Great Sippewisset Salt Marsh	2	–	EM	N/A	Nelson et al. (1982); McHatton et al. (1996); Ahmad et al. (2006)
single filaments (*Beggiatoa alba* B15LD)	N/A	3	–	EM	N/A	Strohl et al. (1982)
single filaments	N/A	ca. 2-3	–	EM	N/A	Drawert and Metzner-Küster (1958)
single filaments	N/A	3	–	EM	N/A	Morita and Stave (1963)
single filaments	Araruama Lagoon	2.4–6.5	–	FITC	N/A	de Albuquerque et al. (2010)
vacuolate attached filaments ('*Ca.* Marithrix spp.')	White Point (hydrothermal vents)	<2–4	–	FITC	N/A	Kalanetra et al. (2004)

N/A Not available.

[a] Morphology of the investigated bacteria. The phylogenetic affiliation given in parentheses is based on 16S rRNA gene sequences published in the referenced study or other studies investigating the same population. The revised taxonomy according to Salman et al. (2011) is used.

[b] Protein to biovolume ratio.

[c] Large vacuoles developed a blue color upon staining of nitrate with diphenyl amine in concentrated sulfuric acid.

[d] One to many vacuoles per cell (de Albuquerque et al., 2010). Many small or few large vacuoles (von Zastrow, 1953).

[e] Polyphosphate staining with DAPI highlighted one to several internal vacuoles.

[f] Diameter range for *Beggiatoa mirabilis* as summarized in Klas (1937).

[g] LM stands for light microscopy, EM for electron microscopy (scanning or transmission). MDY-46, FITC, Newport Green, DCFDA, CMFDA, DAPI, and Neutral Red are stains, the specificity of which is given in the respective publications.

Table S1.3 | Nitrate enrichment in members of the family *Beggiatoaceae*.

Organism[a]	Source	Diameter [μm]	Vacuole	Nitrate concentration Internal [mM]	Ambient [μM]	Reference
bundled trichomes (*Thioploca ingrica*)	Lake Biwa	3–5.6	–	ca. 0.4–2	ca. 15–20	Kojima et al. (2003, 2007)
bundled trichomes (*Thioploca ingrica*)	Hjarbæk fjord	2.3–4	–	ca. 3	> 100[b]	Høgslund et al. (2010)
bundled trichomes (*Thioploca ingrica*)	Lake Baikal (low-temp. hydrothermal vents)	2–5	+	136	10.2	Zemskaya et al. (2001, 2009)
bundled trichomes ('*Ca.* Marithioploca spp.')	Chilean continental shelf	15–20; 30-40	+	≤ 500	25	Fossing et al. (1995)
bundled trichomes ('*Ca.* Marithioploca spp.')	Chilean continental shelf	12–22; 28–42	+	160 ± 150	N/A	Otte et al. (1999)
bundled trichomes	Bay of Concepción	9.4–24.2	N/A	≤ 500	25	Zopfi et al. (2001)
single filaments ('*Ca.* Marithioploca spp.')	Bay of Concepción	35–40	+	42 ± 26.75	10	Teske et al. (1999)
single filaments ('*Ca.* Maribeggiatoa vulgaris')	Carmel Canyon	20–76	+	16.6 ± 4	N/A	Kalanetra et al. (2004)
single filaments ('*Ca.* Maribeggiatoa vulgaris')	Monterey Canyon (Clam Field Seep)	65–85	+	160 ± 20	40–45	McHatton et al. (1996)
single filaments ('*Ca.* Allobeggiatoa salina')	Lake Chiprana[c] (fresh mat sample)	6–8	+	4 ± 8	N/A	Hinck et al. (2007, 2011)
single filaments ('*Ca.* Allobeggiatoa salina')	Lake Chiprana[c] (dark incubated mat)	6–8	+	42 ± 1.9	N/A	Hinck et al. (2007, 2011)
single filaments ('*Ca.* Allobeggiatoa salina')	Lake Chiprana[c] (24 h in 50 μm nitrate)	6–8	+	8 ± 0.7	50	Hinck et al. (2007, 2011)
single filaments ('*Ca.* Allobeggiatoa salina')	Lake Chiprana[c] (48 h in 50 μm nitrate)	6–8	+	44 ± 3.4	50	Hinck et al. (2007, 2011)
single filaments ('*Ca.* Allobeggiatoa salina')	Lake Chiprana[c] (mesocosm, 8% salinity)	6–8	+	4	N/A	Hinck et al. (2011)
single filaments ('*Ca.* Allobeggiatoa salina')	Lake Chiprana[c] (enrichment, 8% salinity)	10	+	430 ± 90	50	Hinck et al. (2011)
single filaments ('*Ca.* Allobeggiatoa salina')	Solar saltern, Ibiza (enrichment, 6% salinity)	8–9	+	470 ± 280	50	Hinck et al. (2011)
single filaments('*Ca.* Allobeggiatoa salina')	Solar saltern, Ibiza (enrichment, 15% salinity)	10	+	650 ± 190	50	Hinck et al. (2011)
single filaments ('*Ca.* Halobeggiatoa spp.')	Tokyo Bay (hypertrophic coastal sediment)	ca. 9	+	105 ± 36	ca. 25	Sayama (2001); Kojima and Fukui (2003)
single filaments ('*Ca.* Para- or Isobeggiatoa spp.')	Limfjorden	9–12	+	156 ± 71	17	Mußmann et al. (2003)
single filaments ('*Ca.* Isobeggiatoa spp.')	Jade Bight (intertidal mud flat)	9–11	+	288 ± 80	N/A	Mußmann et al. (2003)
single filaments ('*Ca.* Isobeggiatoa spp.')	Svalbard/ Smeerenburgfjorden	16–20	+	86	1–10[d]	Jørgensen et al. (2010)
single filaments ('*Ca.* Isobeggiatoa spp.')	Svalbard/ Smeerenburgfjorden	16–20	+	134	1–10[d]	Jørgensen et al. (2010)
single filaments	Svalbard/ Smeerenburgfjorden	13–15	+	260	1–10[d]	Jørgensen et al. (2010)
single filaments	Svalbard/ Ymerbukta	8–10	+	2.7 ± 0.2	1–10[d]	Jørgensen et al. (2010)
single filaments	Guaymas Basin (hydrothermal vents)	88–140	+	130 ± 10	0–40	McHatton et al. (1996)
single filaments	Eckernförde Bay	24 or 30	N/A	100–300	7–8	Preisler et al. (2007)
single filaments	Håkon Mosby Mud Volcano	4.8 ±0.5	N/A	110 ± 36	15	Lichtschlag et al. (2010)
spherical cells (*Thiomargarita* spp.)	Namibian continental shelf	100–300	+	100–800	5–28	Schulz et al. (1999)
spherical cells (*Thiomargarita* spp.)	Gulf of Mexico (hydrocarbon seeps)	180–375	+	460	N/A	Kalanetra et al. (2005)
vacuolate attached filaments ('*Ca.* Marithrix spp.')	White Point (hydrothermal vents)	4–112	+	<0.8[e]	N/A	Kalanetra et al. (2004)
vacuolate attached filaments ('*Ca.* Marithrix spp.')	Juan de Fuca Ridge (hydrothermal vents)	9–96	+	<0.8[e]	12.54 ± 0.32	Kalanetra and Nelson (2010)
single filaments (*Beggiatoa* sp. MS-81-1c)	Great Sippewisset Salt Marsh	4	–	≤0.0003	1000	Nelson et al. (1982); McHatton et al. (1996); Ahmad et al. (2006)

N/A Not available.

[a] Morphology of the investigated bacteria. The phylogenetic affiliation given in parentheses is based on 16S rRNA gene sequences published in the referenced study or other studies investigating the same population. The revised taxonomy according to Salman et al. (2011) is used.

[b] Annual average in adjacent rivers.

[c] Athalassohaline, hypersaline lake.

[d] General value for bottom water in fjords on the west coast of Svalbard.

[e] Internal nitrate concentration was below the given detection limit. The authors suggested passive, transient oxygen storage in the vacuole.

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Chapter 2

Beggiatoaceae genomes

2.1 Oxidative sulfur metabolism in the family Beggiatoaceae: a comparative perspective

Anne-Christin Kreutzmann

Contributions:

I developed the concept of this study, performed and evaluated all experiments and analyses and wrote the report.

Acknowledgements:

I thank Marc Mußmann for kindly providing the draft genome sequences of *Beggiatoa* sp. 35Flor and '*Ca.* Thiomargarita nelsonii'. Barbara J. MacGregor and Matthias Winkel are acknowledged for sending me manuscripts prior to publication.

Abstract

Colorless sulfur bacteria of the family *Beggiatoaceae* can contribute significantly to sulfide detoxification and chemosynthetic primary production in many sulfidic habitats. Accordingly, their sulfur metabolism has been of great interest ever since and many studies have focussed on investigating different aspects of this topic. However, these studies had to rely on only a few and rather closely related strains so that a more comprehensive overview over the sulfur compound oxidation pathways in the family *Beggiatoaceae* could not be given. In this study we provide such an overview by integrating data from six *Beggiatoaceae* draft genomes with previously published physiological and biochemical data from cultured strains. We found that members of the *Beggiatoaceae* encode three distinct types of sulfide-oxidizing flavoproteins, being type I and type VI sulfide:quinone oxidoreductases and flavocytochrome *c*-sulfide dehydrogenases. Thiosulfate is typically oxidized via the branched thiosulfate oxidation pathway by a truncated Sox-multienzyme complex (SoxAXBYZ). In addition, some strains might be capable of oxidizing thiosulfate to tetrathionate. Elemental sulfur, which is deposited intracellularly from the oxidation of sulfide and thiosulfate is further oxidized via the reverse dissimilatory sulfite reductase (rDSR) pathway, but a few strains are apparently not capable of performing this reaction. Sulfite can be oxidized to sulfate either directly or indirectly via adenosine-5'-phosphosulfate (APS), but the direct oxidation of sulfite seems to be more widespread among the *Beggiatoaceae*. However, it is currently unclear which enzyme is used for this purpose, because a SorAB-type sulfite-dehydrogenase was identified only in a single case. All three types of sulfide-oxidizing flavoproteins, the SoxAXBYZ multienzyme complex, and the rDSR were likely inherited from the last common ancestor of the family. APS reductase sequences are currently not available for any strain from the root of the *Beggiatoaceae*, but this enzyme seems to be ancestral at least for the more derived members of the family.

Introduction

Members of the family *Beggiatoaceae* belong to the sulfur bacteria, a physiological group of prokaryotes characterized by the oxidation of inorganic sulfur compounds for dissimilatory purposes. The oxidative sulfur metabolism of sulfur bacteria has been studied extensivey in phototrophic and chemotrophic representatives, which can easily be cultured and modified genetically. Both, cultivation and genetic modification, are however difficult for members of the *Beggiatoaceae*. This family includes only few currently culturable strains and for none of these a genetic system has been established. Physiological studies on sulfur metabolism have been conducted with cultured representatives, but these studies could compare at most a few, rather closely related strains. Two genomic studies on in total three *Beggiatoaceae* draft genomes (Mußmann et al., 2007; MacGregor et al., 2013) provided additional data, which are particularly valuable because they originate from more distantly related members of the family. Nevertheless, both genomic studies focussed primarily on the putative metabolic properties of the sequenced individuals, so that broad-scale comparative studies attempting to identify common pathways of sulfur compound oxidation are not available. This limits not only the general understanding of *Beggiatoaceae* metabolism but also the comparability with other sulfur bacteria and the establishment of a comprehensive model of sulfur compound oxidation in this phylogenetically diverse group.

In this study, we present a comparative analysis of the six so far sequenced *Beggiatoaceae* draft genomes with respect to the pathways involved in oxidative sulfur metabolism. In order to corroborate the identification of the genome-suggested pathways on a different level of evidence, we compared the obtained data with the results of previous physiological and biochemical studies. In addition, we reconstructed phylogenetic trees for key enzymes of several sulfur compound oxidation pathways in order to estimate whether the respective enzymes could have been inherited from a common ancestor of the family. Eventually, we integrated genomic, physiological, biochemical, and phylogenetic data to evaluate how widespread the different sulfur compound oxidation pathways may be among members of the *Beggiatoaceae* and whether these can be regarded as typical for the family.

Materials and Methods

Genome mining and gene identification

The software suite JCoast version 1.7 (Richter et al., 2008) was used to search the draft genomes of *Beggiatoa* sp. 35Flor, *Beggiatoa alba* B18LD, '*Candidatus* Isobeggiatoa sp.', '*Ca.* Parabeggiatoa sp.', '*Ca.* Thiomargarita nelsonii', and that of an orange filament sampled in the Guaymas basin (hereafter named 'Guaymas filament') for genes of interest. The genomes of '*Ca.* Isobeggiatoa sp.', '*Ca.* Parabeggiatoa sp.' (Mußmann et al., 2007), and the Guaymas filament (MacGregor et al., 2013) have been studied previously and details on genome assembly, gene prediction, and gene annotation are given in the respective publications. The genome of *Beggiatoa alba* B18LD was sequenced at the DOE Joint Genome Institute (JGI project ID 16466; NCBI project ID 62137; principal investigator J. A. Müller) and corresponding information is given on the institute's website (www.jgi.doe.gov). The genomes of *Beggiatoa* sp. 35Flor and '*Ca.* Thiomargarita nelsonii' were recently sequenced, assembled, and annotated as described by Winkel et al. (2013).

For this study, we assumed that frame shifts in the identified genes were introduced during the amplification of genomic DNA for sequencing or during the sequencing reaction itself rather than having been present in the original DNA template. Hence, we corrected all detected frame shifts manually and treated the respective genes as being complete and functional. Likewise, we regarded a gene as being present and encoding a functional enzyme if we identified only a fragment residing at the end of a contig. Genes with frame shifts and gene fragments are indicated in **Table S2.2**.

Phylogenetic reconstructions

Phylogenetic reconstructions were performed with subunits of several key enzymes involved in the oxidation of inorganic sulfur compounds. For a better comparability, reference sequences were chosen according to recent publications, i.e.

Gregersen et al. (2011) for the sulfide-oxidizing flavoproteins, Loy et al. (2009) for DsrB, Meyer and Kuever (2007) for AprA, and Kappler and Maher (2013) for SoxA. Amino acid sequences were aligned with MAFFT version 6 or 7 (Katoh et al., 2002; Katoh and Standley, 2013) and tree reconstructions were performed with the rapid bootstrap analysis of the Randomized Axelerated Maximum Likelihood algorithm (RAxML; Stamatakis et al., 2008) using the PROTGAMMA rate distribution and the Jones-Taylor-Thornton amino acid substitution model. Bootstrap values were calculated based on 100 replicate runs. The obtained trees were visualized using the software suite ARB (Ludwig et al., 2004). The 16S rRNA gene tree of the family *Beggiatoaceae* was reconstructed as described previously (Salman et al., 2011). In short, individual trees were calculated in ARB using neighbor joining, maximum parsimony, and maximum likelihood (RAxML) algorithms (each with 0, 30, and 50% positional conservatory filters). Eventually, a consensus tree with multifurcations at unstable nodes was constructed. Further details on tree reconstructions are given in the respective figure legends.

Amplification of rDSR genes

Genomic DNA was extracted with the UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) from axenic cultures of *Beggiatoa alba* B15LD (DSM 1416), *Beggiatoa alba* B18LD (ATCC 33555), and several hypersaline (‘60Ibiz’, ‘150Ibiz’, ‘80Chip’; Hinck et al., 2011) and freshwater (00Aarh’ and ‘00Hann’; brought into culture by H. N. Schulz-Vogt and V. Bondarev, respectively) *Beggiatoaceae* enrichments. The potential of the extracted DNA to serve as a PCR template was tested in amplifications with the primer pair GM3F/ITSReub, which targets the 16S rRNA gene together with the 16S-23S intergenetic spacer (see **Table S2.1** for primer sequences; details on the amplification with GM3F/ITSReub are given in Kreutzmann and Mußmann, 2013). These PCRs produced good results, showing that the extracted DNA was of sufficient quality and quantity. Subsequently, the amplification of genes encoding a reverse dissimilatory sulfite reductase (rDSR) was attempted in temperature gradient PCRs using the rDSR-specific primer pairs rDSR1Fa/rDSR4Rb, rDSR1Fb/rDSR4Rb,

rDSR1Fc/rDSR4Rb (Loy et al., 2009). The reactions were performed in a total volume of 30 μL with 15 μL Promega 2× PCR Master Mix (Promega Corporation, Madison, WI, USA), 13.4 μL PCR water, 0.3 μL of the respective forward and reverse primers (100 pmol μL $^{-1}$), and 1 μL DNA template. The PCR program was as follows: an initial denaturation at 95°C for 5 min was followed by 33 cycles of 95°C for 1 min, 45.0–65.5°C (gradient with 12 temperature steps) for 1 min, and 72°C for 3 min. The final elongation at 72°C lasted 10 min.

Results and Discussion

Phylogenetic position of the analyzed Beggiatoaceae

The presence of (partial) 16S rRNA genes in the studied draft genomes or the retrieval of such genes from cultures of the respective strains allowed the clear identification of all genome source species as members of the family *Beggiatoaceae* (**Figure 2.1**). Four of the analyzed draft genomes were studied previously (Mußmann et al., 2007; MacGregor et al., 2013; Winkel et al., 2013) and the authors of these studies provided 16S rRNA gene-based classifications of the respective source species. Mußmann et al. (2007) classified the source species of two genomes as *Beggiatoa* spp., but according to a recent taxonomic revision of the family *Beggiatoaceae* (Salman et al., 2011) these belong to the newly proposed genera '*Candidatus* Isobeggiatoa' and '*Ca.* Parabeggiatoa'. MacGregor et al. (2013) classified the orange filament from the Guaymas Basin, which they studied, as '*Ca.* Maribeggiatoa sp.', but our phylogenetic analyses do not agree with this affiliation. Rather than clustering consistently with the proposed genus '*Ca.* Maribeggiatoa', the cluster of orange filaments from the Guaymas Basin formed also monophyla with the '*Ca.* Marithioploca' cluster and with a cluster of white filaments from the Guaymas Basin, depending on the method of tree reconstruction. In addition, the 16S rRNA gene sequence from the genome studied by MacGregor and colleagues was more identical to sequences from the genus '*Ca.* Marithioploca' (97.7–98.2%) than to sequences from the genus '*Ca.* Maribeggiatoa' (95.3–95.8%) and the cluster of white filaments from the Guaymas Basin (94.7–97.3%). Future sequencing

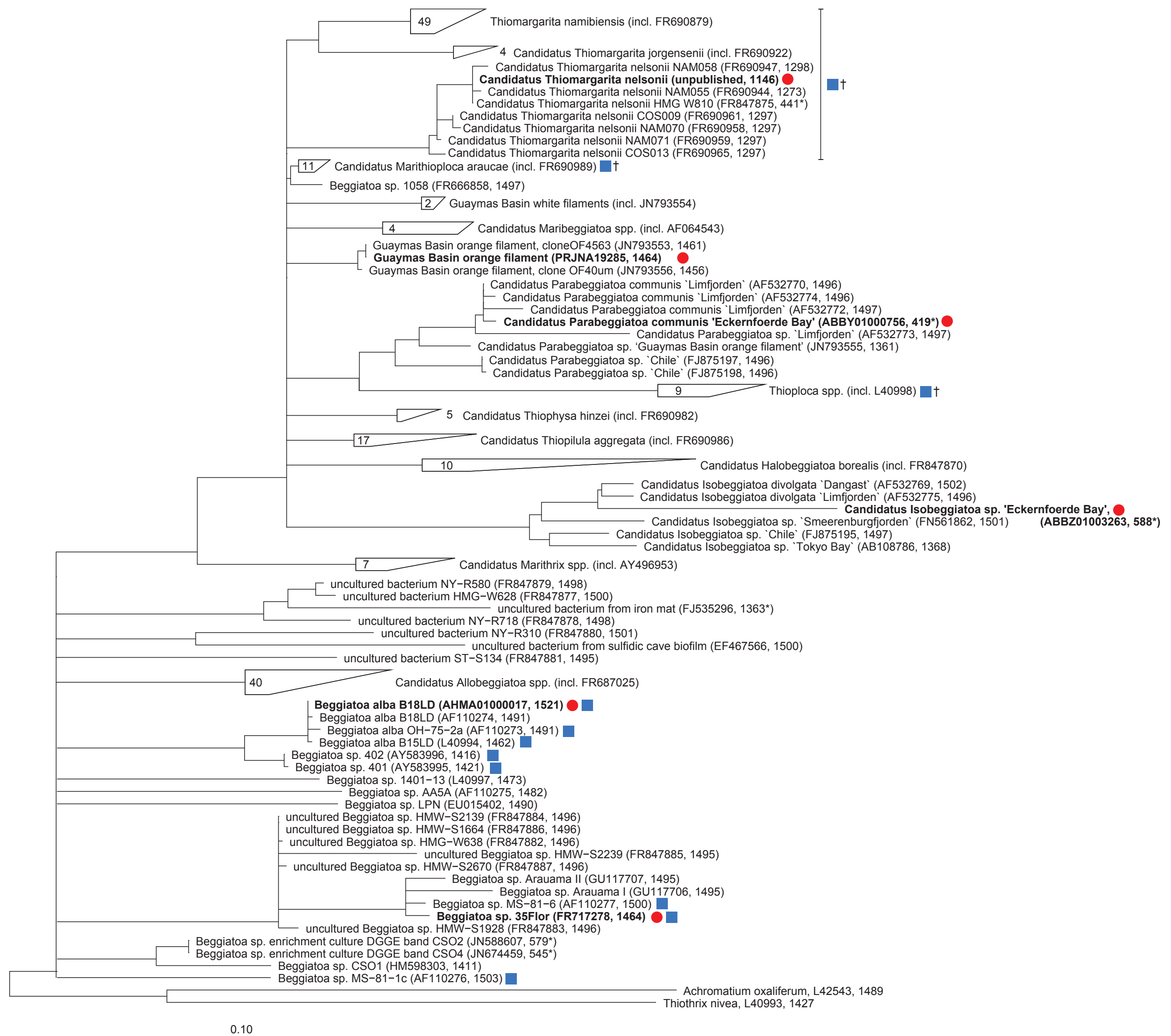


Figure 2.1 | Phylogenetic consensus tree of 16S rRNA genes from members of the family *Beggiatoaceae*. The recent revision of nomenclature within the family *Beggiatoaceae* (Salman et al., 2011; Hinck et al., 2011; Grünke et al., 2012) is adopted. Strains, filaments, or cells, for which partial genome sequences are available, are marked with a red bullett (●). Data from other strains or environmental cell samples were included in the evaluation and these strains are marked with a blue square (■). If 16S rRNA gene sequences were not available for environmental samples the most likely phylogenetic affiliation as inferred from cell morphology and sampling site is given (†). Database accession numbers and sequence lengths are given in parentheses. The tree was constructed according to Salman et al. (2011) with 262 sequences from *Beggiatoaceae* sequences and 99 sequences of different sulfur bacterial genera serving as an outgroup. Only selected sequences are shown. Sequences, which are shorter than the considered segment of the alignment (nucleotide positions 279–1463 according to *E. coli* numbering), are marked with an asterisk (*). The scale bar represents 10% sequence divergence.

may establish the clusters of white and orange filaments from the Guaymas Basin as new genera or allow their sound identification as members of an already proposed genus. However, due to the inconsistent placement in the 16S rRNA gene tree we currently refrain from assigning the orange filaments from the Guaymas Basin to a particular genus. Instead, we will tentatively refer to the source species of the respective genome as 'Guaymas filament'. The single cell, from which the fourth of the previously studied genomes originates, was phylogenerically classified as '*Ca.* Thiomargarita nelsonii' by Winkel et al. (2013) and the results of our phylogenetic reconstructions are in agreement with this affiliation.

The 16S rRNA gene sequence obtained from the *Beggiatoa alba* B18LD draft genome expectedly clustered with previously published sequences of this strain. So far, we did not find a 16S rRNA gene in the *Beggiatoa* sp. 35Flor draft genome, but a sequence from the strain has been published previously (Brock et al., 2012). As Salman et al. (2011) noted, a taxonomic revision of the species still referred to as *Beggiatoa* is required, owing to the paraphyletic nature of this group. Yet, the current resolution in this part of the tree precludes such a revision at the moment.

As a set, the six currently available draft genomes are suited for a first broad-scale comparative genomics study of the *Beggiatoaceae*, as their source species are well distributed over the entire family (**Figure 2.1**). Whenever possible, we included data from previous physiological and biochemical experiments in our considerations. With a few exceptions, such data are however available only for a group of strains from the root of the family, i.e. strains for which the genus name *Beggiatoa* was tentatively retained (**Figure 2.1**; Cluster "XII" in Salman et al., 2011).

Beggiatoaceae encode multiple sulfide-oxidizing enzymes

Sulfide is oxidized by all of the so far studied *Beggiatoaceae* (e.g. *Beggiatoa alba* B15LD, B18LD; *Beggiatoa* sp. B25RD, L1401-15, OH-75-2a, OH-763-B, MS-81-1c, MS-81-6, 35Flor; *Thiomargarita* spp., '*Ca.* Marithioploca spp.'; Mezzino et al., 1984; Nelson and Castenholz, 1981; Nelson et al., 1982; Nelson and Jannasch,

1983; Hagen and Nelson, 1996; Otte et al., 1999; Schulz and de Beer, 2002; Kamp et al., 2008) so that sulfide-oxidizing enzymes are expected to be ubiquitous in the family. Correspondingly, we detected sulfide-oxidizing flavoproteins in all of the here investigated *Beggiatoaceae* genomes (**Table S2.2**; see also Mußmann et al., 2007; MacGregor et al., 2013; Winkel et al., 2013). In fact, a considerable redundancy of these enzymes is evident from the identification of multiple—up to four—homologs in five out of the six available draft genomes. According to a recently proposed classification scheme (Marcia et al., 2010; Gregersen et al., 2011), the predicted sulfide-oxidizing flavoproteins of the *Beggiatoaceae* can be identified as Type I (SqrA) and Type VI (SqrF) sulfide:quinone oxidoreductases (SQRs) and flavoprotein subunits (FccB) of flavocytochrome *c*-sulfide dehydrogenases (FCSDs; **Figure 2.2**). Enzymes of all three types are encoded by *Beggiatoa alba* B18LD and the Guaymas filament, and subsets were identified in the other studied draft genomes (SqrA and FccB in '*Candidatus* Isobeggiatoa sp.'; SqrF and FccB in *Beggiatoa* sp. 35Flor and '*Ca.* Thiomargarita nelsonii'; FccB in '*Ca.* Parabeggiatoa sp.'). This distribution and the monophyletic clustering of most *Beggiatoaceae*-derived sequences within the SqrA, SqrF and FccB clades (**Figures S2.9** to **S2.11**) suggest that all three types of sulfide-oxidizing flavoproteins may have been inherited from a common ancestor of the family and thus could generally be encoded concurrently. Two FccB sequences, BA02_147 from *Beggiatoa alba* B18LD and BOGUAY_2853 from the Guaymas filament, were phylogenetically clearly distinct from other *Beggiatoaceae*-derived FccB sequences and were thus probably acquired via horizontal gene transfer.

Even though a redundancy of sulfide-oxidizing flavoproteins is known from various strains of purple and green sulfur bacteria (e.g. Gregersen et al., 2011), comprehensive models explaining this observation are lacking. Biochemical experiments with *Beggiatoa alba* B18LD grown in presence of ca. 200 μM sulfide (Schmidt et al., 1987) indicated that SQRs are the principal enzymes catalyzing sulfide oxidation in this strain under the tested conditions. Considering the properties of characterized SqrA and SqrF SQRs, it seems probable that these perform sulfide oxidation under different environmental sulfide regimes. All of the so far characterized SqrA enzymes exhibited a high affinity to sulfide (reported K_m values

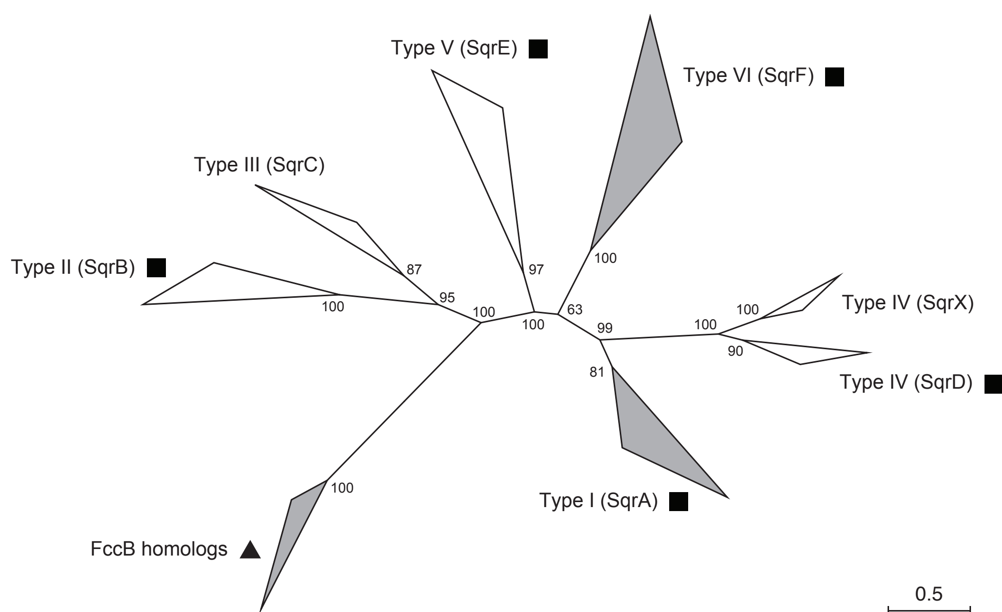


Figure 2.2 | Unrooted phylogenetic tree of sulfide-oxidizing flavoproteins. Clusters including *Beggiatoaceae*-derived sequences are highlighted in grey and are expanded in **Figures S2.9** (Type I SQRs), **S2.10** (Type VI SQRs) and **S2.11** (FccB homologs) on pages 105–107. Clusters, which include enzymes with a proven sulfide:quinone oxidoreductase-activity (■) or flavocytochrome *c*-sulfide dehydrogenase-activity (▲; Gregersen et al., 2011) are marked. The scale bar represents 50% sequence divergence. The tree was reconstructed based on an alignment of 304 sequences. A total of 353 alignment positions was considered for phylogenetic reconstruction; terminal sequence stretches that were not covered by all sequences and positions that were dominated by alignment gaps were excluded. As an exception, sequences of '*Ca.* Thiomargarita nelsonii' that were missing additional sequence information at the C-terminus (THI454_0, THI143717651827) or N-terminus (THI526_0) were included without further reducing the number of considered positions.

are in the micromolar range; Arieli et al., 1994; Schütz et al., 1997; Bronstein et al., 2000; Nübel et al., 2000; Griesbeck et al., 2002; Wakai et al., 2007) and thus appear to be responsible for sulfide oxidation at low concentrations. In contrast, the only studied SqrF enzyme was shown to be crucial for growth only at high sulfide concentrations (≥ 6 mM; Chan et al., 2009). Even though it is currently not feasible to derive a sound model of sulfide oxidation in the *Beggiatoaceae* or in sulfur bacteria in general, it seems likely that the necessity for efficient removal of toxic sulfide under changing environmental conditions favored the presence of multiple, mutually complementing enzymes with distinct functional niches.

Elemental sulfur is oxidized via the rDSR pathway

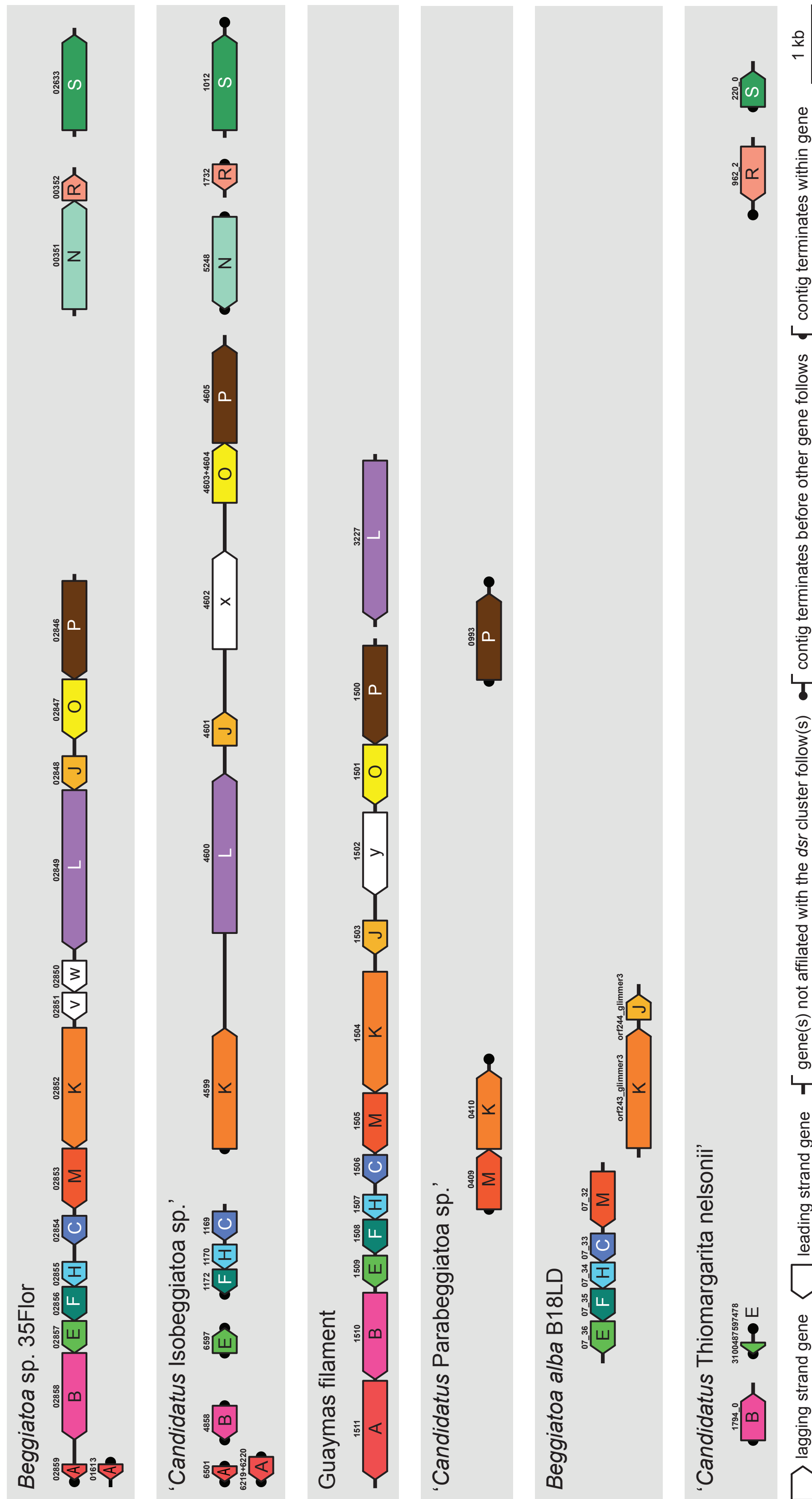
Similar to other sulfur-storing bacteria, members of the family *Beggiatoaceae* appear to oxidize elemental sulfur via the reverse dissimilatory sulfite reductase (rDSR) pathway. Genes encoding rDSR subunits (*dsrAB*), or fragments thereof, were identified in the draft genomes of *Beggiatoa* sp. 35Flor, '*Ca.* Isobeggiatoa sp.', the Guaymas filament, and '*Ca.* Thiomargarita nelsonii' (**Table S2.2**; see also Mußmann et al., 2007; MacGregor et al., 2013; Winkel et al., 2013). In addition, complete or nearly-complete sets of sulfur-bacterial *dsr* core-genes (*dsrABCEFHLMKJOPN*; Sander et al., 2006; Grimm et al., 2008) are contained within the sequenced parts of the genomes from *Beggiatoa* sp. 35Flor, '*Ca.* Isobeggiatoa sp.' (Mußmann et al., 2007), and the Guaymas filament (MacGregor et al., 2013). In contrast to *Allochromatium vinosum*, a purple sulfur bacterium in which the rDSR pathway was studied extensively (Dahl et al., 2005), the *dsr* genes of *Beggiatoaceae* do apparently not form a single, coherent cluster (**Figure 2.3**). Instead, they are distributed over several loci and their arrangement is, even though similarities are evident, not strictly conserved.

The ability to oxidize stored elemental sulfur further to sulfate was one of the central aspects in the initial physiological description of the genus *Beggiatoa* by Winogradsky (1887). Experimental support for a complete oxidation of sulfur was subsequently derived from sulfate production during incubation in presence of reduced sulfur compounds (*Beggiatoa* sp. OH-75-2a, *Beggiatoa* sp. D-402, and '*Ca.* Marithioploca spp.'; Nelson and Castenholz, 1981; Otte et al., 1999; Muntyan et al., 2005) as well as oxygen/sulfide flux balances and the acidification of growth media, presumably through the production of sulfuric acid (*Beggiatoa* sp. MS-81-6; Nelson et al., 1986). Likewise, intracellular sulfate concentrations that exceeded the external levels by up to three orders of magnitude were strongly indicative of sulfate being the end product of sulfur compound oxidation (*Thioploca* spp. and *Beggiatoa* sp. 35Flor; Kojima et al., 2007, and Berg et al., 2013).

However, some members of the *Beggiatoaceae*, such as the *Beggiatoa alba* strains B18LD and B15LD, appeared to be incapable of sulfur oxidation (Schmidt et al.,

1987). Correspondingly, we did not find *dsrAB* genes in the B18LD draft genome nor could we amplify such genes with specific primers (Loy et al., 2009) from any of the two strains (we could, however, amplify a fragment of the expected length from enrichment cultures of hypersaline *Beggiatoaceae*; results not shown). Nevertheless, sulfur oxidation via the rDSR pathway is most likely an ancestral trait of the *Beggiatoaceae*, as indicated by the monophyletic clustering of rDsrB proteins from distantly related members of the family (**Figure 2.4**). A secondary loss of the rDSR in *Beggiatoa alba* B18LD and B15LD may have been facilitated by their chemoorganoheterotrophic lifestlye. This could have relieved the selective pressure on maintaining a functional rDSR by breaking the tight link between the sulfur and energy/carbon metabolisms, which exists in (obligately) chemolithoautrophic *Beggiatoaceae*. Extant genes of the rDSR cluster in the B18LD genome (e.g. *dsrCEFHMKJ*) may be mere relics or the encoded proteins could be involved as sulfur- or electron-transferring elements in other metabolic processes. The latter is particularly likely for *dsrC*, which could not be stably deleted from *Allochromatium vinosum* even if the strain was grown chemoorganoheterotrophically (Cort et al., 2008). In fact, multiple genes encoding for DsrC homologs were identified in the genomes of *Beggiatoa* sp. 35Flor, '*Ca.* Isobeggiatoa sp.' (Mußmann et al., 2007), the Guaymas filament (MacGregor et al., 2013), and '*Candidatus* Thiomargarita nelsonii' (Winkel et al., 2013). An overview of these proteins is given in **Figure S2.12** and their putative functions are discussed in the corresponding figure legend.

Figure 2.3 *(on the next page)* | Schematic overview of putative *dsr* genes and gene fragments identified in the investigated *Beggiatoaceae* draft genomes. Predicted *dsr* genes are labeled with the respective uppercase letter. Predicted genes, which are presumably not affiliated with the *dsr* cluster, are labeled with a lowercase letter (v, x, and y represent annotated hypothetical proteins; w denotes an annotated PilT-domain containing protein). Genes, which putatively encode homologous proteins are displayed in the same color. Further schematic notations used are specified within the figure. All genes, gene fragments and intergenetic spacers but not the termini (— and ─•) are drawn to scale. The numbers displayed above the genes represent the numerical part of the gene locus identifier. In the complete gene locus identifier this number is preceded by a species tag ('FLOR' for *Beggiatoa* sp. 35Flor, 'BGP' for '*Ca.* Isobeggiatoa sp.', 'BOGUAY' for the Guaymas filament, 'BGS' for '*Ca.* Parabeggiatoa sp.', 'BA' for *Beggiatoa alba* B18LD, and 'THI' for '*Ca.* Thiomargarita nelsonii'; compare **Table S2.2**).


Beggiatoa sp. 35Flor
'Candidatus Isobeggiatoa sp.'
Guaymas filament
'Candidatus Parabeggiatoa sp.'
Beggiatoa alba B18LD
'Candidatus Thiomargarita nelsonii'
lagging strand gene
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gene(s) not affilated with the dsr cluster follow(s)
contig terminates before other gene follows
contig terminates within gene
1 kb

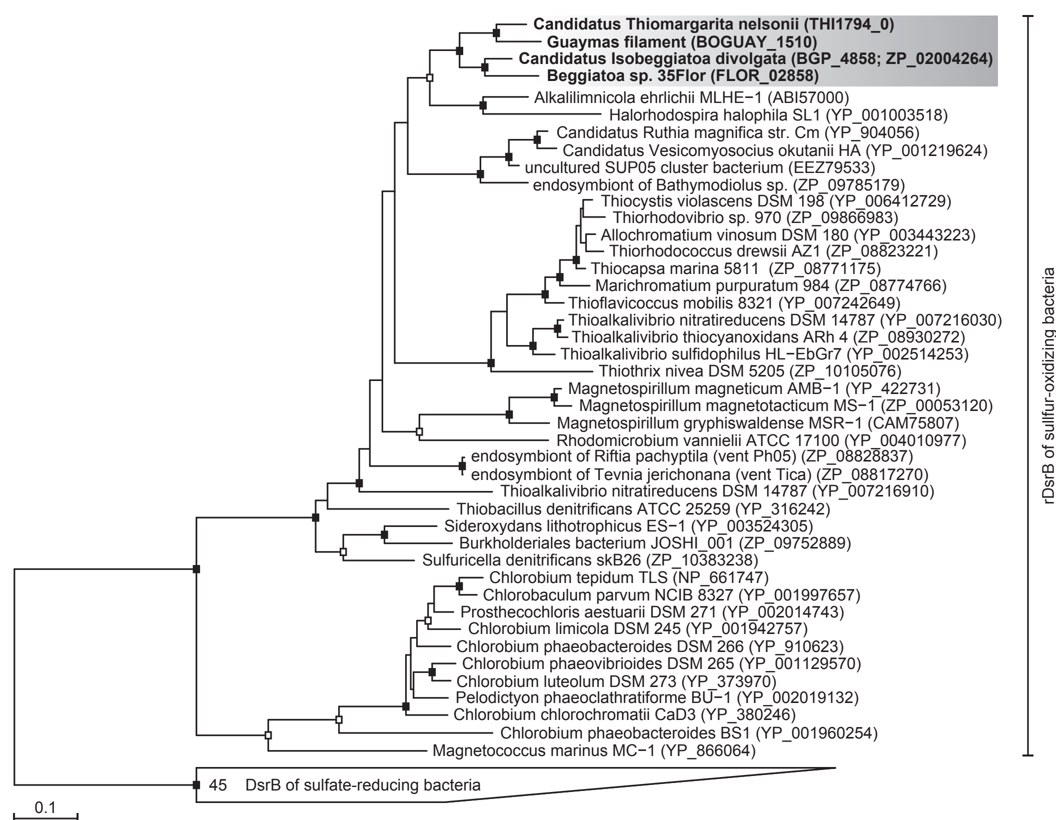


Figure 2.4 | Phylogenetic tree of rDsrB proteins. *Beggiatoaceae*-derived sequences are shown in bold font and the monophyletic cluster formed by these is shaded in grey. Phylogenetically distinct DsrB proteins from sulfate reducers (Loy et al., 2009) are included as an outgroup. Database accession numbers and genome locus identifiers are given in parentheses. The scale bar represents 10% sequence divergence. Plain branching, open boxes (□), and filled boxes (■) denote nodes with bootstrap values of ≤ 50%, 51–75%, and 76–100%, respectively. The phylogenetic reconstruction was performed with an alignment of 88 sequences and 351 considered alignment positions; terminal sequence stretches that were not covered by all sequences and positions that were dominated by alignment gaps were excluded.

Members of the Beggiatoaceae employ different sulfite oxidation pathways

The prokaryotic oxidation of sulfite to sulfate can proceed via two alternative pathways, a direct and an indirect one. Sulfite dehydrogenases of the sulfite oxidase enzyme family were proposed to catalyze the direct oxidation of sulfite. This pathway appears to be more widespread and almost all prokaryotes, which can oxidize

sulfite via only one of the two pathways, use the direct one (Kappler and Dahl, 2001). Correspondingly, all previously tested *Beggiatoaceae* strains were able to oxidize sulfite directly (MS-81-1c, MS-81-6, OH-75-2a, and D-402; Hagen and Nelson, 1997; Grabovich et al., 2001). However, a gene encoding a known directly sulfite-oxidizing enzyme, a periplasmic sulfite:ferricytochrome-*c* oxidoreductase of the SorAB-type (Kappler et al., 2000), was identified only in the Guaymas filament genome (**Table S2.2**; MacGregor et al., 2013). YedY homologs, which likewise belong to the sulfite oxidase enzyme family (Kappler, 2011), are encoded by *Beggiatoa* sp. 35Flor, *Beggiatoa alba* B18LD, and '*Ca.* Isobeggiatoa sp.'. The YedYZ enzyme of *E. coli* is, however, not involved in sulfite oxidation but instead seems to function as a reductase of an unspecified type (Loschi et al., 2004). Accordingly, it remains unclear whether a sulfite dehydrogenase of the SorAB type or a different enzyme is responsible for the direct oxidation of sulfite in most members of the family *Beggiatoaceae*.

A significant heterogeneity in the sulfite metabolism of *Beggiatoaceae* was indicated by the detection of a strong indirect, AMP-dependent sulfite oxidation in the obligately chemolithoautotrohic strain *Beggiatoa* sp. MS-81-1c and the absence of such activity in the facultatively chemolithoautotrophic or mixotrophic strains MS-81-6, OH-75-2a, and D-402 (Hagen and Nelson, 1997; Grabovich et al., 2001). The indirect oxidation of sulfite is a two-step process catalyzed conjointly by the cytoplasmic enzymes adenosine-5'-phosphosulfate (APS) reductase and ATP sulfurylase or APS:phosphate adenylytransferase (APAT; reviewed by Kappler and Dahl, 2001; Meyer and Kuever, 2007; Ghosh and Dam, 2009; Kappler, 2008, 2011). In comparison to the direct oxidation of sulfite, this pathway conserves additional energy via substrate-level phosphorylation and possibly by a more efficient coupling with the electron transport chain. Notably, the indirectly sulfite-oxidizing strain MS-81-1c was able to produce about double the amount of dry weight per unit of sulfide oxidized when compared to strain MS-81-6 grown in identical medium (Hagen and Nelson, 1997). Even though this difference cannot be attributed exclusively to the different sulfite oxidation routes (**Section 5.2.2**), it is suggestive to observe a considerably higher biomass gain in a strain that is capable of employing a more energy-efficient dissimilatory pathway. In support of a heterogeneous sulfite

metabolism in the family *Beggiatoaceae*, genes encoding APS reductase subunits (*aprBA*) could not be amplified from the facultatively chemolithoautotrophic or mixotrophic strains B18LD, D-401, and D-402 with specific primers (Meyer and Kuever, 2007) but were identified in three out of the six draft genomes ('*Ca.* Isobeggiatoa sp.', '*Ca.* Thiomargarita nelsonii', the Guaymas filament; **Table S2.2**; see also Mußmann et al., 2007; MacGregor et al., 2013; Winkel et al., 2013).

Phylogenetically, the encoded AprA proteins and protein fragments of the *Beggiatoaceae* affiliate with the Apr lineage II, in which they form a monophyletic cluster (**Figure 2.5**). Due to the lack of AprA sequence information from the directly sulfite-oxidizing strain MS-81-1c or any other strain from the root of the *Beggiatoaceae*, it remains speculative whether APS reductase as such is ancestral for the family and has been lost secondarily in several strains or whether the enzyme was acquired repeatedly via lateral gene transfer in different branches of the family. Indeed, multiple lateral gene transfer events were proposed to have shaped the present distribution and phylogeny of the lineage II APS reductases and a 16S rDNA-discordant branching pattern of AprA proteins is evident e.g. in the family *Chromatiaceae* (**Figure 2.5**; see Meyer and Kuever, 2007, for an in-depth discussion of lateral *aprBA* transfer).

In accordance with its putative function as the redox partner of APS reductase (Pires et al., 2003; Zane et al., 2010; Rodriguez et al., 2011; Ramos et al., 2012), subunits of a quinone-interacting membrane-bound oxidoreductase (Qmo) complex are encoded in all three of the above mentioned *aprBA*-positive *Beggiatoaceae* genomes (**Table S2.2**). However, only *qmoAB* genes (homologs of *hdrA*) and *hdrCB* genes were detected. A gene coding for a heme *b*-containing, quinone-interacting transmembrane subunit such as HdrE or QmoC (a HdrEC fusion protein encoded in the *qmo* locus of most sulfate reducers and *Chlorobiaceae*; Meyer and Kuever, 2007) is not part of the *qmo* cluster in the studied *Beggiatoaceae*. Notably, such a gene is likewise absent in other Apr II-bearing *Beta*- and *Gammaproteobacteria* (Meyer and Kuever, 2007). The absence of a quinone-interacting transmembrane protein raises the question as to how the Qmo complex and ultimately the APS reductase of these organisms couple to the electron transport chain. Ei-

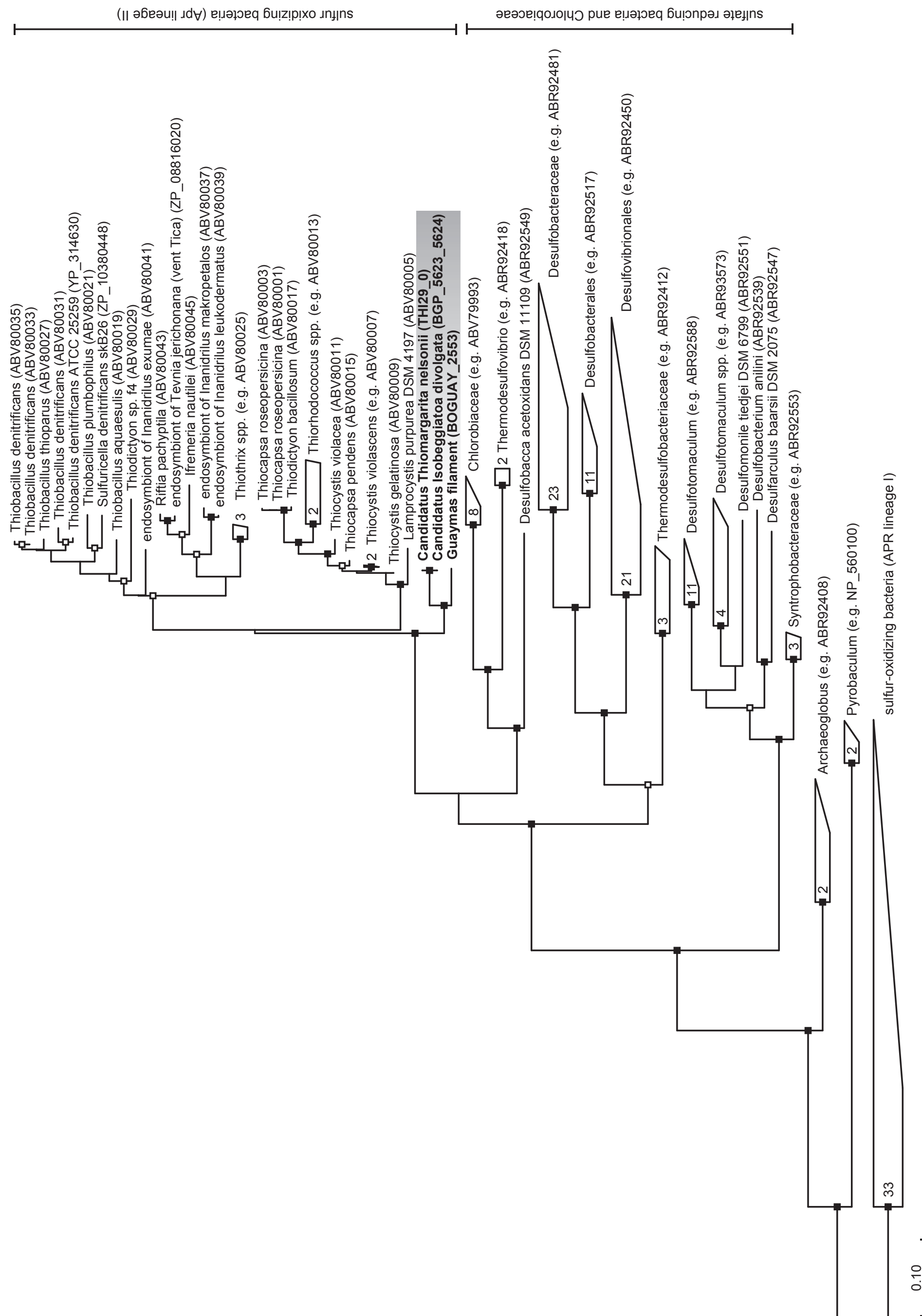


Figure 2.5 | Phylogenetic tree of AprA proteins. Sequences from *Beggiatoaceae* are shown in bold font and the monophyletic cluster formed by these is highlighted in grey. Lineage I AprA proteins from sulfur-oxidizing bacteria (Meyer and Kuever, 2007) were used as an outgroup. Database accession numbers and gene locus identifiers are given in parentheses. The scale bar represents 10% sequence divergence. The tree was calculated based on an alignment of 159 sequences, of which 553 positions were considered for phylogenetic reconstruction; terminal sequence stretches that were not covered by all sequences and positions that were dominated by alignment gaps were excluded. Note that only 43 residues of THI29_0 could be considered for phylogenetic reconstruction.

ther, electrons are not exchanged with the quinone pool and instead reduce an unknown cytoplasmic acceptor or the putative QmoAB-HdrCB complex interacts with a different heme *b*-containing, quinone-interacting transmembrane protein.

Genes or gene fragments encoding an ATP sulfurylase (*sat* or *sopT*), the second enzyme of the APS pathway, were identified in all three of the above mentioned *aprBA*-positive *Beggiatoaceae* genomes and in the genome of *Beggiatoa* sp. 35Flor (**Table S2.2**). Regarding the latter strain, it can only be speculated whether the missing enzymes of the APS pathway are encoded in the not yet sequenced part of the genome or whether the identified Sat-type ATP sulfurylase operates in a different metabolic context. In support of the latter option, all enzymes required for assimilatory sulfate reduction were found to be encoded in the *Beggiatoa* sp. 35Flor draft genome with the exception of a CysDN-type ATP sulfurylase (Kreutzmann and Schulz-Vogt, 2013; see **Section 4.1**). The identified Sat-type ATP sulfurylase might thus substitute for this enzyme during sulfate assimilation.

Using a 600 bp fragment of the *sopT* gene form the sulfur-oxidizing endosymbiont '*Ca.* Endoriftia persephone' as a southern blot probe, a gene encoding a putatively dissimilatorily operating ATP sulfurylase was identified in the strain *Beggiatoa* sp. MS-81-1c, while no hybridization was obtained with DNA of *Beggiatoa* sp. OH-75-2a (Laue and Nelson, 1994). Correspondingly, a high ATP sulfurylase activity was reported for the APS-reductase positive strain MS-81-1c but not for the strains MS-81-6 and OH-75-2a, which assimilate sulfate but oxidize sulfite exclusively via the direct pathway (Hagen and Nelson, 1997). A gene encoding the alternative sulfate-liberating enzyme APAT (*apt*) was not identified in any of the analyzed *Beggiatoaceae* genomes nor was a respective enzyme activity previously detected in any *Beggiatoa* strain (MS-81-1c, MS-81-6, OH-75-2a; Hagen and Nelson, 1997).

Thiosulfate might be oxidized via two pathways

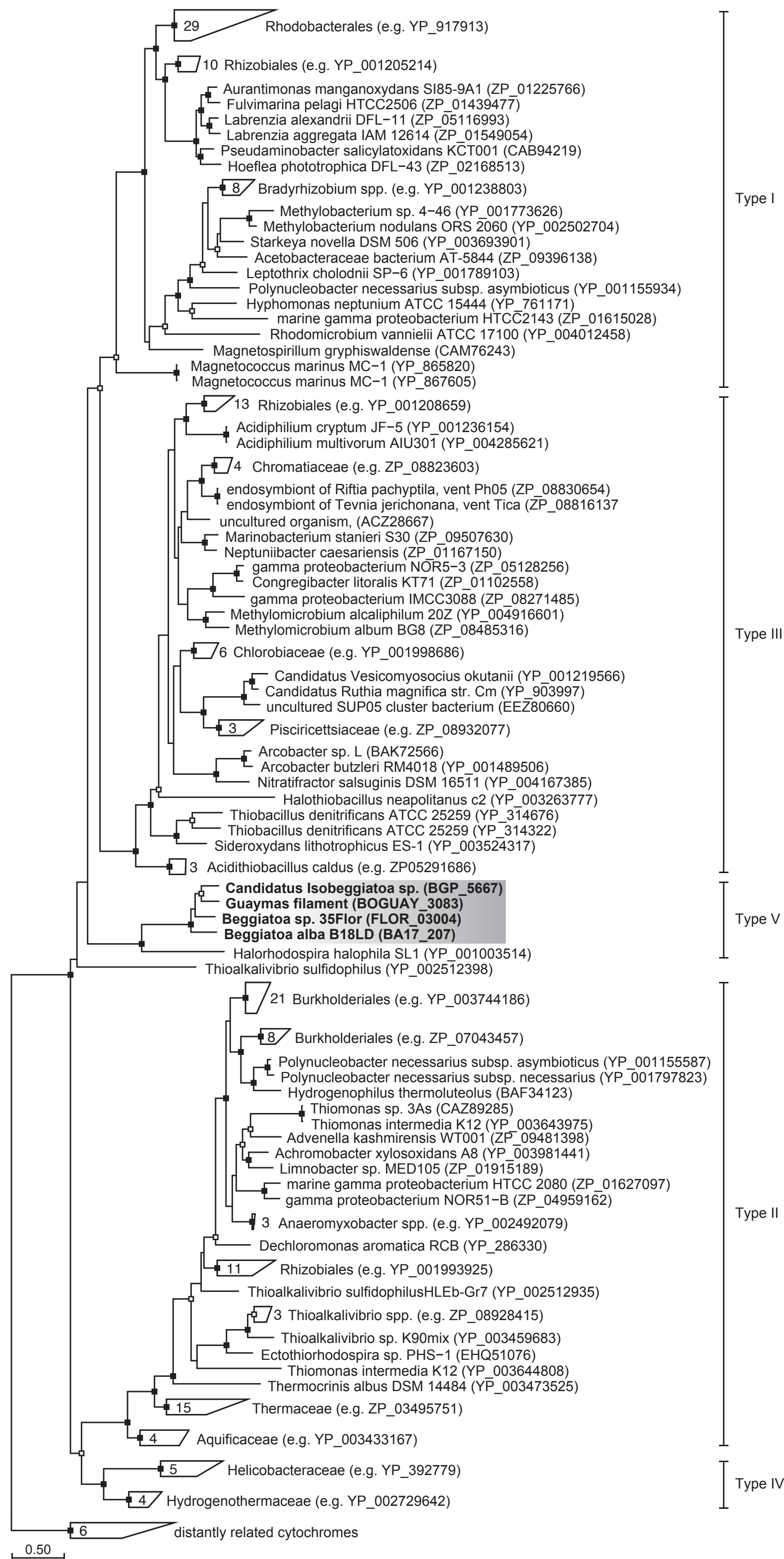
Several strains of the family *Beggiatoaceae* are known to oxidize thiosulfate (e.g. *Beggiatoa alba* B15LD, B18LD; *Beggiatoa* sp. B25RD, L1401-15, OH-75-2a, MS-81-1c, MS-81-6, D-402) and all deposit elemental sulfur when doing so (Güde et al., 1981; Nelson and Castenholz, 1981; Nelson and Jannasch, 1983; Mezzino et al., 1984; Hagen and Nelson, 1996; Muntyan et al., 2005). The appearance of sulfur inclusions during growth on thiosulfate strongly suggests that these strains consume thiosulfate via the branched thiosulfate oxidation pathway (reviewed by e.g. Hensen et al., 2006; Grimm et al., 2008; Kappler and Maher, 2013). The reactions of this pathway strongly discriminate between the two sulfur atoms of thiosulfate, sulfone sulfur (oxidation state +5) and sulfane sulfur (oxidation state -1), which are oxidized to sulfate and elemental sulfur, respectively (Smith and Lascelles, 1966; Trüper and Pfennig, 1966; Kappler and Maher, 2013). Accordingly, a 1:1 ratio of produced sulfur and sulfate is expected for organisms, which employ the branched thiosulfate oxidation pathway. In fact, cultures of *Beggiatoa* sp. OH-75-2a were observed to produce elemental sulfur and sulfate in equimolar amounts when grown on thiosulfate (Nelson and Castenholz, 1981).

In line with the above observations, we identified genes encoding most enzymes involved in the branched thiosulfate oxidation pathway, i.e. SoxA, SoxYZ, and SoxB, in the genomes *Beggiatoa* sp. 35Flor, '*Ca.* Isobeggiatoa sp.' (Mußmann et al., 2007), the Guaymas filament (MacGregor et al., 2013), and *Beggiatoa alba* B18LD (**Table S2.2**). Fragments of *soxY* and *soxB* were further detected in the genome of '*Ca.* Thiomargarita nelsonii' (Winkel et al., 2013). Separate genes encoding for SoxX, the second subunit of the SoxAX protein, were not found (see also Kappler and Maher, 2013). However, the identified SoxA proteins feature an N-terminal extension of roughly 150–200 amino acids, which bears strong resemblance to SoxX and provides, equivalent to SoxX, an additional heme binding site (CXXCH; **Figure S2.13**). Database searches identified a similar extension only in the SoxA proteins of *Halorhodospira halophila* (YP_001003514; see alignment in **Figure S2.13**) and *Bradyrhizobium japonicum* (WP_018321809; not included in the alignment but shares all characteristic sequence features). Thus, the genes

encoding for SoxA and SoxX appear to be fused in members of the *Beggiatoaceae*, *H. halophila*, and *B. japonicum* and this fusion represents an exception among the so far studied SoxAX proteins. Phylogenetic analyses of SoxA proteins demonstrated that all *Beggiatoaceae*-derived sequences are closely related and thus have likely been inherited from a common ancestor of the family (**Figure 2.6**). Notably, the SoxA domain of the *H. halophila* SoxAX fusion protein was identified as the closest relative of the *Beggiatoaceae*-derived SoxA domain sequences (the sequence from *B. japonicum* was not included in the phylogenetic reconstruction). Biochemical data, which confirm the function of the peculiar SoxAX fusion proteins in thiosulfate oxidation, are not available. Nevertheless, it appears likely that the here identified SoxAXYZB proteins constitutes the enzymatic system responsible for thiosulfate oxidation via the branched thiosulfate oxidation pathway in members of the *Beggiatoaceae*.

Corresponding to the accumulation of elemental sulfur during thiosulfate oxidation, *soxCD* genes were so far not identified in any of the *Beggiatoaceae* draft genomes (see also Mußmann et al., 2007; MacGregor et al., 2013). The SoxCD enzyme is a sulfur (sulfane) dehydrogenase, which—when present—allows the complete and direct oxidation of thiosulfate to sulfate via the Sox system (Friedrich et al., 2001, 2005). In fact, the absence of SoxCD appears to distinguish the branched thiosulfate oxidation pathway of sulfur-accumulating thiosulfate oxidizers from the complete Sox pathway found in organisms oxidizing thiosulfate with-

Figure 2.6 ***(on the next page)*** | **Phylogenetic tree of SoxA proteins.** Sequences from *Beggiatoaceae* are shown in bold font and the monophyletic cluster comprising these is highlighted in grey. The given SoxA classification is according to Kappler and Maher (2013), with the exception that the SoxAX fusion proteins of *Beggiatoaceae* and *Halorhodospira halophila* were not included in the Type IV cluster. Instead, we propose a distinct cluster (Type V) for the latter proteins, based on their phylogenetic separation, the present *soxAX* gene fusion, and the conserved substitution of a heme-ligating cysteine with a histidine (**Figure S2.13**). Database accession numbers and genome locus identifiers are given in parentheses. Plain branching, open boxes (□), and filled boxes (■) denote nodes with bootstrap values of ≤ 50%, 51–75%, and 76–100%, respectively. The scale bar represents 50% sequence divergence. The phylogenetic reconstruction was performed with 218 sequences and considered 212 alignment positions that were available for all sequences. The SoxX domain of SoxAX fusion proteins and other terminal sequence stretches were excluded. Likewise, positions that were dominated by alignment gaps were excluded.


29 Rhodobacterales (e.g. YP_917913)
10 Rhizobiales (e.g. YP_001205214)
Aurantimonas manganoxydans SI85-9A1 (ZP_01225766)
Fulvimarina pelagi HTCC2506 (ZP_01439477)
Labrenzia alexandrii DFL-11 (ZP_05116993)
Labrenzia aggregata IAM 12614 (ZP_01549054)
Pseudaminobacter salicylatoxidans KCT001 (CAB94219)
Hoeflea phototrophica DFL-43 (ZP_02168513)
8 Bradyrhizobium spp. (e.g. YP_001238803)
Methylobacterium sp. 4-46 (YP_001773626)
Methylobacterium nodulans ORS 2060 (YP_002502704)
Starkeya novella DSM 506 (YP_003693901)
Acetobacteraceae bacterium AT-5844 (ZP_09396138)
Leptothrix cholodnii SP-6 (YP_001789103)
Polynucleobacter necessarius subsp. asymbioticus (YP_001155934)
Hyphomonas neptunium ATCC 15444 (YP_761171)
marine gamma proteobacterium HTCC2143 (ZP_01615028)
Rhodomicrobium vannielii ATCC 17100 (YP_004012458)
Magnetospirillum gryphiswaldense (CAM76243)
Magnetococcus marinus MC-1 (YP_865820)
Magnetococcus marinus MC-1 (YP_867605)
Type I
13 Rhizobiales (e.g. YP_001208659)
Acidiphilium cryptum JF-5 (YP_001236154)
Acidiphilium multivorum AIU301 (YP_004285621)
4 Chromatiaceae (e.g. ZP_08823603)
endosymbiont of Riftia pachyptila, vent Ph05 (ZP_08830654)
endosymbiont of Tevnia jerichonana, vent Tica (ZP_08816137
uncultured organism, (ACZ28667)
Marinobacterium stanieri S30 (ZP_09507630)
Neptuniibacter caesariensis (ZP_01167150)
gamma proteobacterium NOR5-3 (ZP_05128256)
Congregibacter litoralis KT71 (ZP_01102558)
gamma proteobacterium IMCC3088 (ZP_08271485)
Methylomicrobium alcaliphilum 20Z (YP_004916601)
Methylomicrobium album BG8 (ZP_08485316)
6 Chlorobiaceae (e.g. YP_001998686)
Candidatus Vesicomyosocius okutanii (YP_001219566)
Candidatus Ruthia magnifica str. Cm (YP_903997)
uncultured SUP05 cluster bacterium (EEZ80660)
3 Piscirickettsiaceae (e.g. ZP_08932077)
Arcobacter sp. L (BAK72566)
Arcobacter butzleri RM4018 (YP_001489506)
Nitratifractor salsuginis DSM 16511 (YP_004167385)
Halothiobacillus neapolitanus c2 (YP_003263777)
Thiobacillus denitrificans ATCC 25259 (YP_314676)
Thiobacillus denitrificans ATCC 25259 (YP_314322)
Sideroxydans lithotrophicus ES-1 (YP_003524317)
3 Acidithiobacillus caldus (e.g. ZP05291686)
Type III
Candidatus Isobeggiatoa sp. (BGP_5667)
Guaymas filament (BOGUAY_3083)
Beggiatoa sp. 35Flor (FLOR_03004)
Beggiatoa alba B18LD (BA17_207)
Halorhodospira halophila SL1 (YP_001003514)
Type V
Thioalkalivibrio sulfidophilus (YP_002512398)
21 Burkholderiales (e.g. YP_003744186)
8 Burkholderiales (e.g. ZP_07043457)
Polynucleobacter necessarius subsp. asymbioticus (YP_001155587)
Polynucleobacter necessarius subsp. necessarius (YP_001797823)
Hydrogenophilus thermoluteolus (BAF34123)
Thiomonas sp. 3As (CAZ89285)
Thiomonas intermedia K12 (YP_003643975)
Advenella kashmirensis WT001 (ZP_09481398)
Achromobacter xylosoxidans A8 (YP_003981441)
Limnobacter sp. MED105 (ZP_01915189)
marine gamma proteobacterium HTCC 2080 (ZP_01627097)
gamma proteobacterium NOR51-B (ZP_04959162)
3 Anaeromyxobacter spp. (e.g. YP_002492079)
Dechloromonas aromatica RCB (YP_286330)
11 Rhizobiales (e.g. YP_001993925)
Thioalkalivibrio sulfidophilusHLEb-Gr7 (YP_002512935)
3 Thioalkalivibrio spp. (e.g. ZP_08928415)
Thioalkalivibrio sp. K90mix (YP_003459683)
Ectothiorhodospira sp. PHS-1 (EHQ51076)
Thiomonas intermedia K12 (YP_003644808)
Thermocrinis albus DSM 14484 (YP_003473525)
15 Thermaceae (e.g. ZP_03495751)
4 Aquificaceae (e.g. YP_003433167)
Type II
5 Helicobacteraceae (e.g. YP_392779)
4 Hydrogenothermaceae (e.g. YP_002729642)
Type IV
6 distantly related cytochromes
0.50

out the appearance of intermediates (Hensen et al., 2006). Not accessible for the SoxAXBYZ complex, the accumulated sulfur is thought to be oxidized further via the rDSR pathway (see above).

A third pathway for the oxidation of thiosulfate is the tetrathionate intermediate pathway, in which thiosulfate is oxidized to sulfate via tetrathionate and sulfite (Ghosh and Dam, 2009). The formation of tetrathionate during thiosulfate oxidation is barely studied in *Beggiatoaceae* and we are aware of only one strain for which this possibility was tested. This strain, *Beggiatoa* sp. D-402, deposited elemental sulfur when growing on thiosulfate, but minor quantities of tetrathionate were likewise detected in the medium (Grabovich et al., 2001; Muntyan et al., 2005). The authors, however, failed to show data from measurements in sterile media, so that it is difficult to assess whether tetrathionate arose from chemical or biological thiosulfate oxidation. Biologically, the oxidation of thiosulfate to tetrathionate is catalyzed by the enzyme thiosulfate dehydrogenase and genes encoding different types of this enzyme have recently been identified (Müller et al., 2004; Denkmann et al., 2012). We detected a homolog of the thiosulfate dehydrogenase from *Allochromatium vinosum* (TsdA; Denkmann et al., 2012) in the genome of '*Ca.* Isobeggiatoa sp.' (**Table S2.2**) and found a truncated, apparently dysfunctional version in the genome of '*Ca.* Thiomargarita nelsonii' (THI516_0). An alignment with proven thiosulfate dehydrogenases of the TsdA type (Denkmann et al., 2012), showed that all conserved and likely functionally important residues are present in the protein encoded by '*Ca.* Isobeggiatoa sp.', supporting its function as a thiosulfate dehydrogenase (**Figure S2.14**). A gene encoding a tetrathionate hydrolase, the enzyme catalyzing the oxidative cleavage of tetrathionate to sulfite, was so far not identified in any of the the analyzed *Beggiatoaceae* genomes. Likewise, there are no physiological studies, which investigated whether members of the *Beggiatoaceae* are able to oxidize tetrathionate further.

Together, these data suggest that *Beggiatoaceae* in general may be capable of oxidizing thiosulfate via two pathways, the branched thiosulfate oxidation pathway and possibly the tetrathionate intermediate pathway. The branched thiosulfate oxidation pathway appears to be widespread among members of this family as

judged from the usual deposition of sulfur inclusions under thiosulfate-oxidizing conditions. In contrast, the commonness and importance of the tetrathionate intermediate pathway can currently not be assessed. However, strains, which seem to be capable of oxidizing thiosulfate via tetrathionate (*Beggiatoa* sp. D-402, '*Ca.* Isobeggiatoa sp.') are also likely able to employ the branched thiosulfate oxidation pathway. Thus, the two pathways might function complementary in members of the *Beggiatoaceae*, catalyzing thiosulfate oxidation under different environmental conditions. Physiological studies in the purple sulfur bacterium *Allochromatium vinosum* indicated that thiosulfate oxidation via the branched pathway prevails at pH values above 7.0, while the tetrathionate intermediate pathway dominates at neutral to acidic pH (Smith and Lascelles, 1966; Hensen et al., 2006). Nevertheless, thiosulfate oxidation may not be an universal feature of the *Beggiatoaceae*, as suggested by the absence of such activity in several freshwater strains (*Beggiatoa* sp. OH-763-B, OH-765-B, OH-766-B, OH-767-B; Nelson and Castenholz, 1981; Nelson et al., 1982). Thiosulfate oxidation could so far also not be shown for *Beggiatoa* sp. 35Flor (H. N. Schulz-Vogt, personal communication), even though this strain encodes all components of the SoxAXYZB complex (see above).

Conclusions

By combining data from genomic, biochemical, physiological, and phylogenetic analyses we were able to draw the so far most comprehensive picture of sulfur metabolism in the family *Beggiatoaceae*. Nevertheless, our evaluation suffers—as any comparative study on the metabolism of *Beggiatoaceae* currently would—from the unculturability prevailing in this family, the lack of complete genome sequences and the absence of a genetic system. Despite these limitations, the available data (summarized in **Figures 2.7** and **2.8**) support several hypotheses on the commonness of the different sulfur compound oxidation pathways in extant members of the *Beggiatoaceae* and the presence of these pathways in the last common ancestor of the family.

In short, all of the so far studied *Beggiatoaceae* are capable of oxidizing sulfide to elemental sulfur, irrespective of their carbon and energy metabolisms (listed in

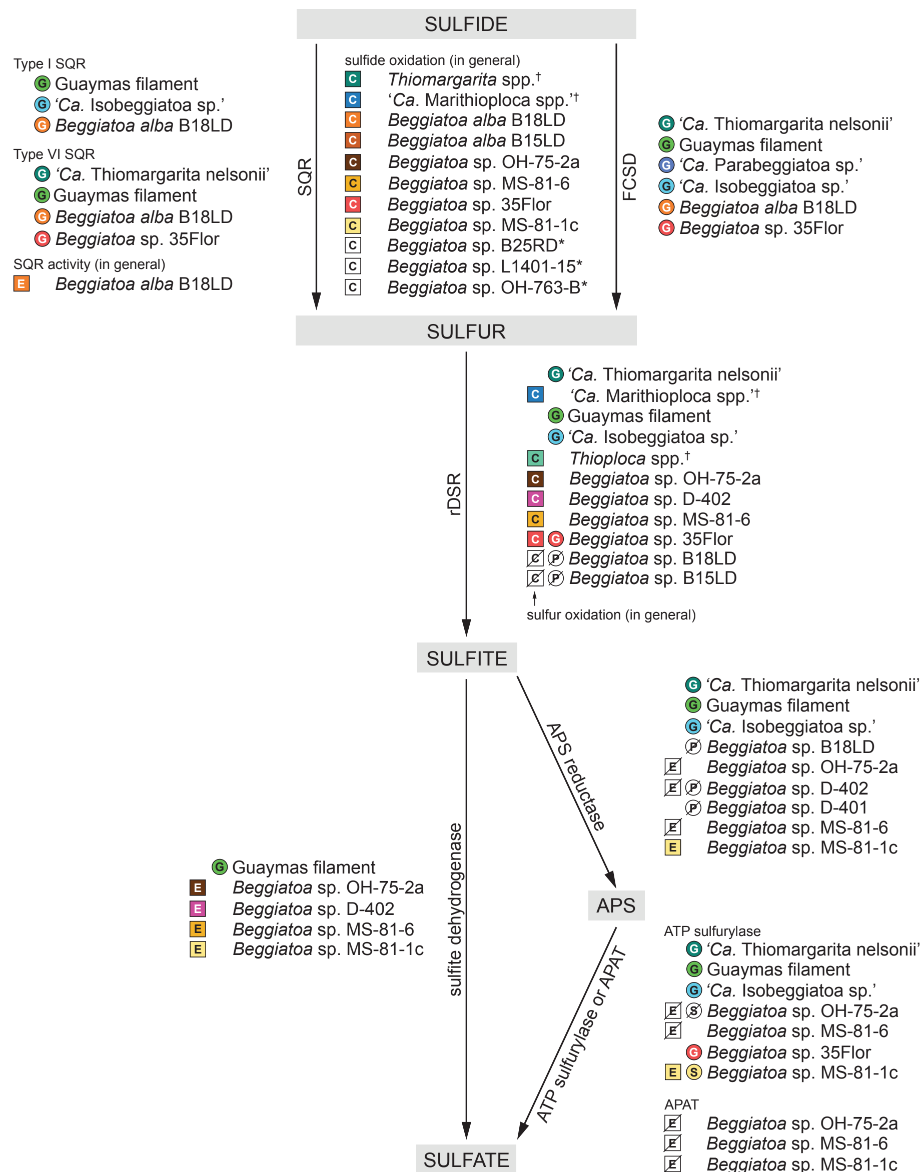


Figure 2.7 | Pathways for the oxidation of sulfide, sulfur, and sulfite in members of the family *Beggiatoaceae*. The figure summarizes the current knowledge about the oxidative sulfur metabolism in the family *Beggiatoaceae* as presented in the text. It is shown for which strain the available evidence suggests the presence (colored symbols) or absence (strikethrough symbols) of a pathway. The colors indicate the approximate phylogenetic position of a strain in the 16S rRNA gene tree (**Figure 2.1**). Blues and greens stand for strains from the upper part of the tree, while reds, yellows and browns stand for strains from the lower part; a dagger (†) indicates an assumed phylogenetic position (see **Figure 2.1**). The shape of the symbols refers to whether the presence or absence of a pathway is indicated on the gene level (○) or the functional level (□). Upper case letters specify the type of supporting evidence; 'G' denotes the identification of respective genes in the draft genome, 'P' indicates PCR amplification of such genes with specific primers, 'S' means southern blot hybridizations targeting such genes, 'E' refers to the measurement of respective enzyme activities, and 'C' to observations and measurements in cultures or environmental samples (references are given in the text).

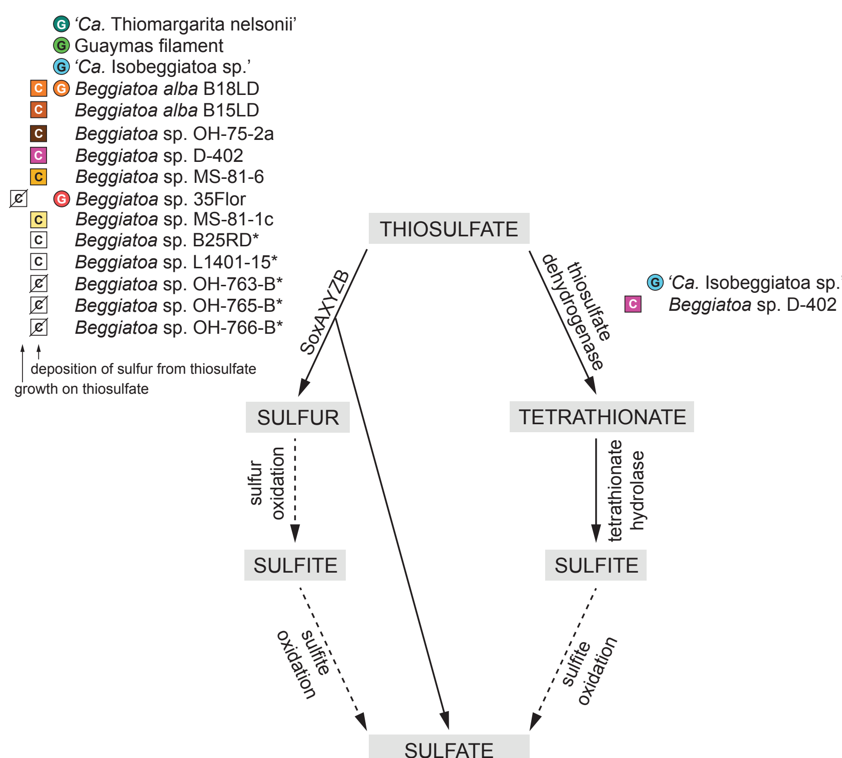


Figure 2.8 | Pathways for the oxidation of thiosulfate in members of the family *Beggiatoaceae*. Thiosulfate oxidation via the branched thiosulfate oxidation pathway is shown on the left side, oxidation via the tetrathionate intermediate pathway on the right side. Dashed arrows refer to pathways specified within **Figure 2.7**. The phylogenetic position of the strains marked with an asterisk (*) is not known as 16S rRNA gene sequences are not available. Further schematic notations used in the figure are explained in the legend of **Figure 2.7**.

Table S2.3). Three distinct sulfide-oxidizing enzymes, which seem to be typically encoded in the genomes of *Beggiatoaceae*, could catalyze this reaction, possibly under different environmental conditions (sulfide:quinone oxidoreductases of the types I and VI, flavocytochrome *c*-sulfide dehydrogenases; **Figures 2.7**). Similarly, most studied *Beggiatoaceae* are capable of oxidizing thiosulfate, typically via the branched thiosulfate oxidation pathway (**Figure 2.8**). There are indications that thiosulfate may also be oxidized to tetrathionate, but the available data are yet insufficient to draw conclusions on the prevalence and importance of this pathway. All three types of sulfide-oxidizing flavoproteins as well as the enzymes of the branched thiosulfate oxidation pathway seem to have been encoded in the

last common ancestor of the family *Beggiatoaceae*. Most studied strains of the family *Beggiatoaceae* are further able to oxidize stored elemental sulfur, likely via the rDSR pathway (**Figures 2.7**). Similar to the above mentioned enzymes, those of the rDSR pathway have apparently been encoded in the last common ancestor of the family. Nevertheless, *Beggiatoa alba* B18LD and the closely related strain *Beggiatoa alba* B15LD are incapable of oxidizing sulfur further. However, the loss of this ability seems to have occurred rather recently, as the likewise closely related strain OH-75-2a (compare **Figure 2.1**) can oxidize sulfur to sulfate. The heterogeneity among different members of the family *Beggiatoaceae* appears to be most pronounced in the pathways used for sulfite oxidation. While all of the so far biochemically studied *Beggiatoaceae* catalyze the direct oxidation of sulfite via sulfite dehydrogenases, indirect sulfite oxidation via the APS pathway appears to be limited to certain strains (**Figure 2.7**). In order to assess whether the presence or absence of the APS pathway is associated with (obligately) litho- or autotrophic modes of growth, more comprehensive studies of the carbon- and energy-acquisition pathways of APS reductase-positive and -negative strains are required. The available evidence shows that both sulfite oxidation pathways are realized in distantly related strains of the *Beggiatoaceae*, but the shortage of sequence information for sulfite dehydrogenase and APS reductase currently precludes any sound conclusions on whether these pathways were or were not encoded in the last common ancestor of the family. Thus, the presented hypotheses can serve as a basis for future experiments and considerations but will need to be tested and re-evaluated as more data become available.

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Supplemental material

Table S2.1 | Sequences of PCR primers used in this study.

Primer (Abbreviation)	Target gene	Sequence (5' → 3')	Reference
GM3F	16S rRNA gene	AGAGTTTGATCMTGGC	Muyzer et al., 1995
ITSReub	23S rRNA gene	GCCAAGGCATCCACC	Cardinale et al., 2004
rDSR1Fa	*dsrA*	AARGGNTAYTGGAARG	Loy et al., 2009
rDSR1Fb	*dsrA*	TTYGGNTAYTGGAARG	Loy et al., 2009
rDSR1Fc	*dsrA*	ATGGGNTAYTGGAARG	Loy et al., 2009
rDSR4Rb	*dsrB*	GGRWARCAIGCNCCRCA	Loy et al., 2009

Table S2.2 | Genes from *Beggiatoaceae* genomes predicted to encode for proteins involved in the oxidative sulfur metabolism. The letter code in the locus name serves as a species identifier ('FLOR' for *Beggiatoa* sp. 35Flor, 'BGP' for '*Ca.* Isobeggiatoa sp.', 'BGS' for '*Ca.* Parabeggiatoa sp.', 'BA' for *Beggiatoa alba* B18LD, 'THI' for '*Ca.* Thiomargarita nelsonii', and 'BOGUAY' for Guaymas filament). Amino acid (AA) counts in parentheses denote truncated sequences, which reside at an end of a contig. An asterisk (*) indicates a frame shift in the sequence, which was corrected manually.

Product	Gene	EC	Locus	Contig	AA
sulfide:quinone oxidoreductase	*sqr*	1.8.5.4	FLOR_01938	RL524	376
			BGP_0667	contig00835_0667	376
			BA07_67	BA07	377
			BA16_158	BA16	323
			THI190_0	THI190	373
			BOGUAY_0181	contig01192	375
			BOGUAY_2390	contig01341	422
flavocytochrome *c*-sulfide dehydrogenase, cytochrome *c* subunit	*fccA*	1.8.2.3	FLOR_01512	RL517	205
			BGP_4977	contig24305_4976–4978	221
			BA02_146	BA02	90
			THI1301_0	THI1301	218
			THI712_2	THI712	198
			THI35_7	THI735	(165)
			BOGUAY_2852	contig00614	190
			BOGUAY_3988	contig00494	182

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Table S2.2 (Continued from previous page.)

Product	Gene	EC	Locus	Contig	AA
flavocytochrome *c*-sulfide	*fccB*	1.8.2.3	FLOR_01513	RL517	430
dehydrogenase,			BGP_0124	contig00141_0124–0126	431
flavoprotein subunit			BGP_4976	contig24305_4976–4978	449
			BGS_0815	contig1041_0814–0815	431
			BA02_147	BA02	431
			THI35_6	THI735	425
			THI454_0	THI454	(210)
			THI526_0	THI526	(217)
			THI143717651827	THI1437	(254)
			BOGUAY_2853	contig00614	428
			BOGUAY_3987	contig00494	431
dissimilatory sulfite	*dsrA*	1.8.99.1	FLOR_02859	RL554	(53)
reductase,			FLOR_01613	RL5197	(50)
alpha subunit			BGP_6219+6220	contig03953_6219–6220	(97)*
			BGP_6501	contig23609_6501–6500	(46)
			BOGUAY_1511	01191	413
dissimilatory sulfite	*dsrB*	1.8.99.1	FLOR_02858	RL554	356
reductase,			BGP_4858	contig24049_4858	(126)
beta subunit			THI1794_0	1794	(209)
			BOGUAY_1510	01191	355
DsrC	*dsrC*		FLOR_02854	RL554	116
			BGP_1169	contig04876_1169–1172	110
			BA07_33	BA07	110
			BOGUAY_1506	01191	110
DsrE	*dsrE*		FLOR_02857	RL554	130
			BGP_6597	contig24910_6597	(79)
			BA07_36	BA07	130
			THI3100487597478	THI3100	(36)
			THI33331369909	THI3333	(122)
			BOGUAY_1509	01191	130
DsrF	*dsrF*		FLOR_02856	RL554	139
			BGP_1172	contig04876_1169–1172	(85)
			BA07_35	BA07	128
			BOGUAY_1508	01191	140
DsrH	*dsrH*		FLOR_02855	RL554	101
			BGP_1170	contig04876_1169–1172	101
			BA07_34	BA07	101
			BOGUAY_1507	01191	101
DsrM	*dsrM*		FLOR_02853	RL554	246
			BGS_0409	Contig992_0409–0410	249
			BA07_32	BA07	230
			BOGUAY_1505	01191	259
			THI21821642981	THI2182	(213)

(Continued on next page.)

Table S2.2 (Continued from previous page.)

Product	Gene	EC	Locus	Contig	AA
DsrK	*dsrK*		FLOR_02852	RL554	504
			BGP_4599	contig23606_4599–4608	475
			BGS_0410	Contig992_0409–0410	(321)
			orf243_glimmer3	BA01	492
			BOGUAY_1504	01191	499
			THI2182652907982	THI2182	(84)
			THI1139_0	THI1139	(44)
DsrJ	*dsrJ*		FLOR_02848	RL554	145
			BGP_4601	contig23606_4599–4608	141
			orf244_glimmer3	BA01	97
			BOGUAY_1503	01191	159
DsrO	*dsrO*		FLOR_02847	RL554	244
			BGP_4603+4604	contig23606_4599–4608	243*
			BOGUAY_1501	01191	247
DsrP	*dsrP*		FLOR_02846	RL554	408
			BGP_4605	contig23606_4599–4608	400
			BGS_0993	Contig814_0993	(357)
			BOGUAY_1500	01191	402
DsrL	*dsrL*		FLOR_02849	RL554	660
			BGP_4600	contig23606_4599–4608	662
			BOGUAY_3227	003278	643
			THI1139_2	THI1139	(238)
DsrN	*dsrN*		FLOR_00351	RL503	459
			BGP_5248	contig24727_5248	(383)
DsrR	*dsrR*		FLOR_00352	RL503	103
			BGP_1732	contig20601_1732–1734	107
			THI962_2	THI962	105
DsrS	*dsrS*		FLOR_02633	RL546	394
			BGP_1012	contig01798_1011–1012	376
			THI220_0	THI220	(138)
sulfite:ferricytochrome-*c* oxidoreductase, molybdenum subunit	*sorA*	1.8.2.1	BOGUAY_2965	contig00500	444
sulfite:ferricytochrome-*c* oxidoreductase, cytochrome subunit	*sorB*	1.8.2.1	BOGUAY_2966	contig00500	237
YedY, molybdenum subunit	*yedY*		FLOR_00411	RL504	327
			BA07_56	BA07	322
			BGP_1786	contig20660_17	(49)
			BGP_1787	contig20660_17	(251)
YedZ, transmembrane subunit	*yedZ*		FLOR_00410	RL504	162
			BA07_57	BA07	167
APS reductase, alpha subunit	*aprA*	1.8.99.2	BGP_5623–5624	contig25275_56	(502)*
			BOGUAY_2553	contig_01044	634
			THI29_0	THI29	(104)
APS reductase, beta subunit	*aprB*	1.8.99.2	BGP_5858	contig25738_58	(134)
			BOGUAY_2554	contig01044	154

(Continued on next page.)

Table S2.2 (Continued from previous page.)

Product	**Gene**	**EC**	**Locus**	**Contig**	**AA**
QmoA	*qmoA*		BGP_0252	contig00284_2	428
			BOGUAY_5266	contig00078	(101)
			BOGUAY_4703–4704	contig00371	(210)*
			THI141793213291590	THI1755	(131)
			THI1755_0	THI1755	(249)
QmoB	*qmoB*		BGP_0253	contig00284_2	725
			BOGUAY_4705	contig00371	(368)
			BOGUAY_2741	contig00469	(364)
			THI175580411251660	THI1755	(106)
			THI8461340616	THI846	(112)
HdrC	*hdrC*		BGP_2616	contig21553_26	204
			BOGUAY_2742	contig00469	202
			THI846_1	THI846	213
HdrB	*hdrB*		BGP_2617	contig21553_26	298
			BOGUAY_2743	contig00469	298
sulfate adenylyltransferase	*sat*	2.7.7.4	FLOR_01554	RL518	390
			BGP_6163	contig00469_61	(98)
			BOGUAY_2370	contig00043	397
			THI2760_1	THI2760	(35)
			THI293711201539	THI2937	(39)
sulfur compound-oxidizing multienzyme complex, SoxXA fusion protein	*soxXA*		FLOR_03004	RL561	407
			BGP_5667	contig25364_5667–5668	413
			BA17_207	BA17	412
			BOGUAY_3083	contig00997	413
sulfur compound-oxidizing multienzyme complex, subunit Z	*soxZ*		FLOR_02369	RL536	105
			BGP_4778	contig23916_4775–4779	102
			BA02_173	BA02	299
			BA14_49	BA14	104
			BOGUAY_0116	contig00632	99
sulfur compound-oxidizing multienzyme complex, subunit Y	*soxY*		FLOR_02368	RL536	155
			BGP_4779	contig23916_4775–4779	(57)
			BA14_48	BA14	155
			BOGUAY_0115	contig00632	160
			THI32931297475	THI3293	(98)
sulfur compound-oxidizing multienzyme complex, subunit B	*soxB*		FLOR_02744	RL550	584
			BGP_2304	contig21202_2303–2304	589
			BA17_315	BA17	583
			BOGUAY_1092	contig00701	622
			THI13761462477	THI1376	(153)
thiosulfate dehydrogenase	*tsdA*	1.8.2.2	BGP_3543	contig22582_3541–3543	306

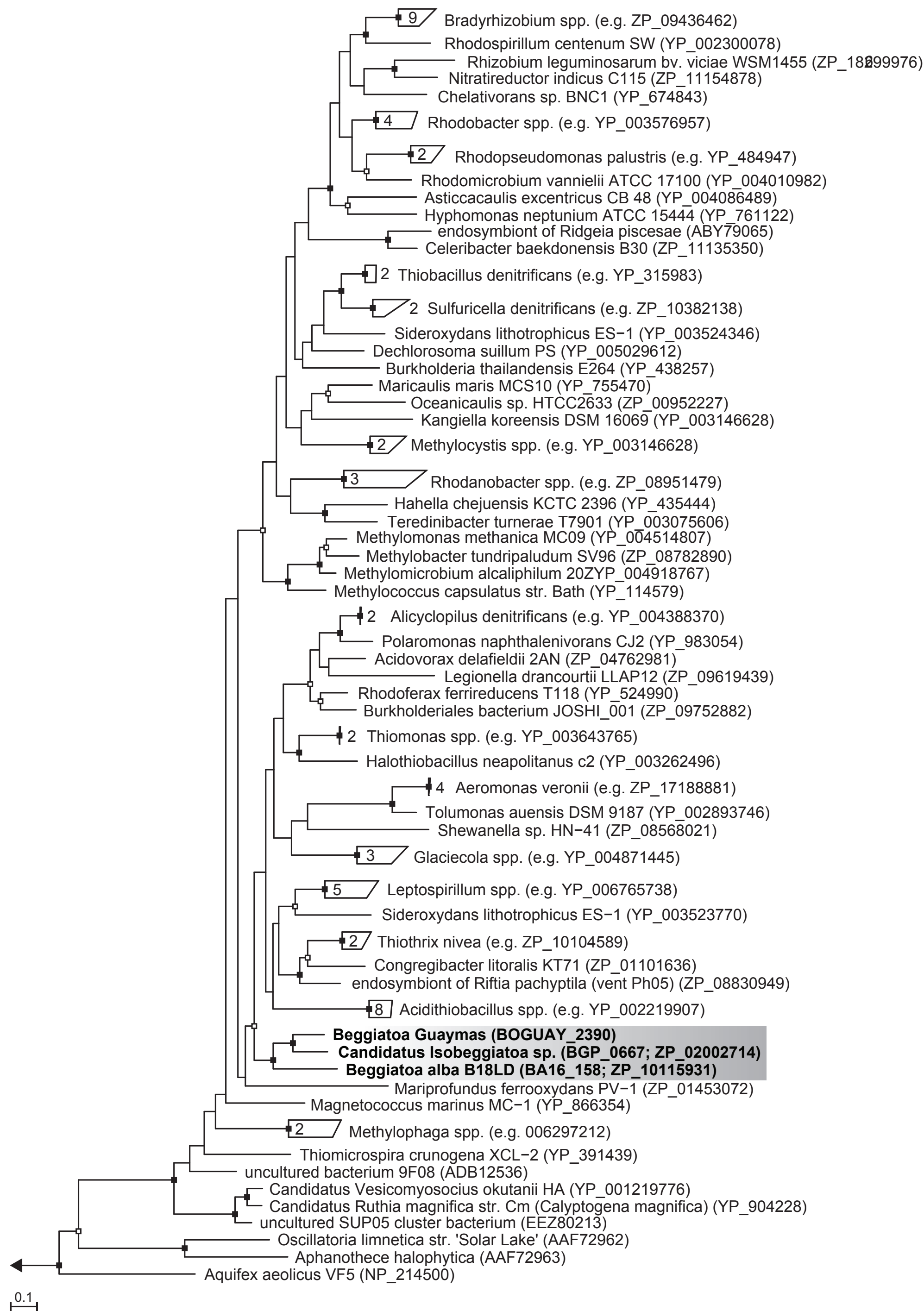

Figure S2.9 | Expanded view of the Type I (SqrA) sulfide:quinone oxidoreductases. The comprehensive tree, from which this excerpt is taken, is presented in Figure 2.2 (page 78; details on tree reconstruction are given there). *Beggiatoaceae* sequences are shown in bold font and the monophyletic cluster comprising these is shaded in grey. Database accession numbers and genome locus identifiers are given in parentheses. The scale bar represents 10% sequence divergence.

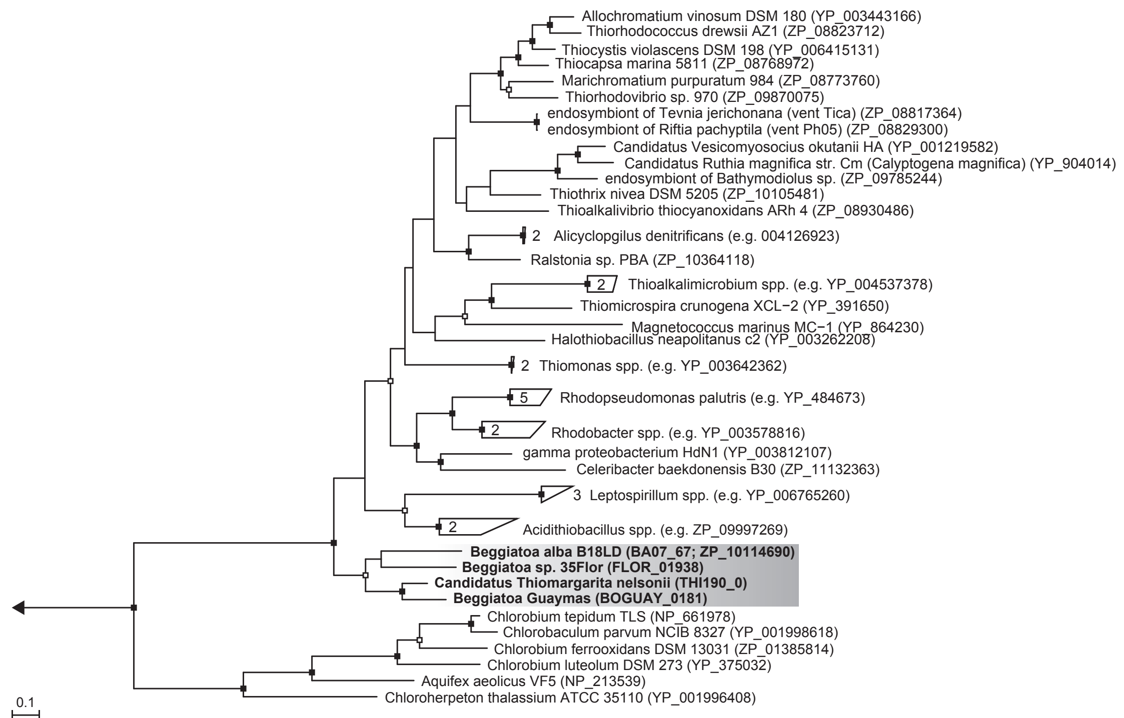

Figure S2.10 | Expanded view of the type VI (SqrF) sulfide:quinone oxidoreductases. The comprehensive tree, from which this excerpt is taken, is presented in Figure 2.2 (page 78; details on tree reconstruction are given there). *Beggiatoaceae* sequences are shown in bold font and the monophyletic cluster comprising these is shaded in grey. Database accession numbers and genome locus identifiers are given in parentheses. The scale bar represents 10% sequence divergence.

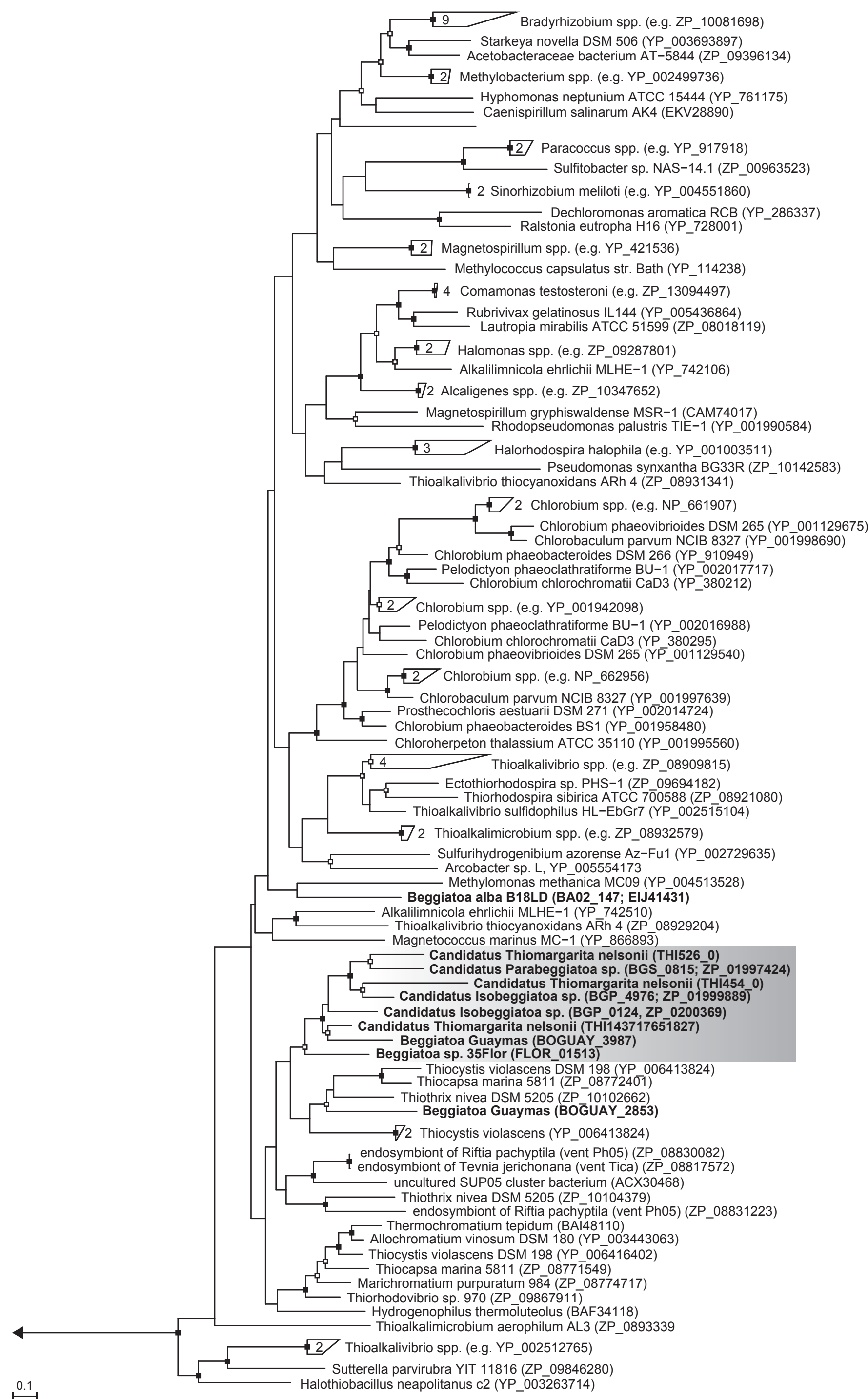

Figure S2.11 | Expanded view of FccB subunit homologs of flavocytochrome *c*-sulfide dehydrogenases. The comprehensive tree, from which this excerpt is taken, is presented in Figure 2.2 (page 78; details on tree reconstruction are given there). *Beggiatoaceae* sequences are shown in bold font. All but two *Beggiatoaceae*-derived FccB sequences (BA02_147; BOGUAY_2853) form a monophyletic cluster (shaded in grey). Database accession numbers and genome locus identifiers are given in parentheses. The scale bar represents 10% sequence divergence.

Figure S2.12 ***(on the next page)*** **| Alignment of DsrC/TusE homologs from *Beggiatoaceae* genomes to selected reference sequences.** The sequences are grouped according to primary structure features, which were proposed to be indicative of particular metabolic functions (detailed in Cort et al., 2008). The cysteine residue at the penultimate position (highlighted in red), which is present in all of the shown sequences, is essential for the function of TusE in thiouridine biosynthesis and is supposed to likewise be indispensable for DsrC (Ikeuchi et al., 2006; Cort et al., 2008). Hence, DsrC/TusE homologs lacking a penultimate cysteine are unlikely to function accordingly and were not included in the alignment (FLOR_01765, BGP_2977, BGP_3375, BGP_4243, THI348_2, THI40_4, THI2578139828, and BOGUAY_1185). DsrC proteins from bacteria, which oxidize sulfur via the reverse dissimilatory sulfide reductase (rDSR) pathway, are characterized by a second C-terminal cysteine (highlighted in yellow) and a seven- to eight-residue insertion (highlighted in blue). Four predicted *Beggiatoaceae* proteins, including the only two known to be encoded in a *dsr* cluster (marked with asterisks), share these primary structure features and thus should be regarded as DsrC proteins involved in the rDSR pathway. The second cysteine residue, which invariably occurs in homologs involved in the DSR or rDSR pathway, enables thiol/disulfide interchanges at the C-terminus, and thereby not only sulfur- but also electron-transfer reactions (Cort et al., 2008). Four *Beggiatoaceae* proteins group with TusE homologs, which consistently lack the second C-terminal cysteine but carry the aforementioned seven-residue insertion. The sequence BOGUAY_1905 cannot clearly be assigned to a particular group, even though the presence of a single C-terminal cysteine residue is reminiscent of TusE homologs. Reference sequences are shown in regular font, sequences from *Beggiatoaceae* genomes in bold font. Dots (.) in the alignment indicate missing sequence information, dashes (-) indicate alignment gaps. The full species names and accession numbers for the reference sequences (Cort et al., 2008) are as follows: Eco: *Escherichia coli* (ZP_00708844), Pmu: *Pasteurella multicida* (NP_245338), Hin: *Haemophilus influenzae* (ZP_01785345), Ype: *Yersinia pestis* (NP_992704), Vch: *Vibrio cholerae* (NP_231000), Son: *Shewanella oneidensis* (NP_717969), Azv: *Azotobacter vinelandii* (ZP_00417496), Pae: *Pseudomonas aeruginosa* (NP_251298), Avi: *Allochromatium vinosum* (AAc35399), Mca: *Methylococcus capsulatus* (YP_113796), Vok: *Candidatus* Vesicomyosocius oktuanii (YP_001219620), Hha: *Halorhodospira halophila* (YP_001003522), Tde: *Thiobacillus denitrificans* (YP_316238), Mma: *Magnetospirillum magnetotacticum* (ZP_00052645), Cch: *Chlorobium chlorochromatii* (YP_380248), Cte: *Chlorobium tepideum* (NP_661745), Pya: *Pyrobaculum aerophilum* (NP_560103), Pyr: *Pyrobaculum arsenaticum* (YP_001056328), Pyi: *Pyrobaculum islandicum* (YP_929693), Dvu: *Desulfovibrio vulgaris* (YP_011988), Tno: *Thermodesulforhabdus norvegica* (CAC36215), Dal: *Desulfatibacillum alkenivorans* (ZP_02133988), Dol: *Desulfococcus oleovorans* (YP_001528350), Dps: *Desulfotalea psychrophila* (YP_064733), Chy: *Carboxydothermus hydrogenoformans* (YP_361199), Mth: *Moorella thermoacetica* (YP_430477), Dha: *Desulfitobacterium hafniense* (YP_516550), Afu: *Archaeoglobus fulgidus* (NP_071053).

TusE homologs

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Eco          ........MLIFEGKEIETDTEGY-LKESSQWSEPLAVVIAKN-E-----GI-SLSPEHWEVVRFVRDFYLEFNTSPAIRMLVKAMANKFGEEKGN-SRYLYRLFPKGPAKQATKIAGLPKPVKCI
Pmu          ........MIEVNQQQIETDAHGY-LLDLTQWDEDVARAIAQQ-E-----NI-DLTDAHWEVIYFVRAFYQEYKTSPAIRMLVKAMAQKLGEDKGN-SRYLQRLFPDGPAKQATKLAGLPKPAKCL
Hin          ........MLNINGIEVETDKDGY-LLNSQQWNEDVAQAIARL-E-----SL-ELTDAHWEVIYFVRDFYQEYNTSPAIRMLVKAMAEKLGADKGN-SRYLQRLFPEGPAKQATKLAGLPKPAKCL
Ype          ........MLEFEGRIIDTDAQGY-LKNSTDWSEALAPVLAEQ-E-----GI-TLTEPHWEVVRFVRAFYQEFNTSPAIRMLVKAMAQKYGEEKGN-SRYLYRLFPKGPAKQATKIAGLPKPVKCI
Vch          ........MLEYQGKTIDTDAQGY-LLDFTQWEEGMIALLAEQ-E-----GI-ELTDAHLEVVHFVRRFYEEFKTSPAIRMLVKAMEKEHGPEKGN-SKYLFKLFPKGPAKQATKLAGLPKPAKCL
Son          MVN-----PLMFNGVDIERDHQGY-LKNIADWHPDMAPLLAQE-E-----NI-ELTSAHWEVINFVRDFYLEYKTSPAIRVLVKAIGQTLGPEKGN-SKYLYTLFPVGPAKQATKIAGLPKPAKCI
Azv          .MS-----TLNVAGRDLALDEDGY-LSDLRDWSEEVAEALARR-E-----EL-ELSVEHWEILRLLREFHAQFQLSPATRPLVKYVALKLGPEKGN-SPHLNRLFKGSPAKLAAKLAGLPKPTNCL
Pae          .MT-----LLMLEGREIRLDKDGY-LAALDDWSEPVAEALAAR-E-----EL-ALTAEHWEILQLLREFYAEFQLSPANRPLIKYVAQRLGPDKGN-SLHLNHLFKGAPAKLGAKLAGLPKPSNCL
BGS_0190     ..M-----ELEINGKSIETDSQGY-LVNLSDWNEELANVLAQQ-N-----DL-PLTEGHWEIINMIRTYYHDHGTAPAMRALIKLVKEELGSDKGN-SKYLYSLFPYGPGKQAARYAGLPKPTGCV
BGP_3768     ..M-----ELEVNGKTVETDPQGY-LVNLSDWSTDLANTLAEK-E-----GF-SLDEPHWEVINMIRGYYQEHGTAPAMRTLTKLAKTELGSDKGD-SKYLYSLFPYGPGKQAARYAGLPKPTGCV
FLOR_02380   ..M-----QLEINGRTIETDDQGY-LADLSDWSQDLAIELAKR-D-----DL-ELSESHWEVINLIRQFYEDNGSAPAMRALTKLAKKELGADKGD-SKYLYTLFPYGPGKQAARYAGLPKPTGCV
THI1718_0    ..M-----QIEVNGTSIETDPQGY-LVNLSDWSEDLANALAEK-D-----EL-VLGEAHWEIINMIRAYYEDHGTAPAMRKLTKLAKSELGAEKGD-SKYLYSLFPYGPGKQGSRYAGLPKPTGCV

BOGUAY_1905  .MD-----YVELK------SENGY-MVDPSQWSKEYAKWRMTD-L-----EI-EITENHWNIVNVFREFIEEKGITPSSRVAQKEAKKRFGVD----SKGFYALFPNGP-KQVAMVAGGIKPSGC.
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DsrC (rDSR) homologs

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Avi          MAD-----TIEVDGKQFAVDEEGY-LSNLNDWVPGVADVMAKQ-D-----NL-ELTEEHWDIINFLREYYEEYQIAPAVRVLTKAVGKKLGKEKGN-SKYLYSLFPYGPAKQACRFAGLPKPTGCV
Mca          ........MDVN-QPLRLGADGF-LLDSHSWDEATAERLAET-I-----GI-RLTDAHWEIVRFIRDYHRRFDHLPNARLFVKAVQKELGETKGN-NRYLHGLFPGGPLKQACLIAGLPKPPGCL
Vok          ........MADILG--AKVDEEGF-LVDLGDWSKEIAEQMAKD-D-----DV-SLSEEHWDVINFLREYFEEYQIAPAVRVLTKAIAKRMGKDKGN-SKYLYSLFPYGPGKQGCRFAGLPKPTGCI
Hha          ..M-----AIQVNGTTIPTDEEGY-IEDLSLWSPEVAEIMAQE-D-----GQ-ELTEQHWEVINFLREYYDEYQIAPAVRVLTKQIGKRLGPEKGN-SKYLYELFPYGPAKQACRYAGLPKPTGCV
Tde          .MP-----MVNIAGKEVEVDEEGY-LVDLSQWNEDIAKYMAVE-E-----KV-ELTDSHWEVVNFLREYYAEYQIAPAVRVLTKAIGKKLGPEKGN-NKYLYELFPYGPAKQACKIAGLPKPTGCI
Mma          ..M-----AYTSNGATIEADEEGY-LTDINQWNEDLAGQIAKD-E-----GI-TLSPEHWEVVNFLREYYAEYQIAPAVRVLTKAIAKKFGADKGN-NKYLYELFPYGPGKQACKIAGLPKPTGCV
BOGUAY_1506* ..M-----AIDVNGKTVDLDEEGY-LTDLKDWNEDVAKVLASQ-D-----EI-ELTESHWEVVKFLRDYYDEYQIAPAVRVLTKAIGKKLGKDKGN-SKYLYELFPYGPAKQACRFAGLPKPTGCI
BA07_33      ..M-----TIEVNGKLLETDEDGY-LTNLSDWSTDVATEMAKQ-D-----GI-TLTDNHWEVVNFLKEYYSEYQIAPAVRVLTKAIGKKLGAEKGN-SKYLYDLFPLGPAKQACRYAGLPKPTGCV
BGP_1169     ..M-----AIEVNGKTIETDEEGY-LVNLAEWNEDCASVLAEQ-D-----EL-TLTESHWEVINFLREYYDEYQIAPAVRVLTKAIGKKLGPEKGN-SKYLYELFPYGPAKQACRFAGLPKPTGCV
FLOR_02854*  .MS-----TIEVGGKSVELDEEGY-LTNLADWSEEVAGILADQ-DKADNPDF-ELTDNHWEVINFLREYYDEYQIAPAVRVLTKAIGKKLGKDKGN-SKYLYELFPYGPAKQACRYAGLPKPTGCI
Cch          ..M-----AIEIGGVRYETDENGY-LVNLEDWSEDVAKILAEG-E-----EI-EMDEVHWDIVNFLRRYYAEYQIAPAVKVLTKAVAAEKGMDKKEASEFLYGLFPKGPGLQACKIAGLPKPTGCV
Cte          ..M-----AIEVNGMSVETDENGY-LVNLDDWTEEVAVKIAED-E-----GI-AMEAGHWDLVKFLRNYYKEYQIAPAVKVLTKAVASEKGMDKKEASEFLYALFPKGPALQACKIAGLPKPTGCV
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DsrC (DSR) homologs

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Pya          .MPVKCPGEYQVDGKKVILDEDCF-MQNPEDWDEKVAEWLARELE-----GIQKMTEEHWKLVKYLREYWETFGSCPPIKMVTKETGFSL--------EKIYQLFPSGPAHGACKVAGAPKPTGCV
Pyc          .MPVKCPGEYTVDGKKVILDEDCF-LQNTEDWDEKIAEWMARELE-----GIQQLTDAHWKVIRYLREYWETYGSCPPIKMLTKETGFTL--------EQIYQLFPSGPAHGACKVAGAPKPTGCV
Pyi          .MPVKCPGEYKVDDVTIILDEECF-LSNPEVWNEKVAEWMARELE-----GIQKMTEDHWKVVKYLREYWETYGVCPPIKMLLKETGFTL--------EQIYQLFPSGPANGACKVAGAPKPTGCV
Dvu          .MA-----EVTYKGKSFEVDEDGF-LLRFDDWCPEWVEYVKES-E-----GISDISPDHQKIIDFLQDYYKKNGIAPMVRILSKNTGFKL--------KEVYELFPSGPGKGACKMAGLPKPTGCV
Tno          MAQ-----TIEFKGKVFEVDEDGF-IQSFDQWCPEWVEYVKQS-E-----GIEELTEAHWKIIHMLQDYYKKHGIAPMVRILTKVTGYKL--------KEIYELFPSGPGKGACKMAGLPKPTGCV
Dal          .MA-----TIEFEGKSFTVDEDGF-IDSFENWSEEWVTYVKGV-E-----GIDELTEEHWKVINVLQDYYKKNGIAPMVRVLSKVTGFKL--------KHIYELFPSGPGKGACKMAGLPKPTGCV
Dol          .MA-----TIEFQGKSFNVDEDGF-IESYSEFSEEWVQWVKKE-E-----GIDELTDEHRQVIKVLQDYYEKNGIAPMVRILSKLTGFKL--------KHIYELFPSGPGKGACKMAGLPKPTGCV
Dps          .MP-----TLEHNGSSFQVDEDGFLLTGMEEFNEDWIEYVSSV-E-----GIEELTDEHRKVIDSLQEYYKKNGIAPMVRILSKTTGFPL--------KRIYELFPSGPGKGACKMAGLPKPTGCV
Chy          .MP-----QIEIDGLVLNVDEDGF-IEDPSIWNEEIAKALAKT-E-----GVTELTEAHWKVVNYIRNYYLQYQIAPMIRKLCKDTGFSL--------KEIYELFPSGPAKGACKIAGLPKPTGCV
Mth          .MP-----TVNLAGMQLEVDEDGF-IADPSKWNEDVARALAEQ-E-----GVTEMTEDHWKLVNYLRQYYLQFGIAPMIRKVCKETGFSL--------KQIYDLFPSGPAKGACKVAGLPKPTGCV
Dha          .MA-----DLVVNGVSYELDEDGF-LEDAAEWNEDVAKALAPN-E-----DVEELTEEHWRVINYLRDYYDQYGVAPMVRKLLKDTGYDQ--------KTIYNLFPTGPGKGACKIAGLPKPTGCV
Afu          .MP-----ELEVKGKKLRLDEDGF-LQDWEEWDEEVAEALAKD-TRFSPQPI-ELTEEHWKIIRYLRDYFIKYGVAPPVRMLVKHCKKEVRPD-CN-LQYIYKLFPQGPAKDACRIAGLPKPTGCV
```

Figure S2.13 *(on the next page)* | Alignment of SoxAX homologs. SoxAX sequences from *Beggiatoaceae* and *Halorhodospira halophila* were aligned with SoxA sequences of the Types I, II, III, and IV (dark grey; classification by Kappler and Maher, 2013) and corresponding SoxX sequences (light grey). The alignment was calculated with MAFFT version 7 (Katoh and Standley, 2013). Regions of the alignment, which correspond to SoxX and SoxA are indicated with light grey and dark grey bars. Dots (.) in the alignment represent missing sequence information, dashes (-) indicate alignment gaps. SoxAX sequences, which were retrieved from *Beggiatoaceae* genomes, are shown in bold font. The SoxAX fusion proteins of *Beggiatoaceae* and *H. halophila* feature three heme *c* binding sites (CXXCH; highlighted in blue). One of these binding sites is located in the putative SoxX domain and methionine (highlighted in green) appears to serve as the second axial of the bound heme, corresponding to other SoxX proteins (Kappler and Maher, 2013). The two other heme groups are located in the putative SoxA domain. The C-terminal heme binding site is present and invariantly ligated by a cysteine (highlighted in red) in all of the so far studied SoxA proteins (Kappler and Maher, 2013), including those of the *Beggiatoaceae* and *H. halophila*. In contrast, the N-terminal heme binding site of SoxA (central binding site of SoxAX) shows substantial variation. This heme binding site is entirely absent from Type II, Type III, and several Type IV SoxA proteins (Kappler and Maher, 2013) and, when present, the nature of its second axial ligand is variable. We found that the usual second axial cysteine ligand (highlighted in red; Kappler and Maher, 2013) is substituted by histidine in the SoxAX sequences of *Beggiatoaceae* and *H. halophila* as well as in the SoxA sequence of *Fulvimarina pelagi* (ZP_01439477; a Type I SoxA sequence not included in the alignment). This is important, because a change in the nature of the second axial ligand can influence the redox potential of the respective heme. The cysteine-ligated hemes usually found in SoxA proteins feature strongly negative redox potentials and thus are likely not available for and electron storage during catalysis (Kappler and Maher, 2013). A transient storage of two electrons is, however, required for the reaction catalyzed by SoxAX, i.e. the oxidative coupling of thiosulfate to the carrier protein SoxYZ. Recently, a copper center has been identified in a type II SoxAX protein and was proposed to serve as a second site of electron storage in addition to the methionine-ligated SoxX heme (Kappler et al., 2008). Thus, it is possible that histidine-ligated hemes of SoxAX proteins fulfill a similar function. However, redox titrations will be required to determine the actual redox potential of the central, histidie-ligated heme. The full species names and accession numbers of the reference sequences are as follows (SoxA/ SoxX or SoxAX): Pde: *Paracoccus denitrificans* (YP_917913/ WP_011750379), Sag: *Stappia aggregata* (EAV42296/ WP_006937184), Cli: *Chlorobium limicola* (AAL68886/ AAL88883), Avi: *Allochromatium vinosum* (YP_003444122/ ABE01360) Sno: *Starkeya novella* (AAR98727/ AAR98728), Dar: *Dechloromonas aromatica* (AAZ47860/ AAZ47859), Sde: *Sulfurimonas denitrificans* (YP_392779/ YP_392776), Saz: *Sulfurihydrogenibium azorense* (YP_002729642/ YP_002729638), Bfl: *Beggiatoa* sp. 35Flor (FLOR_03004), Ibg: '*Ca.* Isobeggiatoa sp.' (BGP_5667), Gfi: Guaymas filament (BOGUAY_3083), Bal: *Beggiatoa alba* B18LD (BA17_207), Hha: *Halorhodospira halophila* (YP_001003514).

Gene Type

SoxX

```
SoxA  I    Pde  .............MPRFTKTKGTLAA-------TALGLA-----LAGAAFAEPAEDE-------------------------------------------
           Sag  ........................ML-------AALMVS-----TSASLAGGPVDEK-------------------------------------------
      III  Cli  ................MKKTIQRGLF-------TGALVL-----LTA-MTSKPAHAAVNYQALVDADVKK----------FQ------------------
           Avi  ...................MTKHGFL-------LATLVL-----AGATLPIGPVTAATP-----EEEQAA----------FQ------------------
      II   Sno  .............MRRFAAGCLALAL-------LVLPFV-----LTGARAAEDES---------------------------------------------
           Dar  ............MIQRLLKTLVGVAT-------IA---A-----FAGSVLAQDSK---------------------------------------------
      IV   Sde  .................MR---KVLQ-------IALSVL-----VASSLAYGSEQFS-------------------------------------------
           Saz  .................MKLKGKVLL-------FGLAVALTTYGVNKSISQEAGQ-A-------------------------------------------
SoxAX      Bfl  ................................................MQASEMSTDVPLEMEMPSYKTP----------WE------------------
           Ibg  ..............MKYFLITSTGLA-------LSL--F-----LPTPLLAAEMSQAVPLELEKPAYVTP----------WK------------------
           Gfi  ...............MNLRTTLSGLL-------IIIVGY-----LPHLVIAAEMSPEVPLELEKPSYSAP----------WQ------------------
           Bal  ..........MRFITSFYLVSVSVLL-------GSI----------PVVWAAEISSAVPLELKKPAYVSP----------WK------------------
           Hha  .......MTARSPTRSTHSAGIAVTL-------GLCTGA-----AAISLLATQGAGAGEEERNIEPRPAPDGMDYHTSETWESLATEPSQGRIVEEDGEK
SoxX  I    Pde  .......M--SSHLWHAAVVAMAIAT-------PAICET-----APKDVDYAEGAVEASLTG--------------------------------------
           Sag  MFKGATQF--SLPAAFAGLAVLAASA-------AAAGTV-----APDSVPIEDMELSQSLTG--------------------------------------
      III  Cli  .................MKSSGIIAA-------AAILLL-----PSLGIAAAPA----------------------------------------------
           Avi  ...........MPLNVSHRSRLTALL-------FGLSAL-----SSGSLPAADLPDDLA-----------------------------------------
      II   Sno  ..............MRFETLLKRAAQ-------VGALVL-----LPLAAHAQEASAVD------PARVDA----------VV------------------
           Dar  ............MKLKFALFFALSAA-------FG-------------ASAQADKPVDAPGGKFAKDAEQ----------MF------------------
      IV   Sde  .......MKRSL--TLSLLLGMSVFAADYS---SVIEVP-----DAQKIIQKDLLPPLGVNKMPTECITT------------------------------
           Saz  .....MKLKKVILTTVATITGISALAFALTLQDAGIENP-----EAKSIMLKDVPPEPRLYAIDSSCNLS------------------------------
```

SoxX

```
SoxA  I    Pde  ------------------------------------------------------------LVIETDDGPVEIATRT------------------------
           Sag  ------------------------------------------------------------LVI---DGELEIDTRV------------------------
      III  Cli  ---GYFLKEFPGV-----------------------------------------------KLEDFGDGVYALDEDS------------------------
           Avi  ---AYFKQRFPNV-----------------------------------------------PEDEFKNGTYAIDPVT------------------------
      II   Sno  -------------------------------------------------------------EKEIERYRQMIED--------------------------
           Dar  ------------------------------------------------------------VADELAKYREALAD--------------------------
      IV   Sde  ------------------------------------------------------------M---------------------------------------
           Saz  ------------------------------------------------------------I---------------------------------------
SoxAX      Bfl  ---RYS--GWKQTDWKQFSTLRQMVSPPVSFMQKLDKSTTSDSNIILNDDESDV------IEGDPVKGKALVADRK---------------R---GGSCY
           Ibg  ---RYP--HWNQSDWKDFSNLKNNIRARSSSFQDIESP----------------------INGNPENGKKLVADRK---------------R---GGSCF
           Gfi  ---RYTGRRWARTDWKNFSNLRHEVLTPVSTYQKIETP----------------------IKGNPENGKKLVADRK---------------R---GGGCV
           Bal  ---RYK--DWSSDNWSNFNSLDKNSSPKVGKIKKIDKL----------------------AAGDAEKGKKLVADRS---------------R---GGSCY
           Hha  RQIRYEFGGFPDEENEDFSEWPTHSYADDADYPEPQEADIPND-----------------LEGDPERGRELFA-KG---------------E---LGPCS
SoxX  I    Pde  ------------------------------------------------------------VPGNPEEGVRVMTTNA-------------------LGNCV
           Sag  ------------------------------------------------------------VPGDPLAGREAFADRK---------------K----GNCL
      III  Cli  ------------------------------------------------------------GESSVEKGKALALDTN---------------K----GNCI
           Avi  ------------------------------------------------------------LDGDAVAGREVATDRA---------------K----GNCV
      II   Sno  ---KTS--F-TKLPEGWESRLQQDETQRICSVTRNNPSPEQAAAIMKAEEVRIKFPA-GPVLGSWKDGAKVAQNGRGGQFS----DPPGTVS---GGNCY
           Dar  ---RSS--FRTDNSKYWMARLDQDETMKTCGLYRDNPPRKLGEKLEKLNAATIRYPVEGKLIGDWKEGEKLAAVGTGGHIGFIQPDPAGKLR---GGNCY
      IV   Sde  -----------------------------------DSKAIARGAYIFHNLNGEN------AKGNAPEGLSKTVENG---------------KPKQYGNCV
           Saz  -----------------------------------DKESI---------------------KKLAEKGKKVFMEVS---------------K----GNCV
```

SoxX

```
SoxA  I    Pde  -----------TP--PAFLADTFDEIYS------------------------------------------------------------------------
           Sag  ---------------PAPEGHPFDELIS------------------------------------------------------------------------
      III  Cli  ----------------------------------------------------------------------------------------------------
           Avi  ----------------------------------------------------------------------------------------------------
      II   Sno  ----------------------------------------------------------------------------------------------------
           Dar  ----------------------------------------------------------------------------------------------------
      IV   Sde  ----------------------------------------------------------------------------------------------------
           Saz  ----------------------------------------------------------------------------------------------------
SoxAX      Bfl  ACHI---LPDASL--PGNVGINISSIGV-----------WGRDDEYLFNYIYDPR-IF-NP--------STVMPPWGAHD--------------------
           Ibg  SCHI---LPDGSM--PGNIGPALSMIGI-----------WNRSDERLFNYIYDAR-QY-NP--------NTVMPPWGAHG--------------------
           Gfi  ACHI---MPDTAM--PGNIGPDLSTIGA-----------WKRPDEFLFNYIYDAR-QF-NP--------YTNMPPWGAHN--------------------
           Bal  ACHV---MPNANL--AGNIAPDLSTVAT-----------WGRTDEHLFNYIYDPR-VY-NP--------ASVMPPWGAHQ--------------------
           Hha  SCHL---VPEAEIDSPGNVGTDLRTVGE-----------WAPDKEWLYQVVYDPR-VFYGE--------DTPMPPFGVNG--------------------
SoxX  I    Pde  ACHQIGALPDVEF--PGTIAPPLDGAAD-----------RW-TEAQLRGIVANAK-MT-FE--------GTFMPAFYKGEGFVRPGDGFTGKAGTEPLAP
           Sag  ACHANADLSDQLF--HGEVGPVLDGAAD-----------RW-SEAQLRAIVVNSKDVF-GD--------QTIMPGFYTLKVGINVDEEFAGKT-------
      III  Cli  ACHM---MGDGEF--PGNYGPPLIQMKE-----------RYPDRAALHKQIGDAT-II-NP--------KSIMPPFGKHG--------------------
           Avi  ACHV---MAGAES--PGTIGPVLVSMQT-----------RFPSKRDLAAQIWDAT-AK-NP--------EAVMPPFGKHE--------------------
      II   Sno  ACHQL--DPKEVS--YGTLGPSLVGYGR-------ERNFSAEDAKIAFAKVYDAQ-AS-LA--------CSSMPRFGVNG--------------------
           Dar  ACHQL--AKQEVA--YGTIGPSLQHFGK-------IRGNSEEIIKYAYDKIYNSN-AF-TA--------CTNMPRFGLHS--------------------
      IV   Sde  ACHN---IEGAVG--GGNIGPDLTNYTEMFVKT------GVRDAQYVVYAKIADPR-VD-NP--------HTDMTVNLTTK--------------------
           Saz  ACHC---APEAKG--CGNIGPDLTGYKNGLFKAPDYRG-EPKTVDWLHQKIADGR-IL-IPKELQSVPYYNIMTVQLTTG--------------------
```

SoxX SoxA

```
SoxA  I    Pde  -------------------------------------GWLFRDDTTRDMERDDFD-NPAMVFVD-R-GLDAWNAAMGSNGESCASCHQG----------P
           Sag  -------------------------------------GWHYRTDETRDLEADSFQ-NPGMLYVE-R-GEEIWNTVDGAAGKSCASCHED----------A
      III  Cli  RKQWKEMEEFPPY----------------------------------------------ELDVE-A-GKALFNKPFANGKSLGSCFSNG----------G
           Avi  RENWEAIEEFPPY----------------------------------------------ENAIS-Q-GETLWNTPFADGQGYADCFPDG----------P
      II   Sno  ----------------------------------------------------PMA-NPGFLNVD-R-GEVLWSEPRGTRNVSLETCDLG----------E
           Dar  ------------------------------------------------------G-NPADLLEV-K-GEGLWSEKRGPKQVSLEQCDLG----------L
      IV   Sde  ------------------------------------------SDADRALYEELLDNNPADMMLA-S-GSEHLDALGGAASLAKYLEQ------------S
           Saz  ------------------------------------------SEEDLALYKSGI--NPGEVFAQ-EVGGALFNKPMGTSNKSCASCH------------S
SoxAX      Bfl  IFSKAEIKDIVAYLKTLTK-------PVRFKNTQENPDTRTVPVETR-DNFDEFV-NPAMMSIE-N-AEDTFGTD-GPKGKSCQSCHEK----------P
           Ibg  LYTKAEIKDIVSYLQTLKQ-------PINFSNPQDNPATRRAPDEDKHSSLDPFE-NTAMFSLD-L-GEELFEMQ-GPNGKSCQDCHEA----------A
           Gfi  LFHQDEIKDIVAYLQTLTE-------PTHFKTPTDDPTKRPVPVETR-DNLDEFE-NPAMFSLE-L-GEELFSTP-GPKNQSCQSCHAE----------A
           Bal  VFSDAEIMDIVAYLKTLNK-------ATQFTDDKENPKTRPVPVETR-ANLDAFE-NPAMFGTE-L-GEKLFKQT-GATGKSCASCHEQ----------A
           Hha  MWSEEQIIDVVAYLMTLEGDEDGEPIAPEGVDRHWDPNDRP-PLQPAGEHLDPFD-NPGLMQAEQI-AVPLWDEP-APNGESCASCHGDLEPADDLRPIG
SoxX  I    Pde  ILNAQQIEDVVAFLVTLKE.................................................................................
           Sag  ILSAQEVEDVVAYLLTLKE-------N.........................................................................
      III  Cli  ILNVSEIDQIVDYLYTL...................................................................................
           Avi  ILTRQEFVDVVEYIWSL...................................................................................
      II   Sno  VLTEQQIKDVVAYLFDPES-------P--------------------VNK..................................................
           Dar  WLTPEQITHIVAFLIDPES-------P--------------------VNKD.................................................
      IV   Sde  LFNEKEICELASYVLSKK..................................................................................
           Saz  QLTAEEVCQLTAYILSLE..................................................................................
```

(Continued on next page.)

(Continued from previous page.)

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Gene Type                                                                                                                     SoxA
SoxA  I    Pde  -ET-MAGLRAALPRVDETSGKLMILEDYVNACVTERMGL-EKW---------GTTSEDMKDMLSLISLQSRGM--AVNVAI-DGP-AAHFWEQGREIYYT
           Sag  -ESFLKGLGASYPKWNEDAGKPFNIELQINQCREQNMQA-EPY---------KFDAPDQKALTTYIKHQSLGM--PMHVDLSEGE-MQAWWEKGKDLYYT
      III  Cli  ------AVRGMYPYFDEKRKEVITLEMAINECRVANGEK-PY----------APKKGDIARVSAYIASISRGQ--KIDVKVKSKA-AYDAYMKGKEMFYA
           Avi  ------AIMNHYPRWDRERGQVMTLPLALNACRTAHGET-PL----------KYKKGPIADLLAYIAFESRGQITRVEIPQDDPR-ALAAYEQGKRFYFA
      II   Sno  GPGKLEGAYAHLPRYFADTGKVMDLEQRLLWCMETIQGR-DTKPLVAKPFSGPGRTSDMEDLVAFIANKSDGV--KIKVALATPQ-EKEMYAIGEALFFR
           Dar  GPGKLEGAYAQLPKYFKDTGKVMDVESRLVHCMVTLQGF-KQEQVTRQWFSKPGQDSDIEALVTFIGAKSNGK--PINVPASHPE-EAKMAKMGEYIFYR
      IV   Sde  -EDSLASYLAGFPRYIDKYKSVVSIDQMLQAF--MHDSGAKAY---------TLKSEEMFNMSSYVKSLGNEQ--AINIDINANKYMQEAYALGKEVFNT
           Saz  -EEKLKKAVGTYPKYEPKLNTVISLQQRIQMCQKLNQGVDKPF---------PLNSQENTALLTYLKYIASGE--KINVDTSSNPVVKEYYEYGKYVFDL
SoxAX      Bfl  -EVEFATWATNMPKVETRMNKVIGIEEFITRHARATTGA-EY----------PSQSEENLDMAIYLRYLANGR--PIAIDKSDRN-TQLAIRRGEDLMSR
           Ibg  LKTQFTTWAATMPKFETRLNQVIGIEEFITRHARATTGA-EY----------PSQSEENLGLAIYLRYLANGQ--PINIDQSDVN-TQAAIKRGNALTQR
           Gfi  --TELKTWAATMPKFETRLNKVIGVEEFVTRHALATTGA-EY----------LAQSKQNLALAIYLRYLANGQ--TIAIDQSDAN-TQAAIKRGKALMER
           Bal  -EQAFKTWAASMPKYEPRLKKVVGVEEFVTRHAMATTGE-SY----------LAQSEENLGLAIYLRYLANGQ--PIQINAGDKA-TKAALKRGEQLMTR
           Hha  VIEDLEGVATEYPKWFDEYDRMMSLEDFLAVHAKEEQDM-EL----------PTQSQENLYMSILVHSQSNGM--VYDLDDDDPN-VQAAIERGEELFHR
SoxX  I    Pde  ....................................................................................................
           Sag  ....................................................................................................
      III  Cli  ....................................................................................................
           Avi  ....................................................................................................
      II   Sno  ....................................................................................................
           Dar  ....................................................................................................
      IV   Sde  ....................................................................................................
           Saz  ....................................................................................................

                                                                                                                              SoxA
SoxA  I    Pde  RYGQLEMSCANCH-E--DNYGNMIRADHLS---------QG------------QVNG--FPTYRLKDAGMVTAQQRFVGCVRDTRAETFKAGSDEFKALE
           Sag  RTGQLNLSCATCH-E--NYNGSYIRADHLS---------QG------------NVNG--FPTYRLKQSEMVSLHNRFRGCIRDTRAAFPPAFSDELMALE
      III  Cli  KRGQLNMSCSGCH-M--EYSGRHLRAEIIS---------PA---LG-------HTTH--FPVFRSKWGEIGTLHRRYAGCNENIGAKPFPAQSKEYRDLE
           Avi  RRGQLNFACAHCH-L--ATSGTKLRTETLS---------PA---YG-------HTTH--WPVYRSEWGEMGTLHRRFAGCNEQVRAKAFEPQGEEYRNLE
      II   Sno  RSSINDFSCSTCH----GAAGKRIRLQALP---------QL---DVPGKDAQLTMAT--WPTYRVSQSALRTMQHRMWDCYRQMRMPAPDYASEAVTALT
           Dar  RSGPQDFSCAICH----GQEGKRIRLQDLG---------NL---TT-KDGAGTAMKT--WPSYRVSQGAVWTMQRRLIDCMRQARWPEPNYLADSIIALE
      IV   Sde  RRGGRGLSCYNCHNS--NVIGAVLRTQPLPDISA-----KG------------NASAATWPAYRMTKSKLATLQKRFQGCMENALLAVIPLGSKEMTALE
           Saz  KRGKRNLSCQTCH-E--FAAGMVLRMQRLTPLGAEYNGIKG------------TNAAAHWPGYRMTQSKVVTIEQRFQQCMSQAGMKILPLGSKEMVALE
SoxAX      Bfl  KIGQLNFACVDCH-E--ASANKWIRGQYLT---------GL---VG-------MMDH--FPTYRTSRGEIWDIRKRFQWCGVAIRANELEPDAAEYGDIE
           Ibg  KMGQLNFACMDCH-G--LLANRWIRGQYLV---------SM---SS-------IYDH--FPTYRTSRGEIWDIRKRFQWCNVSIRANELPPNAPEYGDIE
           Gfi  KIGQINRACVDCH-I--TNVKKWIRGQYLV---------PR---KG-------MYEH--FPTYRTSRGAIWDIRKRIQWCGVSVRANELPPDAPEYGDLE
           Bal  KIGQLNFACNDCH-V--FGANHWVRGQYLS---------GF---DG-------MLDH--FPTYRTSRAEIWDIRKRLQWCGVAVRANELPPDAPEYGDIE
           Hha  PVGQRAHACANCH-TDRGGGDKWLSGRMLA---------NIEADDT-------AMTN--HPYWRTAQSRVWDLRTRFQWCMTPVGTNYLPGDAPEYADLE
SoxX  I    Pde  ....................................................................................................
           Sag  ....................................................................................................
      III  Cli  ....................................................................................................
           Avi  ....................................................................................................
      II   Sno  ....................................................................................................
           Dar  ....................................................................................................
      IV   Sde  ....................................................................................................
           Saz  ....................................................................................................

                              SoxA
SoxA  I    Pde  LYVASRGNGLSVEGVSVRH
           Sag  VYVTWRGSGLDIETPAVRQ
      III  Cli  FFQTVMSNGLKFNGPASRK
           Avi  YFLTYMNNGLELNGPGARK
      II   Sno  LYLTKQAEGGELKVPSIKR
           Dar  TYLQKNATGTVMETPGIKR
      IV   Sde  VYFTHEAKGAPIAIPGLKR
           Saz  LYVTSLANGATIEAPGLVR
SoxAX      Bfl  MYLMAKNNGRILSIPGIRH
           Ibg  IYLATINQGQKLSVPGIRH
           Gfi  IYLATLNNGQILNVPGLGH
           Bal  LYLMQLSNGKNLSVPGIRH
           Hha  TYIVSEQQGEEIIVPRYAH
SoxX  I    Pde  ...................
           Sag  ...................
      III  Cli  ...................
           Avi  ...................
      II   Sno  ...................
           Dar  ...................
      IV   Sde  ...................
           Saz  ...................
```

```
α  Nha   327  GFVGNDLQCSNCHIDR----GRQPNSAPLGAAYLLYPAYRAKNGHVNTFQERLQGCFRFSMNGKAPPFNDKVLVA--LETYAYFLAKGGPTG-VAVKG
   Cme   272  KNVGNGLNCTNCHLSG----GTTAYASPWVGLSGAFPEYRSRSGKLISLQERVNDCFQRSMNGKPLAFDSAEMNA--IMAYMKWLSTGVPVG-TNVTG
β  Tde   352  KYVGNAMNCSNCHLDN----GRRANSAPLWAAYVLYPAYRKKTGTVDTIQSRIQGCFMYSMDGRPPALDSKEMTA--LVTYHYWMSKGAPTG-VKLPG
   Tin   314  DYVGNTLSCVNCHTDA----GRMAGSAPLWAAYVSYPAYRGKNKKVNTFEERLQGCFKFSQNGKAPPLGSKTLVA--LESYSYWLSKGLPVD-EKVAG
   Avi*  270  DFVGNGLACRHCHPGRDGEVGTEANAAPFVGVVGRFPQYSARHGRLITLEQRIGDCFERSLNGRALALDHPALID--MLAYMSWLSQGVPVG-AVVAG
   Hne   317  QYVGNSLNCVNCHTDG----GAMAGSAPLWGAWVSYPAYRGKNKKVNTYEDRLQGCFRYSMNGKMPPLGSDVLVA--LSAYSYWLAKGLPTGDNKIAG
γ  Pse*  307  EYVGNGMNCTNCHLEQ----GRKANSAPLWGAYPMYPAYRKKNDKVNSYAERVQGCFQFSMNGTPPAADSHVINA--LTAYSYWLSTGAPTG-QELPG
   Par*  327  NNVGNQLNCTSCHLGN----GSEAYAAPWNNTPSVYPNYSKRTGRINTIQERINGCFERSLNGKALDLNSDDMNA--MVSYMSWLSQDMPFG-VSPEG
   Ibg   306  RYVGNGLNCTNCHLSE----GRKANAAPLWGAYGMYPQYRGKNREVVTFQERIQDCFKYSLDGIAPTVDSPEMEA--LIAYAHWLSKGVPVG-VLLPG
δ  Dac   274  DLVHSRLRCSNCHLKA----GTVAYAAPWVGVTTRYPRYSRRSAGDVSLPQRIQGCFRRSLNSEAPAVDSEPMQA--IVAYMTWLSEEISEG-YRLEG
ε  Wsu   371  RYSGNNLSCNSCHLGA----GTAKYAAPLVDNHANFPQYRNRENSLGTMAARVNGCMQRSMNGYPLPAEGKEMKA--FLAYIHWLGQGIPVG-AKIEG

α  Nha        QGYPKLKA---PDQPADYDRGAKAYAQHCSLCHGGDGEGQK--SADG---QTVFPPLWGP-RSFNWGAGMASINNAAGFIKANMPLGLGGSLSDQEA   60
   Cme        RGFEKIDT---AL-VPNREHGKAVYAAQCASCHGADGQGMK--NPQG---GYVFPPVWGK-DSFNIGAGMARMYTAAAFVKHNMPLGQGGTLSAQDA  132
β  Tde        QGFIKVPK---PPQTPDLARGEAVYKANCVICHGANGEGI---KVDG---QHAFPPLWGK-ESFNWGAGMHRIDTAAGFIKANMPYGLGGTLSDQEA   78
   Tin        RGYPNLPE---PQQAPDYVRGQKVYEAKCILCHAANGEGQ---YVNG---ETVFPPLWGP-KSFNWGAGMGSYKNAAKFIYANMPYGMSYSLSPQEA   55
   Avi*       HGIPTLTL---ER-EPDGVHGEALYQARCLACHGADGSGTL--DADG---RYLFPPLWGP-RSFNTGAGMNRQATAAGFIKHKMPLGADDSLSDEEA   21
   Hne        RGYPDLPE---PKQAPDFVRGKTVYEANCAICHAANGQGR---VVNG---TVVFPPLWGA-QSFNWGAGMGSIKNAAKFIFANMPYGQSYSLTPQQA   57
γ  Pst*       RAYPEVPQ---PQGGFDIAKGKQIYAEQCAVCHGDDGQGQ---KAGG---GYVFPPLWGK-DSFNWGAGMHRINTAAAFIKESMPLGKGGSLSDADA   53
   Par*       SGFVKVDK---TL-EPNTDNGKKLFAEKCSVCHGATGEGQY--NDDG---TYVYPAIAGD-KSFNDGAGMARTYTAASFIKGKMPFGQGGSLSDQEA   33
   Ibg        NGFTPVNR---TR-APSTENGEILYKTQCAMCHGKDGLGY---KYEDDRPGYMFPPLWGS-DSFNRAAGMNKVKTAAQFIKANMPLGRGFTLTDNEA   32
δ  Dac        WGFPRLAE---MP-PADRQRGEQLFVQRCAVCHGKEGQGRL--DETPQRYPYGFPPLWGK-DSFNIAAGMARLHKAAAFIQRNMPFSSGGILTIQQA   33
ε  Wsu        RSLKTVDRKMVQQNAADVKNGAEVYARDCASCHGAEGEGLRRESKDGKPAGYEFPPLWGSDDTYNTGAGMYRTLKAADFIKSTMPKG-APTLSDKDA   74
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Figure S2.14 | Alignment of thiosulfate dehydrogenase (TsdA) homologs. Proven thiosulfate dehydrogenases (Denkmann et al., 2012) are marked with an asterisk (*). The homolog from '*Ca.* Isobeggiatoa sp.' (Ibg; BGP_3543; bold font) shares all strictly conserved residues (highlighted). Among these are two heme *c* binding sites (CXXCH, shown in blue), two methionines, which could potentially serve as axial heme ligands (shown in green), and a single conserved cysteine residue (shown in red). The latter was proposed to ligate the catalytically active heme axially, in correspondence to other heme groups involved in redox-reactions on sulfur compounds (Denkmann et al., 2012). The numbers of amino acids, which precede and follow the shown TdsA excerpts are given. Dashes (-) indicate alignment gaps. The full species names and the accession numbers (gene locus identifier for '*Ca.* Isobeggiatoa sp.') of the above shown sequences are as follows: Nha: *Nitrobacter hamburgensis* X14 (ABE61094), Cme: *Cupriavidus metallidurans* (ABF12206), Tde: *Thiobacillus denitrificans* ATCC 25259 (AAZ96081), Tin: *Thiomonas intermedia* K12 (YP_003644562), Avi: *Allochromatium vinosum* DSM 180 (ADC61061), Hne: *Halothiobacillus neapolitanus* c2 (YP_003263355), Pst: *Pseudomonas stutzeri* A1501 (YP_001173331), Par: *Psychrobacter arcticus* 273-4 (AAZ19791), Ibg: '*Ca.* Isobeggiatoa sp.' (BGP_3543), Dac: *Desulfuromonas acetoxidans* DSM 684 (EAT14957), Wsu: *Wolinella succiogenes* DSM 1740 (NP_906283)

Table S2.3 | Carbon and energy metabolism of *Beggiatoaceae* strains. 'A' stands for autotrophy, 'H' for heterotrophy, 'LA' for lithoautotrophy, 'LH' for lithoheterotrophy, and 'OH' for organoheterotrophy. Letters in parentheses indicate that the respective carbon acquisition pathway was suggested by genomic data. The listing is not meant to be comprehensive as not all possibilities have been investigated for each strain.

Strain	Modes of growth	References
'*Ca.* Thiomargarita nelsonii'	(A)	Winkel et al. (2013)
Guaymas filament	(A), (H)	MacGregor et al. (2013)
'*Ca.* Isobeggiatoa sp.'	(A)	Mußmann et al. (2007)
Beggiatoa alba B18LD	OH, LH, (A)[a]	Strohl et al. (1981a); Güde et al. (1981); Mezzino et al. (1984); Winkel et al. (2013)
Beggiatoa alba B15LD	OH	Mezzino et al. (1984)
Beggiatoa B25RD	OH	Mezzino et al. (1984)
Beggiatoa L1401-15	OH	Mezzino et al. (1984)
Beggiatoa sp. OH-75-2a	OH	Nelson and Castenholz (1981)
Beggiatoa sp. 402	LH, LA	Grabovich et al. (1998, 2001)
Beggiatoa sp. MS-81-6	OH, LA	Nelson and Jannasch (1983); Hagen and Nelson (1996, 1997)
Beggiatoa sp. 35Flor	LA, (A)	Schwedt et al. (2012); Winkel et al. (2013)
Beggiatoa sp. MS-81-1c	LA[b]	Hagen and Nelson (1996, 1997)

[a] Strohl et al. (1981b) showed that *Beggiatoa alba* B18LD does fix CO_2, but only in minor amounts. Growth in absence of organic carbon compounds was not possible.

[b] Acetate is used only as a supplemental carbon source (Hagen and Nelson, 1996).

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Chapter 3

Sulfur respiration in Beggiatoaceae

3.1 Sulfur respiration in a marine chemolithoautotrophic Beggiatoa strain

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Manuscript published in
Frontiers in Microbiology (2012), Volume 2, Article 276.
doi:10.3389/fmicb.2011.00276

Contributions:
This study was commenced by A. Schwedt and H. N. Schulz-Vogt and the initial experiments were performed by A. Schwedt. Follow-up experiments and data analyses were performed by me, A. Schwedt and L. Polerecky. The manuscript was written by me with the help of the other authors. A previous version of this manuscript was part of the doctoral thesis of A. Schwedt (2011).

Acknowledgements:
We thank Martina Meyer and Kirsten Imhoff for technical support. We also thank the two reviewers for their useful comments and suggestions. The study was funded by the European Research Council (No. 203364-ELNOX), the Max Planck Society and the German National Merit Foundation.

Abstract

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 downwards 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.

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 downwards 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 chemolithoautotropic 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 $MgCl_2 \cdot 6\,H_2O$, 16.6 mM $MgSO_4 \cdot 7\,H_2O$, 4.5 mM $CaCl_2 \cdot 2\,H_2O$ and 13.7 mM KCl; 27.5 g NaCl, 5 g $MgCl_2 \cdot 6\,H_2O$, 4.1 g $MgSO_4 \cdot 7\,H_2O$, 0.66 g $CaCl_2 \cdot 2\,H_2O$ 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 $FeCl_3 \cdot 6\,H_2O$; 555 mg K_2HPO_4, 28.72 mg Na_2MoO_4, 750 mg $Na_2S_2O_5$, 29 mg $FeCl_3 \cdot 6\,H_2O$ 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 one to two 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 (H2S-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 afterwards 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 $1.56 \times 10^{-9}\ m^2\ s^{-1}$.

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 one second 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 minutes 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 hours 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 minutes 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 time-points 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 3.2 A** and **Movie S3.1** in Supplementary Material). In cultures with medium to high sulfide fluxes (**Table 3.1**) 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 3.1**). 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 3.1 and 3.2 B**). 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.

Table 3.1 | Diffusive sulfide fluxes in gradient cultures from this study.

Na_2S [mmol L^{-1}] in bottom agar	Time [days]	Flux [mmol m^{-2} d^{-1}]
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

Migration of filaments in cultures with a high sulfide flux followed a general pattern illustrated in **Figure 3.2 E**. 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 3.2 E**). After reaching a depth of 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 3.2 E**), 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

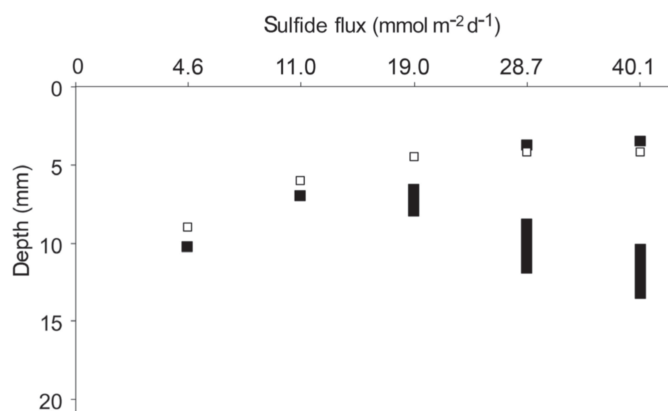

Figure 3.1 | Distribution of *Beggiatoa* sp. 35Flor filaments over depth in gradient cultures with different sulfide fluxes after 6 (open symbols) and 12 (closed symbols) days of cultivation. The flux values were estimated from measurements after 7 days (**Table 3.1**).

sulfide (**Figure 3.2 C,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 3.2 D**). 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 3.2 F**), 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 3.3 A,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 3.3 E**). With

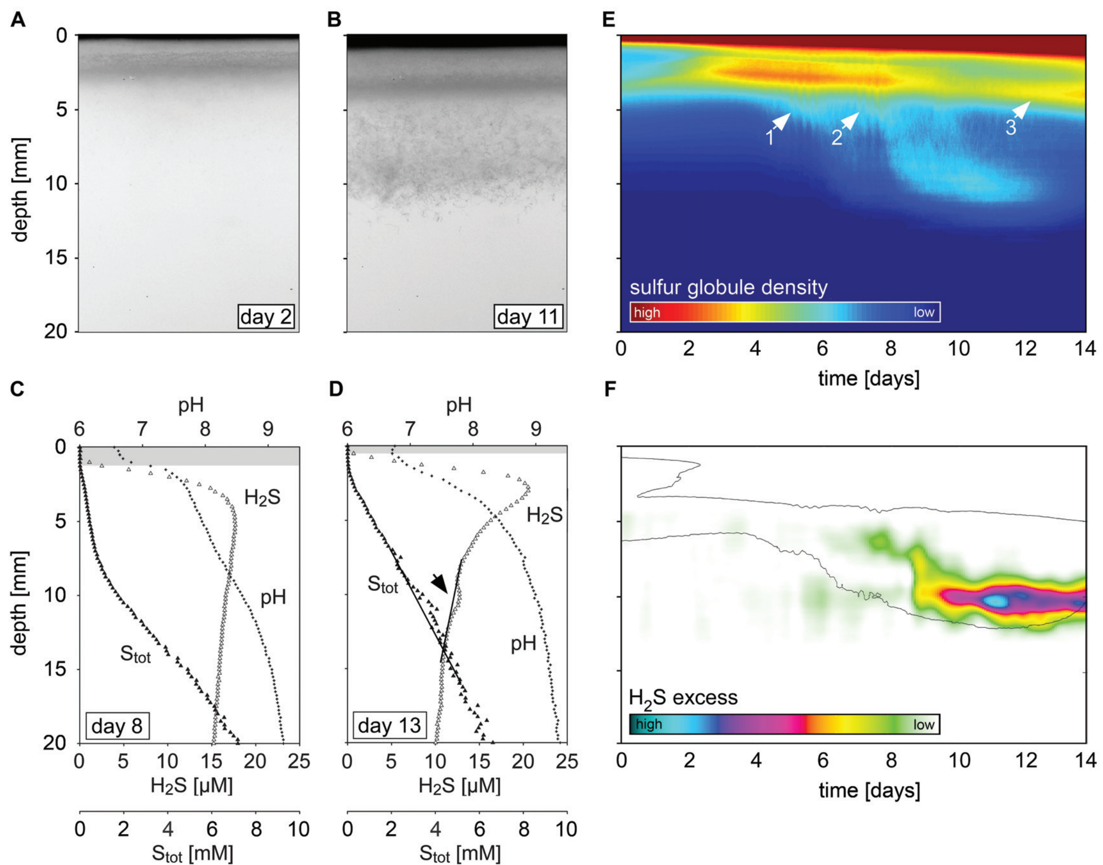

Figure 3.2 | Relationship between the migration of *Beggiatoa* sp. 35Flor filaments and the dynamics of O_2, pH, H_2S, and S_{tot} (total sulfide) in gradient cultures with high sulfide flux. **(A+B)** Images of culture tubes showing the filament distribution after 2 and 11 days. **(C+D)** Examples of 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.

increasing sulfide flux the amount of internal sulfur strongly increased (compare **Figures 3.3 A,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 3.3 B,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 3.3 D,F**).

The proportion of damaged filaments (**Figure 3.3 C**) from the mat at the oxygen-sulfide interface increased with sulfide flux. In cultures growing for one 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 two 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.

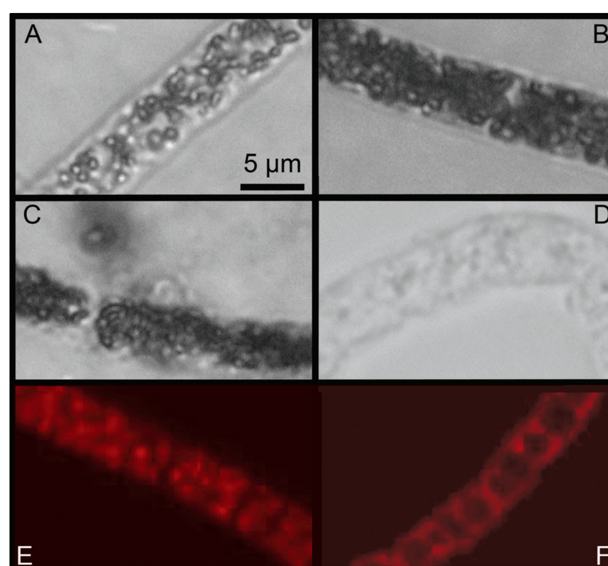

Figure 3.3 | Bright-field and fluorescence micrographs illustrating the typical appearance of *Beggiatoa* sp. 35Flor filaments cultivated under different conditions. (A+B) Filaments from the mat at the oxygen-sulfide interface of 6-days-old cultures growing under low **(A)** and high **(B)** sulfide 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.

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 ($2\,H_2S + O_2 \longrightarrow 2\,S^0 + 2\,H_2O$) further to sulfate ($2\,S^0 + 3\,O_2 + 2\,H_2O \longrightarrow 2\,SO_4^{2-} + 4\,H^+$). 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 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 upwards into the oxic section of the gradient system. Instead, they migrated downwards 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 $[C_4O_2H_6]_n + n \cdot 9\,S^0 + n \cdot 6\,H_2O \longrightarrow n \cdot 4\,CO_2 + n \cdot 9\,H_2S$, 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 ($2\,S + 3\,O_2 + 2\,H_2O \longrightarrow 2\,SO_4^{2-} + 4\,H^+$) or nitrate ($5\,S + 6\,NO_3^- + 2\,H_2O \longrightarrow 5\,SO_4^{2-} + 3\,N_2 + 4\,H^+$ or $4\,S + 3\,NO_3^- + 7\,H_2O \longrightarrow 4\,SO_4^{2-} + 3\,NH_4^+ + 2\,H^+$). Likewise, sulfur disproportionation would produce sulfuric acid ($4\,S + 4\,H_2O \longrightarrow 3\,H_2S + SO_4^{2-} + 2\,H^+$). 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.

Table 3.2 | Diffusive sulfide fluxes in natural *Beggiatoa* spp. mats. 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.

Sediment from	Measured	Flux [mmol m^{-2} d^{-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)

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

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 3.1**) were well within the range of fluxes previously measured in different natural *Beggiatoa* mats (**Table 3.2**), 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 3.4**). However, the conditions at which 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 towards high sulfide fluxes.

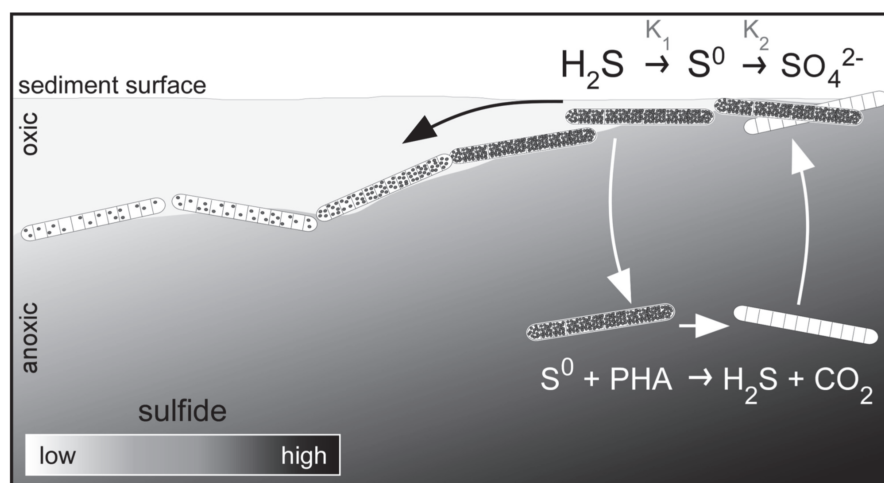

Figure 3.4 | Proposed function of sulfur reduction as a survival strategy of *Beggiatoa* spp. under high sulfide fluxes. In locations with high sulfide fluxes (right side) filaments become excessively filled with sulfur (black dots inside the filaments), because the oxidation rates of sulfide to sulfur (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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Supplemental material

The Movie S3.1 for this article can be found online at http://www.frontiersin.org/Microbial_Physiology_and_Metabolism/10.3389/fmicb.2011.00276/abstract.

Movie S3.1 | 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.

Chapter 4

Hydrogen oxidation by members of the family Beggiatoaceae

This chapter reports on the oxidation of molecular hydrogen by members of the family *Beggiatoaceae*. We combined physiological and genomic approaches to study different aspects of hydrogen metabolism in this family. Physiological experiments showed that a chemolithoautotrophic strain of the family oxidized H_2 at high rates and under different incubation conditions, illustrating that this electron donor can play an important role in the metabolism of *Beggiatoaceae* (**Section 4.1** on page 147). Genes encoding hydrogenases—enzymes catalyzing redox reactions involving the H^+/H_2 couple—were identified in strains throughout the entire family, indicating that H_2 oxidation may indeed be widespread within the *Beggiatoaceae* (**Section 4.2** on page 179). We discuss our results with respect to how hydrogen oxidation could add to the ecological plasticity of the *Beggiatoaceae*, how the different types of hydrogenases we identified could function in the metabolic context, and point out environmental settings, in which members of this family may exploit molecular hydrogen as a source of energy.

4.1 Oxidation of molecular hydrogen by a chemolithoautotrophic Beggiatoa strain

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Manuscript submitted for publication

Contributions:

The concept of this study was developed by me and H. N. Schulz-Vogt. I planned, performed and analyzed all experiments. The manuscript was written by me, including comments from H. N. Schulz-Vogt.

Acknowledgements:

We thank Hans Røy and Rita Dunker for sharing the design of the gas incubation tubes as well as Martina Meyer for technical assistance. Mark Mußmann is thanked for comments on the manuscript and Dirk de Beer is acknowledged for consultation on microsensor measurements. The study was supported by the Max Planck Society and the German National Academic Foundation.

Abstract

A chemolithoautotrophic strain of the family *Beggiatoaceae*, *Beggiatoa* sp. 35Flor, was found to oxidize molecular hydrogen under microoxic and anoxic conditions when grown in oxygen-sulfide gradient medium with a diffusional hydrogen gradient. Microsensor profiles and rate measurements suggested that the strain oxidized hydrogen aerobically in the presence of oxygen, while hydrogen consumption under anoxic conditions was presumably driven by sulfur respiration. *Beggiatoa* sp. 35Flor reached significantly higher biomasses in hydrogen-supplemented oxygen-sulfide gradient media, but hydrogen did not support growth of the strain in the absence of reduced sulfur compounds. Nevertheless, hydrogen oxidation can provide *Beggiatoa* sp. 35Flor with energy for maintenance and assimilatory purposes and support the disposal of internally stored sulfur to prevent physical damage resulting from excessive accumulation. Our knowledge about the exposure of natural populations of *Beggiatoaceae* to hydrogen is very limited, but significant amounts of hydrogen may indeed be available in several of their typical habitats such as photosynthetic microbial mats, submarine sites of hydrothermal fluid flow and terrestrial sulfur springs.

Introduction

Members of the family *Beggiatoaceae* are colorless sulfur bacteria known to oxidize reduced sulfur compounds and organic substrates for chemolithoautotrophic, chemoorganoheterotrophic, and mixotrophic growth (Teske and Nelson, 2006). While the use of various organic substances, such as mono- and dicarboxylic acids, sugars, amino acids, and alcohols, has been studied repeatedly in different strains of the family (e.g. Scotten and Stokes, 1962; Pringsheim, 1964; Burton and Morita, 1964; Nelson and Castenholz, 1981a; Jewell et al., 2008), inorganic electron donors other than reduced sulfur compounds were never reported to support growth. A first hint at the oxidation of molecular hydrogen by a member of the family *Beggiatoaceae* was the hydrogen-stimulated reduction of stored elemental sulfur in a microaerophilic *Beggiatoa* strain that was exposed to short-term anoxic conditions (Schmidt et al., 1987). Hydrogen-supported growth or hydrogen oxidation has been reported for various other well-known sulfur oxidizers such as members of the families *Chromatiaceae* (Imhoff, 2006), *Acidithiobacillaceae* (Drobner et al., 1990; Hallberg and Lindström, 1994), *Aquificaceae* (Bonjour and Aragno, 1986; Kawasumi et al., 1984; Huber et al., 1992b), *Sulfolobaceae* (Huber et al., 1992a), the genus *Sulfurimonas* (Takai et al., 2006; Grote et al., 2012), the SUP05 clade (Anantharaman et al., 2013), and endosymbionts of mussels (Petersen et al., 2011). This prevalence of hydrogen consumption in sulfur oxidizers suggests that the ability to consume H_2 could in fact be a common metabolic trait of this group and as thus also realized in members of the family *Beggiatoaceae*.

Substantial amounts of molecular hydrogen are produced and consumed in many microbial ecosystems, so that H_2 is considered to be an important electron transfer agent in both, oxic and anoxic environments (Schwartz and Friedrich, 2006). Nevertheless, *in situ* studies on hydrogen cycling and availability are difficult, owing to its extraordinarily high diffusivity, usually low concentrations, and high turnover rates (Hoehler et al., 1998). Steep biogeochemical gradients, which are typical for habitats of *Beggiatoaceae*, pose an additional problem because these necessitate a sampling resolution on the micrometer scale for meaningful conclusions. A microsensor for hydrogen is available for more than two decades (Witty,

1991), but has the critical disadvantage of being sensitive to sulfide (Revsbech, 2005). This disqualifies the sensor from many *in situ* applications, in particular from measurements in habitats of sulfur bacteria where the concentrations of sulfide are usually considerably higher than those of hydrogen. Accordingly, there is little information about the environmental exposure of *Beggiatoaceae* populations to hydrogen and the potential importance of hydrogen oxidation for members of the family *in situ*.

In the present study, we investigated the consumption of molecular hydrogen in cultures of a chemolithoautotrophic *Beggiatoa* strain using microsensors. Culture-based experiments allowed us to adjust the concentrations of hydrogen and sulfide to levels at which reliable measurements with the hydrogen microsensor are possible. We discuss our results with respect to how hydrogen oxidation could contribute to the ecophysiological plasticity of the strain, and point out environmental settings in which members of the family *Beggiatoaceae* may be able to use hydrogen as an electron donor.

Experimental Procedures

Organisms and cultivation

All experiments were conducted with the marine, chemolithoautotrophic strain *Beggiatoa* sp. 35Flor, which is maintained in a defined co-culture with *Pseudovibrio* sp. FO-BEG1, a heterotrophic and metabolically versatile bacterium (Bondarev et al., 2013). The co-culture was grown in a medium with opposed oxygen and sulfide gradients as described previously (Nelson et al., 1982; Schwedt et al., 2012). However, the concentration of $NiCl_2$ in top and bottom agar was increased to 7 μM to provide a sufficient amount of nickel for the synthesis of the [NiFe]-hydrogenase cofactor. Bottom agar sulfide concentrations were adjusted to 6 mM (low sulfide flux) or 16 mM (high sulfide flux), depending on the experiment. If a source of fixed nitrogen was required, 200 μM NH_4Cl were added to the top agar.

The setup for cultivation in presence of a diffusional hydrogen gradient was as follows (**Figure 4.1**): a tube with a conical ground cone (NS 29/32; 26 × 130 mm; 22 mm inner diameter; all glassware from Lenz Laborglas GmbH & Co. KG, Wertheim, Germany) was closed towards the cone with a 20 mm high plug of silicone (RTV-2 silicone, 13 ShA; Silikonfabrik.de, Ahrensburg, Germany) and loosely capped on top with a lid of thick aluminum foil. The sterilized tube was placed on the central socket of a 100-mL three-neck flask, while the screwthread adapters on the side necks (NS 14/23) were closed with butyl stoppers and apertured caps. All joints were greased with Baysilone paste (medium viscosity, GE Bayer Silicones GmbH & Co. KG, Leverkusen, Germany) and fixed in place with steel clips. Bottom and top agar layers of 4 and 17 mL, respectively, were poured consecutively on top of the silicone plug. The gas reservoir was flushed with hydrogen or nitrogen gas for 30 minutes immediately after pouring of the top agar and was subsequently refreshed every 3–4 days. A low hydrogen flux was achieved by replacing 12 mL from a nitrogen-filled gas reservoir with hydrogen. Gradients were allowed to establish for one day prior to inoculation with 300 μL filament suspension prepared from mats of 9–16 days-old pre-cultures (Schwedt et al., 2012). The cultures were incubated at room temperature.

Microsensor measurements

Microsensors were purchased from Unisense A/S (Aarhus, Denmark) and calibrated directly before and after the measurements as described by Schwedt et al. (2012). Concentration profiles of total sulfide (H_2S + HS^- + $S_2^=$) were calculated from the corresponding H_2S and pH profiles as described previously (Kühl et al., 1998; Schwedt et al., 2012). The hydrogen sensor was calibrated in artificial seawater by stepwise addition of a hydrogen-saturated stock solution, the concentration of which was calculated according to Gordon et al. (1977). Measured hydrogen profiles were corrected for the H_2S background recorded by the cross-reactive H_2 sensor. This background was estimated from profiles measured in hydrogen-unsupplemented, parallel cultures. In case of cultures with a low sulfide flux, the H_2 sensor was H_2S-calibrated using the H_2S and apparent H_2 concen-

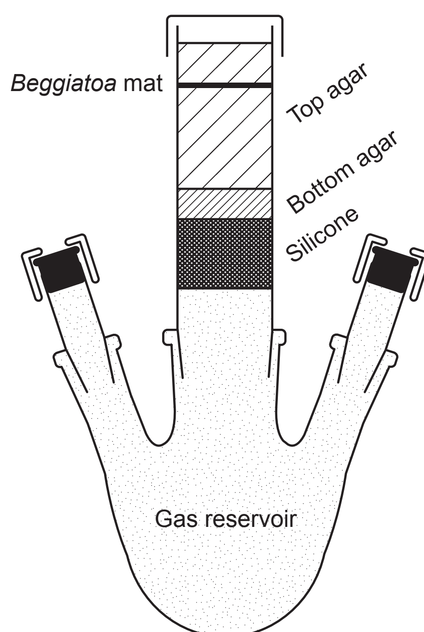

Figure 4.1 | Setup for the incubation of *Beggiatoa* sp. 35Flor in the presence of diffusional gas gradients.

trations measured at the same depths in the very same hydrogen-unsupplemented cultures. The so-determined H_2S background accounted for ≤ 12% of the recorded hydrogen signal in all hydrogen-supplemented cultures and was in most cases even ≤ 5%. In cultures with a high sulfide flux, the congruent shape of H_2S profiles from hydrogen-supplemented and -unsupplemented cultures allowed for a simple subtraction of the average sulfide background profile from the measured H_2 profiles. It has to be noted that both procedures could overestimate the contribution of the background, because genuine H_2 signals present in hydrogen-unsupplemented cultures would wrongly be ascribed to H_2S. It is indeed possible that hydrogen-unsupplemented cultures contained H_2 because *Beggiatoa* sp. 35Flor fixes nitrogen under standard cultivation conditions (A.-T. Henze, unpublished) and this process is associated with the evolution of H_2 (reviewed by Burgess and Lowe, 1996). However, we assume that hydrogen concentrations were not significant in hydrogen-unsupplemented cultures due to the slow growth and fast hydrogen oxidation rates of *Beggiatoa* sp. 35Flor. Correspondingly, there was no notable difference in H_2 profiles measured in hydrogen-supplemented nitrogen-fixing and non-nitrogen-fixing *Beggiatoa* sp. 35Flor cultures (**Figure S4.8**).

Protein determination

Total cell protein was measured as a proxy for *Beggiatoa* biomass as described previously (Nelson et al., 1982; Nelson and Jannasch, 1983; Nelson et al., 1986; Hagen and Nelson, 1997). The semi-liquid top agar of a culture was sampled by pouring the entire volume into a 50-mL polypropylene tube; residual agar, which adhered to the walls of the culture tube, was transferred by rinsing with 10 mL sterile artificial seawater. Centrifugation in a swing-out rotor at 5000 × g (20 min) yielded a dense agar pellet of about 8 mL, in which the entire biomass was concentrated. The density of accompanying *Pseudovibrio* sp. FO-BEG1 cells was determined in triplicate using a subsample of the thoroughly vortexed pellet. The remaining agar was hydrolyzed and the protein was precipitated through incubation in hot trichloroacetic acid (Nelson et al., 1982) followed by cooling at 4°C over night. Four 2-mL subsamples were taken from each sample and centrifuged at 20,817 × g (10 min, 4°C). The supernatant was removed and each pellet was dissolved and incubated in 0.7 mL of a 0.1 M NaOH (20 min, 55°C). The colorimetric protein assay (Bradford, 1976) was composed of 0.5 mL sample or standard in 0.1 M NaOH, 0.5 mL of a 0.15 M HCl and 0.35 mL dye reagent concentrate (Bio-Rad Laboratories, Inc., Hercules, USA). Bovine serum albumin (2–10 μg mL^{-1}; Fluka, Steinheim, Germany) served as a standard. All measured concentrations were corrected for blanks (extractions from sterile top agar) and the contribution of *Pseudovibrio* sp. FO-BEG1 protein, considering the respective *Pseudovibrio* cell densities. In order to determine the average protein content of *Pseudovibrio* sp. FO-BEG1 cells, known amounts of axenically cultivated and washed cells were added to a sterile mix of top agar and artificial sea water and the protein was extracted as described above.

Photography

Photographs of culture tubes were taken with a Sony XCD-X710 digital camera (Sony, Tokyo, Japan), controlled by the image acquisition software IC Capture (The Imaging Source Europe GmbH, Bremen, Germany). Due to better visibility in print, negatives are shown.

Genomic analyses

The software suite JCoast version 1.7 (Richter et al., 2008) was used to search the fully sequenced genome of *Pseudovibrio* sp. FO-BEG1 (Bondarev et al., 2013) for hydrogenase-encoding genes and the draft genome of *Beggiatoa* sp. 35Flor for genes encoding enzymes involved in the assimilatory sulfur metabolism. The draft genome *Beggiatoa* sp. 35Flor was recently sequenced (M. Winkel *et al.*, in preparation) and annotated as previously described (Bondarev et al., 2013).

Results

Migration behavior of Beggiatoa sp. 35Flor filaments grown in hydrogen-supplemented and -unsupplemented media

Beggiatoa sp. 35Flor filaments cultivated in agar-stabilized gradient media grew in dense, opaque mats at the transition from oxic to sulfidic conditions. Irrespective of supplementing with hydrogen, these mats migrated downwards in the course of a four-week incubation period in response to the changing gradients of oxygen and sulfide. However, this movement was considerably less pronounced in the presence of a diffusional hydrogen gradient (**Figure 4.2**). While mats of hydrogen-supplemented cultures had not left the upper third of the top agar even after four weeks of growth, mats of hydrogen-unsupplemented cultures had already reached the bottom agar layer and filaments were dead (**Figure 4.2 D**).

Hydrogen oxidation under different conditions

Oxygen, sulfide, and hydrogen were depleted within mats of hydrogen-supplemented *Beggiatoa* sp. 35Flor cultures during the first three weeks of incubation (**Figure 4.3 A–C**). After four weeks, hydrogen was still oxidized, but the consumption was not complete and some hydrogen diffused through the mat (**Figure 4.3 D**). The zones of hydrogen and oxygen consumption consistently overlapped at all times and microsensor profiles showed no evidence of hydrogen oxidation in the anoxic section of the mat. Presence of ammonium in a concentra-

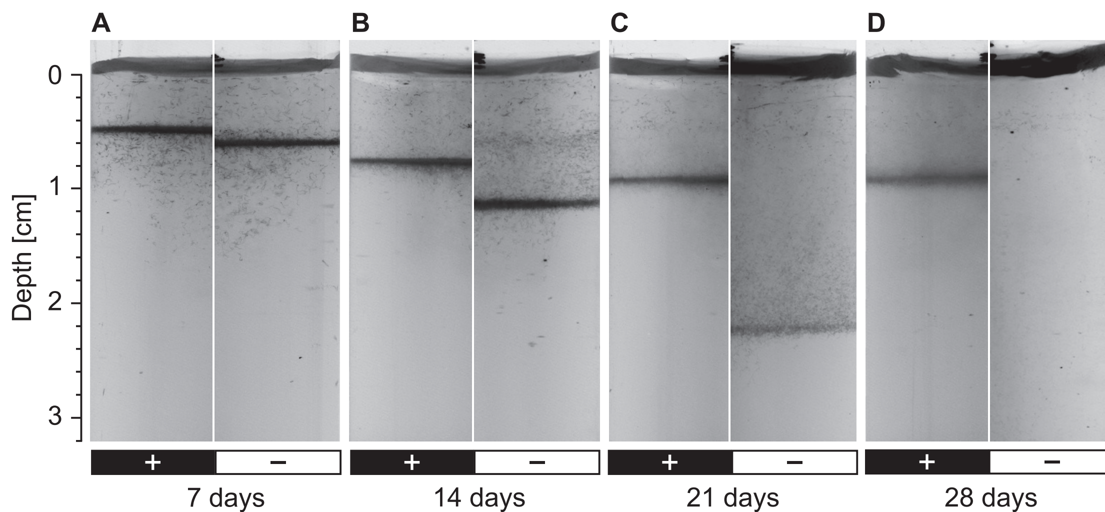

Figure 4.2 | Position and appearance of *Beggiatoa* sp. 35Flor mats incubated in hydrogen-supplemented and -unsupplemented oxygen-sulfide gradient media over four weeks. (**A**–**D**) Culture tubes were photographed after 7, 14, 21, and 28 days of growth in presence (left panels; +) and absence (right panels; –) of a diffusional hydrogen gradient. The scale bar on the left indicates the depth below the air-agar interface.

tion previously shown to inhibit nitrogen fixation in *Beggiatoa* sp. 35Flor (A.-T. Henze, unpublished) did not affect hydrogen consumption in mats at the oxygen-sulfide interface (**Figure S4.8**). When *Beggiatoa* sp. 35Flor cultures were grown in presence of a high sulfide flux, a subpopulation of filaments migrated from the oxygen-sulfide interface into the anoxic section of the gradient medium after about one week of incubation. These filaments aggregated loosely in a horizon ca. 2–4 mm below the oxygen-sulfide interface, in which upward-diffusing hydrogen was consumed (**Figure 4.4**). Nitrate was not available as an electron acceptor for hydrogen oxidation in the medium nor was nitrate stored by the filaments (Schwedt et al., 2012).

Beggiatoa sp. 35Flor grows in a defined co-culture with *Pseudovibrio* sp. FO-BEG1 but several lines of evidence suggest that the *Pseudovibrio* strain did not contribute to the consumption of H_2. Hydrogen was not oxidized in axenic gradient cultures of *Pseudovibrio* sp. FO-BEG1, while being consumed efficiently in *Beggiatoa* sp. 35Flor/*Pseudovibrio* sp. FO-BEG1 co-cultures (**Figure S4.9 B**), in which the average *Pseudovibrio* cell density was only 13% higher (p = 0.12;

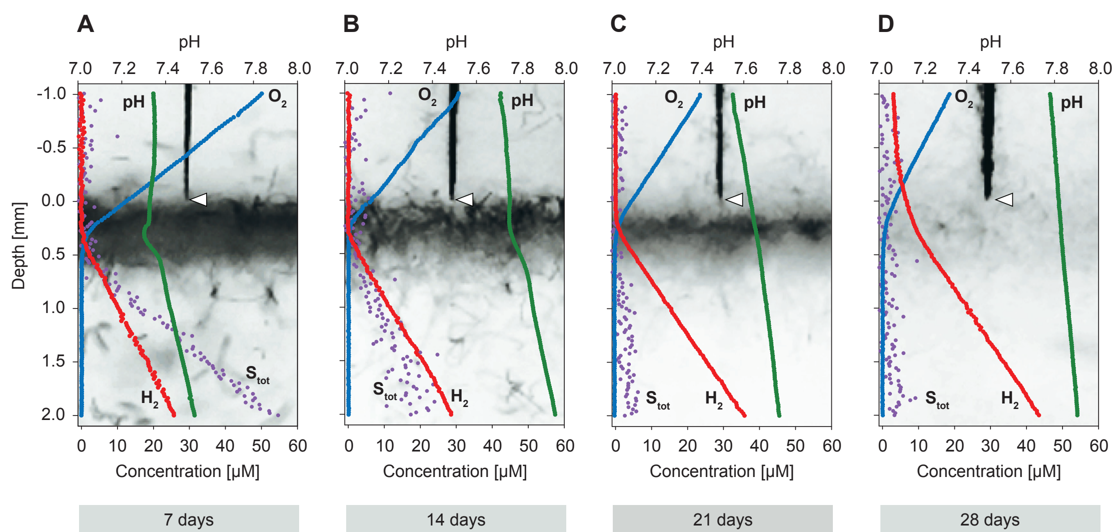

Figure 4.3 | Development of chemical gradients in hydrogen-supplemented *Beggiatoa* sp. 35Flor cultures over four weeks of incubation. (**A–D**) Microsensor profiles were recorded 7, 14, 21, and 28 days after inoculation. Profiles of oxygen (blue), hydrogen (red), pH (green), and total sulfide (purple) were determined at a resolution of 20 μm. Photographs of the profiled mat sections are shown in the background. Mats appeared almost transparent by day 28, as 70–80% of the *Beggiatoa* sp. 35Flor filaments were devoid of internal sulfur globules and the remainder contained only a low number (counts in three parallel cultures; 126–134 inspected filaments per culture). For each set of profiles, the tip of the microsensor (◁) indicates the arbitrarily defined position zero at the mat surface to which all sensors were aligned. Note that the y-axis gives the depth relative to this point and does not relate to the depth below the air-agar interface.

Figure S4.9 A). Correspondingly, hydrogen oxidation was never observed in liquid cultures of *Pseudovibrio* sp. FO-BEG1, irrespective of the incubation conditions tested (V. Bondarev, unpublished). In addition, we could not identify any hydrogenase gene in the fully sequenced genome of *Pseudovibrio* sp. FO-BEG1 (Bondarev et al., 2013) nor could we retrieve such a gene from genomic DNA of *Pseudovibrio* sp. FO-BEG1 in hydrogenase-specific PCRs (Kreutzmann and Mußmann, 2013).

Consumption rates of oxygen, sulfide, and hydrogen

Average consumption rates of oxygen, total sulfide (H_2S + HS^- + S^{2-}), and hydrogen were determined in hydrogen-supplemented and -unsupplemented

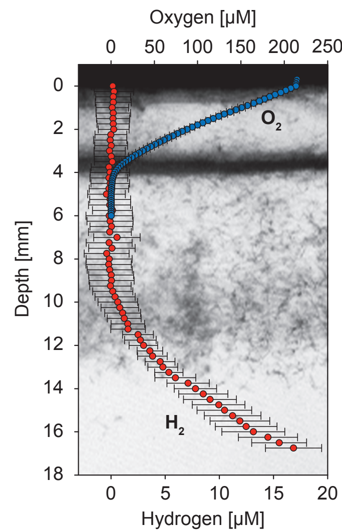

Figure 4.4 | Gradients of oxygen and hydrogen in hydrogen-supplemented *Beggiatoa* sp. 35Flor cultures with a high sulfide flux. Average oxygen (blue) and hydrogen (red) profiles (± standard deviations; $n = 3$) were measured 9 days after inoculation with resolutions of 100 μm and 250 μm, respectively. The diffusional hydrogen gradient was lowered and resulted from the addition of only 12 mL H_2 to a nitrogen-filled gas reservoir. A photograph of the profiled mat section is shown in the background. Due to a dissimilar distribution of filaments in the anoxic sections of hydrogen-supplemented and -unsupplemented media, we could not correct the sulfide profiles from hydrogen-supplemented cultures for carbon-fueled sulfide production. Hence, the setup was not suited to reliably measure the sulfide production likely associated with hydrogen uptake under anoxic conditions.

Beggiatoa sp. 35Flor cultures over four weeks (**Figure 4.5**). The average oxygen consumption rate in hydrogen-supplemented cultures was significantly higher at all times ($p \leq 1.5 \times 10^{-5}$) and decreased less pronouncedly in the course of the incubation (**Figure 4.5 A**). Measurements conducted within the first three weeks in fact suggested a leveling off at about 3–4 × 10^{-3} nmol O_2 cm^{-2} s^{-1}, but a pronounced drop to circa 2.5 × 10^{-3} nmol O_2 cm^{-2} s^{-1} occurred between week three and four. The average consumption rate of total sulfide in hydrogen-supplemented cultures was always similar to or slightly higher than in hydrogen-unsupplemented cultures (**Figure 4.5 B**). The average hydrogen consumption rate increased slightly within the first three weeks in hydrogen-supplemented cultures, but dropped markedly by week four (**Figure 4.5 C**), corresponding to the diffusion of hydrogen through the mat (**Figure 4.3 D**). The average contribution of hydrogen oxidation to the consumption of oxygen increased gradually from 38.5% after one week to 104.6%

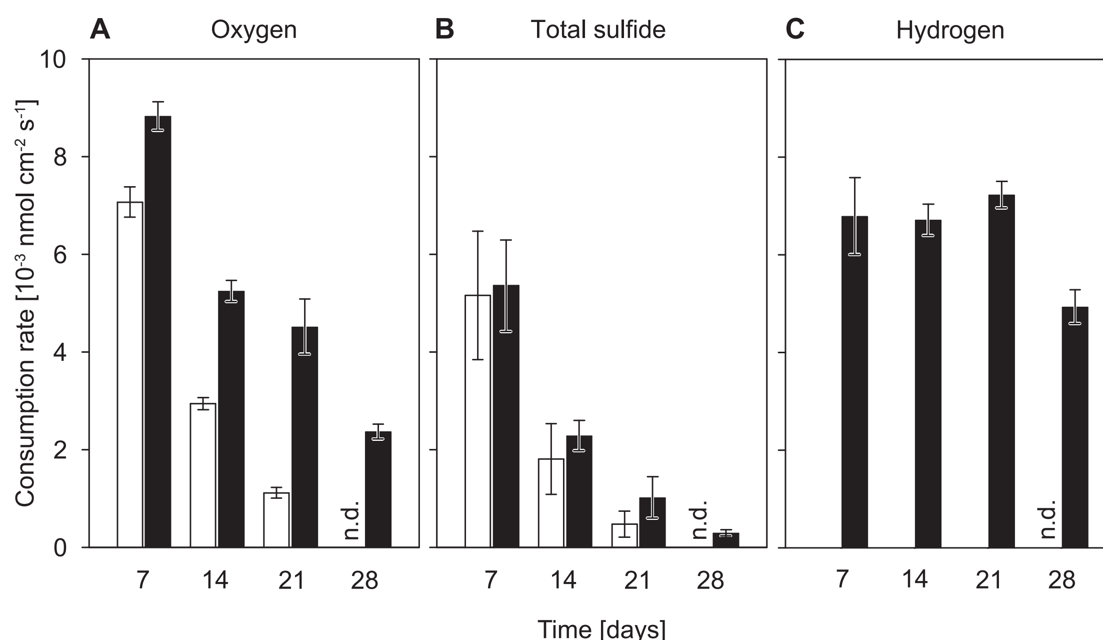

Figure 4.5 | Average consumption rates of oxygen, total sulfide, and hydrogen in *Beggiatoa* sp. 35Flor cultures over four weeks of incubation. (**A–C**) Average consumption rates were determined weekly for cultures grown in presence (■) and and absence (□) of a diffusional hydrogen gradient. Fluxes of oxygen, total sulfide, and hydrogen into *Beggiatoa* sp. 35Flor mats were calculated from profiles, which were measured with a vertical resolution of 100–250 μm and covered a distance of ca. 11 mm around the mat. Values for 7, 14, and 21 days represent averages of consumption rates (± standard deviation) measured in six replicate cultures of two independent cultivations; values for 28 days are averages of consumption rates (± standard deviation) measured in triplicate cultures. Since mats were absent from 28 days-old, hydrogen-unsupplemented cultures consumption rates cannot be given.

after four weeks (**Figure 4.6 A**). Thus, the oxygen and hydrogen consumption rates reached the molar ratio of the knallgas reaction ($2\,H_2 + O_2 \longrightarrow 2\,H_2O$) when the pools of external sulfide and internal sulfur were essentially exhausted.

Budget calculations (**Figure 4.6 A**) showed that the absolute contribution of sulfide oxidation ($2\,H_2S + O_2 \longrightarrow 2\,S^0 + 2\,H_2O$) to the consumption of oxygen was similar in both types of cultures. Hydrogen oxidation contributed to an overall higher respiration rate, but the increase in oxygen consumption was lower than expected from the stoichiometry of the knallgas reaction. Because hydrogen and sulfide were evidentially oxidized, less oxygen was left in the budget for the oxidation of internally stored sulfur ($2\,S^0 + 3\,O_2 + 2\,H_2O \longrightarrow 2\,SO_4^{2-} + 4\,H^+$) in hydrogen-

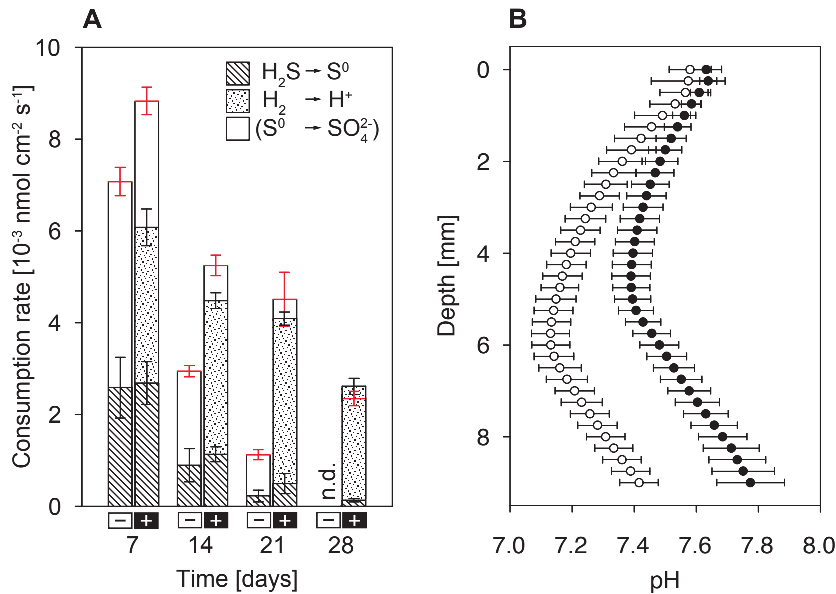

Figure 4.6 | Influence of hydrogen consumption on the oxidation rates of sulfide and sulfur in *Beggiatoa* sp. 35Flor cultures. (**A**) Total oxygen consumption rates in hydrogen-supplemented (+) and -unsupplemented (−) cultures are shown in red (see **Figure 4.5** for details on the measurements). Hatched areas indicate the contribution of sulfide oxidation ($2\,H_2S + O_2 \longrightarrow 2\,S^0 + 2\,H_2O$) to the observed oxygen consumption; dotted areas represent the contribution of hydrogen oxidation ($2\,H_2 + O_2 \longrightarrow 2\,H_2O$). The oxygen consumption, which cannot be accounted for by the above reactions is assumed to result from sulfur oxidation ($2\,S^0 + 3\,O_2 + 2\,H_2O \longrightarrow 2\,SO_4^{2-} + 4\,H^+$). Electron flow into CO_2 fixation was not considered. (**B**)Average pH profiles (± standard deviation; $n = 6$) measured in hydrogen-supplemented (●) and -unsupplemented (○) cultures after 7 days of incubation. Mats in hydrogen-supplemented cultures were situated 5.2–6.2 mm below the air-agar interface; mats in hydrogen-unsupplemented cultures resided between 6.6–7.5 mm depth.

supplemented cultures. Hydrogen-suppelmented and -unsupplemented cultures showed an acidification of the medium in the region of the mat, corresponding to the production of sulfuric acid (**Figure 4.6 B**). However, this acidification was less pronounced in hydrogen-supplemented cultures.

Influence of hydrogen oxidation on the growth of Beggiatoa sp. 35Flor

Hydrogen-supplemented cultures grew faster and contained at least double the amount of *Beggiatoa* protein as hydrogen-unsupplemented cultures did at all time

points (**Figure 4.7**). In addition, hydrogen-supplemented cultures maintained growth for about three weeks, while hydrogen-unsupplemented cultures reached the stationary phase after already two weeks.

Genes encoding enzymes involved in sulfate assimilation

In search for a reason as to why hydrogen is not able to support growth of *Beggiatoa* sp. 35Flor in the absence of reduced sulfur compounds, we hypothesized that the strain might not be capable of assimilating sulfate. However, most of the enzymes involved in assimilatory sulfate reduction were identified in the *Beggiatoa* sp. 35Flor draft genome, such as an adenylylsulfate kinase (*cysC*; FLOR_01349), a phosphoadenylyl-sulfate reductase (*cysH*; FLOR_03186), and the beta subunit of an assimilatory sulfite reductase (*cysI*; FLOR_03184). Genes encoding the subunits of an assimilatory sulfate adenylyltransferase (*cysND*) were not found, but a gene encoding a Sat-type sulfate adenylyltransferase (FLOR_01554) was identified.

Discussion

Beggiatoa sp. 35Flor oxidizes hydrogen under microoxic conditions

We showed that a chemolithoautotrophic strain of the family *Beggiatoaceae*, *Beggiatoa* sp. 35Flor, consumed molecular hydrogen under microoxic conditions. Microsensor profiles and rate measurements suggested that *Beggiatoa* sp. 35Flor oxidized hydrogen aerobically, a reaction that could have been catalyzed by its Hup-type hydrogenase (Kreutzmann and Mußmann, 2013). With 36–45 nmol H_2 per cm^{-3} mat volume and hour or 5–14 nmol H_2 per μg protein and hour (weeks 1–3), the hydrogen oxidation rates were substantial and in fact exceeded the sulfide oxidation rates at all times. Moreover, the measured hydrogen oxidation rates did most likely not even represent the possible maximum, as hydrogen oxidation was diffusion-limited.

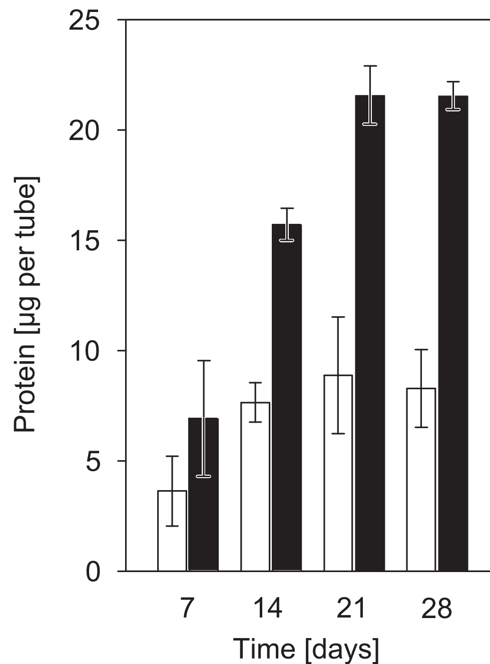

Figure 4.7 | Growth of *Beggiatoa* sp. 35Flor in hydrogen-supplemented and -unsupplemented cultures. *Beggiatoa* sp. 35Flor protein was measured as a proxy for filament biomass. Hydrogen-supplemented (■) and -unsupplemented (□) oxygen-sulfide gradient media were prepared in parallel and inoculated with the very same homogeneous filament suspension. The top agar from triplicate cultures was sampled weekly to assay the total cellular protein and to determine the density of accompanying *Pseudovibrio* sp. FO-BEG1 cells. Measured total protein amounts were subsequently corrected for the contribution of *Pseudovibrio* sp. FO-BEG1 protein, which accounted for 20–50% of the measured values.

Hydrogen is a valuable electron donor for *Beggiatoa* sp. 35Flor, as illustrated by the significantly higher growth rates of hydrogen-supplemented cultures (**Figure 4.7**). Correspondingly, hydrogen uptake in this strain is regulated to scavenge all available pools of H_2. Similar to other members of the family *Beggiatoaceae* (Nelson et al., 1982; Polman and Larkin, 1988), *Beggiatoa* sp. 35Flor is capable of nitrogen fixation (A.-T. Henze, unpublished). Because this process releases hydrogen as a by-product (Burgess and Lowe, 1996), many diazotrophs express uptake-hydrogenases for H_2 recycling under nitrogen-fixing conditions (Brito et al., 1997; Axelsson et al., 1999; Elsen et al., 2000; Happe et al., 2000). However, hydrogen oxidation under nitrogenase repression (**Figure S4.8**) illustrated that the hydrogenases of *Beggiatoa* sp. 35Flor are not merely regulated to recycle internally produced H_2 but instead allow to exploit H_2 as a genuine electron donor.

The influence of hydrogen oxidation on the sulfur metabolism of *Beggiatoa* sp. 35Flor further points to a very efficient and purposeful use of the different electron donors in an environment, in which sulfide toxicity, competition for resources, and fluctuating supplies with oxidants and reductants are the major challenges. Budget calculations indicated that *Beggiatoa* sp. 35Flor populations oxidized less sulfur to sulfuric acid under low sulfide flux conditions when hydrogen was available (**Figure 4.6 A**). Correspondingly, the acidification of the medium was less pronounced in hydrogen-supplemented cultures (**Figure 4.6 B**). However, it has to be noted that this pH difference cannot alone be attributed to a lower production of sulfuric acid, as higher CO_2 fixation rates in hydrogen-supplemented cultures will likewise result in a higher pH. In contrast to sulfur, the oxidation rate of sulfide was not influenced by hydrogen. Thus, sulfide and hydrogen, which cannot be stored, appear to be oxidized immediately when available, while sulfur is kept in reserve when the current energy requirement can be met by using other electron donors.

Hydrogen oxidation clearly influenced the mat position, oxygen consumption and growth rates of *Beggiatoa* sp. 35Flor cultures. This is of particular importance for environmental studies, because it illustrates that measurements of oxygen and sulfide gradients alone do not necessarily suffice to gain a comprehensive picture of *Beggiatoaceae* metabolism. In contrast, the use of alternative electron donors such as hydrogen or electron acceptors such as nitrate (Sweerts et al., 1990; Mußmann et al., 2003; Sayama et al., 2005; Kamp et al., 2006; Hinck et al., 2007) can significantly influence both, biogeochemical gradients and the position of *Beggiatoaceae* populations with respect to these.

Hydrogen serves as an electron donor for sulfur respiration in Beggiatoa sp. 35Flor

Under anoxic conditions, the strains *Beggiatoa* sp. 35Flor, *Beggiatoa alba* B18LD and *Beggiatoa* sp. OH-75-2a reduce internally stored sulfur to sulfide with carbon reserve compounds or acetate serving as electron donors (Nelson and Castenholz,

1981b; Schmidt et al., 1987; Schwedt et al., 2012). This process has been suggested to serve two purposes, being energy generation under short-term anoxic conditions (Nelson and Castenholz, 1981b; Schmidt et al., 1987) and the disposal of internal sulfur, which accumulates excessively in the presence of high sulfide fluxes and can eventually cause bursting of filaments (Schwedt et al., 2012). Molecular hydrogen can serve as an alternative electron donor in this process, as shown by the increase of sulfide production in the presence of hydrogen (*Beggiatoa alba* B18LD; Schmidt et al., 1987) and hydrogen uptake under sulfur-reducing conditions (*Beggiatoa* sp. 35Flor, this study). Importantly, the use of hydrogen enables an uncoupling of sulfur respiration from the availability of carbon compounds. Thus, energy can be generated and sulfur can be disposed of even if carbon reserve compounds such as polyhydroxyalkanoates (Kowallik and Pringsheim, 1966; Güde et al., 1981; Strohl et al., 1981) or glycogen (Schulz and Schulz, 2005) are exhausted and oxidizable external carbon compounds are not accessible.

Metabolic pathways, which allow a more flexible handling of the internal sulfur pool, are particularly valuable for members of the family *Beggiatoaceae*, as sulfur plays a central role in their metabolism. Indeed, different lines of evidence suggest that hydrogen-fueled sulfur respiration is widespread within the family. Two *Beggiatoa* strains, *Beggiatoa* sp. 35Flor and *Beggiatoa alba* B18LD, which differ in both, their trophic modes (lithoautotrophy vs. organoheterotrophy) and their habitats (marine vs. freshwater), evidentially couple sulfur reduction to hydrogen oxidation. In addition, Hyn-type hydrogenases, which are thought to catalyze the reduction of S–S bonds with H_2 (Pandelia et al., 2012), are widespread among members of the family (Kreutzmann and Mußmann, 2013). Thus, hydrogen has the potential of increasing the ecophysiological plasticity of *Beggiatoa* sp. 35Flor and possibly other members of the family *Beggiatoaceae* in two ways, both of which are tightly coupled to the sulfur metabolism. In presence of a low sulfide flux and redox-positive electron acceptors such as oxygen, hydrogen can partially replace sulfur as an electron donor and thereby increase the amount of sulfur available for storage. Under high sulfide flux conditions and anoxia, hydrogen can, in contrast, support sulfur respiration and disposal in order to provide energy and prevent physical damage from excessive sulfur accumulation.

Hydrogen is used as an accessory electron donor

The presented results clearly show that *Beggiatoa* sp. 35Flor used hydrogen as an energy source for growth under microoxic conditions. As long as external sulfide and internally stored sulfur were available, hydrogen was consumed, and hydrogen-supplemented cultures grew significantly better than hydrogen-unsupplemented controls. However, as soon as the pools of sulfide and sulfur were exhausted, both, hydrogen oxidation and growth ceased (**Figures 4.3** and **4.7**). The inability of hydrogen to support growth of *Beggiatoa* sp. 35Flor as an exclusive electron donor is unexpected, given that H_2-uptake hydrogenases, such as those responsible for aerobic hydrogen oxidation, are thought to reduce the quinone pool (Vignais et al., 2001; Pandelia et al., 2012). Enzymes oxidizing sulfide and other reduced sulfur compounds appearing en route to sulfate are likewise thought to reduce quinones (sulfide:quinone oxidoreductase, adenosine-5'-phosphosulfate reductase, SoeABC-type sulfite-oxidizing enzyme) or *c*-type cytochromes (flavocytochrome *c*-sulfide dehydrogenase, SorAB-type sulfite dehydrogenase; Dahl et al., 2008a; Kappler, 2011; Dahl et al., 2013). In case of the reverse dissimilatory sulfite reductase, different models of electron transfer reactions are being discussed (Dahl et al., 2005, 2008b; Grein et al., 2010), so that it is not clear how electrons from the oxidation of sulfide to sulfite enter the electron transport chain. However, with a redox potential of $E_0' = -116$ mV (HSO_3^-/HS^-; Thauer et al., 1977) an entry above the level of quinones is unlikely. Accordingly, electrons from hydrogen will enter the electron transport chain on the same level or upstream of electrons from reduced sulfur compounds and should be able to support at least the same metabolic processes.

Possibly, *Beggiatoa* sp. 35Flor could not grow under sulfide- and sulfur-depleted conditions, because it lacks the ability to reduce sulfate for assimilatory purposes. Similar to other chemolithoautotrophic members of the family *Beggiatoaceae*, *Beggiatoa* sp. 35Flor is constantly exposed to reduced sulfur compounds when growing and hence may have lost the ability to assimilate sulfate. However, genes encoding most of the enzymes required for assimilatory sulfate reduction are present in the *Beggiatoa* sp. 35Flor draft genome. Only genes encoding the subunits of an as-

similatory sulfate adenylyltransferase (*cysND*) were not detected, but these may be located in the not yet sequenced part of the genome or could be functionally substituted by the encoded Sat-type sulfate adenylyltransferase. Hence, it appears likely that the ability to assimilate sulfate is retained in *Beggiatoa* sp. 35Flor.

A mechanism for hydrogen uptake, which would indeed require the presence of internally stored sulfur, is the oxidation of hydrogen by sulfur reduction ($H_2 + S^0 \longrightarrow H_2S$) followed by a re-oxidation of sulfide to sulfur with oxygen serving as an electron acceptor ($2\,H_2S + O_2 \longrightarrow 2\,S^0 + 2\,H_2O$). Such a two-step 'sulfur-catalyzed' hydrogen oxidation could in principle take place in *Beggiatoa* sp. 35Flor mats, being masked by a rapid re-oxidation of sulfide. Accordingly, both, the nature of the direct electron acceptor for hydrogen oxidation under microoxic conditions as well as the reason for discontinued growth in the absence of reduced sulfur compounds, remain unclear.

Environmental relevance of hydrogen oxidation for members of the family Beggiatoaceae

A variety of biotic and abiotic environmental processes are associated with the production of molecular hydrogen (Schwartz and Friedrich, 2006). Nevertheless, significant amounts of hydrogen are probably available to members of the family *Beggiatoaceae* only in certain environments. The hypersaline microbial mats of the Guerrero Negro evaporation lagoons (Baja California Sur, Mexico) are a prominent example of such environments. At the surface of these mats exceptionally high H_2 concentrations were measured (Hoehler et al., 2001) and filamentous *Beggiatoaceae* were regularly identified within the upper few millimeters. At daytime, these filaments are present in a layer directly below and partially overlapping with the uppermost horizon, which is dominated by cyanobacteria (e.g Jørgensen and Des Marais, 1986; Garcia-Pichel et al., 1994; Des Marais, 1995; Dillon et al., 2009). During the day, the cyanobacteria engage in photosynthesis, thereby producing large amounts of organic matter and simultaneously supplying the top few millimeters of the mat with oxygen (Canfield and Des Marais, 1993; Des Marais,

1995). At night, when the oxygen-sulfide interface moves upward and the mat surface becomes anoxic and sulfidic (Canfield and Des Marais, 1993; Des Marais, 1995; Dillon et al., 2009), this organic matter can fuel oxygen-sensitive and hydrogen-evolving processes such as fermentation and nitrogen fixation. Correspondingly, H_2 concentrations peaked at the surface of the Guerrero Negro mats and were highest in the dark, reflecting the location of cyanobacteria and the time of their highest diazotrophic and fermentative activities (Hoehler et al., 2001; Omoregie et al., 2004). It is difficult to estimate whether nitrogen fixation or fermentation is the main H_2-evolving reaction in the Guerrero Negro mats, but nitrogen fixation will prevail when H_2 concentrations exceed the low nanomolar range and inhibit fermentative processes thermodynamically (Schwartz and Friedrich, 2006). Following the upward movement of the oxygen-sulfide interface, the filamentous *Beggiatoaceae* of the Guerrero Negro lagoons are likewise present at the very top of the mat at night (Jørgensen and Des Marais, 1986; Garcia-Pichel et al., 1994) and thus are regularly exposed to high hydrogen concentrations or production rates. Extensive cyanobacterial mats resembling those of the Guerrero Negro lagoons, were present on earth for most of life's history, once dominating the biosphere (Des Marais, 1995, 2003; Jørgensen, 2001). Substantial genetic exchange between cyanobacteria and *Beggiatoaceae* strikingly evidences a historically frequent co-occurrence of both taxa on the molecular level (Mußmann et al., 2007; MacGregor et al., 2013), suggesting that hydrogen transfer from nitrogen-fixing and fermenting cyanobacteria to members of the family *Beggiatoaceae* could indeed be an ancient and once widespread process.

Filamentous *Beggiatoaceae* from the Guerrero Negro mats could not be analyzed for the presence of uptake hydrogenases, but Hyb- and Hyn-type hydrogenases were consistently identified in filaments originating from three comparable hypersaline mats (Kreutzmann and Mußmann, 2013). Since filaments from all of these sites are closely related, constituting the proposed genus '*Candidatus* Allobeggiatoa' (Hinck et al., 2011; Kreutzmann and Mußmann, 2013), it is reasonable to assume that filaments from the Guerrero Negro lagoons encode similar hydrogenases. Hyb- and Hyn-hydrogenases would serve the filaments well at nighttime, when hydrogen is most abundant (Hoehler et al., 2001) and the mat surface anoxic

and sulfidic (Dillon et al., 2009). Under these conditions, '*Ca.* Allobeggiatoa spp.' filaments could employ Hyb-hydrogenases for coupling hydrogen uptake to the dissimilatory reduction of nitrate (Kreutzmann and Mußmann, 2013), which they store in internal vacuoles (Hinck et al., 2007, 2011; Beutler et al., 2012). Concurrently, Hyn-type enzymes could be used for the hydrogen-driven reduction of elemental sulfur (Kreutzmann and Mußmann, 2013) to counteract its excessive internal accumulation due to high sulfide concentrations (Schwedt et al., 2012).

In contrast to photosynthetic mats, the oxygen-sulfide interface is usually well and permanently separated from the zone of hydrogen production in organic-rich sediments. Even though large quantities of hydrogen are produced by fermentative processes in deeper, anoxic layers, H_2 is rapidly and efficiently re-oxidized by the local community of hydrogenotrophic prokaryotes (Hoehler et al., 1998). Hence, *Beggiatoaceae*, which usually populate the oxygen-sulfide interface, are unlikely to experience high hydrogen concentrations or fluxes in such systems. Nevertheless, members of the family, which are residing in or traveling through fermenting sediment layers, could exploit hydrogen as an electron donor.

In addition, chemosynthetic ecosystems in the deep sea are sites at which hydrogen, specifically H_2 of geothermal origin, could potentially serve as a source of energy for *Beggiatoaceae*. Members of the family *Beggiatoaceae* are regularly encountered in the deep sea at sites of hydrothermal fluid flow (e.g. Jannasch et al., 1989; Kalanetra et al., 2005; de Beer et al., 2006; Girnth et al., 2011; Grünke et al., 2011, 2012), and hydrogen is extruded in several of such places (Welhan and Craig, 1979; Lilley et al., 1982; Petersen et al., 2011). In fact, H_2 of geothermal origin was suggested to be a key energy source in deep-sea water masses (Anantharaman et al., 2013) and has been shown to fuel CO_2 fixation in sulfide-oxidizing endosymbionts of deep sea mussles (Petersen et al., 2011). Yet, seep-dwelling populations of *Beggiatoaceae* have apparently never been tested for an exposure to or even a consumption of H_2. Similar to sub-marine sites of hydrothermal fluid flow, members of the *Beggiatoaceae* thrive in terrestrial sulfidic springs (Cohn, 1875; Caldwell et al., 1975; Nelson and Castenholz, 1981a; Teske and Nelson, 2006), sites at which where molecular hydrogen is frequently emitted (Aragno, 1992). However, further

studies are necessary to evaluate the importance of molecular hydrogen for members of the family *Beggiatoaceae* on a broader scale. These studies will need to investigate the availability of H_2 to environmental populations as well as the ability of different strains to oxidize this electron donor.

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Supplemental material

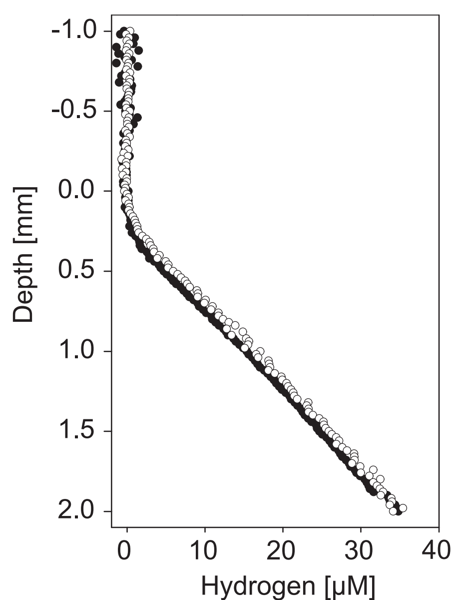

Figure S4.8 | Hydrogen oxidation by *Beggiatoa* sp. 35Flor in presence of a fixed nitrogen source. Oxygen-sulfide gradient media with a diffusional hydrogen gradient were prepared without the addition of fixed nitrogen compounds (○) or with an initial ammonium concentration of 200 μM in the top agar (●), a concentration which has previously been shown to inhibit nitrogen fixation in *Beggiatoa* sp. 35Flor cultures (A.-T. Henze, unpublished). Hydrogen profiles were recorded after 7 days of incubation. The position zero denotes the mat surface.

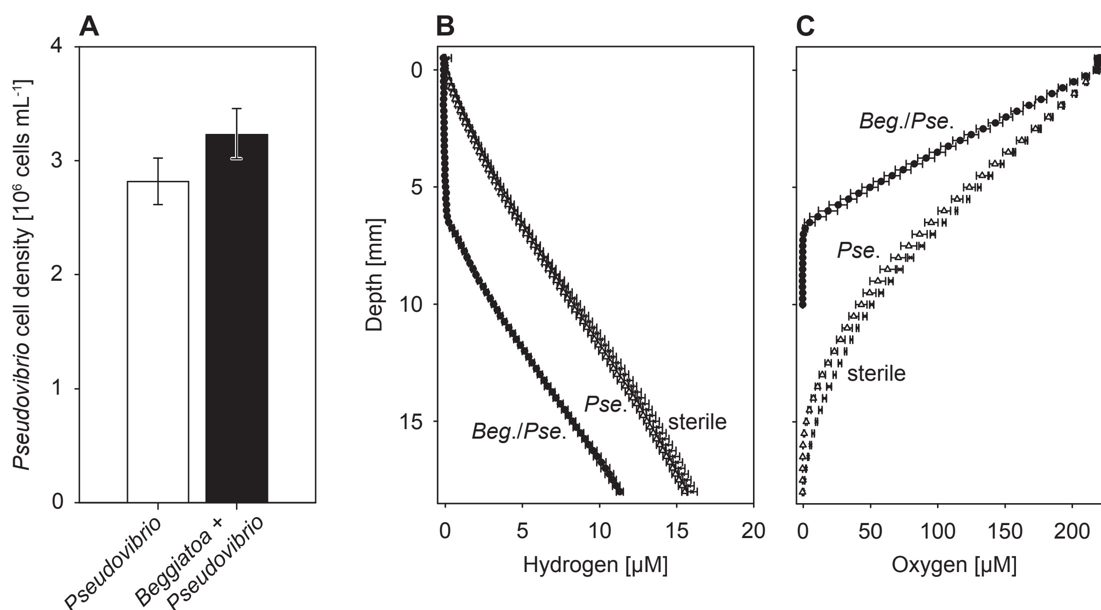

Figure S4.9 | Test for hydrogen oxidation by *Pseudovibrio* sp. FO-BEG1 in hydrogen-supplemented oxygen-sulfide gradient media. Oxygen-sulfide gradient media with a diffusional hydrogen gradient were inoculated with either a mixed *Beggiatoa* sp. 35Flor/*Pseudovibrio* sp. FO-BEG1 suspension or a *Pseudovibrio* sp. FO-BEG1 suspension of the same cell density. Sterile media were kept as controls and measurements were performed after eight days of growth. (**A**) *Pseudovibrio* cell densities are shown as averages ± standard deviations of counts in three parallel cultures. The cell densities were normalized to the entire top agar but cells concentrate in both, *Beggiatoa*/*Pseudovibrio* (■) and *Pseudovibrio* (□) cultures, at the oxic-anoxic interface (A. Fink, unpublished). (**B+C**) Hydrogen and oxygen profiles measured in *Beggiatoa*/*Pseudovibrio* (●), *Pseudovibrio* (△) and sterile cultures (×) are shown as averages ± standard deviations ($n = 4$). Hydrogen profiles were corrected for the sulfide background recorded by the cross-reactive sensor. Moderately higher oxygen consumption rates in *Pseudovibrio*-inoculated cultures relative to sterile media are evident but were previously observed also in hydrogen-unsupplemented media and are likely resulting from the oxidation of reduced sulfur compounds or agar impurities by *Pseudovibrio* sp. FO-BEG1 (A. Fink, unpublished). The y-axis indicates the depth below the air-agar interface.

4.2 Diversity of hydrogenase genes in the family Beggiatoaceae

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Manuscript submitted for publication

Contributions:
I developed the concept of this study and I designed, performed, and analyzed all experiments. The genome sequences of *Beggiatoa* sp. 35Flor and '*Candidatus* Thiomargarita nelsonii' were provided by M. Mußmann. The manuscript was written by me, including comments from M. Mußmann.

Acknowledgements:
We thank Heide N. Schulz-Vogt for comments on the manuscript and Susanne Menger for technical assistance. The study was supported by the Max Planck Society and the German National Academic Foundation.

Abstract

Members of the family *Beggiatoaceae* often form conspicuous mats at sediment surfaces, where they oxidize sulfide. While two strains of the genus *Beggiatoa* are known to oxidize also molecular hydrogen, most members of this highly diverse family are not culturable yet and nothing is known about their hydrogen oxidation potential. To fill this gap, we examined six draft genomes from distantly related members of the family for hydrogenase-encoding genes and additionally screened filaments from eight cultures and enrichments with [NiFe]-hydrogenase-specific PCRs. [NiFe]-hydrogenases were identified in the majority of the tested organisms, suggesting a widespread capacity for hydrogen oxidation within the *Beggiatoaceae*. In addition, the identified enzymes belong to four phylogenetically different clades, which presumably represent hydrogenases with different metabolic functions. The hydrogenases of three clades belong to the H_2-uptake hydrogenases. These enzymes likely couple hydrogen oxidation to oxygen, nitrate, and sulfur respiration and the generation of a proton motive force. The fourth clade comprises NADP-reducing hydrogenases, which are thought to couple hydrogen oxidation to the reduction of pyridine nucleotides, a reaction that otherwise requires a costly reverse electron transport in chemolithotrophic *Beggiatoaceae*. Accordingly, hydrogen metabolism in the family *Beggiatoaceae* appears to be not only widespread but also versatile and thus may help these gradient organisms to adapt more easily to the fluctuating conditions in microbial mats.

Introduction

Large sulfur bacteria of the family *Beggiatoaceae* (Salman et al., 2011) typically thrive in oxygen-sulfide transition zones, in which they often occur in high biomass and substantially influence the local carbon, sulfur, nitrogen, and phosphorus cycles (e.g. Fossing et al., 1995; Schulz and Schulz, 2005; Prokopenko et al., 2013). In addition, we showed in a recent study that a marine, chemolithoautotrophic *Beggiatoa* strain oxidizes also molecular hydrogen at high rates and under various conditions (Kreutzmann and Schulz-Vogt, 2013). Likewise, a heterotrophic freshwater *Beggiatoa* strain is known to oxidize hydrogen under short-term anoxic conditions (Schmidt et al., 1987) and many members of the *Beggiatoaceae* can fix nitrogen (Nelson et al., 1982), a process, which releases H_2 as a by-product (Burgess and Lowe, 1996). In other organisms, nitrogen fixation is therefore often associated with the expression of hydrogenases for H_2 recycling (Brito et al., 1997; Axelsson et al., 1999; Elsen et al., 2000; Happe et al., 2000). Together, these observations suggest that *Beggiatoaceae* could in general be involved in the consumption of hydrogen, coupling the H^+/H_2 redox reaction to the above mentioned element cycles. However, the lack of suitable cultures hampers physiological studies on hydrogen metabolism in most genera of this family. In contrast, most *Beggiatoaceae* are convenient subjects for culture-independent genetic analyses. Their large and conspicuous cells or filaments can readily be separated in a sterile fashion from environmental samples or enrichment cultures and can thus serve as templates for the amplification of specific genes or genomes (e.g. Mußmann et al., 2007; Salman et al., 2011; MacGregor et al., 2013).

Currently, three phylogenetically unrelated classes of hydrogenases—enzymes designated to catalyze redox reactions involving the H^+/H_2 couple—are recognized, which differ in amino acid sequence and the metal cofactor integral to their active site (Wu and Mandrand, 1993; Vignais et al., 2001; Vignais and Billoud, 2007). Hydrogenases of the [NiFe] class are thought to be the most widespread (Vignais and Billoud, 2007) and all hydrogenases yet identified in members of the *Beggiatoaceae* are of this type. [NiFe]-hydrogenases differ considerably in their quaternary structure, cellular localization, and the type of electron carriers they

interact with, but all share a common heterodimeric core. This core is composed of a large subunit (LSU), which carries the active site, and a small subunit, which provides an electron relay of three iron-sulfur clusters. Phylogenetic trees based on sequences of either core subunit revealed numerous distinct lineages of [NiFe]-hydrogenases, which correlate well with the structural and functional properties of characterized enzymes (Vignais et al., 2001; Vignais and Billoud, 2007; Pandelia et al., 2012). Accordingly, the phylogenetic position of a [NiFe]-hydrogenase core subunit sequence is regarded as an appropriate predictor of the enzyme's metabolic function.

We analyzed six draft genomes from members of the family *Beggiatoaceae* for the presence of hydrogenase-encoding genes. Additionally, we retrieved [NiFe]-hydrogenase large subunit genes from filaments of several cultures and enrichments using specific PCRs. The distribution, phylogeny, and gene neighborhood of the identified [NiFe]-hydrogenases suggested that hydrogen oxidation in the *Beggiatoaceae* is both widespread and active under different environmental and metabolic conditions. We propose how the different types of hydrogenases could be integrated in the overall metabolism of the *Beggiatoaceae* and hypothesize how hydrogen oxidation could add to their ecophysiological plasticity.

Materials and Methods

Sample preparation and primers for the amplification of 16S rRNA and hydrogenase LSU genes

Genomic DNA was extracted from axenic cultures of the *Beggiatoa alba* strains B15LD (DSM 1416) and B18LD (ATCC 33555) using the UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). The 16S rRNA gene together with the 16S-23S intergenetic spacer was amplified from these extracts with the primer pair GM3F/ITSReub (see **Table S4.1** for primer sequences). Primers published by Csáki et al. (2001) were used for the amplification of [NiFe]-hydrogenase large subunit (LSU) genes. Appropriate primer pairs were selected

for each DNA extract by screening all possible primer combinations in temperature gradient PCRs for their ability to retrieve a desired fragment. Clone libraries were constructed from amplicons obtained with the primer pairs HUPLX1/HUPLW2 and HUPLW1/HUPLXF.

DNA was extracted from the *Beggiatoa* sp. 35Flor/ *Pseudovibrio* sp. FO-BEG1 co-culture (Kreutzmann and Schulz-Vogt, 2013) and a primer pair (HUPLX1/HUPLXF) was chosen for the amplification of [NiFe]-hydrogenase LSU genes as described above. This and all other primer pairs were also tested on a DNA extract from an axenic *Pseudovibrio* sp. FO-BEG1 culture, but no [NiFe]-hydrogenase LSU gene was amplified. *Beggiatoa* sp. 35Flor filaments were picked manually and washed two to three times in sterile, artificial seawater to remove contaminating *Pseudovibrio* sp. FO-BEG1 cells. Single cleaned filaments were transferred into 0.2-mL tubes containing 10μL of water and served as PCR templates.

Filaments from hypersaline ('60Ibiz', '150Ibiz', '80Chip'; Hinck et al., 2011) and freshwater ('00Aarh', '00Hann'; brought into culture by H. N. Schulz-Vogt and V. Bondarev) enrichment cultures were cleaned in sterile water of the respective salinity as described above. Several washed filaments from each freshwater enrichment culture were pooled in 10 μL of water. [NiFe]-hydrogenase LSU genes were amplified from these filaments with the primer pair HUPLX1/HUPLXF. Washed, single filaments from hypersaline enrichment cultures (filaments I27, Z31, and C31 from enrichments '60Ibiz', '150Ibiz', and '80Chip', respectively) were cut into halves and each half was transferred into a separate 0.2-mL tube containing 10 μL of water. From one half 16S rRNA genes were amplified using the general bacterial 16S rRNA gene primer pair 8-27F/1507R. From the other half [NiFe]-hydrogenase LSU genes were amplified using the primer pair HUPLX1/HUPLXF.

PCR conditions and sequencing

PCRs were performed using the Promega 2× PCR Master Mix (Promega Corporation, Madison, WI, USA) and 1 μM of the respective forward and reverse primers.

Either 1 μL of the diluted DNA extract or 10 μL of water with one or several suspended filaments served as a template in a total reaction volume of 30 μL. The PCR program was as follows: initial denaturation at 95°C for 5 min, 33 cycles of 95°C for 1 min, 42°C (8-27F/1507R) or 59°C (HUPLX1/HUPLXF) or 63.6°C (HUPLX1/HUPLW2 and HUPLW1/HUPLXF) for 1 min, and 72°C for 3 min, and a final elongation at 72°C for 10 min. The PCR program used for amplification with the primer pair GM3F/ ITSReub is given by Salman et al. (2011). All PCR products were purified by Sephadex gel filtration prior to downstream processing. Purified 16S rRNA gene amplicons from filaments of hypersaline enrichment cultures were sequenced directly. Direct sequencing was chosen because it allowed to simultaneously check for possible bacterial contaminations, which would be evident from a number of double peaks in the chromatograms. However, such double peaks were not detected. Clone libraries were constructed from all other amplicons. These were screened by partial sequencing and inserts of interest were fully sequenced. Sequencing was performed with the BigDye Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the ABI Prism 3130x Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The chromatrograms were quality-checked with BioEdit version 7.2 (Hall, 1999) and full-length sequences were assembled with Sequencher version 4.6 (Gene Codes Corporation, Ann Arbor, Mi, USA). Primer sequences were removed prior to *in silico* translation of the assembled sequences with BioEdit version 7.2.

Retrieval of hydrogenase genes from draft genomes

Using the software suite JCoast version 1.7 (Richter et al., 2008), sequences of hydrogenases were retrieved from the draft genomes of *Beggiatoa alba* B18LD (BioProject numbers PRJNA163695, PRJNA62137), *Beggiatoa* sp. 35Flor, '*Candidatus* Thiomargarita nelsonii', and an orange filament collected in the Guaymas Basin (here referred to as 'Guaymas filament'; MacGregor et al., 2013). The genomes of *Beggiatoa* sp. 35Flor and '*Ca.* Thiomargarita nelsonii' were recently sequenced, found to be free of contamination (M. Winkel *et al.*, in preparation) and were annotated as described elsewhere (e.g. Bondarev et al., 2013). The draft

genomes of '*Ca.* Isobeggiatoa sp.' and '*Ca.* Parabeggiatoa sp.' (Mußmann et al., 2007) were also analyzed but no hydrogenase-encoding genes were found.

Phylogenetic reconstruction of hydrogenase LSU and 16S rRNA gene trees

A seed alignment of hydrogenase proteins was computed with MAFFT version 7 (Katoh and Standley, 2013) using a selection of 62 [NiFe]-hydrogenase LSU sequences from all four subgroups of this class (Vignais et al., 2001); 15 [FeFe]-hydrogenase sequences served as an outgroup. Further sequences, such as (i) the retrieved [NiFe]-hydrogenase sequences from *Beggiatoaceae*, (ii) nearly full-length sequences from the PFAM family of nickel-dependent hydrogenases (PF00374), (iii) the 100 closest BLAST hits for representatives of the retrieved *Beggiatoaceae* hydrogenases, (iv) the 100 closest BLAST hits for representatives of the [NiFe]-hydrogenase subgroups 2a–b, 3a–d, and 4, as well as (v) additional outgroup sequences were added to the alignment with MAFFT version 7 using the 'seed' option. Duplicate entries were removed and the resulting alignment of 2038 sequences was used to reconstruct a rough guide tree with the rapid bootstrap analysis of the Randomized Axelerated Maximum Likelihood algorithm (RAxML; Stamatakis et al., 2008; 414 valid columns; 10 replications) using the PROTGAMMA rate distribution model and the Jones-Taylor-Thornton amino acid substitution model. Subtrees for Group 1 and Group 3 hydrogenases were subsequently reconstructed with selected sequences (302 ingroup/ 99 outgroup and 233 ingroup/ 101 outgroup sequences, respectively) using the RAxML algorithm with 100 replicate runs. Phylogenetic reconstruction of a 16S rRNA gene tree with 262 sequences from *Beggiatoaceae* and 99 outgroup sequences from sulfur bacteria of different families was performed as described previously (Salman et al., 2011).

Results and Discussion

Diversity of Beggiatoaceae hydrogenases

[NiFe]-hydrogenase large subunit (LSU) genes were identified in four out of the six analyzed *Beggiatoaceae* draft genomes and could be amplified from filaments of all eight tested cultures and enrichments (**Table S4.2**). This suggests that the capacity for hydrogen oxidation is indeed widespread within the family. Phylogenetic analyses and gene cluster composition showed that the identified proteins affiliate with five distinct clades of [NiFe]-hydrogenases (**Figures 4.10**, **4.11**), which are thought to comprise hydrogenases of different functions (reviewed by e.g. Vignais et al., 2001; Vignais and Billoud, 2007; Pandelia et al., 2012). Three of these clades belong to the membrane-bound H_2-uptake hydrogenases (Group 1 hydrogenases according to Vignais et al., 2001), and more specifically to the 6C- (Hup), Isp- (Hyn), and HybA- (Hyb) hydrogenases (classification system proposed by Pandelia et al., 2012; **Figure 4.10**). The remaining two clades belong the Group 3 hydrogenases (**Figure 4.11**). One of these two clades comprises the NADP-reducing hydrogenases (Hyh; Vignais and Billoud, 2007), while the other is represented by only a few sequences from *Geobacter* species. These *Geobacter* enzymes were originally described as methyl viologen-reducing hydrogenases (Group 3c) based on the phylogeny of the small subunit, but this grouping is neither supported by the phylogeny of the large subunit nor by the gene cluster composition (Coppi, 2005). Little is known about these hydrogenases apart from the fact that they do not support hydrogen-dependent growth (Coppi, 2005). Hence, the potential function of the THI43_4 hydrogenase, the only *Beggiatoaceae*-derived sequence of this cluster (**Figure 4.11**), will not be discussed further.

Distribution of hydrogenases within the family Beggiatoaceae

[NiFe]-hydrogenases of the Hup-, Hyn-, Hyb-, and Hyh-clades were repeatedly identified in the investigated *Beggiatoaceae* (**Table S4.2**) and thus seem to make up the general hydrogenase inventory of the family. Studies on the distribution and

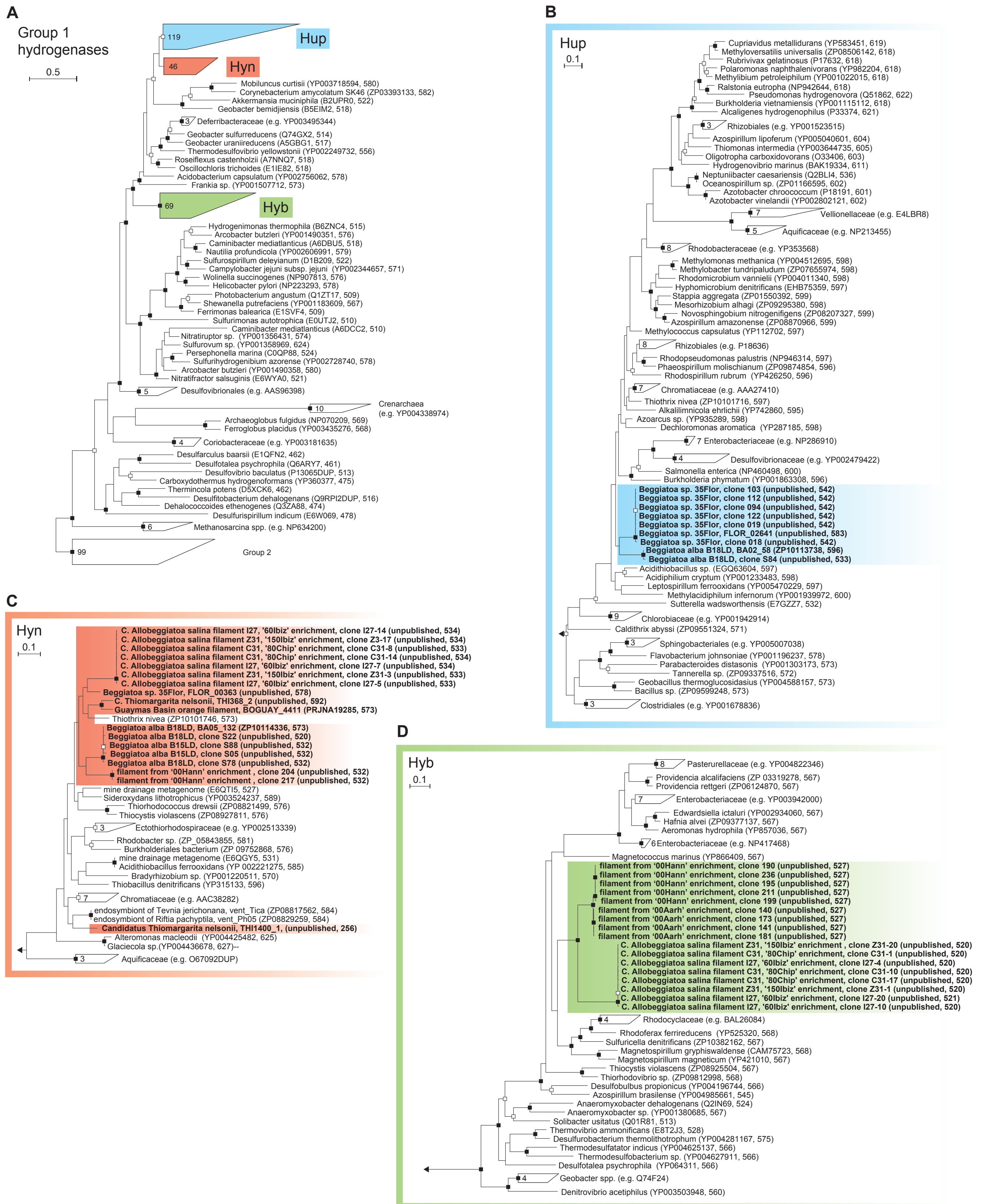


Figure 4.10 | Phylogenetic tree of Group 1 [NiFe] hydrogenases. The tree was reconstructed using the maximum likelihood (RAxML) algorithm and the Jones-Taylor-Thornton amino acid substitution model. Only selected sequences are shown. Plain branching, open boxes (□), and filled (■) boxes denote nodes with bootstrap values of ≤ 50%, 51–75%, and 76–100%, respectively.

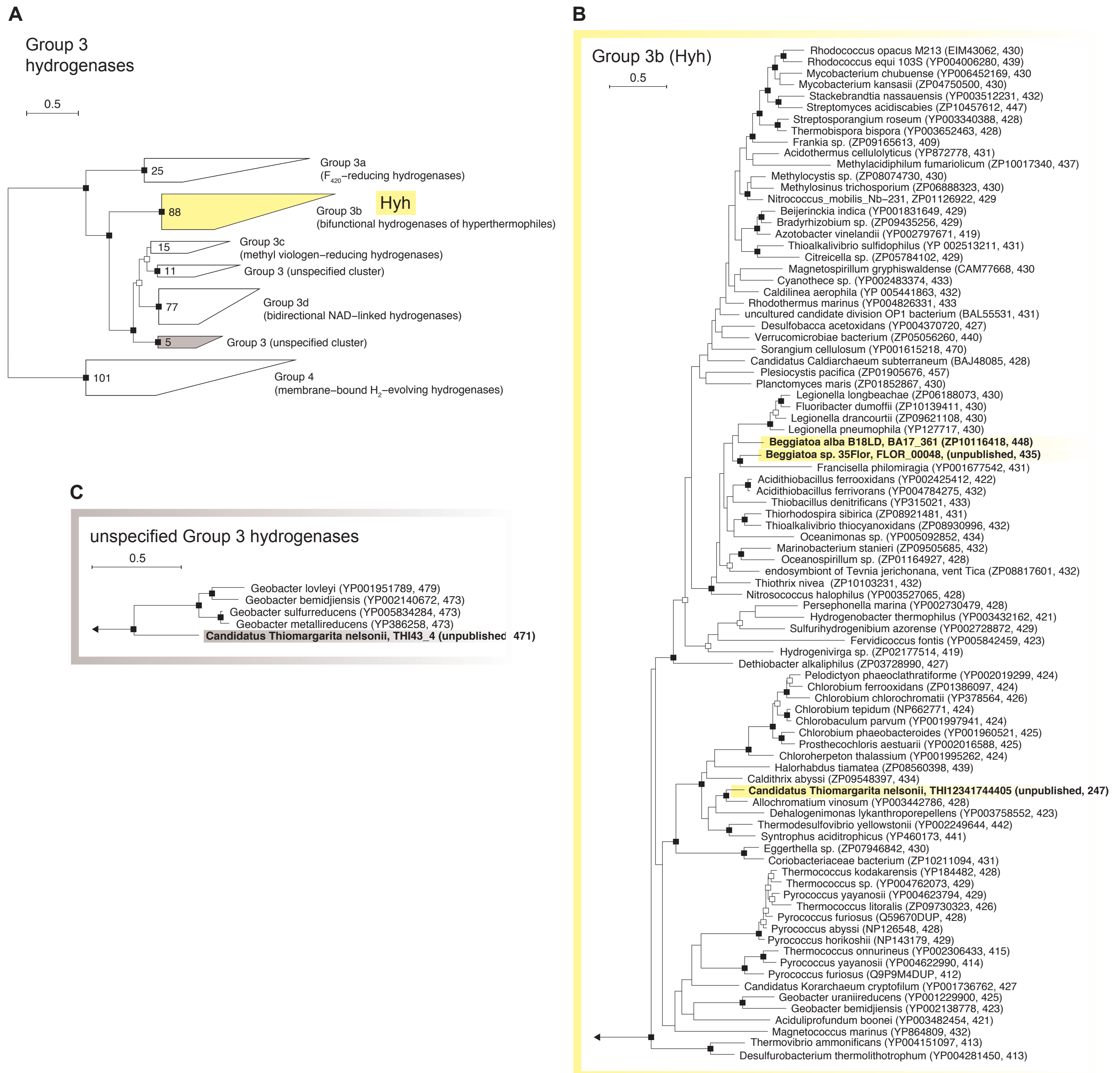


Figure 4.11 | Phylogenetic tree of Group 3 [NiFe] hydrogenases. Details on tree reconstruction are given in **Figure 4.10**.

diversity of hydrogenases in the family *Beggiatoaceae* are limited by incompletely sequenced genomes and PCR-inherent amplification biases. However, the *Beggiatoaceae*-derived hydrogenase sequences from each of the four clades are closely related (**Figures 4.10**, **4.11**). This indicates that hydrogenases from all of these clades could be ancestral either for the entire family or at least for a certain subgroup thereof.

Hyn-hydrogenases were found in nine out of the twelve investigated strains from throughout the entire family (**Figure S4.15**) and constitute, with one exception, a monophyletic cluster. Accordingly, Hyn-hydrogenases were likely inherited from a common ancestor of the family (**Figure 4.10 C**). The only phylogenetically distinct Hyn-hydrogenase (THI1400_1) is one out of two Hyn-homologs from '*Ca.* Thiomargarita nelsonii'. In contrast to the bacterium's authentic Hyn-hydrogenase, this homolog was probably acquired via horizontal gene transfer from a member of the *Chromatiaceae*. A sequence from *Thiothrix nivea* further clustered with the authentic Hyn-hydrogenases of the *Beggiatoaceae*, suggesting that *Thiothrix nivea* acquired the gene from a member of the *Beggiatoaceae* via horizontal gene transfer. In contrast to Hyn-hydrogenases, the currently available data do not allow to assess, whether hydrogenases of the Hup, Hyb-, and Hyh-clades were inherited from a common ancestor of the entire family, as enzymes of these clades were so far only identified in more closely related strains (**Figures 4.10 A**, **B**, **4.11 B**). An exception are the Hyh-hydrogenases, which were not only retrieved from the rather closely related strains *Beggiatoa* sp. 35Flor and *Beggiatoa alba* B18LD but also from the more distantly related '*Ca.* Thiomargarita nelsonii' (**Figure 4.11 B**). However, the Hyh-homolog of '*Ca.* Thiomargarita nelsonii' (THI12341744405) did not cluster with those of the other two strains in the phylogenetic tree and was probably likewise acquired via horizontal gene transfer from a member of the family *Chromatiaceae* (**Figure 4.11 B**). Thus, the phylogenetic trees point at a rather frequent exchange of hydrogenase genes between different sulfide-oxidizing bacteria, i.e. the *Beggiatoaceae*, *Chromatiaceae*, and *Thiothrix nivea*. This suggests that their possible co-occurrence in similar ecological niches could have facilitated the horizontal transfer of hydrogenase genes, a rationale previously called on also in other contexts (Smillie et al., 2011; Kleiner et al., 2012).

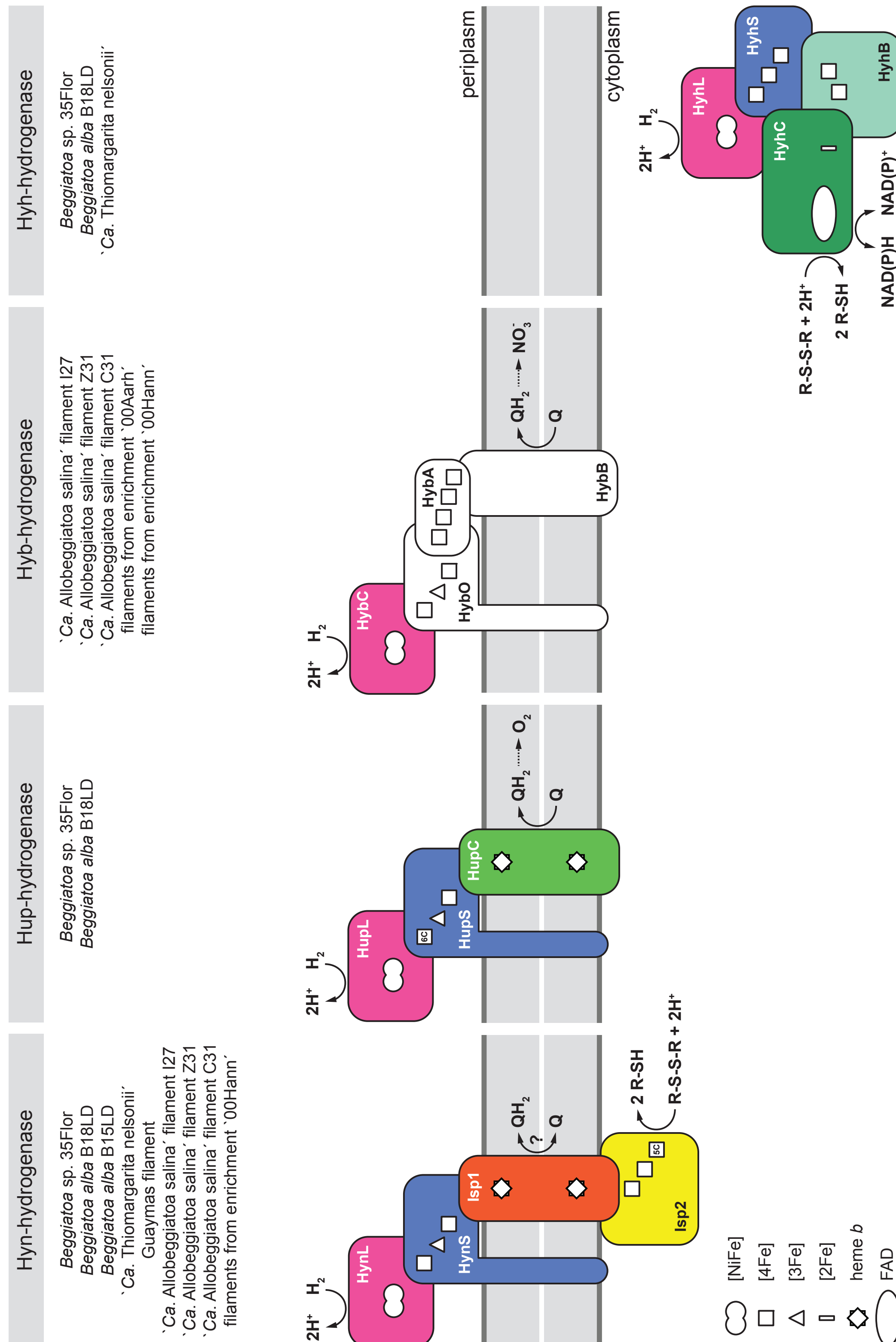
Possible metabolic functions of the encoded hydrogenases

The co-occurrence of multiple [NiFe]-hydrogenases is known from many other bacteria and archaea (reviewed by Vignais and Billoud, 2007) and the functional differentiation of these enzymes has been subject to numerous studies. Accordingly, a considerable amount of biochemical, biophysical, and genetic data has been gathered, which we refer to when proposing possible functions for the four hydrogenases types identified in members of the family *Beggiatoaceae* (**Figure 4.12**).

Beggiatoaceae may employ Hyn-hydrogenases to couple hydrogen oxidation to sulfur reduction

Hyn-hydrogenases were proposed to be directly linked to the sulfur metabolism and are thought to be active under anoxic conditions (Pandelia et al., 2012). Corresponding to other hydrogenases of this type, the core subunits (HynSL) of the *Beggiatoa alba* B18LD Hyn-hydrogenase are co-localized with genes encoding a putatively quinone-interacting transmembrane *b*-type cytochrome (Isp1) and a hydrophilic iron-sulfur protein (Isp2) featuring a characteristic, cysteine-rich 5C sequence motif (**Figure 4.13**; Rakhely et al., 1998; Dahl et al., 1999; Pandelia et al., 2012). Modules resembling Isp1Isp2 are known from various enzymes (e.g. HdrDE, HmeCD, DsrMK) and Isp2 homologs were shown to consistently carry an iron-sulfur cluster that is coordinated by the 5C motif or a derivative thereof

Figure 4.12 ***(on the next page)*** | **Putative quaternary structures and proposed biochemical functions of hydrogenases identified in members of the *Beggiatoaceae*.** The putative subcellular localizations of hydrogenases as well as their proposed redox partners are shown. Proteins evidentially encoded in *Beggiatoaceae* are colored corresponding to the respective genes shown in **Figure 4.13**; proteins, which were so far not identified in the draft genomes are depicted in white. Redox cofactors are shown as given by Pandelia et al. (2012) and Silva et al. (1999) for enzymes of the respective clades. Iron-sulfur clusters with the peculiar 5C and 6C coordinations are indicated. The unspecified Group 3 hydrogenase encoded in the genome of '*Ca.* Thiomargarita nelsonii' is not included in the scheme. The source species of the respective hydrogenase types are given.


Hyn-hydrogenase
Beggiatoa sp. 35Flor
Beggiatoa alba B18LD
Beggiatoa alba B15LD
´Ca. Thiomargarita nelsonii´
Guaymas filament
´Ca. Allobeggiatoa salina´ filament I27
´Ca. Allobeggiatoa salina´ filament Z31
´Ca. Allobeggiatoa salina´ filament C31
filaments from enrichment ´00Hann´
Hup-hydrogenase
Beggiatoa sp. 35Flor
Beggiatoa alba B18LD
Hyb-hydrogenase
´Ca. Allobeggiatoa salina´ filament I27
´Ca. Allobeggiatoa salina´ filament Z31
´Ca. Allobeggiatoa salina´ filament C31
filaments from enrichment ´00Aarh´
filaments from enrichment ´00Hann´
Hyh-hydrogenase
Beggiatoa sp. 35Flor
Beggiatoa alba B18LD
´Ca. Thiomargarita nelsonii´
periplasm
cytoplasm
2H+ H2
HynL
HynS
Isp1
Isp2
6C
5C
QH2
Q
?
2 R-SH
R-S-S-R + 2H+
HupL
HupS
HupC
QH2 → O2
HybC
HybO
HybA
HybB
QH2 → NO3-
HyhL
HyhS
HyhC
HyhB
R-S-S-R + 2H+
2 R-SH
NAD(P)H
NAD(P)+
[NiFe]
[4Fe]
[3Fe]
[2Fe]
heme b
FAD

(Künkel et al., 1997; Pott and Dahl, 1998; Mander et al., 2002). In all cases, this iron-sulfur cluster is known or proposed to be directly involved in redox-reactions on S–S bonds (Duin et al., 2003; Mander et al., 2004; Grimm et al., 2008). In line with this hypothesis, hydrogenases of the Hyn-cluster originate almost exclusively from sulfur-metabolizing bacteria (**Figure 4.10 C**). Experimental support for the function of Hyn-hydrogenases comes from the purple sulfur bacterium *Thiocapsa roseopersicina*, which was shown to use this enzyme for coupling hydrogen oxidation to the reduction of sulfur (Laurinavichene et al., 2007). The Hyn-type hydrogenases of *Beggiatoaceae* are therefore good candidates for catalyzing hydrogen oxidation at the expense of stored sulfur (**Figure 4.12**), a reaction previously demonstrated in *Beggiatoa alba* B18LD and *Beggiatoa* sp. 35Flor (Schmidt et al., 1987; Kreutzmann and Schulz-Vogt, 2013).

Hup-hydrogenases are indicative of aerobic hydrogen oxidation in Beggiatoaceae

Hydrogen oxidation under oxic conditions is thought to be catalyzed by hydrogenases of the Hup-clade (Pandelia et al., 2012). Hup-hydrogenases are unique among the H_2-metabolizing enzymes in featuring a profound oxygen tolerance, a characteristic, which is brought about by a supernumerary cysteine coordination of an iron-sulfur cluster in the small subunit (6C cluster; Fritsch et al., 2011; Shomura et al., 2011; Goris et al., 2011). Being strictly conserved among Hup-type hydrogenases (Pandelia et al., 2012), including those of the *Beggiatoaceae*, this coordination was suggested to enable a reductive removal of oxygen from the active site (Pandelia et al., 2011; Goris et al., 2011). Oxygen tolerance and relatively higher redox potentials of the [NiFe] and iron-sulfur centers in Hup-hydrogenases argue for an electron transfer to oxygen or other high-potential acceptors (Laurinavichene et al., 2002; Pandelia et al., 2012). Indeed, the Hup-hydrogenases of e.g. *Thiocapsa roseopersicina* and *Escherichia coli* were shown to be responsible for oxygen-dependent hydrogen oxidation (Laurinavichene and Tsygankov, 2001; Laurinavichene et al., 2007). Therefore, it is likely that the corresponding enzymes of the *Beggiatoaceae*, a family in which aerobic respiration is widespread

(Teske and Nelson, 2006), function accordingly (**Figure 4.12**). The core subunits of Hup-hydrogenases (HupSL) are generally co-localized with HupC, a transmembrane di-heme *b*-type cytochrome, which is thought to channel electrons from the small subunit into the quinone pool (Pandelia et al., 2012). HupC homologs were also identified in the genetic vicinity of the *Beggiatoaceae* HupSL genes, suggesting a corresponding link of aerobic hydrogen oxidation to quinone reduction in members of this family.

Hyb-hydrogenases may allow Beggiatoaceae to oxidize hydrogen with nitrate

Hyb-hydrogenases are thought to functionally complement Hup-hydrogenases by catalyzing hydrogen oxidation preferentially under anoxic conditions. We repeatedly retrieved Hyb-hydrogenase LSU sequences with [NiFe]-hydrogenase-specific primers from washed filaments of different enrichment cultures but did not yet identify a corresponding enzyme in any of the *Beggiatoaceae* draft genomes. Accordingly, we lack direct information about the subunits probably associated with the Hyb-hydrogenases of the *Beggiatoaceae*. In other organisms, however, an iron-sulfur cluster-bearing electron transfer protein (HybA) and a transmembrane-protein (HybB) are typically encoded in the vicinity of the Hyb-hydrogenases core subunits (HybOC). HybB is thought to reduce quinones despite being devoid of established heme binding sites (Dubini et al., 2002; Pandelia et al., 2012). Redox-titrations with cell-free extracts of *Escherichia coli* mutants suggested a preference of Hyb-hydrogenases for low-potential electron acceptors (Laurinavichene et al., 2002). Correspondingly, *in vivo* studies showed that the *E. coli* Hyb-hydrogenase (hydrogenase 2) preferentially catalyzed hydrogen oxidation with more redox-negative electron acceptors such as nitrate, dimethyl sulfoxide, and fumarate (Laurinavichene and Tsygankov, 2001). Nitrate is an important electron acceptor in many *Beggiatoaceae* (Schulz, 2006; Teske and Nelson, 2006) and we propose that these employ Hyb-hydrogenases for hydrogen oxidation under nitrate-respiring conditions (**Figure 4.12**). Correspondingly, Hyb-hydrogenases were so far identified only in those *Beggiatoaceae* known to respire with nitrate (Kamp et al., 2006; Hinck et al., 2007, 2011; Beutler et al., 2012).

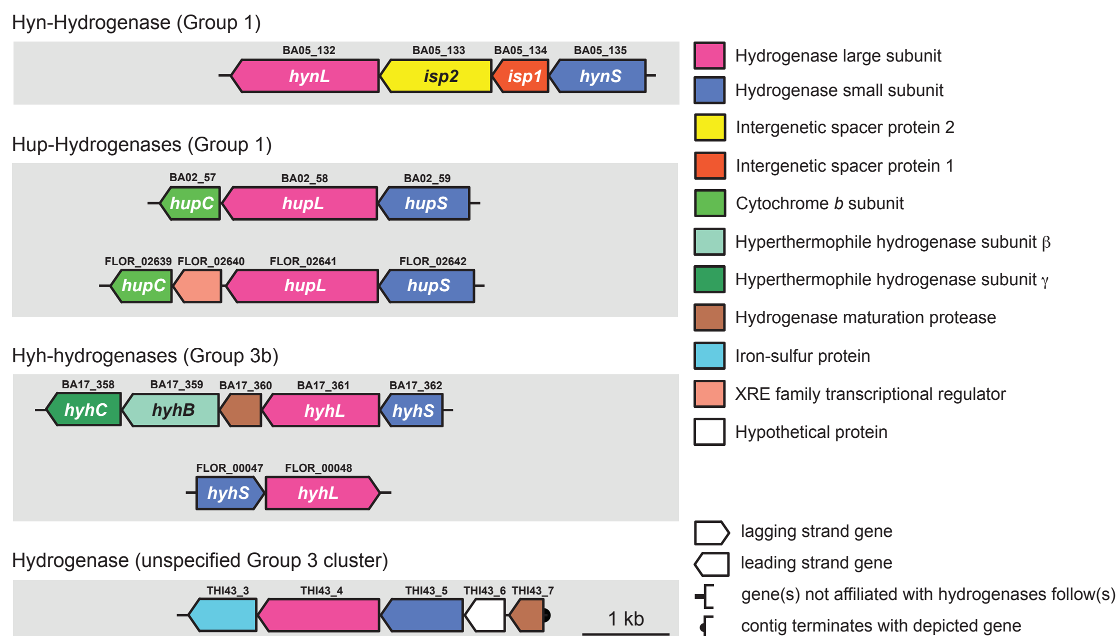


Figure 4.13 | Gene arrangements in clusters encoding [NiFe] hydrogenase large subunits. Gene clusters are sorted by hydrogenase type and genes putatively encoding homologous proteins are displayed in the same color. Gene locus identifiers are given above the schematic representations with the epithets BA, FLOR, and THI denoting genes from *Beggiatoa alba* B18LD, *Beggiatoa* sp. 35Flor, and '*Ca.* Thiomargarita nelsonii', respectively. Hydrogenase large subunit genes, for which no contextual information was available or which did apparently not cluster with other hydrogenase-affiliated genes, are not shown. Genes encoding the β and γ subunits (HyhBC) of the *Beggiatoa* sp. 35Flor Hyh-hydrogenase were missing in the genetic vicinity of the *hyhSL* genes, but were identified on a different contig (FLOR_02158 and FLOR_02159).

Cytoplasmic Hyh-hydrogenases may allow Beggiatoaceae to generate reducing equivalents without a reverse electron flow

Formerly named 'sulfhydrogenases', the cytoplasmic Hyh-hydrogenases are reversible and bifunctional enzymes capable of exchanging electrons between $NAD(P)^+/NAD(P)H$, S^0/H_2S and H^+/H_2 (Ma et al., 1993, 1994, 2000). These hydrogenases are heterotetrameric enzymes (Ma et al., 1993; Pedroni et al., 1995; Silva et al., 1999) and genes encoding all four subunits have been identified in the genomes of *Beggiatoa alba* B18LD and *Beggiatoa* sp. 35Flor (**Figure 4.13**). Initially found in fermenting, hyperthermophilic prokaryotes, Hyh-hydrogenases were suggested to dispose of excess electrons and recycle pyridine nucleotides by

reducing elemental sulfur or protons (Ma et al., 1993, 1994; Silva et al., 2000). Later, gene disruption studies in the hyperthermophilic archaeon *Thermococcus kodakarensis* demonstrated that its Hyh-hydrogenase is in fact responsible for the production of significant quantities of NADPH, rather than the reduction of protons (Kanai et al., 2011). We assume a similar function for the Hyh-hydrogenases, which we found in members of the family *Beggiatoaceae* (**Figure 4.12**). By reducing pyridine nucleotides, these enzymes could capture the low redox potential of the H^+/H_2 couple more efficiently than any of the other identified hydrogenases.

The encoded hydrogenases could enable Beggiatoaceae to use molecular hydrogen in different metabolic contexts

The hydrogenases of the *Beggiatoaceae* could serve different metabolic purposes such as energy conservation in a transmembrane proton gradient, the production of reducing equivalents, as well as the disposal of excess electrons and stored sulfur (**Figure 4.14**). Membrane-bound, periplasmic hydrogenases of the Hup-, Hyb-, and Hyn-clades are likely to conserve energy in a proton motive force. In contrast, cytoplasmic Hyh-hydrogenases can reduce pyridine nucleotides, which could serve either as electron donors for the electron transport chain (NADH) or as reducing equivalents in assimilatory reactions (NADPH). The reduction of $NAD(P)^+$ with H_2 would be highly advantageous, in particular for chemolithoautotrophic *Beggiatoaceae*, as it would provide reducing equivalents without the costly but otherwise necessary reverse electron transport.

In addition to providing energy and reducing equivalents for maintenance and anabolic purposes, the encoded hydrogenases could function in the disposal of excess electrons and overabundant internal sulfur under certain conditions (**Figure 4.14**). All hydrogenases could in principle run backwards and catalyze the reduction of protons with unwanted electrons under strongly reducing conditions. In *E. coli*, however, hydrogen evolution under such circumstances was preferentially catalyzed by its Hyb-type hydrogenase (Lukey et al., 2010). Cell rupture as a result of excessive sulfur accumulation occurred in a chemolithoautotrophic *Beggiatoa* strain, when sulfide was oxidized at high rates (Schwedt et al., 2012). Probably

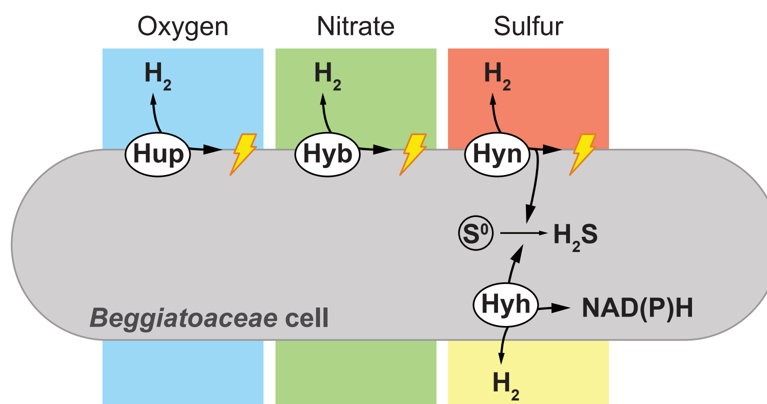


Figure 4.14 | Hypothetical model of hydrogenase function in the *Beggiatoaceae*. The figure summarizes the functions of the four different hydrogenases as proposed in the text. The bolt stands for the generation of an electrochemical potential across the cytoplasmic membrane.

to avoid this fate, the strain reduced stored sulfur to sulfide with molecular hydrogen or carbon reserve compounds such as polyhydroxyalkanoates serving as electron donors (Schwedt et al., 2012; Kreutzmann and Schulz-Vogt, 2013). Hyn- and Hyh-hydrogenases may function as safety valves for internal sulfur under such conditions as they likely have the ability to reduce disulfide bonds. While Hyn-hydrogenases will conserve energy in course of a hydrogen-driven sulfur reduction, Hyh-type enzymes will not. However, the ability to uncouple sulfur reduction from the proton motive force might, in fact, be advantageous for *Beggiatoaceae*. When sulfide oxidation rates are high, excessive sulfur accumulation and a strongly energized cytoplasmic membrane co-occur. Under these conditions, Hyh-hydrogenases would be able to dispose of sulfur, ignoring any backpressure from the proton gradient. Besides reducing sulfur with hydrogen, Hyh-type enzymes could also couple sulfur reduction to the degradation of carbon storage compounds. PHA breakdown generates NADH (Uchino et al., 2007) and Hyh-hydrogenases have been shown to reduce sulfur with electrons from both, H_2 and NADH (Ma et al., 1993, 1994).

Fulfilling different functions, the encoded hydrogenases will play different ecological roles for members of the family *Beggiatoaceae*. The importance of the probably oxygen- and nitrate-reducing Hup- and Hyb-hydrogenases ultimately depends on the ability of a strain to respire with these terminal oxidants as well as on

the availability of H_2 under conditions when either oxidant is used. In contrast, the sulfur-reducing Hyn-hydrogenase appears to be of general metabolic importance as suggested by its wide distribution in many extant members of the family (**Figures 4.13** and **S4.15**). Sulfur is a central metabolite in the *Beggiatoaceae* so that any enzyme, which supports a more flexible and adaptable sulfur metabolism, will be highly valuable. As shown recently (Kreutzmann and Schulz-Vogt, 2013), molecular hydrogen can influence the sulfur metabolism of *Beggiatoaceae* twofold: hydrogen may partially replace sulfur as an electron donor when the supply with reduced sulfur compounds is limited and, alternatively, hydrogen can be used to reduce sulfur under anoxic and highly sulfidic conditions. Hyn-hydrogenases could in fact support both reactions. Sulfur could not only be reduced with hydrogen when sulfur accumulates excessively but also under sulfur-compound limited conditions. In the latter case, the produced sulfide could then immediately be re-oxidized with oxygen or nitrate to sulfur, resulting in a 'sulfur-catalyzed' net consumption of hydrogen (Kreutzmann and Schulz-Vogt, 2013).

Overall, the presented data support a model of hydrogen metabolism in the family *Beggiatoaceae*, which takes into consideration that the H^+/H_2 redox reaction can likely be coupled to the overall metabolism in numerous ways and thus can serve very different ecophysiological purposes (**Figure 4.14**). Even though hydrogen consumption under some of the proposed conditions has previously been shown in single strains of the *Beggiatoaceae* (Schmidt et al., 1987; Kreutzmann and Schulz-Vogt, 2013), further studies will be necessary to understand the importance of hydrogen metabolism and the function of the different hydrogenases in these sulfur bacteria. Yet, the prevalence and diversity of hydrogenase genes within the family suggests that such studies could uncover an important but so far disregarded aspect of their metabolism.

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Supplemental material

Table S4.1 | Sequences of PCR primers used in this study. Abbreviations used are given in parentheses.

Primer (Abbreviation)	Target gene	Sequence (5' → 3')	Reference
GM3F	16S rRNA gene	AGAGTTTGATCMTGGC	Muyzer et al., 1995
8-27F	16S rRNA gene	AGAGTTTGATYMTGGCTCAG	Edwards et al., 1989
1507R	16S rRNA gene	TACCTTGTTACGACT	Muyzer et al., 1995
ITSReub	23S rRNA gene	GCCAAGGCATCCACC	Cardinale et al., 2004
HUPLX1 (X1)	[NiFe] H_2ase LSU gene	GACCCSGTBACSCGNATYGARGG	Csáki et al., 2001
HUPLW2 (W2)	[NiFe] H_2ase LSU gene	RCANGCNAGRCASGGGTCGAA	Csáki et al., 2001
HUPLW1 (W1)	[NiFe] H_2ase LSU gene	GACCCSGTSACSCGNATCGAGGGSCA	Csáki et al., 2001
HUPLXF (XF)	[NiFe] H_2ase LSU gene	CASGCVARRCASGGRTCRAA	Csáki et al., 2001

Table S4.2 | Hydrogenase large subunit genes identified in members of the family *Beggiatoaceae*. Sequences were retrieved from *Beggiatoaceae* draft genomes (see Material and Methods) or were amplified with the specified primer pairs (see **Table S4.1** for full primer names and sequences) from cleaned filaments. The phylogenetic classification of the identified hydrogenases is given along with the type of supporting evidence ('P' and 'OS' refer to LSU phylogeny and operon structure, respectively). If a sequence was clearly distinct from the other *Beggiatoaceae*-derived sequences of the respective clade this is indicated with a star (*). For each strain (*Beggiatoa* sp. 35Flor and *Beggiatoa alba* B18LD), individual ('*Ca.* Thiomargarita nelsonii', '*Ca.* Allobeggiatoa salina' filaments I27, Z31, C31 and the Guaymas filament), and pool of filaments (enrichment cultures '00Hann' and '00Aarh') the identified hydrogenase types are shown as bullets (●) of the respective color. Sequences lengths are given in amino acids counts (AA) with those of partial sequences being enclosed in parentheses.

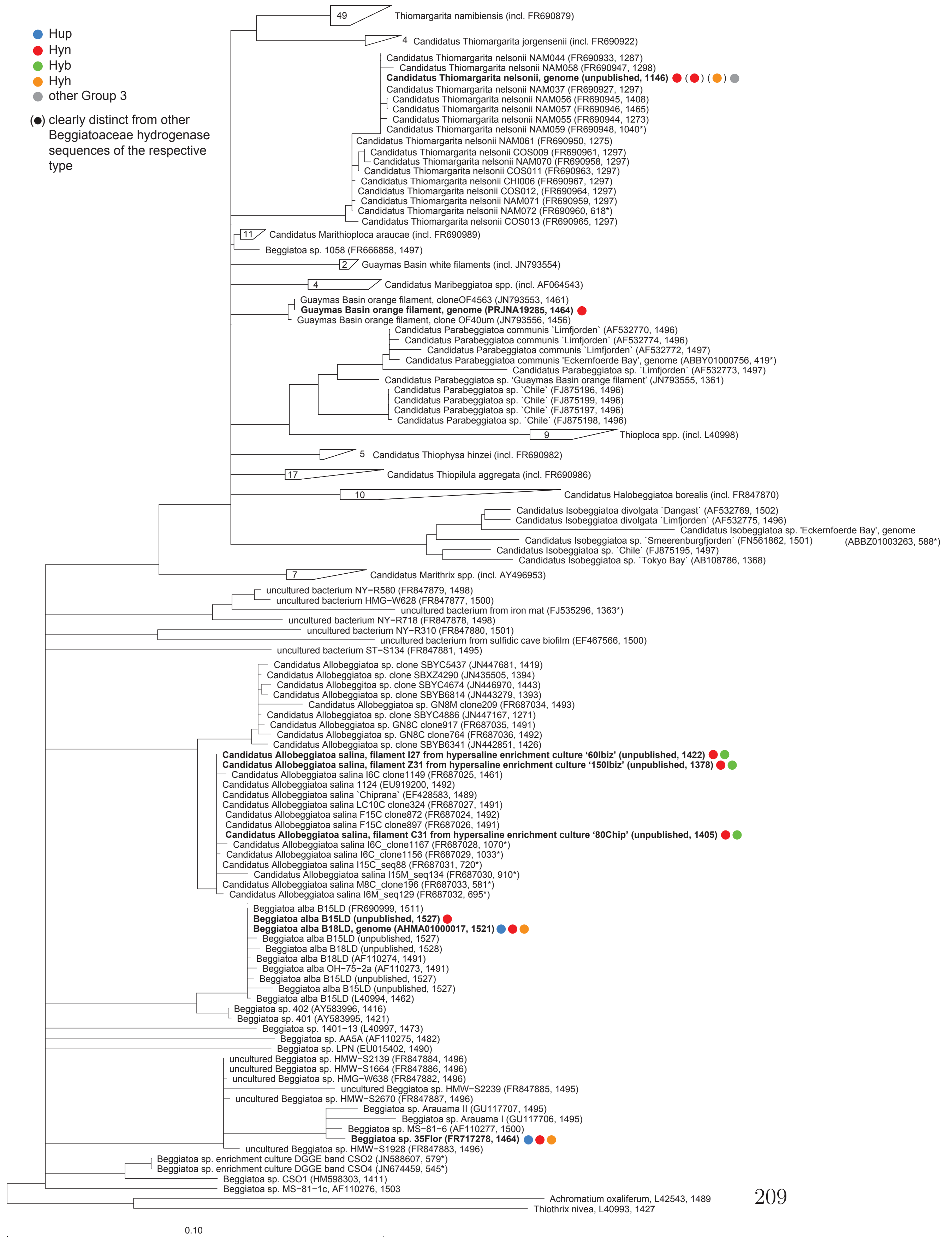
Origin and sequence	Affiliation					AA	Obtained from
	Group 1			Group 3			
	Hup	Hyn	Hyb	Hyh	Other		
	●	●	●	●	●		
***Beggiatoa* sp. 35Flor**	●	●		●			
FLOR_02641	P;OS	-	-	-	-	583	Genome
FLOR_00363	-	P;OS	-	-	-	578	Genome
FLOR_00048	-	-	-	P	-	435	Genome
clone 018	P	-	-	-	-	(542)	PCR (X1/XF)
clone 019	P	-	-	-	-	(542)	PCR (X1/XF)
clone 094	P	-	-	-	-	(542)	PCR (X1/XF)
clone 103	P	-	-	-	-	(542)	PCR (X1/XF)
clone 112	P	-	-	-	-	(542)	PCR (X1/XF)
clone 122	P	-	-	-	-	(542)	PCR (X1/XF)
***Beggiatoa alba* B18LD**	●	●		●			
BA02_58 (ZP10113738)	P;OS	-	-	-	-	596	Genome
BA05_132 (ZP10114336)	-	P;OS	-	-	-	573	Genome
BA17_361 (ZP10116418)	-	-	-	P;OS	-	448	Genome
clone S84	P	-	-	-	-	(533)	PCR (W1/XF)
clone S22	-	P	-	-	-	(520)	PCR (X1/W2)
clone S78	-	P	-	-	-	(532)	PCR (W1/XF)
***Beggiatoa alba* B15LD**		●					
clone S05	-	P	-	-	-	(532)	PCR (X1/W2)
clone S88	-	P	-	-	-	(532)	PCR (W1/XF)
'*Ca.* Thiomargarita nelsonii'		●		●	●		
THI1400_1	-	P*	-	-	-	(256)	Genome
THI368_2	-	P	-	-	-	592	Genome
THI12341744405	-	-	-	P*	-	(247)	Genome
THI43_4	-	-	-	-	P	471	Genome
orange Guaymas filament		●					
BOGUAY_4411	-	P	-	-	-	573	Genome

(Continued on next page.)

Table S4.2 (Continued from previous page.)

Origin and sequence	Affiliation					AA	Obtained from
	Group 1			Group 3			
	Hup	Hyn	Hyb	Hyh	Other		
	●	●	●	●	●		
'*Ca.* Allobeggiatoa salina' I27 (enrichment '60Ibiz')		●	●				
clone I25-5	-	P	-	-	-	(533)	PCR (X1/XF)
clone I27-7	-	P	-	-	-	(534)	PCR (X1/XF)
clone I27-14	-	P	-	-	-	(534)	PCR (X1/XF)
clone I27-4	-	-	P	-	-	(520)	PCR (X1/XF)
clone I27-10	-	-	P	-	-	(520)	PCR (X1/XF)
clone I27-20	-	-	P	-	-	(520)	PCR (X1/XF)
'*Ca.* Allobeggiatoa salina' Z31 (enrichment '150Ibiz')		●	●				
clone Z31-3	-	P	-	-	-	(533)	PCR (X1/XF)
clone Z31-7	-	P	-	-	-	(534)	PCR (X1/XF)
clone Z31-1	-	-	P	-	-	(520)	PCR (X1/XF)
clone Z31-20	-	-	P	-	-	(520)	PCR (X1/XF)
'*Ca.* Allobeggiatoa salina' C31 (enrichment '80Chip')		●	●				
clone C31-8	-	P	-	-	-	(533)	PCR (X1/XF)
clone C31-14	-	P	-	-	-	(534)	PCR (X1/XF)
clone C31-1	-	-	P	-	-	(520)	PCR (X1/XF)
clone C31-10	-	-	P	-	-	(520)	PCR (X1/XF)
clone C31-17	-	-	P	-	-	(520)	PCR (X1/XF)
filaments (enrichment '00Hann')		●	●				
clone 204	-	P	-	-	-	(532)	PCR (X1/XF)
clone 217	-	P	-	-	-	(532)	PCR (X1/XF)
clone 190	-	-	P	-	-	(527)	PCR (X1/XF)
clone 195	-	-	P	-	-	(527)	PCR (X1/XF)
clone 199	-	-	P	-	-	(527)	PCR (X1/XF)
clone 211	-	-	P	-	-	(527)	PCR (X1/XF)
clone 236	-	-	P	-	-	(527)	PCR (X1/XF)
filaments (enrichment '00Aarh')			●				
clone 140	-	-	P	-	-	(527)	PCR (X1/XF)
clone 141	-	-	P	-	-	(527)	PCR (X1/XF)
clone 173	-	-	P	-	-	(527)	PCR (X1/XF)
clone 181	-	-	P	-	-	(527)	PCR (X1/XF)

Figure S4.15 *(on the next page)* | Phylogenetic 16S rRNA gene consensus tree of the family *Beggiatoaceae* with highlighted hydrogenase source species. The revised nomenclature of the family *Beggiatoaceae* according to Salman et al. (2011), Hinck et al. (2011), and Grünke et al. (2012) is used. Only representative sequences are shown. 16S rRNA gene sequences shorter than the segment of the alignment considered for phylogenetic reconstruction (nucleotide positions 279–1463 according to *E. coli* numbering) are marked with an asterisk (*). Strains or filaments for which hydrogenase large subunit (LSU) sequences are available are marked with a bullet (●), the color of which indicates the type of hydrogenase identified. If a hydrogenase LSU sequence is clearly distinct from other *Beggiatoaceae* hydrogenase sequences of the same type, the bullet is enclosed in parentheses. In the case of filaments from hypersaline enrichment cultures, 16S rRNA and hydrogenase genes were amplified concurrently from the very same individuals so that the phylogenetic affiliations of the hydrogenase source species could be identified unequivocally. Further hydrogenase LSU sequences were amplified from filaments of the freshwater enrichment cultures '00Aarh' and '00Hann' but corresponding 16S rRNA gene sequences are not available. Database accession numbers and sequence lengths are given in parentheses. The scale bar represents 10% sequence divergence.



Hup
Hyn
Hyb
Hyh
other Group 3
(●) clearly distinct from other Beggiatoaceae hydrogenase sequences of the respective type
49 Thiomargarita namibiensis (incl. FR690879)
4 Candidatus Thiomargarita jorgensenii (incl. FR690922)
Candidatus Thiomargarita nelsonii NAM044 (FR690933, 1287)
Candidatus Thiomargarita nelsonii NAM058 (FR690947, 1298)
Candidatus Thiomargarita nelsonii, genome (unpublished, 1146)
Candidatus Thiomargarita nelsonii NAM037 (FR690927, 1297)
Candidatus Thiomargarita nelsonii NAM056 (FR690945, 1408)
Candidatus Thiomargarita nelsonii NAM057 (FR690946, 1465)
Candidatus Thiomargarita nelsonii NAM055 (FR690944, 1273)
Candidatus Thiomargarita nelsonii NAM059 (FR690948, 1040*)
Candidatus Thiomargarita nelsonii NAM061 (FR690950, 1275)
Candidatus Thiomargarita nelsonii COS009 (FR690961, 1297)
Candidatus Thiomargarita nelsonii NAM070 (FR690958, 1297)
Candidatus Thiomargarita nelsonii COS011 (FR690963, 1297)
Candidatus Thiomargarita nelsonii CHI006 (FR690967, 1297)
Candidatus Thiomargarita nelsonii COS012, (FR690964, 1297)
Candidatus Thiomargarita nelsonii NAM071 (FR690959, 1297)
Candidatus Thiomargarita nelsonii NAM072 (FR690960, 618*)
Candidatus Thiomargarita nelsonii COS013 (FR690965, 1297)
11 Candidatus Marithioploca araucae (incl. FR690989)
Beggiatoa sp. 1058 (FR666858, 1497)
2 Guaymas Basin white filaments (incl. JN793554)
4 Candidatus Maribeggiatoa spp. (incl. AF064543)
Guaymas Basin orange filament, cloneOF4563 (JN793553, 1461)
Guaymas Basin orange filament, genome (PRJNA19285, 1464)
Guaymas Basin orange filament, clone OF40um (JN793556, 1456)
Candidatus Parabeggiatoa communis `Limfjorden` (AF532770, 1496)
Candidatus Parabeggiatoa communis `Limfjorden` (AF532774, 1496)
Candidatus Parabeggiatoa communis `Limfjorden` (AF532772, 1497)
Candidatus Parabeggiatoa communis 'Eckernfoerde Bay', genome (ABBY01000756, 419*)
Candidatus Parabeggiatoa sp. `Limfjorden` (AF532773, 1497)
Candidatus Parabeggiatoa sp. 'Guaymas Basin orange filament' (JN793555, 1361)
Candidatus Parabeggiatoa sp. `Chile` (FJ875196, 1496)
Candidatus Parabeggiatoa sp. `Chile` (FJ875199, 1496)
Candidatus Parabeggiatoa sp. `Chile` (FJ875197, 1496)
Candidatus Parabeggiatoa sp. `Chile` (FJ875198, 1496)
9 Thioploca spp. (incl. L40998)
5 Candidatus Thiophysa hinzei (incl. FR690982)
17 Candidatus Thiopilula aggregata (incl. FR690986)
10 Candidatus Halobeggiatoa borealis (incl. FR847870)
Candidatus Isobeggiatoa divolgata `Dangast` (AF532769, 1502)
Candidatus Isobeggiatoa divolgata `Limfjorden` (AF532775, 1496)
Candidatus Isobeggiatoa sp. 'Eckernfoerde Bay', genome (ABBZ01003263, 588*)
Candidatus Isobeggiatoa sp. `Smeerenburgfjorden` (FN561862, 1501)
Candidatus Isobeggiatoa sp. `Chile` (FJ875195, 1497)
Candidatus Isobeggiatoa sp. `Tokyo Bay` (AB108786, 1368)
7 Candidatus Marithrix spp. (incl. AY496953)
uncultured bacterium NY−R580 (FR847879, 1498)
uncultured bacterium HMG−W628 (FR847877, 1500)
uncultured bacterium from iron mat (FJ535296, 1363*)
uncultured bacterium NY−R718 (FR847878, 1498)
uncultured bacterium NY−R310 (FR847880, 1501)
uncultured bacterium from sulfidic cave biofilm (EF467566, 1500)
uncultured bacterium ST−S134 (FR847881, 1495)
Candidatus Allobeggiatoa sp. clone SBYC5437 (JN447681, 1419)
Candidatus Allobeggiatoa sp. clone SBXZ4290 (JN435505, 1394)
Candidatus Allobeggitoa sp. clone SBYC4674 (JN446970, 1443)
Candidatus Allobeggiatoa sp. clone SBYB6814 (JN443279, 1393)
Candidatus Allobeggiatoa sp. GN8M clone209 (FR687034, 1493)
Candidatus Allobeggiatoa sp. clone SBYC4886 (JN447167, 1271)
Candidatus Allobeggiatoa sp. GN8C clone917 (FR687035, 1491)
Candidatus Allobeggiatoa sp. GN8C clone764 (FR687036, 1492)
Candidatus Allobeggiatoa sp. clone SBYB6341 (JN442851, 1426)
Candidatus Allobeggiatoa salina, filament I27 from hypersaline enrichment culture '60Ibiz' (unpublished, 1422)
Candidatus Allobeggiatoa salina, filament Z31 from hypersaline enrichment culture '150Ibiz' (unpublished, 1378)
Candidatus Allobeggiatoa salina I6C clone1149 (FR687025, 1461)
Candidatus Allobeggiatoa salina 1124 (EU919200, 1492)
Candidatus Allobeggiatoa salina `Chiprana` (EF428583, 1489)
Candidatus Allobeggiatoa salina LC10C clone324 (FR687027, 1491)
Candidatus Allobeggiatoa salina F15C clone872 (FR687024, 1492)
Candidatus Allobeggiatoa salina F15C clone897 (FR687026, 1491)
Candidatus Allobeggiatoa salina, filament C31 from hypersaline enrichment culture '80Chip' (unpublished, 1405)
Candidatus Allobeggiatoa salina I6C_clone1167 (FR687028, 1070*)
Candidatus Allobeggiatoa salina I6C_clone1156 (FR687029, 1033*)
Candidatus Allobeggiatoa salina I15C_seq88 (FR687031, 720*)
Candidatus Allobeggiatoa salina I15M_seq134 (FR687030, 910*)
Candidatus Allobeggiatoa salina M8C_clone196 (FR687033, 581*)
Candidatus Allobeggiatoa salina I6M_seq129 (FR687032, 695*)
Beggiatoa alba B15LD (FR690999, 1511)
Beggiatoa alba B15LD (unpublished, 1527)
Beggiatoa alba B18LD, genome (AHMA01000017, 1521)
Beggiatoa alba B15LD (unpublished, 1527)
Beggiatoa alba B18LD (unpublished, 1528)
Beggiatoa alba B18LD (AF110274, 1491)
Beggiatoa alba OH−75−2a (AF110273, 1491)
Beggiatoa alba B15LD (unpublished, 1527)
Beggiatoa alba B15LD (unpublished, 1527)
Beggiatoa alba B15LD (L40994, 1462)
Beggiatoa sp. 402 (AY583996, 1416)
Beggiatoa sp. 401 (AY583995, 1421)
Beggiatoa sp. 1401−13 (L40997, 1473)
Beggiatoa sp. AA5A (AF110275, 1482)
Beggiatoa sp. LPN (EU015402, 1490)
uncultured Beggiatoa sp. HMW−S2139 (FR847884, 1496)
uncultured Beggiatoa sp. HMW−S1664 (FR847886, 1496)
uncultured Beggiatoa sp. HMG−W638 (FR847882, 1496)
uncultured Beggiatoa sp. HMW−S2239 (FR847885, 1495)
uncultured Beggiatoa sp. HMW−S2670 (FR847887, 1496)
Beggiatoa sp. Arauama II (GU117707, 1495)
Beggiatoa sp. Arauama I (GU117706, 1495)
Beggiatoa sp. MS−81−6 (AF110277, 1500)
Beggiatoa sp. 35Flor (FR717278, 1464)
uncultured Beggiatoa sp. HMW−S1928 (FR847883, 1496)
Beggiatoa sp. enrichment culture DGGE band CSO2 (JN588607, 579*)
Beggiatoa sp. enrichment culture DGGE band CSO4 (JN674459, 545*)
Beggiatoa sp. CSO1 (HM598303, 1411)
Beggiatoa sp. MS−81−1c, AF110276, 1503
Achromatium oxaliferum, L42543, 1489
Thiothrix nivea, L40993, 1427
0.10

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Chapter 5

General discussion of the present work and perspectives

Large sulfur bacteria of the family *Beggiatoaceae* are frequently encountered in sulfidic habitats, where they can contribute significantly to chemosynthetic (primary) production and sulfide detoxification. Accordingly, their most prominent attribute, which is referred to in virtually all studies dealing with these bacteria, is their ability to oxidize reduced sulfur compounds, using either oxygen or nitrate as an electron acceptor. Yet, previous studies have shown that the metabolisms of *Beggiatoaceae* are in fact a lot more complex and able to adapt dynamically to changing environmental conditions. This thesis focusses on exploring different aspects of the dissimilatory metabolism of these sulfur bacteria and thereby contributes to a more comprehensive understanding of their physiology and ecology (summarized in **Figure 5.1**). The main findings of the included studies have been discussed in the previous sections and will only be recapitulated shortly where appropriate. Rather, this chapter will present a synthesizing discussion of the obtained results and supported hypotheses in the context of energy acquisition.

Reduced sulfur compounds are surely the most important electron donors for members of the family *Beggiatoaceae* and the first of the here included studies (**Section 2**) is centered on the oxidative sulfur metabolism of these bacteria. Considering genomic, phylogenetic, biochemical, and physiological data, we pointed

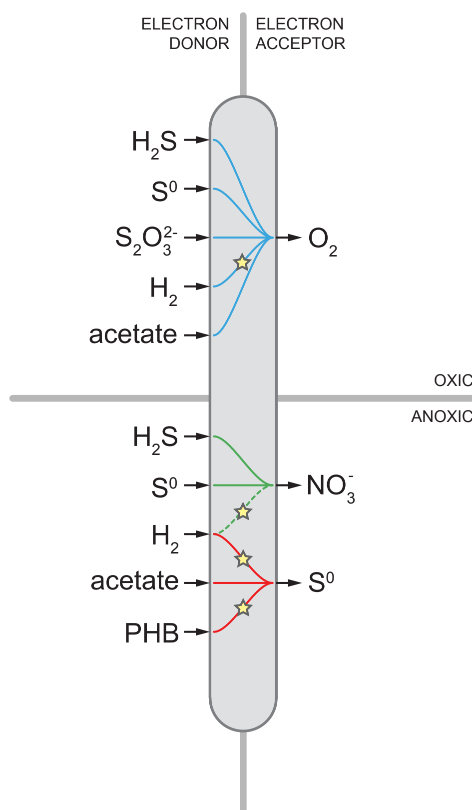

Figure 5.1 | Dissimilatory reactions known or thought to be performed by members of the family *Beggiatoaceae* under oxic and anoxic conditions. The schematic drawing does not consider the stoichiometries of the reactions; CO_2 and N_2 assimilation (Nelson et al., 1982; Nelson and Jannasch, 1983) are not included as electron-accepting processes. Solid lines represent reactions, which are experimentally supported, dashed lines reactions, which are suggested by genomic data. Stars indicate reactions, which are discussed in this thesis. Several other organic electron donors, such as pyruvate, lactate, malate, succinate, fumarate, methanol, and ethanol, are used by different members of the *Beggiatoaceae* (e.g. Pringsheim, 1964; Burton and Morita, 1964; Nelson and Castenholz, 1981a; Mezzino et al., 1984; Jewell et al., 2008) but these are not included in the scheme.

out metabolic pathways, which have likely been present in the last common ancestor of the family, pathways, which apparently make up the common metabolic core of the extant *Beggiatoaceae*, as well as pathways, which seem to contribute to the metabolic distinctiveness of the family's different strains. Using this information we will give an estimate of how much energy members of the family *Beggiatoaceae* could gain from the oxidation of reduced sulfur compounds (**Sections 5.1.2** and **5.2.2**).

In the past, physiological studies have reported on several metabolic properties of *Beggiatoaceae*, which were often disregarded or mentioned only in passing in later publications. Among these properties is the anaerobic respiration with stored elemental sulfur (Nelson and Castenholz, 1981b; Schmidt et al., 1987) and the oxidation of molecular hydrogen under anoxic conditions (Schmidt et al., 1987). We studied sulfur respiration and hydrogen oxidation in members of the family *Beggiatoaceae* in more detail (**Sections 3**, **4.1**, and **4.2**) and provided evidence that both—alone and in combination—have the potential of significantly increasing the ecophysiological plasticity of these bacteria. The oxidation of molecular hydrogen is the major topic of this work and the energy, which *Beggiatoaceae* could gain from this reaction will be compared with the estimated energy yield from the oxidation of sulfur substrates (**Sections 5.1.2** and **5.2.2**).

5.1 Thermodynamic considerations

If the amount of energy a chemical reaction can supply to an organisms is to be expressed in quantitative terms, the reaction's change in Gibbs energy (ΔG^{0}') is usually referred to. Reactions with a negative ΔG^{0}' are exergonic and thus theoretically able to supply an organism with energy under the given conditions. Reactions with a positive ΔG^{0}' are endergonic and will not proceed spontaneously. However, changes in the concentrations of reactants and products (as well as temperature and pressure) can render an endergonic reaction exergonic and vice versa, in particular if the reaction is close to the thermodynamic equilibrium. **Table 5.1** gives an overview over the ΔG^{0}' values of different redox reactions involving sulfur compounds or molecular hydrogen, which have been discussed in the previous sections. In all cases, the ΔG^{0}' value of the complete reaction is shown as well as ΔG^{0}' values normalized to (i) one mole of electrons transferred, (ii) one mole of electron donor oxidized, and (iii) one mole of electron acceptor reduced. These normalized values can be used to compare the energy yields of the tabulated reactions from different perspectives. In the first set of reactions (**Table 5.1**) oxygen serves as the electron acceptor; sulfur is the electron acceptor in the second set and these reactions will be considered at first.

Table 5.1 | Change in Gibbs energy (ΔG^{0}') for the oxidation of sulfide, sulfur, hydrogen, and 3-hydroxybutyrate with oxygen and elemental sulfur. Values for ΔG^{0}' (25°C, pH 7) were calculated with ΔG_f^0 values listed in Thauer et al. (1977) according to the equation ΔG^{0}' $= \Sigma\ \Delta G_f^0$ (products) - $\Sigma\ \Delta G_f^0$ (reactants); ΔG_f^0 values for $O_{2(aq)}$ and $H_{2(aq)}$ are from Stumm and Morgan (1996). The number of electrons transferred in a reaction (n) is given. The formula $C_4O_3H_7^-$ stands for 3-hydroxybutyrate, the monomer of PHB, i.e. the reaction does not include the depolymerization of PHB.

Reaction		n	ΔG^{0}' in kJ normalized to			
			Reaction	mol e⁻	mol e⁻ donor	mol e⁻ acceptor
$HS^- + 2\,O_2$	$\rightarrow SO_4^{2-} + H^+$	8	-829.19	-103.65	-829.19	-414.60
$2\,HS^- + O_2$	$\rightarrow 2\,S^0 + 2\,OH^-$	4	-435.04	-108.76	-217.52	-435.04
$2\,S^0 + 3\,O_2 + 2\,H_2O$	$\rightarrow 2\,SO_4^{2-} + 4\,H^+$	12	-1223.34	-101,95	-611,67	-407,78
$2\,H_2 + O_2$	$\rightarrow 2\,H_2O$	4	-525.82	-131.45	-262.91	-525.82
$C_4O_3H_7^- + 9\,S^0 + 9\,H_2O$	$\rightarrow 4\,HCO_3^- + 9\,HS^- + 12\,H^+$	18	-76.49	-4.25	-76.49	-8.50
$H_2 + S^0$	$\rightarrow HS^- + H^+$	2	-45.39	-22.70	-45.39	-45.39

5.1.1 Sulfur respiration

Sections 3 and **4.1** describe the reduction of stored elemental sulfur with stored poly(3-hydroxybutyrate) (PHB) and molecular hydrogen, respectively. Under standard conditions the reduction of sulfur either hydrogen or 3-hydroxybutyrate, the monomer of PHB, is exergonic but rather close to the thermodynamic equilibrium (**Table 5.1**). Thus, it appears that these reaction will at most be able to support a rudimentary energy metabolism in order to allow the survival of *Beggiatoaceae* under short-term anoxic conditions, but will not be able to fuel a substantial growth. However, the *Beggiatoa* strain OH-75-2a was reported to grow well with externally supplied acetate when respiring with stored sulfur (Nelson and Castenholz, 1981b) and this reaction is likewise close to the thermodynamic equilibrium under standard conditions ($C_2H_3O_2^-+4\,H_2O+4\,S^0 \longrightarrow 2\,HCO_3^-+4\,HS^-+5\,H^+$; ΔG^{0}' = –6.73 kJ/ reaction). This suggests that *Beggiatoaceae* could in fact also conserve energy from the reduction of sulfur with 3-hydroxybutyrate or molecular

hydrogen *in vivo*. Even if any of these reactions would yield less energy than the oxidation of acetate in the above experiment, the conserved energy might still be sufficient. This is because, from an ecological perspective, actual growth under conditions requiring sulfur respiration will most likely not be important for the *Beggiatoaceae* at large. Most members of the family will not be exposed to such conditions for a prolonged time either because the biogeochemical gradients change constantly, e.g. on a daily basis, or because nitrate can be used as electron acceptor instead. Moreover, long-term growth by sulfur respiration would ultimately be precluded by the eventual exhaustion of stored sulfur. Energy for maintenance and motility purposes will, however, be required also under short-term anoxic conditions for survival and the ability to move within the biogeochemical gradients in order to find a position with more favorable conditions. If this energy cannot be provided by nitrate respiration, sulfur respiration might still suffice to meet this energy demand.

A different electron-donating reaction, which is coupled to sulfur reduction and energy conservation has been described in *Chromatium* sp. 6412 (van Gemerden, 1986). The strain generates storage carbohydrates via photosynthesis in the light and converts these to PHB in the dark. This reaction produces ATP and releases electrons, the latter of which are transferred to elemental sulfur, producing sulfide. Glycogen storage has been reported for *Thiomargarita* spp. (Schulz and Schulz, 2005) and genes encoding glycogen-metabolizing enzymes have been found in the genomes of '*Candidatus* Isobeggiatoa sp.' and '*Ca.* Parabeggiatoa sp.' (Mußmann et al., 2007). Thus, the endogenous energy metabolism of *Beggiatoaceae* under conditions requiring sulfur respiration might not be limited to the breakdown of stored PHB but could likewise involve stored glycogen. However, we have not tested this hypothesis, so far.

Besides supplying *Beggiatoaceae* with energy, all of these sulfur-reducing reactions may contribute to the maintenance of cell integrity under highly sulfidic conditions by disposing of excess elemental sulfur (discussed in **Sections 3** and **4**). Hydrogenases, which most probably catalyze the oxidation of hydrogen with sulfur, seem to be ubiquitous in *Beggiatoaceae* (**Section 4.2**), suggesting that the ability to

perform this reaction is advantageous for probably all members of the family, even those, which can respire with nitrate. The dual function of hydrogen-fueled sulfur respiration could be an explanation for the apparent importance of this enzyme in the *Beggiatoaceae*.

5.1.2 Oxidation of sulfur substrates and molecular hydrogen with oxygen

Many sulfur bacteria of various phylogenetic origins and life strategies have been demonstrated to consume molecular hydrogen (see page 149) so that it appears that hydrogen oxidation could, in fact, be a common metabolic trait of this group. In order to estimate the importance of molecular hydrogen for the *Beggiatoaceae*—and sulfur bacteria in general—from an energetic point of view, the changes in Gibbs energy of these reactions will be compared.

The aerobic oxidation of both, sulfur substrates and molecular hydrogen, is strongly exergonic under standard conditions (**Table 5.1**) and thus very likely able to support growth also *in situ*. When normalized to the amount of electron acceptor reduced, the ΔG^{0}' values of these reactions are rather similar (about -400 to -530 kJ mol^{-1} oxygen under the given conditions), but the most exergonic reaction is the oxidation of molecular hydrogen with –525 kJ mol^{-1} oxygen. This suggests that hydrogen consumption may allow for the conservation of more energy than the oxidation of sulfide or elemental sulfur with a given amount of oxygen. Further, the very low redox potential of the H^{+}/H_2 couple (E_0' = –414 mV; Thauer et al., 1977) suggests that electrons from hydrogen could be used to reduce pyridine nucleotides directly (E_0' = –320 mV for NAD^{+}/NADH and E_0' = –327 mV for $NADP^{+}$/NADPH; Barton, 2005). A direct reduction of NAD^{+} or $NADP^{+}$ is not possible with reduced sulfur compounds (E_0' = –250 mV for S^{0}/H_2S, E_0' = +50 mV for SO_3^{2-}/S^{0}, and E_0' = –280 mV for SO_4^{2-}/SO_3^{2-}; Barton, 2005) and would thus render the oxidation of hydrogen more energy efficient.

It has to be kept in mind, however, that values like ΔG^{0}' and E_0' describe a reaction appropriately in physicochemical terms but do not adequately reflect it in a biochemical context. While setting the limits for which reactions are possible, i.e. occur spontaneously, under a given set of conditions, these values provide merely an orientation with respect to how much energy an organism is in fact able to conserve from a given reaction. In the following sections, the energy gain from the oxidation of reduced sulfur compounds and molecular hydrogen will be discussed on a more biochemical basis.

5.2 Biochemical considerations

Cells conserve energy from substrate oxidation via two different procedures, substrate level phosphorylation and electron transport phosphorylation. During substrate level phosphorylation, ATP is formed by direct transfer of a phosphoryl group from an high-energy reaction intermediate to ADP. In contrast, electron transport phosphorylation at ATP synthases is driven by an electrochemical potential, in which the energy from substrate oxidation is transiently stored. The electrochemical potential is usually realized in a proton gradient over the cytoplasmic membrane, but protons are replaced by Na^+ in some bacteria (Dimroth et al., 2006). Substrate level phosphorylation happens only in a single reaction of the oxidative sulfur metabolism in the family *Beggiatoaceae*, the phosphorolysis of APS to sulfate and ATP, and does not occur during the oxidation of molecular hydrogen. Hence, the largest part of the energy from the oxidation of sulfur substrates and molecular hydrogen is initially conserved in an electrochemical potential, which will here be referred to as a proton gradient. In order to estimate to which extent the oxidation of a substrate contributes to the proton gradient, and eventually to electron transport phosphorylation, several aspects have to be considered:

- What are the substrate-oxidizing enzymes? Where are their active sites localized, and does the reaction produce or consume protons in the periplasm or cytoplasm?

- To which electron carrier are the electrons transferred?
- Through which complexes of the electron transport chain do the electrons pass and at which H^+/e^- ratio do these complexes translocate protons?
- At which ATP/H^+ ratio does the ATP synthase generate ATP?

It is obvious that such an estimation of energy conservation from a given reaction requires a lot of knowledge about the pathways and the organisms in question. We presented information on the enzymes, which members of the family *Beggiatoaceae* use for oxidizing sulfur substrates and molecular hydrogen, in **Sections 2** and **4.2**, respectively. Information on the usual subcellular localization of these enzymes as well as the type of electron carriers they typically interact with are given either there or in the introduction (**Sections 1.3** and **1.5**). The proton-translocating electron transport complexes of the respiratory chain, which are present in *Beggiatoaceae*, will be discussed in the following section.

5.2.1 Membrane complexes involved in electron transport and proton motive force generation

We limit our estimation of energy yield to the aerobic oxidation of reduced sulfur compounds and molecular hydrogen; other electron acceptors will not be considered. Electrons from both, reduced sulfur compounds and molecular hydrogen, most likely enter the electron transport chain of *Beggiatoaceae* at the levels of quinone or cytochrome *c*. Accordingly, quinol-oxidases and cytochrome *c*-oxidases will contribute to the generation of a proton motive force when these substrates are oxidized. It should be mentioned, that some types of hydrogenases have the potential of catalyzing redox reactions between molecular hydrogen and pyridine nucleotides. Such hydrogenases have been identified in members of the family *Beggiatoaceae* (Hyh-hydrogenases; **Section 4.2**), but they are usually not considered to support growth on hydrogen. Nevertheless, we included the channeling of electrons from hydrogen via NADH into the respiratory chain for comparison reasons in **Table 5.3** (assuming a translocation of $2H^+/e^-$ at the NADH dehydrogenase (Nuo); Friedrich et al., 1995).

Table 5.2 lists genes from the six available *Beggiatoaceae* draft genomes, which putatively encode subunits of quinol- and cytochrome *c*-oxidases. Genes encoding subunits of a cytochrome *c*-reducing quinol oxidase (cytochrome bc_1 complex), were identified five out of the six analyzed draft genomes (*Beggiatoa* sp. 35Flor, '*Ca.* Isobeggiatoa sp.', *Beggiatoa alba* B18LD, the Guaymas filament, '*Ca.* Thiomargarita nelsonii'); genes encoding for subunits of an oxygen-reducing quinol-oxidase were so far not found. Two protons per transferred electron are thought to be translocated at the cytochrome bc_1 complex (Mitchell, 1975a,b; Brown and Brand, 1985).

Genes encoding two distinct types of cytochrome *c* oxidases, aa_3 and cbb_3, were identified in the investigated *Beggiatoaceae* genomes (see also Mußmann et al., 2007) and a gene encoding the subunit I of a cbb_3-type oxidase has further been amplified from genomic DNA of *Beggiatoa* sp. D-402 (Muntyan et al., 2005). Studies with other bacteria showed that cbb_3-type oxidases are typically expressed only under micro-oxic conditions and have an about 10–100-fold higher affinity to oxygen than aa_3-type oxidases (reviewed by Pitcher and Watmough, 2004). Correspondingly, the cbb_3-type oxidase of *Beggiatoa* sp. D-402 was expressed preferentially under micro-oxic conditions, while the aa_3-type oxidase was induced at higher oxygen levels (Muntyan et al., 2005). Similar to other bacteria, the two terminal oxidases of *Beggiatoaceae* are thus likely responsible for aerobic respiration under high and low oxygen concentrations, respectively (Muntyan et al., 2005; Mußmann et al., 2007). Many of the so far studied *Beggiatoaceae* prefer micro-oxic growth conditions (Teske and Nelson, 2006) and a negative chemotactic response of filamentous *Beggiatoaceae* to oxygen has been reported (Møller et al., 1985; Hüttel et al., 1996). Consistent with this preference for micro-oxic conditions, cbb_3-type oxidases appear to be ubiquitous in *Beggiatoaceae* as suggested by the identification of respective genes in five of the six investigated draft genomes (*Beggiatoa* sp. 35Flor, '*Ca.* Isobeggiatoa sp.', *Beggiatoa alba* B18LD, the Guaymas filament, and '*Ca.* Thiomargarita nelsonii'; **Table 5.2**; see also Mußmann et al., 2007; Winkel et al., 2013). Even though favoring micro-oxic conditions, members of the *Beggiatoaceae* typically live in very steep oxygen gradients and thus are likely to experience substantial changes in oxygen concentrations when environmental

parameters fluctuate only moderately. Accordingly, it appears advantageous to be equipped with cytochrome *c* oxidases optimized to function under higher oxygen concentrations. Subunits of an aa_3-type oxidases were so far only detected in the genomes of '*Ca.* Isobeggiatoa sp.' and '*Ca.* Thiomargarita nelsonii' (**Table 5.2**; Mußmann et al., 2007; Winkel et al., 2013) and biochemical evidence suggests the presence of a respective enzyme in *Beggiatoa* sp. D-402 (Muntyan et al., 2005). We do not know whether the presence of aa_3-type oxidases is limited to strains, which experience high oxygen concentrations on a more regular basis, but this is most likely the case for *Thiomargarita* spp., which are regularly exposed to high oxygen concentrations e.g. during resuspension events in the water column (Schulz et al., 1999). The H^+/e^- pumping ratio of cbb_3- and aa_3-type cytochrome *c*-oxidases is discussed in **Section 5.2.2**.

Table 5.2 | Genes from *Beggiatoaceae* genomes predicted to encode for proteins involved in quinol and cytochrome *c* oxidation. The letter code in the locus or contig name serves as a species identifier ('FLOR' for *Beggiatoa* sp. 35Flor, 'BGP' for '*Ca.* Isobeggiatoa sp.', 'BGS' for '*Ca.* Parabeggiatoa sp.', 'BA' for *Beggiatoa alba* B18LD, 'THI' for '*Ca.* Thiomargarita nelsonii', and 'BOGUAY' for the Guaymas filament). Amino acid (AA) counts in parentheses denote truncated sequences, which reside at an end of a contig.

Product	Gene	EC	Locus	Contig	AA
quinol-cytochrome-*c* reductase	*petA*	1.10.2.2	FLOR_00183	RL501	203
(bc_1 complex),			BGP_0838	contig01043_0838–0839	(58)
iron-sulfur subunit			BA01_14	BA01	199
			BOGUAY_0396	contig01232	216
			THI736_1	THI736	(121)
ubiquinol-cytochrome-*c* reductase	*petB*	1.10.2.2	FLOR_00184	RL501	404
(bc_1 complex),			BGP_0839	contig01043_0838–0839	(115)
cytochrome *b* subunit			orf15_glimmer3	BA01	419
			BOGUAY_0395	contig01232	408
			THI736_0	THI736	408
quinol-cytochrome *c* reductase,	*petC*	1.10.2.2	FLOR_00185	RL501	243
cytochrome *c* subunit			BGP_6663	contig25875_6663	(94)
			orf16_glimmer3	BA01	251
			THI361431872	THI736	(142)
			THI471_0	THI471	100
cytochrome-*c* oxidase aa_3,	*coxA*	1.9.3.1	BGP_2866	contig21849_2863–2866	525
subunit I			THI509_0	THI509	(236)
cytochrome *c* oxidase aa_3,	*coxB*	1.9.3.1	BGP_2865	contig21849_2863–2866	256
subunit II			THI2043_0	THI2043	260
cytochrome-*c* oxidase aa_3,	*coxC*	1.9.3.1	BGP_2863	contig21849_2863–2866	270
subunit III			TH195312791821	THI1953	(92)

(Continued on next page.)

Table 5.2 (Continued from previous page.)

Product	Gene	EC	Locus	Contig	AA
cytochrome-*c* oxidase *cbb*$_3$, subunit I	*ccoN*	1.9.3.1	FLOR_00924	RL5118	478
			orf210_glimmer3	BA01	490
			BOGUAY_3546	contig00628	483
			THI267_0	THI267	(132)
			THI97913441283	THI979	(113)
cytochrome-*c* oxidase *cbb*$_3$, subunit II	*ccoO*	1.9.3.1	FLOR_00573	RL506	(134)
			FLOR_00923	RL5118	192
			BGP_3209	contig22214_3205–3209	205
			orf211_glimmer3	BA01	205
			BOGUAY_3547	contig00628	205
			THI267_1	THI267	205
cytochrome-*c* oxidase *cbb*$_3$, subunit III	*ccoP*	1.9.3.1	FLOR_00571	RL506	308
			orf213_glimmer3	BA01	309
			BGP_3207	contig22214_3205–3209	308
			BOGUAY_3549	contig00628	312
			THI267_3	THI267	301
cytochrome-*c* oxidase *cbb*$_3$, subunit IV	*ccoQ*	1.9.3.1	FLOR_00572	RL506	35
			orf212_glimmer3	BA01	63
			BGP_3208	contig22214_3205–3209	60
			BOGUAY_3548	contig00628	44
			THI267_2	THI267	59

5.2.2 Estimation of the energy yield from the oxidation of sulfur substrates and molecular hydrogen in ATP equivalents

We were able to base our estimation of the biochemically possible energy yield from the aerobic oxidation of sulfur substrates and molecular hydrogen on a substantial amount of data, which we derived from our and previous studies on members of the family *Beggiatoaceae* and other organisms. The results of our calculations are shown in **Table 5.3**, but it has to be mentioned that the actual energy yield may be different due to variables which are unknown or which we could not account for adequately. Before considering the results of our calculations, we discuss the three most speculative aspects of our calculations and explain how we dealt with these.

The first speculative aspect concerns the process by which periplasmically stored, elemental sulfur is activated to enter the rDSR pathway. The mode of sulfur activa-

tion is important for our calculations, because it directly influences the net energy gain of the rDSR-catalyzed reaction. Despite extensive research on the rDSR pathway, the mechanism of sulfur activation has still not been fully resolved. The current model envisages a reductive activation of sulfur to the formal oxidation state of sulfide (S^{2-}) and free sulfide or a DsrC-bound persulfide have been proposed to represent this activated form (Cort et al., 2008). The electrons required for activation were suggested to be delivered by the putative NADH:acceptor oxidoreductase DsrL, which uses NADH as an electron donor (Dahl et al., 2005) or the DsrMKJOP transmembrane complex, which is thought to derive electrons from an unspecified periplasmic sulfur substrate (Grein et al., 2010a,b). In both cases, we do not know if and how much energy would be turned over for sulfur activation. If NADH served as an electron donor, the generation of two activation electrons would likely require at least four protons from the proton gradient to flow in the reverse direction through the NADH dehydrogenase (the amount of protons translocated by two electrons transported forward). We used this energy for the provision of two activation electrons, but the mechanistic details of the reverse electron flow are unknown (Barton, 2005) and the required energy could well be higher. **Figure 5.2** explains how we included the energy required for sulfur activation in our calculations.

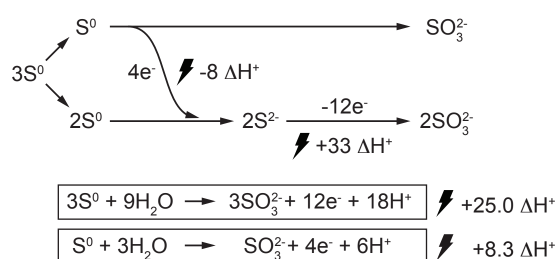

Figure 5.2 | Calculation of the net energy gain of sulfur oxidation via the rDSR pathway. For calculation of the net energy gain of the rDSR pathway, we treat the activation electrons as originating from sulfur itself. The activation energy ($\Delta H^+ = -8$) represents the minimum energy likely required to pump four electrons in reverse through the NADH-dehydrogenase (see text). The contribution of sulfur oxidation via the rDSR ($\Delta H^+ = +33$) to the proton motive force results from the release of $18H^+$ in the cytoplasm ($\Delta H^+ = -9$; compare **Table 5.3**) and the translocation of $24H^+$ at the cytochrome bc_1 complex ($\Delta H^+ = +24$). At the terminal oxidase 12 protons are translocated ($\Delta H^+ = +12$) and another 12 are consumed in the cytoplasm ($\Delta H^+ = +6$). Thus, the net contribution of sulfur oxidation via the rDSR to the proton motive force would be 25 ΔH^+ (33–8) for the oxidation of $3S^0$ and 8.33 ΔH^+ (25/3) for the oxidation of $1S^0$.

Table 5.3 | Estimated energy yield from the aerobic oxidation of reduced sulfur compounds and molecular hydrogen by members of the family *Beggiatoaceae*. Reactions catalyzed by sulfide:quinone oxidoreductase (SQR), flavocytochrome *c*-sulfide dehydrogenase (FCSD), reverse dissimilatory sulfite reductase (rDSR), sulfite dehydrogenase (SorAB type), APS reductase, ATP sulfurylase, the truncated thiosulfate-oxidizing multienzyme complex (SoxAXBYZ), and hydrogenases of the Hup- and Hyh-types are shown. The first five numbers in each row state how much electrons are transferred to which electron acceptors, how the production of protons during substrate oxidation contributes to the proton motive force, and how much ATP is produced by substrate level phosphorylation. The following three numbers give the amount of protons translocated at the NADH-dehydrogenase (Nuo), the quinol:cytochrome *c* oxidoreductase (bc_1), and the terminal oxidases (aa_3 or cbb_3). The next three rows give the total contribution of a reaction to the proton motive force, the ATP production possible from this ΔH^+ as well as the total net energy yield of the reaction in ATP equivalents. The enzymes are numbered according to the principal reaction, which they catalyze, i.e. sulfide to sulfur (1), sulfur to sulfite (2), sulfite to sulfate (3), thiosulfate to sulfur/sulfate (4), and hydrogen to protons (5); alternative pathways are specified with small letters. If alternative enzymes are listed, the one which seems to be more likely to catalyze the reaction in the *Beggiatoaceae* at large is marked with an asterisk (*).

#	Enzyme	Reaction		From reaction					ΔH^+ from ETC			Total	ATP from	Total
				NAD^+	Q	cyt *c*	ΔH^{+} [a]	ATP	Nuo	bc_1	aa_3/cbb_3	ΔH^+	ΔH^+	ATP
1a	SQR*	H_2S	$\rightarrow S^0 + 2\,e^- + 2\,H^+$	–	2	–	1	–	–	4	3	8	2.42	**2.42**
1b	FCSD	H_2S	$\rightarrow S^0 + 2\,e^- + 2\,H^+$	–	–	2	1	–	–	–	3	4	1.21	**1.21**
2	rDSR	$S^0 + 3\,H_2O$	$\rightarrow SO_3^{2-} + 4\,e^- + 6\,H^+$	–	4	–	-3	–	-2.67 [b]	8	6	8.33	2.52	**2.52**
3a	sulfite dehydrogenase*	$SO_3^{2-} + H_2O$	$\rightarrow SO_4^{2-} + 2\,e^- + 2\,H^+$	–	–	2	1	–	–	–	3	4	1.21	**1.21**
3b	APS reductase	$SO_3^{2-} + AMP$	$\rightarrow APS + 2\,e^-$	–	2	–	–	–	–	4	3	7	2.12	**2.12**
3b	ATP sulfurylase	$APS + PP_i$	$\rightarrow SO_4^{2-} + ATP$	–	–	–	–	1	–	–	–	–	–	**1.00**
4	SoxAXBYZ	$S_2O_3^{2-} + H_2O$	$\rightarrow SO_4^{2-} + S^0 + 2\,e^- + 2\,H^+$	–	–	2	1	–	–	–	3	4	1.21	**1.21**
5a	Hydrogenase (Hup)*	H_2	$\rightarrow 2\,e^- + 2\,H^+$	–	2	–	1	–	–	4	3	8	2.42	**2.42**
5b	Hydrogenase (Hyh)	H_2	$\rightarrow 2\,e^- + 2\,H^+$	2	–	–	-1	–	4	4	3	10	3.03	**3.03**

[a] In contrast to proton translocation, the production of a proton in the periplasm or cytoplasm contributes only with $\pm\frac{1}{2}H^+$ to the proton motive force.
[b] See **Figure 5.2**.

The second aspect associated with some uncertainty is the channeling of electrons from the rDSR and the APS reductase into the electron transport chain. Usually, quinones are assumed to be the electron acceptors of both enzymes (Dahl et al., 2008) but the mechanisms of the respective electron transfer pathways are unclear (discussed in **Sections 1.3.2** and **2**). We tentatively assume quinones as electron acceptors for our calculations, as the cytoplasmic localization of both enzymes favors quinones over cytochrome *c*. Nevertheless, electron transfer to other cytoplasmic electron carriers is still possible.

The third somewhat vague assumption concerns the efficiency of energy conservation at the electron transport complexes of the respiratory chain, in particular the cytochrome *c* oxidases. For both types of cytochrome *c* oxidases, which were identified in members of the *Beggiatoaceae*, i.e. aa_3 and cbb_3, variable H^+/e^- pumping stoichiometries with a maximum of 1 were reported (e.g. Capitanio et al., 1996; de Gier et al., 1996; Pitcher and Watmough, 2004). This variability is likely associated with the presence of different electron transfer pathways within each complex. In the case of the cytochrome aa_3 complex the presence of a coupled and an uncoupled electron transfer pathways was demonstrated by Capitanio et al. (1996), who showed that the H^+/e^- pumping ratio varied between 0 and 1, depending on the rate of electron flow through the enzyme and the transmembrane pH gradient. Thus, the energy conservation at the terminal oxidase is not a fixed value but will depend on the type of terminal oxidase used (energy conservation by cbb_3-type oxidase appears to be lower; Pitcher and Watmough, 2004) as well as the physiological status of the cell. For our calculations we assumed the maximum observed H^+/e^- pumping ratio of 1; the cytoplasmic consumption of protons associated with the production of water will contribute with an additional $\frac{1}{2}H^+/e^-$ to the proton motive force. Likewise, the ATP/H^+ ratio might be different than the here assumed value of 3.3 (Stock et al., 1999; 3–4 H^+/ATP are generally estimated).

With these uncertainties in mind, we estimated the energy yields of sulfur compound and hydrogen oxidation (**Table 5.3**). According to our calculations, the maximum energy yield from the oxidation of sulfide to sulfate (SQR, rDSR, APS reductase, ATP sulfurylase) would be 8.06 ATP equivalents, the minimum yield

4.94 ATP equivalents, i.e. 39% less energy (FCSD, rDSR, sulfite dehydrogenase). For two steps of the oxidative sulfur metabolism, alternative enzymes exist in members of the *Beggiatoaceae*. If FCSD instead of SQR catalyzes the oxidation of sulfide to sulfur, 50% less energy is conserved in this step (1.21 instead of 2.42 ATP equivalents); the oxidation of sulfite by sulfite dehydrogenase instead of APS reductase and ATP sulfurylase conserves 61% less energy (1.21 instead of 3.12 ATP equivalents). With respect to the complete oxidation of sulfide to sulfur the replacement of SQR by FCSD would result in an energy yield, which is 15% lower (6.85 ATP equivalents) than the maximum, while replacement of APS reductase and ATP sulfurylase by sulfite dehydrogenase would result in a 24% lower energy conservation (6.15 ATP equivalents). Hagen and Nelson (1997) reported on two *Beggiatoa* strains, which differed in growth yield as well as the pathways used for sulfite oxidation. One of these strains used a sulfite dehydrogenase for the oxidation of sulfite to sulfate, while the other one expressed an APS reductase and an ATP sulfurylase, in addition. Our calculations suggest, that the use of a more energy efficient sulfite oxidation pathway alone cannot result in the 2–3 times higher growth yield (normalized to oxygen) observed in the latter strain. Considering the data presented in **Section 2**, sulfide oxidation via SQR, rDSR, and sulfite dehydrogenase (6.15 ATP eqiovalents) may be regarded as the default pathway in the family *Beggiatoaceae* and we will use this pathway for comparing the oxidation of sulfur substrates with hydrogen oxidation.

Hydrogen oxidation via Hup-type hydrogenases is expected to yield 2.42 ATP equivalents. A 20% higher energy yield would be possible if the electrons were channeled into the electron transport chain via NADH by a Hyh-hydrogenase, but these enzymes are thought to produce NADPH for assimilatory purposes rather than NADH for respiration (Kanai et al., 2011). Assuming that Hup-hydrogenases are responsible for hydrogen oxidation, the reaction would yield exactly the same amount of metabolic energy as the oxidation of sulfide to sulfur (SQR), in contrast to what is suggested by the respective ΔG^{0}' and E_0' values (**Section 5.1.2**). The oxidation of 1 mol sulfide to sulfate (SQR, rDSR, sulfite dehydrogenase) would conserve even about 254% more energy than the oxidation of 1 mol H_2; i.e. about 2.5 mol hydrogen would need to be oxidized to yield the same energy as 1 mol

sulfide that is oxidized completely. In contrast, hydrogen oxidation is more energy efficient when normalized to the amount of oxygen consumed. By reducing 1 mol oxygen, hydrogen oxidation would yield 4.84 mol ATP equivalents, while the complete oxidation of sulfide to sulfate would yield only 3.08 mol ATP equivalents (36% less).

Figure 5.3 illustrates the relation of oxygen consumption and energy production by the oxidation of sulfide, sulfur, and molecular hydrogen in cultures of *Beggiatoa* sp. 35Flor (subfigure **A** is shown in **Section 4.1** on page 159). If energy production (**Figure 5.3 B**, **D**) rather than oxygen consumption (**Figure 5.3 A**, **C**) is considered, the overall pattern does not change pronouncedly. However, the relative importance of sulfur oxidation is lower while the relative importances of sulfide and hydrogen oxidation increase proportionally (**Figure 5.3 C**, **D**). This illustrates that the comparison of oxygen consumption rates can, in this case, be regarded as a reasonably good measure for the importance of each electron donor in energetic terms and we used this approach in **Section 4.1**. Nevertheless, the comparison of oxygen consumption rates takes a rather environmental, biogeochemical view of the organisms and their metabolisms while the comparison of energy yield is more adequate in biological terms. Yet, our calculation is an estimation, which will need to be improved when more is known about the pathways and enzymes involved.

5.3 Electron confurcation at Hyh-hydrogenases

An interesting, though entirely speculative, possibility of how hydrogen and sulfur metabolisms could interact in *Beggiatoaceae* is associated with their Hyh-hydrogenases. These enzymes have been discussed in **Section 4.2**, where we proposed that they produce NADPH for assimilatory purposes from the oxidation of molecular hydrogen, according to their function in *Thermococcus kodakarensis* (Kanai et al., 2011). However, Hyh-hydrogenases are enzymes, which can exchange electrons not only between two but three redox couples, $NAD(P)^+/NAD(P)H$, H^+/H_2, and S^0/H_2S (Ma et al., 1993, 1994, 2000), and the proposed scenario leaves

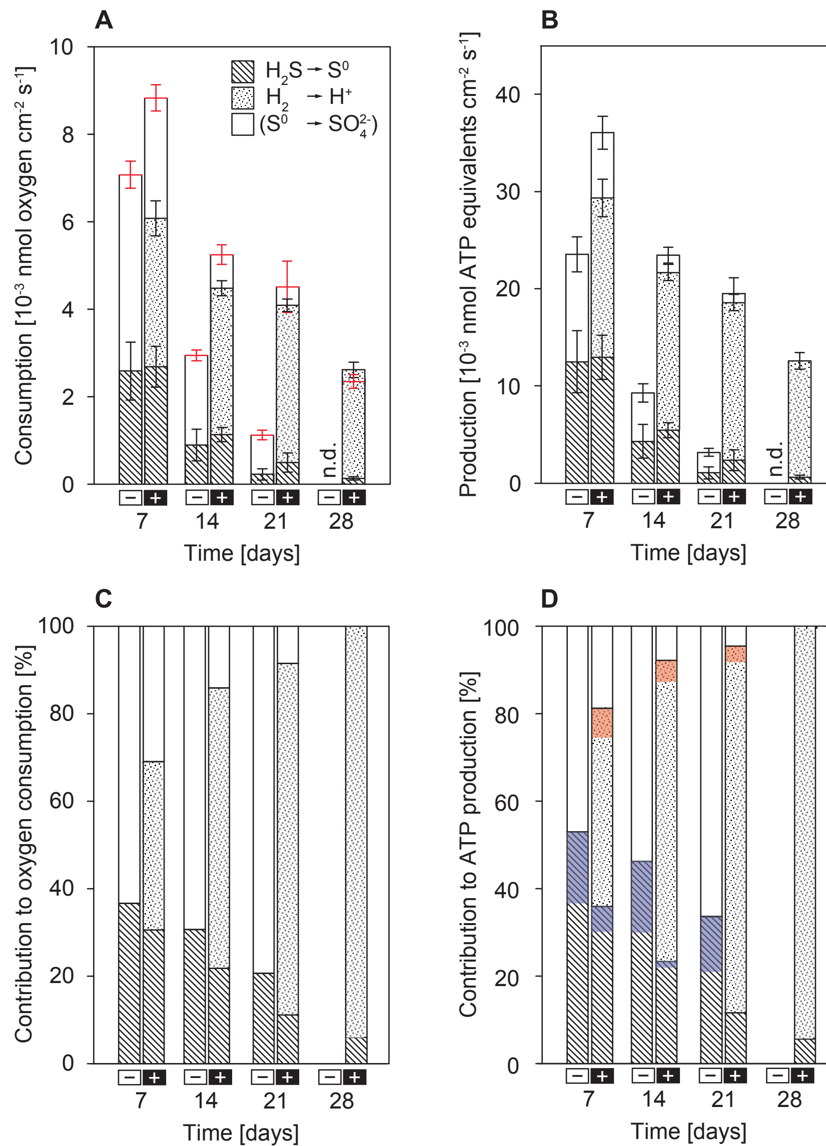

Figure 5.3 | Comparison of oxygen consumption by and energy yield in ATP equivalents from the oxidation of sulfide, sulfur, and hydrogen in *Beggiatoa* sp. 35Flor cultures. Data from hydrogen-supplemented (+) and -unsupplemented (–) cultures are shown. Hatched areas indicate the contribution of sulfide oxidation ($2\,H_2S + O_2 \longrightarrow 2\,S^0 + 2\,H_2O$), dotted areas represent the contribution of hydrogen oxidation ($2\,H_2 + O_2 \longrightarrow 2\,H_2O$), and white areas the contribution of sulfur oxidation ($2\,S^0 + 3\,O_2 + 2\,H_2O \longrightarrow 2\,SO_4^{2-} + 4\,H^+$; compare **Figure 4.6** on page 159). (**A**) Absolute contribution of each reaction to the measured oxygen consumption rate (indicated in red). (**B**) Absolute energy gain in ATP equivalents from each reaction. The energy gain was estimated from the oxygen consumption rates considering the stoichiometry of the reaction and the data presented in **Table 5.3**. (**C**) Relative contribution of each reaction to the total oxygen consumption. (**D**) Relative contribution of each reaction to the total energy yield. Colored areas denote the relative increase in the contribution of sulfide (blue) and hydrogen (red) oxidation when energy yield rather than oxygen consumption is considered.

no purpose for the enzyme's sulfur reductase activity. Hence, it is intriguing to speculate about an alternative route, in which the exergonic reduction of $NADP^+$ by H_2 is coupled to and drives the endergonic reduction of $NADP^+$ by reduced sulfur compounds. Such an electron confurcation process, i.e. the joint reduction of a compound with electrons from a favorable and an unfavorable donor, as well as the reverse reaction, electron bifurcation, have previously been discussed for a variety of enzyme systems (Herrmann et al., 2008; Li et al., 2008; Thauer et al., 2008; Schut and Adams, 2009; Wang et al., 2010; Kaster et al., 2011; Huang et al., 2012; Ramos et al., 2012). A detailed mechanistic review of electron bifurcation has recently been published by Nitschke and Russel (2011). Both processes are believed to rely on the presence of a flavin cofactor as a two-electron center and coupling site. Indeed, a flavin-adenine dinucleotide (FAD) cofactor was identified in the HyhC subunit of the *Pyrococcus furiosus* Hyh-hydrogenase (Silva et al., 1999) and respective binding sites were predicted for the putative HyhC proteins, which are encoded in the *Beggiatoaceae* genomes (BA17_358 and FLOR_02175). A spatial proximity of the flavin-cofactor and the second (unfavourable) electron-donating compound was proposed to be required for an immediate reaction of the latter with the highly reactive, semi-reduced flavin (Nitschke and Russel, 2011). In fact, the FAD cofactor and the active site of the sulfur reductase are located on the same subunit of the *Pyrococcus furiosus* Hyh-hydrogenase (Silva et al., 1999), but a crystal structure is not available for any HyhC protein so that the absolute distance is unknown. From an ecological perspective, such a reaction would be advantageous, in particular when hydrogen is less abundant than reduced sulfur compounds. In this case, hydrogen oxidation would not only provide additional energy, but allow the production of reducing equivalents from reduced sulfur compounds without a costly reverse electron transport. Thus, electron confurcation at Hyh-hydrogenases could allow *Beggiatoaceae* and other sulfide-oxidizing prokaryotes to use the environmental resources of electron donors more efficiently.

5.4 Perspectives

The culture-based physiological experiments and genomic analyses conducted within the scope of this thesis allowed to put forward several hypotheses on the dissimilatory metabolism and the ecophysiology of the family *Beggiatoaceae*. In addition, we suggested environmental conditions, under which the studied and proposed traits could be beneficial. Yet, we did not perform any *in situ* studies, which would allow for a better estimation of whether, where, and how sulfur respiration and hydrogen oxidation could play a role for environmental *Beggiatoaceae* populations.

Section 3 reports on the migration of a *Beggiatoa* strain into the anoxic and sulfidic section of a gradient medium under high sulfide fluxes. We showed that the strain respired with stored sulfur under these conditions and proposed that it did so to dispose of excess sulfur in order to maintain cell integrity. However, it is technically challenging to demonstrate this reaction to high sulfide fluxes *in situ*. This is because nitrate respiration under anoxic condition must be ruled out and the production of sulfide in the presence of a high and possibly dynamic sulfide background has to be shown. Nevertheless, a thorough screen of environmental *Beggiatoaceae* populations for a corresponding migrational response to high sulfide fluxes could provide indications for the prevalence of this reaction.

An intriguing, though not directly environment-related aspect of this study was that the downward movement of *Beggiatoa* sp. 35Flor filaments appeared to happen in a highly coordinated manner (**Figure 3.2**). So far, we could not identify whether and how the filaments communicate with each other, but it is possible that the blue-light activated adenylylcyclase, which is encoded in the genome of *Beggiatoa* sp. 35Flor (FLOR_03291), is involved in the process. The exposure of *Beggiatoa* sp. 35Flor cultures to blue light induced a similar movement (Hohmann, 2009) and the chemical oxidation of sulfide in seawater is known to produce light (Tapley et al., 1999). As the share of chemical sulfide oxidation in a *Beggiatoa* mat will increase when *Beggiatoa* filaments burst due to overfilling with sulfur or when the sulfide flux exceeds the oxidation capacity of the mat, (blue) light

could serve as an indicator of too high sulfide fluxes. Under these conditions the blue-light activated adenylylcyclase could produce cyclic AMP (cAMP), which could accumulate in the mat and serve as a quorum sensing compound for downward migration once a critical threshold concentration is reached. Such a system would explain both, the coordinated movement and the two consecutive waves of migration in cultures with a high sulfide flux (**Figure 3.2**). In order to test this hypothesis, cAMP concentrations should first be monitored in *Beggiatoa* sp. 35Flor mats incubated in presence of high sulfide fluxes. Subsequently, respective cAMP concentrations could be applied to stationary *Beggiatoa* mats to induce migration. This way, it should be possible to judge whether cAMP is involved in a signaling cascade leading to the coordinated movement of *Beggiatoa* filaments in response to an environmental trigger.

In contrast to sulfur respiration, the study of hydrogen oxidation by environmental *Beggiatoaceae* populations offers several promising possibilities. As discussed in **Section 4.1**, the standard H_2 microsensor is sensitive to sulfide and thus not suited for an application in most *Beggiatoaceae* habitats. However, a sulfide-insensitive H_2 microsensor is currently developed by M. Nielsen (Åarhus University) so that reliable measurements of hydrogen concentrations in the presence of sulfide could be possible soon. With this sensor, high resolution profiles of hydrogen could be measured in hypersaline cyanobacterial mats to determine hydrogen concentrations in the immediate vicinity of the '*Ca.* Allobeggiatoa spp.' filaments, which inhabit these mats. Likewise, it would be possible to routinely measure hydrogen profiles in *Beggiatoaceae* mats, which thrive at sites of hydrothermal venting or seeping, to determine whether these are exposed to and oxidize hydrogen of geothermal origin. Further, hydrogen oxidation by *Thiomargarita* cells from environmental samples could be tested by measuring hydrogen profiles towards single cells. As '*Ca.* Thiomargarita nelsonii' cells can be removed in a sterile manner from their mucous sheath, it should be possible to conduct the measurements without a significant influence of contaminating, possibly H_2-oxidizing bacteria. I developed a system which allows to measure hydrogen profiles towards single *Thiomargarita* cells using a conventional H_2 microsensor. However, I could not perform the final experiments so far because the available samples were old and the *Thiomargarita* cells not active enough.

A detailed analysis of the diversity of hydrogenase genes present in members of the family *Beggiatoaceae* is given in **Section 4.2**. Possible functions of the four encoded hydrogenases were proposed according to literature data available for enzymes from other organisms. Currently, it is not possible to test the proposed functions in *Beggiatoaceae* by the construction of deletion mutants, because a genetic system is not available for any member of the family. Instead, transcriptomic or proteomic studies could be conducted to test the proposed hypotheses and identify under which conditions a certain hydrogenase is expressed. Such studies could for example help to determine whether *Beggiatoa* sp. 35Flor uses a Hup-hydrogenase for hydrogen oxidation under microoxic conditions or whether hydrogen uptake occurs in fact via a 'sulfur-catalyzed' reaction involving Hyn- or Hyh-hydrogenases (outlined in **Section 4.1**). The expression of hydrogenases under sulfur-respiring conditions could likewise be studied in *Beggiatoa* sp. 35Flor, but the strain is not suited to investigate whether members of the *Beggiatoaceae* employ Hyb-hydrogenases to couple hydrogen oxidation to nitrate reduction. This hypothesis could, however, be tested in '*Ca.* Allobeggiatoa spp.', which likely consume hydrogen when respiring with nitrate (**Section 4.1**). Enrichment cultures of '*Ca.* Allobeggiatoa spp.' are available and the corresponding populations can reach high densities *in situ*, so that expression studies could be conducted with material from both, enrichment cultures and environmental samples. In fact, the high local abundances and the probably flexible hydrogen metabolism could make members of the family *Beggiatoaceae* convenient model organisms for studying hydrogen oxidation by sulfur bacteria in the environment.

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// Acknowledgements

I thank my *Doktormutter* Heide Schulz-Vogt very much for offering support and guidance but also scientific freedom so that I could follow my interests. I am especially grateful for your support I could count on when becoming a *Mutter* while pursuing my *Doktor*.

I thank Professor Ulrich Fischer for reviewing my dissertation and Professor Nicole Dubilier and Dr. Tim Ferdelman for joining my defense committee. Of course, this committee would not be complete without Julia and Sascha. Thank you!

A huge thank you goes to the once-upon-a-time Ecophysiology Group. Jörg, Anne, Vladi, Verena, Sandra, Stefano, and Artur, I enjoyed the great team spirit we all could rely on. Susanne, Martina, and Christin, thank you so much for all your support, may it be technical or moral.

Richard, thank you a lot for your patience in answering every single one of my sometimes stupid, occasionally repetitive, but often confusing questions. You saved my day more than once.

Lubos, I am very grateful for the challenging but so very helpful questions you asked. I enjoyed working with you a lot!

Marc, I thank you for your timely comments and the helpful discussions.

Artur, Vladi, and Stefano—we probably had an unhealthy amount of coffee together, but I always looked forward to the conversations we had during our breaks when we shared joy, pain, jokes, frustration, and ideas.

I thank my parents a lot for providing an environment in which I could pursue my interests without having to justify how they could possibly pay my bills. Vielen Dank für eure liebevolle Unterstützung!

Arne, thank you so much for all the moral, physical, and computer support you offered. Thank you for letting me work long hours when writing this thesis, the great food and [illegible] but [illegible] shared, and of course for letting me win most arguments, rightfully or not.

Finn, thank you for reminding me of the important things in life!

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Publication list

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Girnth, A.-C., Grünke, S., Lichtschlag, A., Felden, J., Knittel, K., Wenzhöfer, F., et al. (2011) A novel mat-forming *Thiomargarita* population associated with a sulfidic fluid flow from a deep-sea mud volcano. *Environmental Microbiology* 13:495–505

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Accepted

Berg, J.S., Schwedt, A., **Kreutzmann, A.-C.**, Kuypers, M.M.M., and Milucka, J. (2013) Polysulfides as intermediates in the oxidation of sulfide to sulfate by *Beggiatoa*. Accepted for publication in *Applied and Environmental Microbiology*

Submitted

Kreutzmann, A.-C. and Schulz-Vogt, H.N. (2013) Oxidation of molecular hydrogen by a chemolithoautotrophic *Beggiatoa* strain. Submitted for publication

Kreutzmann, A.-C. and Mußmann, M. (2013) Diversity of hydrogenase genes in the family *Beggiatoaceae*. Submitted for publication

Erklärung der selbstständigen Erarbeitung

Erklärung gemäß § 6 Abs. 5 der Promotionsordnung der Universität Bremen vom 14.03.2007 für die mathematischen, natur- und ingenieurwissenschaftlichen Fachbereiche

Hiermit versichere ich, dass ich die vorliegende Dissertation mit dem Titel "Electron donors and acceptors for members of the family *Beggiatoaceae*"

1. ohne unerlaubte fremde Hilfe angefertigt habe
2. keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe und
3. die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Bremen, den 6. November 2013

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(Anne-Christin Kreutzmann)