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Summary

Wetlands are currently net carbon sinks that store about a third of the world's organic soil carbon. Nevertheless, they are still responsible for 25–30% of the global methane budget. Therefore, it is critical to understand the biogeochemical and microbiological processes involved in carbon mineralization. Among other factors, sulfur cycling exerts control over carbon mineralisation by diverting carbon and energy flow away from methanogenesis to emission the of CO₂. This thesis aimed to identify the microorganisms involved in sulfur cycling and their syntrophic partners in wetland ecosystems. In addition, their metabolic repertoire, ecophysiology, and potential and realized niches were characterized. Soil from a long-term peatland study site in Germany was used to inoculate anoxic microcosm experiments. Microcosms were incubated for 50 days with micromolar amounts of formate, acetate, propionate, lactate, or butyrate, and were either stimulated with the addition of sulfate or incubated without external sulfate amendment. Responding microorganisms were identified and quantified with 16S rRNA gene and transcript sequencing, and quantitative PCR. In parallel, draft genomes of microorganisms encoding DsrAB-type dissimilatory sulfite reductases were obtained using metagenomics. Activity of selected annotated functions was analyzed by metatranscriptome sequencing of microcosm samples from different time points. This resulted in novel insights in the sulfur cycling microorganisms in this wetland. The sulfate reducer *Candidatus Desulfosporosinus infrequens* is an autochthonous low-abundance member of the microbial community (<0.1% relative abundance) that remained rare over 50 days of microcosm incubations. At the same time, it was highly active as shown by increased transcriptional and metabolic activity. This novel strategy by *Ca. D. infrequens* highlights the importance of studying the rare biosphere. On the other hand, members of the alphaproteobacterial genus *Telmatospirillum* became highly abundant in the microcosm incubations. While not previously known to be involved in sulfur cycling, they responded rapidly to sulfate stimulation with transcriptional activity and growth. Genome sequencing of the closest relative revealed genes for a partial Sox-type sulfur oxidation machinery, indicating *Telmatospirillum* species were likely involved in wetland sulfur oxidation. Furthermore, several metagenome-assembled genomes were reconstructed from phyla which were not previously known to contain sulfur compound-cycling microorganisms. Members of acidobacterial subdivisions 1 and 3 contained the new *Candidatus* genera *Sulfotelmatobacter*, *Sulfotelmatomonas*, and *Sulfopaludibacter* that are tentatively involved in dissimilation of sulfur compounds with either complex organic matter or oxygen. Surprisingly, their genomes encode the marker genes *dsrD* and *dsrL*, which were previously considered to be mutually exclusive and indicative for reductive and oxidative sulfur dissimilation, respectively. Additionally, a member of the *Verrucomicrobia* together with members of two novel phyla formed a deep branching cluster in DsrAB phylogeny, which could give new insights in the evolution of sulfur metabolism. In conclusion, this thesis extends the known diversity and metabolic potential of sulfur cycling microorganismes and reveals their potential roles in wetland

carbon mineralisation. Further research will be required to fully elucidate the physiology and ecology of all microorganism that tie together element cycles in these complex environments and thereby understand how wetlands will react to our ever changing climate.

Zusammenfassung

Feuchtgebiete sind momentan noch eine Kohlenstoffsene und speichern ungefähr ein Drittel alles organischen Kohlenstoffs. Trotzdem sind sie für 25–30% des globalen Methanhaushalts verantwortlich. Daher ist es essentiell, alle biogeochemischen und mikrobiellen Prozesse zu verstehen welche eine Rolle in der Kohlenstoff-Mineralisierung spielen. Indem er den Kohlenstoff- und Energiefluß von Methanogenese auf CO₂ Emission umleitet, hat der Schwefelkreislauf einen erheblichen Einfluss darauf, wie Kohlenstoff mineralisiert wird. Das Ziel dieser Doktorarbeit war es, alle Mikroorganismen die in den Schwefelkreislauf von Feuchtgebieten involviert sind, zu identifizieren. Zusätzlich wurden ihre metabolische Fähigkeiten, Ökophysiologie, und potentielle als auch realisierte Nischen charakterisiert. Boden aus einem ausführlich studierten Moor in Deutschland wurde verwendet um Inkubationsexperimente anzusetzen. Mikrokosmen wurden 50 Tage mit mikromolaren Mengen von Formiat, Acetat, Propionat, Lactat, oder Butyrat inkubiert, und zusätzlich durch Sulfat stimuliert, sowie auch ohne zusätzliche Schwefelquelle inkubiert. Angesprungene Mikroorganismen wurde durch 16S rRNA Gene und Transkript Sequenzierung, und quantitative PCR quantifiziert. Mit Hilfe von Metagenomik wurden gleichzeitig Genome von Mikroorganismen, welche eine DsrAB-Typ Dissimilatorische Sulfit-Reduktase kodieren, erforscht. Die Aktivität ausgewählter annotierter Funktionen zu verschiedenen Zeitpunkten wurde mit Metatranskriptomik analysiert. Dies ergab neue Einblicke in die Mikroorganismen des Schwefelkreislaufs von Feuchtgebieten. Der Sulfatreduzierer *Candidatus Desulfosporosinus infrequens* ist ein rares, autochthones Mitglied der mikrobiellen Gemeinschaft (<0.1% relative Abundanz), welches auch über die gesamte Dauer der Mikrokosmos-Inkubationen selten geblieben ist. Zugleich gab es aber auch ansteigende transkriptionelle und metabolische Aktivität. Diese neue Strategie von *Ca. D. infrequens* zeigt, warum es so wichtig ist, die "seltene Biosphere" zu erforschen. Gleichzeitig waren Mitglieder des alphaproteobakteriellen Genus *Telmatospirillum* in den Mikrokosmos-Inkubationen sehr häufig. Obwohl diese bis jetzt nicht mit dem Schwefelkreislauf in Verbindung gebracht wurden, reagierten sie sowohl mit Wachstum als auch mit transkriptioneller Aktivität auf Sulfat Stimulation. Durch die Genom-Sequenzierung des nächsten Verwandten hat sich herausgestellt, dass dieser Gene für einen Teil des Sox-Typ Schwefeloxidaions-Komplex kodiert. Daraus lässt sich folgern, dass *Telmatospirillum* Spezies möglicherweise bei Schwefeloxidation in Feuchtgebieten mitwirken. Weiters wurden aus Metagenomen auch solche Genome rekonstruiert, die zu Phyla gehören in welchen bis jetzt keine Gene für einen dissimilatorischen Schwefelmetabolismus gefunden wurden. Mitglieder der acidobakteriellen Subdivisionen 1 und 3 beinhalten die *Candidatus* Genera *Sulfotelmatobacter*, *Sulfotelmatomonas*, und *Sulfopaludibacter*, welche wahrscheinlich im Schwefelkreislauf involviert sind. Dies könnte kombiniert mit Umsatz von organischem Kohlenstoff oder mit Sauerstoff Atmung geschehen. Überraschenderweise besitzen deren Genome die Gene *dsrD* und *dsrL*, welche bis jetzt nie zusammen in einem Genom gefunden wurde, weshalb vermutet wurde, dass sie

spezifisch für Sulfat-/Sulfitreduktion oder Schwefeloxidation sind. Zusätzlich formte ein Mitglied der *Verrucomicrobia* zusammen mit Mitgliedern zweier kürzlich erst entdeckten Phyla einen neuen tief zweigenden Ast in der DsrAB Phylogenie. Dies könnte neue Einsichten in die Evolution von Schwefelmetabolismus geben. Diese Doktorarbeit erweitert das Wissen über die Diversität und die potentiellen metabolischen Fähigkeiten von Mikroorganismen im Schwefelkreislauf, und welche Rolle sie bei der Kohlenstoff-Mineralisierung in Feuchtgebiete spielen. Weiterführende Forschung ist nötig, um die Physiologie und Ökologie aller Mikroorganismen aufzudecken, die in Element-Kreisläufen in diesem komplexen Ökosystem zusammenwirken. Nur dadurch kann man verstehen wie sich Feuchtgebiete im immer wandelnden Klima verhalten werden.

Introduction

In 1896, Svante Arrhenius proposed a relationship between atmospheric carbon dioxide concentrations and surface temperature and thereby introduced the concept of global warming (Arrhenius, 1896), which started the multidisciplinary field of climate research (Weart, 2008). Today, this phenomenon is known as the greenhouse effect and the atmospheric concentrations of the three most concerning greenhouse gases (carbon dioxide, methane, and nitrous oxide) are ever increasing (IPCC, 2013). Even though atmospheric methane is less abundant than atmospheric carbon dioxide, its global warming potential is 28 times higher (100 year window; IPCC, 2013). Of the global methane budget, wetlands alone are responsible for 25–30% (Saunio *et al.*, 2016), despite covering only approximately 4% of the earth's surface area (Bridgman *et al.*, 2006). Wetlands store about a third of the world's organic soil carbon and, while they are currently net carbon sinks, this may change in response to climate changes. Therefore, the fate of carbon in wetlands has worldwide implications as they have the potential to release even larger amounts of greenhouse gases (Limpens *et al.*, 2008).

Carbon and sulfur cycles are linked in wetlands

Wetlands are rich in complex organic matter derived from plants (e.g., cellulose, hemicellulose, lignin) that is hydrolysed by microorganisms to monomers (e.g., monosaccharides). In the absence of oxygen, these monomers can then be fermented for energy generation, which results in the formation of short-chain fatty acids (e.g., formate, acetate, propionate, butyrate) and other small organic (e.g., lactate) or inorganic (e.g., H₂, CO₂) compounds (Limpens *et al.*, 2008). Under anoxic conditions, organic carbon is not only mineralised to carbon dioxide, but also to methane in the microbially mediated process of methanogenesis (Serrano-Silva *et al.*, 2014). However, methanogens can only use acetate, H₂ and CO₂, and a few methylated compounds as substrates (Serrano-Silva *et al.*, 2014). In addition, methanogenesis is one of the least thermodynamically favourable processes for microbial energy generation. Therefore, methanogens can be outcompeted in the presence of more favourable electron acceptors. For example, iron and sulfate reducers utilize iron(III) and sulfate, respectively, to oxidize organic matter or H₂, thereby depriving methanogens of electron donors (e.g., Roden and Wetzel, 1996; Loy *et al.*, 2004; Liu and Conrad, 2017). Even without external supply of sulfur compounds, active sulfate reduction can be maintained in low-sulfate wetlands. Reduced sulfur species are rapidly re-oxidized, which can sustain sulfate reduction rates that are as high as in sulfate-rich marine surface sediments (Pester *et al.*, 2012). In this so-called cryptic sulfur cycle, sulfur re-oxidation could be either coupled to O₂ respiration or under anoxic conditions to the reduction of iron (Hansel *et al.*, 2015), nitrate (Haaijer *et al.*, 2007), and/or humic matter (Heitmann and Blodau, 2006; Yu *et al.*, 2015). Despite the observation of cryptic sulfur cycling for three decades now, the geochemical and biological contributions to the re-oxidation of sulfur are

not fully resolved and remain to be studied further in respect to its biogeochemistry, microbial key players, and ecological impact (Pester *et al.*, 2012).

On the detection of sulfate-reducing microorganisms

Sulfate reduction by microorganisms is studied extensively in wetlands using an array of methods from molecular biology. Potential sulfate (and sulfite) reducers are screened for by amplification of the dissimilatory sulfite reductase genes (*dsrAB*; Wagner *et al.*, 2005). DsrAB is a key enzyme in the last step of sulfate reduction, the reduction of sulfite to sulfide (Figure I-1). However, there are some considerations when using *dsrAB* as a marker for sulfate reducers: (1) Both sulfate and sulfite reducers use the DsrAB enzyme. These can be differentiated using additional amplification of the adenylyl-sulfate reductase genes (*aprBA*), encoding an enzyme essential for sulfate but not sulfite reduction (Wagner *et al.*, 2005; Figure I-1). (2) Some syntrophic bacteria harbour *dsrAB* genes without any apparent dissimilatory sulfur metabolism, i.e., they are pseudogenes (Müller *et al.*, 2015). (3) The sulfate reduction pathway is used in reverse by some sulfide-oxidizing microorganisms (Müller *et al.*, 2015; Thorup *et al.*, 2017; Figure I-1). Notably, when sulfur compounds are used as electron donors and energy sources in anoxic environments, microorganisms need an electron acceptor besides oxygen. Which electron acceptors are indeed utilized and which are relevant in carbon turnover remains to be elucidated (Pester *et al.*, 2012).

DsrAB phylogeny and taxonomy is used to classify *dsrAB* genes into family-level lineages and larger clusters and branches. However, a large fraction of the known DsrAB diversity belongs to phylogenetic lineages without any cultivated relatives (Pester *et al.*, 2012; Müller *et al.*, 2015). This makes it challenging to link *dsrAB* gene sequences from uncultured lineages to their taxonomic identity and to identify the type and direction of the dissimilatory sulfur metabolism (Müller *et al.*, 2015). Further hindering the prediction of the direction of the DsrAB enzyme from phylogenetic information is that even closely related microorganism, e.g., members of the *Desulfobulbaceae* family, can perform both oxidative and reductive sulfur metabolism (Dannenberg *et al.*, 1992; Lovley and Phillips, 1994; Fuseler and Cypionka, 1995; Fuseler *et al.*, 1996; Holmes *et al.*, 2004; Pagani *et al.*, 2011; Thorup *et al.*, 2017). Another approach is to use more specific marker genes. For example, the genes encoding the Sox machinery are only used in oxidative sulfur metabolism (Dahl and Friedrich, 2008). However, microorganisms oxidizing sulfide and not thiosulfate do not require the Sox machinery. Furthermore, the presence of the *dsrD* and *dsrL* genes as indications of reductive or oxidative metabolisms was proposed due to their exclusivity in the genomes of microorganism performing these functions, respectively (Hittel and Voordouw, 2000; Mizuno *et al.*, 2003; Dahl *et al.*, 2005; Lübke *et al.*, 2006; Sander *et al.*, 2006; Dahl and Friedrich, 2008; Ghosh and Dam, 2009; Rabus *et al.*, 2015). However, while DsrL is considered to be essential for sulfur oxidation (Lübke *et al.*, 2006), the function of neither DsrD nor DsrL was proven so far.

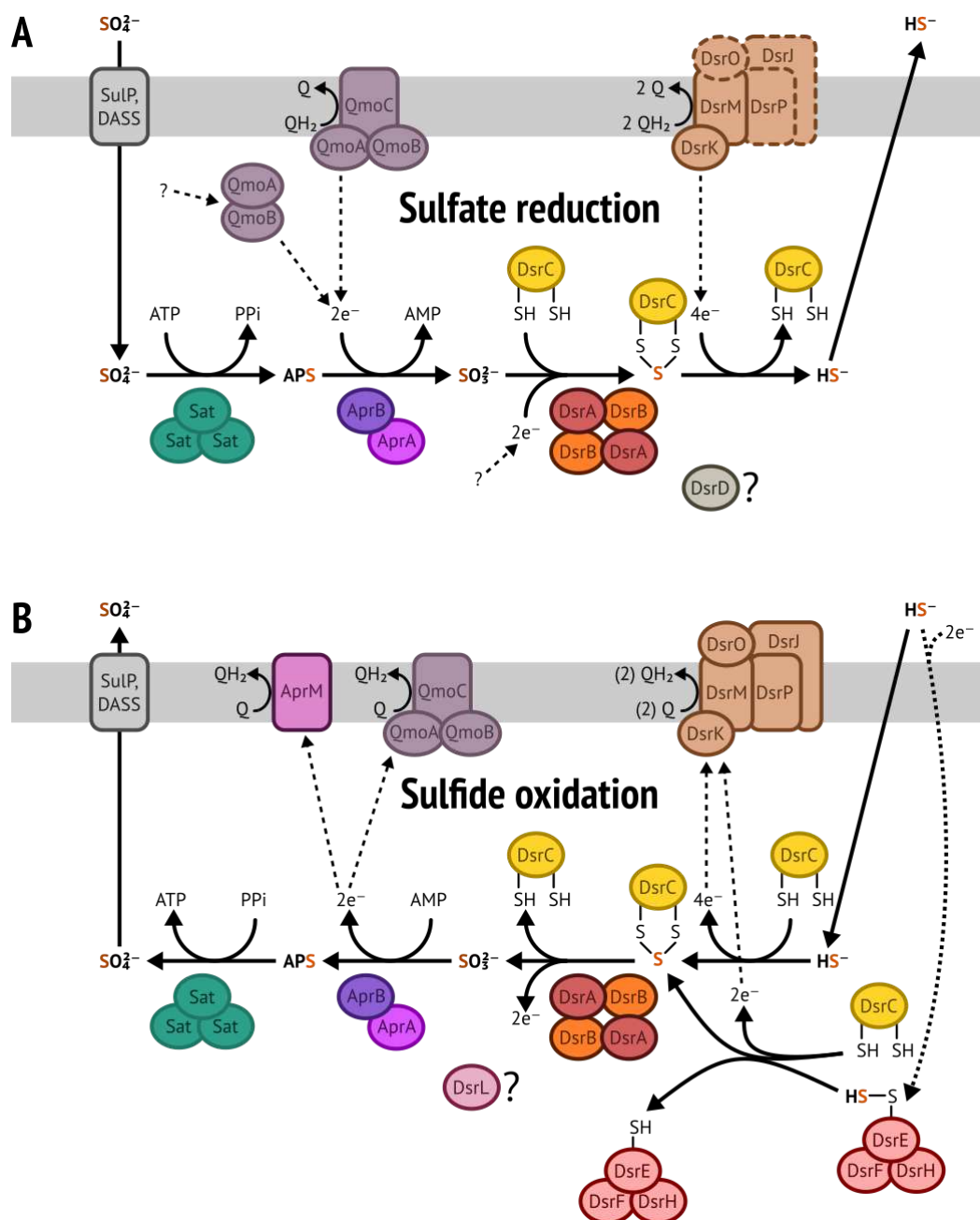


Figure I-1. Dissimilatory sulfur metabolism pathways involving DsrAB. (A) Complete sulfate reduction pathway (Santos *et al.*, 2015). Sulfite reduction does not require Sat, Apr, and Qmo. QmoC is not ubiquitous in sulfate reducers, therefore a cytoplasmic electron transport mechanisms could be possible. DsrJOP is not ubiquitous in sulfate/sulfite reducers. (B) Complete sulfide oxidation pathway. The mechanism how sulfide is transferred to DsrC-trisulfide is not resolved. Sulfide oxidation was observed without any known additional enzymes (Thorup *et al.*, 2017). Alternatively, sulfide could be transferred to DsrC via a sulfur transport system ending in DsrEFH (Dahl, 2017). AprM is found instead of QmoABC in some sulfide oxidizers (Dahl and Friedrich, 2008). SulP/DASS: sulfate permease/divalent anion:sodium symporter; Sat: sulfate adenylyltransferase; AprBA: adenylyl-sulfate reductase; AprM: adenylyl-sulfate reductase, membrane-bound subunit; *qmoAB(C)*: quinone-interacting membrane-bound oxidoreductase complex; DsrAB: dissimilatory sulfite reductase; DsrMK(JOP): sulfite reduction-associated membrane complex ([DsrC]-trisulfide reductase); DsrD: unknown function (putative regulatory protein); DsrL: unknown, but essential, function; DsrEFH: sulfur transfer proteins; Q/QH₂: membrane quinone pool.

The Schlöppnerbrunnen wetlands as a model system for low-sulfate sulfur cycling

The acidic Schlöppnerbrunnen wetland system (located in Bavaria, Germany) is a long-term field site for studying microorganisms involved in sulfur cycling and the anoxic degradation of carbon. The biogeochemistry and microbiology of the wetlands in this forested catchment were extensively studied in the past (e.g., Loy *et al.*, 2004; Knorr and Blodau, 2009; Knorr *et al.*, 2009; Pester *et al.*, 2010; Steger *et al.*, 2011). Measured sulfate concentrations are generally low (< 1 mM), however, sulfate reduction under anoxic conditions was observed as part of the cryptic sulfur cycle (Knorr *et al.*, 2009). Continued sulfate turnover was shown to be ongoing for up to 240 days in a closed anoxic mesocosm study conducted with soil from this wetland (Knorr and Blodau, 2009). Additionally, numerous well-known and tentative sulfate reducers were previously detected in the Schlöppnerbrunnen wetland using 16S rRNA gene and *dsrAB* gene sequencing (Loy *et al.*, 2004; Pester *et al.*, 2010; Steger *et al.*, 2011).

Challenging the view that only abundant microorganisms can drive important ecological functions, a study by Pester *et al.* (2010) applied DNA-stable isotope probing (DNA-SIP) to show that members of the low abundant genus *Desulfosporosinus* are the only active sulfate reducers in microcosm incubations with soil from the Schlöppnerbrunnen wetland (at *in situ*-like conditions). When tested with quantitative PCR, the relative abundance of the *Desulfosporosinus* genus was below 0.1%, i.e., members of the rare biosphere (Pedrós-Alió, 2012). Despite its low abundance, estimated cell-specific sulfate reduction rates were comparable to measurements observed with axenic sulfate reducers and therefore the rare *Desulfosporosinus* was considered to be highly active (Pester *et al.*, 2010). Not many examples for low-abundance key players that significantly influence element cycles were known (Lynch and Neufeld, 2015), therefore this environment and this sulfate reducer is worth studying.

Research objectives and approach

This thesis aimed to elucidate the identity and ecophysiology of microorganisms responsible for anoxic sulfur cycling and degradation of organic matter in the model wetland Schlöppnerbrunnen:

- Low-abundance members of the genus *Desulfosporosinus* were previously observed as sulfate reducers of major importance when a mix of fermentation products (formate, acetate, propionate, and lactate) was provided (Pester *et al.*, 2010). However, little was known about their ecophysiology, i.e., which substrates were indeed utilized. To answer this question, I aimed to elucidate the response of the natural *Desulfosporosinus* population in greater detail. Cultivated relatives reduce sulfate with formate, lactate and butyrate, but not acetate or propionate as electron donors. Which of these substrates can the low-abundance *Desulfosporosinus* members use when integrated into their natural complex microbial community? What is their genetic potential, i.e., which substrate degradation pathways are encoded in their genomes? Which of these pathways are transcribed when corresponding substrates and sulfate are utilized? Does *Desulfosporosinus* start growing when continuously active under permanently favourable conditions?
- Formate, acetate, propionate, lactate, and butyrate are important intermediates in the food web of wetlands and their fate affects the amount of produced methane. Importantly, each substrate can be directly used by sulfate reducers. I aimed to deduce which microorganisms (in addition to *Desulfosporosinus*) respond to sulfate stimulation with these substrates within their natural complex microbial community. Which known sulfate reducers use which substrate? Which other microorganisms respond to sulfate stimulation and what is their potential role in or connection to the sulfur cycle? Are these novel sulfate reducers? How fast do these microorganisms respond to favorable conditions?
- A large fraction of DsrAB diversity in Schlöppnerbrunnen and other wetlands is of unknown taxonomy. This is hampering the interpretation of obtained *dsrAB* sequences from wetland and other environments. I aimed to link unclassified DsrAB sequences with their taxonomic identity. To which taxa do the uncultivated DsrAB family lineages, previously observed in Schlöppnerbrunnen, belong to? Are the identified DsrAB enzymes potentially used for sulfate/sulfite reduction, for sulfur oxidation, both, or not at all (pseudogenes)? What are the corresponding electron donors/electron acceptors used by these DsrAB-encoding microorganisms?

To answer these questions, this thesis applied a combination of laboratory manipulations of soil samples with in-depth analysis of the microbial response:

- Microcosm incubations: To elucidate the response of microorganism to specific single substrates within the food web, a clear separation of such is required. I chose to incubate soil slurries under anoxic conditions in microcosms. Replicate microcosms were incubated with the substrate formate, acetate, propionate, lactate, butyrate separately, and without any external carbon source. Acetate is of importance, as some sulfate reducers incompletely oxidized propionate, lactate, and/or butyrate to acetate, while others use acetate as the electron donor. In addition, formate was used as a proxy for H_2 , as it can be interconverted by many H_2 -consuming sulfate reducers. To detect which microorganisms were stimulated with sulfate, triplicate microcosm for each substrate were amended with sulfate, or incubated without external sulfate additions. Microcosms were incubated for 50 days and sampled regularly (twice a week) to resolve the temporal response. To mimic *in situ* conditions, substrates and sulfate were added regularly (1–3 times a week) at concentrations in the same range as measured in the field.
- High-throughput sequencing: I chose a several high-throughput sequencing methods to answer my research questions: (1) 16S rRNA gene and transcript amplicon sequencing (supported by quantitative PCR) was used to observe the growth and transcriptional response of the total microbial community in the microcosm incubations. This method has sufficient resolution to observe the response of low-abundance microorganisms. (2) Metagenomics was used to obtain draft metagenome-assembled genomes (MAGs) of as many DsrAB-encoding microorganisms as possible. In addition to the DNA from native soil, DNA from a previous DNA-stable isotope probing study (Pester *et al.*, 2010) was used for metagenomic sequencing. Combining these differing metagenomes is required when using differential coverage binning and to obtain MAGs from the natively rare microorganisms. (3) To infer metabolic activity, the transcription of the obtained MAGs in the microcosm incubations was analyzed using metatranscriptomics. Compared to transcription studies using axenic cultures, this approach reveals the transcriptional patterns of organisms in response to different treatments while interacting with the total microbial community.

To summarize, I aimed to extend and specify our knowledge of which microorganisms are involved at which specific steps in the sulfur and carbon cycles of the model wetland Schlöppnerbrunnen. These findings will help us to better understand microbial sulfur and carbon cycling in general.

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Chapter 1

Consortia of low-abundance bacteria drive sulfate reduction-dependent degradation of fermentation products in peat soil microcosms

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Author contributions

BH, AL, and MP designed research. MP performed field sampling. BH, KHK, and MP performed microcosm experiments. BH, KHK, and MP performed and analyzed biogeochemical measurements. KS performed physiological characterization of *Telmatospirillum siberiense*. BH extracted nucleic acids. SGT and TG contributed amplicon sequencing. BH analysed the sequencing data and performed statistical analysis. BH performed and analysed qPCR assays. MP performed phylogenetic analysis. BH, AL, and MP wrote the manuscript. KHK, KS, SGT, and TG revised the manuscript.

ORIGINAL ARTICLE

Consortia of low-abundance bacteria drive sulfate reduction-dependent degradation of fermentation products in peat soil microcosms

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Dissimilatory sulfate reduction in peatlands is sustained by a cryptic sulfur cycle and effectively competes with methanogenic degradation pathways. In a series of peat soil microcosms incubated over 50 days, we identified bacterial consortia that responded to small, periodic additions of individual fermentation products (formate, acetate, propionate, lactate or butyrate) in the presence or absence of sulfate. Under sulfate supplementation, net sulfate turnover (ST) steadily increased to 16–174 nmol cm⁻³ per day and almost completely blocked methanogenesis. 16S rRNA gene and cDNA amplicon sequencing identified microorganisms whose increases in ribosome numbers strongly correlated to ST. Natively abundant ($\geq 0.1\%$ estimated genome abundance) species-level operational taxonomic units (OTUs) showed no significant response to sulfate. In contrast, low-abundance OTUs responded significantly to sulfate in incubations with propionate, lactate and butyrate. These OTUs included members of recognized sulfate-reducing taxa (*Desulfosporosinus*, *Desulfopila*, *Desulfomonile*, *Desulfovibrio*) and also members of taxa that are either yet unknown sulfate reducers or metabolic interaction partners thereof. Most responsive OTUs markedly increased their ribosome content but only weakly increased in abundance. Responsive *Desulfosporosinus* OTUs even maintained a constantly low population size throughout 50 days, which suggests a novel strategy of rare biosphere members to display activity. Interestingly, two OTUs of the non-sulfate-reducing genus *Telmatospirillum* (*Alphaproteobacteria*) showed strongly contrasting preferences towards sulfate in butyrate-amended microcosms, corroborating that closely related microorganisms are not necessarily ecologically coherent. We show that diverse consortia of low-abundance microorganisms can perform peat soil sulfate reduction, a process that exerts control on methane production in these climate-relevant ecosystems.

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Introduction

Peatlands currently store about one-third of all terrestrial carbon (Limpens *et al.*, 2008), which is predicted to be partially released as the greenhouse gases CO₂ and CH₄ because of climate change (Hodgkins *et al.*, 2014; Schuur *et al.*, 2015). In these water-saturated soils, steep gradients in redox conditions sustain a complex network of biogeochemical processes (Limpens *et al.*, 2008), with organic matter degradation being carried out by different functional guilds of microorganisms. This

diversity of simultaneously occurring organic carbon degradation pathways is favored by frequently fluctuating concentrations of electron acceptors (Schmalenberger *et al.*, 2007; Küsel *et al.*, 2008; Knorr and Blodau, 2009) and donors (Schmalenberger *et al.*, 2007; Küsel *et al.*, 2008; Wüst *et al.*, 2009). A changing water table that steadily shifts the oxic–anoxic interface (Knorr *et al.*, 2009; Reiche *et al.*, 2009) and complex flow paths of infiltrating and exfiltrating water create distinct spatial and temporal patterns (hot spots and hot moments) of various biogeochemical processes, making peatlands very dynamic ecosystems (Jacks and Norrström, 2004; Knorr *et al.*, 2009; Knorr and Blodau, 2009; Frei *et al.*, 2012).

Below the water table, where anoxic conditions persist, the processes of fermentation and subsequent methanogenesis are competing with energetically more favorable degradation pathways such as

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anoxic respiration coupled to nitrate, Fe(III), humic matter or sulfate reduction (Loy *et al.*, 2004; Beer *et al.*, 2008; Hamberger *et al.*, 2008; Küsel *et al.*, 2008; Drake *et al.*, 2009; Keller *et al.*, 2009; Knorr and Blodau, 2009; Knorr *et al.*, 2009; Reiche *et al.*, 2009; Wüst *et al.*, 2009; Hunger *et al.*, 2011; Pester *et al.*, 2012b). The importance of sulfate reduction for peatland biogeochemistry is often neglected because of the low prevailing sulfate concentrations (μM range). This is contrasted by a highly active but cryptic sulfur cycle that rapidly reoxidizes reduced sulfur species and thus sustains sulfate reduction rates that are as high as in sulfate-rich marine surface sediments (Pester *et al.*, 2012b). Here, (bio)geochemical sulfur reoxidation can be either coupled to the reduction of O_2 at the oxic–anoxic interphase or to the reduction of Fe(III) (Hansel *et al.*, 2015) and/or humic matter (Heitmann and Blodau, 2006; Yu *et al.*, 2015) under completely anoxic conditions. As sulfate-reducing microorganisms (SRM) generally outcompete methanogens and syntrophically associated fermenters (Muyzer and Stams, 2008), they exert an important intrinsic control function on CH_4 production in peatlands (Gauci *et al.*, 2004, 2005; Gauci and Chapman, 2006).

Microbial communities in soils are typically composed of a large number of species (e.g., Roesch *et al.*, 2007; Serkebaeva *et al.*, 2013). Few of these taxa are very or moderately abundant (arbitrarily classified at $\geq 1\%$ and $\geq 0.1\%$ relative abundance, respectively). The remaining majority of species have an individual relative abundance of $< 0.1\%$ and are summarized as the rare biosphere (Pedrós-Alió, 2012; Lynch and Neufeld, 2015). Because of its species richness and genetic diversity, the rare biosphere functions as a microbial seed bank for the recruitment of dormant cells to become active and numerically dominant under favorable environmental conditions. For example, singular disturbance events such as oil spills resulted either in the recruitment of specific taxa (Teira *et al.*, 2007) or even widespread changes of the total microbial community (Newton *et al.*, 2013). Furthermore, periodic environmental filtering through seasonal changes led to reoccurring temporal shifts between rare and abundant population sizes in marine bacterioplankton species (Campbell *et al.*, 2011; Vergin *et al.*, 2013; Alonso-Sáez *et al.*, 2015). Dormant microorganisms may also be introduced to environments by transport mechanisms such as ocean currents (Müller *et al.*, 2014) or wind (Hervàs and Casamayor, 2009; Hervàs *et al.*, 2009), where they stay inactive and eventually undergo taphonomy (Lynch and Neufeld, 2015). However, there is increasing evidence that the rare biosphere is not just a seed bank but also harbors active populations. Seasonal patterns in marine bacterial and archaeal plankton revealed that many taxa that displayed reoccurring annual abundance changes were actually rare biosphere members and even during their bloom periods never reached abundant numbers (Campbell *et al.*, 2011; Hugoni *et al.*, 2013; Alonso-Sáez *et al.*, 2015).

In addition, microorganisms with a specialized metabolism often fulfill gatekeeper functions in an ecosystem while sustaining low-abundance populations (Lynch and Neufeld, 2015), for example, N_2 -fixing microorganisms in the ocean (Großkopf *et al.*, 2012) or nitrifiers in wastewater treatment plants (Wang *et al.*, 2014). The reasons for their constantly low abundance are not yet resolved but most likely vary between different taxa and may include a decreased efficiency in the competition for resources, predation and viral attack, or an energy metabolism sustaining little growth (Lynch and Neufeld, 2015).

Recognized SRM are typically members of the rare biosphere in peatlands (Loy *et al.*, 2004; Costello and Schmidt, 2006; Dedysh *et al.*, 2006; Kraigher *et al.*, 2006; Pester *et al.*, 2010; Steger *et al.*, 2011; Tveit *et al.*, 2013). Previous research showed that rare peatland *Desulfosporosinus* spp. have the potential for high cell-specific sulfate reduction rates (Pester *et al.*, 2010) that are at the upper limit of cell-specific rates observed for pure cultures (Detmers *et al.*, 2001). This provided indirect evidence that rare biosphere members could contribute substantially to biogeochemical cycling in peatlands. In the same peatland, a high diversity of deep-branching lineages of the *dsrAB* genes (subunit A and B of the dissimilatory sulfite reductase) was identified previously (Loy *et al.*, 2004; Schmalenberger *et al.*, 2007; Pester *et al.*, 2010; Steger *et al.*, 2011). Although *dsrAB* generally serve as functional marker genes for SRM (Müller *et al.*, 2015), the taxonomic identity of these novel lineages and their function with regard to sulfur and carbon metabolism remain unclear (Pester *et al.*, 2012b; Müller *et al.*, 2015). Here, we set up controlled incubation experiments supplemented with single substrates and sulfate to simulate sulfate-reducing hot spots and gain insights into the discrepancy between the low abundance of SRM and the high sulfate reduction rate regularly observed in peatlands.

Materials and methods

Peat microcosms

The acidic peatland Schlöppnerbrunnen II is located in Germany (50°07'54.8"N, 11°52'51.8"E). Main site characteristics and soil core sampling are described in the Supplementary Methods. Peat soil microcosms were set up by mixing 30 g of fresh soil (10–20 cm depth) with 60 ml of filter-sterilized (0.2 μm) and anoxic peat water in 250 ml sterile glass bottles. Microcosms were sealed under 100% N_2 with butyl rubber septa and incubated for 53 days in the dark at 14 °C, which is within the observed air temperature range of this peatland (Schmidt *et al.*, 2015). Microcosms were periodically amended from the first day of incubation with formate, acetate, propionate, lactate or butyrate, or incubated without any external substrate (Supplementary Figure S1). Added substrates generally did not exceed a final concentration

of 100–200 μM (Supplementary Figure S2a). A side aspect of the formate additions was its potential to target H_2 -using microorganisms as well. Interconversion of formate to $\text{H}_2 + \text{CO}_2$ is at thermodynamic equilibrium (-4 kJ mol^{-1}) and has been documented in peat soil incubations of the analyzed peatland (Hunger *et al.*, 2011). Half of the substrate-amended and -unamended microcosms additionally received periodic amendments of sulfate (Supplementary Figure S1). These microcosms were initially spiked with sulfate to a final concentration of 190–387 μM , and, thereafter, were periodically amended with small amounts of sulfate equivalent to 79–161 μM (Supplementary Figure S2b). Triplicate microcosms were set up per incubation condition (36 microcosms in total) and periodically sampled for substrate/sulfate concentrations directly after amendment (Supplementary Figure S1). Identical microcosms were set up to measure CO_2 and CH_4 production (Supplementary Methods).

Quantification of sulfate and substrate turnover

Sulfate and substrate concentrations at days 0, 4, 7, 11, 14, 18, 21, 25, 28, 35, 42 and 49 were determined by capillary electrophoresis (P/ACE MDQ; Beckman Coulter, Brea, CA, USA) directly after amendment using the CEofix Anions 5 Kit (Analisis, Suarlée, Belgium). To obtain substrate-specific sulfate turnover (ST) rates, we determined how much sulfate was used in the substrate- and sulfate-amended microcosms in comparison with the sulfate-amended controls without external substrate. This served to discriminate against sulfate dissimilation caused by SRMs using endogenous substrates and sulfate assimilation in general. The calculation was performed by subtracting average sulfate concentrations of the sulfate-amended no-substrate controls from the individual substrate- and sulfate-amended microcosms at each corresponding time point. A nonparametric regression was fitted to the obtained values in R (R Core Team, 2015), and the slope of the regression curve was used to calculate net ST rates. Sulfate concentrations were previously converted from μM to nmol cm^{-3} of fresh peat soil using the measured soil water content (78%; Supplementary Methods) and the bulk density of peat soil (0.29 g cm^{-3} ; Goldberg *et al.*, 2008) to allow comparison with results obtained in other studies (e.g., Knorr *et al.*, 2009; Knorr and Blodau, 2009).

Amplicon sequencing and qPCR

After 5, 26 and 50 days of incubation, total nucleic acids were extracted from soil samples, purified, separated into RNA and DNA fractions and quantified (Supplementary Methods). Complete removal of DNA in RNA samples was verified with a quantitative PCR (qPCR) assay targeting the 16S rRNA genes of most *Bacteria* and *Archaea* (Supplementary Methods). The V4 region of the 16S rRNA gene and its cDNA was amplified as described previously (Caporaso *et al.*, 2011) and sequenced in three Illumina MiSeq

runs at the Joint Genome Institute (genome portal projects 1 016 201, 1 016 203 and 1 031 338) and deposited at NCBI (SRA277144, SRA277986 and SRA277988). All reads were subjected to quality control (iTagger; https://bitbucket.org/berkeleylab/jgi_itagger/), UPARSE clustering (including *de novo* chimera filtering) (Edgar, 2013), singleton filtering (iTagger) and UCHIME chimera filtering (Edgar *et al.*, 2011) (Supplementary Methods). This resulted in 10.0 and 11.3 million high-quality reads from DNA and RNA samples, respectively, which formed a total of 7435 species-level operational taxonomic units (OTUs) (97% sequence identity). The library size varied between 17 795–323 368 and 46 764–341 948 reads per DNA and RNA sample, respectively, with a median of 84 629 and 93 245 reads (Supplementary Figure S3a). Taxonomic identity was assigned with the Ribosomal Database Project (RDP) classifier 2.9 and the RDP 16S rRNA training set 10 using a confidence threshold of 0.5 (Wang *et al.*, 2007). Relative abundances obtained from 16S rRNA gene samples were corrected for copy number bias using *rrnDB* 4.3.3 (Stoddard *et al.*, 2015) in R (R Core Team, 2015), resulting in estimated relative genome abundance values for each OTU (Supplementary Methods). This was previously shown to improve diversity and abundance estimates (Kembel *et al.*, 2012). For selected OTUs, the taxonomic assignment was verified by phylogenetic tree reconstruction as outlined in the Supplementary Methods. qPCR assays targeting 16S rRNA genes or cDNA of the genus *Desulfosporosinus*, most *Bacteria* and *Archaea*, and selected *dsrA* variants were performed as described in the Supplementary Methods. Reliability of relative abundance shifts in our amplicon sequencing approach was verified by a mock community analysis as internal control (Supplementary Figure S4; Herbold *et al.*, 2015).

Statistical analysis

Differential abundance of OTUs between individual treatments and time points was tested in R (R Core Team, 2015) using the package edgeR (Robinson *et al.*, 2010) based on the recommendations of McMurdie and Holmes (2014). Pairwise comparisons were performed by testing independently for the effect of sulfate, substrate and incubation time (Supplementary Methods). Each test included three replicates per treatment, with the exception of the propionate- and sulfate-amended microcosms, where one replicate was excluded from all analyses because of its inconsistent ST (Figure 1). OTUs entering statistical analysis required >10 reads in at least two out of six samples per pairwise comparison. Significantly responding OTUs were assigned to sulfate-stimulated or -deterred conditions with respect to a given substrate and response time. For association network analysis, regularized log transformation was applied to OTU abundance data to stabilize variance heterogeneity, as implemented in the DESeq2 package (Love *et al.*, 2014). Resulting variance homogeneity was verified with the Brown–Forsythe

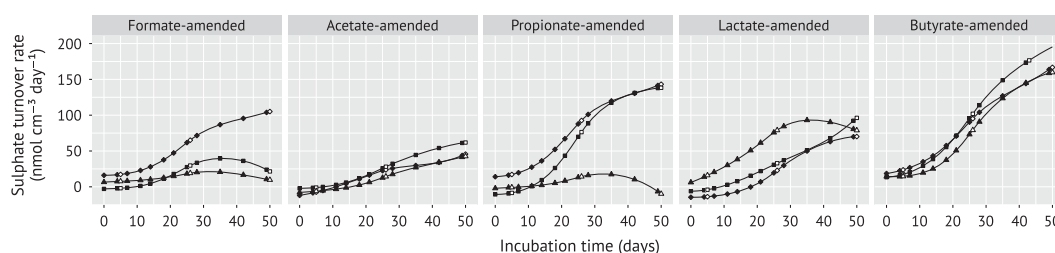


Figure 1 Substrate-specific net ST rates in sulfate-stimulated microcosm. Individual microcosms are depicted by different symbols (and are consistent in all figures that show triplicate data). Solid symbols indicate time points where sulfate concentrations were measured, and open symbols indicate time points selected for 16S rRNA (gene) amplicon sequencing. Minor negative values are because of variations caused by manual sulfate addition to individual microcosms in comparison with the corresponding no-substrate controls and as such can be neglected. Sulfate pools were never depleted in any sulfate-amended microcosm.

test (P -value > 0.1) in R (R Core Team, 2015). Transformed OTU abundances of responsive OTUs were correlated pairwise to each other and to ST using Pearson's correlation. Significant (false discovery rate-corrected P -value < 0.05) OTU–ST and OTU–OTU pairwise correlations were used for network construction.

Physiological characterization of *Telmatospirillum siberiense*

Telmatospirillum siberiense 26-4b1^T (DSM-18240; Sizova *et al.*, 2007) was incubated anoxically with DSMZ medium 1126 (DSMZ, Braunschweig, Germany). Sulfate was added to the medium to a final concentration of 1 mM. Anaerobic growth at 28 °C was inferred from measuring optical density (600 nm) in parallel incubations in medium supplemented with different carbon sources, that is, citrate (positive control for growth of *T. siberiense*), formate, propionate, lactate or butyrate (5 mM each). DNA was extracted from biomass grown on the citrate-amended medium with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and tested for the presence of the *dsrAB* genes with the primer pairs DSR190F/DSR916R (*dsrA*), DSR1762F/DSR2107R (*dsrB*) and DSR190F/DSR2107R (both) (Pelikan *et al.*, 2015).

Results

Fermentation products differ in their impact on ST

Anoxic peat microcosms were set up to reflect naturally occurring concentrations of substrates and generally did not exceed 100–200 μ M (Supplementary Figure S2a). For this purpose, microcosms were periodically amended with small amounts of formate, acetate, propionate, lactate or butyrate—substrates that represent typical organic carbon degradation intermediates in peatlands (Schmalenberger *et al.*, 2007; Küsel *et al.*, 2008; Limpens *et al.*, 2008). Microcosms without external substrate served as controls for the impact of endogenous substrates. To mimic a sulfate reduction hot spot in the peat (Jacks and Norrström, 2004; Knorr and Blodau, 2009; Knorr *et al.*, 2009; Frei *et al.*, 2012), half of the microcosms were periodically amended with sulfate

in the lower μ M range (accumulating over time to a maximum of ~ 0.5 to 2 mM depending on the substrate supplemented in parallel; Supplementary Figure S2b). In sulfate-stimulated incubations, supplemented substrates were completely turned over after each addition. The same was true for incubations without external sulfate with the exception of butyrate-amended microcosms, where butyrate started to accumulate slightly after 30 days (Supplementary Figure S2a).

Net ST increased over time but differed substantially between different substrates. Highest ST was observed for butyrate at the end of the incubations (averaging 174 nmol cm⁻³ per day), followed by propionate, lactate, acetate and formate (averaging 16 nmol cm⁻³ per day) in decreasing order (Figure 1). In formate- and propionate-amended microcosms, one replicate each showed a clear deviation in ST from the other two, being either substantially higher or lower, respectively. As the peat soil had not been homogenized before separation into individual microcosms, this likely reflects the spatially heterogeneous distribution of the microbiota in the peat soil matrix. ST increased overincubation time in each of the individual substrate-amended microcosms. Little ST at early time points is possibly due to a presence of alternative, energetically more favorable electron acceptors such as nitrate, Fe(III) or humic matter (Thauer *et al.*, 1977) as observed previously in this peatland (Küsel *et al.*, 2008; Knorr and Blodau, 2009). The presence of such alternative electron acceptors was corroborated by a parallel microcosm setup, where over the total incubation time of 27 days only a minor carbon flow towards methanogenesis (0.1–0.2%) was observed in incubations not amended with sulfate (Supplementary Table S1). In sulfate-stimulated microcosms, CH₄ production was reduced by 83–100% as compared with controls without additional sulfate (based on the time frame between 18 and 27 days; Supplementary Figure S2c). In the same parallel microcosm setup, sulfate amendment did not alter total CO₂ production (Supplementary Figure S2c), but shifted 43–100% of organic carbon mineralization towards sulfate reduction at the very end of the incubation time (assuming that equivalents of acetate are completely oxidized to two CO₂ with the eight released reducing equivalents

being used to reduce sulfate to sulfide, detailed in Supplementary Methods and Results).

Only low-abundance OTUs responded to sulfate stimulation

We used changes in relative genome abundance (i.e., relative 16S rRNA gene abundance corrected for *rrn* operon copy numbers) per OTU to estimate microbial growth. In addition, the protein synthesis potential of an OTU population was determined by calculating its relative ribosome abundance (i.e., relative 16S rRNA cDNA abundance). The abundant community (OTUs with $\geq 0.1\%$ relative genome abundance) of the native soil was dominated by members of the *Acidobacteria*, *Alphaproteobacteria*, *Actinobacteria* and *Planctomycetes* (Supplementary Figure S3b). None of these abundant OTUs was stimulated by sulfate in any of the treatments. In contrast, 18 low-abundance OTUs each responded positively (P -value < 0.05) to either sulfate-stimulated or -unstimulated conditions under different substrate scenarios (Figure 2a). Most of these OTUs stayed below or reached levels slightly above 0.1% relative genome abundance throughout the incubation period. Only *Telmatospirillum* OTU0029 exceeded 1% relative genome abundance towards the end of the incubation period in butyrate incubations without sulfate amendments (Figure 2b).

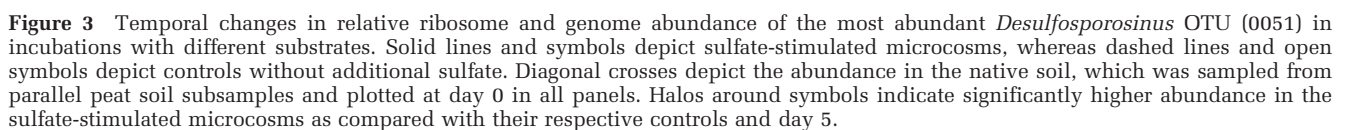
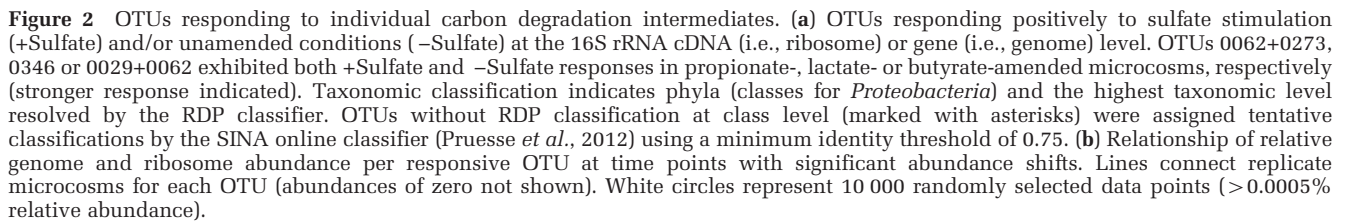
In accordance to the high ST observed under butyrate, propionate and lactate, 5–11 OTUs responded positively to sulfate stimulation under each of these substrates, whereas none responded significantly to the addition of formate or acetate. Responsive OTUs were affiliated with taxa containing known SRM (*Desulfosporosinus*, *Desulfomonile*, *Desulfopila* and *Desulfovibrio*) and also to taxa not known to harbor SRM. The latter were affiliated to *Alphaproteobacteria*, *Acidobacteria* and *Gammaproteobacteria*, and tentatively also to candidate phylum TM6, *Fibrobacteres*, ‘Parcubacteria’ (candidate phylum OD1; Rinke et al., 2013) and *Verrucomicrobia* (Figure 2a). Our stringent statistical approach did not reveal any OTUs responding positively to sulfate stimulation with formate. However, although ST only increased minimally in two of the formate-amended microcosms, it clearly increased in the third microcosm (Figure 1). Interestingly, these differences in ST were mirrored in concurrent response patterns at the ribosome level for several OTUs that responded also positively to the sulfate addition under other substrates (*Desulfosporosinus* OTU0051, *Telmatospirillum* OTU0062, *Desulfomonile* OTU0144, *Desulfopila* OTU0256, *Magnetospirillum* OTU0339 and unclassified *Rhodospirillaceae* OTU0577; Figure 3 and Supplementary Figures S5a–e).

Low-abundance bacteria differ in their response strategy towards favorable conditions

The majority of OTUs with significantly increased ribosome abundance showed only a weak and some even no significant increase in genome abundance

over a period of 50 days (Figure 2a). These relative abundance shifts were not obscured by changes in absolute microbial abundance as the number of bacterial and archaeal 16S rRNA genes cm^{-3} soil remained constant throughout all incubations as measured by qPCR (Supplementary Figure S6a). Members of the genus *Desulfosporosinus* (Supplementary Figure S7a) were major responders to the addition of sulfate and substrate at the ribosome level but apparently did not grow. Two *Desulfosporosinus* OTUs were detected with a relative genome abundance of 0.003% (OTU0051) and 0.001% (OTU0228) in the native soil. Their relative genome abundance was slightly higher at the first measured time point in the microcosms (day 5, on average 0.022% and 0.009%, respectively), which could simply be due to the different soil subsamples used to characterize the native peat soil community and for setting up the microcosms. Alternatively, this might be explained by germination of a *Desulfosporosinus* sub-population (Stackebrandt, 2014), as the nucleic acids extraction from spores is less efficient than from active cells. Thereafter, the relative genome abundance of both OTUs did not change significantly over 50 days of incubation, as confirmed with a qPCR assay targeting the genus *Desulfosporosinus* (Supplementary Figure S6b). Also here, no growth was observed at a median abundance of 1.2×10^5 genomes cm^{-3} soil with one small outlier under lactate at day 50 that reached an abundance of 6.9×10^5 genomes cm^{-3} soil. In contrast, the relative ribosome abundance of both OTUs increased significantly (P -value < 0.001) from 0.30% to 3.69% (OTU0051) and 0.03% to 0.48% (OTU0228) between days 5 and 50 in microcosms with butyrate and sulfate amendment (Figure 3 and Supplementary Figure S5f). The more abundant *Desulfosporosinus* OTU0051 also responded in the same manner to additions of lactate and propionate. These findings were corroborated by qPCR analysis, which showed that the ribosome per genome ratio of the genus *Desulfosporosinus* increased from 2600–7300 to 57 000–84 000 throughout the incubation in sulfate-stimulated microcosms amended with propionate, lactate or butyrate (Supplementary Figure S6b). Such high cellular ribosome contents have been reported for exponentially growing pure cultures (Bremer and Dennis, 1996) but so far have not been observed in low-abundance microorganisms keeping a steady population size. Among recognized SRM OTUs, *Desulfosporosinus* OTU0051 correlated best with substrate-specific ST (Supplementary Figure S8) and dominated the average relative ribosome abundance of this metabolic group (Figure 2b).

In contrast to *Desulfosporosinus* and other slow- or non-growing taxa, OTUs of the genus *Telmatospirillum* (Supplementary Figure S7b) responded with both increased relative ribosome and genome abundance (Supplementary Figure S5a). The two most abundant *Telmatospirillum* OTUs were standing out among the responsive OTUs that are not



affiliated with recognized SRM because of the magnitude of their response as well as their ecologically incoherent behavior, that is, they showed an opposite response to sulfate under butyrate amendment. Although OTU0062 responded stronger in sulfate-stimulated as compared with -unstimulated microcosms, the opposite response was observed for OTU0029 (Figure 4). OTU0029 dominated the response under butyrate without sulfate amendment, reaching at the end of the incubation an average relative genome abundance of 1.1% and an average relative ribosome abundance of 12.9%. In comparison, in butyrate incubations with sulfate amendment OTU0062 reached an average relative genome abundance of 0.4% and an average relative ribosome abundance of 5.0%.

To assess the possibility that *Telmatospirillum* OTU0062 could represent a so far unrecognized SRM, we performed growth experiments with *T. siberiense*, which is the only cultured species of this genus (97.2% sequence similarity). *T. siberiense* was previously reported to ferment various dicarboxylic acids, pyruvate and glucose under anoxic conditions. It was further tested negative for sulfate reduction, but only with acetate as electron donor (Sizova et al., 2007). Here, we show that *T. siberiense* is also unable to grow anaerobically with either formate, propionate, lactate or butyrate in the presence of sulfate as electron acceptor (Supplementary Figure S9). In addition, all attempts to amplify the SRM marker genes *dsrAB* from DNA

extracts of *T. siberiense* using specific PCR assays failed.

Discussion

Low-abundance microorganisms drive a biogeochemically relevant process

In this study, we performed a series of controlled microcosm experiments that constituted a window into naturally fluctuating biogeochemical conditions in peat soils favorable for sulfate reduction. We show that SRM were clearly involved in the degradation of the intermediate fermentation products propionate, lactate and butyrate. It is possible that at least some of these substrates were not directly used by SRM but initially degraded by secondary fermenters to acetate, formate or H_2/CO_2 with SRM thriving on the latter. However, lower ST under formate and acetate as compared with propionate, lactate and butyrate rather indicated a direct utilization of these C3 and C4 compounds (Figure 1).

Responsive OTUs affiliated with ST were all composed of low-abundance OTUs in the native soil, most of which stayed below or around 0.1% relative genome abundance throughout the incubation period and were permanent members of the rare biosphere in our experiments. The only major exceptions were *Desulfomonile* OTU0144 and *Telmatospirillum* OTUs 0029 and 0062, which increased in sulfate-amended incubations to maximum relative genome abundances of 0.26–0.46% (Supplementary Figures S5a and b). As all three OTUs were of low abundance in the native soil, they classify as conditionally rare biosphere members (Lynch and Neufeld, 2015). Some responsive OTUs were affiliated to the sulfate-reducing genera *Desulfovibrio*, *Desulfomonile* and *Desulfopila* (Deltaproteobacteria), and *Desulfosporosinus* (Firmicutes), and showed a strong correlation in their response to ST ($r=0.71$ – 0.94 ; Supplementary Figure S8). In addition, several responsive OTUs not related to recognized SRM were also strongly associated with ST and/or SRM (Supplementary Figure S8). Here, two OTUs were standing out, with *Telmatospirillum* OTU0062 correlating strongly under butyrate to ST ($r=0.87$) as well as to all recognized SRM ($r=0.90$ – 0.96) and *Rhodospirillaceae* OTU0577 correlating to ST under propionate, lactate and butyrate ($r=0.83$ – 0.89). In the light of the large diversity of novel *dsrAB* variants, which have been repeatedly detected in the analyzed peatland (Loy et al., 2004; Pester et al., 2010; Steger et al., 2011), we speculated whether these OTUs may represent so far unrecognized SRM (detailed in Supplementary Results). Because *Telmatospirillum* OTU0062 had the highest 16S rRNA identity to a cultivated species, we used the type species *T. siberiense* as a case example to evaluate this hypothesis. However, *T. siberiense* tested negative for growth under sulfate-reducing conditions with all five substrates investigated in this study (Sizova et al., 2007; and this

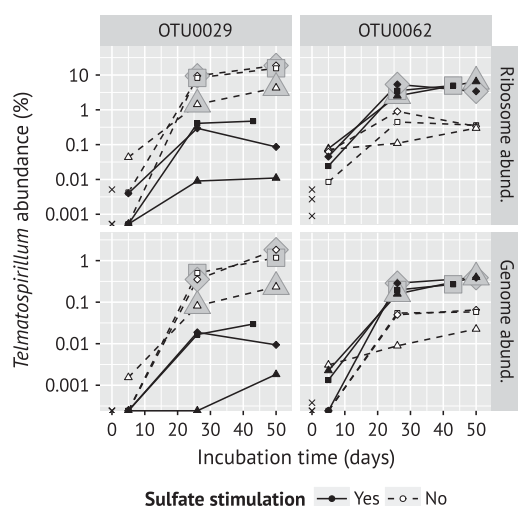


Figure 4 Temporal changes in relative ribosome and genome abundance of the two most abundant *Telmatospirillum* OTUs (0029 and 0062) in butyrate-amended incubations. Solid lines and symbols depict sulfate-stimulated microcosms, whereas dashed lines and open symbols depict controls without additional sulfate (note the logarithmic scale on the y axis). Diagonal crosses depict the abundance in the native soil. Halos around symbols indicate significantly higher abundance in the unstimulated or sulfate-stimulated microcosms, respectively, as compared with their respective controls and day 5. Both *Telmatospirillum* OTUs showed a significant increase in relative ribosome abundance over time, irrespective of sulfate stimulation. Data points drawn directly on the x axis represent relative abundance values of zero.

study, Supplementary Figure S9) and for the presence of *dsrAB*. Hence, it seems more likely that *Telmatospirillum* OTU0062, and possibly also *Rhodospirillaceae* OTU0577, fermented the provided substrates, whereas SRM made syntrophic use of the formed formate and/or H₂. However, this would not explain why both OTUs also responded to formate in the one microcosm that showed pronounced ST. As such, it may also be possible that these OTUs represent anaerobic sulfur oxidizers. As sulfide oxidation in wetlands can be coupled to the reduction of Fe(III) (Hansel *et al.*, 2015) or potentially also humic matter (Pester *et al.*, 2012b), *Telmatospirillum* OTU0062 and *Rhodospirillaceae* OTU0577 may represent microorganisms that are responsible for this so far missing link in the cryptic sulfur cycle, either by directly being involved in catalyzing these reactions or using elemental sulfur resulting from chemical oxidation of sulfide with Fe(III) compounds. Recently, correlation of sulfate-reducing activity and anaerobic CH₄ oxidation was reported for three North American wetlands (Segarra *et al.*, 2015). We did not observe any evidence for the presence and activity of typical microorganisms mediating sulfate-dependent anaerobic CH₄ oxidation in our incubations, despite the good coverage of the used primers for ANME archaea (ANME-1: 77%; ANME-2a/2b: 89%; ANME-2c: 76%; and ANME-3: 82%; Quast *et al.*, 2013) and the detection of related archaea within the orders *Methanosarcinales* (five OTUs) and *Methanocellales* (five OTUs).

A novel mechanism to display metabolic activity

The relationship between non-growth activity and cellular rRNA content is not understood but important for the interpretation of environmental rRNA data to characterize microbial communities (Blazewicz *et al.*, 2013). Our results provide strong evidence that a rare *Desulfosporosinus* species can follow an ecological strategy to increase its cellular rRNA content while maintaining its population size over a period of 50 days. As *Desulfosporosinus* OTU0051 always correlated best in its ribosome response to ST among all responsive SRM ($r = 0.89\text{--}0.94$) and contributed the highest relative ribosome abundance of this metabolic group, our results strongly suggest that this increase in protein synthesis potential was also translated into metabolic activity. All microorganisms have to divide at some time in their lives to avoid extinction. Population growth of a *Desulfosporosinus* species was observed in a DNA-stable isotope probing study of the same peatland after a longer period of incubation (73 days) (Pester *et al.*, 2010). Direct comparisons between the two studies are difficult because of the different incubation conditions; that is, peat soil microcosm incubations with a substrate mixture of formate, acetate, lactate and propionate, and two starvation phases in the previous study (Pester *et al.*, 2010). Whether the addition of a substrate mixture, especially in the presence of

acetate that is a commonly supplemented building block for biomass production in anoxic cultivation media, was responsible for the observed growth remains uncertain, but may explain the different growth response as compared with this study. The previous study also showed that *Desulfosporosinus* stayed rare in snapshot samples of the native soil spanning over a time period of 3 years. The combined results of this and the previous study thus indicate that rare peatland *Desulfosporosinus* species (i) maintain a stable, low-abundance population in the studied peatland and (ii) respond to favorable environmental conditions first by increasing their ribosome content and metabolic activity and only after prolonged time by growth. This is supported by the fact that *Desulfosporosinus* species contain an exceptionally high number of ribosomal (*rrn*) operons in their genomes (8–10 copies; Pester *et al.*, 2012a), which would enable them to react very quickly to favorable conditions by an increase in ribosomes. This strategy would make sense in the highly fluctuating conditions of peatlands in space and time (Jacks and Norrström, 2004; Knorr *et al.*, 2009; Reiche *et al.*, 2009; Frei *et al.*, 2012) and would be beneficial in escaping grazing pressure or viral attack (Lynch and Neufeld, 2015). In addition, such a strategy may be widespread in different habitats as suggested by the high ribosome to genome ratios of certain rare biosphere members in a 3-year survey of marine bacterioplankton (Campbell *et al.*, 2011) and in a snapshot analysis of a hypersaline lake sediment (Weigold *et al.*, 2015).

Rare biosphere members are either considered as conditionally rare taxa that eventually grow to large population sizes upon favorable conditions or as permanently rare taxa that express rapid, periodic small abundance changes upon activity (Lynch and Neufeld, 2015). Here, we provide conclusive evidence that the *Desulfosporosinus* species in the studied peatland soil is prototypical of a new strategy of rare biosphere members, namely to postpone growth and remain at a constant low population size while maintaining increased metabolic activity over prolonged time spans. As *Desulfosporosinus* species are members of the rare biosphere in permafrost soil, natural wetlands and rice paddies worldwide (Supplementary Figure S10), their sulfate-reducing activity and ecological strategy may have a widespread effect on ecosystem functioning in these environments. In conclusion, our results underscore the importance of the rare microbial biosphere not only as a seed bank of dormant microorganisms but also as an active mediator of biogeochemical processes that buffer against climate change.

Conflict of Interest

The authors declare no conflict of interest.

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Chapter 2

Growth arrest in the active rare biosphere

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Author contributions

BH, AL, and MP designed research. BH performed laboratory work and data processing. TR contributed computational resources. BH, CP, AL, and MP analyzed data. BH, AL, and MP wrote the manuscript. CP and TR revised the manuscript.

Growth arrest in the active rare biosphere

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Significance

The microbial rare biosphere represents the largest pool of biodiversity on Earth and constitutes, in sum of all its members, a considerable part of a habitat's biomass. Dormancy or starvation are typically used to explain a low-abundance state. We show that low-abundance microorganisms can be highly metabolically active while being growth-arrested over prolonged time periods. We show that this is true for microbial keystone species, such as a cosmopolitan but low-abundance sulfate reducer in wetlands that is involved in counterbalancing greenhouse gas emission. Our results challenge the central dogmas "metabolic activity translates directly into growth" as well as "low abundance equals little ecosystem impact" and provide an important step forward in understanding rare biosphere members relevant for ecosystem functions.

Abstract

Microbial diversity in the environment is mainly concealed within the rare biosphere, which is arbitrarily defined as all species with <0.1% relative abundance. While dormancy explains a low-abundance state very well, the cellular mechanisms leading to rare but active microorganisms are not clear. We used environmental systems biology to genomically and metabolically characterize a cosmopolitan sulfate reducer that is of low abundance but highly active in peat soil, where it contributes to counterbalance methane emissions. We obtained a 98%-complete genome of this low-abundance species, *Candidatus Desulfosporosinus infrequens*, by metagenomics. To test for environmentally relevant metabolic activity of *Ca. D. infrequens*, anoxic peat soil microcosms were incubated under diverse *in situ*-like conditions for 36 days and analyzed by metatranscriptomics. Compared to the no-substrate control, transcriptional activity of *Ca. D. infrequens* increased 56- to 188-fold in incubations with sulfate and acetate, propionate, lactate, or butyrate, revealing a versatile substrate use. Cellular activation was due to a significant overexpression of genes encoding ribosomal proteins, dissimilatory sulfate reduction, and carbon-degradation pathways, but not of genes encoding DNA or cell replication. We show for the first time that a rare biosphere member transcribes metabolic pathways relevant for carbon and sulfur cycling over prolonged time periods while being growth-arrested in its lag phase.

Introduction

The vast majority of microbial diversity worldwide is represented by the rare biosphere (Sogin *et al.*, 2006; Pedrós-Alió, 2012; Lynch and Neufeld, 2015; Jousset *et al.*, 2017). This entity of microorganisms consists of all microbial species that have an arbitrarily defined relative population size of $<0.1\%$ in a given habitat at a given time (Sogin *et al.*, 2006; Pedrós-Alió, 2012; Lynch and Neufeld, 2015; Jousset *et al.*, 2017). The rare biosphere is opposed by a much smaller number of moderately abundant or very abundant microbial species ($\geq 0.1\%$ and $\geq 1.0\%$ relative abundance, respectively, Hausmann *et al.*, 2016), which are thought to be responsible for the major carbon and energy flow through a habitat as based on their cumulative biomass. However, there is accumulating experimental evidence that the rare biosphere is not just a so-called “seed bank” of microorganisms that are waiting to become active and numerically dominant upon environmental change (Müller *et al.*, 2014; Lynch and Neufeld, 2015), but also harbors metabolically active microorganisms with important ecosystem functions (Jousset *et al.*, 2017).

First hints for metabolically active rare biosphere members were evident from seasonal patterns of marine bacterioplankton species. Here, many taxa that displayed recurring annual abundance changes were of low abundance and even during their bloom periods never reached numerically abundant population sizes (Campbell *et al.*, 2011; Hugoni *et al.*, 2013; Alonso-Sáez *et al.*, 2015). In soil environments, removal of low-abundance species by dilution-to-extinction had a positive effect on intruding species, suggesting that active low-abundance species pre-occupy ecological niches and thus slow down invasion (van Elsas *et al.*, 2012; Vivant *et al.*, 2013; Mallon *et al.*, 2015). Soil microorganisms of low relative abundance were also shown to play a role in community-wide species interactions, e.g. by being involved in the production of antifungal compounds that protect plants from pathogens (Hol *et al.*, 2015) or by constituting the core of microorganisms that respond to the presence of a particular plant species (Dawson *et al.*, 2017). Other examples include microorganisms with a specialized metabolism that sustain stable low-abundance populations in an ecosystem (Lynch and Neufeld, 2015). For example, N_2 -fixing microorganisms in the ocean (Großkopf *et al.*, 2012) or sulfate-reducing microorganisms (SRM) in peatlands (Pester *et al.*, 2010, 2012b; Hausmann *et al.*, 2016) were shown to fulfill such gatekeeper functions.

A peatland *Desulfosporosinus* species was one of the first examples identified as an active rare biosphere member contributing to an important ecosystem function (Pester *et al.*, 2010). This SRM is involved in the cryptic sulfur cycle of peatlands (Pester *et al.*, 2010; Hausmann *et al.*, 2016), which in turn controls the emission of the greenhouse gas CH_4 from these globally relevant environments (Pester *et al.*, 2012b). Although porewater sulfate concentrations are

typically quite low in peatlands (<300 μM , Pester *et al.*, 2012b), these environments are characterized by temporally fluctuating high sulfate reduction rates (up to 1800 $\text{nmol cm}^{-3} \text{ day}^{-1}$, Pester *et al.*, 2012b). These rates can be in the same range as in sulfate-rich marine surface sediments, where sulfate reduction is one of the major anaerobic carbon degradation pathways (e.g., Jørgensen, 1982; Leloup *et al.*, 2009; Holmkvist *et al.*, 2011a, 2011b). In low-sulfate peatlands, such high sulfate reduction rates can only be maintained by rapid aerobic or anaerobic re-oxidation of reduced sulfur species back to sulfate (Pester *et al.*, 2012b). Since SRM generally outcompete methanogens and syntrophically associated fermenters (Muyzer and Stams, 2008), they exert an important intrinsic control function on peatland CH_4 production (Gauci *et al.*, 2004, 2005; Gauci and Chapman, 2006). This is important, since natural wetlands, such as peatlands, are estimated to be responsible for 30% of the annual emission of this potent greenhouse gas (Ciais *et al.*, 2013; Kirschke *et al.*, 2013; Saunois *et al.*, 2016).

Little is known about the ecophysiology of metabolically active but low-abundance microorganisms. This lack of knowledge is clearly founded in their low numerical abundance making it inherently difficult to study their metabolic responses or even to retrieve their genomes directly from the environment. In a preceding study, we could show that the low-abundance peatland *Desulfosporosinus* species mentioned above follows an ecological strategy to increase its cellular ribosome content while maintaining a stable population size when exposed to favorable, sulfate-reducing conditions (Hausmann *et al.*, 2016). This was unexpected since metabolic activity in bacteria and archaea is typically immediately followed by growth. Furthermore, this *Desulfosporosinus* species can be found worldwide in a wide range of low-sulfate wetlands including not only peatlands but also permafrost soils and rice paddy fields (Hausmann *et al.*, 2016), which emphasizes its importance as a model organism for active rare biosphere members. In this study, we used an environmental systems biology approach to deepen our understanding of the ecophysiology of this rare biosphere member. In particular, we retrieved its genome directly from a combination of native and incubated peat soil and followed its transcriptional responses in peat soil microcosms, which were exposed to different environmental triggers that mimicked diverse *in situ* conditions.

Results

Recovery of a near complete genome of a rare biosphere member: *Desulfosporosinus* MAG SbF1 represents a novel species

We obtained the population genome of the low-abundance *Desulfosporosinus* species by co-assembly and differential coverage binning of metagenomes obtained from native peat soil and ¹³C-fractions of a DNA-stable isotope probing experiment (Fig. S1) (Hausmann *et al.*, 2018). The high quality metagenome-assembled genome (MAG) SbF1 had a size of 5.3 Mbp (on 971 scaffolds), a G+C content of 42.6%, a checkM-estimated completeness of 98.0%, a potential residual contamination of 3.9%, and 10% strain heterogeneity. Besides 16S and 23S rRNA genes, SbF1 carried 6440 protein-coding genes (CDS), five 5S rRNA gene copies, 59 tRNAs, and 37 other ncRNAs, making a total of 6543 predicted genomic features. Scaffolds encoding rRNA genes had a higher coverage compared to the average coverage of all scaffolds (Fig. S1), indicating multiple *rrn* operon copies, as is known from other *Desulfosporosinus* genomes. The 16S rRNA gene was 100% identical to *Desulfosporosinus* OTU0051, which was previously shown to correlate strongest among all recognized SRM to sulfate turnover in microcosms of the analyzed peat soil (Hausmann *et al.*, 2016). The genome size and G+C content was in the same range as observed for genomes of cultured *Desulfosporosinus* species (3.0–5.9 Mbp and 42–44%, respectively; Abicht *et al.*, 2011; Pester *et al.*, 2012a; Abu Laban *et al.*, 2015; Petzsch *et al.*, 2015; Mardanov *et al.*, 2016).

In the 16S rRNA tree, SbF1 formed a well supported clade with *Desulfosporosinus* sp. 44a-T3a (98.3% sequence identity), *Desulfosporosinus* sp. OT (98.8%), and *Desulfosporosinus* sp. 5apy (98.1%). The most similar validly described species was *D. lacus* with a sequence identity of 97.5% (Fig. S2a). Phylogenomics confirmed *Desulfosporosinus* sp. OT as the closest relative (Fig. S2b) with average amino and nucleic acid identities (AAI and ANI) of 77% and 79%, respectively (Fig. S3). The intra-genus AAI variability of *Desulfosporosinus* species was 69–93% (Fig. S3). MAG SbF1 represents a novel species in this genus based on species-level thresholds of 99% for the 16S rRNA gene (Stackebrandt and Ebers, 2006) and 96.5% for ANI (Varghese *et al.*, 2015).

The versatile energy metabolism of the low-abundance *Desulfosporosinus*

Desulfosporosinus MAG SbF1 encoded the complete canonical pathway for dissimilatory sulfate reduction (Fig. 1, Table S1). This encompassed the sulfate adenylyltransferase (Sat), adenylyl-sulfate reductase (AprBA), dissimilatory sulfite reductase (DsrAB), and the sulfide-releasing DsrC, which are sequentially involved in the reduction of sulfate to sulfide. In addition, genes encoding the electron-transferring QmoAB and DsrMKJOP complexes were detected, with their

subunit composition being typical for *Desulfosporosinus* species (Abicht *et al.*, 2011; Pester *et al.*, 2012a; Petzsch *et al.*, 2015; Mardanov *et al.*, 2016). Other *dsr* genes included *dsrD*, *dsrN*, and *dsrT* (Rabus *et al.*, 2015), with hitherto unvalidated function, as well as *fdxD*, which encodes a [4Fe4S]-ferredoxin, and a second set of DsrMK-family encoding genes (*dsrM2* and *dsrK2*). SbF1 also encoded the trimeric dissimilatory sulfite reductase AsrABC (anaerobic sulfite reductase) (Huang and Barrett, 1991).

SbF1 carries genes for both complete and incomplete oxidation of propionate and lactate. In addition, the ability to utilize acetate, formate, or H₂ as electron donors was encoded (Fig. 1). All enzymes necessary for propionate oxidation to the central metabolite pyruvate (including those belonging to a partial citric acid cycle) were encoded on two scaffolds (Table S1). For lactate utilization, SbF1 carried three paralogs of glycolate/D-lactate/L-lactate dehydrogenase family genes. However, the substrate specificity of the encoded enzymes could not be inferred from sequence information alone. The transcription of *lutDF* and *lutD_2* was stimulated by the addition of L-lactate (Fig. 1), which indicates that these genes encode functional lactate dehydrogenases (LDH). The third paralog (*glcDF*, Table S1) was not stimulated by lactate. *LutDF* was organized in an operon with a lactate permease (*LutP*) and a lactate regulatory gene (*lutR*). *LutD_2* was organized in a operon with an electron-transferring flavoprotein (*EtfBA_2*), which resembled the electron-confurcating LDH/Etf complex in *Acetobacterium woodii* (Weghoff *et al.*, 2015). LDHs have been shown to utilize both L- and D-lactate (e.g., Weghoff *et al.*, 2015; Zhang *et al.*, 2016). However, SbF1 also encoded a lactate racemase (*LarA*) and a lactate racemase-activating system (*LarEBC*) for interconversion of both stereoisomers (Desguin *et al.*, 2014).

Pyruvate, the intermediate product in propionate and lactate degradation, can be further oxidized to acetyl-CoA with either one of several pyruvate-ferredoxin oxidoreductases (*PfoA*) or formate C-acetyltransferase (*PfID*). Acetyl-CoA can then be completely oxidized to CO₂ via the Wood-Ljungdahl pathway (Pierce *et al.*, 2008), which is complete in SbF1 (Fig. 1, Table S1) and present in the genomes of all other sequenced *Desulfosporosinus* species (Abicht *et al.*, 2011; Pester *et al.*, 2012a; Petzsch *et al.*, 2015; Mardanov *et al.*, 2016). Alternatively, acetyl-CoA may be incompletely oxidized to acetate via acetyl-phosphate by phosphate acetyltransferase (*Pta*) and acetate kinase (*AckA*). *Pta* and *AckA* are bidirectional enzymes, opening the possibility that acetate could be degraded via these two enzymes and the downstream Wood-Ljungdahl pathway to CO₂.

Formate and H₂ represented additional potential electron donors for SbF1. Its genome encoded three formate dehydrogenases (FDH). FDH-1 consists of three subunits (*fdhCBA*) while FDH-2 (*FdhA_2*) and FDH-3 (*FdhA-3*) are monomeric enzymes. In addition, [NiFe] hydrogenases of

group 1 and 4f, as well as [FeFe] hydrogenases of group A (Greening *et al.*, 2016) were encoded. Homologs of genes for butyrate oxidation were missing in SbF1 (Schmidt *et al.*, 2013), which is in contrast to other *Desulfosporosinus* species (e.g., *D. orientis*). Both glycolysis and gluconeogenesis were complete. However, neither a glucokinase or a phosphotransferase system was found (PTS). Coupling of electron transfer to energy conservation could be mediated in SbF1 by a H⁺/Na⁺-pumping Rnf complex (RnfCDGEAB) (Buckel and Thauer, 2013) and a NADH dehydrogenase (respiratory complex I, NuoABCDEFGHJKLMN). In addition, the complete gene set for ATP synthase (AtpABCDEFGH) was identified (Fig. 1, Table S1).

Activation of energy metabolism is uncoupled from cell division initiation

We used metatranscriptomics to analyse gene expression changes of *Desulfosporosinus* MAG SbF1 in anoxic peat microcosms, which mimicked diverse *in situ*-like conditions. Total transcriptional activity of *Desulfosporosinus* MAG SbF1 was clearly stimulated by individual additions of acetate, propionate, lactate, and butyrate in combination with sulfate. In these incubations, total mRNA counts of SbF1 increased by 56-, 80-, 62-, and 188-fold as compared to the no-substrate-control, respectively, and constituted between $0.11 \pm 0.13\%$ (acetate) and $0.36 \pm 0.02\%$ (butyrate) of all transcripts in the respective metatranscriptomes after 36 days (Fig. 2a). This substrate-specific activity was mirrored in the increased transcription of genes encoding ribosomal proteins as general activity markers (Fig. 2b) and of all dissimilatory sulfate reduction genes, except the alternative pathway via *asrABC* (Fig. 3). For example, Spearman's rank correlation coefficients of *dsrA* and *dsrB* transcripts as compared to total mRNA counts were 0.91 and 0.90, respectively (FDR-adjusted *p*-value < 0.001). Other enzyme complexes involved in the central metabolism of SbF1 such as the ATP synthase, the NADH dehydrogenase (complex I), and ribosomal proteins followed the same transcriptional pattern (Fig. 3) with an average Spearman's rank correlation coefficients of 0.79 ± 0.07 (*n* = 72, FDR-adjusted *p*-value < 0.05) to total mRNA counts. Interestingly, transcription of genes encoding proteins involved in general stress response were stimulated as well. In particular, genes encoding the universal stress promotor *UspA* and the GroSL chaperonin showed an increased transcription (Fig. 3) with an average Spearman's rank correlation coefficients of 0.76 ± 0.06 (*n* = 3, FDR-adjusted *p*-value < 0.05) to total mRNA counts.

In addition, we screened the COG categories D, L, and M for indicator genes that encode functions in cell division (e.g., *ftsZ* or *minE*), DNA replication (e.g., *gyrBA*, *dnaC*, and *dnaG*) and cell envelope biogenesis (e.g., *murABCDEFGI*), respectively, and followed their expression patterns. Genes that unambiguously encoded such functions (Table S1) showed either no or only barely detectable but insignificant (FDR-adjusted *p*-value > 0.05) increases in transcripts under these conditions (Fig. 2b, detailed in Fig. S4). Extension of this analysis to all genes belonging to COG D (*n* = 73), L (*n* = 280), and M (*n* = 215), which included also genes with

ambiguous classification or unknown function, revealed that also here 96%, 99%, and 99%, respectively, were not significantly overexpressed under acetate, propionate, lactate, and butyrate in combination with sulfate (Table S1, Table S3). Here, the average Spearman's rank correlation coefficients to total mRNA counts was only 0.45 ± 0.13 (FDR-adjusted p -value < 0.05 , Table S1).

We also analysed genes reported to be upregulated immediately after phage infection, as a potential ecological driver that controls bacterial population size. Respective genes in *Bacillus subtilis* encode, e.g., functions in DNA and protein metabolism and include the ribonucleoside-diphosphate reductase (*nrdEF*), aspartyl/glutamyl-tRNA amidotransferase (*gatCAB*), and the proteolytic subunit of ATP-dependent Clp protease (*clpP*) (Mojardín and Salas, 2016). However, homologs in SbF1 were not significantly overexpressed (FDR-adjusted p -value > 0.05), which was reflected in an average Spearman's rank correlation coefficient of 0.63 ± 0.08 ($n = 5$, FDR-adjusted p -value < 0.05) to total mRNA counts. The same was true when screening for active sporulation of a *Desulfosporosinus* subpopulation as an alternative explanation for a low population size. The identified sporulation genes (*spo0A-spoVT*) did not show any significant increase in transcript numbers as well, with the only exception of *spoIIIAD*. This stage III sporulation gene was significantly more abundant when stimulated with propionate and sulfate, however did not correlate to total mRNA levels (Table S1). Again, genes involved in sporulation had a low average Spearman's rank correlation coefficient of 0.44 ± 0.13 ($n = 22$, FDR-adjusted p -value < 0.05) to total mRNA counts.

The individual incubation regimes additionally triggered transcriptional activation of the respective substrate degradation pathways of *Desulfosporosinus* MAG SbF1. For example, all genes necessary for the conversion of propionate to pyruvate were overexpressed only upon addition of propionate and sulfate but not in any other incubation type. The same was true for lactate degradation, where genes encoding the lactate permease, lactate racemase and two of the detected lactate dehydrogenases were overexpressed upon addition of both lactate and sulfate, but not in incubations with lactate only (Fig. 3). Although genes encoding phosphotransacetylase and acetate kinase were overexpressed under lactate and propionate, the complete Wood-Ljungdahl pathway was overexpressed as well, which indicates that at least part of these substrates were completely degraded to CO₂ rather than to acetate and CO₂. This conclusion was supported by the overexpression of the Wood-Ljungdahl pathway in incubations amended with acetate and sulfate. Interestingly, the Wood-Ljungdahl pathway was also overexpressed upon addition of butyrate and sulfate. Under such conditions, *Desulfosporosinus* MAG SbF1 apparently relies on a syntrophic lifestyle based on acetate uptake as it lacked the capability for butyrate oxidation; albeit failed recovery of the butyrate degradation pathway during binning cannot be excluded.

Discussion

Current knowledge on the mechanisms that interconnect energy metabolism, gene expression, cell division, and population growth of microorganisms are mainly based on pure cultures that can be easily maintained in the laboratory. Here, the typical lifecycle of a metabolically active microorganisms would go through an activating lag phase, an exponential growth phase, and a stationary phase upon limitation of substrate, nutrient, or space. Under ideal conditions, a single *Escherichia coli* cell would grow to a population with the mass of the Earth within 2 days. Clearly, this does not occur, but the discrepancy between potential and actual growth underscores that microorganisms spend the vast majority of their time not dividing (Bergkessel *et al.*, 2016). A large fraction of these microorganisms is part of the rare biosphere. For example, in the studied peatland, the sum of all low-abundance species made up approximately 12% of the total bacterial and archaeal 16S rRNA genes (Hausmann *et al.*, 2016). In other soils, low-abundance *Alphaproteobacteria* and *Bacteroidetes* alone constituted in sum 10% and 9% of the total bacterial population, respectively, while all low-abundance populations summed up to 37% of all bacteria (Dawson *et al.*, 2017). Upon strong environmental change, low-abundance microorganisms often grow to numerically abundant populations and replace dominant species as observed for microbial community changes after an oil spill (Teira *et al.*, 2007; Newton *et al.*, 2013) or in the response of soil microorganisms towards the presence of plants (Dawson *et al.*, 2017). However, subtle environmental changes (Hausmann *et al.*, 2016) or recurring seasonal shifts (Campbell *et al.*, 2011; Vergin *et al.*, 2013; Alonso-Sáez *et al.*, 2015) often lead to rather small shifts in low-abundance populations without rare biosphere members becoming numerically dominant.

The low-abundance *Desulfosporosinus* MAG SbF1 represents an interesting case of the latter response type. When exposed to favorable, sulfate-reducing conditions in peat soil microcosms, it did not increase its population size but drastically increased its cellular ribosome content by one order of magnitude to 57,000–84,000 16S rRNA molecules per cell (Hausmann *et al.*, 2016). Throughout the incubation period of 50 days, it correlated best in its 16S rRNA response to sulfate turnover among all identified SRM (Hausmann *et al.*, 2016). In this study, we expanded upon this observation by genome-centric metatranscriptomics to test whether the increase in cellular ribosome content is indeed translated into increased transcriptional and, as a consequence, metabolic activity of *Desulfosporosinus* MAG SbF1. As expected, increases in cellular 16S rRNA content clearly corresponded to increased transcription of genes coding for ribosomal proteins (Fig. 2b; Hausmann *et al.*, 2016). This cellular ribosome increase under sulfate-reducing conditions was correlated to an increase in total mRNA counts (Fig. 2). This is the first time that changes in population-wide 16S rRNA levels are proven to be directly linked to transcriptional activity for a rare biosphere member.

Analyzing the transcriptional response of a rare biosphere member under *in situ*-like conditions opens the unique opportunity to gain insights into its ecophysiology. *Desulfosporosinus* MAG SbF1 clearly overexpressed its sulfate reduction pathway under sulfate amendment when supplied with either acetate, lactate, propionate, or butyrate as compared to the no-substrate and the methanogenic controls (Fig. 3). Detailed analysis of the transcribed carbon degradation pathways showed that *Desulfosporosinus* MAG SbF1 is able to oxidize propionate, lactate, and acetate completely to CO₂. Under butyrate-amended conditions it presumably relied on syntrophic oxidation of acetate supplied by a primary butyrate oxidizer. This shows that *Desulfosporosinus* MAG SbF1 is capable of utilizing diverse substrates that represent the most important carbon degradation intermediates measured in peatlands (Schmalenberger *et al.*, 2007; Küsel *et al.*, 2008). Such a generalist lifestyle is of clear advantage in peat soil given the highly variable nutrient and redox conditions (Schmalenberger *et al.*, 2007; Küsel *et al.*, 2008). These fluctuations are caused by the periodically changing water table that steadily shifts the oxic-anoxic interface (Knorr *et al.*, 2009; Reiche *et al.*, 2009). In addition, the complex flow paths of water create distinct spatial and temporal patterns (hot spots and hot moments) of various biogeochemical parameters, to which peat microorganisms have to adapt (Jacks and Norrström, 2004; Knorr *et al.*, 2009; Knorr and Blodau, 2009; Frei *et al.*, 2012).

The question remains, which mechanisms are at work that keep the *Desulfosporosinus* MAG SbF1 population in a stable low-abundance state? Population sizes can be kept low by actively restricting growth. Alternatively, ongoing growth could be hidden by continuous predation, viral lysis, or active sporulation of a major subpopulation. To answer this question, we analysed expression patterns of growth-specific genes. Compared to the strong overexpression of metabolic or ribosomal protein genes, transcription of genes essential for DNA replication, cell division, and cell envelope biogenesis did not increase or only marginally (Fig. 2b, Fig. S4). Genes encoding DNA replication or cell division typically show a largely invariable transcription in the exponential and stationary phase (e.g., Sumby *et al.*, 2012; Brudal *et al.*, 2013; Sihto *et al.*, 2014). However, there is experimental evidence that in the lag phase transcription of growth-specific genes is not stable but increases due to the overall activation of cellular processes (Rolfe *et al.*, 2012). In this context, the lack of an increasing transcription of growth-specific genes would clearly indicate a state of no growth rather than an actively dividing population that is kept stable by an equally high growth and mortality or sporulation rate. This conclusion is further corroborated by the lack of overexpressed sporulation genes or genes upregulated directly after phage attack. Nevertheless, the ATP generated by the induced energy metabolism has to be utilized somehow. This could be mediated by the production of storage compounds or by counterbalancing environmental stress. We found no indication for the former scenario but observed overexpression of the universal stress promotor UspA, which is one of the most abundant proteins in growth-arrested cells (Kvint *et al.*, 2003), and the

chaperonin GroSL, which was linked previously to stress response such as low pH (Silva *et al.*, 2005). Since the pH in the analyzed peat soil incubations varied between 4.1–5.0 (Hausmann *et al.*, 2016), coping with a low pH would be the most likely reason that deviates ATP away from growth towards stress response. Based on the integrated findings of our previous study (stable population over 50 days as based on 16S rRNA gene counts; Hausmann *et al.*, 2016), and this study (no activation of the DNA replication and cell division machinery within 36 days), we propose that *Desulfosporosinus* MAG SbF1 was growth-arrested in the lag phase over a period of at least 50 days while being a metabolically active rare biosphere member. This finding shows that growth arrest is not restricted to starving or otherwise limited microorganisms that persist in the environment (Bergkessel *et al.*, 2016) but can also occur in metabolically highly active microorganisms.

Our results are important in the context of the increasing awareness that the microbial rare biosphere is not only the largest pool of biodiversity on Earth (Sogin *et al.*, 2006; Pedrós-Alió, 2012; Lynch and Neufeld, 2015; Jousset *et al.*, 2017) but in sum of all its low-abundance members constitutes also a large part of the biomass in a given habitat (e.g., Hausmann *et al.*, 2016; Dawson *et al.*, 2017). Understanding the mechanisms governing this low-abundance prevalence and its direct impact on ecosystem functions and biogeochemical cycling is thus of utmost importance. *Desulfosporosinus* MAG SbF1 has been repeatedly shown to be involved in cryptic sulfur cycling in peatlands (Pester *et al.*, 2010; Hausmann *et al.*, 2016) — a process that counterbalances the emission of the greenhouse gas methane due to the competitive advantage of SRM as compared to microorganisms involved in the methanogenic degradation pathways (Muyzer and Stams, 2008). This species can be found worldwide in low-sulfate environments impacted by cryptic sulfur cycling including not only peatlands but also permafrost soils, rice paddies, and other wetland types (Hausmann *et al.*, 2016). Here, we provided proof that *Desulfosporosinus* MAG SbF1 is indeed involved in the degradation of important anaerobic carbon degradation intermediates in peatlands while sustaining a low-abundance population. It has a generalist lifestyle in respect to the usable carbon sources, re-emphasizing its importance in the carbon and sulfur cycle of peatlands. Our results provide an important step forward in understanding the microbial ecology of biogeochemically relevant microorganisms and show that low-abundance keystone species can be studied “in the wild” using modern environmental systems biology approaches.

Proposal of *Candidatus Desulfosporosinus infrequens*

Based on its phylogenetic placement and novel ecophysiological behaviour, we propose that *Desulfosporosinus* MAG SbF1 represents a novel species with the provisional name *Candidatus Desulfosporosinus infrequens* sp. nov. (in.fre'quens. L. adj. *infrequens*, rare, referring to its low relative abundance). Based on its genome-derived metabolic potential and support from metatranscriptomics, *Ca. D. infrequens* is capable of complete oxidation of acetate, propionate and lactate with sulfate as the electron acceptor, with further potential for oxidation of molecular hydrogen (Fig. 1).

Materials and Methods

Genome assembly, binning, and phylogenetic inference

Sampling of peat soil from the acidic peatland Schlöppnerbrunnen II (Germany), DNA-stable isotope probing (DNA-SIP), total nucleic acids extraction, metagenome sequencing and assembly, and coverage-based binning was described previously (Pester *et al.*, 2010, Hausmann *et al.* (2016); Hausmann *et al.*, 2018). In brief, DNA from native peat soil (10–20 cm depth) and DNA pooled from 16 ¹³C-enriched fractions (density 1.715–1.726 g mL⁻¹) of a previous DNA-SIP experiment with soil from the same site (Pester *et al.*, 2010) was sequenced using the Illumina HiSeq 2000 system. DNA-SIP was performed after a 73-day incubation (again 10–20 cm depth) that was periodically amended with small dosages of sulfate and first a mixture of unlabeled formate, acetate, propionate, and lactate for two weeks and thereafter a mixture of ¹³C-labeled formate, acetate, propionate, and lactate (all in the lower μM-range) (Pester *et al.*, 2010). Raw reads were quality filtered, trimmed, and co-assembled into one metagenomic assembly using the CLC Genomics Workbench 5.5.1 (CLC Bio). Differential coverage binning was applied to extract the *Desulfosporosinus* metagenome-assembled genome (MAG) (Albertsen *et al.*, 2013). A side effect of sequencing a DNA-SIP sample is an apparent G+C content skew, which was normalized arbitrarily to improve binning using the following formula (Herbold *et al.*, 2017; Hausmann *et al.*, 2018):

$$\frac{\text{Coverage}}{\text{G+C content}^9} \times 10^{15}$$

Scaffolds encoding the 16S and 23S rRNA genes were successfully identified using paired-end linkage data (Albertsen *et al.*, 2013). Completeness, contamination, and strain heterogeneity was estimated using CheckM 1.0.6 (Parks *et al.*, 2015).

Phylogenomic analysis of the *Desulfosporosinus* MAG was based on a concatenated set of 34

phylogenetically informative marker genes as defined by Parks *et al.* (2015) and the Bayesian phylogeny inference method PhyloBayes using the CAT-GTR model (Lartillot *et al.*, 2009). 16S rRNA gene-based phylogeny was inferred using the ARB SILVA database r126 as a reference (Quast *et al.*, 2013), the SINA aligner (Pruesse *et al.*, 2012), and the substitution model testing and maximum likelihood treeing method IQ-TREE (Trifinopoulos *et al.*, 2016). Pairwise 16S rRNA gene sequence identities were calculated with T-Coffee 11 (Notredame *et al.*, 2000). Pairwise average nucleic and amino acid identities (ANI, AAI, Varghese *et al.*, 2015) between protein-coding genes of the *Desulfosporosinus* MAG and reference genomes were calculated as described previously (Hausmann *et al.*, 2018)

Genome annotation

The genome was annotated using the MicroScope annotation platform (Vallenet *et al.*, 2017). Annotation refinement for selected genes was done as follows: proteins with an amino acid identity $\geq 40\%$ (over $\geq 80\%$ of the sequence) to a Swiss-Prot entry (The UniProt Consortium, 2017), curated MaGe annotation (Vallenet *et al.*, 2017), or protein described in the literature were annotated as true homologs of known proteins. The same was true, if classification according to InterPro families (Mitchell *et al.*, 2015; Jones *et al.*, 2014), TIGRFAMs (Haft *et al.*, 2003), and/or FIGfams (Overbeek *et al.*, 2014) led to an unambiguous annotation. Proteins with an amino acid identity $\geq 25\%$ (over $\geq 80\%$ of the sequence) to a Swiss-Prot or TrEMBL (The UniProt Consortium, 2017) entry were annotated as putative homologs of the respective database entries. In addition, classification according to COG (Galperin *et al.*, 2015) or InterPro superfamilies, domains, or binding sites were used to call putative homologs in cases of an unambiguous annotation. Membership to syntenic regions (operons) was considered as additional support to call true or putative homologs.

Metatranscriptomics from single-substrate incubations

We analysed total RNA from anoxic peat soil slurry microcosms that were described previously (Hausmann *et al.*, 2016, 2018). In brief, anoxic microcosms were incubated at 14 °C in the dark for 50 days and regularly amended with either low amounts of sulfate (76–387 μM final concentrations) or incubated without an external electron acceptor. Formate, acetate, propionate, lactate, butyrate ($<200 \mu\text{M}$), or no external electron donor was added to biological triplicates each. RNA was extracted from the native soil, and after 8 and 36 days of incubations, followed by sequencing with the Illumina HiSeq 2000/2500 system. Raw reads were quality-filtered as described previously (Hausmann *et al.*, 2018) and mapped to the combined metagenomic assembly using Bowtie 2 (Langmead and Salzberg, 2012). Counting of mapped reads to protein-coding genes (CDS) was performed with featureCounts 1.5.0 (Liao *et al.*, 2014). We used an unsupervised approach to identify CDS stimulated by sulfate and the

different substrates regimes. First, we applied the DESeq2 R package (Love *et al.*, 2014; R Core Team, 2017) to identify differentially expressed CDS. Treatments without external sulfate added and samples after 8 days of incubations had too little transcript counts to be used for a statistical approach. Therefore, we limited our analysis to pairwise comparison of sulfate-stimulated microcosms after 36 days of incubations. We compared each substrate regime to the no-substrate controls and each other. The set of all significantly differentially expressed CDS (FDR-adjusted p -value < 0.05) were further clustered into response groups. For clustering, we calculated pairwise Pearson's correlation coefficients (r) of variance stabilized counts (cor function in R), transformed this into distances ($1-r$), followed by hierarchical clustering (hclust function in R). Variance stabilisation was performed using the rlog function of the DESeq2 package.

Sequence data availability

The MAG SbF1 is available at MicroScope (<https://www.genoscope.cns.fr/agc/microscope/>) and is also deposited under the ENA accession number OMOF01000000. Metagenome and -transcriptomic data is available at the Joint Genome Institute (<https://genome.jgi.doe.gov/>) and is also deposited under the NCBI accession numbers PRJNA412436 and PRJNA412438, respectively.

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Figures

Fig. 1

Metabolic model of *Desulfosporosinus* MAG SbF1. Gene expression stimulated by specific substrates in combination with sulfate is indicated by coloured points. Paralogous genes are indicated by an underscore followed by a number. Plus signs indicates proposed protein complexes. Details for all genes are in given in Table S1 and transcription patterns are shown in Fig. 3. For the citric acid cycle and anaplerotic reactions, carriers of reducing equivalents and further by-products are not shown. The following abbreviations were used. X: unknown reducing equivalents, e.g., NAD⁺ or ferredoxin. WL: Wood-Ljungdahl pathway consisting of enzymes encoded by the *acs* operon, MetF, F_oD, F_hA, and F_hS. TCA: citric acid cycle. FDH: formate dehydrogenase. Hase: hydrogenase. NDH-1: NADH dehydrogenase 1. LDH: lactate dehydrogenase.

Fig. 2

Time-resolved transcriptional changes of *Desulfosporosinus* MAG SbF1 in anoxic peat soil microcosms under various *in situ*-like conditions. (a) Total mRNA of all CDS and (b) selected genes encoding the sulfate-reduction pathway (*sat*, *dsrA*), ribosomal proteins of the large (*rplA*) and small subunit (*rpsC*), cell division (*ftsZ*), DNA replication (*gyrB*), and peptidoglycan synthesis (*murA*). Solid lines and symbols represent sulfate-stimulated microcosms whereas dashed lines and open symbols represent control microcosms without external sulfate. Panels represent the various substrate incubations, native stands for native peat soil. Different symbols represent replicates and are consistent throughout all panels.

Fig. 3

Transcription patterns of whole pathways and central enzyme complexes involved in the carbon and energy metabolism of *Desulfosporosinus* MAG SbF1 under *in situ*-like conditions. In addition, transcription patterns of general stress response proteins are shown. Mean abundance for the native soil (—) and each incubation treatment and time point is shown. Supplemented substrates are indicated by initials and addition of external sulfate is depicted by –S/+S (columns). Abundance values are normalized variance-stabilized counts x , which were scaled from 0 to 1 for each CDS using the formula $[x - \min(x)] / \max[x - \min(x)]$. Incompletely assembled genes are indicated by _a, _b, and _c.

Fig. 1.

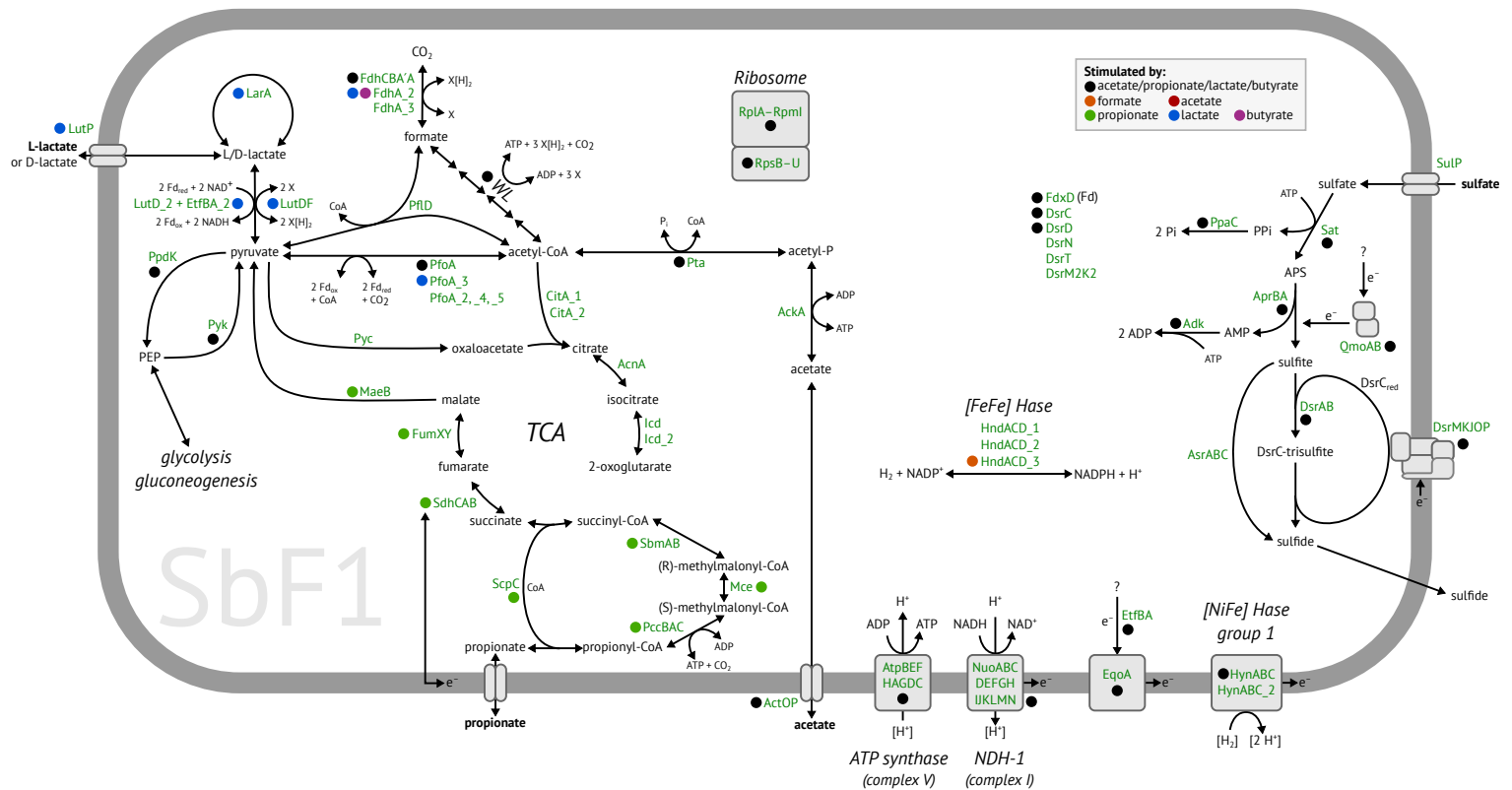


Fig. 2.

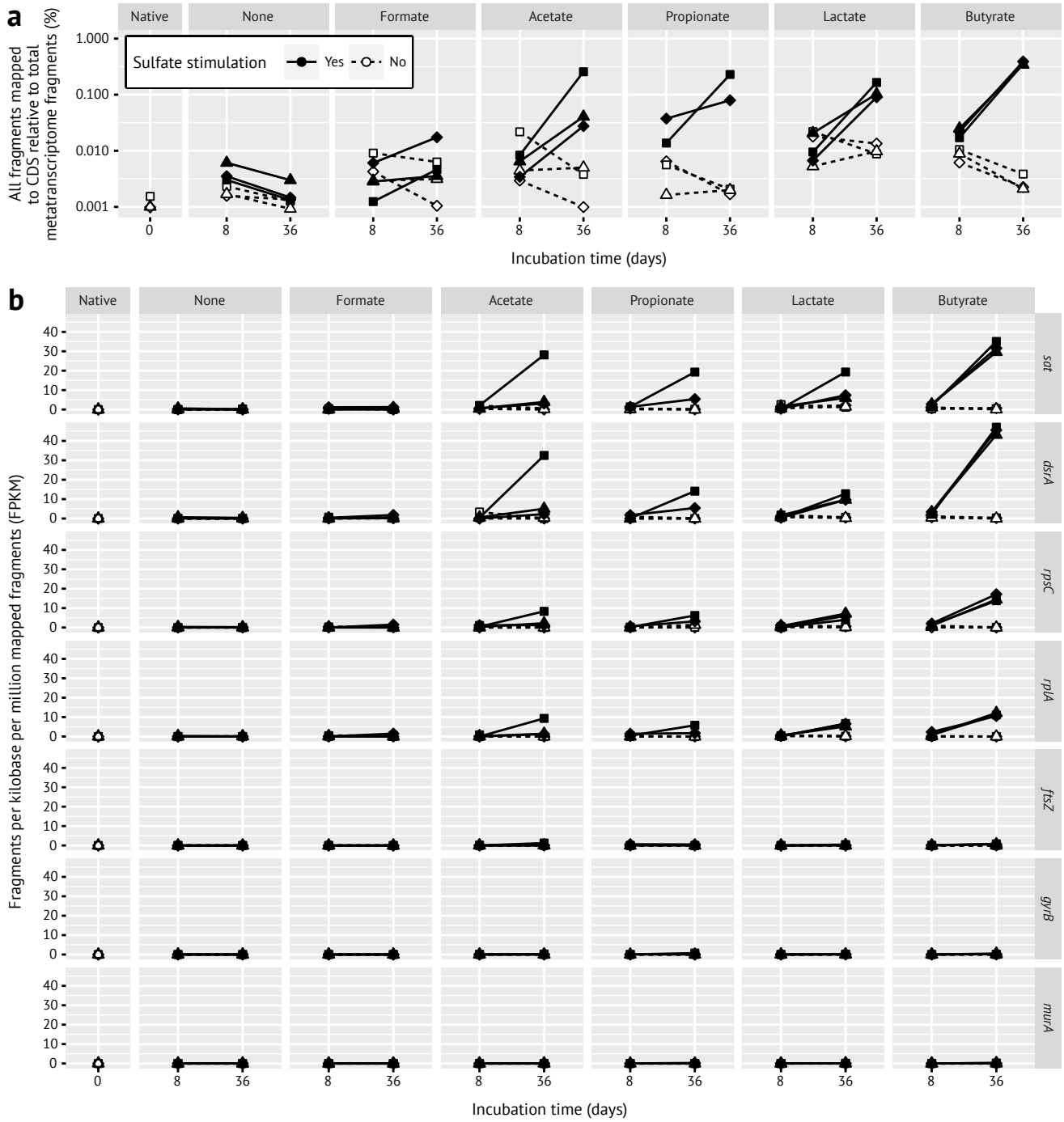
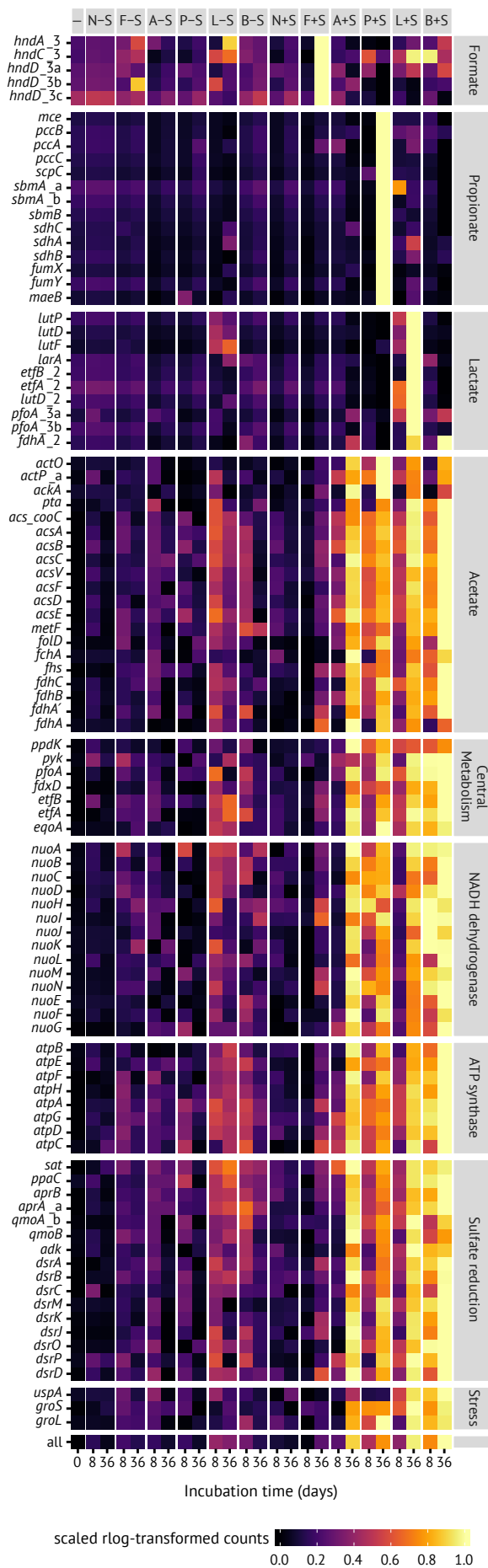


Fig. 3.



Chapter 3

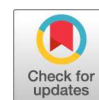
Draft genome sequence of *Telmatospirillum siberiense* 26-4b1, an acidotolerant peatland alphaproteobacterium potentially involved in sulfur cycling

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Author contributions

BH, PP, and AL designed research. BH and KS performed strain cultivation and extracted DNA. PP and CWH assembled the genome. BH and PP annotated and analysed the genome. BH, PP, and AL wrote the manuscript. KS, CWH, HD, and MW revised the manuscript.



Draft Genome Sequence of *Telmatospirillum siberiense* 26-4b1, an Acidotolerant Peatland Alphaproteobacterium Potentially Involved in Sulfur Cycling

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ABSTRACT The facultative anaerobic chemoorganoheterotrophic alphaproteobacterium *Telmatospirillum siberiense* 26-4b1 was isolated from a Siberian peatland. We report here a 6.20-Mbp near-complete high-quality draft genome sequence of *T. siberiense* that reveals expected and novel metabolic potential for the genus *Telmatospirillum*, including genes for sulfur oxidation.

All three validly described strains of *Telmatospirillum siberiense* were isolated from a mesotrophic Siberian peatland (1). In addition, closely related 16S rRNA gene sequences were recovered from other peatlands (2–8). Uncultured members of the genus *Telmatospirillum* have been associated with the anaerobic degradation of glucose (2), butyrate (8), acetate, propionate, and lactate (3, 7) in peat soils. Furthermore, in the literature, uncultured *Telmatospirillum* spp. were stimulated by propionate and butyrate under sulfate-reducing conditions (7), indicating a yet-unresolved role in sulfur cycling.

We obtained the draft genome sequence of *Telmatospirillum siberiense* 26-4b1 (DSM 18240), the type strain of the only validly described species of this genus (1). DNA was isolated using the DNeasy blood and tissue kit (Qiagen), and sequencing libraries were prepared using the Nextera XT kit (Illumina) and sequenced with the Illumina HiSeq 2000 platform. Raw reads were assembled using SPAdes (version 3.6.2) (9) and subsequently iteratively ($n = 4$) reassembled with SPAdes (version 3.11.1) using contigs >1 kb from the previous assembly as the “trusted contigs” input. The draft genome sequence consists of 81 scaffolds, with a total size of 6,202,994 bp, a G+C content of 62.3%, and an N_{50} value of 131,736 bp. Based on CheckM (10), the completeness of the draft genome is 99.5%. The genome was annotated using Rapid Annotation of microbial genomes using Subsystems Technology (RAST) (11) and the NCBI Prokaryotic Genome Annotation Pipeline. The draft genome contains 5,405 coding sequences (CDSs) and 48 tRNAs. Furthermore, the rRNA genes are carried on a small scaffold, with a coverage of 1,210 $\times$, although the average genome coverage was 216 $\times$, indicating the presence of 5 to 6 rRNA operons.

T. siberiense was reported to grow anaerobically, utilizing organic acids and sugars as energy and carbon sources, with the capability for nitrogen fixation. Autotrophic growth on hydrogen and tolerance to low oxygen pressure (up to 5 kPa) were also observed (1). As expected, the draft genome contains the genetic repertoire for these physiological traits. *T. siberiense* encodes the Embden-Meyerhof-Parnas (glycolysis) pathway, pentose phosphate pathway, Entner-Doudoroff pathway, and oxidative tricarboxylic acid cycle. It possesses genes of lactate dehydrogenases, for mixed acid fermentation, and several pathways for monosaccharide degradation. Three nitrogenase operons (two [FeMo]nitrogenases and one [FeFe]nitrogenase) and three [NiFe]hydrogenases (groups 1c, 1d, and 2b) (12) were identified. Respiratory complexes I to IV

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are present, including high-affinity terminal oxidases (cytochrome *bd* and *cbb₃* types) that enable the aerobic growth of *T. siberiense* at low oxygen concentrations. The motility of *T. siberiense* is explained by flagellar genes and autotrophic growth by genes encoding the complete Calvin-Benson-Bassham cycle. Acidotolerance (1) is reflected by the presence of genes coding for a potassium-transporting ATPase (*kdpABCD*) and a potassium uptake system (*ktrAB*) (13).

Surprisingly, the genome analysis revealed a possible metabolic potential of *T. siberiense* for sulfur oxidation. A partial thiosulfate-oxidizing machinery (*soxEFYZA*) and the sulfur-shuttling system DsrEFH were identified. The partial *sox* operon is syntenic with the operon structure of the alphaproteobacterial thiosulfate oxidizer *Starkeya novella* (14), in which *soxBCD* is located separately. No other *sox* or *dsr* genes are present in the draft genome. Experimental evidence is needed to confirm a potential role of *T. siberiense* in sulfur cycling.

Accession number(s). The draft genome sequence of *Telmatospirillum siberiense* 26-4b1 was deposited in GenBank under the accession number [PIUM00000000](#).

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Chapter 4

Peatland *Acidobacteria* with a dissimilatory sulfur metabolism

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Author contributions

BH, MP, and AL designed the research. MP performed field sampling. BH and MP performed microcosm experiments. BH extracted nucleic acids. TG, US, and SGT contributed metagenomic and -transcriptomic sequencing. TR contributed computational resources. BH and MA analysed metagenomic data. BH analysed metatranscriptomic data. BH and DT annotated genomes. CP and CWH performed phylogenetic analysis. SK performed genome comparisons. MH and CW performed quantitative PCR. BH, MP, and AL wrote the manuscript. CP, CWH, SK, MA, SAE, TG, MH, PHN, TR, US, SGT, DT, CW, and DW revised the manuscript.



Peatland *Acidobacteria* with a dissimilatory sulfur metabolism

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Abstract

Sulfur-cycling microorganisms impact organic matter decomposition in wetlands and consequently greenhouse gas emissions from these globally relevant environments. However, their identities and physiological properties are largely unknown. By applying a functional metagenomics approach to an acidic peatland, we recovered draft genomes of seven novel *Acidobacteria* species with the potential for dissimilatory sulfite (*dsrAB*, *dsrC*, *dsrD*, *dsrN*, *dsrT*, *dsrMKJOP*) or sulfate respiration (*sat*, *aprBA*, *qmoABC* plus *dsr* genes). Surprisingly, the genomes also encoded DsrL, which so far was only found in sulfur-oxidizing microorganisms. Metatranscriptome analysis demonstrated expression of acidobacterial sulfur-metabolism genes in native peat soil and their upregulation in diverse anoxic microcosms. This indicated an active sulfate respiration pathway, which, however, might also operate in reverse for dissimilatory sulfur oxidation or disproportionation as proposed for the sulfur-oxidizing *Desulfurivibrio alkaliphilus*. *Acidobacteria* that only harbored genes for sulfite reduction additionally encoded enzymes that liberate sulfite from organosulfonates, which suggested organic sulfur compounds as complementary energy sources. Further metabolic potentials included polysaccharide hydrolysis and sugar utilization, aerobic respiration, several fermentative capabilities, and hydrogen oxidation. Our findings extend both, the known physiological and genetic properties of *Acidobacteria* and the known taxonomic diversity of microorganisms with a DsrAB-based sulfur metabolism, and highlight new fundamental niches for facultative anaerobic *Acidobacteria* in wetlands based on exploitation of inorganic and organic sulfur molecules for energy conservation.

Introduction

Specialized microorganisms oxidize, reduce, or disproportionate sulfur compounds of various oxidation states (–II to

+VI) to generate energy for cellular activity and growth and thereby drive the global sulfur cycle. The capability for characteristic sulfur redox reactions such as dissimilatory sulfate reduction or sulfide oxidation is not confined to single taxa but distributed across different, often unrelated taxa. The true extent of the taxon-diversity within the different guilds of sulfur microorganisms is unknown [1]. However, ecological studies employing specific sulfur metabolism genes (e.g., dissimilatory adenylyl-sulfate

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reductase-encoding *aprBA*, dissimilatory sulfite reductase-encoding *dsrAB*, or *soxB* that codes for a part of the thiosulfate-oxidizing Sox enzyme machinery) as phylogenetic and functional markers have repeatedly demonstrated that only a minor fraction of the sulfur metabolism gene diversity in many environments can be linked to recognized taxa [2–4]. A systematic review of *dsrAB* diversity has revealed that the reductive bacterial-type enzyme branch of the DsrAB tree contains at least thirteen family-level lineages without any cultivated representatives. This indicates that major taxa of sulfate-/sulfite-reducing microorganisms have not yet been identified [3].

Wetlands are among those ecosystems that harbor a diverse community of microorganisms with reductive-type DsrAB, most of which cannot be identified because they are distant from taxonomically classified DsrAB sequences [5]. Sulfur-cycling microorganisms provide significant ecosystem services in natural and anthropogenic wetlands, which are major sources of the climate-warming greenhouse gas methane [6, 7]. While inorganic sulfur compounds are often detected only at low concentration (lower μM range), fast sulfur cycling nevertheless ensures that oxidized sulfur compounds such as sulfate are rapidly replenished for anaerobic respiration. The activity of sulfate-reducing microorganisms (SRM) fluctuates with time and space, but at peak times can contribute considerably to the anaerobic mineralization of organic carbon in wetlands [5]. Simultaneously, SRM prevent methane production by rerouting carbon flow away from methanogenic archaea. Peat microorganisms that are affiliated to known SRM taxa, such as *Desulfosporosinus*, *Desulfomonile*, and *Syntrophobacter*, are typically found in low abundance [8–15]. In contrast, some microorganisms that belong to novel, environmental *dsrAB* lineages can be considerably more abundant in wetlands than species-level *dsrAB* operational taxonomic units of known taxa [13]. However, the taxonomic identity of these novel *dsrAB*-containing microorganisms and their role in sulfur and carbon cycling has yet to be revealed.

To identify these unknown DsrAB-encoding organisms and further investigate their fundamental ecological niches, we recovered thirteen metagenome-assembled genomes (MAGs) encoding DsrAB from a peat soil through a targeted, functional metagenomics approach. We analyzed expression of predicted physiological capabilities of the MAGs by metatranscriptome analyses of anoxic peat soil microcosms that were periodically stimulated by small additions of individual fermentation products with or without supplemented sulfate [9]. Here, we show that facultatively anaerobic members of the diverse *Acidobacteria* community in wetlands employ one or more types of dissimilatory sulfur metabolism.

Materials and methods

Anoxic microcosm experiments, stable isotope probing, and nucleic acids isolation

DNA and RNA samples were retrieved from a previous peat soil microcosm experiment [9]. Briefly, soil from 10–20 cm depth was sampled from an acidic peatland (Schlößnerbrunnen II, Germany) in September 2010, and stored at 4 °C for one week prior to nucleic acids extractions and set-up of soil slurry incubations. Individual soil slurry microcosms were incubated anoxically (100% N_2 atmosphere) in the dark at 14 °C, and regularly amended with low amounts (<200 μM) of either formate, acetate, propionate, lactate, butyrate, or without any additional carbon sources (six replicates each). In addition, half of the microcosms for each substrate were periodically supplemented with low amounts of sulfate (initial amendment of 190–387 μM with periodic additions of 79–161 μM final concentrations). DNA and RNA were extracted from the native soil and RNA was additionally extracted from the soil slurries after 8 and 36 days of incubation.

Furthermore, DNA was obtained from the heavy, ^{13}C -enriched DNA fractions of a previous DNA-stable isotope probing (DNA-SIP) experiment with soil from the same site [12]. Analogous to the single-substrate incubations, anoxic soil slurries were incubated for two months with low-amounts of sulfate and a ^{13}C -labelled mixture of formate, acetate, propionate, and lactate. DNA was extracted, separated on eight replicated density gradients, and DNA from a total of 16 heavy fractions (density 1.715–1.726 g mL^{-1}) was pooled for sequencing.

Additional DNA was obtained from soils that were sampled from different depths in the years 2004 and 2007 [13].

Quantitative PCR and metagenome/-transcriptome sequencing

Abundances of *Acidobacteria* subdivision 1, 2, and 3 in soil samples from different years and depths were determined by newly-developed 16S rRNA gene-targeted real-time quantitative PCR (qPCR) assays (Supplementary Methods). Native soil DNA (two libraries), heavy ^{13}C -enriched DNA (three libraries), and native soil RNA, and RNA samples from the microcosms were sequenced on an Illumina HiSeq 2000/2500 system (Supplementary Methods).

Binning, phylogeny, and annotation of DsrAB-encoding genomes

The differential coverage binning approach by Albertsen et al. [16] was applied to extract MAGs of interest. The raw FASTQ paired-end reads were imported into the CLC

Genomics Workbench 5.5.1 (CLC Bio) and trimmed using a minimum Phred quality score of 20 with no ambiguous nucleotides allowed. TruSeq adapters were removed and a minimum length filter of 50 nt was applied. This resulted in 214, 171, 233, 49, and 294 million reads after quality filtering and trimming for the two native soil and three SIP metagenomes, respectively (84–95% of the raw reads). All reads were co-assembled using CLCs de novo assembly algorithm (kmer size 63, bubble size 50, minimum scaffold size 1000 nt). The reads from all five metagenomes were independently mapped to the assembled scaffolds using CLCs map to reference function (minimum length 0.7, minimum similarity 0.95) to obtain the scaffold coverage. The SIP metagenomes were merged into one mapping. 137, 112, and 376 million reads could be mapped to the two native soil metagenomes and the SIP metagenome, respectively (64–66% of quality filtered reads). Gene prediction of the complete assembly was performed using prodigal [17]. In addition to the detection and taxonomic classification of 105 essential marker genes [16], *dsrA* and *dsrB* homologs were identified using TIGRFAM's hidden Markov model (HMM) profiles TIGR02064 and TIGR02066, respectively, with HMMER 3.1 [18] and the provided trusted cut-offs. Additional *dsrAB*-containing scaffolds were identified by using tblastn with the published DsrAB database as a query against the assembly [3]. DsrAB sequences were classified by phylogenetic analysis (Supplementary Methods; [3]). Binning and decontamination was finalized utilizing the G +C content and tetramer frequencies of the scaffolds, as well as paired-end information, as described and recommended in Albertsen et al. [16]. Completeness, contamination, and strain heterogeneity was estimated using CheckM 1.0.6 [19] with lineage-specific marker sets selected at phylum rank (or class rank for *Proteobacteria*). MAGs were taxonomically classified by phylogenomic analysis of concatenated marker sequences and calculation of average nucleic and amino acid identities (ANI, AAI, Supplementary Methods). MAGs were annotated using the MicroScope annotation platform [20] and eggNOG [21]. Genes of interest (Supplementary Table S2) were manually curated using the full range of tools integrated in MicroScope annotation platform (Supplementary Methods).

Genome-centric activity analysis: iRep and metatranscriptomics

The index of replication (iRep) was calculated for each MAG with the combined native soil metagenomes. Settings and thresholds were applied as recommended [22] using bowtie2 [23] and the iRep script with default settings. Quality-filtered metatranscriptome reads were mapped to all genomes using bowtie2 and counted with featureCounts [24]. To determine gene expression changes, we applied the

DESeq2 pipeline with recommended settings [25] (Supplementary Methods).

Data availability

Metagenomic and -transcriptomic data were deposited under the BioProject accession numbers PRJNA412436 and PRJNA412438, respectively, and can also be obtained via the JGI's genome portal (JGI Proposal ID 605). MAGs are available at MicroScope (<https://www.genoscope.cns.fr/abc/microscope/>) and were deposited at the European Nucleotide Archive (PRJEB24926). DsrAB sequences were deposited at NCBI GenBank under the accession numbers MG182080–MG182141.

Results

Functional metagenomics: recovery of *dsrAB*-containing genomes from native soil and ^{13}C -DNA fraction metagenomes

This study was conducted with soil samples from the Schlöppnerbrunnen II peatland in Germany, which is a long-term study site with active sulfur cycling and harbors a large diversity of unknown microorganisms with divergent *dsrAB* genes [5, 13]. We initially generated co-assembled metagenomes from native peat soil DNA (53 Gb) and a pool of DNA extracts from the heavy fractions of a previous DNA-stable isotope probing (DNA-SIP) experiment with soil from the same peat (101 Gb). The heavy fractions, which were obtained from anoxic peat incubations with periodically supplemented sulfate and a mixture of ^{13}C -labelled formate, acetate, propionate, and lactate at low concentrations, were enriched in DNA from *Desulfosporosinus* and also harbored DNA from yet unidentified *dsrAB*-containing microorganisms [12]. Based on the metagenome data, the native peat was dominated by *Acidobacteria* (61%), but also had *Actinobacteria*, *Alphaproteobacteria*, and *Deltaproteobacteria* as abundant (>5%) phyla/classes (Fig. 1). Dominance of *Acidobacteria*, *Alpha*- and *Delta*-*proteobacteria* is typical for peatlands [26]. Quantitative PCR confirmed that *Acidobacteria* subdivisions 1, 2, and 3 persistently dominated the Schlöppnerbrunnen II peat microbiota in oxic and anoxic soil layers (Supplementary Methods, Fig. 1), as observed in other peatlands [27–29].

We identified 36 complete or partial *dsrAB* genes on scaffolds of the co-assembled metagenome and subsequently recovered thirteen MAGs of DsrAB-encoding bacteria by differential coverage binning (Supplementary Table S1, [16]). Twenty-eight *dsrAB* sequences were part of the reductive bacterial-type DsrAB family branch and were closely related to previously recovered sequences from this

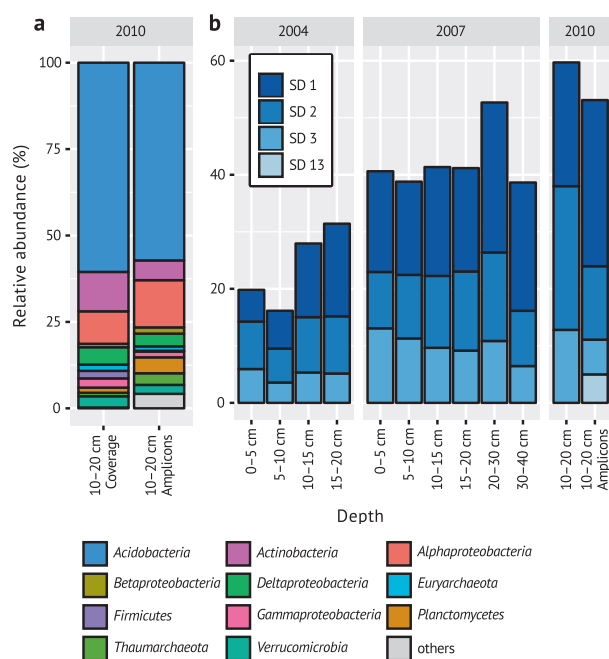


Fig. 1 Microbial community composition in Schlöppnerbrunnen II peatland in samples from different years and soil depths. **a** Abundance of phyla and proteobacterial classes in the native soil (relative to all classified reads/amplicons). Taxa less abundant than 1% are grouped in grey. Coverage abundance is based on metagenomic reads mapped to classified scaffolds. Amplicon abundance is based on *rrn* operon-copy number-corrected abundance of 16S rRNA gene operational taxonomic units [9]. **b** Relative abundance of acidobacterial subdivisions (SD) in the native soil samples as determined by 16S rRNA gene qPCR assays. In addition, all subdivisions more abundant than 1% in a 16S rRNA gene amplicon dataset are shown [9]

and other wetlands (Supplementary Figure S1). These *dsrAB* sequences were affiliated to the known SRM genera *Desulfosporosinus* (Firmicutes, $n = 1$, one MAG) and *Syntrophobacter* (Deltaproteobacteria, $n = 3$, two MAGs), the *Desulfobacca acetoxidans* lineage ($n = 1$), and the uncultured DsrAB family-level lineages 8 ($n = 19$, seven MAGs) and 10 ($n = 4$). Six sequences grouped with the oxidative bacterial-type DsrAB family and were distantly affiliated with *Sideroxydans lithotrophicus* (Betaproteobacteria, $n = 5$, two MAGs) or *Rhodomicrobium vannielii* (Alphaproteobacteria, $n = 1$) (Supplementary Figure S2). Interestingly, two of our sequences ($n = 2$, one MAG) and a DsrAB sequence from the candidate phylum *Rokubacteria* [30] formed a completely novel basal lineage outside the four previously recognized DsrAB enzyme families (Supplementary Figure S2) [3]. The thirteen partial to near complete *dsrAB*-containing MAGs had moderate to no detectable contamination as assessed by CheckM and manual curation (Supplementary Table S1) [19] and derived from *Acidobacteria* subdivisions 1 and 3 (SbA1–7), *Desulfosporosinus* (SbF1), *Syntrophobacter* (SbD1, SbD2), *Betaproteobacteria* (SbB1, SbB2), and *Verrucomicrobia* (SbV1), as inferred by

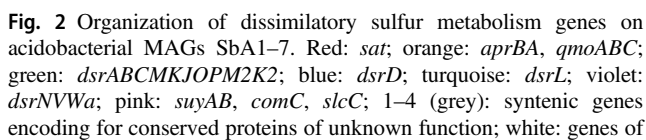
phylogenetic analysis of DsrAB sequences (Supplementary Figures S1 and S2) and concatenated sequences of single-copy, phylogenetic marker genes (Supplementary Figure S3). Only the *Desulfosporosinus* and *Syntrophobacter* MAGs contained rRNA gene sequences.

Phylogenomic analysis showed that *Acidobacteria* MAGs SbA1, SbA5, and SbA7 are affiliated with subdivision 1, while SbA3, SbA4, and SbA6 are affiliated with subdivision 3 (Supplementary Figure S3). The partial MAG SbA2 lacked the marker genes used for phylogenomic treeing, but was unambiguously assigned to *Acidobacteria* using an extended marker gene set [16] and DsrAB phylogeny. The two near complete (96%) MAGs SbA1 and SbA5 have a size of 5.4 and 5.3 Mb, respectively. The G+C content of all acidobacterial MAGs ranges from 58 to 63% (Supplementary Table S1). This is in accordance with genome characteristics of acidobacterial isolates, which have genome sizes of 4.1–10.0 Mb and G+C contents of 57–62% [31, 32]. SbA1 and SbA7 form a monophyletic clade in the *Acidobacteria* subdivision 1 with an AAI [33] of 63% (Supplementary Figure S3) and DsrAB identity of 80% as was calculated with T-Coffee 11 [34] using the unfiltered reference alignment without the intergenic region [3]. They have 56% AAI to their closest relative, *Ca. Koribacter versatilis*, which is lower than AAIs among members of known acidobacterial genera (60–71%). The third MAG from subdivision 1, SbA5, is affiliated with *Terracidiphilus gabretensis* with an AAI of 61%. DsrAB identity of SbA5 to SbA1 and SbA7 is 79%. The three subdivision 3 MAGs form a monophyletic clade with *Ca. Solibacter usitatus* (Supplementary Figure S3). SbA3, SbA4, and SbA6 have AAIs of 59–73% amongst them and 61–62% to *Ca. S. usitatus*. DsrAB identity amongst the three MAGs is 80–94% and 74–79% to the subdivisions 1 MAGs.

The DsrAB sequences encoded on all seven MAGs belong to the uncultured DsrAB family-level lineage 8 (Supplementary Figure S1), which so far only consisted of environmental *dsrAB* sequences of unknown taxonomic identity [3]. Based on these MAGs and metatranscriptome analyses of anoxic peat soil microcosms, we describe the putative metabolic capabilities of these novel DsrAB-encoding *Acidobacteria*. Details on the other MAGs will be described elsewhere (Hausmann et al., unpublished; Anantharaman et al., unpublished). Functional interpretations of the recovered MAGs are made under the premise that the genomes are not closed, and thus it is unknown if genes are absent in these organisms or are missing due to incomplete sequencing, assembly, or binning.

Dissimilatory sulfur metabolism

Although *Acidobacteria* are abundant in diverse environments with active sulfur cycling [28, 29, 35, 36], this is the



unknown function or not involved in sulfur metabolism. In SbA2 all genes are on one scaffold (scaffold 0lkb). Gene fragments at contig borders are indicated by an asterisk. *aprB* in SbA6, indicated by two asterisks, is truncated, which indicates a pseudogene or is due to an assembly error. Scaffolds are separated by two slashes

cytoplasmic DsrC-trisulfide in SRM, the final step in sulfate reduction [37]. DsrC encoded on the MAGs have the two essential cysteine residues at the C-terminal end for full functionality [43]. Interestingly, *dsrC* forms a gene duo with *dsrL* downstream of *dsrAB* in all seven MAGs. This is surprising, because *dsrL* is not found in SRM but in sulfur oxidizers. DsrL is highly expressed and essential for sulfur oxidation by the purple sulfur bacterium *Allochromatium vinosum* [40, 44]. DsrL is a cytoplasmic iron-sulfur flavo-protein with proposed NAD(P)H: acceptor oxidoreductase activity and was copurified with DsrAB from the soluble fraction of *A. vinosum* [45]. The acidobacterial DsrL sequences are shorter than their homolog in *A. vinosum* (Supplementary Table S2a), but have the same functional domains (Supplementary Figure S6). Given the possible role of DsrL in sulfur oxidation, we sought to detect additional genes indicative of oxidative sulfur metabolism in the acidobacterial MAGs. However, genes for Sox enzyme machinery (*soxABXYZ*), thiosulfate dehydrogenase (*tsdA*), sulfide:quinone reductase (*sqr*), adenylyl-sulfate reductase membrane anchor subunit (*aprM*), flavocytochrome c sulfide dehydrogenase (*fccAB*), sulfur reductase (*sreABC*), thiosulfate reductase (*phsABC*), polysulfide reductase (*psrABC*), membrane-bound sulfite oxidizing enzyme (*soeABC*), cytoplasmic sulfur trafficking enzymes (*tusA*, *dsrE2*, *dsrEFH*), or DsrQ/DsrU (unknown functions) were absent [1, 41, 46, 47]. SbA1, SbA3, SbA4, and SbA6 contain genes that have only low homology to *soxCD/sorAB*, periplasmic sulfite-oxidizing enzymes (Supplementary Results) and, thus, might have another function [48].

Despite ongoing sulfur cycling, concentrations of inorganic sulfur compounds such as sulfate are low (lower μM

range) in the Schlöppnerbrunnen II peatland [49–51]. Enzymatic release of inorganic sulfur compounds from organic matter might thus represent a significant resource for sulfur-dissimilating microorganisms. Therefore, we specifically searched for genes coding for known organo-sulfur reactions that yield sulfite [1]. Genes for cystate sulfo-lyase (*cuyA*), methanesulfonate monooxygenase (*msmABCD*), sulfoacetaldehyde acetyltransferase (*xsc*), and taurine dioxygenase (*tauD*) were absent. However, *suyAB*, coding for the (*R*)-sulfolactate sulfo-lyase complex that cleaves (*R*)-sulfolactate into pyruvate and sulfite [52], were present in SbA4 and SbA5 (Supplementary Table S2a). Intriguingly, SbA4 and SbA5 only have capability for sulfite reduction. SbA5 also encodes the racemase machinery for (*S*)-sulfolactate to (*R*)-sulfolactate, (*S*)-sulfolactate dehydrogenase (*slcC*) and (*R*)-sulfolactate dehydrogenase (*comC*); the regulator gene *suyR* or the putative importer SlcHFG were absent [52]. Pyruvate may be used as an energy and carbon source, while sulfite could be used as an electron acceptor for anaerobic respiration [53].

Respiration

Cultivated *Acidobacteria* of subdivisions 1 and 3 are strict aerobes or facultative anaerobes (e.g., [54–59]). Accordingly, we found respiratory chains encoded in all acidobacterial MAGs (Fig. 3, Supplementary Results), with (near) complete operons for NADH dehydrogenase 1, succinate dehydrogenase (lacking in SbA2), one or both types of quinol–cytochrome-*c* reductase, low-affinity terminal oxidases, and ATP synthase (lacking in SbA2) (Supplementary Tables S2b–h). High-affinity terminal oxidases, putatively involved in detoxification of oxygen [60, 61], are limited to four MAGs (Supplementary Table S2g). Genes for dissimilatory nitrogen or iron metabolisms are absent, with the exception of a putative metal reductase in SbA2 of unclear physiological role (Supplementary Results).

Hydrogen utilization and production

We identified [NiFe] hydrogenases of groups 1, 3, and 4 [62] in SbA1–7 (Supplementary Table S2j). Membrane-bound group 1 hydrogenases (SbA1, SbA3, SbA5) consume hydrogen from the periplasm as an electron donor to generate energy, possibly coupled to sulfate/sulfite reduction. In contrast to other *Acidobacteria*, no group 1h/5 hydrogenases, which are coupled to oxygen respiration, were identified [63]. Cytoplasmic group 3 hydrogenases (all MAGs) are bidirectional and proposed to be involved in energy-generating hydrogen oxidation and/or fermentative hydrogen production. Membrane-bound group 4 hydrogenases (SbA1, SbA5, SbA4, SbA6) produce H₂ and are postulated to conserve energy by proton translocation by

oxidizing substrates like formate (group 4a) or carbon monoxide (via ferredoxin, group 4c) (Fig. 3).

A versatile heterotrophic physiology

Acidobacteria are known for their capability to degrade simple and polymeric carbohydrates [31, 55–59, 64, 65], supported by many diverse carbohydrate-active enzymes encoded on their genomes [31, 32]. Accordingly, the MAGs recovered in our study also contain many genes encoding diverse carbohydrate-active enzymes (Supplementary Methods, Fig. 4). These include glycoside hydrolases (GH, 1.0–4.0% of all genes), polysaccharide lyases (0.07–0.3%), and carbohydrate esterases (0.7–1.4%) that are generally involved in degradation of complex sugars, but also glycosyltransferases (0.9–1.4%) for biosynthesis of carbohydrates. Functional GH families (assigned by EC number) putatively involved in cellulose and hemicellulose degradation were most prevalent (Supplementary Table S4). Specifically, the most often encountered EC numbers encompassed by the different GH families represented cellulose (EC 3.2.1.4, e.g., GH5, GH74), xyloglucan (EC 3.2.1.150, EC 3.2.1.151, e.g., GH5, GH74), or xylan (EC 3.2.1.8, EC 3.2.1.37, e.g., GH5) degradation, which is similar to the situation found in other members of *Acidobacteria* subdivision 1 and 3 [31, 32]. Further EC numbers that were often encountered in the various detected GH families were associated with oligosaccharide degradation (EC 3.2.1.21, e.g., GH2) or α -*N*-acetylglactosaminidase activity (EC 3.2.1.49, e.g., GH109). Degradation of cellulose and hemicellulose yields glucose and all MAGs encode glycolysis and pentose phosphate pathways (Fig. 3, Supplementary Results). α -*N*-acetylglactosaminidase releases *N*-acetylglactosamine residues from glycoproteins that are commonly found in microbial cell walls and extracellular polysaccharides [66]. *N*-acetylglactosamine can not be directly utilized via glycolysis, however the additionally required enzymes are present (Fig. 3; Supplementary Results). Under oxic conditions, organic carbon could be completely oxidized to CO₂ via the citric acid cycle (Fig. 3). Alternatively, we also identified fermentative pathways. SbA3 encodes the bifunctional aldehyde-alcohol dehydrogenase AdhE that yields ethanol (Fig. 3). All MAGs encode additional aldehyde and alcohol dehydrogenases without clear substrate specificity that could also ferment acetyl-CoA to ethanol. SbA7 and SbA5 encode a L-lactate dehydrogenase (Ldh) yielding lactate from pyruvate, while six MAGs encode L-lactate dehydrogenases (LldD, GlcDEF, LutABC) that presumably perform the reverse reaction (Fig. 3). Similarly, we identified pathways for acetate and/or propionate production or utilization in all MAGs (Fig. 3; Supplementary Results). SbA1 and SbA3 potentially produce H₂ via formate C-acetyltransferase PfID,

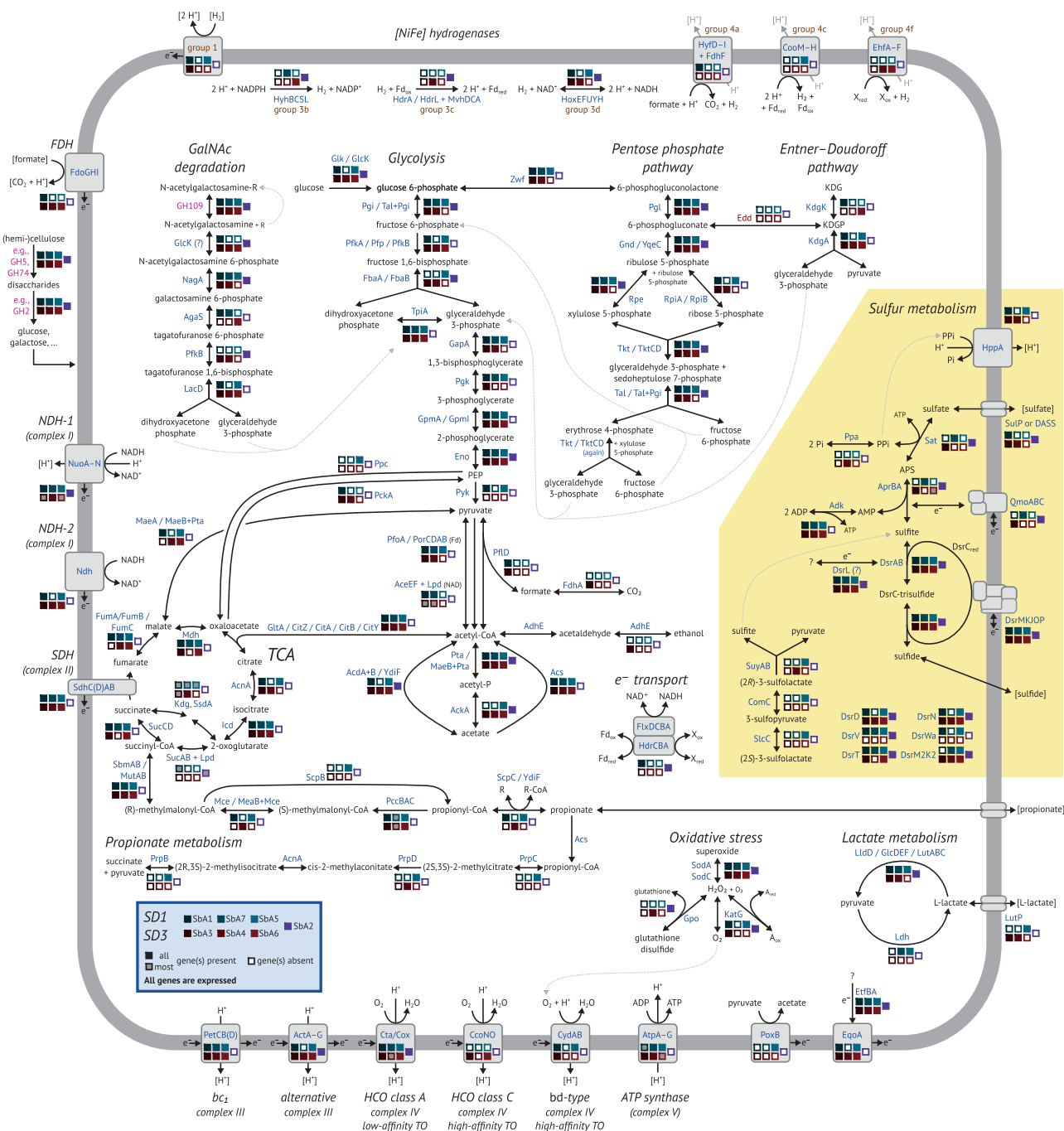


Fig. 3 Metabolic model as inferred from analysis of acidobacterial MAGs SbA1–7. Sulfur metabolism is highlighted in yellow. Enzymes and transporters are shown in blue font. Glycoside hydrolases are shown in pink font (Supplementary Table S2). Extracellular compounds are in parentheses. A slash (/) indicates isozymes, i.e., enzymes that perform the same function, but are distinctly different or have more than one established name. AcdA+B, MaeB+Pta, MeaB+Mce,

Tal+Pgi: bifunctional fusion genes/proteins. Otherwise the plus sign (+) indicates protein complexes. TCA: tricarboxylic acid cycle, FDH: formate dehydrogenase, Hse: hydrogenase, NDH: NADH dehydrogenase, HCO: haem-copper oxidase, TO: terminal oxidase, KDG: 2-dehydro-3-deoxy-D-gluconate, KDGP: 2-dehydro-3-deoxy-D-gluconate 6-phosphate. Expression of at least one copy of every enzyme and transporter was observed in the incubation samples

which cleaves pyruvate into acetyl-CoA and formate. SbA1 encodes for the membrane-bound formate hydrogenlyase complex (*fdhF*, *hyf* operon) that produces H_2 and might also translocate protons. SbA3 harbours an uncharacterized,

cytoplasmic, monomeric FDH (*fdhA*) to transform formate to H_2 . SbA1, SbA3, SbA4, and SbA6 also encode membrane-bound, periplasmic FDH (*fdo* operon) that transfers electrons into the membrane quinol pool, as a non-

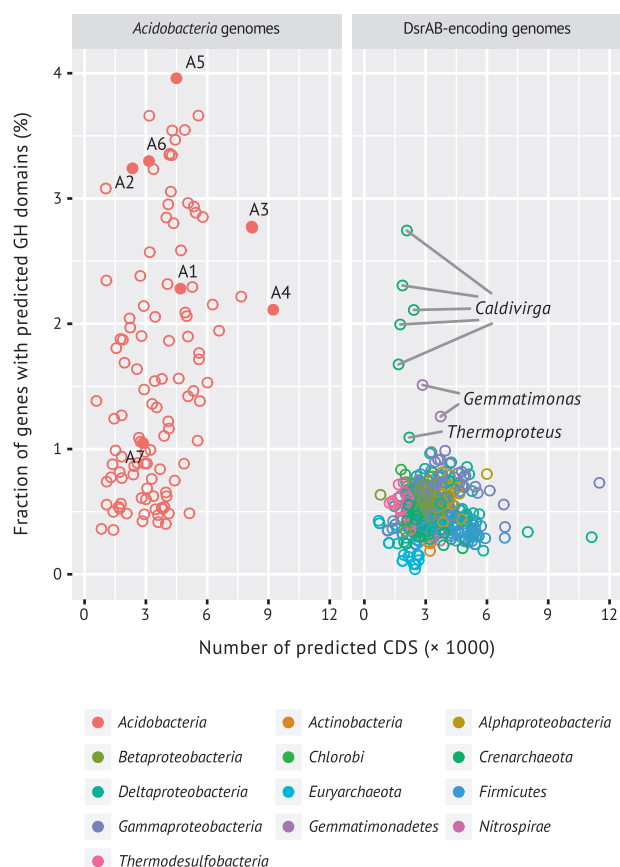


Fig. 4 Glycoside hydrolase genes are enriched in acidobacterial genomes/MAGs compared to genomes from other taxa that encode DsrA/DsrB. DsrAB-containing MAGs SbA1–7 are shown as solid symbols and numbered accordingly. X-axis shows the total number of predicted CDS per genome/MAG

fermentative alternative of formate oxidation (Fig. 3, Supplementary Table S2j).

DsrAB-encoding *Acidobacteria* are metabolically active under anoxic conditions

We calculated the iRep based on the native peat soil metagenomes to assess whether DsrAB-encoding *Acidobacteria* were active in situ [22]. SbA1 and SbA5, which were sufficiently complete ($\geq 75\%$) for a reliable estimate, had iRep values of 1.21 and 1.19, respectively. This shows that a fraction of each population was metabolically active, i.e., on average 21% of SbA1 and 19% of SbA5 cells were actively replicating at the time of sampling. Concordantly, SbA1–7 were also transcriptionally active in the same native soil samples. 35–46% of the SbA1–7 genes were expressed in at least one replicate. SbA1 and SbA5 contributed a considerable fraction (0.4% and 1.8%, respectively, Supplementary Table S1) of the total mRNA reads in the native soil metatranscriptome. These data likely

underestimate the metabolic activity of SbA1–7 in situ because freshly sampled soil was stored at 4 °C for one week prior to nucleic acids extraction.

We further analyzed metatranscriptome data from a series of anoxic incubations of the peat soil with or without individual substrates (formate, acetate, propionate, lactate, or butyrate) and with or without supplemental sulfate [9]. While the incubations were not designed to specifically test for the MAG-inferred metabolic properties, they still allowed us to evaluate transcriptional response of the DsrAB-encoding *Acidobacteria* under various anoxic conditions (Supplementary Methods and Results). All treatments triggered shifts in genome-wide gene expression; more genes were significantly ($p < 0.05$) upregulated (73–933) than downregulated (14–81) as compared to the native soil. Upregulated genes included sulfur metabolism enzymes, high-affinity terminal oxidases, group 1 and 3 hydrogenases, aldehyde-alcohol dehydrogenase AdhE, glycoside hydrolases, and other carbon metabolism enzymes (Supplementary Table S3, Supplementary Figure S7). Significantly upregulated glycoside hydrolase genes belonged to GH family 2, 3, 5, 9, 10, 18, 20, 23, 26, 28, 29, 30, 33, 35, 36, 38, 43, 44, 50, 51, 55, 74, 76, 78, 79, 88, 95, 97, 105, 106, 109, and 129 in MAGs SbA1–6. None of the GH genes were significantly downregulated in the incubations. Noteworthy genes that were significantly downregulated were superoxide dismutases (*sodA*) in SbA2 and SbA4 (Supplementary Table S3a).

Discussion

Diverse members of the phylum *Acidobacteria* are abundant in various ecosystems, particularly in soils and sediments with relative abundances typically ranging from 20–40% [67]. *Acidobacteria* are currently classified in 26 subdivisions based on their 16S rRNA phylogeny [68]. Given their phylogenetic breadth, comparably few isolates and genomes are available to explore their metabolic capabilities. Currently isolated species of subdivisions 1, 3, 4, and 6 are aerobic chemoorganotrophs that grow optimally at neutral or low pH [26, 64, 65, 69]. Furthermore, subdivision 4 contains an anoxygenic phototroph [70, 71], subdivisions 8 and 23 contain anaerobes [72–74], subdivisions 1, 3, and 23 fermenters [56, 59, 73, 75] and subdivision 4, 8, 10, and 23 thermophiles [71, 73, 76, 77].

Acidobacteria are known as dominant inhabitants of wetlands worldwide, in particular members of subdivision 1, 3, 4, and 8 [26]. Strains in the genera *Granulicella* [57], *Telmatobacter* [59], *Bryocella* [58] and *Bryobacter* [55] have been isolated from acidic wetlands and are presumably active in plant-derived polymer degradation

(such as cellulose) [26, 78–80], and in nitrogen and iron cycling [50, 56].

Here, we provide metagenomic and metatranscriptomic evidence that the newly discovered species represent at least three novel genera in *Acidobacteria* subdivision 1 and 3 (Supplementary Figure S3) and possess a dissimilatory sulfur metabolism. The seven acidobacterial MAGs from the Schlöppnerbrunnen II peatland encode the complete canonical pathway for dissimilatory sulfite or sulfate reduction. The sulfate reduction pathway, however, could also operate in reverse as proposed for a sulfur-oxidizing deltaproteobacterium [81]. The phylogenetic separation into two subdivisions as based on the concatenated marker gene tree is also apparent in the DsrAB phylogeny (Supplementary Figure S1). The acidobacterial DsrAB sequences are distributed among two monophyletic clades within the uncultured family-level lineage 8, which is part of the reductive, bacterial-type DsrAB branch [3]. The phylogenetic breadth of the acidobacterial DsrAB sequences is representative for the intra-lineage sequence divergence within uncultured DsrAB lineage 8, which suggests that this entire lineage represents yet uncultivated bacteria of the phylum *Acidobacteria*. Members of this uncultured DsrAB lineage are widespread in freshwater wetlands (Supplementary Figure S1) [5]. In particular, they represented an abundant fraction of the DsrAB diversity and were permanent autochthonous inhabitants of oxic and anoxic soil layers in the analyzed Schlöppnerbrunnen II peatland [12, 13].

Presence of the complete gene set for canonical dissimilatory sulfate reduction suggests that the pathway is functional, as the genetic capability for sulfate reduction can be rapidly lost by adaptive evolution if unused [82]. Except for a truncated *aprB* on SbA6, we found no indications of pseudogenes, i.e., unexpected internal stop codons or reading frame shifts, for any of the sulfate/sulfite reduction genes on the acidobacterial MAGs [3]. In addition, sulfur metabolism genes of each MAG were expressed in the native soil and the anoxic microcosms (Supplementary Table S3a). Many sulfur metabolism genes were even significantly upregulated in the anoxic microcosms, with *dsrC* and *aprBA* among the top 10 most expressed genes in SbA7 (Supplementary Table S3a). These findings further support full functionality of the acidobacterial dissimilatory sulfur pathways under anoxic condition.

Known SRM typically couple sulfate respiration to oxidation of fermentation products such as volatile fatty acids, alcohols, or hydrogen [83]. While other microorganisms in the Schlöppnerbrunnen II soil, such as *Desulfosporosinus*, showed sulfate-specific and substrate-specific responses in our microcosms, hundreds of acidobacterial 16S rRNA phylotypes did not (with the exception of two) [9]. Gene expression patterns of DsrAB-encoding *Acidobacteria* in

the individual anoxic microcosms as analyzed in the present study were ambiguous. Genes for putative oxidation of the supplemented substrates (formate, acetate, propionate, lactate, butyrate) were not specifically upregulated, neither without nor with supplemental sulfate. However, sulfur metabolism genes were upregulated in several incubations as compared to no-substrate-controls, suggesting indirect stimulation of a sulfur-based metabolism (Supplementary Results, Supplementary Table S3a). Indirect changes in microbial activity after the addition of fresh organic matter is often observed in soils (priming effects, [84]). One explanation for this priming effect is the co-metabolism theory stating that easily available substrates provide the energy for microorganisms to produce extracellular enzymes to make immobile carbon accessible, which is then also available to other microorganisms. The DsrAB-encoding *Acidobacteria* have a large genetic repertoire to utilize carbohydrates and monomeric sugars (Fig. 3). This is in accordance with the carbohydrate utilization potential previously described for subdivision 1 and 3 *Acidobacteria* [31, 32]. Yet utilization of monomeric sugars is a rare feature of known SRM [85, 86] and utilization of polysaccharides or oligosaccharides by sulfate-reducing bacteria was not yet reported. While the studied *Acidobacteria* expressed many of their glycoside hydrolase genes in our anoxic peat soil microcosms, further experiments are required to confirm if DsrAB-encoding *Acidobacteria* couple degradation of carbohydrate polymers or monomers to sulfate reduction.

It is intriguing to propose that MAGs SbA2, SbA3, and SbA7 derive from acidobacterial SRM as they lack known sulfur oxidation genes, except *dsrL* (Supplementary Figure S6), and express the complete dissimilatory sulfate reduction pathway (Supplementary Table S2a), including reductive, bacterial-type *dsrAB*, and *dsrD* that may be exclusive to SRM [39, 48, 87, 88]. However, the functions of DsrL and DsrD are yet unresolved, which prevents functional predictions based only on these genes. The proposal of an alternative hypothesis that these novel *Acidobacteria* reverse the sulfate reduction pathway for dissimilatory sulfur oxidation or sulfur disproportionation, bases on findings with the deltaproteobacterium *Desulfurivibrio alkaliphilus* [81]. *D. alkaliphilus* also lacks known sulfur oxidation genes (including *dsrL*), except for *sqr*, and is proposed to gain energy by coupling sulfide oxidation via a reversed sulfate reduction pathway (with a reductive-type DsrAB) to the dissimilatory reduction of nitrate/nitrite to ammonium. Sulfide oxidation in acidobacterial MAGs SbA2, SbA3, and SbA7 could proceed analogous to the pathway models proposed by Thorup et al. [81] and Christiane Dahl [89]. Briefly, hydrogen sulfide might react with DsrC either spontaneously [90] or via an unknown sulfur transfer mechanism to form persulfated DsrC.

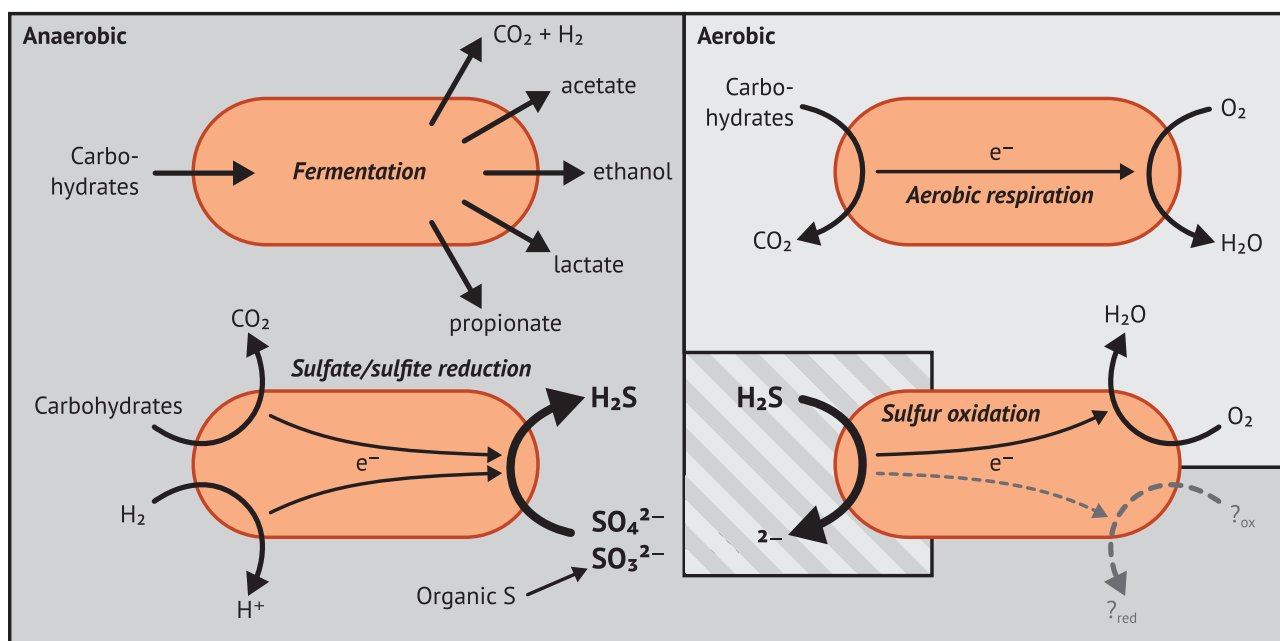


Fig. 5 Putative lifestyles of DsrAB-encoding *Acidobacteria*

Persulfated DsrC is then oxidized by DsrMKJOP, thereby transferring electrons into the membrane quinone pool, and releasing a DsrC-trisulfide, which is the substrate for DsrAB [37, 89]. It was hypothesized that electrons released during DsrC-trisulfide oxidation to sulfite and DsrC are transferred to DsrL [89]. Further sulfite oxidation to sulfate would be catalyzed by AprBA-QmoABC and Sat.

The acidobacterial MAGs have the genomic potential to use oxygen as terminal electron acceptor and might thus couple sulfide oxidation to aerobic respiration. Alternative electron acceptors for biological sulfur oxidation in wetlands could include nitrate/nitrite and metals such as Fe(III) [50]. However, known genes for dissimilatory nitrate reduction and metal reduction [91] were absent from these acidobacterial MAGs. Only SbA2 encodes a putative metal reduction complex that was recently characterized in *Desulfotomaculum reducens* [92]. At this time, it is unclear whether DsrAB-encoding *Acidobacteria* are capable of Fe (III) respiration, as seen in *Geothrix fermentans* [74] and certain isolates in subdivision 1 [56, 93].

Proposal of the acidobacterial *Candidatus* genera *Sulfotelmatobacter*, *Sulfotelmatomonas*, and *Sulfopaludibacter*

Based on combined interpretation of phylogeny (concatenated phylogenetic marker genes, DsrAB), genomic (ANI, AAI) and genetic (DsrAB) distances, and characteristic genomic features of dissimilatory sulfur metabolism (Fig. 3), in accordance with Konstantinidis et al. [94], we classify MAGs SbA1, SbA7, SbA5, SbA3, SbA4, and

SbA6 into three new acidobacterial *Candidatus* genera, including *Candidatus* species names for the >95% complete MAGs SbA1 and SbA5. In-depth phylogenomic analysis of SbA2 was not possible and therefore it is tentatively assigned to *Acidobacteria* subdivision 3.

Acidobacteria subdivision 1

- *Ca.* genus *Sulfotelmatobacter* (Sul.fo.tel.ma.to.bac'ter. L. n. *sulfur*, sulfur; Gr. n. *telma*, -tos, swamp, wetland; N.L. masc. n. *bacter*, bacterium; N.L. masc. n. *Sulfotelmatobacter*, a bacterium from a swamp metabolizing sulfur) with *Ca.* *Sulfotelmatobacter kueseliae* MAG SbA1 (kue.se'li.ae. N.L. gen. n. *kueseliae*, of Kuesel, honouring Kirsten K  sel, for her work on the geomicrobiology of wetlands) and *Ca.* *Sulfotelmatobacter* sp. MAG SbA7.
- *Ca.* *Sulfotelmatomonas gaucii* MAG SbA5 (Sul.fo.tel.ma.to.mo.nas. L. n. *sulfur*, sulfur; Gr. n. *telma*, -tos, swamp, wetland; N.L. fem. n. *monas*, a unicellular organism; N.L. fem. n. *Sulfotelmatomonas*, a bacterium from a swamp metabolizing sulfur; gau'.ci.i. N.L. gen. n. *gaucii*, of Gauci, in honour of Vincent Gauci, for his pioneering work on the interplay of wetland sulfate reduction and global methane emission).

Acidobacteria subdivision 3

- *Ca.* genus *Sulfopaludibacter* (Sul.fo.pa.lu.di.bac'ter. L. n. *sulfur*, sulfur; L. n. *palus*, -udis, L. swamp; N.L. masc. n. *bacter*, bacterium; N.L. masc. n. *Sulfopaludibacter*, a bacterium from a swamp metabolizing sulfur)

with *Ca. Sulfopaludibacter* sp. MAG SbA3, *Ca. Sulfopaludibacter* sp. MAG SbA4, and *Ca. Sulfopaludibacter* sp. MAG SbA6.

- *Acidobacteria* bacterium MAG SbA2.

Conclusion

Sulfur cycling exerts important control on organic carbon degradation and greenhouse gas production in wetlands, but knowledge about sulfur microorganisms in these globally important ecosystems is scarce [5]. Here, we show by genome-centric metagenomics and metatranscriptomics that members of the phylum *Acidobacteria* have a putative role in peatland sulfur cycling. The genomic repertoire of these novel *Acidobacteria* species encompassed recognized acidobacterial physiologies, such as a facultative anaerobic metabolism, oxygen respiration, fermentation, carbohydrate degradation, and hydrogen metabolism, but was additionally augmented with a DsrAB-based dissimilatory sulfur metabolism (Fig. 5). Based on their genetic repertoire and previous findings on reversibility of the dissimilatory sulfate reduction pathway [81, 95–97], it is intriguing to speculate that the described peatland *Acidobacteria* could use the same pathway for both sulfate reduction and sulfide oxidation. The described DsrAB-carrying *Acidobacteria* that only encoded the pathway for dissimilatory sulfite reduction had additional genes for sulfite-producing enzymes, which suggests that organosulfonates might be their primary substrate for sulfur respiration. Our results not only extend the current understanding of the genetic versatility and distribution of dissimilatory sulfur metabolism among recognized microbial phyla, but also underpin the challenge to unambiguously differentiate between reductive or oxidative sulfur metabolism solely based on (meta-)genome/transcriptome data [81].

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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Chapter 5

Expanded diversity of microbial groups that shape the dissimilatory sulfur cycle

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Author contributions

BH contributed the *Verrucomicrobia* genome, phylogenetic analysis of DsrAB sequences, and revised the manuscript.



Expanded diversity of microbial groups that shape the dissimilatory sulfur cycle

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Abstract

A critical step in the biogeochemical cycle of sulfur on Earth is microbial sulfate reduction, yet organisms from relatively few lineages have been implicated in this process. Previous studies using functional marker genes have detected abundant, novel dissimilatory sulfite reductases (DsrAB) that could confer the capacity for microbial sulfite/sulfate reduction but were not affiliated with known organisms. Thus, the identity of a significant fraction of sulfate/sulfite-reducing microbes has remained elusive. Here we report the discovery of the capacity for sulfate/sulfite reduction in the genomes of organisms from 13 bacterial and archaeal phyla, thereby more than doubling the number of microbial phyla associated with this process. Eight of the 13 newly identified groups are candidate phyla that lack isolated representatives, a finding only possible given genomes from metagenomes. Organisms from *Verrucomicrobia* and two candidate phyla, *Candidatus* Rokubacteria and *Candidatus* Hydrothermarchaeota, contain some of the earliest evolved *dsrAB* genes. The capacity for sulfite reduction has been laterally transferred in multiple events within some phyla, and a key gene potentially capable of modulating sulfur metabolism in associated cells has been acquired by putatively symbiotic bacteria. We conclude that current functional predictions based on phylogeny significantly underestimate the extent of sulfate/sulfite reduction across Earth's ecosystems. Understanding the prevalence of this capacity is integral to interpreting the carbon cycle because sulfate reduction is often coupled to turnover of buried organic carbon. Our findings expand the diversity of microbial groups associated with sulfur transformations in the environment and motivate revision of biogeochemical process models based on microbial community composition.

Introduction

The cycling of sulfur is one of Earth's major biogeochemical processes. Sulfate reduction in conjunction with sulfur

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disproportionation may be an early evolved microbial metabolism, given evidence for biological fractionation of sulfur isotopes around 3.5 billion years ago [1, 2], and it remains an important energy metabolism for anaerobic life [3]. In natural ecosystems, human microbiomes, and engineered systems, this process is important because the product hydrogen sulfide (H_2S) can be toxic [4], can corrode steel [5], and sour oil reservoirs [6]. Overall, sulfate reduction is a primary driver in the carbon cycle, and is responsible for a large part of the organic carbon flux to CO_2 in marine sedimentary environments [7, 8] and in wetlands [9]. Importantly, the coupling of sulfate/sulfite reduction to oxidation of H_2 , small chain fatty acids, or other carbon compounds limits the availability of these substrates to other organisms like methanogens and alters the energetics via syntrophic interactions [10, 11]. All of these processes also impact methane production. Given the many reasons why the biological conversion of sulfate/sulfite to sulfide is important, it is vital that we understand which organisms can carry out the reactions and the pathways involved.

The canonical microbial pathway for dissimilatory sulfate reduction involves the initial reduction of sulfate to sulfite by a combination of sulfate adenylyltransferase (Sat) and adenylyl-sulfate reductase (AprBA) followed by reduction of sulfite by sulfite reductases. Sulfite reductase genes catalyze the rate-limiting steps in the global sulfur cycle [12, 13] and confer bacteria and archaea the ability to grow via reduction of sulfite, and can function in reverse in some organisms that disproportionate or oxidize elemental sulfur [14–16]. Four different groups of sulfite reductases function in dissimilatory sulfur metabolism. Of these, siroheme-dependent dissimilatory sulfite reductase (dsr), siroheme-dependent anaerobic sulfite reductase (asr) genes, and octaheme cytochrome *c* sulfite reductase (*mccA*) catalyze the reduction of sulfite to sulfide, while reverse dissimilatory sulfite reductase genes (rdsr) are involved in sulfur oxidation. All of these sulfite reductases except for *mccA* constitute an ancient lineage of enzymes that may predate the separation of Bacteria and Archaea [17].

The taxonomic distribution of dissimilatory sulfite reductases has been considered to be restricted to organisms from selected bacterial and archaeal phyla [18]. Only organisms from nine microbial phylum-level lineages, namely *Deltaproteobacteria*, *Firmicutes*, *Thermodesulfobacteria*, *Actinobacteria*, *Nitrospirae*, *Caldiseica*, *Euryarchaeota*, *Crenarchaeota*, and *Aigarchaeota* are known to possess the genetic capacity to reduce sulfite to sulfide using the dsr system. The asr enzymes have a far more limited distribution and are known to be present only in organisms from four phylum-level lineages, *Gammaproteobacteria*, *Firmicutes*, *Spirochaetes*, and

Fusobacteria. The distribution of *MccA* enzymes is restricted to organisms from *Epsilonproteobacteria* [19] and *Gammaproteobacteria* [20]. Finally, the rdsr enzyme complex for sulfur oxidation is associated with organisms from five phylum-level lineages including *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, and *Chlorobi*. This diversification of sulfite reductases was likely driven by speciation and functional divergence, and to a lesser extent, lateral gene transfer (LGT) [21].

The recent availability of thousands of genomes from organisms belonging to many newly sampled phyla has provided the opportunity to test for the presence of sulfite reductase genes in bacteria and archaea that have not previously been associated with dissimilatory sulfur metabolism [22]. Here we use shotgun metagenomic sequencing and recovery of metagenome-assembled genomes (MAGs) from a diverse set of marine and terrestrial environments to show that organisms from novel lineages contain sulfite reductases that implicate them in the dissimilatory cycling of sulfur. In total, we more than doubled the number of microbial lineages that can catalyze dissimilatory sulfate/sulfite reduction or sulfur oxidation in the environment. We shed light on the complicated evolutionary history of dissimilatory sulfite reductases and show that LGT of catalytic sulfite reductase genes is much more common than previously thought.

Materials and methods

Sample collection and data processing

Details of sample collection (Sampling, DNA Extraction), individual sample geochemical measurements, and data processing (DNA sequencing, assembly, annotation, binning, genome completion estimates) are described in detail elsewhere [23–27].

Identification of selected sulfate/sulfite reduction genes

Genome-specific metabolic potential for sulfate/sulfite reduction was determined in an iterative manner by (A) searching all predicted ORFs in a genome with HMM profiles for *dsrA* and *dsrB* from TIGRFAM [28], and *dsrD* from Pfam [29] using hmmscan v3.1b2 [30], and (B) searching against custom hmm profiles for *dsrA*, *dsrB*, and *dsrD* using hits generated from step (A) and searching all predicted ORFs again for the above genes. (C) Identification of anaerobic sulfite reductase genes was conducted by searching all predicted ORFs against *asrA*, *asrB*, and *asrC* hmm databases from TIGRFAM [28]. (D) Identification of genes for the reduction of sulfate to

sulfite was conducted by searching all predicted ORFs against *aprA*, *aprB*, and *sat* hmm databases from TIGRFam [28].

Taxonomic confirmation

The taxonomy of organisms represented by the 123 identified genomes was determined by using a concatenated RP tree. Briefly 16 RPs (L2, L3, L4, L5, L6, L14, L15, L16, 18, L22, L24, S3, S8, S10, S17, S19) were each aligned separately using MUSCLE v3.8.31 [31]. All alignments were end trimmed manually and all columns with >95% gaps were trimmed. The individual alignments were concatenated and the phylogenetic tree was inferred by RAxML v8.0.26 [32] implemented by the CIPRES Science Gateway [33]. This analysis sampled a total of 204 bootstrap replicates before being stopped by the autoMRE algorithm. The complete RP tree is presented as Data File S1.

RAxML was called as follows:

```
raxmlHPC-HYBRID -s input -N autoMRE -n result -f a -p 12345 -x 12345 -m PROTCATLG
```

Other *dsr* genes

To study the *dsr* operon structure in the newly identified organisms, we identified other *dsr* genes, namely C, E, F, H, M, K, J, O, P, N, L, R, and S. These genes were identified in the vicinity of *dsrAB* using domain similarity identified by hits to TIGRFam [28], COG [34], and Pfam databases [29], as well certain key traits and conserved residues in specific genes.

Sequence alignment and phylogeny

Phylogenetic analyses were performed as follows:

Each individual gene (*dsrA*, *dsrB*, *dsrC*, *dsrD*, *aprA*, *aprB*, *dsrT*, *qmoA*, *qmoB*, *sat*) was aligned along with reference sequences using MUSCLE [31] with default parameters. All alignments were manually refined by trimming the start and ends and removing all columns with >95% gaps. For generation of concatenated alignments (*dsrAB*, *qmoAB*, and *aprBA*), individual alignments were concatenated in Geneious version 7 [35]. In construction of the concatenated *qmo* tree, only subunits A and B were used since subunit C is not universally present in sulfate/sulfite-reducing organisms. All phylogenetic analyses were inferred by RAxML v8.0.26 [32] implemented by the CIPRES Science Gateway [33]. RAxML was called as follows:

For AsrABC, DsrD, Sat, DsrT, QmoAB, DsrEFH trees:
raxmlHPC-PTHREADS -s input -N 1000 -n result -f a -p 12345 -x 12345 -m PROTGAMMAGTR.

For DsrAB, AprBA trees:

```
raxmlHPC-HYBRID -s input -N autoMRE -n result -f a -p 12345 -x 12345 -m PROTGAMMAGTR.
```

The complete DsrAB tree is presented as Data File S2.

Conserved residues and motifs

Conserved residues and motifs in DsrA, DsrB, DsrC, AsrC, DsrD, and DsrE proteins were identified by aligning the identified genes from all 123 genomes in this study with reference proteins [36–38]. All conserved residues identified by us were also compared with the model sulfate-reducing organism, *Desulfovibrio vulgaris* [39].

Rooting of *dsrA/dsrB* tree

A reference alignment was calculated de novo using MAFFT based on a non-redundant (90% identity clustering with uclust [40]) set of length-filtered (300–500 nt) sulfite reductase superfamily sequences from this study [18], and additional sequences collected from UniProt and UniParc [41] using the eggNOG COG2221 and TIGRFAM DsrA/DsrB HMM models. The reference alignment was end trimmed and filtered with noisy [42]. Selected full-length DsrA, DsrB, and outgroup sequences were reference-aligned and used for phylogenetic inference using Phylo-Bayes with the CAT-GTR model [43].

Trees for inferring LGT by comparison of 16S rRNA and reductive-type DsrAB

16S rRNA genes were aligned with the SINA aligner [44] with default parameters. Alignments were then manually refined by trimming the start and ends and removing all columns with gaps >95%. DsrA and DsrB sequences were aligned as described above. 16S rRNA and DsrAB were sourced from the same organisms where possible. In the case of MAGs not containing 16S genes, the closest related organisms with 16S rRNA genes were chosen for this analysis. Organisms with oxidative-type DsrAB were excluded from this analysis. 16S rRNA trees were calculated using maximum likelihood (RAxML) and neighbor-joining (Geneious) methods. The neighbor joining tree was constructed using the Jukes-Cantor Genetic distance model with 1000 bootstrap replicates.

RAxML was run as follows:

```
raxmlHPC-PTHREADS -f a -s input -n result -m GTRGAMMA -x 12345 -# autoMRE -p 12345 -T 4.
```

The 16S rRNA consensus tree was constructed using the majority (extended) consensus rule setting using CONSENSE and branch lengths were adjusted using DNAML implemented in the PHYLIP package [45]. Similarly, DsrAB trees were combined into a consensus tree by using the majority (extended) consensus rule in CONSENSE and

Table 1 Details of lineages involved in dissimilatory sulfur cycling as identified in this study

Phylum-level lineage	No. of genomes reported	Potential contribution to sulfur cycle	Mechanism	Source	Electron donor		
					Hydrogen	Fatty acid metabolism	Organic C
<i>Acidobacteria</i>	3	Sulfate/sulfite reduction	dsr	A, D	Yes	Yes	Yes
<i>Actinobacteria</i>	3	Sulfate/sulfite reduction, or sulfur oxidation, or both	dsr	–	Yes	No	Yes
<i>Armatimonadetes</i>	1	Sulfate/sulfite reduction	dsr	C	Yes	No	Yes
<i>Candidatus Desantisbacteria</i>	4	Sulfate/sulfite reduction	dsr	B, C	Yes	No	Yes
<i>Candidatus Falkowbacteria</i>	8	Unknown	Unknown	A	Yes	No	Yes
<i>Candidatus Hydrothermarchaeota</i>	4	Sulfate/sulfite reduction, or sulfur oxidation, or both	dsr	E	Yes	No	Yes
<i>Candidatus Lambdaproteobacteria</i>	5	Sulfite reduction, or sulfur oxidation, or both	dsr	A	Yes	Yes	Yes
<i>Candidatus Muproteobacteria</i>	14	Sulfur oxidation	rdsr	A	Yes	Yes	Yes
<i>Candidatus Omnithrophica</i>	2	Sulfite reduction	asr	A, B	Yes	Yes	Yes
<i>Candidatus Riflebacteria</i>	4	Sulfite reduction	asr	A, B	Yes	Yes	Yes
<i>Candidatus Rokubacteria</i>	8	Sulfur oxidation	dsr	A	No	Yes	Yes
<i>Candidatus Schekmanbacteria</i>	1	Sulfate/sulfite reduction	dsr	A	No	Yes	Yes
<i>Candidatus Zixibacteria</i>	2	Sulfate/sulfite reduction	dsr	B	Yes	Yes	Yes
<i>Chloroflexi</i>	2	Sulfate/sulfite reduction	dsr	A	Yes	Yes	Yes
<i>Deltaproteobacteria</i>	34	Sulfate/sulfite reduction	dsr	A, C	Yes	Yes	Yes
<i>Ignavibacteria</i>	5	Sulfate/sulfite reduction	dsr	A, B	Yes	Yes	Yes
<i>Nitrospirae</i>	3	Sulfur oxidation	rdsr	A	Yes	Yes	Yes
<i>Nitrospirae</i>	2	Sulfur oxidation	rdsr	A	No	Yes	Yes
<i>Nitrospirae</i>	19	Sulfate/sulfite reduction	dsr	A, C	Yes	No	Yes
<i>Planctomycetes</i>	1	Sulfate/sulfite reduction	dsr, asr	A, B	Yes	Yes	Yes
<i>Verrucomicrobia</i>	1	Sulfate/sulfite reduction, or sulfur oxidation, or both	dsr	F	Yes	No	Yes

Sampling sources are indicated by letters: A—Aquifer at Rifle, Colorado, USA; B—Deep subsurface in Japan; C—CO₂ geyser at Green River, Utah, USA; D—Glencore Mine, Canada; E—Juan de Fuca ridge flank marine subsurface fluids; F—Natural Peatland in Germany. Newly identified lineages are shown in bold. Contribution to sulfur cycle for DsrAB-containing organisms were decided as described in Table 2.

branch lengths of the consensus tree were inferred by using PROML (JTT model).

Structural models

We selected the DsrT proteins identified in *Desulfovibrio vulgaris* (WP_012611240) and *Candidatus Rokubacteria* CSP1-6 (KRT71371) for structural modeling. Protein models were predicted using the I-TASSER suite [46]. Only the top predicted models out of the top five I-TASSER simulations are shown. Both DsrT proteins used the identical top threading template from the sporulation inhibitor protein pXO1-118 from *Bacillus anthracis* [47].

Analyses of electron donors for sulfate/sulfite reduction

Analyses of putative electron donors and other metabolic potential were centered around identification of genes for

hydrogen oxidation, complex carbon compounds (carbohydrates), fatty acid metabolism, and carbon fixation. For identification of the potential for hydrogen oxidation, hmm searches were conducted by searching all predicted ORFs against individual HMM profiles for nickel–iron hydrogenases from Groups I, IIa, IIb, IIIa, IIIb, IIIc, and IIId, and Fe–Fe hydrogenases. All hits above the noise cutoffs were inspected manually. The details are described in detail previously [24, 26, 48].

For identification of carbohydrate substrates for sulfate/sulfite reduction, all predicted ORFs were searched against the CAZy HMM database [49]. Pre-filtering of hits was conducted using the following cutoffs: coverage: 0.40; e-value: 1e–18. To determine the specificity of enzymes, we established a set of 84 distinct reactions involving 189 enzyme families that allowed us to track specific substrates and products. All hits to glycosyltransferases (GT) and Carbohydrate Binding Modules (CBM) were excluded from this analysis due to high incidence of false

positives and/or difficulty in determining substrate specificity.

Generation of custom hmm models

For generation of custom HMM profiles, reference sequences and identified genes from the 123 genomes in this study were aligned using MUSCLE [31] with default parameters followed by manually trimming the start and ends of the alignment. The alignment was converted into Stockholm format and databases were built using hmmscan [30]. Individual noise and trusted cutoffs for all HMMs were determined by manual inspection and are built into the custom HMM profiles.

Results and discussion

To investigate the diversity of microorganisms that contain sulfite reductases involved in dissimilatory sulfate/sulfite reduction or sulfur oxidation in the environment, we analyzed genomes reconstructed from metagenomic sequence datasets recovered from six distinct terrestrial and marine subsurface environments where geochemical conditions have suggested active microbial sulfur cycling. Our sampling sites included an aquifer adjacent to the Colorado River, USA [24, 26], a deep subsurface CO₂ geyser in Utah, USA [27], a deep borehole in Japan [23], an acidic sulfide mine waste rock site in Canada, deep seafloor basaltic crustal fluids of the hydrothermally active Juan de Fuca ridge flank in the Pacific Ocean [25], and an acidic peatland in Germany [50]. In total, we searched in excess of 4000 near-complete MAGs for the presence of sulfite reductase genes.

Identification of dissimilatory sulfur cycling organisms from MAGs

We identified sulfite reductase genes in 123 near-complete microbial genomes (Supplementary Table 1). Phylogenetic analyses using a set of 16 concatenated ribosomal proteins (RP) and the small subunit ribosomal (SSU) RNA gene show that these genomes belong to organisms from 20 distinct phylum-level lineages (Table 1), 13 of which were not known to have *dsr* genes [18]. In addition, we identified anaerobic sulfite reductase (*asr*) genes required for sulfite reduction in three bacterial groups not previously reported to have this capacity [51]. All of the identified catalytic protein subunits (*DsrA*, *DsrB*, and *AsrC*) contained all conserved sulfite reductase residues and secondary structure elements for the formation of α helices and β sheets [36] (Supplementary Figs. 1–3).

Dissimilatory sulfite reductase containing organisms

Given our interest in identifying organisms with the capacity to produce sulfide, we initially searched the genomes for operons that contained genes encoding *DsrD* [52]. This gene was considered a marker for sulfite reduction because it is absent in bacteria that use the *rdsr* pathway for sulfur oxidation [53]. It is however important to note that the *dsrD* gene is present and highly expressed in sulfur disproportionating organisms like *Desulfurivibrio alkaliphilus* that cannot be distinguished from canonical sulfate-reducing bacteria using gene synteny or other genomic features [16]. Although the exact function of the *DsrD* protein is unclear, the presence of winged-helix domains in its structure and its association with other core proteins of the *dsr* complex (*dsrABC*) suggest a regulatory role in bacterial sulfite reduction [37]. We identified 78 genomes that encode at least *dsrABCD* (Supplementary Fig. 4). A multiple alignment of *DsrD* sequences confirmed highly conserved residues, indicating that the proteins are likely active (Supplementary Fig. 5). These putative sulfate/sulfite-reducing microorganisms affiliate with eight distinct phyla not previously reported to be capable of these processes. Four are phyla with isolated representatives (*Acidobacteria*, *Armatimonadetes*, *Ignavibacteria*, *Planctomycetes*) and four are uncultivated candidate phyla (*Candidatus Zixibacteria*, *Candidatus Schekmanbacteria*, *Candidatus Desantisbacteria*, *Candidatus Lambdaproteobacteria*) (Fig. 1a).

Importantly, organisms from *Verrucomicrobia* and two candidate phyla, *Candidatus Rokubacteria* and *Candidatus Hydrothermarchaeota*, lack *dsrD* genes and their *dsrAB* sequences form completely novel lineages outside the four known main phylogenetic *DsrAB* clusters, namely the reductive bacterial-type, the oxidative bacterial-type, the reductive archaeal-type, and the second *dsrAB* copies of *Moorella* species (Fig. 1a). To determine the earliest evolved and most basal lineages in the *DsrAB* tree, we performed paralogous rooting analysis on a representative subset of sequences. In accordance with previous reports, our results show that the second copies of *dsrAB* in *Moorella* spp. likely represent the most basal *DsrAB* branch [18]. This was followed by the newly identified sequences from *Candidatus Rokubacteria*, *Verrucomicrobia*, and *Candidatus Hydrothermarchaeota* (Fig. 2). Interestingly, *Candidatus Hydrothermarchaeota* sequences were not monophyletic with one sequence (JdFR-18 JGI24020J35080_1000005) clustering with *Verrucomicrobia* and *Candidatus Rokubacteria*, while the remaining were affiliated with bacterial-type *DsrAB*. Other organisms lacking *dsrD* genes cluster together with organisms known to be sulfur oxidizers in the *dsrAB* tree. Based on this clustering, the group implicated in sulfur oxidation using

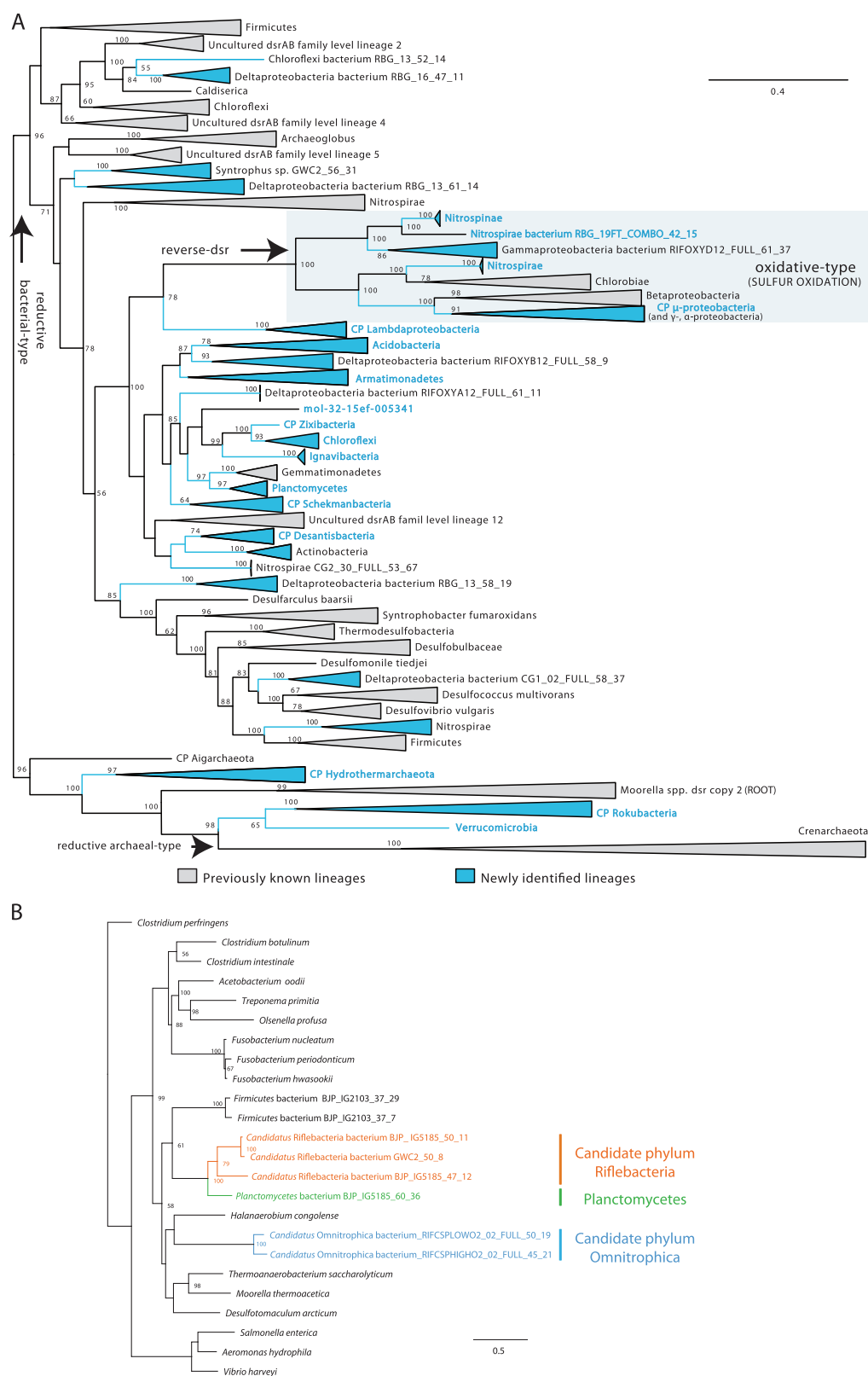


Fig. 1 **A.** Concatenated DsrAB protein tree showing the diversity of organisms involved in dissimilatory sulfur cycling using the dsr system. Lineages in blue contain genomes reported in this study. Phylum-level lineages with first report of evidence for sulfur cycling are indicated by blue letters. Only bootstrap values >50 are shown. The complete tree is available with full bootstrap support values as Additional Data File S2. **B.** Concatenated AsrABC protein tree showing the diversity of organisms that possess the anaerobic sulfite reductase system. Lineages in colors were identified in this study. Only bootstrap values >50 are shown

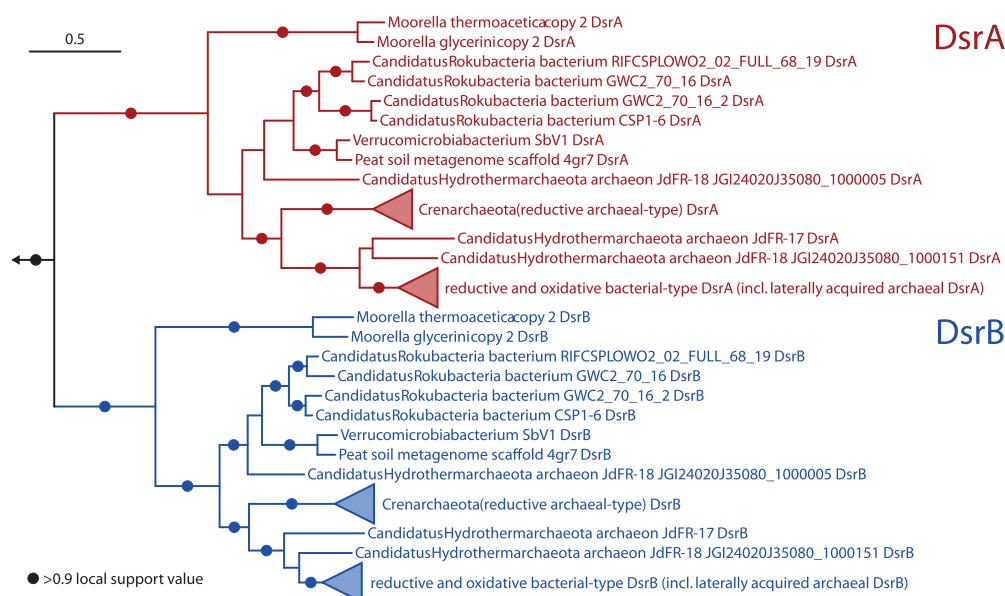


Fig. 2 Paralogous rooting analysis of DsrAB. Bayesian inference tree showing the phylogenetic relationship between DsrA and DsrB (50 sequences, 377 alignment positions). Arrow indicates outgroup of other sulfite, non-DsrAB reductase superfamily (COG2221)

sequences. Branch supports (posterior probability) higher than 0.9 are indicated in bold. DsrA/DsrB sequences from this study are marked in bold. Assignment of oxidative/reductive, bacterial/archaeal-type DsrAB is according to Müller et al. [18]

the *rdsr* pathway now includes bacteria from three additional phylum-level lineages: *Nitrospirae*, *Nitrospinae*, and *Candidatus* Muproteobacteria (Fig. 1a).

Anaerobic sulfite reductase-encoding organisms

The *asr* pathway for sulfite reduction was found in three bacterial phyla not previously known to possess this pathway, namely *Planctomycetes* and members of two candidate phyla, *Candidatus* Omnitrophica and *Candidatus* Riflebacteria. Concatenated protein trees of all three subunits AsrA, AsrB, and AsrC showed that sequences from these phyla clustered with those from *Firmicutes*, suggesting that they were acquired by LGT (Fig. 1b). Investigations into the operon structure of the *asr* complex revealed that while organisms from *Planctomycetes* and *Candidatus* Riflebacteria had a canonical gene organization in the order *asrA*, *asrB*, and *asrC*, *Candidatus* Omnitrophica had a fourth gene (*asrD*) as an insertion between *asrB* and *asrC* subunits. Analyses of conserved domains show that AsrD is related to the family of formate and nitrite transporters (pfam01226, COG2116, TIGRfam00790). We hypothesize that this may in fact serve as a bisulfide channel associated with dissimilatory sulfite reduction using the *asr* enzyme complex as observed in *Clostridium difficile* [54].

dsrD genes in candidate phyla radiation organisms

Surprisingly, we identified *dsrD* genes in eight genomes of organisms affiliating with *Candidatus* Falkowbacteria,

putatively symbiotic bacteria within the Parcubacteria superphylum of the candidate phyla radiation (CPR) [55]. There is no indication of the presence of other *dsr* genes in these genomes. Given the predicted close physical and metabolic interactions between CPR bacteria and their hosts, we suggest that this small protein could modulate host metabolism, as sometimes occurs with viruses/phage and their hosts [56]. CPR organisms are common in aquifers where conditions oscillate between oxic and anoxic [24, 26]. The predicted Falkowbacteria DsrD protein sequences cluster with sequences from well-characterized *Deltaproteobacteria* capable of sulfate reduction, suggesting that *deltaproteobacterial* sulfate reducers served as *dsrD*-donors during LGT to these CPR bacteria (Supplementary Fig. 6). Considering the presence of *dsrD* genes in CPR organisms and putative sulfur-oxidizing/sulfur disproportionating bacteria [16], we propose that *dsrD* is not a good marker for sulfite reduction. Therefore, we suggest an alternate set of rules for utilizing a combination of *dsr* genes to distinguish DsrAB-based sulfite reduction from sulfur oxidation on the basis of genomic features (Table 2).

Lateral gene transfer of DsrAB sulfite reductases

Prior analyses have suggested that LGT has influenced the evolution of *dsrAB* among extant microorganisms but only by comparably few events among major taxonomic lineages [14, 18, 57]. We used a comparison of 16S ribosomal RNA and concatenated DsrAB protein trees to reevaluate the extent to which LGT has influenced the organismal

Table 2 Suggested rules for determination of direction of dissimilatory sulfur metabolism for uncultivated organisms

Contribution to sulfur cycle	Suggested rules
Sulfate reduction (to sulfide)	<i>aprBA</i> , <i>sat</i> are present, DsrAB cluster with reductive DsrAB, <i>dsrD</i> gene is present, and <i>dsrEFH</i> are absent
Sulfite reduction (to sulfide)	DsrAB cluster with reductive DsrAB, <i>dsrD</i> gene is present, and <i>dsrEFH</i> are absent
Sulfate reduction (to sulfite)	<i>aprBA</i> and <i>sat</i> are present
Sulfur oxidation (to sulfite)	DsrAB do not cluster with reductive DsrAB, <i>dsrEFH</i> are present, and <i>dsrD</i> is absent
Sulfur oxidation (to sulfate)	DsrAB do not cluster with reductive DsrAB, <i>dsrEFH</i> are present, and <i>dsrD</i> is absent, <i>aprBA</i> and <i>sat</i> are present
Sulfite reduction, or sulfur oxidation, or both	<i>dsrD</i> and <i>dsrEFH</i> are present.
Sulfur oxidation (to sulfite) as a part of sulfur disproportionation	DsrAB cluster with reductive DsrAB, <i>dsrD</i> gene is present, and <i>dsrEFH</i> are absent

Sulfur disproportionating organisms cannot be differentiated from sulfite-reducing organisms on genomic features alone. Details of individual *dsr* genes are specified in Supplementary Table 6.

distribution of *dsrAB* genes (Fig. 3). Mismatching branching pattern between the two trees indicates that *dsrAB* has been introduced into most of the candidate phyla members by multiple independent LGT events. Our analyses show that organisms from five bacterial and archaeal phyla, *Deltaproteobacteria*, *Nitrospirae*, *Candidatus* Hydrothermarchaeota, *Actinobacteria*, *Chloroflexi* likely acquired *dsrAB* genes in multiple events. Amongst these, *Nitrospirae* and *Deltaproteobacteria* displayed the highest number of LGT involving five independent events spanning across both reductive and oxidative branches of the DsrAB tree. These findings provide evidence for the complex evolutionary history of *dsr* genes. Currently, it may not be possible to identify the specific lineage in which sulfite reduction first appeared; however, our extensive dataset sets the stage for future studies to investigate the evolution of dissimilatory sulfur metabolism.

Sulfate vs. sulfite reduction in organisms

To determine whether these newly identified organisms reduce sulfate vs. sulfite to sulfide we looked for the genes involved in the reduction of sulfate to sulfite, specifically adenylyl-sulfate reductase subunits A and B (*aprBA*), sulfate adenylyl transferase (*sat*), and quinone-interacting membrane-bound oxidoreductase subunits A, B, and C (*qmoABC*) [58–60]. Organisms from three phyla, the *dsr*-containing *Candidatus* Lambdaproteobacteria, and *asr*-containing *Candidatus* Riflebacteria, and *Candidatus* Omnitrophica, lacked genes for the reduction of sulfate to sulfite suggesting that they were sulfite reducers. Sulfite utilized by these organisms may derive from the environment or is produced inside the cell as part of other sulfur metabolism pathways such as tetrathionate or thiosulfate reduction, sulfur disproportionation, or by organosulfonate respiration. This suggests that recent genome-based

observations supporting potential “metabolic handoffs” between organisms (transfer of metabolites associated with energy metabolism) in the oxidative cycle of sulfur [24, 26, 61] likely extend to the reductive cycle as well [62]. Interestingly, *Candidatus* Rokubacteria whose DsrAB sequences represent a novel deep-branching lineage in the DsrAB tree also have *apr*, *sat*, and *qmo* genes that are required for sulfate reduction or sulfite oxidation. Phylogenetic analyses of the individual *dsr* proteins shows that the sulfate reduction system of *Candidatus* Rokubacteria is of mosaic evolutionary origin (Fig. 4) (Supplementary Figs. 7–9).

Prevalence of *dsrT* in *dsrAB*-containing microorganisms

In addition to *dsrD*, we sought evidence for hypothetical genes in proximity to known *dsr* genes that may help in distinguishing between DsrAB-based sulfate/sulfite reduction and sulfur oxidation pathways. We identified a hypothetical gene that encodes for the N-terminal domain of an anti-sigma factor antagonist protein [63] that almost always occurs within the operon encoding *dsr* genes (Fig. 5; Supplementary Fig. 4). This hypothetical protein is part of a protein family that includes the *Bacillus subtilis* RsbT co-antagonist protein rsbRD, which are important components of the stressosome and function as negative regulators of the general stress transcription factor sigma-B [64]. This gene is unique to DsrAB-based sulfite-reducing organisms and is mostly absent in recognized sulfur-oxidizing organisms, except for those within the phylum *Chlorobi* (Supplementary Fig. 10). We refer to this gene as ‘*dsrT*’ in accordance with homologous genes in phototrophic green sulfur bacteria from the phylum *Chlorobi* [65]. This gene always precedes the electron transport components encoded by *dsrMKJOP* genes [66] and is fused with *dsrM* in some

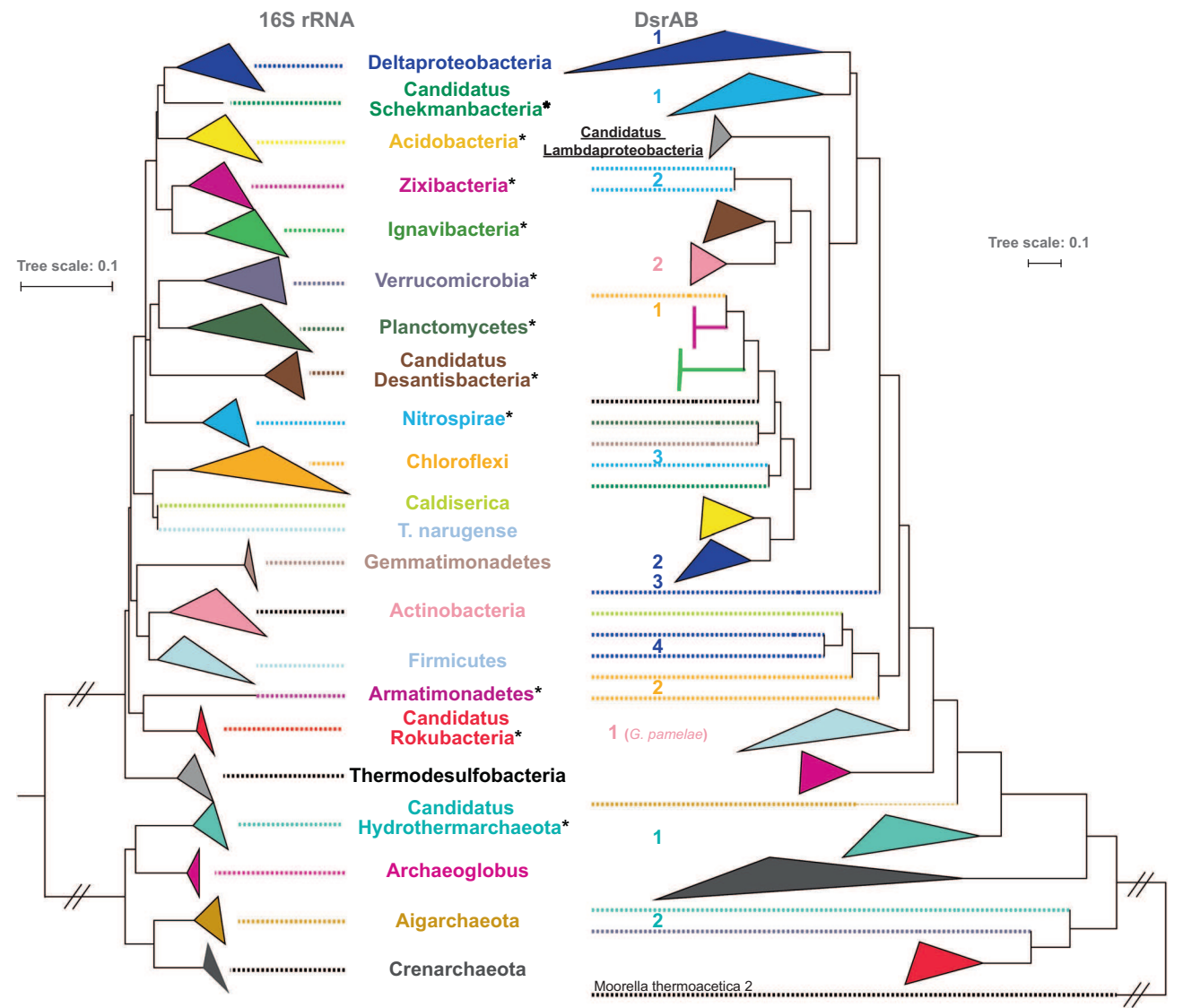


Fig. 3 Comparison of 16S rRNA and concatenated DsrAB trees for sulfate/sulfite-reducing microorganisms. Sequences are grouped at the phylum level. Trees were constructed using a consensus of neighbor-joining and maximum-likelihood phylogenies with 1000 bootstrap re-samplings each. Each phylum is colored differently to identify LGT based on inconsistent branching patterns. Phylum names with an asterisk represent sulfate/sulfite-reducing lineages that were discovered in this study. Numbers indicate number of independent LGT events associated with the specific phylum. Complete phylogenetic trees with bootstrap values are available in Data Files S3–S6. LGT events involving oxidative-type DsrAB for Nitrospirae (2 events) and Deltaproteobacteria (1 event) are not shown

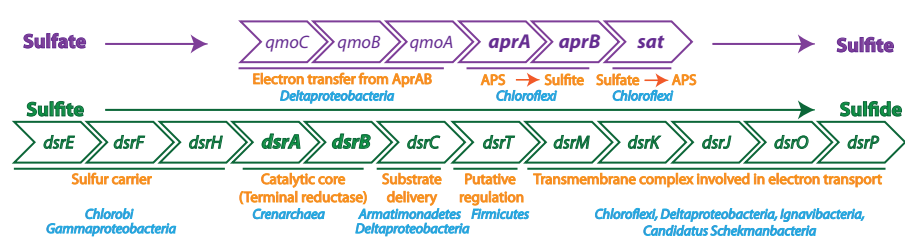


Fig. 4 Dsr operon structure and enzymatic roles of proteins involved in sulfate reduction in *Candidatus Rokubacteria*. Purple: genes involved in sulfate reduction to sulfite. Orange: putative enzymatic roles of genes; blue: microbial lineages with closest homologs as determined by phylogeny/BLAST against NCBI GenBank. APS refers to adenosine-5'-phosphosulfate. Green: genes involved in sulfite reduction to sulfide. This is the first case in which *dsrE*, *dsrF*, and *dsrH* genes are present in organisms other than sulfur-oxidizing bacteria

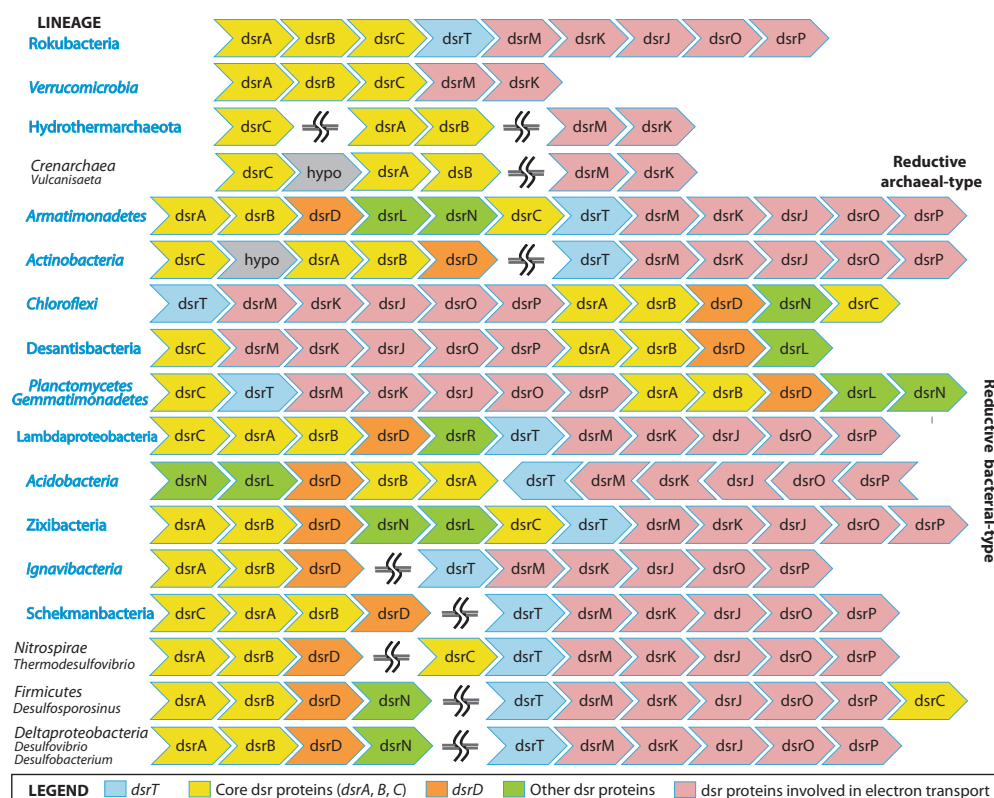


Fig. 5 Dsr operon structure in previously reported (black names) and newly reported groups (blue names). Interestingly, and in contrast to the previously studied organisms for which the operon is interrupted

(=SS=), the entire dsr pathway (including electron transport chain and ancillary proteins) is often encoded in a single genomic region

organisms (Supplementary Fig. 11). Fused *dsrT-dsrM* genes are oriented with *dsrT* in the N-terminal and *dsrM* in the C-terminal, thereby maintaining the gene order observed in canonical dsr operons: *dsrT*, *dsrM*, *dsrK*. From structural predictions and conserved motifs, we hypothesize that it likely performs a regulatory function (Supplementary Fig. 12).

***dsrEFH* in the newly discovered *dsrAB*-containing microorganisms**

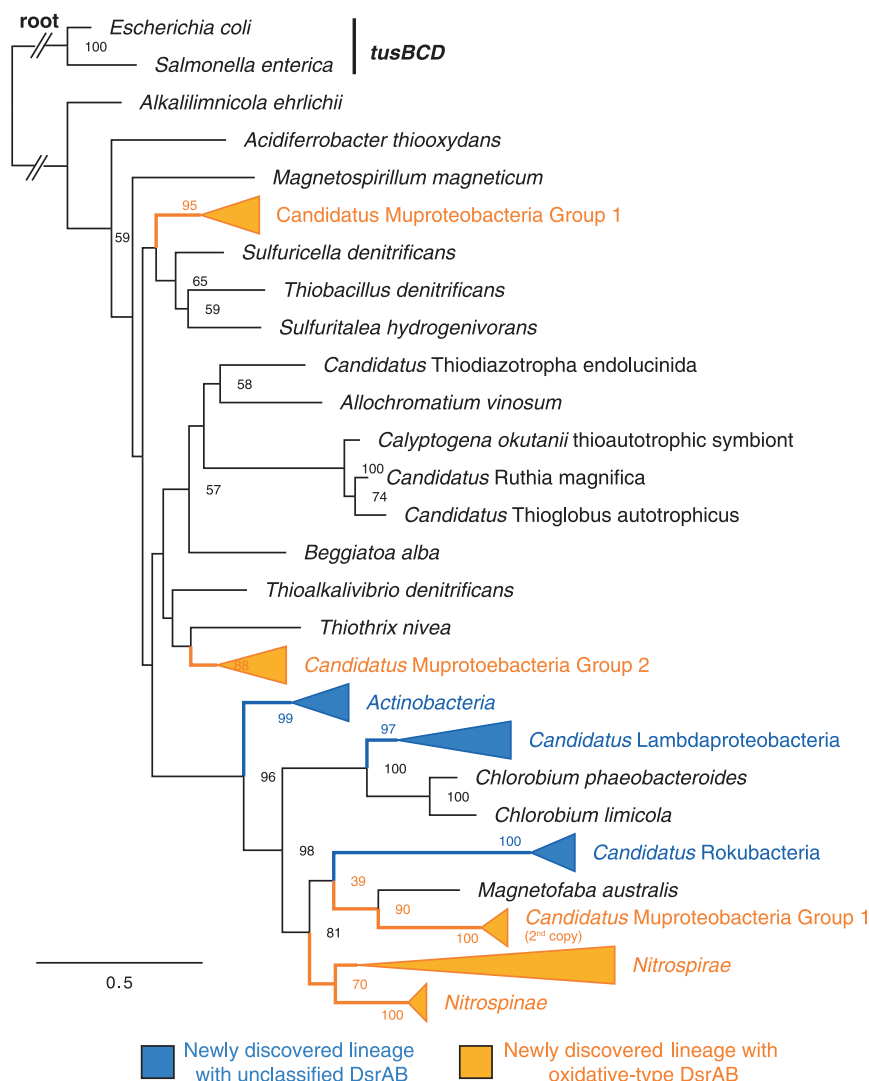
Recent studies looking into the distribution of genes associated with the dsr operon have suggested that *dsrE*, *dsrF*, and *dsrH* are unique to sulfur oxidizing microorganisms and are absent in sulfate/sulfite-reducing and putative sulfur disproportionating microorganisms [16, 38, 67]. In sulfur-oxidizing microorganisms, DsrEFH can serve as an effective sulfur donor for DsrC [68]. On the other hand, co-located *dsrE*, *dsrF*, and *dsrH* genes are present in ~24% of the newly identified DsrAB-encoding microorganisms (30 out of 123 genomes) (Supplementary Fig. 4). These *dsrEFH* genes were identified in organisms from six phylum-level lineages, *Actinobacteria*, *Candidatus Rokubacteria*, *Candidatus Lambdaproteobacteria*, *Candidatus Muproteobacteria*, *Nitrospirae*, and *Nitrospinae*. Phylogenetic

analysis of all identified DsrEFH shows that they cluster with well-characterized sulfur-oxidizing organisms (Fig. 6). The presence of *dsrEFH* genes (with well-known roles in sulfur oxidation) in organisms from *Actinobacteria* and *Candidatus Lambdaproteobacteria* is perplexing since these organisms also possess the *dsrD* gene that is unique to DsrAB-containing sulfite-reducing organisms. Further, the presence of *dsrEFH* genes in organisms within the phylum-level lineage *Candidatus Rokubacteria* (with a novel deep-branching clade of DsrAB) suggests that these organisms are likely involved in sulfur oxidation rather than sulfite reduction. Finally, sequences from *Candidatus Muproteobacteria* formed two distinct clades (Group 1, Group 2) and clustered with two separate groups, *Alphaproteobacteria* and *Gammaproteobacteria* respectively. All *Candidatus Muproteobacteria* with *dsrEFH* sequences in Group 1 also possessed a second copy of these genes that clustered with sequences from *Magnetofaba australis* and *Candidatus Rokubacteria*.

Electron donors and other metabolic potential associated with sulfur cycling

In order to better understand the energy metabolism and ecology of these newly identified organisms, we

Fig. 6 Concatenated DsrEFH protein tree inferred by maximum likelihood. Phylum-level lineages with first report of the presence of *dsrEFH* genes are shown in blue (from organisms with unknown-type DsrAB) and orange (from organisms with oxidative type DsrAB). Homologous TusBCD from *E. coli* and *S. enterica* were used to root the tree. Only bootstrap values >50 are shown



investigated potential electron donors for putative sulfate/sulfite reduction. Specifically, we targeted genes involved in the oxidation of hydrogen [69] (Ni–Fe hydrogenase groups I, IIa, IIb, IIIa, IIIb, IIIc, IIId, Fe–Fe hydrogenase groups A, B1/B2) and transformation of organic carbon compounds (genes involved in breakdown of cellulose, hemicellulose, chitin, pectin, starch, amino sugars, other monosaccharides, and polysaccharides) [49] and short chain fatty acids. Our analyses show that organisms from 12 of the 13 newly identified putative sulfate/sulfite-reducing DsrAB-containing lineages identified in this study possess the ability to utilize hydrogen as an electron donor (Supplementary Table 2). On the other hand, organisms from all 13 lineages possessed the ability to breakdown complex carbon compounds although the diversity of genes encoding for specific carbohydrate-active enzymes varied greatly across phyla (Supplementary Table 3). Organisms from 8 of the 13 putative sulfate/

sulfite-reducing lineages possessed the ability to oxidize short chain fatty acids by beta-oxidation (Supplementary Table 4). In order to understand if these newly identified organisms were heterotrophs or autotrophs, we looked at the carbon fixation potential encoded in the genomes. We identified three different carbon fixation mechanisms, the Calvin–Benson cycle (CBB), the reverse (reductive) tri-carboxylic acid (rTCA) cycle, and the Wood–Ljungdahl pathway in ~50% of all organisms (Supplementary Table 5). In total, 11 organisms contained genes encoding for the CBB cycle with 10 possessing the Form I RuBisCO and 1 organism possessed the Form II RuBisCO. Genes for the Wood–Ljungdahl pathway were encoded in 42 genomes while genes for the rTCA cycle were encoded in 7 genomes. We propose that sulfate/sulfite reduction by organisms from these newly identified lineages likely serves an important control on carbon cycling in the terrestrial and marine subsurface.

Conclusions

By the Proterozoic Eon, sulfate reduction had become a significant biological process in the oceans [70, 71]. Based on phylogenomic arguments and isotopic records, it was suggested that the capacity to reduce sulfite to sulfide emerged in thermophilic archaea around 3.5 billion years ago, and that mesophilic sulfate reducers evolved only after the rise in atmospheric oxygen level [2, 72]. Our findings indicate a complex evolutionary history of this capacity involving extensive LGT of *dsr* genes. Consequently, it may be impossible to constrain the specific lineage in which this metabolism first appeared. The ability for a DsrAB-based dissimilatory sulfur metabolism is now predicted in a much wider diversity of mesophilic bacterial and archaeal groups than was recognized previously. We conclude that many groups of microorganisms now known to have genes involved in dissimilatory sulfur metabolism impact biogeochemical processes in marine and terrestrial sediments, aquifers, wetlands, methane seeps, coastal marshes and estuaries, as well as agricultural and human microbiomes. Many are organisms from well-studied phyla, but still novel at the genus to class levels, but others are organisms from candidate phyla known only based on their genomes. The results underline the value of genomic analyses for prediction of key ecosystem capacities that cannot be made based on rRNA gene surveys and motivate targeted cultivation strategies for organisms currently lacking laboratory tractable representatives. Finally, these findings will better inform future microbial trait-based ecosystem models that can predict the outcomes of global change on biogeochemical processes and planetary elemental cycles [73].

Data availability

NCBI Genbank, BioProject, BioSample, and Taxonomy ID (TaxID) accession numbers for individual genomes are listed in Supplementary Table 1. Genomes are also available through ggKbase: http://ggkbase.berkeley.edu/novel_sulfate_reducers (ggKbase is a 'live' site, genomes may be updated after publication). The JdFR-17, JdFR-18, and JdFR-19 genomes are also available through the Integrated Microbial Genomes and Microbiomes database (IMG) through Genome IDs: 2728369317, 2728369320, 2728369322. HMM databases used in this study are available from <https://github.com/banfieldlab/metabolic-hmms>. The authors declare that all other data supporting the findings of this study are available within the article and its supplementary information files, or from the corresponding author on request.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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Conclusions

This thesis contributed to the identification of low- and high-abundance microorganisms and taxa involved or putatively involved in wetland sulfur cycling. This resulted in an extended diversity and revised interpretation of sulfur compound-cycling microorganisms in general and specifically on their role in wetlands. It revealed their potential niches, which will help to build a comprehensive model of microbial carbon and sulfur cycling in food web of wetlands.

Ecophysiology of the permanently low-abundance sulfate reducer *Candidatus Desulfosporosinus infrequens*

Candidatus Desulfosporosinus infrequens SbF1 encodes the expected genetic features required of a sulfate reducer utilizing fermentative intermediates as electron donors, i.e., a complete sulfate reduction pathway and substrate-specific degradation pathways. However, when comparing the proposed ecophysiology of *Ca. D. infrequens* to the physiological observations from axenic *Desulfosporosinus* isolates, key differences should be highlighted. Described type strains of the genus *Desulfosporosinus* can utilize a wide array of substrate, including formate, lactate, and butyrate (Lee *et al.*, 2009; Alazard *et al.*, 2010; Mayeux *et al.*, 2013; Sánchez-Andrea *et al.*, 2015; Vandieken *et al.*, 2017). However, no indication for degradation of acetate or propionate was observed (Lee *et al.*, 2009; Alazard *et al.*, 2010; Mayeux *et al.*, 2013; Sánchez-Andrea *et al.*, 2015; Vandieken *et al.*, 2017). In addition, only incomplete oxidation of organic matter to acetate, but not complete oxidation to CO₂, was observed previously, except with formate (Alazard *et al.*, 2010; Mayeux *et al.*, 2013; Sánchez-Andrea *et al.*, 2015; Vandieken *et al.*, 2017). Contrarily, some *Desulfosporosinus* strains can grow autotrophically with H₂/CO₂ (Alazard *et al.*, 2010; Sánchez-Andrea *et al.*, 2015; Vandieken *et al.*, 2017), therefore it is unexpected that they only incompletely oxidize substrates to acetate (Rabus *et al.*, 2013). In addition, the lack of complete oxidation to CO₂ is not due to missing genes, as these are present in the published genomes of *Desulfosporosinus* strains.

Ca. D. infrequens not only encodes, but also expresses the pathways for the complete oxidation of acetate, lactate, and propionate (Chapter 1; Figure C-2). However, propionate oxidation differs from the proposed pathway in *Desulfotomaculum kuznetsovii* (Visser *et al.*, 2013). The initial transfer of CoA onto propionate is presumably performed by a propionyl-CoA:succinate CoA transferase instead of a propionyl-CoA:acetate CoA transferase, thereby removing the need for a succinate—CoA ligase. In addition, malate gets presumably converted to pyruvate by the malate dehydrogenase MaeB instead of to oxaloacetate by the malate dehydrogenase Mdh. Acetate may be oxidized by acetate kinase (AckA) and phosphate acetyltransferase (Pta) to acetyl-CoA which can be completely oxidized to CO₂ by the Wood—Ljungdahl pathway. Lactate and propionate degradation also

result in acetyl-CoA which can be further oxidized by either complete (Wood–Ljungdahl pathway) or incomplete (acetate kinase, phosphate acetyltransferase) oxidation. Expression of genes for both pathways was observed, therefore the true fate of lactate and propionate is unclear. The transcription of the Wood–Ljungdahl pathway concomitant with both the propionate or lactate degradation pathway in *Ca. D. infrequens* clearly shows that microorganisms behave differently in their (mimicked) natural settings as compared to what would be expected based on laboratory observation. Under substrate limitation, as is typically observed in wetlands, it makes sense to maximize the gain of ATP yield rather than the rate of ATP production as is predicted from the kinetic theory of optimal pathway length (Costa *et al.*, 2006). A prominent example of such a strategy are the recently discovered comammox bacteria (van Kessel *et al.*, 2015; Daims *et al.*, 2015), which unify the processes of ammonia and nitrite oxidation in one microorganism at the expense of higher costs to be invested into a longer energy-generating pathway. The complete oxidation of propionate and lactate to CO₂ in *Ca. D. infrequens* would follow the same reasoning. Cultivation media are normally not substrate-limited, which could explain why *Desulfosporosinus* isolates exhibit only incomplete oxidation when grown under laboratory conditions. Alternatively, complete oxidation may be a feature unique to the wetland strain. In addition it should be considered that the microcosms may have included two populations, one preferring complete and one incomplete oxidation, results in a mixed transcription patterns. Surprisingly, a pathway that could perform oxidation of butyrate was not identified. One reason would be that butyrate degradation is encoded as one operon, as observed in *Syntrophomonas wolfei* (Schmidt *et al.*, 2013), but is missing. Either because it was not assembled or the scaffold containing it was not binned. This is not in contradiction with the high checkM completeness estimates, as these are based on conserved marker genes and not functional marker genes. Alternatively, *Ca. D. infrequens* may indeed not be capable of butyrate degradation. However, as a strong response to addition of butyrate and sulfate was observed, *Ca. D. infrequens* was, at least indirectly, involved in butyrate degradation. For example, butyrate could be degraded by another microorganism to acetate, which in turn could be utilized by *Ca. D. infrequens*.

The different manifestations of rare biosphere microorganisms, their functional roles, and their ecological relevance have been discussed for over a decade (Sogin *et al.*, 2006; Lynch and Neufeld, 2015; Jousset *et al.*, 2017). Vigneron *et al.* (2018) demonstrated that a major fraction of DsrB species-level OTUs made <0.1% of all OTUs, and thereby were even less abundant as compared to the total microbial community and part of the rare biosphere. Here, the sulfate reducer *Ca. D. infrequens* showed a permanently low abundance (<0.1% relative abundance) in the native soil and in peat soil microcosm incubations for up to 50 days as demonstrated with 16S rRNA gene amplicon sequencing and verified by specific quantitative PCR assays (Chapter 1). Despite being rare, it showed high levels of activity as indicated by a significant increase in ribosome content (16S rRNA transcript amplicon sequencing) when amended with sulfate and propionate, lactate, or butyrate (Chapter 1). Further support for metabolic activity was given by observation of a significant upregulation

of general and/or substrate-specific gene expression when *Ca. D. infrequens* was amended with sulfate and acetate, propionate, lactate, or butyrate using metagenomics combined with metatranscriptomics (Chapter 2). Therefore, *Ca. D. infrequens* was highly active while at the same time being growth-arrested over an incubation period of 50 days (Chapter 1; Chapter 2). This differentiates *Ca. D. infrequens* from other permanently rare (sulfate-reducing) microorganisms that, when favorable conditions arrive, increase activity but also abundance (“seed bank” microorganisms). This raises the question, what are the mechanisms keeping *Ca. D. infrequens* at a low abundance even when active? One possibility is that an active and growing population is kept at a constant abundance by continuous predation, viral lysis, or active sporulation. However, no evidence was found for these hypotheses (Chapter 2). Alternatively, cellular mechanism may be active in response to environmental stresses. Growth arrest with non-rare microorganisms was observed previously (Bergkessel *et al.*, 2016). For example, under substrate-limiting conditions, *Pseudomonas aeruginosa* actively induces growth arrest, which in turn increases antibiotic tolerance. Growth arrest is achieved by influencing gene expression via the starvation-signaling stringent response (Nguyen *et al.*, 2011). However, starvation conditions are unlikely as sulfate and substrate were regularly added. This is supported by the observed transcription of catabolic genes. Growth arrest (of subpopulations) in response to antibiotics and other stress factors was also shown to be controlled by toxin-antitoxin systems that inhibit genome replication and translation when triggered (Page and Peti, 2016). *Ca. D. infrequens* encodes and transcribes a type II toxin-antitoxin system (*ndoA-ndoAI*) that could potentially perform this function in response to, e.g., pH stress. Finally, the strategy of growth arrest has its limits, as all living microorganisms need to divide at some point to survive, and indeed, after 73 days incubating microcosms, an increase of abundance by the *Desulfosporosinus* population was observed (Pester *et al.*, 2010). Genome replication could also be observed using the DNA-stable isotope probing metagenome from the same experiment. The iRep value (Brown *et al.*, 2016) of *Ca. D. infrequens* in this metagenome was 1.98, i.e., on average, 98% of cells were replicating their genomes at the sampling time point. In conclusion, this thesis establishes *Ca. D. infrequens* as a model organism to study the active rare biosphere. This sulfate reducer is driving sulfur cycling, a globally important process, while staying at a consistently low abundance showing that ecosystem impact does not have to depend on numbers alone.

Telmatospirillum species are associated with sulfur cycling

A species-level OTU classified as a member of the genus *Telmatospirillum* was linked to substrate degradation in sulfate-stimulated microcosm incubations (Chapter 1; Figure C-2). Its strong response in the incubation experiment was surprising, as *Telmatospirillum* was never observed to have any type of sulfur metabolism, although 16S rRNA genes sequences of that genus were often found in wetlands (Hamberger *et al.*, 2008; Pester *et al.*, 2010; Hunger *et al.*, 2011; Kanokratana *et al.*, 2011; Pankratov *et al.*, 2011; Hausmann *et al.*, 2016; Schmidt *et al.*, 2016). The described *Telmatospirillum siberiense* type species was tested for

the capability of sulfate reduction with lactate as the electron donor (Sizova *et al.*, 2007), and then again tested for sulfate reduction with the electron donors formate, acetate, propionate, and butyrate (Chapter 1), all resulting in a negative outcome. Initial *dsrAB*-specific PCR analysis revealed the strain to be missing those genes, which was later confirmed by sequencing its genome (Chapter 3). However, other genes involved in dissimilarity sulfur oxidation were identified: *soxEFXYZA* and *dsrEFH*. As the Sox machinery is incomplete, it is unclear what type of sulfur oxidation metabolism *Telmatospirillum siberiense* can perform. The related wetland OTU may harbour the same set of sulfur metabolism genes or even encode a complete Sox machinery. Together with sulfate-specific response in the incubation experiment, it is likely *Telmatospirillum* microorganisms are involved in sulfur cycling in wetlands, however, their exact function in the sulfur cycle remains to be elucidated. If *Telmatospirillum* members would oxidizing sulfur compounds, it would be an important step towards completing our understanding of the cryptic sulfur cycle in this wetland.

Activity of *Telmatospirillum* microorganisms was detected previously in the studied wetland using DNA-stable isotope probing (Pester *et al.*, 2010). However, the applied T-RFLP method did not have the resolution to distinguish different phylotypes. As shown in Chapter 1, additional *Telmatospirillum*-classified OTUs responded to additions of substrates, but significantly stronger when no additional sulfate was added (Figure C-2). An opposing response of these closely related microorganisms shows that members of the same genus do not necessarily have similar ecophysiologicals. Similarly, niche differentiation within one genus was observed previously (e.g., Bayer *et al.*, 2016). This brings up the question, what is an ecologically relevant unit? Even observations beyond species level were observed to be ecologically distinct. For example, two organisms with 16S rRNA genes of 99.2% sequence identity show ecological different behavior (Tikhonov *et al.*, 2015). This emerging pattern highlights that fine-scale phylogenetic resolution will be required to resolve the ecological role of some microorganisms in their environment. In this thesis, a total of 17 species-level OTUs were classified as members of the genus *Telmatospirillum* with some showing varying and opposite response patterns to different substrate and sulfate treatments. Further research using methods with sub-species resolution (e.g.; Tikhonov *et al.*, 2015; Callahan *et al.*, 2016) may give new insights in the ecophysiology of members of this genus and ecologically relevant units in general.

Metabolically diverse, DsrAB-encoding acidobacterial *Candidatus* genera *Sulfotelmatobacter*, *Sulfotelmatomonas*, and *Sulfopaludibacter*

Based on 16S rRNA gene-based surveys and data from high-throughput sequencing methods, the ubiquity and high abundance of *Acidobacteria* in many soils and especially wetlands is known (Dedysh *et al.*, 2006; Hartman *et al.*, 2008; Ausec *et al.*, 2009; Dedysh, 2011; Serkebaeva *et al.*, 2013; Bragina *et al.*, 2014; Etto *et al.*, 2014; Sun *et al.*, 2014;

Urbanová and Bárta, 2014; Pankratov *et al.*, 2011; Tsitko *et al.*, 2014; Elliott *et al.*, 2015; Hunger *et al.*, 2015; Ivanova *et al.*, 2016; Jeong and Ham, 2017). In the studied wetland, *Acidobacteria* have a relative abundance of more than 50% (Chapter 4). Until now, they were largely considered to be aerobic degraders of complex carbon substrates or anaerobic fermenters. However, their ecosystem function can only be inferred based on what is observed for cultured and/or genome-sequenced relatives. Therefore, it is of great importance to know all potential metabolic traits exhibited within a phylum (or higher taxonomic levels). Genome- and transcriptome-based analysis of the seven DsrAB-encoding acidobacterial wetland MAGs generated multiple testable hypotheses (Chapter 4; Figure C-2):

Sulfate reducers with unusual traits? Three of the seven acidobacterial MAGs encode and transcribe the complete canonical sulfate reduction pathway. Sulfate as the terminal electron acceptor completely replaces aerobic respiration in the anaerobic oxidation of organic matter (or H₂). The recovered MAGs, as well as other genomes of *Acidobacteria*, encode a wide range of pathways to degrade complex and simple carbon molecules, e.g., polysaccharides, amino sugars, propionate, or lactate. This enables them to interact at different levels of the microbial food web, greatly influencing how carbon gets mineralized. Complete oxidation of organic matter to CO₂ could deplete methanogens of possible substrates (e.g., acetate) thereby potentially influencing greenhouse gas emissions. In addition, degradation of polysaccharides with sulfate as the electron acceptor would also be a novel trait for sulfate-reducing bacteria, as this trait was only observed in sulfate-reducing archaea so far (Rabus *et al.*, 2013). This could also explain why sulfate-reducing *Acidobacteria* were not yet isolated, as polysaccharide as an enrichment substrate favours fermentative bacteria due to their faster growth in batch cultures (Rabus *et al.*, 2013).

Electron acceptors besides sulfate? Four of the seven acidobacterial MAGs are missing genes encoding sulfate adenylyltransferase and adenylyl-sulfate reductase, making them still genetically capable to reduce sulfite but not sulfate. It is important to note, that from analysing MAGs it is nearly impossible to know if genes are missing because of incomplete sequencing, assembly, or binning, or because these microorganisms' genomes are indeed deficient for those traits. Assuming the later, the lack of sulfate reduction genes can be explained by the presence of genes for the utilization of organic sulfur compounds. Two of the adenylyl-sulfate reductase-deficient MAGs, but none of the adenylyl-sulfate reductase-encoding MAGs, harbour a (2R)-sulfolactate sulfo-lyase, which catalyzes the last step in the degradation of organosulfonate compounds. a process that releases sulfite (Chapter 4).

A missing link in the cryptic sulfur cycle? So far, the identity of the microbial players performing the oxidative steps of the cryptic (anoxic) sulfur cycling in wetlands is unknown (Pester *et al.*, 2012). DsrAB-encoding *Acidobacteria* could perform this function, as the canonical sulfate reduction pathway is reversible. It is impossible to predict the direction of

this pathway based on the sequence of DsrAB, and even with an established DsrAB phylogeny (Müller *et al.*, 2015) their classification into reductive-type and oxidative-type has always to be seen as tentative. This was clearly demonstrated with the deltaproteobacterium *Desulfurivibrio alkaliphilus* (Thorup *et al.*, 2017) that uses the reverse sulfate reduction pathway despite being indistinguishable from a sulfate reducer based on its taxonomic affiliation and genetic makeup. However, anaerobic oxidation of sulfur compounds requires an electron acceptor that replaces oxygen. In wetlands, possible candidates are nitrate/nitrite and metals such as iron (Pester *et al.*, 2012), however, no genes encoding enzymes that could oxidize these compounds were identified. It cannot be excluded, that the microorganisms from which the MAGs originate still perform these functions, as there could be undiscovered enzymatic pathways for these lifestyles.

Alternation of sulfate reduction and sulfide oxidation? However, coupling sulfur oxidation to aerobic respiration is a well documented and favourable lifestyle (Dahl and Friedrich, 2008). The concept of sulfide-oxidizing *Acidobacteria* is supported by (near) complete respiration chains in six of the seven acidobacterial MAGs, including sulfide-resistant terminal oxidases in three of the MAGs (Forte *et al.*, 2016). Going one step further, one can speculate that a switch of aerobic and anaerobic lifestyles is viable in the studied wetland. In this scenario, organic matter is degraded anaerobically coupled to sulfate reduction, and the (possible internally stored) oxidized sulfur compounds can then be used as the energy substrate if the environment becomes oxic. These MAGs were extracted from a depth of 10–20 cm where temporal fluctuations of the water table result in regular switching between oxic and anoxic (Paul *et al.*, 2006). An alternation of sulfate reduction and sulfur oxidation also implies that the *dsrD* and *dsrL* genes are still indicative to their respective proposed processes.

Phylogenetic analysis of DsrAB revealed that all seven acidobacterial wetland MAGs fall into the uncultured family-level DsrAB lineage 8 (Figure C-1). Together with lineage 9, lineage 12, and unclassified sequences these make up the DsrAB environmental supercluster 1, which contains no cultivated representatives. Based on environmental distribution analysis, lineage 8 is dominated by sequences originating from soil and industrial samples. Contrarily, the closely related lineage 9 is dominated by sequences from marine and freshwater sources (Müller *et al.*, 2015). Because of their phylogenetic closeness, it stands to reason that DsrAB lineages 8 and 9 originate from the phylum *Acidobacteria*. This is supported by the identification of a lineage 9 DsrAB-encoding MAG from an aquifer identified as *Acidobacteria* (Chapter 5). While the wetland lineage 8 DsrAB sequences belong to subdivision 1 and 3 of the *Acidobacteria*, the lineage 9 DsrAB sequence belongs to a MAG from *Acidobacteria* subdivision 17. It is also likely that the dissimilatory sulfur metabolism genes found on fosmids from the Wadden Sea (Mussmann *et al.*, 2005) originate from marine *Acidobacteria* due to the conserved synteny of the operons and the phylogenetic closeness of the DsrAB sequences to lineage 8 and 9 DsrAB sequences.



Figure C-1. Phylogenetic tree of DsrAB sequences calculated by maximum likelihood following the established taxonomy (Müller *et al.*, 2015; Wasmund *et al.*, 2017). Sequences from novel Schlöppnerbrunnen MAGs identified in this thesis are in bold. Uncultured family-level DsrAB lineages are shown in red.

Novel deep-branching DsrAB sequences found in bacteria and archaea

DsrA and DsrB are part of the dissimilatory sulfite reductase family (COG2221) that also contains other dissimilatory sulfite reductases, e.g., trimeric dissimilatory sulfite reductase catalytic subunit AsrC and coenzyme F₄₂₀-dependent sulfite reductase Fsr (Huang and Barrett, 1991; Johnson and Mukhopadhyay, 2005). However, *dsrA* and *dsrB* are paralogs always encoded together in one operon. Single *dsrA* or single *dsrB* gene copies in closed genomes were not observed, therefore it is deduced that these originate from an ancient gene duplication. This is unique among the dissimilatory sulfite reductases and brings advantages in resolving their phylogeny as the DsrA/DsrB tree can be rooted by paralogous rooting analysis. Previous analyses revealed that a second DsrAB copy found in members of the genus *Moorella* as the most basal branch (Müller *et al.*, 2015). This still holds true when additional DsrAB sequences of all novel MAGs were added, however, another basal branch containing DsrA/DsrB sequences from *Ca. Rokubacteria*, *Verrucomicrobia*, and a part of *Ca. Hydrothermarchaeota* was created (Chapter 5; Figure C-1). Surprisingly, these differ in their encoded sulfur metabolism genes. While some of the *Ca. Rokubacteria* MAGs encode a complete sulfur oxidation pathway up to sulfate, including the unambiguous marker genes *dsrEFH*, *Verrucomicrobia* and *Ca. Hydrothermarchaeota* MAGs only encode *dsrABCMK* (Chapter 5). The DsrAB enzyme with the DsrC sulfur carrier in combination with the membrane complex DsrMK could potentially convert sulfite to sulfate (Figure C-2). Sulfite reduction without the additional membrane complex subunits DsrJOP is possible, as was observed in well-studied sulfate reducers, e.g., *Desulfotomaculum* (Junier *et al.*, 2010). A non-monophyletic distribution of reductive and oxidative metabolisms was observed before. For example, crenarchaeotal sulfate reducers are a sister branch to reductive and oxidative bacterial DsrAB sequences (Müller *et al.*, 2015). In addition, several sequences within the deltaproteobacterial sulfate reducer DsrAB cluster originate from sulphide oxidizers, e.g. *Desulfurivibrio alkaliphilus* (Thorup *et al.*, 2017). It remains to be elucidated if this polyphyletic distribution of different sulfur metabolism types is caused by lateral gene transfer, or if the DsrAB enzyme evolved to change direction multiple times?

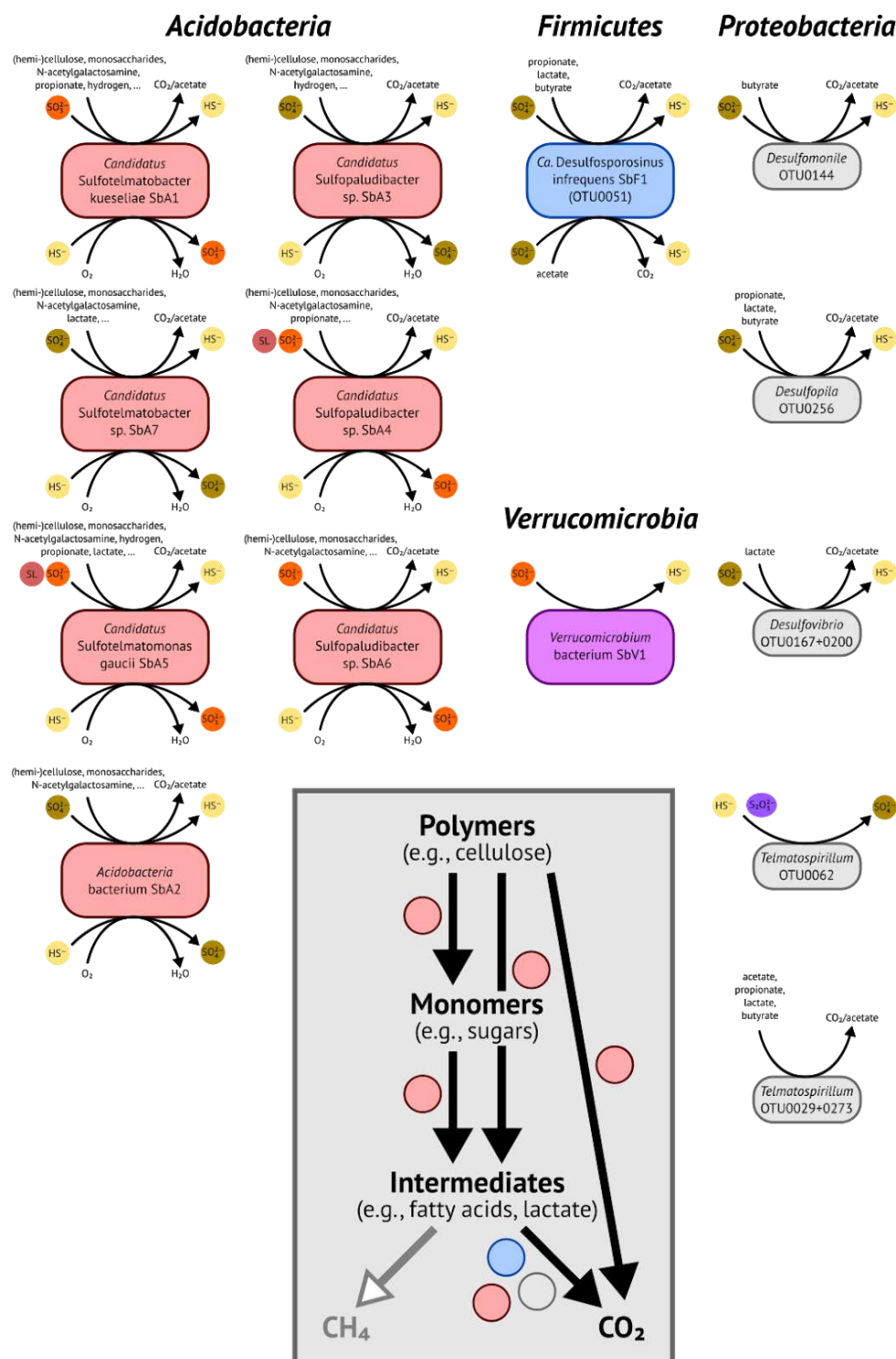


Figure C-2. Overview of the proposed ecophysiology of discovered microbial key players in Schlöppnerbrunnen. Sulfur and carbon cycling organisms are shown as schematic cells (separated by phylum). Proposed metabolic reactions are shown as double arrows (electron donors and acceptors). Sulfur compounds are marked by coloured circles (SL, sulfolactate). If electron donor or acceptor was unknown, only one arrow is shown. Inference is based on 16S rRNA (gene) amplicon sequencing (grey cells; Chapter 1; Chapter 3), metagenomics/-transcriptomics (coloured larger cells; Chapter 2; Chapter 4; Chapter 5), or both (*Ca. D. infrequens* SbF1). Proposed places of organisms in the wetland carbon food web is indicated in the grey box (colours circles correspond to coloured cells).

Further research

Genome-centric metagenomics has enabled us to greatly extend our knowledge about the genetic potential of uncultured organisms. However, functional gene predictions, on which physiological predictions and thereby the proposed ecological relevance of microorganisms are based, are – no matter how well supported – only predictions. While it is not possible to prove all hypotheses generated by high-throughput sequencing and the resulting boom of novel MAGs, it will be required to selectively pick out new model microorganisms and elucidate them and their roles in their environments in more detail. One important step towards this achievement will be to obtain new representative axenic cultures of *Acidobacteria* and other microorganisms that carry dissimilatory sulfur metabolism genes. One challenge will be that *Acidobacteria* are hard to isolate, and that most cultivation efforts were focused on ambient oxygen concentrations, as demonstrated by the bias in aerobic vs anaerobic isolates (Kielak *et al.*, 2016). Attempts will have to be made to isolate DsrAB-encoding *Acidobacteria* by using sulfur compounds as electron donors or electron acceptors under anoxic conditions. The amino sugar *N*-acetylgalactosamine would be a promising substrate to isolate potential acidobacterial sulfate reducers, as the MAGs are rich in glycoside hydrolases that were shown to degrade this substrate. Under the assumption that the DsrAB-encoding *Acidobacteria* utilized sulphide as an electron acceptor, enrichment attempts can still be made under oxic conditions, however, one new approach could be to use microoxic conditions, as the MAGs encode high-affinity terminal oxidases presumably used under low oxygen pressure. While there are methods to improve isolation efficiency of *Acidobacteria* (e.g., plate wash PCR, Stevenson *et al.*, 2004), isolating representatives from Candidate phyla may prove even more challenging. Until proof on the type of sulfur metabolism is delivered by cultivation efforts and cell biology, all plausible lifestyles will have to be considered while interpreting microbial community data obtained in environments containing these microorganisms. While there are model microorganisms for sulfate reduction and sulfur oxidation, each containing *dsrD* and *dsrL*, respectively, there are no cultivated representatives containing both these genes. To exhaustively evaluate the role of these tentative marker genes, such isolates will be required as well. Simulating a fluctuating water table in micro- or mesocosms experiments could lead to enrichment of such microorganisms. Testing for sulfur storage compounds could be performed using Raman spectroscopy (Berg *et al.*, 2014). However, axenic strains may not behave in pure culture as they would *in situ*, nor can all microorganisms native to wetlands be isolated due to practical considerations alone. Therefore, incubation studies mimicking *in situ* conditions using substrates and conditions informed by MAG annotations are worth pursuing. As with the mentioned enrichment strategies, using untested substrates such as *N*-acetylgalactosamine could lead to informative responses when used soil slurry microcosms. These can be performed under anoxic, microoxic, or oxic conditions, or even alternations of these to test for a mixture of reductive and oxidative sulfur metabolism within one microorganism.

On the other hand, the insights and hypotheses gained from metagenomics analyses are invaluable. While this thesis focused on dissimilatory sulfite reductases as markers for sulfur metabolism, additional established and putative marker genes are available. Required in sulfate reduction and sulfur oxidation is the adenylyl-sulfate reductase AprBA (Watanabe *et al.*, 2016). Specific for thiosulfate oxidation are the *sox* genes (Meyer *et al.*, 2007). Both have established phylogenies, taxonomies, and collected metadata. Many more dissimilatory sulfur metabolism genes are known (Wasmund *et al.*, 2017), however, work is needed to establish them as usable marker genes. In addition, the role of organosulfur as part of the sulfur cycle should not be neglected, which adds additional genetic complexity (e.g., Denger *et al.*, 2014; Felux *et al.*, 2015). All these genes can and should be used to identify microorganisms directly and indirectly in the sulfur cycle in existing and upcoming metagenomes, which in turn will improve the size and quality of functional gene databases. This approach was demonstrated with DsrAB as an (ongoing) success story. DsrAB was established as a set of marker genes two decades ago (Wagner *et al.*, 1998). Sequencing of *dsrAB* gene clones then quickly revealed a large novel diversity not covered by cultured representatives (Pester *et al.*, 2012; Müller *et al.*, 2015). Now, slowly, with metagenomics (and new cultivated strains) these lineages are being assigned to taxonomic groups, while in parallel still extending the diversity of the *dsrAB* gene by revealing new phylogenetic clades and branches. With the size and number of available metagenomes, manual binning of MAGs, as was done in this study, will become infeasible, therefore automated binning should be used. Resulting MAGs can then be screened for the presence of any of the dissimilatory sulfur metabolism gene and further analysed in detail. In addition, phenotypic classification of sulfur metabolism should be a focus of further research. For example, such classifiers exist for traits like aerobic vs anaerobic lifestyles (Feldbauer *et al.*, 2015), which could be extended to test for DsrAB-based sulfate reduction vs sulphide oxidation.

Ca. D. infrequens is now a well established model for a rare sulfate-reducing microorganisms (Pester *et al.*, 2010; Chapter 1; Chapter 2). Still, the mechanisms that are in place to regulate its growth arrest will need to be further elucidated, e.g., is it direct regulation of expression, toxin-antitoxins, or a novel mechanism? In addition, the relevance of this genus and also that of other rare microorganisms in global wetlands will need to be researched. As a first indication of global relevance, 16S rRNA gene sequences related to the rare *Desulfosporosinus* are found world wide (Chapter 1). Further research will need to be dedicated to evaluate the impact of this and other rare microorganisms on the carbon and sulfur cycles of wetlands and other environments. This means the rare biosphere can not be overlooked any more. The necessary sequencing depth or other appropriate methods will need to be applied to fully elucidate the diversity and interplay of microbes of all abundances in complex environments. That will include stopping using arbitrary abundance cutoffs as filtering or evaluation criteria on an microorganism's impact on an ecosystem. Rare microorganisms are a great example of why axenic culture alone are not enough to fully understand an microorganism or its natural habitat.

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