EXPLORATION OF SULFUR-CYCLING MICROORGANISMS FROM ANOXIC BLACK SEA WATERS AND SEDIMENT

Daan M. van Vliet

PROPOSITIONS

1

One plus one equals three when it comes to using cultivation and 'omics' techniques for elucidating microbial metabolism. (this thesis)

2

'You are what you eat' applies to bacteria living on fucoidan. (this thesis)

3

In biology, explorative, descriptive and hypothesis-driven research are equally important.

4

Repeated use of Occam's razor can lead to tunnel vision and loss of creativity.

5

Scientists fulfill societal roles formerly held by clergy and monastics.

6

Microorganisms as well as humans do better in mixed communities.

7

Life is sulfuring.

Propositions belonging to the thesis, entitled:
"Exploration of sulfur-cycling microorganisms from anoxic Black Sea waters and sediment"

Daan M. van Vliet
Wageningen, 8 September 2021

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EXPLORATION OF SULFUR-CYCLING MICROORGANISMS FROM ANOXIC BLACK SEA WATERS AND SEDIMENT

Daan M. van Vliet

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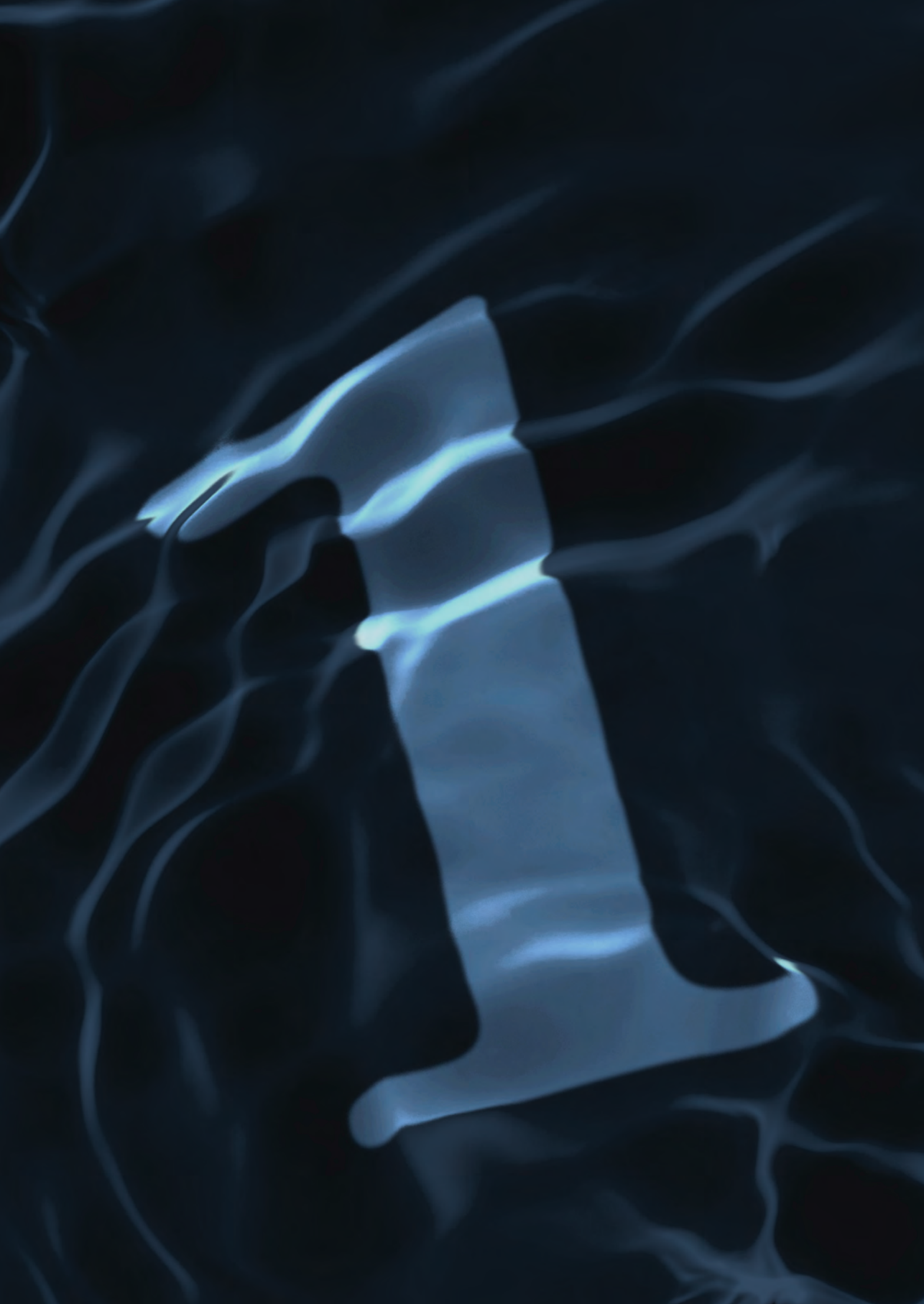

CHAPTER 1

General introduction and thesis outline

1 Marine microbiology and biogeochemical cycles

Marine ecosystems range from mangrove forests and coastal estuaries to the open oceans. Together they cover the majority, about 70%, of the Earth's surface. Tremendous numbers of microbes can be found in marine ecosystems. For instance, a liter of seawater from the ocean surface commonly contains a billion (10^9) microbial cells. Sediments commonly contain even higher cell densities. A single grain of sand from the North Sea can host around 100,000 cells (Probandt et al., 2018).

Marine microbes are diverse and have various roles within their ecosystem, and numerous ways of obtaining energy for growth. In surface waters, photosynthetic phytoplankton capture energy from light and use it to convert inorganic carbon (CO_2, HCO_3^-) and nutrients to biomass. Phytoplankton are by far the largest source of marine primary production, surpassing kelp, influx of terrestrial organic matter, and chemosynthesis at hydrothermal vents. Marine phytoplankton account for half of the global net primary production, and produce half of the oxygen in the Earth's atmosphere (Field et al., 1998).

Many other marine microorganisms rely on organic carbon for energy and biomass. Organisms with such a metabolism are commonly referred to as heterotrophs, but are more accurately described as organoheterotrophs. Organoheterotrophic microbes convert organic matter back to inorganic carbon and nutrients, a process known as mineralization. However, they do not do a perfect job. On geological timescales, around 0.5% of the marine primary production is buried in the seafloor instead of mineralized, making marine sediments the Earth's biggest carbon sink (Burdige, 2007). In general, marine microbes are vital to consider in the context of global biogeochemistry and climate change (Cavicchioli et al., 2019). From this perspective, central questions in marine microbiology and in this thesis are:

- Which microbes are present?
- What is their energy metabolism?
- What is their biogeochemical role and impact?

the carbon cycle was central in the aforementioned examples of microbial groups. However, microbes also drive other elemental cycles, such as the cycling of nitrogen, phosphorus, iron or sulfur. These elements are essential constituents of amino acids, nucleic acids and other biomolecules, and are thus essential to living organisms. Elemental cycling is more complex than just the interconversion between inorganic nutrient and constituent of organic biomolecules. Nitrogen, sulfur and carbon occur

naturally in various redox states, with an eight-electron difference between their most oxidized forms (nitrate, sulfate and CO_2/HCO_3^-, respectively) and reduced forms (ammonium, sulfide and methane, respectively). In the absence of oxygen, dissimilatory redox reactions between these boundaries by microbes form the basis for elemental cycling. Oxygen-depleted habitats abound the marine environment, in the form of anoxic sediments, or as expansive "oxygen-minimum zones" in the water column (Wright et al., 2012). Elemental cycles tend to occur sequentially over sediment or water column depth, which is directly related to the associated redox potentials (Figure 1). This thesis focuses on microbes involved in different aspects of the marine sulfur cycle, including mineralization of sulfur-containing organic matter as well as redox reactions.

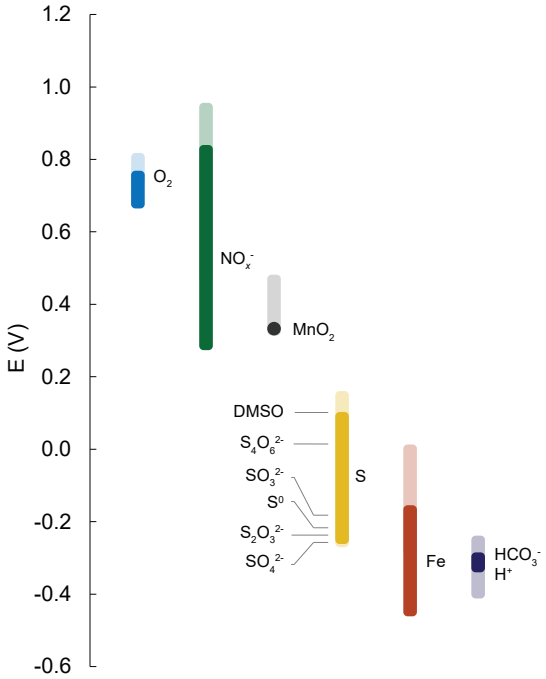

Figure 1. A redox tower comprising redox potentials of electron acceptors from different elemental cycles relevant in the marine environment. Opaque colors indicate conditions representative of the marine environment (T = 8°C, pH = 8, P[O2] between 0.21 atm and 0.1 Pa, P[N2] = 0.8 atm, P[H2] = 0.1 Pa, P[CH4] = 10 Pa, a[S0] = 1, C[SO42-] = 20 mM, all other concentrations 1 mM). Transparent colors indicate standard biological conditions (T = 25°C, pH = 7, reactants present at 1 M or 1 atm). For the nitrogen cycle, I took into account denitrification and ammonification of nitrate or nitrite, as well as reduction of nitrate to nitrite. Sulfur electron acceptor taken into account are indicated in the figure and are all reduced to sulfide, except for tetrathionate reduced to thiosulfate and dimethylsulfoxide (DMSO) reduced to dimethylsulfide (DMS). As ferric iron is usually only present in insoluble mineral form, only Fe(OH)3 and α-FeOOH (goethite) were considered. Bicarbonate was included as electron acceptor for both methanogenesis and homoacetogenesis.

Although the topic of this thesis is the marine sulfur cycle, it is necessary to first give a short overview of its well-studied sibling, the marine nitrogen cycle, as these two cycles are interconnected. Within the marine nitrogen cycle, ammonia-oxidizing *Thaumarchaeota* and nitrite-oxidizing *Nitrospinae* with a chemolithoautotrophic metabolism drive the aerobic oxidation of ammonium to nitrate (nitrification; Bristow et al., 2016; Sun et al., 2019). Nitrate is reduced again to ammonium by phytoplankton for assimilation into proteins and other biomolecules. However, in oxygen-depleted environments, nitrate can also serve as electron acceptor for various nitrate-reducing denitrifying or ammonifying microbes. These microbes can derive energy from various sources including organic carbon, H_2 or sulfur compounds (Kraft et al., 2014). Both ammonium oxidation and nitrate reduction produce nitrite. Nitrite thus forms an intermediate in nitrification, denitrification as well as ammonification. It is the electron acceptor for anaerobic ammonium oxidation (anammox). In marine environments, anammox is driven by chemolithoautotrophic anammox bacteria of the *Scalindua* genus (Woebken et al., 2008). Both denitrification and anammox yield dinitrogen gas (N_2) as end product, leading to a loss of bioavailable 'fixed' nitrogen from the marine environment. Since nitrogen – together with phosphate and/or iron – is limiting for photosynthetic primary production in the open oceans, nitrogen loss and fixation have been the incentive and focus for many microbiological marine studies. Such studies have concluded sulfur-cycling microbes to have an important role in nitrogen loss (Canfield et al., 2010), and possibly also in nitrogen fixation (Bonnet et al., 2013; Loescher et al., 2014).

2 Sulfur-cycling marine microbes

Sulfur is essential for life, since it is an important constituent of amino acids and metabolic cofactors. Sulfur plays a major role in our perception of marine environments, as the characteristic smell of the sea is caused by the volatile sulfur compound dimethylsulfide (DMS; Stefels, 1997). DMS is derived from the osmolyte dimethylsulfoniopropionate produced in large quantities by phytoplankton. This smelly compound is the main natural source of atmospheric sulfur aerosols (Lovelock et al., 1972). In the atmosphere, DMS promotes cloud condensation, raising the Earth's albedo. Marine phytoplankton could thus counteract climate change (Charlson et al., 1987). This peculiar example is only one of the ways in which the marine sulfur cycle is connected with global biogeochemistry and the conditions on the surface of our planet.

2.1 Sulfate-reducing microorganisms

In the most simplified form, the marine sulfur cycle can be viewed as the interconversion of sulfate and sulfide (Figure 2). Sulfate is present at 28 mM in the oceans, making it the second-most abundant anion after chloride (Canfield and Farquhar, 2009). Moreover, marine sulfate constitutes the largest pool of dissolved sulfur on the planet, with an oxidation capacity of an order of magnitude larger than that of atmospheric oxygen (Hayes and Waldbauer, 2006). Organisms reduce sulfate either for assimilatory or dissimilatory purposes.

Dissimilatory sulfate reduction to sulfide is mediated exclusively by sulfate-reducing microorganisms (SRM). All known SRM reduce sulfate through the same pathway in which dissimilatory (bi)sulfite reductase (Dsr) is central (Rabus et al., 2015). Dsr genes are therefore used to detect and quantify SRM in microbial ecology. SRM are found in various bacterial and some archaeal lineages, most notably the bacterial lineages *Deltaproteobacteria*[1], *Firmicutes* and *Nitrospirae* (Müller et al., 2015; Chernyh et al., 2020). The typical substrates of marine SRM are the fermentation products of other microbes, such as ethanol, lactate, acetate or hydrogen gas (Muyzer and Stams, 2008). Dissimilatory sulfate reduction is the dominant route for the mineralization of organic matter in shallow marine sediments with high organic matter deposition rates (Jørgensen, 1982; Canfield et al., 1993a), and accounts for 12-29% of the total organic carbon mineralization in marine sediments (Bowles et al., 2014). SRM thus play an important role in the marine sulfur cycle as well as the carbon cycle.

1 The class *Deltaproteobacteria* was recently reclassified into several novel phyla, and is effectively obsolete. The sulfate-reducing members of *Deltaproteobacteria* as well as the obsolete phylum *Thermodesulfobacteria* are now part of the novel phylum *Desulfobacterota* Waite, D.W., Chuvochina, M., Pelikan, C., Parks, D.H., Yilmaz, P., Wagner, M., Loy, A., Naganuma, T., Nakai, R., Whitman, W.B., Hahn, M.W., Kuever, J., and Hugenholtz, P. (2020). Proposal to reclassify the proteobacterial classes *Deltaproteobacteria* and *Oligoflexia*, and the phylum *Thermodesulfobacteria* into four phyla reflecting major functional capabilities. *Int J Syst Evol Microbiol* 70(11), 5972-6016. doi: 10.1099/ijsem.0.004213..

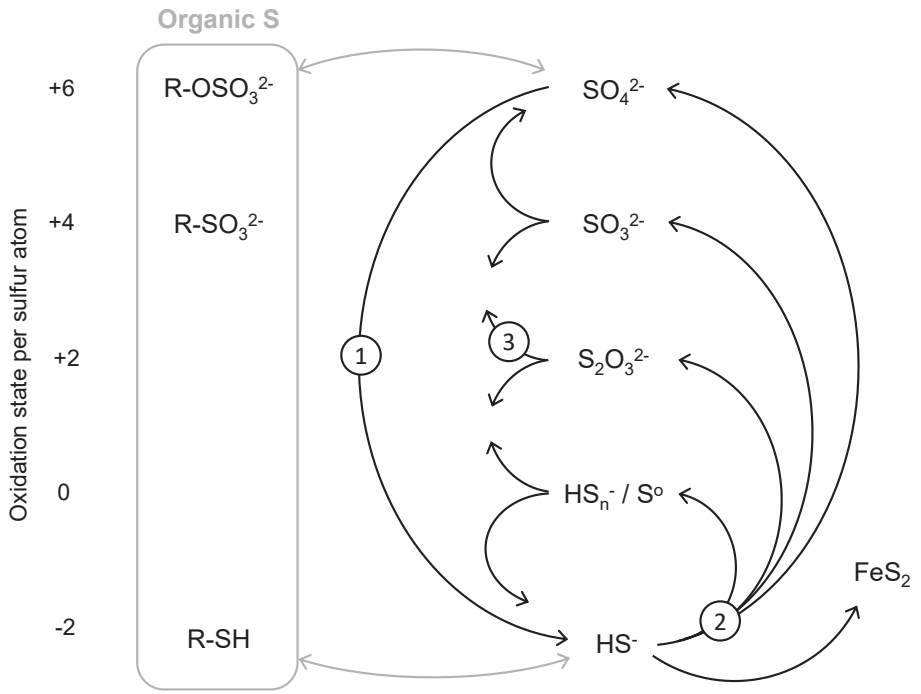

Figure 2. Overview of the marine sulfur cycle. Indicated with numbers are 1) dissimilatory sulfate reduction, 2) sulfide oxidation, and 3) reduction, oxidation or disproportionation of sulfur cycle intermediates.

2.2 Sulfur-oxidizing microorganisms

Most sulfide produced in sediments and water columns is re-oxidized to sulfate (Jørgensen et al., 1991). Oxidation of sulfide can be catalyzed by sulfur-oxidizing microorganisms (SOM) in the presence of oxygen, nitrate, MnO_2, or light, but also abiotically by oxygen or metal oxides (Canfield et al., 2005). While sulfide oxidation can produce sulfate, it can also yield sulfur cycle intermediates such as elemental sulfur, polysulfides, thiosulfate and sulfite. These intermediates can continue oxidation to sulfate, or they can be microbially reduced to sulfide. Moreover, they can function simultaneously as electron donor and acceptor for microbes in a process termed 'disproportionation' (reviewed by Finster, 2008, and by Slobodkin and Slobodkina, 2019).

Sulfide and sulfur cycle intermediates are energy-rich electron donors. This energy is the basis for some spectacular marine life-forms. At hydrothermal vents on the seafloor, bright-red *Riftia* tube worms rely completely on sulfur for energy through their endosymbiotic chemolithoautotrophic SOM. Temperate sulfidic habitats such as cold

seeps and sediments are also home to a diversity of symbiotic clams, worms, nematodes and protists (Dubilier et al., 2008). SOM themselves are sometimes also quite conspicuous. Phototrophic SOM are colored green or purple by their photopigments. Colorless sulfur-oxidizing *Beggiatoa, Thioploca* and *Thiomargarita* bacteria form thick white mats on the seafloor, and can reach large cell sizes with a diameter of up to 1 mm mostly thanks to big nitrate-filled vacuoles (Fossing et al., 1995; Schulz et al., 1999; Jørgensen and Boetius, 2007). Furthermore, in the last decade so-called cable bacteria were discovered, which form centimeter-long, multicellular, conductive 'cables' in marine sediments. These conductive cables span redox zones to connect sulfide oxidation to aerobic respiration through an electrical current (Meysman, 2018). However, ordinary unicellular SOM are probably more relevant from a global biogeochemical perspective. Chemolithoautotrophic SOM are thought to be responsible for the bulk of inorganic carbon fixation in oxygen minimum zones (0.4–5 Pg carbon y^{-1}) and marine sediments (<0.4 Pg carbon y^{-1}), which adds up to an estimated 2-11% of marine primary production by phytoplankton (Middelburg, 2011; Hawley et al., 2014). These SOM mostly belong to various lineages in the class *Gammaproteobacteria* (Glaubitz et al., 2013; Dyksma et al., 2016). In oxygen minimum zones, SOM are dominated by the gammaproteobacterial 'SUP05' clade within the genus *Thioglobus* (Lavik et al., 2009; Canfield et al., 2010).

2.3 Ancient Earth

Some sulfur leaves the sulfur cycle through burial in marine sediments, either as gypsum ($CaSO_4$), mineral sulfides such as pyrite (FeS_2; Neretin et al., 2004) or as sulfurized organic matter (Raven et al., 2016; Schmidt et al., 2017). On geological time scales these processes are important factors determining the atmospheric oxygen content (Berner et al., 2000). Furthermore, the isotopic signatures of buried pyrite are a window into the biogeochemistry of ancient Earth. Sulfur isotopes and microfossils in the geological record indicate SRM already existed in the early Archaean era, 3.5 billion years (Ga) ago (Shen et al., 2001; Ueno et al., 2008; Wacey et al., 2011). This is supported by the ancient origin of reductive DsrAB proteins, around the time of the evolutionary split between Bacteria and Archaea (Wagner et al., 1998; Müller et al., 2015). However, the very first DsrAB proteins probably had a role in sulfite reduction, from which dissimilatory sulfate reduction later developed (Chernyh et al., 2020). Other dissimilatory sulfur metabolisms such as elemental sulfur disproportionation (Philippot et al., 2007) and anoxygenic phototrophy (Brocks et al., 2005) are thought to be similarly ancient. Despite the onset of oxygen in the atmosphere at 2.4 Ga ago, the deep ocean remained anoxic until 0.57 Ga ago (Canfield and Farquhar, 2012). Thus, sulfur-cycling marine microbes for most of their existence enjoyed an even larger habitat than they currently do.

3 Sulfur cycling in the Black Sea

The Black Sea is the world's largest anoxic basin. It has a surface area of $4.2 \cdot 10^5$ km^2 and a volume of $5.3 \cdot 10^5$ km^3 (Özsoy and Ünlüata, 1997). It is a semi-enclosed sea with no direct connection to the oceans. In the southwest, the narrow and shallow Bosporus strait forms a connection to the Sea of Marmara and the Mediterranean Sea (Figure 3A). The Black Sea has a large positive water balance, receiving about 400 km^3 y^{-1} more freshwater through rivers and precipitation than it loses through evaporation (Ludwig et al., 2009). Besides freshwater, rivers have also transported large amounts of nutrients from sources such as agricultural runoff into the Black Sea during the last decades, causing eutrophication and persistent ecological damage (Mee, 1992; Mee et al., 2005; Oguz and Velikova, 2010). Due to the large input of freshwater and limited water exchange with the Mediterranean Sea, the salinity of the Black Sea is relatively low ranging from 18‰ at the surface to 22‰ at the bottom (Spencer and Brewer, 1971; Murray et al., 1991), versus 35‰ in the open ocean. Most of its seafloor is a flat abyssal plain with depths of 2,000 to over 2,200 m (Figure 3A). The upper sediment layers are organic-rich sapropel and coccolith ooze and support high sulfate reduction rates (<38 nmol cm^{-3} d^{-1}; Holmkvist et al., 2011).

3.1 Anoxia and stratification

The anoxia in the Black Sea is caused by vertical stratification, or layering, of the water column, restricting mixing and oxygen transport to deep waters. This is also a common phenomenon in lakes. The stratification in the Black Sea is based on a steep difference in salinity and thus in density between the less saline surface water layer, which receives freshwater, and the more saline deeper waters, which receives saline water from the Mediterranean Sea. Unlike in most lakes, in the Black Sea the majority of the anoxic waters are also sulfidic (Figure 3B) due to the abundance of sulfate and the activity of SRM. Anoxic, sulfidic conditions are also referred to as euxinic conditions, after "Pontos Euxeinos" (Πόντος Εὔξεινος), the ancient Greek name for the Black Sea.

Just decades ago, oxygen and sulfide overlapped in the Black Sea water column (Spencer and Brewer, 1971; Gagosian and Heinzer, 1979; Fashchuk and Ayzatullin, 1986; Vinogradov et al., 1986). However, the depth of oxygen depletion has unexpectedly shoaled whereas the depth of the onset of sulfide has not (Murray and Izdar, 1989; Murray et al., 1989). This phenomenon has created a 'suboxic' zone in between the oxic and euxinic waters. Here, a redox sequence or 'redoxcline' of nitrogen, manganese and sulfur occurs, which can be inferred from chemical profiles (Figure 3C). Due to continuing atmospheric warming and eutrophication, shoaling of the redoxcline is expected to continue (Capet et al., 2016). The observed shoaling is possibly not a new phenomenon, since sedimentary biomarkers of anoxygenic phototrophs suggest that

euxinia has been at current depths for substantial periods during the last 6000 years (Sinninghe Damste et al., 1993).

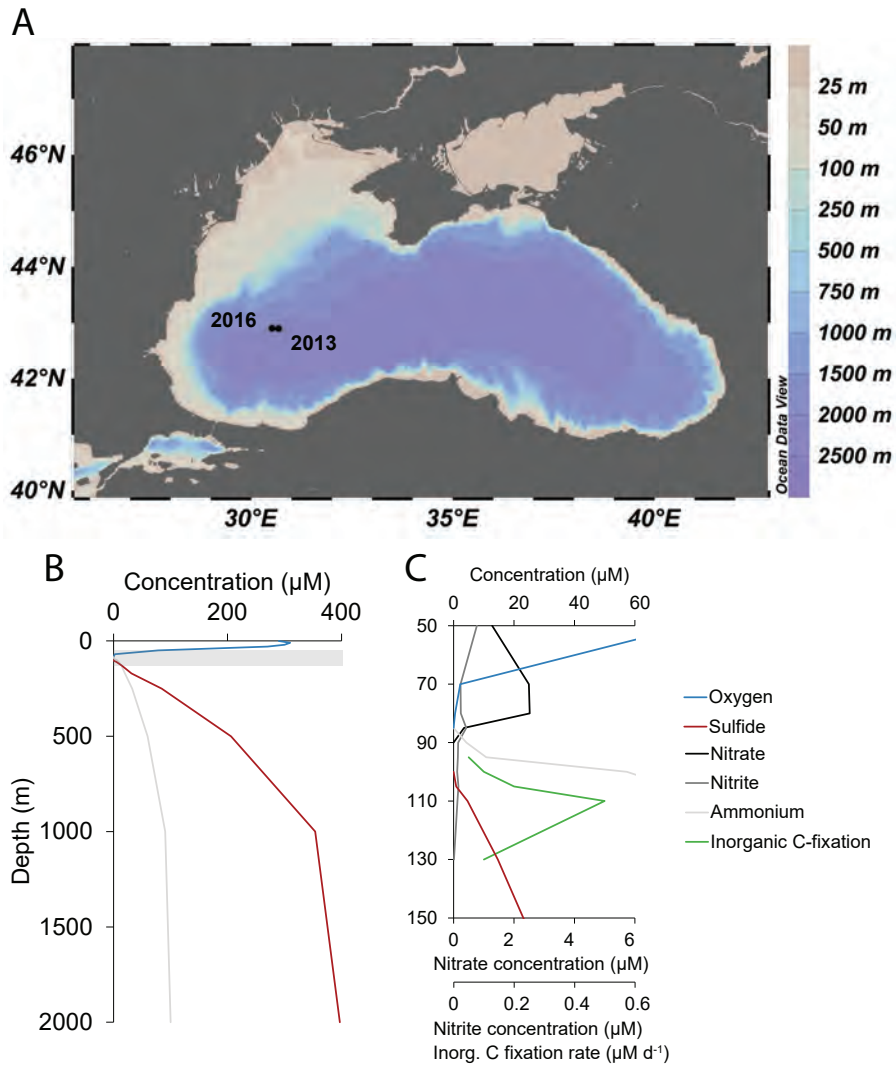

Figure 3. Sampling locations in the Black Sea and representative biogeochemical water column profile. (*A*) Bathymetric map of the Black Sea marking station 2 sampled during the 2013 PHOXY and the 2016 64PE408 research cruises aboard R/V Pelagia. The map was constructed in ODV 5.3.0 (Schlitzer, Reiner, Ocean Data View, odv.awi.de, 2020). (*B*) Chemical profile of the water column showing data from the 2013 cruise (Sollai et al., 2019). (*C*) Chemical profile in the redoxcline, shaded gray in *B*. The inorganic carbon fixation rate profile gives an impression of observations by Grote et al. (2008) and Jørgensen et al. (1991) transposed to match their vertical location relative to the sulfide profile observed in 2013.

3.2 The redoxcline

The Black Sea redoxcline is a model environment for gaining insight into the intricate redox-based linking of elemental cycles by marine microorganisms. Redoxclines also occur in other enclosed marine systems, but fluctuate with seasons (Canfield et al., 2005), and in coastal marine sediments on a millimeter scale (Froelich et al., 1979). In contrast, the Black Sea redoxcline is stable and stretches out over tens of meters (Murray et al., 1995), and hosts a conveniently stratified microbial community (Fuchsman et al., 2011). The redoxcline has a rich history of microbiological research, including the discovery of *Scalindua* anammox bacteria using cultivation-independent methods (Kuypers et al., 2003). This was a first and important step in the recognition of anammox as an important pathway for nitrogen loss in marine environments (Lam and Kuypers, 2011).

Central research questions are how sulfide is oxidized in the absence of oxygen and nitrate, and how microorganisms are involved. Rates of sulfide oxidation are high in the upper euxinic zone, and coincide with a peak in inorganic carbon fixation, which implies activity of autotrophic SOM (Figure 3C; Jørgensen et al., 1991). The anoxygenic green sulfur bacterium *Chlorobium phaeobacteroides* strain BS-1 was initially hypothesized to be the main SOM responsible (Repeta et al., 1989; Overmann et al., 1992). Later studies concluded their contribution to sulfide oxidation rates was minor, but did reveal this bacterium to be very adapted to low light intensity (Manske et al., 2005) and to have exceptionally low maintenance energy requirements (Marschall et al., 2010). Instead, a consensus arose that sulfide is mainly oxidized by MnO_2 and complexed Mn(III) (Luther III et al., 1991; Konovalov et al., 2003; Trouwborst et al., 2006), occurring through abiotic reactions yielding sulfur cycle intermediates such as S^0 and thiosulfate (Sorokin, 1972; Jørgensen et al., 1991; Luther III et al., 1991). However, this model still does not explain inorganic carbon fixation rates. Furthermore, the recent description of a manganese-reducing sulfide-oxidizing *Sulfurimonas* species from the Black Sea (Henkel et al., 2019) indicates this abiotic model should be reconsidered. Nevertheless, as further discussed in **Chapter 2**, advanced insight into marine SOM has been achieved through many cultivation-based and cultivation-independent studies in the Black Sea and similar marine systems such as the Baltic Sea and Cariaco Basin over the last decade.

4 Prior knowledge gaps

The study of marine sulfur-cycling microbes relies on a strong fundamental understanding of the dissimilatory pathways and physiologies associated with dissimilatory sulfur oxidation (Dahl, 2017; Götz et al., 2019) and dissimilatory sulfate reduction (Rabus et al., 2015; Santos et al., 2015). Cultivation-independent techniques such as marker gene-based amplicon sequencing have been used extensively to map the diversity of SRM in marine sediments (Wasmund et al., 2017). This confirmed the *in situ* abundance of known cultivated deltaproteobacterial SRM as well relatively unknown deltaproteobacterial sulfate-reducing lineages such as *Desulfatiglans* (Jochum et al., 2018) and Sva0081 (Dyksma et al., 2018). In water columns, the diversity of SRM has been less well studied (Vetriani et al., 2003; Canfield et al., 2010; Carolan et al., 2015; Suter et al., 2018). The total diversity of marine SRM may well extend beyond the canonical lineages such as *Deltaproteobacteria* (Müller et al., 2015; Anantharaman et al., 2018). Modern genome-resolved 'omics' methodologies have the potential to greatly advance our insight into the diversity of SRM *in situ*. The euxinic water column and sediments of the Black Sea house a community of anaerobic marine microorganisms which have crucial roles in sulfate reduction (Albert et al., 1995; Holmkvist et al., 2011) and the degradation of marine organic matter (Suominen et al., 2019). However, little is known about most of the anaerobic microbes detected in the euxinic column water and sediment through cultivation-independent methods (Vetriani et al., 2003; Suominen et al., 2019). Although the functional genes of SRM have been detected (Leloup et al., 2007; Neretin et al., 2007; Blazejak and Schippers, 2011), the current insight into SRM identity and diversity in the Black Sea is limited (Vetriani et al., 2003; Leloup et al., 2007; Blazejak and Schippers, 2011), and there are no reports of cultivated SRM. The fundamental microbiological value of studying the poorly explored anaerobes living in the Black Sea is illustrated by a recent cultivation-independent study which provides evidence that one of the dominant microbial lineages there, *Cloacimonetes* bacteria, produce both bacterial and archaeal membrane lipids (Villanueva et al., 2021).

Anaerobic marine conversions of organic sulfur compounds constitute a large microbiological knowledge gap. Sulfate groups are a common 'decoration' of marine polysaccharides (Helbert, 2017). For instance, sulfated polysaccharides such as carrageenan and fucoidan are abundantly produced by macroalgae, also known as kelp or seaweed. Various aerobic marine *Planctomycetes* bacteria are specialized to use these compounds as energy and carbon source (Wegner et al., 2013), a property which is thought to be important in their association with macroalgae (Bondoso et al., 2017). However, there is scarce information on microorganisms that degrade sulfated organic compounds in anoxic marine environments. Microbiological exploration of this functional niche may provide another "puzzle piece" to understand the greater question of how marine polysaccharides are mineralized, and why some are buried instead of mineralized (Arnosti et al., 2021).

5 Thesis outline

With this PhD thesis, I have aimed to advance what is known about the microorganisms involved in the marine sulfur cycle, with the Black Sea as model environment. The research presented here addresses some of the aforementioned knowledge gaps through an explorative, descriptive approach, combining cultivation-dependent and -independent methods. In **Chapter 2**, the literature on sulfur-cycling bacteria in oxygen-deficient marine water columns is critically reviewed. Additionally, conclusions from a metagenome analysis of the Black Sea water column are presented. This metagenome yielded several metagenome-assembled genomes (MAGs) of hitherto unknown lineages of putative SRM. **Chapter 3** further explores SRM and sulfur-reducing or -disproportionating microbes in the Black Sea through a cultivation-based approach with sediment as inoculum. Although different bacteria were enriched than were detected in the water column metagenome, a novel *Desulfopila* species was isolated with the unusual capacity for dissimilatory manganese reduction.

In **Chapter 4**, a similar cultivation approach is reported, here targeting anaerobic marine microorganisms that degrade sulfated polysaccharides. This resulted in the isolation of two novel fucoidan-degrading *Kiritimatiellales* strains belonging to the same superphylum as the aforementioned *Planctomycetes* bacteria. These bacteria encoded remarkably high numbers of sulfatases genes in their genomes, presumably serving to cleave off sulfate groups from polysaccharides. In **Chapter 5**, we propose the names *Pontiella desulfatans* and *Pontiella sulfatireligans* and the novel family *Pontiellaceae* based on phenotypic and phylogenetic characterization. Furthermore, this chapter includes an analysis of the extracellular polymeric substances of these bacteria, which revealed production of sulfated glycosaminoglycan-like exopolymers, not reported before for bacteria in pure culture. In **Chapter 6**, the enzymatic mechanism of fucoidan degradation by *Pontiella desulfatans* F1^{T} is investigated through gene expression analysis with transcriptomics. This revealed organization of some of the many genes encoding sulfatases and other polysaccharide-depolymerizing enzymes into expressed 'polysaccharide utilization loci' originally known from polysaccharide-degrading *Bacteroidetes* bacteria. Furthermore, a novel pathway for degradation of the fucoidan backbone monosaccharide L-fucose is hypothesized.

Chapter 7 is a general discussion of the research presented in this thesis. I place the research in context of recent developments in the field of environmental microbiology, critically evaluate the presented research, and give recommendations for future research. Lastly, I discuss the relevance of the presented research on *Pontiella* spp. for potential future applications.

CHAPTER 2

The bacterial sulfur cycle in expanding dysoxic and euxinic marine waters

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Abstract

Dysoxic marine waters (DMW, < 1 μM oxygen) are currently expanding in volume in the oceans, which has biogeochemical, ecological and societal consequences on a global scale. In these environments, distinct bacteria drive an active sulfur cycle, which has only recently been recognized for open-ocean DMW. This review summarizes the current knowledge on these sulfur-cycling bacteria. Critical bottlenecks and questions for future research are specifically addressed. Sulfate-reducing bacteria (SRB) are core members of DMW. However, their roles are not entirely clear, and they remain largely uncultured. We found support for their remarkable diversity and taxonomic novelty by mining metagenome-assembled genomes from the Black Sea as model ecosystem. We highlight recent insights into the metabolism of key sulfur-oxidizing SUP05 and *Sulfurimonas* bacteria, and discuss the probable involvement of uncultivated SAR324 and BS-GSO2 bacteria in sulfur oxidation. Uncultivated *Marinimicrobia* bacteria with a presumed organoheterotrophic metabolism are abundant in DMW. Like SRB, they may use specific molybdoenzymes to conserve energy from the oxidation, reduction or disproportionation of sulfur cycle intermediates such as S^0 and thiosulfate, produced from the oxidation of sulfide. We expect that tailored sampling methods and a renewed focus on cultivation will yield deeper insight into sulfur-cycling bacteria in DMW.

1 Introduction

Oxygen deficiency is a rather common phenomenon in marine waters caused by microbial aerobic respiration coupled to the degradation of organic matter, combined with insufficient supply of oxygen through water circulation or diffusion (Canfield et al., 2005). Oxygen-minimum zones (OMZs) are waters in the open ocean containing <20 μM oxygen occurring between 100 and 1,500 m depth. The largest OMZs are found in the Eastern Tropical North Pacific (ETNP), the Eastern Tropical South Pacific (ETSP) and the Arabian Sea (Figure 1, Table S1). Together, OMZs amount to 10 million km^3 or approximately 1% of the ocean's volume (Paulmier and Ruiz-Pino, 2009). When there is sufficient input of sinking phytoplankton biomass, oxygen concentrations in OMZs can drop to below the common detection level of 1 μM (Ulloa et al., 2012). Such conditions are often interpreted as anoxic. However, using a highly sensitive STOX oxygen sensor, Revsbech and colleagues (2009) and Thamdrup and colleagues (2012) showed that there still may be traces of oxygen (<50 nM) in supposedly anoxic OMZ waters, which will here be termed 'OMZ core'. Yet, these sensors have not yet been widely applied in marine sampling campaigns. Coastal waters can similarly experience oxygen deficiency and anoxia, for example in the Namibian Upwelling, Chesapeake Bay and the Pacific South-American coastal waters (Figure 1, Table S1).

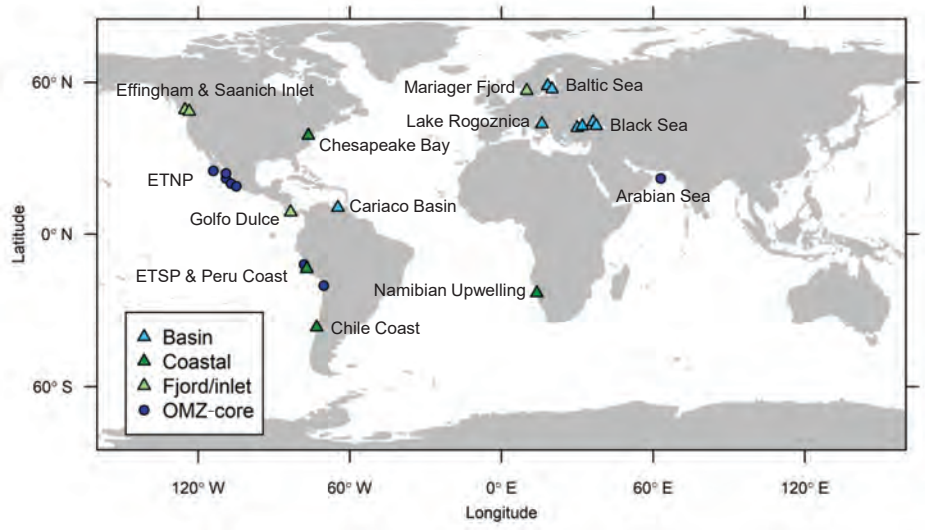


Figure 1. Dysoxic marine waters studied with respect to microorganisms driving the sulfur cycle. Triangles indicate locations that are permanently, seasonally or incidentally euxinic. Abbreviations: ETNP, Eastern Tropical North Pacific; ETSP, Eastern Tropical South Pacific; OMZ, oxygen-minimum zone. For a list of studies per location, see Table S1.

In enclosed marine basins and fjords, as well as in coastal waters, stratification is a common factor in the development and persistence of oxygen deficiency (Canfield et al., 2005). Stratification can even lead to euxinia (Meyer and Kump, 2008), here defined as anoxic conditions with >0.1 μM sulfide. Several euxinic marine basins, fjords and inlets have been studied with respect to their sulfur cycle and the associated microorganisms (Figure 1, Table S1). The development of euxinia in OMZs is prevented by a relatively high advection of oxygenated water compared to enclosed environments, and by a negative feedback loop centered around nitrogen loss. Denitrifying and anaerobic ammonia-oxidizing bacteria in the OMZ convert fixed forms of nitrogen such as ammonium and nitrate into N_2 at such high rates that OMZs are responsible for 30-50% of the total loss of fixed nitrogen from the ocean (Lam and Kuypers, 2011). This causes surface phytoplankton to be limited in nitrogen, which in turn limits the input of organic matter to the OMZs, preventing the depletion of nitrate and nitrite (Canfield, 2006; Boyle et al., 2013). Hence, the ensuing development of euxinia is halted, as denitrifying bacteria outcompete sulfate-reducing ones (Froelich et al., 1979; Chen et al., 2017b).

OMZ core waters, despite being dominated by nitrogen cycling, can also harbor an active sulfur cycle, which has long been overlooked due to the absence of detectable sulfide (Canfield et al., 2010; Johnston et al., 2014; Carolan et al., 2015). Similar conditions are found in some stratified environments where the oxic and euxinic zones are separated by a suboxic zone (Murray et al., 1989; Lavik et al., 2009; Hawley et al., 2014; Findlay et al., 2017), here defined to contain no detectable oxygen (<1 μM) or sulfide (<0.1 μM) using standard methods. The exact concentration of oxygen in suboxic zones is still unclear. However, the detection of sulfur-cycling microorganisms suggests an active sulfur cycle in suboxic zones, for instance in the Black Sea and Cariaco Basin (Neretin et al., 2007; Rodriguez-Mora et al., 2016). For the purpose of this review, we use the term 'dysoxic marine water' (DMW, <1 μM of oxygen) to describe all marine suboxic zones, OMZ core waters, anoxic waters and euxinic waters (Box 1).

Box 1. Glossary of definitions for marine oxygen-deficient environments.

Environmental terminology	**definition**
Dysoxic water	<1 μM oxygen
Anoxic water	Not containing oxygen
Euxinic water	Anoxic, >0.1 μM sulfide
Oxygen-minimum zone (OMZ)	Open ocean water with <20 μM oxygen
OMZ core, also known as 'anoxic marine zone'	Open ocean water with <50 nM oxygen
Suboxic zone	<1 μM oxygen, <0.1 μM sulfide, in between oxic and euxinic zones of stratified waters

Over the last 60 years, DMW has expanded in volume more than fourfold (Schmidtko et al., 2017) because of oceanic warming – reducing oxygen solubility – and eutrophication [reviewed by Breitburg et al. (2018)]. This process is expected to continue. In addition, Ulloa and colleagues (2012) have predicted that the deposition of anthropogenically fixed nitrogen will cause OMZ cores to develop euxinia, since it counteracts the nitrogen-loss-based negative feedback loop. Potential long-term, global consequences of expanding marine dysoxia and euxinia include changes in availability of key nutrients (iron, phosphorus, etc.) and trace metals (cadmium, copper, zinc, etc.), and loss of fishery stocks, affecting coastal economies and food security (Breitburg et al., 2018). Furthermore, since DMW environments are biogeochemical hotspots for microbial production of the greenhouse gas nitrous oxide (Naqvi et al., 2010), their expansion provides a feedback loop that in turn contributes to global warming. In the geological past, the rise of euxinic conditions has led to several mass extinction events such as during the end-Permian (Meyer and Kump, 2008) and the mid-Cretaceous (Kamyshny et al., 2009).

The biogeochemical sulfur cycle in DMW consists of abiotic and biologically mediated reactions (Figure 2; Ehrlich et al., 2015), with the latter providing energy to many different microorganisms. Sulfate-reducing bacteria (SRB) reduce sulfate (SO_4^{2-}) to sulfide (HS^-), coupled to the oxidation of small organic compounds or H_2 (Muyzer and Stams, 2008). Most of this sulfide is re-oxidized by oxidized metals or sulfur-oxidizing bacteria (SOB), either completely to sulfate (Jørgensen et al., 1991) or to different sulfur cycle intermediates (SCIs) including elemental sulfur (S^0), polysulfides (HS_n^-), thiosulfate ($S_2O_3^{2-}$), tetrathionate ($S_4O_6^{2-}$) and sulfite (SO_3^{2-}; Zopfi et al., 2001; Kamyshny et al., 2011; Findlay, 2016). These SCIs can be used as electron donor or acceptor by various microorganisms including SRB and SOB (Rabus et al., 2013; Han and Perner, 2015; Dahl, 2017).

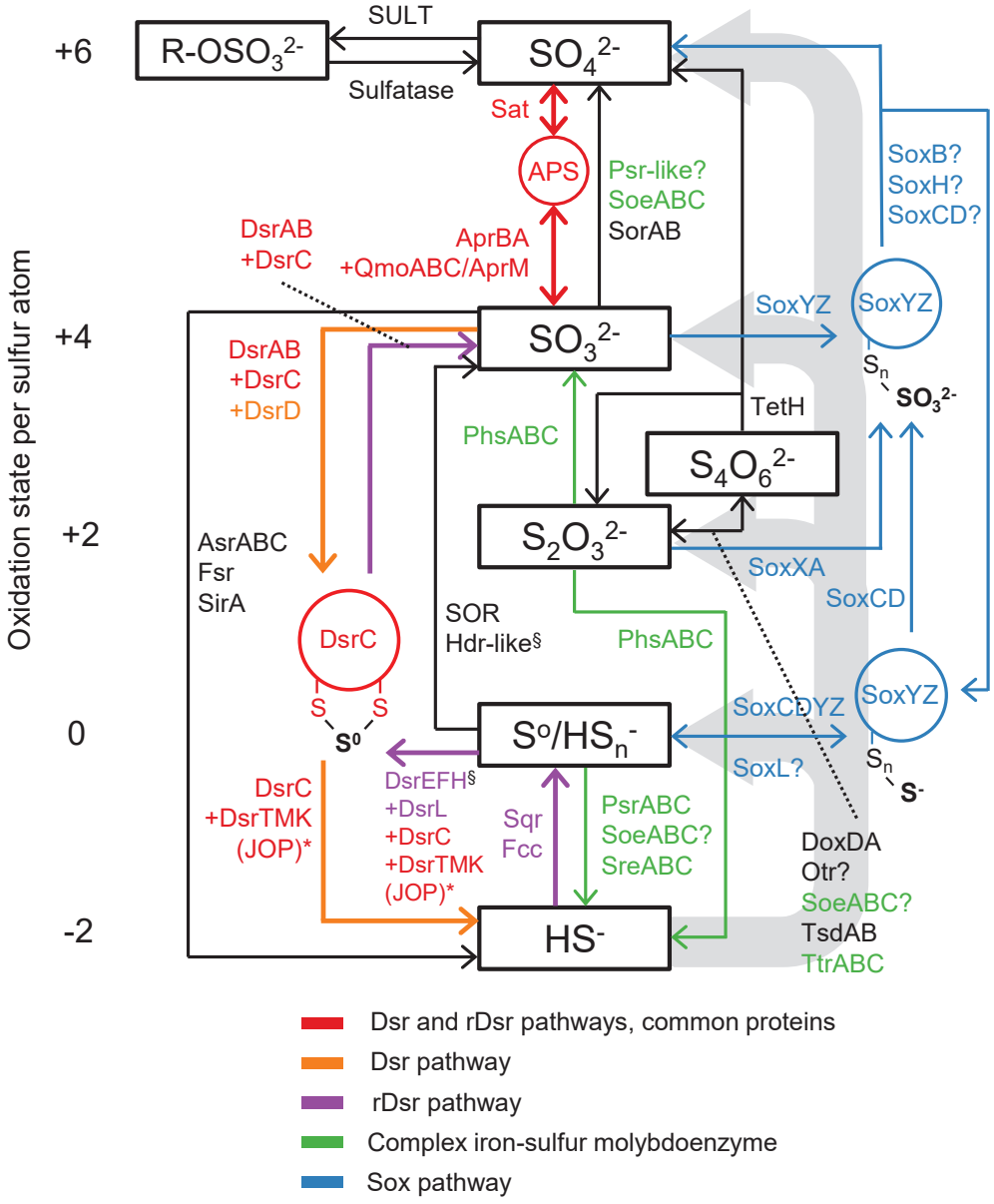


Figure 2. The dissimilatory conversions within the marine sulfur cycle. The oxidation state of the inorganic species is indicated at the left. Abiotic and assimilatory reactions are not indicated, except for the abiotic oxidation of sulfide which is illustrated by wide gray arrows. The S^0 in DsrC-trisulfide is considered zero-valent (Santos et al., 2015). The sulfur atom in APS has an oxidation state of +6, and those in tetrathionate have an oxidation state of +2.5. Key to abbreviations used: Sat, sulfate adenylyltransferase; Apr, APS reductase; Asr, anaerobic sulfite reductase; Dox, thiosulfate quinone oxidoreductase; Dsr, dissimilatory sulfite reductase; Fcc, flavocytochrome c sulfide dehydrogenase; Fsr, F420-dependent sulfite reductase; Hdr, heterodisulfide reductase; Otr, octoheme tetrathionate reductase; Phs, thiosulfate reductase; Psr,

polysulfide reductase; Qmo, quinone-interacting membrane-bound oxidoreductase; Sir, sulfite reductase; Soe, sulfite-oxidizing enzyme; SOR, sulfur oxygenase/reductase; Sor, sulfite-acceptor oxidoreductase; Sox, sulfur-oxidizing multienzyme complex; Sqr, sulfide-quinone oxidoreductase; Sre, sulfur reductase; SULT, sulfotransferase; Tet, tetrathionate hydrolase; Tsd, thiosulfate dehydrogenase; Ttr, tetrathionate reductase. Key to symbols used: ?) involvement is uncertain. *) DsrT is required for sulfide oxidation in green sulfur bacteria (Holkenbrink et al., 2011), but is also found in SRB. Protein complexes other than DsrTMK(JOP) can also transfer electrons to DsrC to enable this reaction (Venceslau et al., 2014). §) The rhodanese sulfurtransferases Rhd-TusA-DsrE2 are also essential in the reaction mediated by this complex (Dahl, 2017).

The detection and analysis of sulfur-cycling genes, transcripts and proteins in DMW yields a powerful perspective on the diversity and activity of sulfur-cycling microorganisms (Figure 2), and more so when applied to metagenome-assembled genomes (MAGs) or single-cell amplified genomes (SAGs). Various 'omics' studies have yielded insight into the dominant SOB in DMW (Lavik et al., 2009; Walsh et al., 2009; Callbeck et al., 2018; Plominsky et al., 2018). However, only few studies have addressed the broader diversity of sulfur-cycling microorganisms (Canfield et al., 2010; Stewart et al., 2012; Schunck et al., 2013; Hawley et al., 2014), without tapping into the larger potential of genome-centric metagenomics and the available metagenome data. To fill this knowledge gap, we screened MAGs from DMW environments for sulfur-cycling marker genes (S.I. Methods). Part of the MAGs was assembled from metagenomes of the Arabian Sea and ETSP OMZ cores produced by Tara Oceans (Parks et al., 2017; Tully et al., 2018). Other MAGs were assembled from metagenomes of 15 different water depths of the Black Sea (Suominen et al., 2019; Villanueva et al., 2021; S.I. Methods), which served as model for enclosed DMW environments.

Despite the central role of the sulfur cycle in DMW, current biogeochemical and microbiological knowledge has not been comprehensively reviewed so far. Therefore, we herein provide an overview of sulfur cycle processes in DMW, and we discuss the diversity, metabolism and physiology of the bacteria involved in these processes. In the following section, we will discuss current knowledge on SRB, who form an essential part of the sulfur cycle through the production of sulfide. The second section treats SOB, covering well-studied groups such as SUP05 and *Sulfurimonas,* less explored groups such as BS-GSO2, and putative sulfur oxidizers such as SAR324 members. The final section discusses which bacteria could be involved in sulfur reduction or disproportionation.

2 Sulfate-reducing bacteria

The presence and activity of SRB in the dysoxic water column has been demonstrated through sulfate reduction rate measurements with isotopically labelled sulfate ($^{35}SO_4^{2-}$) performed in euxinic settings such as the Black Sea with sulfate reduction rates up to 36 nmol L^{-1} d^{-1} (Sorokin, 1972; Jørgensen et al., 1991; Albert et al., 1995; Pimenov et al., 2000) and Mariager Fjord with rates up to 140 nmol L^{-1} d^{-1} (Sørensen and Canfield, 2004), but also in the ETSP OMZ core with rates up to 16.9 nmol L^{-1} d^{-1} (Canfield et al., 2010). More extensively conducted taxonomic marker studies point to a universal presence of SRB in DMW since the 16S rRNA genes of canonical SRB lineages of the class *Deltaproteobacteria* have been widely detected (Madrid et al., 2001; Vetriani et al., 2003; Lin et al., 2006; Fuchsman et al., 2011; Wright et al., 2012; Schunck et al., 2013; Ganesh et al., 2014; Rodriguez-Mora et al., 2015; Suter et al., 2018; Callbeck et al., 2019).

2.1 Presence and diversity

All known SRB reduce sulfate through the dissimilatory (bi)sulfite reductase (Dsr) pathway (Rabus et al., 2015). This consistency has facilitated functional marker investigations of the ecology of SRB. Such a functional marker approach is more appropriate than 16S rRNA gene surveys, as it does not require metabolic assumptions based on taxonomy. Although the core proteins of the Dsr pathway, Sat, AprBA and DsrAB, are also present in the reversed Dsr (rDsr) sulfur oxidation pathway (Dahl, 2017; Figure 2), their reductive and oxidative versions are phylogenetically distinguishable (Meyer and Kuever, 2007; Loy et al., 2009; Müller et al., 2015; Pelikan et al., 2016). Reductive Dsr genes have been identified in both the suboxic and euxinic waters of the Black Sea (Neretin et al., 2007) and the Cariaco Basin (Rodriguez-Mora et al., 2016), in sulfidic coastal waters off Peru (Schunck et al., 2013), in the core of the ETNP and ETSP OMZs (Canfield et al., 2010; Carolan et al., 2015), and in the Gdansk Deep within the Baltic Sea (Korneeva et al., 2015). Moreover, reductive Dsr genes were shown to be transcribed into mRNA in several of these environments (Stewart et al., 2012; Ulloa et al., 2012; Schunck et al., 2013; Rodriguez-Mora et al., 2016; Saunders et al., 2019), providing evidence for activity of SRB. Although powerful, the results of Dsr markers should be cautiously interpreted (Anantharaman et al., 2018), as the Dsr pathway does not only facilitate dissimilatory sulfate reduction but can also mediate dissimilatory reduction and disproportionation of SCIs (Rabus et al., 2015; Florentino et al., 2017), and in some rare cases sulfur oxidation (Sigalevich and Cohen, 2000; Slobodkina et al., 2017; Thorup et al., 2017). Thus, although bacteria with reductive Dsr pathways are core members of the microbial community of DMW, their sulfur metabolism is not necessarily restricted to dissimilatory sulfate reduction. We therefore refer to them as putative SRB.

Surveys based on 16S rRNA or functional marker genes and metagenomic studies of DMW have revealed a high diversity of putative SRB, most of which are only distantly related to described species. The deltaproteobacterial putative SRB detected in 16S rRNA gene datasets are rarely affiliated with established genera (Fuchsman et al., 2011; Wright et al., 2012; Ganesh et al., 2014; Rodriguez-Mora et al., 2015; Suter et al., 2018). Of all canonical SRB lineages, *Desulfobacteraceae* species are thought to be dominant due to the prevalence of their sequences in 16S rRNA datasets (Fuchsman et al., 2011; Wright et al., 2012; Rodriguez-Mora et al., 2015; Suter et al., 2018) and metagenomic datasets (Canfield et al., 2010; Schunck et al., 2013). However, *Desulfobulbaceae* species have also been detected, specifically including the 16S rRNA genes of the genera *Desulfocapsa* and *Desulforhopalus* (Neretin et al., 2007; Canfield et al., 2010; Fuchsman et al., 2011; Fuchsman et al., 2012; Rodriguez-Mora et al., 2015; Suter et al., 2018). Furthermore, bacteria related to the genus *Desulfatiglans* seem widespread in DMW since *Desulfatiglans*-related sequences were retrieved from the Black Sea (16S rRNA genes; Vetriani et al., 2003; Neretin et al., 2007), coastal DMW off Peru (metagenomics; Schunck et al., 2013), the Gdansk Deep in the Baltic Sea (*dsrB* fragments; Korneeva et al., 2015) and the Cariaco Basin (*dsrA* fragments; Rodriguez-Mora et al., 2016). Metagenomics applied to the ETSP OMZ core indicated an even larger diversity of putative SRB beyond the *Deltaproteobacteria,* including *Thermodesulfovibrio*-related bacteria in the ETSP OMZ core (Canfield et al., 2010) and diverse unknown putative SRB in euxinic basins (Korneeva et al., 2015; Rodriguez-Mora et al., 2016).

The *dsrD* gene, encoding a small protein with a possible regulatory function (Mizuno et al., 2003; Venceslau et al., 2014), is an alternative functional marker gene for detection of SRB (Mußmann et al., 2005; Rabus et al., 2015). It has been used to investigate bacteria with genomes lacking *dsrAB* genes with a clear oxidative/reductive affiliation (Anantharaman et al., 2018), since *dsrD* forms a reliable marker for the reductive Dsr pathway when present together with other Dsr genes (Rabus et al., 2015). We detected the *dsrD* gene – in the context of other *dsr* genes – in metagenomes and MAGs from the Black Sea throughout the euxinic and suboxic zones (Figure 3A-B), confirming previously reported distributions of putative SRB (Neretin et al., 2007). We could not obtain any assembled reductive *dsrA* genes from publicly available OMZ metagenomes (Canfield et al., 2010; Ganesh et al., 2014; Fuchsman et al., 2017; Tully et al., 2018; Saunders et al., 2019), likely due to sampling bias (see following subsection) and insufficient sequencing depth. However, many complete *dsrA* genes could be retrieved from the Black Sea metagenome (S.I. Methods). An analysis of these *dsrA* sequences supported the view emerging from previous studies: a large diversity of putative SRB, with a somewhat distant relationship to canonical SRB belonging to *Desulfobacula*, *Desulfococcus*, *Desulfocapsa, Desulfatiglans* and *Thermodesulfovibrio*, and to non-canonical lineages other than the *Deltaproteobacteria* or *Nitrospirae* (Figure 4). This view mirrors the overly large diversity of SRB that can generally be found in marine sediments (Muyzer and Stams, 2008; Müller et al., 2015). Despite the wealth of knowledge on

the metabolism of SRB, the ecophysiological causes behind this diversity are currently poorly understood.

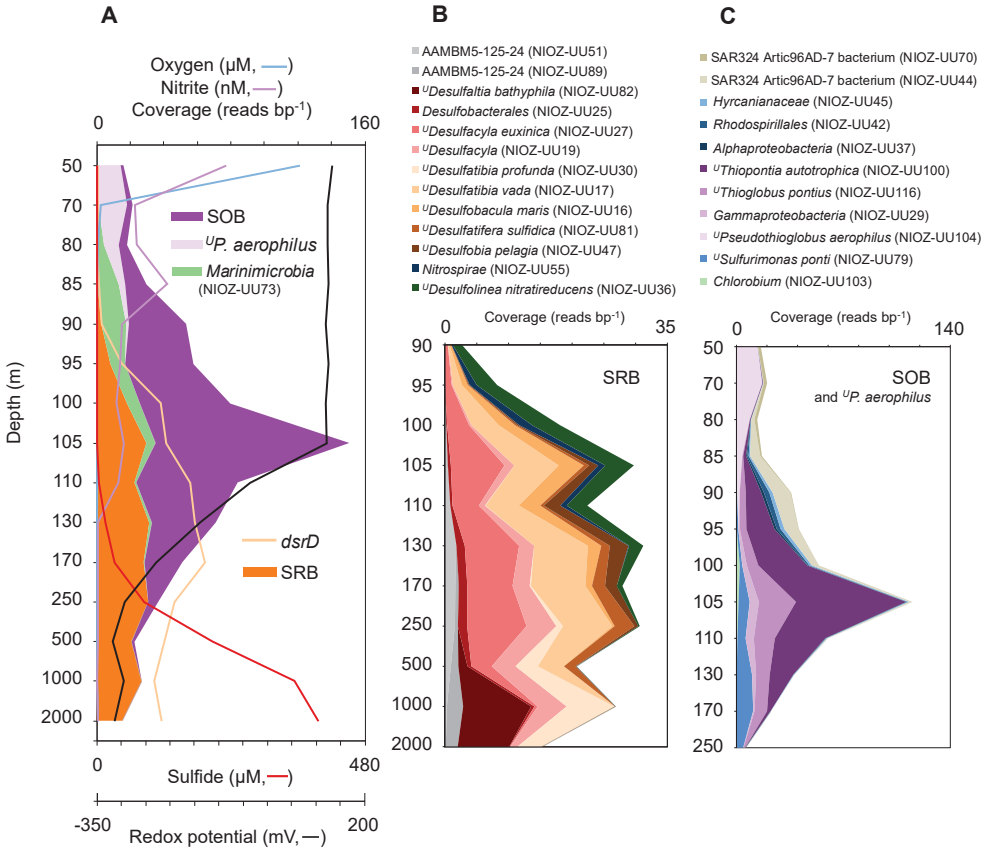


Figure 3. Black Sea water column distribution of metagenome-assembled genomes (MAGs) of sulfur-cycling bacteria based on their genetic capacity. A) Physicochemical measurements and normalized cumulative metagenome coverage of all MAGs of putative sulfur-oxidizing bacteria (SOB) combined, *UP. aerophilus* (NIOZ-UU104), *Marinimicrobia* (NIOZ-UU73), all *dsrD* genes combined and all MAGs of putative sulfate-reducing bacteria (SRB) combined in samples of 15 different depths of the Black Sea. The oxygen, nitrite and sulfide data correspond to the PHOXY cruise of June-July 2013 (Sollai et al., 2019). The Black Sea metagenome was also constructed from samples taken during this cruise as detailed in S.I. Methods and Villanueva et al. (2021). Redox potential was measured during the 64PE408 NESSC/SIAM cruise of January-February 2016 from samples with a closely agreeing sulfide profile. Relative abundances of MAGs of B) putative SRB and C) putative SOB and *UP. aerophilus* were based on normalized metagenome coverage. See Supporting Information Methods for details on the methodology and data processing.

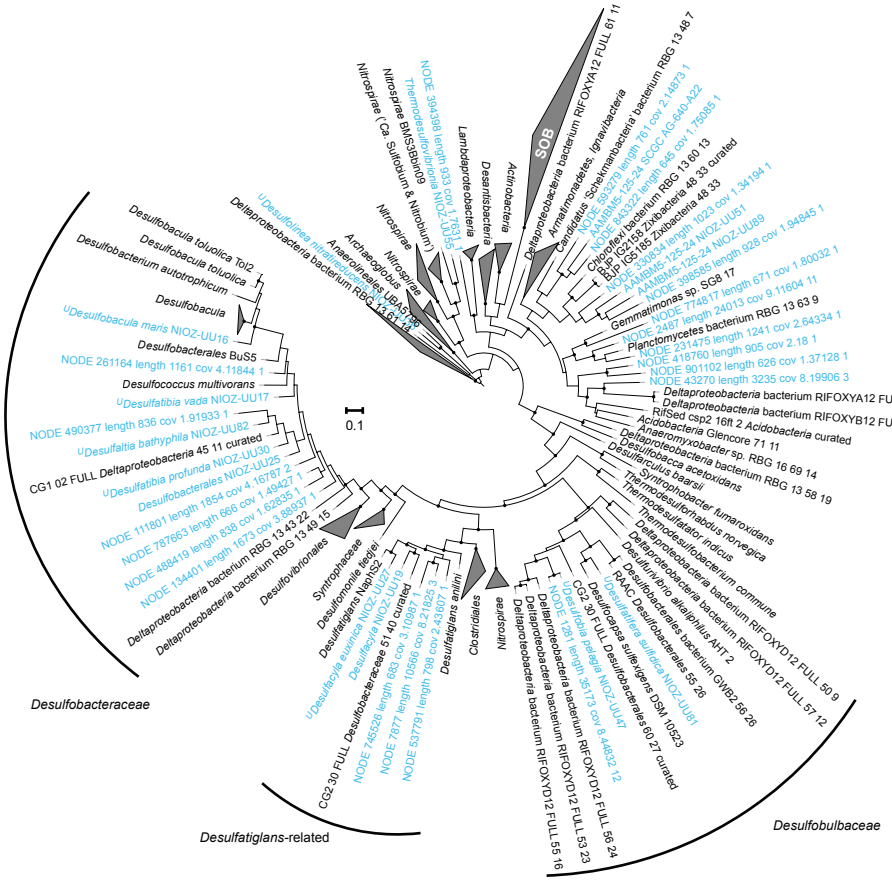


Figure 4. Maximum-likelihood phylogenetic reconstruction based on bacterial reductive DsrA proteins predicted from Black Sea MAGs and unbinned contigs (blue) and reference genomes (Anantharaman et al., 2018). Black dots indicate support of >95% out of 1,000 ultra-fast bootstraps. The scale bar indicates substitutions per site. See Supporting Information Methods for methodology and Data S1.

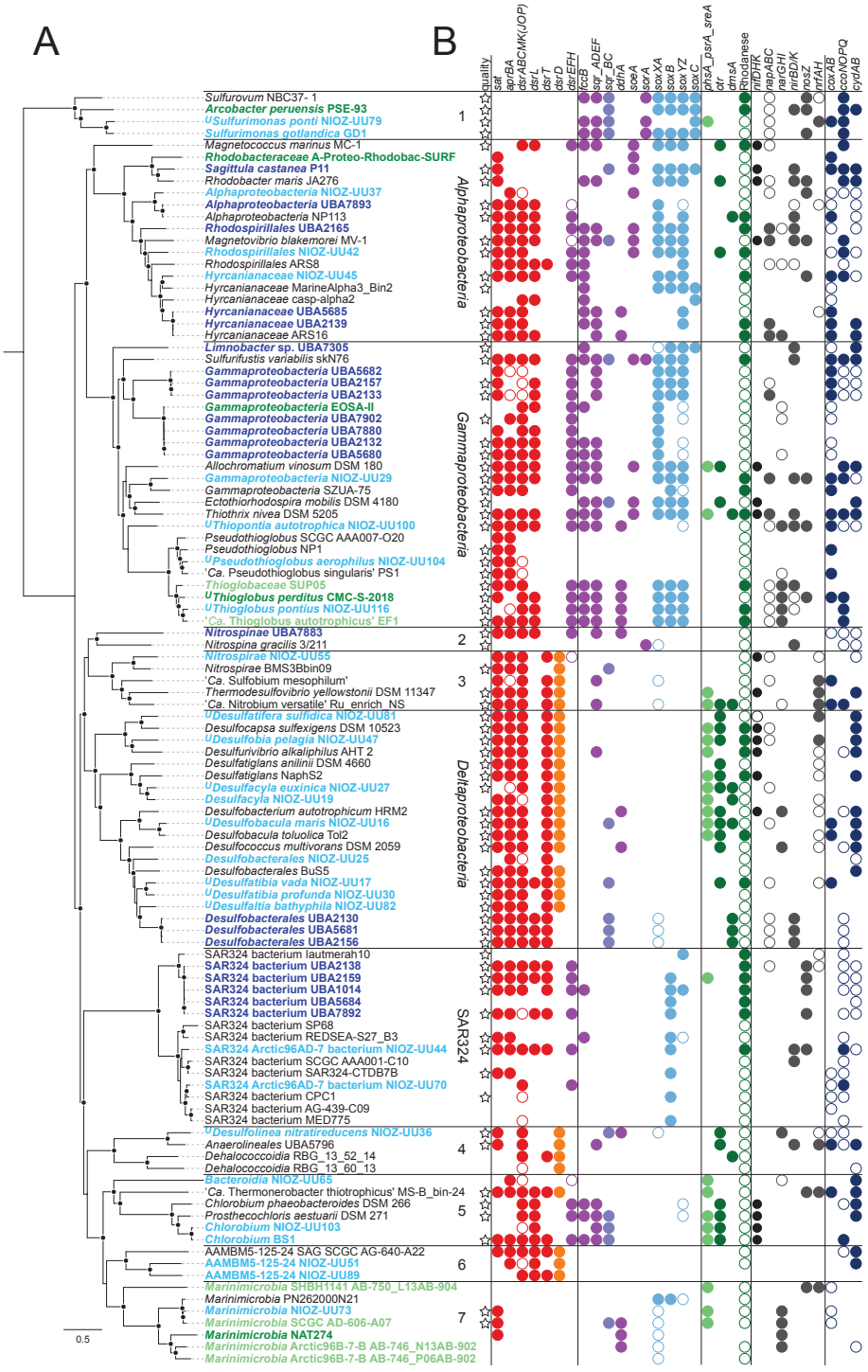
2.2 Physiology and metagenomics

We have a considerable understanding of the physiology of SRB from marine sediments, owing to a rich diversity of isolated SRB that are available for laboratory research (Muyzer and Stams, 2008; Rabus et al., 2015). In contrast, no SRB have been isolated from DMW, except for two subspecies of *Desulfovibrio oceani* from the ETSP OMZ (Finster and Kjeldsen, 2010). It can be reasonably assumed that most of the deltaproteobacterial putative SRB detected in DMW adhere to the general metabolism of dissimilatory reduction of sulfate and oxidation of small organic compounds or H_2. However, the lack of closely related described SRB does not allow further constraining of metabolic niches based on taxonomic affiliation, nor does it allow hypotheses on the many other variable physiological aspects.

These challenges can be addressed by genome-centric metagenomics, exemplified by recent explorations of the potential metabolism of *Desulfatiglans*-related SAGs from marine sediments (Jochum et al., 2018) and of the identity and potential metabolism of non-canonical putative SRB from various environments (Wasmund et al., 2016; Anantharaman et al., 2018; Hausmann et al., 2018; Thiel et al., 2018; Meier et al., 2019). With these aims, we mined metagenome data to obtain thirteen MAGs of putative SRB from the Black Sea, encoding complete or incomplete reductive Dsr pathways (Figures 3A-B and 5, Table S2, S.I. Methods). In agreement with previous diversity studies, most of the putative SRB MAGs were affiliated with *Desulfobacterales*, *Desulfobulbales* and *Nitrospirae* but did not classify within established genera, except for *^{U}Desulfobacula maris*. Four of the putative SRB MAGs from the Black Sea (52-93% complete, 1-3% contaminated) affiliated with the non-canonical phyla *Nitrospirae*, *Chloroflexi* and candidate phylum AAMBM5-125-24. Other novel putative SRB within the phylum *Nitrospirae*, '*Candidatus* Sulfobium mesophilum' (Zecchin et al., 2018) and '*Candidatus* Nitrobium versatile' (Arshad et al., 2017), were only distantly related to *Nitrospirae* MAG NIOZ-UU55 (<51% amino acid identity [AAI], Figure 5, Table S2). Another phylogenetically related SAG containing sulfate-reducing genes (AAMBM5-125-24) has been retrieved from the euxinic Zodletone Spring (Figure 5; Youssef et al., 2019). Despite the novelty revealed by this genome-centric approach, it should be noted that it did not encompass the complete diversity of putative SRB in the Black Sea detected according to the *dsrD* (Figure 3A) and *dsrA* diversity (Figure 4).

More so than taxonomic affiliation, functional gene annotation offers insight into the possible energy metabolism(s) of these microorganisms. Metagenome mining yielded three *Desulfobacterales* MAGs from the Arabian Sea OMZ core (95-96% complete, 0.7% contaminated) with a complete Dsr pathway but lacking the reductive marker *dsrD* (Figure 5, Table S2, S.I Methods). Moreover, they harbored oxidative instead of reductive *dsrA* genes possibly horizontally transferred from *Chlorobia* or SAR324 bacteria (Data S1). Together with the absence of *dsrD* and the presence of *sqr* (Figure 5), this suggests

a sulfur-oxidizing rather than sulfate-reducing metabolism. These MAGs thus question the common assumption that all *Desulfobacterales* reduce sulfate, and undermine taxonomy-based physiological assumptions in general. Habitat profiling can further support metabolic hypotheses based on functional annotation of the genes in different MAGs. For instance, the SRB that reside exclusively in the deeper euxinic waters of the Black Sea (*[U]Desulfatibia profunda*, *[U]Desulfaltia bathyphila*, *Desulfacyla* NIOZ-UU19; 95-97% complete) apparently lack the genes to detoxify oxygen (*cydAB*) or hydrogen peroxide (catalase; Table S2) and to utilize alternative electron acceptors (Figures 3B and 5), reflecting their probably purely euxinic and strictly sulfate-reducing lifestyle. In contrast, the MAGs of SRB relatively abundant in suboxic waters (*[U]Desulfolinea nitratireducens*, *Nitrospirae* NIOZ-UU55, *[U]Db. maris*, *[U]Desulfatibia vada*, *[U]Desulfacyla euxinica*; 73-93% complete, 1-4% contaminated) encode a plethora of genes for the energy-conserving reduction of alternative electron acceptors such as S^0 or thiosulfate (*psrA/phsA/sreA*), tetrathionate (*otr*), nitrate (*napABC, narGHI*) and nitrite (*otr, nirBD, nirK, nrfAH*). They also encode terminal oxidases (*coxAB, ccoNOPQ, cydAB*; Figures 3B and 5), which could be part of complete oxygen respiratory chains. These metabolic potentials are in line with a complex sulfur cycle interlinked with nitrogen cycling and oxygen intrusions (see the following sections). Whether the same SRB species as herein detected in the Black Sea are also present in other DMW requires additional research. However, the metagenomic data from the Black Sea offer a basis for such investigations ranging from 16S rRNA surveys to genome-centric genomics. Expression studies are required to investigate the metabolism of SRB in DMW, and whether they shift their metabolism in response to changing conditions. For instance, the expression of *Desulfocapsa*-related nitrogen fixation (*nif*) genes in the suboxic and upper euxinic zones of the Black Sea (Kirkpatrick et al., 2018) suggests that the *nif*-encoding SRB *[U]Desulfatifera sulfidica* (99% complete, 2% contaminated) and *[U]Desulfobia pelagia* (96% complete, no contamination) could be actively fixing nitrogen. These genes are also encoded by *Nitrospirae* NIOZ-UU55 (73% complete, 1% contaminated). This supports a growing body of evidence for nitrogen fixation by putative SRB in DMW (Jayakumar et al., 2012; Bonnet et al., 2013; Loescher et al., 2014; Christiansen and Loescher, 2019).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cydAB
Sulfurovum NBC37- 1
Arcobacter peruensis PSE-93
USulfurimonas ponti NIOZ-UU79
Sulfurimonas gotlandica GD1
Magnetococcus marinus MC-1
Rhodobacteraceae A-Proteo-Rhodobac-SURF
Sagittula castanea P11
Rhodobacter maris JA276
Alphaproteobacteria NIOZ-UU37
Alphaproteobacteria UBA7893
Alphaproteobacteria NP113
Rhodospirillales UBA2165
Magnetovibrio blakemorei MV-1
Rhodospirillales NIOZ-UU42
Rhodospirillales ARS8
Hyrcanianaceae NIOZ-UU45
Hyrcanianaceae MarineAlpha3_Bin2
Hyrcanianaceae casp-alpha2
Hyrcanianaceae UBA5685
Hyrcanianaceae UBA2139
Hyrcanianaceae ARS16
Limnobacter sp. UBA7305
Sulfurifustis variabilis skN76
Gammaproteobacteria UBA5682
Gammaproteobacteria UBA2157
Gammaproteobacteria UBA2133
Gammaproteobacteria EOSA-II
Gammaproteobacteria UBA7902
Gammaproteobacteria UBA7880
Gammaproteobacteria UBA2132
Gammaproteobacteria UBA5680
Allochromatium vinosum DSM 180
Gammaproteobacteria NIOZ-UU29
Gammaproteobacteria SZUA-75
Ectothiorhodospira mobilis DSM 4180
Thiothrix nivea DSM 5205
UThiopontia autotrophica NIOZ-UU100
Pseudothioglobus SCGC AAA007-O20
Pseudothioglobus NP1
UPseudothioglobus aerophilus NIOZ-UU104
'Ca. Pseudothioglobus singularis' PS1
Thioglobaceae SUP05
UThioglobus perditus CMC-S-2018
UThioglobus pontius NIOZ-UU116
'Ca. Thioglobus autotrophicus' EF1
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Nitrospina gracilis 3/211
Nitrospirae NIOZ-UU55
Nitrospirae BMS3Bbin09
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'Ca. Nitrobium versatile' Ru_enrich_NS
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Desulfocapsa sulfexigens DSM 10523
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Desulfatiglans NaphS2
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SAR324 bacterium UBA1014
SAR324 bacterium UBA5684
SAR324 bacterium UBA7892
SAR324 bacterium SP68
SAR324 bacterium REDSEA-S27_B3
SAR324 Arctic96AD-7 bacterium NIOZ-UU44
SAR324 bacterium SCGC AAA001-C10
SAR324 bacterium SAR324-CTDB7B
SAR324 Arctic96AD-7 bacterium NIOZ-UU70
SAR324 bacterium CPC1
SAR324 bacterium AG-439-C09
SAR324 bacterium MED775
UDesulfolinea nitratireducens NIOZ-UU36
Anaerolineales UBA5796
Dehalococcoidia RBG_13_52_14
Dehalococcoidia RBG_13_60_13
Bacteroidia NIOZ-UU65
'Ca. Thermonerobacter thiotrophicus' MS-B_bin-24
Chlorobium phaeobacteroides DSM 266
Prosthecochloris aestuarii DSM 271
Chlorobium NIOZ-UU103
Chlorobium BS1
AAMBM5-125-24 SAG SCGC AG-640-A22
AAMBM5-125-24 NIOZ-UU51
AAMBM5-125-24 NIOZ-UU89
Marinimicrobia SHBH1141 AB-750_L13AB-904
Marinimicrobia PN262000N21
Marinimicrobia NIOZ-UU73
Marinimicrobia SCGC AD-606-A07
Marinimicrobia NAT274
Marinimicrobia Arctic96B-7-B AB-746_N13AB-902
Marinimicrobia Arctic96B-7-B AB-746_P06AB-902
1
2
3
4
5
6
7
Alphaproteobacteria
Gammaproteobacteria
Deltaproteobacteria
SAR324
0.5

Figure 5. A phylogenomic and genetic overview of important microbial players in the marine sulfur cycle of dysoxic marine water (DMW) environments. A) An unrooted phylogenomic maximum-likelihood tree constructed from a concatenated alignment of 120 single-copy household genes (S.I. Methods). Phylogenetic clades were identified, with numbers indicating the following lineages: 1, *Campylobacterota*; 2, *Nitrospinae*; 3, *Nitrospirae*; 4, *Chloroflexi*; 5, *Fibrobacteres, Chlorobi, and Bacteroidetes*; 6, candidate phylum AAMBM5-125-24; 7, *Marinimicrobia*. Black dots indicate support by >95% out of 1,000 ultra-fast bootstraps. The scale bar indicates substitutions per site. The tree includes data from all available genomes (March 2020) from DMW sites (bold, colored by environment following the color code of Figure 1) that contain dissimilatory sulfur genes, and relevant reference genomes (black). The superscript prefix '*U*' indicates uncultured species for which a taxonomy has been proposed based on a high-quality genome, functional annotation and environmental distribution (Konstantinidis et al., 2017) with the genome sequences as type material (Chuvochina et al., 2019; Murray et al., 2020; see also S.I. Protologue). B) An overview of the presence of functional genes enabling conversions of sulfur, nitrogen and oxygen, following the color scheme of Figure 2. Shortly, red indicates core genes of the Dsr/rDsr pathways, orange indicates *dsrD*, purple indicates *dsrEFH* and various oxidative sulfur genes, light-blue indicates *sox* genes, light-green indicates *phs/psr/sre* genes, dark-green indicates various (potentially) reductive sulfur genes, black/dark-grey indicates nitrogen genes, dark-blue indicates oxygen reduction genes. The presence of the indicated functional genes or gene clusters is shown with filled circles; open circles reveal incomplete gene clusters. For 'Rhodanese', filled circles indicate ten or more rhodanese domains (S.I. Methods). Stars distinguish the high-quality genomes (>80% complete, <5% contaminated) from the medium-quality genomes (>50% complete, <10% contaminated) analyzed. Only two low-quality metagenome-assembled genomes, i.e. that of *Dehalococcoidia* RBG_13_52_14 (35% complete, 2% contaminated) and the population genome of *Gammaproteobacteria* EOSA-II composed of multiple combined single-cell amplified genomes (63% complete, 21% contaminated), were also included. A comprehensive overview of genome origin, quality, classification, annotation and average amino acid identity (AAI) between genomes can be found in Table S2.

2.3 Particles as microhabitat

Sulfate is thermodynamically an inferior electron acceptor to nitrate and nitrite (Table S3), implying that denitrifying microorganisms will outcompete SRB for electron donors in suboxic waters and OMZ cores. How then is dissimilatory sulfate reduction sustained, especially in nitrite- and nitrate-rich OMZ cores? It has been postulated that SRB occupy microhabitats inside organic particles, in which nitrate and nitrite have already been depleted (Fuchsman et al., 2011; Wright et al., 2012). These organic particles or 'marine snow' (Alldredge and Silver, 1988) are particularly abundant in suboxic waters (Karl and Knauer, 1991; Taylor et al., 2001; Sorokin, 2002) and OMZs (Whitmire et al., 2009; Roullier et al., 2014), compared to other regions of the oceanic water column. When oxygen levels drop below ~25 μM, particles of the predominant size range (100-200 μM in diameter; Roullier et al., 2014) develop inner anoxic microhabitats due to limitation of oxygen diffusion (Shanks and Reeder, 1993; Klawonn et al., 2015; Ploug and Bergkvist, 2015). This implies that in nitrate-rich dysoxic waters, particles will develop a nitrate-depleted core, as nitrate rarely exceeds a concentration of 25 μM in DMW and diffuses slower than oxygen (Fuchsman et al., 2019). Thus, sulfate-reducing microhabitats may be abundant in non-sulfidic DMW.

SRB in seawater seem to be more abundant in particles than in free suspension. The 16S rRNA gene sequences of canonical deltaproteobacterial SRB were found to be predominantly particle-associated (>30 μm) in marine suboxic zones (Fuchsman et al., 2011; Suter et al., 2017; Suter et al., 2018) and the ETNP OMZ core (Fuchsman et al., 2017). Moreover, reductive *dsrA* genes in the ETNP OMZ core were almost exclusively detected in the particle (>30 μm) fraction, whereas oxidative *dsrA* genes showed no specific particle association (Saunders et al., 2019). This particle-bound lifestyle causes SRB to be significantly underrepresented in some molecular ecological studies. It is common practice to employ a pre-filter step for the collection of biomass to remove eukaryotes (1.6 to 10 μM pore size cut-off), thus also removing particles and particle-associated SRB. This methodology has been applied for investigations in DMW (Canfield et al., 2010; Stewart et al., 2012; Ulloa et al., 2012; Ganesh et al., 2014; Hawley et al., 2014), which may explain why SRB sequences were present in low abundance or absent. In contrast, studies omitting a pre-filter have identified SRB sequences in substantial proportion (Neretin et al., 2007; Fuchsman et al., 2011; Carolan et al., 2015; Rodriguez-Mora et al., 2016; Saunders et al., 2019). Particle sinking in standard Niskin sampling bottles also caused bias against particles and SRB sequences (Suter et al., 2017). Thus, complete circumvention of these potential biases requires *in-situ* filtration devices, which have occasionally been used in DMW (Lavik et al., 2009; Marschall et al., 2010; Sollai et al., 2019). *In-situ* filtration was also applied for obtaining the Black Sea MAGs presented herein, indeed resulting in higher estimated relative abundances of SRB than found by Neretin and colleagues (2007) in both the suboxic zone (<13% versus <2% of all bacteria) and the euxinic zone (<20% versus <5%, S.I. Methods). Since these estimated fractional abundances may still be biased by DNA extraction methods, ideally an extraction-independent method should also be applied such as fluorescent *in-situ* hybridization of functional genes (Barrero-Canosa et al., 2017). To achieve comparability between different studies, similar sampling methodology with minimal bias is essential and should be carefully evaluated.

3 Sulfur-oxidizing bacteria

The oxidative part of the sulfur cycle starts with the competition between SOB and abiotic reactions for sulfide (Luther et al., 2011). Depending on the sulfide oxidation route, a range of possible sulfide oxidation products can be formed (Figure 2), having a significant effect on the rest of the biogeochemistry in DMW. Oxidized metals such as manganese oxide (MnO_2) or ferric (oxy)hydroxides are so efficient in catalyzing sulfide oxidation (Yao and Millero, 1993; Ma et al., 2006) that even at micromolar concentrations MnO_2 is thought to be abiotically responsible for the bulk of the sulfide oxidation in systems with broad stable chemoclines and low oxygen flux such as the Black Sea (Jørgensen et al., 1991; Konovalov et al., 2003; Trouwborst et al., 2006; Stanev et al., 2018) and Cariaco Basin (Ho et al., 2004). Chemical oxidation of sulfide produces SCIs such as S^0 and thiosulfate, which are commonly detected in euxinic marine waters (Jørgensen and Bak, 1991; Zopfi et al., 2001; Li et al., 2008b; Kamyshny et al., 2013; Findlay et al., 2014). Like sulfide, SCIs can be converted by SOB to sulfate as energy source.

Microorganisms that oxidize sulfur compounds possess widely differing metabolisms, including auto- or heterotrophy, and chemo- or phototrophy (Dahl, 2017). Some SOB oxidize a wide range of sulfur compounds as primary energy source, whereas others facultatively oxidize specific sulfur compounds such as thiosulfate as supplementary energy source (Sorokin, 2003). Photolithotrophic SOB – green or purple sulfur bacteria – can become dominant if euxinic marine waters overlap with the photic zone in shallow waters (Findlay et al., 2015; Pjevac et al., 2015; Findlay et al., 2017; Pjevac et al., 2019). The deeper the chemocline, the smaller the population and role of phototrophic SOB, exemplified by low-light-adapted *Chlorobium* bacteria in the Black Sea (approximately 100 m depth; Overmann et al., 1992; Manske et al., 2005; Marschall et al., 2010). They are outnumbered by chemolithotrophic SOB, with gammaproteobacterial SUP05 bacteria (Lavik et al., 2009; Canfield et al., 2010; Glaubitz et al., 2013) and sulfur-oxidizing *Campylobacterales* bacteria such as *Sulfurimonas* species (Grote et al., 2008; Schunck et al., 2013; Callbeck et al., 2019) as foremost examples. However, the biogeochemical impact on element cycling is not always related to cellular abundance (Pester et al., 2012; Hausmann et al., 2019), exemplified by magnetotactic *Magnetococcus*-related bacteria that shuttle the scarcely available phosphate from the Black Sea chemocline into the euxinic zone (Schulz-Vogt et al., 2019), which suggests a sulfur-oxidizing physiology akin to other *Magnetococcus* species (Bazylinski et al., 2013). This underscores the importance of using multiple approaches when studying functional groups of microorganisms, including sulfur-cycling bacteria. Here, we will mainly discuss well-studied chemolithotrophic SOB specifically abundant in OMZ core waters and other deep DMW.

3.1 SUP05 bacteria

Based on 16S rRNA gene surveys, specific gammaproteobacterial bacteria belonging to the SUP05 clade and closely related to known sulfur-oxidizing symbionts have been identified as abundant putative SOB in DMW [reviewed by Wright et al. (2012)]. The capacity of these bacteria for chemolithoautotrophic nitrate reduction – most probably coupled to the oxidation of sulfide and/or SCIs – in DMW was strongly indicated by stable isotope probing experiments with labelled inorganic carbon (Grote et al., 2008; Glaubitz et al., 2010) and correlations with rate measurements of nitrate reduction and dark carbon fixation (Lavik et al., 2009; Schunck et al., 2013). Direct cell counts with fluorescent probes showed that SUP05 bacteria may form a dominant group of the microbial community; they comprised up to 50% of the microbial population in euxinic shelf waters off Namibia and Peru ($<3{\cdot}10^6$ cells mL^{-1}; Lavik et al., 2009; Callbeck et al., 2018), up to 17% in the ETSP OMZ core ($5{\cdot}10^5$ cells mL^{-1}; Callbeck et al., 2018), up to 30% at an oxic-euxinic interface in the Baltic Sea ($4{\cdot}10^5$ cells mL^{-1}, Landsort Deep) and up to 10% and 13% in the suboxic and euxinic zone of the Black Sea, respectively ($<7{\cdot}10^4$ cells mL^{-1}; Glaubitz et al., 2013).

The presumed physiology of SUP05 bacteria was supported by the presence in their MAGs of genes for sulfide oxidation (*sqr, fccAB*), the 'Sox' thiosulfate oxidation pathway (*soxXABYZ*), the rDsr sulfur oxidation pathway (*sat, aprBA, dsrABCMK, dsrEFH*; Figures 2 and 5), nitrate reduction (*narGHIJ*) and inorganic carbon fixation through the Calvin-Benson-Bassham cycle (Walsh et al., 2009; Canfield et al., 2010; Murillo et al., 2014; Callbeck et al., 2018). This physiology has been confirmed by cultivation experiments of the only current SUP05 isolate, '*Candidatus* Thioglobus autotrophicus' (Shah et al., 2017), which showed growth with sulfide, thiosulfate, thiotaurine and stored S^0 as energy source (Shah et al., 2019). The four available SUP05 genomes from DMW show variation in the presence of other nitrogen-respiration genes (*nirK, nirS, nirBD, norCB, nosZ*; Figure 5) and oxidative phosphorylation genes (*coxBAC, ccoNOPQ*, cytochrome bc_1 complex genes; Figure 5), indicating metabolic diversification of the strains within this clade. Corresponding with their high abundance, SUP05 bacteria generally dominate the detection of rDsr pathway genes, transcripts, and proteins in DMW (Canfield et al., 2010; Stewart et al., 2012; Hawley et al., 2014). The sister clade ARCTIC96BD-19 is represented by '*Candidatus* Thioglobus singularis' (Marshall and Morris, 2013), but ARCTIC96BD-19 genomes are too divergent from SUP05 genomes to consider them the same genus (63-66% AAI, Table S2). '*Ca.* T. singularis' has an organoheterotrophic aerobic lifestyle and does not oxidize sulfur (Spietz et al., 2019). These features are probably representative of all ARCTIC96BD-19 bacteria, based on the absence of most sulfur oxidation genes in their genomes (Swan et al., 2011; Figure 5) and a preference for oxic waters (Wright et al., 2012; Figure 3A, *Pseudothioglobus aerophilus*). To reflect their distinct taxonomy and physiology, we suggest to rename the ARCTIC96BD-19 clade to *Pseudothioglobus* (S.I. Protologue).

The affinity of SUP05 bacteria for sulfide is higher than reported for any other bacterium or substrate (Crowe et al., 2018), demanding a re-evaluation of the existing definition of euxinia. Currently, the sulfide concentration threshold to distinguish non-sulfidic from euxinic conditions commonly falls in the range of 0.5-1 μM. This threshold is similar to the *in-vitro* Michaelis-Menten half-saturation constant (K_m) of 2 μM of purified high-affinity Sqr proteins (Schutz et al., 1997; Brito et al., 2009) and the K_m found for phototrophic SOB (>0.8 μM; Van Gemerden, 1984). However, the estimated K_m of SUP05 bacteria is much lower (25-340 nM; Crowe et al., 2018). The most widely used method for determining sulfide concentrations has a sulfide detection limit of 0.1 μM (Cline, 1969; Jørgensen et al., 1991; Zopfi et al., 2001), hence falling within this estimated range. Thus, we suggest it is biologically sound and practically feasible to use a sulfide threshold of at most 0.1 μM to define euxinia, at least in marine environments. However, an even lower threshold would be more accurate, as SUP05 bacteria consume sulfide at <5 nM (Crowe et al., 2018). Such low concentrations can be detected and quantified with sensitive voltammetric sensors (Luther III et al., 1991; Luther et al., 2008). The findings of Crowe and colleagues (2018) illustrate the value of studies that quantify properties such as substrate affinity and should motivate further investigation, for instance with respect to sulfide toxicity or substrate affinity for sulfide of other key SOB. Such studies are required to advance biogeochemical models that explicitly take microbial community composition and function into account by integrating omics data (Reed et al., 2014; Louca et al., 2016).

3.2 *Campylobacterota*

The SOB of the phylum *Campylobacterota* (formerly *Epsilonproteobacteria*; Waite et al., 2017; 2018) are more diverse and environment-specific than the common SUP05 bacteria. The most widespread *Campylobacterota* genus in DMW is *Sulfurimonas*. Members of this genus dominate the upper euxinic zone of the Black Sea and Baltic Sea at a count of 15-30% of all microorganisms (<$2{\cdot}10^5$ cells per mL) and outnumber SUP05 bacteria (Brettar et al., 2006; Grote et al., 2008; Glaubitz et al., 2010). Members of another *Campylobacterota* genus, *Arcobacter,* are generally less abundant in euxinic basins (Glaubitz et al., 2008; Fuchsman et al., 2012; Rodriguez-Mora et al., 2013), but proliferate in euxinic shelf waters during sulfidic events (<25% of all cells; <$1{\cdot}10^6$ cells per mL)(Lavik et al., 2009; Schunck et al., 2013; Callbeck et al., 2019). Four *Campylobacterota* isolates have thus far been obtained from DMW, all facultative anaerobes capable of sulfur oxidation and nitrate reduction: *Sulfurimonas gotlandica* (Grote et al., 2012) and '*Candidatus* Sulfurimonas baltica' (Henkel, 2019), both from a redoxcline in Gotland Basin; '*Candidatus* Sulfurimonas marisnigri' from the Black Sea euxinic zone (Henkel et al., 2019); and *Arcobacter peruensis* from euxinic coastal waters off Peru (Callbeck et al., 2019). *A. peruensis* was only demonstrated to use sulfide as energy source (Callbeck et al., 2019), whereas the *Sulfurimonas* species were

grown with sulfide, SCIs and H_2 (Labrenz et al., 2013; Henkel, 2019). As is common in *Campylobacterota* members, the genomes of *S. gotlandica* and *A. peruensis* lack rDsr genes and these SOB are presumed to oxidize sulfur compounds through a variant of the Sox pathway encoded by two operons (*soxXY$_1$Z$_1$AB* and *soxCDY$_2$Z$_2$*; Meier et al., 2017; Pjevac et al., 2018; Götz et al., 2019; Figure 2 and 5). We recovered a *Sulfurimonas* MAG from the Black Sea (*^{U}Sulfurimonas ponti*), which also lacks most Sox genes despite being virtually complete (97% completeness, 4% contamination, Figure 5). *^{U}S. ponti* may oxidize sulfide incompletely to S^0, or use an alternative route such as the Hdr-like sulfur oxidation pathway (Boughanemi et al., 2016).

The heterotrophic *A. peruensis* was not capable of fixing inorganic carbon and requires an organic carbon source such as acetate (Callbeck et al., 2019), whereas the autotrophic *Sulfurimonas* species fixed inorganic carbon for growth, presumably through the reverse tricarboxylic acid cycle (Grote et al., 2008; Henkel, 2019). In euxinic coastal waters, sufficient organic carbon can be available to allow *A. peruensis* to successfully compete for sulfur substrate with autotrophic SOB by achieving a higher carbon assimilation rate and therefore probably also a higher growth rate (Callbeck et al., 2019). *Sulfurovum* species of the *Campylobacterota* phylum were highly abundant during sulfidic events in coastal waters (Lavik et al., 2009; Schunck et al., 2013; Callbeck et al., 2019) and possibly outnumber *Sulfurimonas* in Cariaco Basin (Rodriguez-Mora et al., 2013; Rodriguez-Mora et al., 2016; Taylor et al., 2018). Previous cultivation- and metagenomics-based studies of *Sulfurovum* members have primarily addressed hydrothermal vent habitats. They revealed metabolic similarity to *Sulfurimonas* species with respect to sulfur oxidation, carbon fixation, and nitrate reduction (Yamamoto et al., 2010; Giovannelli et al., 2016; Jeon et al., 2017; Meier et al., 2017; Mori et al., 2018). However, one notable exception is *Sulfurovum aggregans*, which cannot oxidize sulfur but instead reduces it (Mino et al., 2014). As such, multiple biochemical roles are possible for *Sulfurovum* species in DMW.

Sulfur-oxidizing autotrophs such as *Sulfurimonas* and SUP05 bacteria compete for very similar niches through different strategies. The metabolically specialized, streamlined (<1.5 Mbp genomes) and non-motile SUP05 bacteria prefer stable conditions, while the motile and more adaptable *Sulfurimonas* species benefit from a less stable chemocline with more mixing of sulfide, nitrate and oxygen (Rogge et al., 2017; Taylor et al., 2018). Furthermore, SUP05 bacteria are most abundant at low-sulfidic conditions (<5 µM; Glaubitz et al., 2013; Rogge et al., 2017), which may be due to their unparalleled high affinity for sulfide (Crowe et al., 2018) and their capability to store S^0 for later usage when external substrates are absent (Shah et al., 2019). In euxinic basins, *Sulfurimonas* species can thrive simultaneously with SUP05 bacteria, but have a relative abundance peak in slightly deeper, more sulfidic waters (median 17 µM; Figure 3C; Rogge et al., 2017). Here, the electron acceptors oxygen and nitrate are irregularly available (Konovalov et al., 2003; Glaubitz et al., 2010; Glaubitz et al., 2013). *Sulfurimonas* species have adapted

to these conditions through motility and chemotaxis towards nitrate-rich conditions (Grote et al., 2012), which is sustained by energy storage in the form of polyphosphate (Möller et al., 2019). Furthermore, *Sulfurimonas* species probably conserve more energy from nitrate than SUP05 bacteria, since instead of partial denitrification to nitrite (Shah et al., 2017) or possibly nitrous oxide (Walsh et al., 2009; Hawley et al., 2014), *S. gotlandica* can perform complete denitrification to nitrogen gas (Labrenz et al., 2013) and ^{U}*S. ponti* could perform ammonification (*nrfAH*, Figure 5).

Intriguingly, '*Ca.* S. marisnigri' is the first bacterium demonstrated to couple sulfur oxidation to the reduction of MnO_2 to Mn^{2+} for growth (Henkel et al., 2019). This trait could be highly beneficial in euxinic basins such as the Black Sea since in contrast to oxygen and nitrate, MnO_2 is in constant supply - albeit at low concentrations – since it is particulate and sinks (Tebo, 1991; Konovalov et al., 2004; Trouwborst et al., 2006). This metabolic capacity could also answer the long-pending question of how high carbon fixation rates are sustained in euxinic waters without sufficient nitrate, nitrite or oxygen (Jørgensen et al., 1991; Taylor et al., 2001; Ho et al., 2004; Jost et al., 2010; Kirkpatrick et al., 2018). Indeed, a reaction-diffusion model by (Yakushev et al., 2007) required coupling of MnO_2 reduction to carbon fixation to reproduce the observed chemical profiles. There are indications that the reaction rates of abiotic and microbial sulfide oxidation by Mn in euxinic basins are in the same order of magnitude (Jørgensen et al., 1991; Sorokin et al., 1995; Henkel, 2019). However, there is currently no insight into the *in-situ* abundance of '*Ca.* S. marisnigri'. Like *S. gotlandica* (Grote et al., 2012), it does not affiliate with the locally abundant *Sulfurimonas* GD17 subclade (95-96% 16S rRNA gene sequence similarity). Further investigation through molecular studies is currently challenging, as the enzymatic pathway allowing MnO_2 reduction is unknown. Nevertheless, these findings have large consequences for our view on euxinic biogeochemistry, as sulfide-driven denitrification and nitrogen loss may effectively be bypassed. Mn-dependent sulfide oxidation could even result in a fixed nitrogen gain, since both *S. marisnigri* and *S. baltica* can apparently fix nitrogen (Henkel, 2019), confirming a recently published hypothesis (Kirkpatrick et al., 2018).

3.3 Other sulfur-oxidizing lineages

As described above, the physiology of some of the key SOB has been explored in some detail, but other microbial players are waiting to be described. Predominantly genomic studies point to a wide phylogenetic diversity of poorly studied SOB for which important ecological or biogeochemical roles in DMW have been demonstrated or are strongly indicated. The class *Gammaproteobacteria* probably contains relevant SOB that do not affiliate with the SUP05 clade, notwithstanding their key role. Firstly, genomes of the EOSA-II lineage were retrieved from coastal waters and the OMZ in Southern Pacific waters (Figure 5), actively expressing sulfur oxidation genes in the ETSP OMZ

core (Plominsky et al., 2018). Secondly, the gammaproteobacterial BS-GSO2 clade was detected in the Black Sea as autotrophic lineage with a peak in relative abundance at the euxinic interface together with SUP05, suggesting it uses sulfur as energy source (Glaubitz et al., 2010). This clade is especially noteworthy since sequencing studies indicate that BS-GSO2 bacteria may outnumber SUP05 bacteria in the Black Sea (Fuchsman et al., 2011; Kirkpatrick et al., 2018; Figure 3C) and Cariaco Basin (Suter et al., 2018; Taylor et al., 2018). The only currently available BS-GSO2 MAG is that of *[U]Thiopontia autotrophica* obtained from the Black Sea metagenomes analyzed herein (NIOZ-UU100, 93% complete, 0.1% contamination) with 99% 16S rRNA gene identity with the original BS-GSO2 sequence reported by Glaubitz and colleagues (2010). Indeed, *[U]T. autotrophica* possesses the genes for sulfur oxidation (rDsr) and the Calvin-Benson-Bassham cycle, but it differs from SUP05 bacteria in lacking most Sox genes and encoding a complete denitrification pathway (Figure 5). It thus seems the BS-GSO2 clade has been overshadowed by SUP05, yet may successfully compete for the same niche.

Bacteria of the uncultivated SAR324 candidate phylum have been abundantly and ubiquitously detected in DMW (Fuchsman et al., 2011; Wright et al., 2012; Beman and Carolan, 2013; Lüke et al., 2016; Suter et al., 2018; Figure 3C). These SAR324 bacteria encode the rDsr sulfur oxidation pathway, which could enable them to oxidize sulfur for energy (Swan et al., 2011; Sheik et al., 2014; Figure 5). Notably, they may couple this process to the reduction of the greenhouse gas nitrous oxide as they encode nitrous oxide reductase genes (*nosZ*, Figure 5). The uncultured alphaproteobacterial family *Hyrcanianaceae* also harbors putative SOB with genomes retrieved from hydrothermal vent plumes (Zhou et al., 2020), the Arabian Sea OMZ core, and the Black Sea (Figure 5). Low-abundance SOB could still significantly alter their environment, for instance through diazotrophy or N_2-fixation. Examples of such SOB are the heterotrophic alphaproteobacterium *Sagitulla castanea* isolated from euxinic Peru shelf waters (Martínez-Pérez et al., 2018) or photolithoautotrophic *Chlorobium* strains (Overmann et al., 1992; Manske et al., 2005; Marschall et al., 2010; *nifDHK*; Figure 3C, 5). Finally, marine *Nitrospinae* members have been shown to oxidize nitrite (Lücker et al., 2013; Sun et al., 2019; Kitzinger et al., 2020), but the presence of a complete rDsr pathway in a *Nitrospinae* MAG from the ETSP OMZ core (UBA7883, 97% complete, 1% contaminated) opens up the possibility that some members may use sulfur as additional or alternative energy source (Figure 5).

4 Sulfur-reducing and sulfur-disproportionating bacteria

The SCIs formed or introduced in DMW could form the substrate for further oxidation by SOB, but could also be used as electron acceptor by sulfur-reducing microorganisms, or as substrate for disproportionation (Figure 2), thus shortcutting the sulfur cycle as has been suggested for other aquatic ecosystems (Tonolla et al., 2004; Wilbanks et al., 2014; Bhatnagar et al., 2020). Similar to abiotic sulfide oxidation, SOB may introduce SCIs in DMW through oxidation of sulfide to S^0 (Dahl, 2017), which is stored intracellularly by the abundant SUP05 bacteria (Shah et al., 2019). Part of this S^0 may be released into the environment due to grazing of SUP05 bacteria by protists (Lin et al., 2007; Glaubitz et al., 2008; Anderson et al., 2013) or due to lysis by SUP05-infecting viruses (Cassman et al., 2012; Anantharaman et al., 2014; Roux et al., 2014; Roux et al., 2016). Additionally, S^0 has been observed to be introduced into OMZs through the drifting off of S^0 produced in coastal waters experiencing sulfidic events (Callbeck et al., 2018). These findings highlight the importance of considering full models of the sulfur cycle and avoid simplified two-reaction representations consisting only of dissimilatory sulfate reduction and chemolithotrophic re-oxidation of sulfide to sulfate (Ulloa et al., 2012; Hawley et al., 2014). The extent of the other fluxes is currently a major unknown factor in DMW, with a large impact on the routes of sulfur-driven carbon fixation and on the occurrence of other linkages with the carbon and nitrogen cycles.

4.1 Sulfur-reducing bacteria and *Marinimicrobia*

The consumption of SCIs in DMW is thought to proceed through a combination of oxidation, reduction and disproportionation (Sorokin et al., 1995; Zopfi et al., 2001; Sørensen and Canfield, 2004). The relative importance of these consumption routes is currently unknown. Sulfur isotope fractionation studies offer little insight, since the measurements in various euxinic marine waters can be explained by sulfate reduction as well as sulfur reduction or disproportionation (Li et al., 2010; Kamyshny et al., 2011). The reduction or disproportionation of S^0 and thiosulfate is more exergonic than sulfate reduction under the conditions found in DMW (S.I. Methods, Table S3), implying that SRB could gain more energy through these reactions. Many cultured SRB are able to reduce or disproportionate thiosulfate (Rabus et al., 2015) through the Dsr pathway and thiosulfate reductase (*phsABC*, Figure 2; Burns and DiChristina, 2009) and may prefer this electron acceptor over sulfate (Jørgensen, 1990). Many anaerobic microorganisms use S^0 as electron acceptor (Rabus et al., 2013) mediated by polysulfide reductase (*psrABC*) or sulfur reductase (*sreABC*, Figure 2; Laska et al., 2003; Sorokin et al., 2015). These three protein complexes (Phs, Psr, Sre) are complex iron-sulfur molybdoenzymes with such a close phylogenetic relationship and with

so few characterized representatives, that distinction based on sequence is currently impossible (Hedderich et al., 1998; Hinsley and Berks, 2002; Laska et al., 2003; Duval et al., 2008; Burns and DiChristina, 2009). Furthermore, various SOB also encode genes with similarity to *psrABC* (Wright et al., 2014), of which the resulting enzymes may well act in reverse (Eddie and Hanson, 2013; Weissgerber et al., 2013). Thus, the presence of *psr*-like genes in a genome suggests the capability of some form of dissimilatory sulfur conversion, but this requires further investigation.

The reduction of SCIs in DMW was used as energy metabolism by an organoheterotrophic *Shewanella* strain isolated from the Black Sea (Perry et al., 1993). However, related microorganisms are unlikely to play a big role in DMW, as they have not been detected in microbial ecology studies. SRB remain probable candidates for mediating sulfur reduction, as several genomes of putative SRB retrieved from the Black Sea encoded *psr*-like genes and a tetrathionate reductase gene (*otr*) in addition to their sulfate-reducing genes (Figure 5). Other MAGs from DMW metagenomes also showed possibly reductive *psr*-like genes, such as *Bacteroidia* NIOZ-UU65 from the Black Sea, a MAG of uncultivated clade SAR324 from the Arabian Sea OMZ core and three *Marinimicrobia* genomes (Figure 5). Bacteria of the uncultivated candidate phylum *Marinimicrobia* (formerly known as Marine Group A and clade SAR406) are prevalent in DMW and contain genomic signatures of organoheterotrophy (Wright et al., 2014; Bertagnolli et al., 2017; Hawley et al., 2017), a metabolism supported by DNA-based stable isotope probing incubations from the Black Sea (Suominen et al., 2019). It has been suggested that *Marinimicrobia* may reduce S^0 based on the presence of *psrABC* genes (Wright et al., 2014; Hawley et al., 2017). As explained, we think it would be more accurate and unambiguous to broaden the hypothesis to *Marinimicrobia* having an unspecified dissimilatory sulfur metabolism. Furthermore, the preference for shallow waters with relatively oxidizing conditions by *Marinimicrobia* NIOZ-UU73 (Figure 3A, 90% complete, no contamination) suggests that for this specific member, a facultative sulfur-oxidizing lifestyle is more likely. Another *Marinimicrobia* MAG (PN262000N21, 98% complete, no contamination) encodes an almost-complete 'Sox' pathway conferring the potential for dissimilatory thiosulfate oxidation (Figures 2 and 5). The most straightforward path to revealing the energy metabolism of these uncultivated bacteria would be cultivation, isolation and characterization. Like genomes of SAR11 and SUP05 bacteria, *Marinimicrobia* genomes are extensively streamlined (Hawley et al., 2017) implying that these bacteria are highly adapted to *in-situ* conditions. Hence, cultivation may require natural seawater as medium, or recently designed synthetic alternatives (Henson et al., 2016).

4.2 Sulfur-disproportionating bacteria

Sulfur disproportionation is the simultaneous oxidation and reduction of an SCI, typically leading to the production of both sulfate and sulfide, which is an uncommon microbial trait (Finster, 2008; Slobodkin and Slobodkina, 2019). The biochemistry of sulfur disproportionation is unresolved and may involve the Dsr pathway in *Deltaproteobacteria* such as *Desulfurivibrio alkaliphilus* (Thorup et al., 2017) and *Desulfocapsa sulfexigens* (Finster, 2008; Finster et al., 2013), and Psr-like molybdoenzymes and rhodanese sulfurtransferases in other microorganisms such as *Desulfurella amilsii* (Florentino et al., 2019). Although uncommon, disproportionation is probably influential in euxinic marine waters, as microorganisms growing through disproportionation of S^0 or thiosulfate could be cultivated from the euxinic Mariager Fjord (Sørensen and Canfield, 2004), and a diffusion-reaction model of Chesapeake Bay required the inclusion of S^0 disproportionation or reduction to explain the observed S^0 concentration profiles (Findlay et al., 2017). In euxinic basins *Desulfocapsa* species could be involved, as their 16S rRNA genes were detected in the Cariaco Basin (Rodriguez-Mora et al., 2015) as well as the Black Sea (Neretin et al., 2007; Fuchsman et al., 2011; Fuchsman et al., 2012). This hypothesis is difficult to test with genomic data due to the unclear biochemistry behind S^0 disproportionation. Out of the MAGs obtained from the Black Sea, *^{U}Df. sulfidica* is the most closely related to *Desulfocapsa*. Yet, it has a markedly different gene repertoire than *Dc. sulfexigens*, without molybdoenzymes or high numbers of rhodanese genes. However, plenty other sulfur-disproportionating bacterial candidates with Dsr pathways, molybdoenzymes and high numbers of rhodanese genes remain (Figure 5). Like *Dv. alkaliphilus* (Thorup et al., 2017), some might oxidize sulfide in a disproportionation-dependent pathway including sulfide oxidation by Sqr (*^{U}Db. maris*, *^{U}Db. vada*, *^{U}Dl. nitratireducens*, Arabian Sea OMZ core *Desulfobacterales*).

Since most characterized sulfur-disproportionating microorganisms can grow autotrophically (Finster et al., 1998; Florentino et al., 2016; Mardanov et al., 2016; Slobodkin and Slobodkina, 2019), their presence could spell a role in the high rates of carbon fixation that are generally observed within euxinic marine waters just below the euxinic interface (Jørgensen et al., 1991; Taylor et al., 2001). This phenomenon is commonly attributed solely to sulfur oxidation by chemolithoautotrophic SOB (Grote et al., 2008; Glaubitz et al., 2010). Our hypothesis is in agreement with the stimulation of carbon fixation measured upon the addition of thiosulfate or polysulfide to euxinic samples from the Baltic Sea (Labrenz et al., 2005; Jost et al., 2010). However, these findings could be influenced by artificial introduction of oxygen (De Brabandere et al., 2012) and are in need of further testing. In general, more dedicated experimental work is needed to quantitatively constrain SCI-consuming reactions, such as was done for a freshwater lake (Findlay and Kamyshny, 2017). Another unexplored factor in the marine sulfur cycle is the cycling of organic sulfur compounds, which has been highlighted for marine sediments (Wasmund et al., 2017). Such processes may be important in

DMW, as dimethylsulfide oxidation genes (*ddhA*) were detected in genomes of SUP05 bacteria, [U]*T. autotrophica* and *Marinimicrobia*, and dimethylsulfoxide reductase genes (*dmsA*) in MAGs of *Desulfacyla* species, [U]*Db. maris* and OMZ *Desulfobacterales*. Future studies should address to what extent reactions of SCIs and organic sulfur compounds contribute to the overall sulfur cycle, and whether this is affected by environmental conditions.

5 Conclusions and future perspectives

This review has presented a compendium of the current insights into sulfur-cycling bacteria in DMW (Figure 6). Based on current experimental evidence, it is difficult to investigate *in-situ* sulfur reactions beyond sulfide oxidation and sulfate reduction. Euxinic marine waters are thought to host a complex network of reactions, whereas this remains more uncertain for suboxic waters and OMZ cores. Molecular studies have revealed a high diversity of putative SRB and SOB, which we expect to be explored further and consolidated over the coming years, specifically with the use of improved genome-centric metagenomics. Sampling methods without bias against particle-associated microorganisms can give an accurate and intercomparable view on diversity and abundance across DMW, specifically of SRB. With this in mind, it could be evaluated whether the community of novel putative SRB genomically revealed by us in the Black Sea is representative of euxinic marine basins and perhaps DMW in general. Together, the diverse sulfur-cycling bacteria form a myriad of connections with other elemental cycles. SUP05 bacteria fix inorganic carbon with energy from very low sulfide concentrations, warranting a biologically meaningful reshaping of our concept of euxinia. These insights into sulfide affinity are crucial building blocks for biogeochemical modelling efforts, which could be further improved by estimations of critical biological parameters including biomass yield and sulfide tolerance. Metatranscriptomics and metaproteomics experiments might play a role in testing under which conditions SRB use their genomic potential for respiration of oxygen and diverse nitrogen compounds. Notably, genomic classifications of some bacteria as 'SRB' are for now putative, as the Dsr pathway on which this is based could also confer other forms of sulfur metabolism, such as sulfur reduction, disproportionation or oxidation. Similarly, the lack of fundamental insight into the relation of sequence and function of prevalent Psr-related molybdoenzymes hinders metabolic predictions. Thus, genomic-based research can find strong support in cultivation experiments and the *in-vitro* study of heterologously expressed sulfur enzymes. The power of cultivation has been showcased by the isolation of SUP05, *Sulfurimonas* and *Arcobacter* bacteria, and specifically that of the Mn-reducing and probably nitrogen-fixing '*Ca.* S. marisnigri'. The cultivation and isolation of SRB

from DMW is also feasible (Teske et al., 1996; Zopfi et al., 2001; Sørensen and Canfield, 2004; Finster and Kjeldsen, 2010), but more challenging than cultivation from sediment due to rapid oxidation of sampled water. These efforts could be facilitated by genome-guided cultivation (Gutleben et al., 2018) or a reverse genomic approach (Cross et al., 2019). Finally, the factors controlling nitrogen fixation by SOB and SRB require further investigation, as this may be an important factor in the expected development of open-ocean euxinia (Ulloa et al., 2012). The advances as summarized and predicted herein will enable the construction of biogeochemical models of the sulfur cycle from meta-omics data, as has been done for the nitrogen cycle in the Arabian Sea (Reed et al., 2014) and in the Saanich Inlet (Louca et al., 2016). In the future, such endeavors could aid in predicting the biogeochemical response to expanding dysoxia and euxinia.

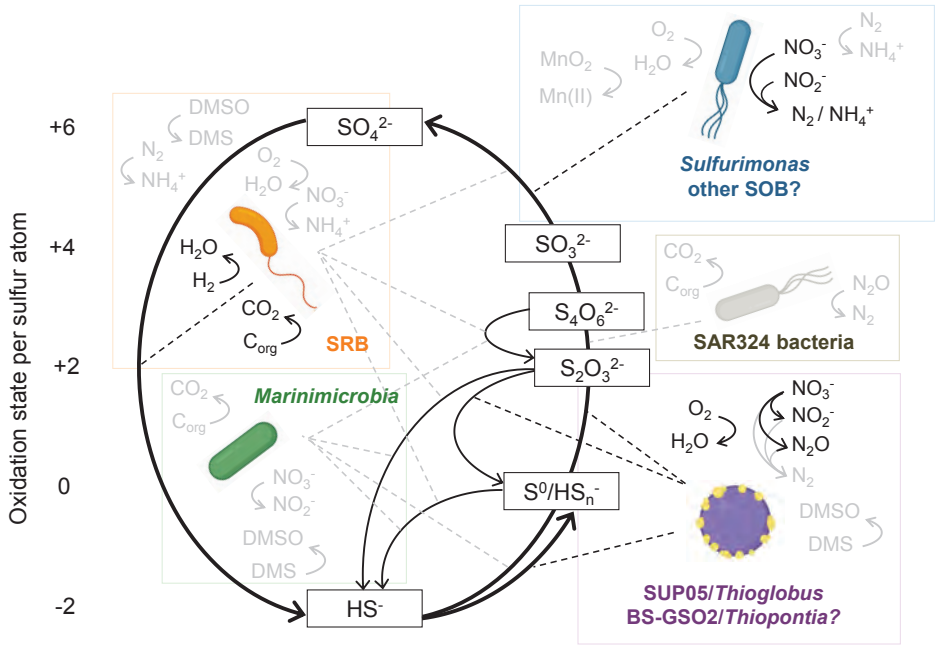


Figure 6. Conceptual ecophysiological model of the sulfur cycle and the involved microorganisms in DMW. Question marks and the color light-gray is used when there are indications for involvement of specific microorganisms and/or processes but definitive proof is lacking. Bacterial images were created with BioRender.

6 Supplementary material

Supplementary tables and other supplementary material can be accessed at: https://sfamjournals.onlinelibrary.wiley.com/doi/full/10.1111/1462-2920.15265.

Data S1. Maximum-likelihood phylogenetic reconstruction of *dsrA* genes in newick format. See S.I. Methods for methodology.

Table S1. Microbiological and biogeochemical studies of the sulfur cycle in dysoxic marine waters, grouped by environment. Volumes based on the work of Paulmier and Ruiz-Pino (2009) correspond to the estimated volume of waters containing >0.5 μM nitrite. The maximum volume of anoxic water off the Namibian coast was calculated from the largest observed extent of sulfidic bottom waters (7,000 km^2; Lavik et al., 2009) and an assumed sulfidic layer thickness of 10 m.

Table S2. Origin, quality, classification, annotation, and average amino acid identity (AAI) of the analyzed genomes in Figure 5. Methods are described in S.I. Methods. The AAI values were calculated with an *enveomics* script using Diamond because of computational limitations, which could lead to significantly overestimated AAI values between 50-60% (https://rodriguez-r.com/blog/aai-blast-vs-diamond/). Following the thresholds proposed by Konstantinidis et al. (2017), values exceeding the 65% genus-level lower threshold are colored green, and values exceeding the 45% family-level lower threshold are colored yellow.

Table S3. Gibbs free energies [ΔG (kJ/e^-)] of common dissimilatory conversions mediated by anaerobic microorganisms under conditions representative of the upper euxinic zone of the Black Sea and the core of the ETSP OMZ. Calculation methodology and variables used can be found in S.I. Methods.

6.1 Supporting Information Methods

6.1.1 Physicochemical data of the Black Sea

The methodology of the physicochemical measurements from sampling station 2 (N42°53.8', E30°40.7, 2,107m depth) in the Black Sea western gyre during the Phoxy cruise 64PE371 (BS2013) on the 9th and 10th June 2013 on board the *R/V Pelagia* were described by Sollai et al. (2019). Redox potential was measured at the same station in 2016 on the 1st and 2nd of February 2016 during the 64PE408 'NESSC/SIAM' cruise aboard the *R/V Pelagia*. Sampling was performed with a rosette sampler equipped with Go-Flow bottles (General Oceanics, Miami, FL, USA) and a conductivity-temperature-density (CTD) unit (SBE 911 plus, Sea-Bird Electronics, Bellevue, WA, USA). On

deck, the bottles were pressurized with N_2 to ensure anoxic conditions throughout the sampling procedure. Redox potential was measured with a ProSense QR400X-6MM Epoxy ORP redox electrode (Oosterhout, The Netherlands). Several hours were taken for equilibration, as is necessary for samples with neutral pH to have reproducible measurements (Boulegue and Michard, 1979). Sulfide was also measured from $ZnCl_2$-complexed samples using the photometric methylene blue method (Cline, 1969), revealing a linear correlation with depth in the upper 500 m down to a concentration of 0.1 μM similar to previous reports (Jørgensen et al., 1991). Furthermore, the depth and potential density of the non-sulfidic/sulfidic interface was similar in all three profiles: 102 m in 2013, and 106 and 107 m in the two 2016 casts, corresponding to potential density values of 16.12, 16.16 and 16.13 kg m^{-3} respectively. Therefore, the physicochemical data of 2013 and the redox potential measurements of 2016 are combined in one plot in Figure 3A.

6.1.2 Metagenomics and phylogenetic reconstruction

Suspended particulate matter (SPM) collection, sequencing, metagenome assembly and binning are described by Villanueva et al. (2021). In short, SPM was collected from 15 depths across the water column of sampling station 2 during the 2013 Phoxy cruise with *in-situ* pumps. Unamplified DNA extracts were sequenced with Illumina MiSeq, generating 45 million 2x250 bp paired-end reads. After quality control with FastQC v0.11.3[2] and trimming with Flexbar v2.5 (Dodt et al., 2012), reads were cross-assembled with metaSPAdes v3.8.0 (Bankevich et al., 2012) and mapped back to the assembly with BWA-MEM v0.7.12 (Li, 2013). The same methodology was applied to assemble ETSP OMZ metagenomes (Canfield et al., 2010; Ganesh et al., 2014). Scaffold read coverage profiles over different water column depths were based on the depth file generated during the binning step with the jgi_summarize_bam_contig_depths script. A biologically meaningful normalization of coverage was performed using single-copy genes (SCGs), similarly to for instance the normalization of read counts using the single-copy *rpoB* gene by Lüke et al. (2016). We analyzed the coverage of the 41 Pfam SCGs used by CheckM by querying the scaffolds as described in the following subsection with the Pfam HMM profiles (Finn et al., 2016) using the 'trusted' bitscore cutoff. The total coverage, i.e. the summed coverage across all sampling depths, was consistent for most SCGs. The coverage of SCGs at different water column water depths was averaged, and the average SCG coverage was used to normalize the coverage of scaffolds as follows:

Black Sea scaffolds were binned into metagenome-assembled genomes (MAGs) based on coverage profile across samples and tetra-nucleotide frequency with MetaBAT v0.32.4 (Kang et al., 2015) with the '—superspecific' preset. Coverage of Black Sea MAGs was inferred from the normalized coverage of representative scaffolds encoding

2 https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

dsr or *sox* genes. In previous research, MAGs have been retrieved from marine metagenomes sampled during the TARA Oceans project (Parks et al., 2017; Tully et al., 2018). Based on physicochemical metadata, metagenomes and MAGs were subselected from the Arabian Sea OMZ core (TARA stations 37, 38, 39), the ETSP OMZ core (TARA stations 100, 102, 109, 110) and anoxic Mexican coastal waters (TARA station 111). The Black Sea and TARA MAGs were screened for dissimilatory sulfur genes as described in the following subsection. Additional genomes for phylogenetic reconstruction and functional annotation were selected from dysoxic marine water literature. Reference genomes were selected from GTDB r89 using AnnoTree v1.2.0 (Mendler et al., 2019). Quality of the genomes was assessed with CheckM v1.0.7 (Parks et al., 2015) in '--lineage_wf' mode. Black Sea MAGs were taxonomically classified with BAT (von Meijenfeldt et al., 2019) with the 'f' parameter set to 0.2, using the NCBI nr database dated January 8, 2019 and the dependencies Prodigal v2.6.3 (Hyatt et al., 2010) and Diamond v.0.9.21 (Buchfink et al., 2014). Additionally, all genomes were classified with GTDB-Tk v1.0.2 (Chaumeil et al., 2019) using GTDB r89 (Parks et al., 2017). They were screened for 16S rRNA genes with the 'ssu_finder' utility of CheckM. For phylogenetic reconstruction, genomes were selected based on >50% completeness, <10% contamination. A trimmed concatenated alignment of 120 single-copy household genes was constructed with the '–identify' and '–align' functionalities of GTDB-Tk. This alignment was used to construct a phylogenetic tree with IQ-TREE v1.6.10 (Nguyen et al., 2015) using the LG+C40+F+R4 evolutionary model determined to be the most suitable by ModelFinder based on the Akaike information criterion (Kalyaanamoorthy et al., 2017). Branch support was determined based on 1,000 bootstraps calculated by UFBoot2 (Hoang et al., 2018). Trees were inspected with FigTree v1.4.2[3] and visualized with iTOL (Letunic and Bork, 2019). Average amino acid identity (AAI) between genomes was calculated with the 'aai-matrix-diamond.sh' script from the enveomics package (Rodriguez-R and Konstantinidis, 2016).

6.1.3 Data availability

Raw DNA sequencing reads, cross-assembled metagenome sequences, MAG sequences and metadata from the Black Sea have been deposited at the European Nucleotide Archive as part of BioProject PRJNA649215.

6.1.4 Detection of functional marker genes

Homologs of functional marker genes were detected with Hidden Markov Models (HMMs), in part obtained from the PFAM (El-Gebali et al., 2018) and TIGRFAM (Haft et al., 2012) databases. HMMs for the detection of *dsrA*, *dsrB*, *dsrD*, *dsrE*, *dsrF*, *dsrH*, *dsrM*, *dsrK*, *dsrJ*, *dsrO*, *dsrP* and *dsrT* were kindly provided by Dr. Karthik

3 https://github.com/rambaut/figtree/releases

Anantharaman (Anantharaman et al., 2018). The remaining HMMs[4] for detection of *dsrC*, *dsrL*, *fccB/soxF*, *fsr*, *mccA_sirA*, *phsA/psrA/sreA*, *rdlA*, *sat*, *soeA*, *sorA*, *sorT*, *soxL*, *sqrA/sqrD*, *sqrB/sqrC*, *sqrE/sqrF*, *tetH*, *tsdA* and *ttrA* were constructed with HMMER version 3.1b2 (Eddy, 2009) following selection of reliable, divergent protein sequences based on literature, SwissProt and InterPro family descriptions, multiple alignment with Clustal Omega (Sievers et al., 2011), and threshold evaluation with HMMER searches against the SwissProt and UniProtRefProt databases using the EBI HMMER web server (Finn et al., 2015). Because of the close homology of the alpha subunits of polysulfide reductase (*psrA*), thiosulfate reductase (*phsA*) and sulfur reductase (*sreA*), we constructed an HMM for the detection of this molybdoenzyme subfamily encompassing *phsA*, *psrA* and *sreA*. Notably, this subfamily includes *psrA*-like genes which are present in cultivated sulfur-oxidizing microorganisms. Similarly, the flavoprotein disulfide reductase family includes six different types of sulfide-quinone oxidoreductase (*sqrA-F*; Gregersen et al., 2011) as well as flavocytochrome *c* sulfide dehydrogenase (known as *fccB*, *soxF* or *FCSD*; Marcia et al., 2010). However, in contrast to *psrA*-like genes, the extensive functional and phylogenetic investigations into *sqr* and *fccB* genes (Han and Perner, 2015; Shuman and Hanson, 2016) allowed us to construct HMMs for the separate detection of *sqrA/sqrD*, *sqrE/sqrF* and *fccB/soxF*. For detection of *dsrD* in the metagenome assembly, the scaffolds were first six-frame translated into protein sequences with the *transeq* tool. Coding sequences of individual genomes were predicted with Prodigal (Hyatt et al., 2010). The translated metagenome scaffolds and genomic coding sequences were queried with the HMM profiles with *hmmsearch* using default parameter settings. Subsequently, scaffolds or coding sequences with hits that passed the bitscore cutoff ('noise' or 'trusted', depending on the profile) were extracted from the *hmmsearch* output using the Bio.SearchIO module of the Biopython package (Cock et al., 2009). Calculations were done within the Pandas package data analysis framework (McKinney, 2010). A phylogenetic reconstruction was made with the *dsrA* genes from all genomes from dysoxic marine waters, the Black Sea metagenome and reference *dsrA* genes from Anantharaman et al. (2018). To extract *dsrA* genes from the Black Sea metagenome, coding sequences were first predicted with Prodigal using the '-p meta' option (Hyatt et al., 2010). HMM matches of less than 200 amino acids were discarded. The remaining sequences were aligned with Clustal Omega (Sievers et al., 2011). The alignment was curated by removing *dsrA* sequences with >70% gaps and dereplicating identical sequences. Subsequently, a bootstrapped phylogenetic tree was constructed with IQ-TREE v1.6.10 as described before using the LG+R4+F evolutionary model.

4 https://github.com/dmvvliet/protein-HMMs

6.1.5 Thermodynamic calculations

Gibbs free energies (ΔG [kJ/e^-], Table S3) were calculated as follows. Gibbs free energies of formation from Thauer et al. (1977) were used to calculate the standard Gibbs free energies (ΔG^0 [kJ/half-reaction]) of half-reactions. These ΔG^0 values were corrected to a pH of 8 with the Nernst equation assuming an *in situ* temperature of 283 K. The concentrations of and ammonium, nitrite and nitrate in the ETSP OMZ core at 80 m depth were obtained from Canfield et al. (2010), and the concentrations of those compounds as well as sulfide in the Black Sea at 110 m depth were obtained from Sollai et al. (2019). Other concentrations were estimated (Table S4). Activities were calculated using activity coefficients from Millero and Schreiber (1982) estimated for 25‰ salinity and 298 K: ammonium, 0.64; monovalent anions, 0.58; divalent anions, 0.109; gases, 1.2. The activity of S^0 in the Black Sea in the upper euxinic zone was previously estimated to be around 1 (Helz, 2014). For the ETSP OMZ core, we assumed S^0 to be mostly colloidal elemental sulfur and thus used an activity of 1.2. The Gibbs free energies (ΔG [kJ/half-reaction]) were corrected for these activities, again with the Nernst equation, and divided by the number of electron per half-reaction to obtain the Gibbs free energy per electron (ΔG [kJ/e^-]) for each half-reaction. The ΔG (kJ/e^-) values for complete reactions were obtained by subtracting the ΔG (kJ/e^-) of the electron donor half-reaction from that of the electron acceptor half-reaction (Table S3).

Table S4. Concentrations and partial pressures used for thermodynamic calculations. SCIs represent thiosulfate, tetrathionate and sulfite.

	Black Sea (110 m depth)	**ETSP OMZ core station 3 (80 m depth)**
Acetate (M)	1.00E-06	1.00E-06
Ammonium (M)	8.80E-06	1.0E-07
Bicarbonate (M)	3.0E-03	3.0E-03
H_2 (atm)	1.0E-06	1.0E-06
Methane (atm)	1.0E-05	1.0E-05
N_2 (atm)	0.8	0.8
Nitrate (M)	2.70E-08	1.2E-05
Nitrite (M)	1.30E-08	5.0E-06
SCIs (except S^0; M)	1.00E-08	1.00E-08
Sulfate (M)	2.0E-02	2.0E-02
Sulfide (M)	4.60E-06	1.00E-08

6.2 Supporting Information Protologue

This protologue contains descriptions of novel taxa proposed in this study. For uncultured taxa, high-quality metagenome-assembled genomes (MAGs) serve as type material as outlined by Chuvochina et al. (2019). This description has been updated to be consistent with the *Deltaproteobacteria* reclassification by Waite et al. (2020). MAG sequences were deposited at the European Nucleotide Archive as part of BioProject PRJNA649215.

Description of ^{U}*Sulfurimonas ponti* sp. nov.
^{U}*Sulfurimonas ponti* (pon'ti. L. gen. masc. n. *ponti*, of the sea). Genome analysis predicts the capacity for dissimilatory sulfide oxidation, nitrate reduction, ammonification and oxygen-based respiration. Appears to be a marine chemolithoautotrophic sulfur-oxidizing bacterium preferring euxinic waters, based on its distribution in the partially euxinic Black Sea water column. The type material is genome assembly NIOZ-UU79^{T} with accession number JACNLH000000000.

Description of ^{U}*Thiopontia* gen. nov., sp. nov.
^{U}*Thiopontia* (Thi.o.pon'tia. Gr. neut. n. *theion/θεῖον*, sulfur; Gr. fem. adj. *pontia/ποντιά*, from the sea; N.L. fem. n. *Thiopontia*, sulfur oxidizer from the sea). Member of the class *Gammaproteobacteria*. Type species is ^{U}*Thiopontia autotrophica*.

Description of ^{U}*Thiopontia autotrophica* sp. nov.
^{U}*Thiopontia autotrophica* (au.to.tro'phi.ca. Gr. masc. pron. *autos/αὐτός*, self; Gr. masc. adj. *trophikos/τροφικός*, one who feeds; N.L. fem. adj. *autotrophica*, one who feeds herself, autotrophic). Genome analysis predicts the capacity for dissimilatory oxidation of sulfide, elemental sulfur and dimethylsulfide, complete denitrification, oxygen-based respiration, and autotrophy. Appears to be a marine chemolithoautotrophic sulfur-oxidizing bacterium, based on its distribution in the partially euxinic Black Sea water column. The type material is genome assembly NIOZ-UU100^{T} with accession number JACNFK000000000.

Description of '*Candidatus* Pseudothioglobus' gen. nov.
^{U}*Pseudothioglobus* (Pseu.do.thi.o.glo'bus. Gr. masc. adv. *pseudos/ψεῦδος*, false; Gr. neut. n. *theion/θεῖον*, sulfur; L. masc. n. *globus*, ball, sphere; N.L. masc. n. *Pseudothioglobus*, false sulfur-oxidizing sphere). Member of the class *Gammaproteobacteria*. Type species is '*Candidatus* Pseudothioglobus singularis' (Marshall and Morris, 2013) comb. nov.

Description of '*Candidatus* Pseudothioglobus singularis' comb. nov.
Candidatus 'Pseudothioglobus singularis' (basionym, '*Candidatus* Thioglobus singularis' Marshall and Morris 2013; sin.gu.la'ris. L. adj. *singularis*, alone, singular). Type material is strain PS1^{T}.

Description of ^{U}*Pseudothioglobus aerophilus* sp. nov.
^{U}*Pseudothioglobus aerophilus* (ae.ro.phi'lus. Gr. n. *aer*/*ἀήρ*, air; Gr. masc. adj. *philos*/*φίλος*, loving; N.L. masc. adj. *aerophilus* air-loving, referring to a strict preference for oxygenated water). Appears to be a strictly aerobic marine bacterium, based on its distribution in the partially euxinic Black Sea water column. The type material is genome assembly NIOZ-UU104^{T} with accession number JACNFO000000000.

Emended description of the genus '*Candidatus* Thioglobus' (Marshall and Morris, 2013)
Member of the class *Gammaproteobaceria*. The type species is '*Candidatus* Thioglobus autotrophicus' (Shah et al., 2017).

Description of ^{U}*Thioglobus pontius* sp. nov.
^{U}*Thioglobus pontius* (pon'ti.us. Gr. masc. adj. *pontios*/*πόντιος*, from the sea; N.L. masc. adj. *pontius*, from the sea). Genome analysis predicts the capacity for dissimilatory oxidation of sulfide, elemental sulfur, thiosulfate and dimethylsulfide, incomplete denitrification of nitrate to nitric/nitrous oxide and microaerobic respiration. Appears to be a marine chemolithoautotrophic sulfur-oxidizing bacterium, based on its distribution in the partially euxinic Black Sea water column. The type material is genome assembly NIOZ-UU116^{T} with accession number JACNGB000000000.

Description of ^{U}*Desulfatifera* gen. nov.
^{U}*Desulfatifera* (De.sul.fa.ti.fe'ra. L. pref. *de,* off; N.L. masc. n. *sulfas –atis,* sulfate; L. fem. n. *fera*, a wild beast; N.L. fem. n. *Desulfatifera,* a wild sulfate reducer). Member of the family *Desulfocapsaceae*. Type species is ^{U}*Desulfatifera sulfidica*.

Description of ^{U}*Desulfatifera sulfidica* sp. nov.
^{U}*Desulfatifera sulfidica* (sul.fi'di.ca. N.L. fem. adj. *sulfidica*, sulfidic, referring to a high relative abundance in the sulfidic part of the Black Sea). Genome analysis predicts the capacity for the dissimilatory reduction of sulfate and tetrathionate, the ammonification of nitrite, and potentially diazotrophy. Appears to be have a preference for euxinic marine waters, based on its distribution in the partially euxinic Black Sea water column. The type material is genome assembly NIOZ-UU81^{T} with accession number JACNLK000000000.

Description of ^{U}*Desulfobia* gen. nov.
^{U}*Desulfobia* (De.sul.fo.bi'a. L. pref. *de,* off; L. neut. n. *sulfur,* brimstone, sulfur; Gr. masc. n. *bios*/*βίος*, life; N.L. fem. n. *Desulfobia,* a sulfur-reducing life-form). Member of the family *Desufurivibrionaceae*. Type species is ^{U}*Desulfobia pelagia*.

Description of ^{U}*Desulfobia pelagia* sp. nov.
^{U}*Desulfobia pelagia* (pe.la'gi.a. Gr. fem. adj. *pelagia*/*πελάγια*, from the sea). Genome analysis predicts the capacity for the dissimilatory reduction of sulfate, tetrathionate, the ammonification of nitrite, and diazotrophy. May reduce and/or disproportionate elemental sulfur and/or thiosulfate, based on its genetic capacity and distribution in the partially euxinic Black Sea water column. The type material is genome assembly NIOZ-UU47^{T} with accession number JACNJZ000000000.

Description of ^{U}*Desulfacyla* gen. nov.
^{U}*Desulfacyla* (De.sul.fa'cy.la. L. pref. *de,* off; N.L. neut. n. *sulfur,* brimstone, sulfur; L. fem. n. *akylos*/*ἄκυλος*, acorn; N.L. fem. n. *Desulfacyla,* a sulfur-reducing acorn). Member of the order *Desulfatiglandales*. Type species is ^{U}*Desulfacyla euxinica*.

Description of ^{U}*Desulfacyla euxinica* sp. nov.
^{U}*Desulfacyla euxinica* (eu.xi'ni.ca. Gr. masc. adj. *euxeinos*/*εὔξεινος*, hospitable; N.L. fem. adj. *euxinica*, euxinic, referring to a high relative abundance in the euxinic part of the Black Sea). Genome analysis predicts the capacity for the dissimilatory reduction of tetrathionate, dimethylsulfoxide, and possibly elemental sulfur, thiosulfate and nitrate. Thrives in both suboxic and euxinic marine waters, based on its distribution in the Black Sea water column. The type material is genome assembly NIOZ-UU27^{T} with accession number JACNJD000000000.

Description of ^{U}*Desulfobacula maris* sp. nov.
^{U}*Desulfobacula maris* (ma'ris. L. gen. neut. n. *maris*, of the sea). Genome analysis predicts the capacity for the dissimilatory reduction of sulfate, tetrathionate, dimethylsulfoxide, and possibly elemental sulfur, thiosulfate, nitrate, and oxygen. May oxidize sulfide and disproportionate elemental sulfur and/or thiosulfate, based on its genetic capacity and distribution in the Black Sea water column. The type material is genome assembly NIOZ-UU16^{T} with accession number JACNHV000000000.

Description of ^{U}*Desulfatibia* gen. nov.
^{U}*Desulfatibia* (De.sul.fa.ti.bi'a. L. pref. *de,* off; N.L. masc. n. *sulfas –atis,* sulfate; Gr. masc. n. *bios*/*βίος*, life; N.L. fem. n. *Desulfatibia,* a sulfate-reducing life-form). Member of the order *Desulfobacterales*. Type species is ^{U}*Desulfatibia profunda*.

Description of ^{U}*Desulfatibia profunda* sp. nov.
^{U}*Desulfatibia profunda* (pro.fun'da. L. fem. adj. *profunda,* deep, referring to a high relative abundance in the deep, euxinic part of the Black Sea). Appears to be a sulfate-reducing bacterium with a strictly euxinic habitat, based on its genetic capacity and occurrence in exclusively the euxinic part of the Black Sea. The type material is genome assembly NIOZ-UU30^{T} with accession number JACNJH000000000.

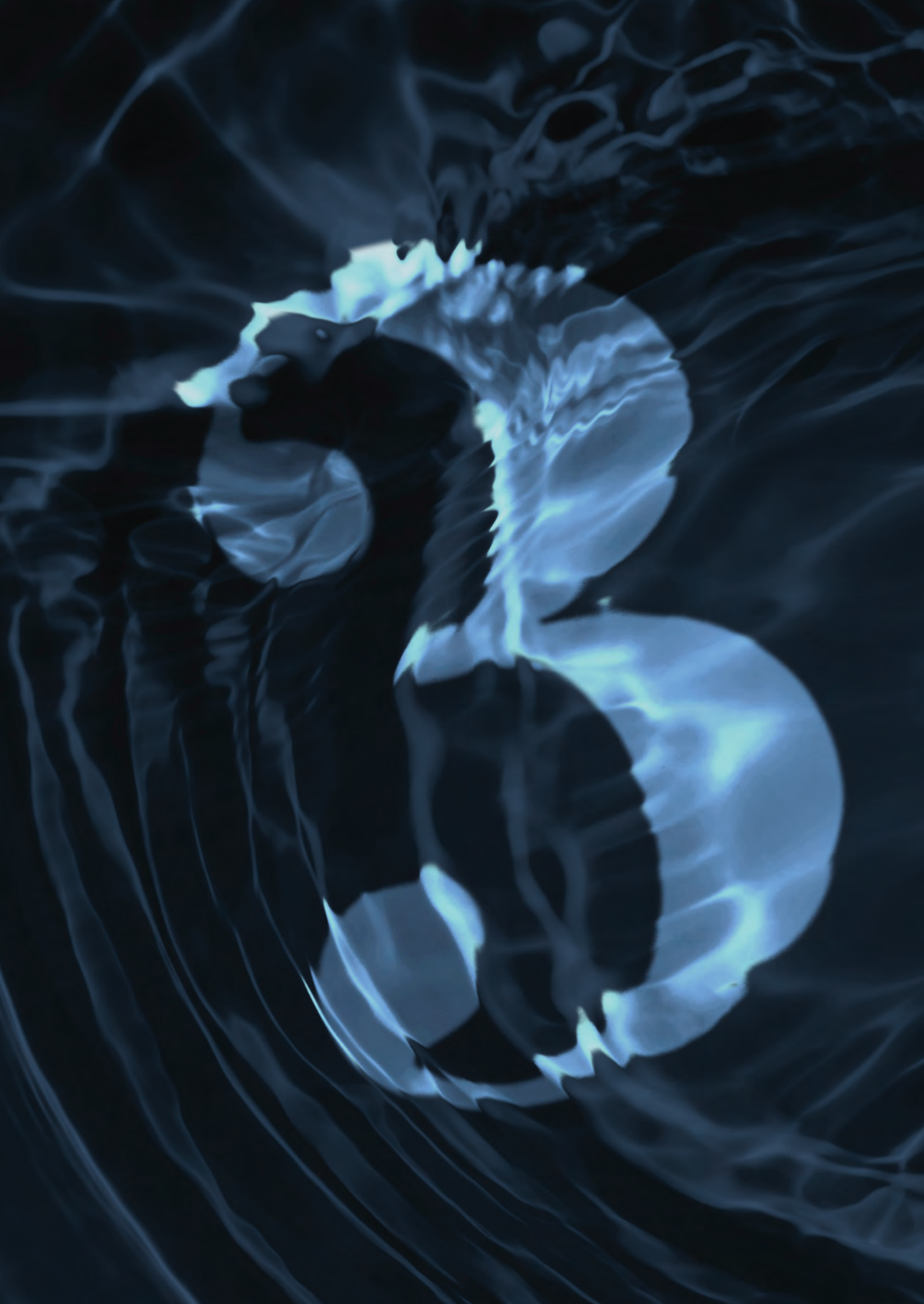
Description of ^{U}*Desulfatibia vada* sp. nov.
^{U}*Desulfatibia vada* (va'da. L. fem. adj. *vada,* shallow, referring to a high relative abundance in the suboxic zone of the Black Sea, which is more shallow than the euxinic zone). Genome analysis predicts the capacity for the reduction of sulfate, tetrathionate, and possibly nitrate (to nitrite or ammonia) and oxygen. May oxidize sulfide and/or disproportionate elemental sulfur, based on its genetic capacity and occurrence in exclusively the euxinic part of the Black Sea. The type material is genome assembly NIOZ-UU17^{T} with accession number JACNIG000000000.

Description of ^{U}*Desulfaltia* gen. nov.
^{U}*Desulfaltia* (De.sul.fal'ti.a. L. pref. *de,* off; L. neut. n. *sulfur,* brimstone, sulfur; L. masc. adj. *altus*, deep; N.L. fem. n. *Desulfaltia,* a sulfate reducer from the deep). Member of the order *Desulfobacterales*. Type species is ^{U}*Desulfaltia bathyphila*.

Description of ^{U}*Desulfaltia bathyphila* sp. nov.
^{U}*Desulfaltia bathyphila* (ba.thy.phi'la. Gr. masc. adj. *bathys*/*βαθύς*, deep; Gr. masc. adj. *philos*/*φίλος*, loving; N.L. fem. adj. *bathyphila*, deep-loving, referring to a high relative abundance in the deep, euxinic part of the Black Sea). Appears to be a sulfate-reducing bacterium with a strictly euxinic habitat, based on its genetic capacity and occurrence in exclusively the euxinic part of the Black Sea. The type material is genome assembly NIOZ-UU82^{T} with accession number JACNLL000000000.

Description of ^{U}*Desulfolinea* gen. nov.
^{U}*Desulfolinea* (De.sul.fo.li'ne.a. L. pref. *de,* off; L. neut. n. *sulfur,* brimstone, sulfur; L. fem. n. *linea*, line, string; N.L. fem. n. *Desulfolinea,* a sulfur-reducing string or line). Member of the order *Anaerolineales*. Type species is ^{U}*Desulfolinea nitratireducens*.

Description of ^{U}*Desulfolinea nitratireducens* sp. nov.
^{U}*Desulfolinea nitratireducens* (ni.tra.ti.re.du'cens. N.L. masc. n. *nitras*, *-atis*, nitrate; L. v. *reducere*, *-o*, to bring back; N.L. part. adj. *nitratireducens*, nitrate-reducing). Genome analysis predicts the capacity for the reduction of sulfite, tetrathionate and nitrate, and the oxidation of dimethylsulfide. May oxidize sulfide, ammonify nitrite and respire oxygen microaerobically, based on its genetic capacity and its high relative abundance in the suboxic zone of the Black Sea. The type material is genome assembly NIOZ-UU36^{T} with accession number JACNJN000000000.



CHAPTER 3

Sulfidogenic bacteria enriched from deep anoxic Black Sea sediment and the description of *Desulfopila canfieldii* sp. nov.

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Abstract

Sulfidogenesis encompasses microbial reduction of sulfate and other sulfur compounds. It is the main mineralization process in organic-rich surface sediments in the deep anoxic basin of the Black Sea. Here, sulfate reduction is thought to be mediated by deltaproteobacterial sulfate-reducing bacteria (SRB) and other, currently unidentified sulfidogens. We present a cultivation-based study to investigate the diversity and identity of sulfidogens in sediment samples from the Black Sea at 2,100 m depth. We set up enrichments with combinations of the electron donors H_2, formate, acetate, propionate, butyrate, or lactate, and the electron acceptors sulfate, thiosulfate or S^0, and analyzed the enriched communities with 16S rRNA gene amplicon sequencing. This resulted in enriched putative sulfidogens (*Desulfobacteraceae*, *Desulfobulbaceae*) as well as fermentative bacteria. In sulfate-reducing, acetate-degrading enrichments, *Aegiribacteria*/*Fermentibacteria* ssp. were enriched. In sulfur-reducing, acetate-degrading dilution enrichments, *Desulfuromonadales* Sva1033 bacteria became abundant. Sulfur-disproportionating enrichments contained canonical *Desulfocapsa* spp., but also other putatively disproportionating *Desulfobulbaceae* members. We isolated and characterized a novel SRB of the *Desulfopila* genus, strain LS5B^T, with lactate as original substrate. Strain LS5B^T was an incomplete oxidizer producing acetate from lactate during sulfate reduction, despite encoding the presumably bidirectional acetyl-CoA pathway, similar to its facultatively lithoautotrophic close relative *Desulfopila inferna* JS_SRB250Lac^T. Strain LS5B^T had the capacity for dissimilatory manganese reduction, an unusual trait among SRB of which the underlying biochemical mechanism is unknown. We propose strain LS5B^T represents the novel species *Desulfopila canfieldii*.

1 Introduction

The Black Sea is the world's largest anoxic basin, with 87% of its volume having anoxic, sulfidic conditions (Sorokin, 2002). Sulfide concentrations reach 0.4 mM in the water column (Murray et al., 1991) and 3 mM in sediments (Leloup 2007). Because of the reducing conditions in the water column, the deeper sediments (>150 m water depth) are not as biogeochemically diverse as marine sediments with oxic waters overlying them. They lack influx of oxygen or nitrate as potential electron acceptors, and therefore sulfate reduction is the main mineralization pathway (Sorokin, 1962). Sulfate reduction rates of 4-20 nmol cm^{-3} d^{-1} were recorded over the upper 15 cm of anoxic Black Sea sediments in the 2,100-m-deep western basin (Albert et al., 1995; Weber et al., 2001), accounting for >65% of the combined sedimentary sulfate reduction rates over an area of sediment (Jørgensen et al., 2001). Sulfate reduction is mediated by sulfate-reducing bacteria (SRB), of which cell numbers have been estimated using molecular surveys of the dissimilatory (bi)sulfite reductase alpha subunit marker gene (*dsrA*) and adenylyl 5'-phosphosulfate reductase alpha subunit marker gene (*aprA*). Putative SRB amounted up to $5 \cdot 10^7$ cells mL^{-1} in the upper 10 cm of western basin sediment at 1,000 m water depth (Leloup et al., 2007), and up to 10^8 cells mL^{-1} in surface sediment at 2,000 m water depth (Schippers et al., 2012). Overall, this amounted to approximately 5% of all cells.

Marine sediments generally harbor a high diversity of putative SRB mainly belonging to the class *Deltaproteobacteria*[5], based on *dsrAB* surveys (Müller et al., 2015; Wasmund et al., 2017). More specifically, 16S rRNA gene surveys indicate the dominant SRB in surface sediments belong to the *Desulfobacteraceae* and *Desulfobulbaceae* lineages (Robador et al., 2016). *Desulfatiglans*-related putative SRB are additionally abundant in subsurface sediments (Jochum et al., 2018). These groups were also detected in the the upper 50 cm of anoxic Black Sea sediment from 1,000 m water depth by a *dsrAB* survey (Leloup et al., 2007). However, novel non-deltaproteobacterial SRB are also thought to be present in marine sediments, indicated by the presence of the dissimilatory sulfate reduction pathway in metagenome-assembled genomes (MAGs) affiliating with *Dehalococcoidia* (Wasmund et al., 2016), *Gemmatimonadetes* and *Nitrospirae* (Baker et al., 2015). Such novel SRB may also play a role in Black Sea sediments, since they harbor *dsrA* sequences without clear affiliation (Leloup et al., 2007; confirmed by reanalysis, data not shown).

Besides dissimilatory sulfate reduction, additional types of sulfidogenesis from inorganic compounds are probably relevant in anoxic Black Sea sediment. Here, the term 'sulfidogenesis' refers to generation of sulfide as part of microbial energy metabolism.

5 The class *Deltaproteobacteria* was recently proposed to be reclassified into four new phyla, along with reclassification of many lower taxa (Waite et al., 2020). Current work adheres to the taxonomic framework prior to this restructuring.

In the western central basin of the Black Sea, oxidized metals introduced by turbidite sediments react abiotically with sulfide to form elemental sulfur (S^0; Yücel et al., 2010), and possibly also thiosulfate. The reduction and disproportionation of such sulfur cycle intermediates are biogeochemically important sulfidogenesis reactions in marine sediments (Jørgensen, 1990; Jørgensen and Bak, 1991). Furthermore, the dissimilatory sulfate reduction pathway can be involved in their reduction, disproportionation or even oxidation (Finster, 2008; Rabus et al., 2015; Thorup et al., 2017; Slobodkin and Slobodkina, 2019), implying that the putative SRB detected by molecular studies do not necessarily reduce sulfate. S^0 can also be reduced or disproportionated by non-SRB sulfidogens through several other, mostly poorly explored pathways (Rabus et al., 2013; Sánchez-Andrea et al., 2018). Cultivation using dilution series demonstrated the presence of sulfidogens growing through disproportionation of S^0 and thiosulfate in Danish marine sediments at reasonable abundance (up to 10^6 cells mL^{-1}; Jørgensen and Bak, 1991; Canfield et al., 1993b; Thamdrup et al., 1993). However, the identity of the dominant sulfur-reducing and sulfur-disproportionating sulfidogens in marine sediments has not been studied, in part due to the lack of proper functional marker genes.

The most common substrates used by SRB, and presumably also by other sulfidogens, are hydrogen (H_2) and low-molecular weight organic compounds such as formate, acetate, propionate, butyrate, ethanol or lactate (Muyzer and Stams, 2008). These compounds are produced in marine sediments by fermentation of organic matter. The inhibition of sulfate reduction with molybdate in sulfidic coastal sediments resulted in accumulation of mostly acetate, but also propionate, butyrate and hydrogen, indicating that these are important substrates *in situ* (Sørensen et al., 1981; Winfrey and Ward, 1983). Similar experiments with various marine and freshwater sediments indicated that lactate and amino acids also play an important role, albeit irregular between sediments (Smith and Klug, 1981; Parkes et al., 1989). Accordingly, all of these substrates have been used in studies aiming to isolate dominant SRB from anoxic marine sediment through dilution cultivation, either separately (Llobet-Brossa et al., 2002; Gittel et al., 2008; Kraft et al., 2013) or as mixture (Köpke et al., 2005; Kraft et al., 2013). Many SRB have been isolated and described from various marine sediments, including representatives of *Desulfobacteraceae* and *Desulfobulbaceae* (Widdel and Bak, 1992; Rabus et al., 2013). However, SRB have not been cultivated from deep Black Sea sediments, although their activity has been repeatedly demonstrated (Sorokin, 1962; Lein and Ivanov, 1990; Lein et al., 1990; Albert et al., 1995; Weber et al., 2001). S^0 is abundantly present in anoxic Black Sea sediments (Yücel et al., 2010), but whether it is used by sulfidogens is not known. In this study, we address the presence, identity and physiology of sulfidogens in Black Sea surface sediments by cultivation with different electron donors and different inorganic sulfur compounds as electron acceptors and by 16S rRNA gene amplicon sequencing of enrichments. Moreover, we describe the isolation and characterization of SRB strain LS5B^T and propose the name *Desulfopila canfieldii* sp. nov..

2 Materials and methods

2.1 Enrichment

The sediment serving as inoculum for this study was sampled from the Black Sea western basin in February 2016 (Van Vliet et al., 2019). A previously described marine bicarbonate-buffered basal medium was used (Van Vliet et al., 2019). All chemicals were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), except for S^0 (Riedel-de Haën, Seelze, Germany). All enrichments and dilution series were carried out in duplicate in 120-mL serum vials containing 50 mL of medium and a 1.5 atm N_2/CO_2 or H_2/CO_2 headspace (80:20 v/v), sealed with butyl rubber stoppers (Rubber BV, Hilversum, Netherlands) and aluminum caps. All substrates were added from anoxic, sterile, separately autoclaved stocks.

Enrichment cultures were set up for conditions that were expected to yield relatively slow growth (acetate, propionate or butyrate; disproportionation), whereas dilution series were used for conditions expected to yield relatively fast growth (lactate, formate & acetate, H_2 & acetate). Enrichment cultures and dilution series were started with 3 mL of sediment slurry that effectively contained 0.5 mL of sediment, resulting in a 100-fold volumetric dilution of the sediment. Enrichment cultures with acetate (7.5 mM), propionate (5 mM) or butyrate (3.75 mM) as electron donor for sulfate reduction were incubated at 15°C and regularly sampled for chemical analyses as described previously (Van Vliet et al., 2019). Disproportionation enrichment cultures contained basal medium with thiosulfate (10 mM) or S^0 (30 mmol/L) and amorphous ferric oxyhydroxide (20 mmol/L) as sulfide sink. They were incubated at 20°C. Sulfide was monitored using the methylene blue method (Cline, 1969), and sulfate by chromatography as described previously (Van Vliet et al., 2019).

Dilution series consisted of eight subsequent tenfold diluted cultures, thus reaching a final dilution factor of 10^{10}. Lactate (10 mM), formate (10 mM) and acetate (5 mM), or H_2 (1.2 atm) and acetate (5 mM) was added as energy and carbon source. As electron acceptor, either sulfate (10 mM), thiosulfate (10 mM) or S^0 (20 mmol L^{-1}) was added to sulfate-free medium, from which $MgSO_4$ was omitted and replaced by the same molarity (17 mM) of $MgCl_2$. The dilution series were incubated at 20°C and monitored by optical density (OD_{600}) measurements and sulfide measurements. The basal medium contained sulfide as reducing agent, present at 1.0-1.5 mM in reduced media. An increase in sulfide concentration to >3.5 mM was interpreted as positive indication for growth.

2.2 Community analysis

DNA for community analysis was extracted from samples of 5 mL with the FastDNA Spin Kit for Soil (MP Biomedicals, OH, USA) and purified with the Zymo DNA Clean & Concentrator it (Zymo Research, CA, USA) as described in more detail previously (Van Vliet et al., 2019). Part of the V4-V5 region of the 16S rRNA genes was amplified through polymerase chain reaction (PCR) with primers 515f and 806rB (Supplementary Material). The 16S rRNA genes in technical duplicate DNA extractions of acetate, propionate and butyrate enrichments were amplified with a two-step PCR for initial amplification and barcode attachment and sequenced as described previously (Van Vliet et al., 2019). Other samples were processed with a one-step PCR using barcode-primer fusions. Duplicate PCRs of 50 µL were performed, each containing 1 µL of the extracted DNA as template, HF PCR buffer, 0.2 mM dNTPs, 0.02 U mL^{-1} Phusion Hot Start II DNA polymerase (Thermo Scientific, Waltham, MA, USA), and 0.2 mM of forward and reverse primer. The amplification program consisted of an initial denaturation step at 98°C for 30 seconds, followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 50°C for 10 seconds and elongation at 72°C for 10 seconds, followed by a final extension step at 72°C for 7 minutes. A negative control PCR without template DNA was included. Duplicate PCR products were pooled and the length of the amplicons (approximately 400 bp) was examined by gel electrophoresis in a 2.2% w/v agarose gel containing the nucleic acid stain SYBR Safe (Thermo Scientific, Waltham, MA, USA). Pooled PCR products were then purified with the HighPrep PCR kit (MagBio Genomics Inc., Gaithersburg, MD, USA). Their DNA concentration was measured with the Qubit dsDNA BR assay kit (Invitrogen, Carlsbad, CA, USA) and a Qubit 2.0 fluorometer (Life Technologies, Darmstadt, Germany).

The amplicons were sequenced with the Illumina HiSeq platform using NovaSeq 6000 S2 sequence mode (GATC Biotech, Konstanz, Germany), yielding paired end 150-bp reads. All amplicon data was processed with the pipeline NG-Tax v1.0 (Ramiro-Garcia et al., 2016). Dominant operational taxonomic units (OTUs) were taxonomically classified with the SILVA ACT service (Pruesse et al., 2012) and SILVA database Ref NR SSU r132 (Quast et al., 2013). The demultiplexed amplicon data of the acetate, propionate and butyrate enrichments were deposited at the European Nucleotide Archive (ENA) under accession numbers SAMEA4717800-SAMEA4717811. Demultiplexed amplicon data of other amplicon data was deposited under study accession PRJEB37825.

2.3 Isolation

For isolation of strain LS5B^T, the pour plate technique was used. The basal medium was amended with 1.5% w/v SeaPlaque agarose (Lonza, Basel, Switzerland), and phosphate

salts were autoclaved separately to increase cultivability as reported by (Tanaka et al., 2014). The inoculated and solidified pour plates were incubated in anaerobic jars with an N_2/CO_2 (80:20 v/v) gas phase in the dark. Liquid cultures were made with picked colonies as inoculum, and pour plating was repeated with the aim of ensuring isolate purity. This was confirmed by full-length 16S rRNA gene Sanger sequencing and analysis using the 27F and 1492R primers (Supplementary Material) as described by Van Vliet et al. (2019), with negative control cultures containing 20 mM of D-glucose and/or 1 g L^{-1} yeast extract incubated at 20°C and 37°C, and by inspecting morphology through phase contrast microscopy with a Leica DM2000 microscope (Leica Microsystems, Wetzlar, Germany). The full-length 16S rRNA gene of strain LS5B^T was deposited at the ENA with accession number LR792819. Pure cultures of strain LS5B^T were deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany) under accession number DSM 108673^T, and at the Korean Collection for Type Cultures (KCTC; Jeongeup-si, South Korea) under accession number KCTC 15796^T.

2.4 Physiological tests

All cultures of strain LS5B^T were incubated statically at 25°C in the dark with 20 mM lactate as electron donor and 17 mM sulfate as electron acceptor, unless mentioned otherwise. The effect of temperature on growth was tested in triplicate cultures incubated at 4, 10, 15, 20, 25, 30 and 37°C. The effect of salinity on growth was tested in triplicate cultures containing 0%, 0.5%, 1.1%, 1.7%, 2.4%, 3.0%, 3.6%, 4.2%, 4.8% and 5.4% NaCl. The effect of pH on growth was tested in triplicate cultures buffered with 50 mM of MES (pH 5.5, 5.8, 6.1, 6.4), HEPES (pH 6.7, 7.0, 7.3, 7.6) and Tris (pH 7.9, 8.2, 8.5). Growth rates were calculated by fitting OD_{600} measurements to a modified Gompertz model (Zwietering et al., 1990).

The following substrates were tested (10 mM unless indicated otherwise): H_2/CO_2 (80:20 v/v, 1.5 atm), H_2/CO_2 with acetate (5 mM), formate (20 mM), formate with acetate (5 mM), acetate (15 mM), propionate, butyrate (5 mM), lactate, pyruvate, malate, succinate, fumarate, methanol, ethanol, propanol, butanol, glycerol, L-glycine, L-alanine, L-serine, D-glucose (5 mM) and yeast extract (1 g L^{-1}). The following electron acceptors were tested in duplicate cultures (concentration in mM): sulfate (17), sulfite (2), thiosulfate (20), biologically produced S^0 (50), nitrate (5), nitrite (2), ferric iron citrate (20), manganese oxide (MnO_2, 10), dimethylsulfoxide (10). Amorphous MnO_2 was prepared from $KMnO_4$ and $MnCl_2$ as previously described (Burdige and Nealson, 1985). Disproportionation was tested in sulfate-free cultures containing thiosulfate (20 mM) or S^0 (50 mM) as substrate, ferric iron citrate (20 mM) or ferric iron oxyhydroxide (20 mM) as sulfide sink and acetate (5 mM) as carbon source. Cultures were incubated for up to four months.

Analysis of respiratory quinones was carried out by the Identification Service of the DSMZ. Catalase activity was tested by applying drops of 15% v/v hydrogen peroxide onto a cell pellet obtained by centrifuging active biomass at 13,400 *g* for five minutes. Oxidase was tested by applying a cell pellet to a filter paper soaked with a tetramethyl-p-phenylenediamine solution. Gram staining was performed following standard protocols and confirmed by applying a drop of 1 M NaOH solution onto a cell pellet, which should lead to slimy wire formation within ten seconds for Gram-negative microorganisms. Scanning electron microscopy (SEM) was performed as previously described (Van Vliet et al., 2019). Cell size was determined from SEM micrographs. Methods used for the analysis of intact polar lipids (IPLs) and cellular fatty acids (CFAs) have been described previously (Van Vliet et al., 2020).

2.5 Genome sequencing, assembly and annotation

Genomic DNA was extracted from a cell pellet, which was obtained from a 100-mL culture centrifuged at 10,000 x *g* for ten minutes, using a chloroform:isoamyl alcohol extraction method (Salvà Serra et al., 2018) modified from Marmur (1961). The quality of the extracted DNA was evaluated with the Qubit dsDNA BR assay (Thermo Fisher Scientific, Waltham, MA, USA) and through electrophoresis on a 1% w/v agarose gel. The genomic DNA was sequenced with the Illumina HiSeq platform (Novogene, Beijing, China) yielding paired-end reads of approximately 250 bp. Quality and length of the reads were inspected with FastQC version 0.10.1 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). A genome assembly was made with SPAdes v3.13.0 (Bankevich et al., 2012). All contigs with shorter than 2,000 bp or having a coverage lower than 60x were removed with Bandage version 0.8.1 (Wick et al., 2015). The size and G+C content of the resulting draft genome were determined with QUAST v4.2 (Gurevich et al., 2013).

The draft genome was annotated with Prokka version 1.12 (Seemann, 2014) and with HMMer v3.1b2 (Eddy, 2009) using a set of Hidden Markov Models (HMMs) from the PFAM (El-Gebali et al., 2018) and TIGRFAM (Haft et al., 2001) databases (Table S1), specific HMMs for *dsr* genes (Anantharaman et al., 2018), as well as custom-made HMMs (https://github.com/dmvvliet/protein-HMMs). Putative hydrogenase genes annotated by prokka were classified with HydDB (Søndergaard et al., 2016). Homologous genes of the reductive acetyl-CoA pathway were confirmed with InterProScan version 5.47-82.0 (Jones et al., 2014) and with blastp queries against the UniProtKB/Swiss-Prot and RefSeq databases. Raw reads were deposited at ENA under study accession PRJEB37825. The draft genome assembly and the Prokka annotation have been deposited at 4TU.ResearchData under digital object identifier (DOI) 10.4121/13317545 and can be accessed with the following link: https://figshare.com/s/efdffafc61ed017b4eb3. For comparison, The *Desulfopila aestuarii* draft

genome with accession number GCA_900143695.1 was annotated using the same methodology.

2.6 Phylogenetic reconstruction

A maximum-likelihood phylogenetic tree of 16S rRNA gene sequences of cultured *Desulfobulbaceae* from the SILVA Ref NR SSU r132 database was made in ARB version 6.0.2 (Westram et al., 2011) using RAxML v7.7.2 (Stamatakis, 2014) with the GTRGAMMAI model, vertical gaps compression, a 'termini' filter and 100 rapid bootstraps (Stamatakis et al., 2008). An identity matrix was calculated with the ARB distance matrix tool. A maximum-likelihood phylogenomic tree of previously described *Desulfobulbaceae* spp. was constructed with IQ-TREE v1.6.12 (Nguyen et al., 2015) from a filtered concatenated alignment of single-copy genes generated with GTDB-Tk v.1.0.2 (Chaumeil et al., 2019). The LG+C40+F+R4 model was selected as optimal choice from a broad selection of models with ModelFinder (Kalyaanamoorthy et al., 2017). The robustness of the tree was evaluated with 1,000 ultra-fast bootstraps (Hoang et al., 2018). The tree was inspected with FigTree v1.4.2 (https://github.com/rambaut/figtree) and visualized with iTOL (Letunic and Bork, 2019). Average amino acid identity (AAI) between genomes was calculated with the 'aai.rb' and 'aai-matrix.sh' scripts from the enveomics package (Rodriguez-R and Konstantinidis, 2016) using versions of blastp (Camacho et al., 2009) or DIAMOND (Buchfink et al., 2014). A bootstrapped hierarchical clustering dendrogram of cellular fatty acid profiles was constructed in R version 3.6.3 (R Core Team, 2020) with the *pvclust* package (Suzuki et al., 2019) using Bray-Curtis dissimilarity implemented with the *vegan* package (Oksanen et al., 2019), single-linkage clustering and 1,000 bootstraps.

3 Results

3.1 Acetate, propionate and butyrate enrichments

The fatty acid enrichment cultures showed degradation of acetate, propionate and butyrate coupled to sulfate reduction within 51, 81 and 41 days, respectively (Figure S1). Acetate enrichments showed increased abundance of *Desulfobacter*, *Desulfuromonas*, *Marinifilum, Marinilabiliaceae* and *Aegiribacteria* (Figure 1). Propionate enriched for *Desulfobacterium* and an uncultured *Clostridiales* group. Propionate enrichment A also contained groups of *Bacteroidetes*. Butyrate enriched for mostly undescribed

Desulfobacteraceae spp. and *Clostridiales* groups. Technical duplicates were reproducible ($r > 0.99$), except for those of acetate enrichment B ($r = 0.46$), which showed a difference in the relative abundance of *Desulfobacter* sequences between the two duplicates (Figure S2). Some taxa detected in the enrichments were also detected in high proportion in the inoculum, such as *Desulfatiglans* (9.1%), and *Thermoflexus* (4.6%, Figure 1). Thus, these taxa were not necessarily enriched in our cultures since they could have persisted instead.

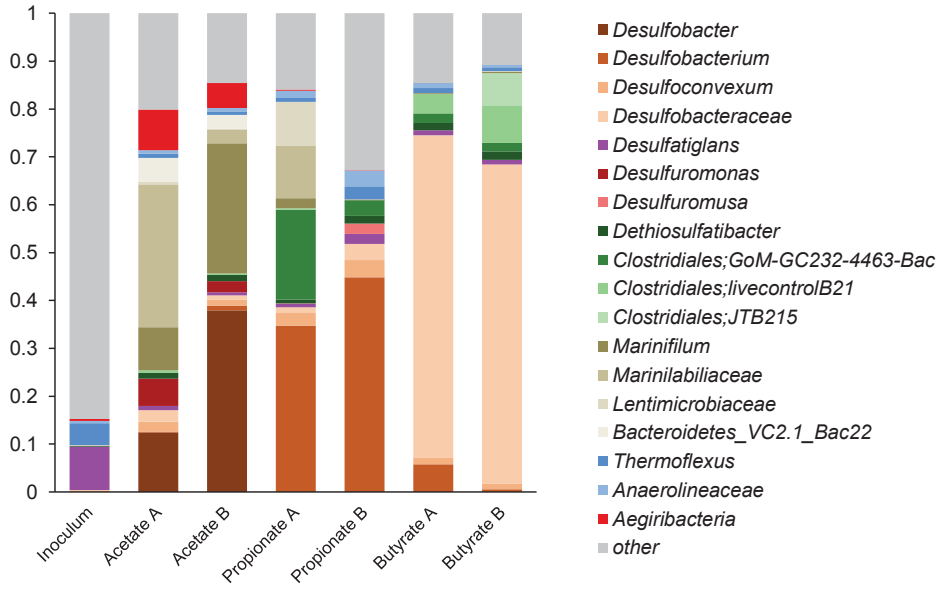


Figure 1. Community analysis of sulfate-reducing enrichment cultures by 16S rRNA gene amplicon sequencing. Shown percentages are the average of two technical duplicate analyses from the same culture. Names on the x-axis indicate electron donor and replicate 'A' or 'B'.

3.2 Dilution series and disproportionation enrichments

After three months of incubation, dilution series with different combinations of electron donors (H_2 and acetate, formate and acetate, or lactate) and acceptors (S^0, thiosulfate, or sulfate) showed growth in cultures with a high dilution factor (Figure 2). The highest dilution factor showing growth with sulfate as electron acceptor was 10^7 with lactate as electron donor. The highest dilution factor showing growth with S^0 or thiosulfate as electron acceptor was 10^6 with H_2 or formate as electron donor in combination with acetate. In the dilution enrichments and subsequent transfer cultures, acetate was

completely consumed (5 mM) whereas H_2 and formate were incompletely consumed. Thus, although intended as carbon source, acetate served as substituting or additional electron donor. After two months of incubation, disproportionation enrichments showed production of sulfide and sulfate. Although optical density measurements were not possible due to the iron suspension, microscopy suggested a substantial increase in the number of cells.

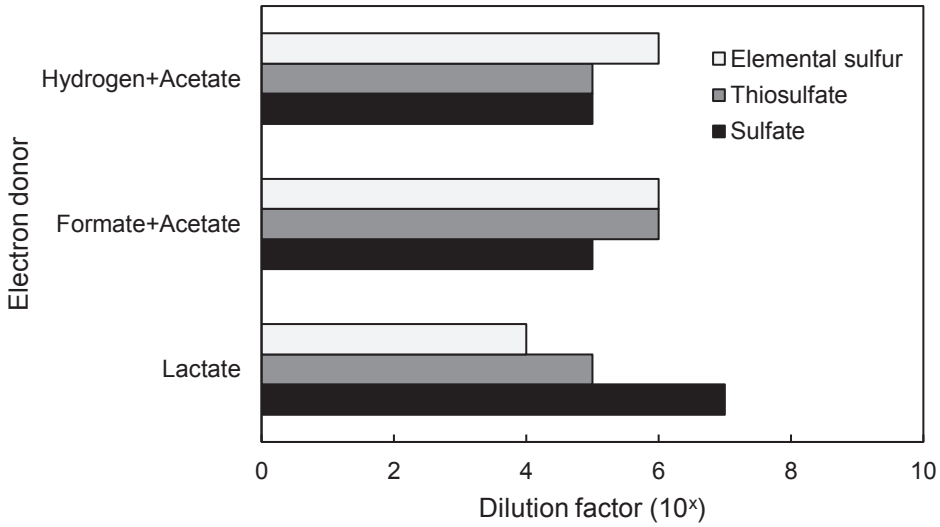


Figure 2. The highest dilution factor in which growth was observed in both replicate liquid dilution series with different electron donors and acceptors.

Dilution enrichments with lactate as electron donor mostly yielded different communities, even between replicate conditions (Figure 3). A single *Desulfobulbaceae* OTU dominated the sequences from the fifth dilution of lactate/sulfate dilution series B, whereas the fifth dilution of the parallel dilution series A showed a more diverse community in which *Desulfoconvexum* sequences were dominant and *Desulfobulbaceae* were absent. The specific *Desulfobulbaceae* OTU was also dominant in the third dilution of the H_2 and acetate/sulfate series A. *Desulfoconvexum* OTUs were dominant in all formate+acetate dilutions with S^0 or thiosulfate as electron acceptor, and in all H_2 and acetate dilutions with sulfate or thiosulfate as electron acceptor. Subculturing of the lactate/sulfate *Desulfoconvexum* dilution lead to a loss of *Desulfoconvexum* sequences. Instead, *Dethiosulfatibacter* became dominant. This taxon was also dominant in a lactate/thiosulfate subculture, but the community of the respective dilution enrichment was not analyzed. The second dilution of lactate/sulfur series A was dominated by *Desulfovibrio*

and *Dethiosulfatibacter* sequences, whereas the third dilution of lactate/sulfur series B was dominated by a *Halodesulfovibrio* OTU.

In contrast to the poor reproducibility in enrichments and subculture with other conditions, microbial communities were similar in all dilutions and subcultures with S^0 as electron acceptor and H_2 or formate in combination with acetate as electron donor. These conditions consistently resulted in the dominance of the *Desulfuromonadales* Sva1033 clade. Sequences from disproportionation enrichments showed a high relative abundance of *Desulfocapsa,* but also of other *Desulfobulbaceae* from not yet defined genera. Presumed fermentative bacteria were enriched across all conditions (*Lachnospiraceae, Marinifilaceae, Prolixibacteraceae, Marinilabiliaceae, Spirochaetaceae, Synergistaceae, Izimaplasmataceae*).

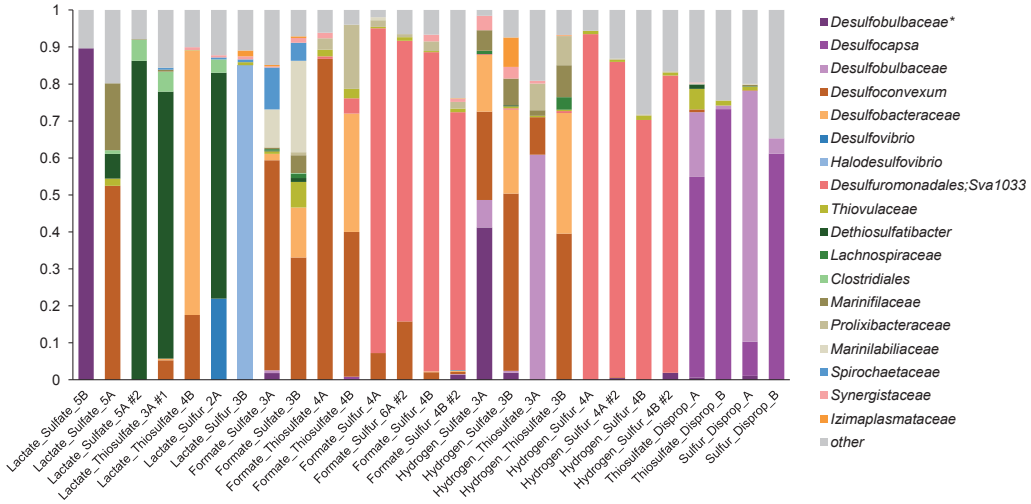


Figure 3. Community analysis of dilution cultures and disproportionation enrichments. Culture names indicate electron donor, electron acceptor, the number of the dilution culture in the dilution series, 'A' or 'B' indicating duplicate dilution series, and optionally a '#' symbol followed by a number, indicating this sample is not the initial dilution enrichment, but a culture propagated/transferred # times. The full-length 16S rRNA gene of isolate LS5B^T was blasted against the OTUs, resulting in one OTU displaying 100% identity. This OTU was marked with an asterisk as '*Desulfobulbaceae**'.

3.3 Isolation and phenotypic characterization of strain LS5B^T

Strain LS5B^T was isolated from the fifth dilution of lactate/sulfate dilution series B. It represents the OTU dominant in this enrichment (Figure 3). Based on the full-length 16S rRNA gene, strain LS5B^T is most closely related to *Desulfopila aestuarii* MSL86^T, *Desulfotalea psychrophila* LSv54^T and *Desulfotalea arctica* LSv514^T (96% identity, Table

1). Colonies were visible within two weeks of incubation of the plates. Strain LS5B^T cells were Gram-negative, non-motile rods of 0.6-0.7 µm wide and 1.4-6.0 µm long (Figure 4). The draft genome of strain LS5B^T consisted of 52 contigs with a mean sequencing depth of 74 and a genome size of 7.0 Mbp. Strain LS5B^T required yeast extract (0.1 g L^{-1}) for growth in pure culture. No sporulation was observed. Catalase and oxidase activity tests were negative.

The optimal temperature for growth was 30°C, with a doubling time of 17 h. Growth was optimal at a NaCl concentration of 1.1-2.4% (w/v) and a pH of 7.0-7.3. Lactate was oxidized incompletely to acetate and CO_2. Apart from lactate, growth was supported (with sulfate as electron acceptor) with H_2/CO_2, formate, pyruvate, malate, succinate, fumarate, ethanol, propanol, butanol, glycerol and L-alanine (Table 1). Strain LS5B^T grew fermentatively in sulfate-free medium with pyruvate as substrate, but not with lactate. Fermentative growth with fumarate or glycerol was not tested. Growth was possible without acetate as carbon source, as strain LS5B^T was grown with H_2/CO_2 or formate as electron donor in five subsequent subcultures. This was consistent with the presence of genes encoding periplasmic [NiFe] hydrogenases, formate dehydrogenases and the reductive acetyl-CoA pathway (Table S1).

Strain LS5B^T grew in cultures supplied with sulfite, thiosulfate or DMSO as alternative electron acceptor to sulfate, although these cultures had a lag phase of two to three months. Sulfite and thiosulfate cultures produced sulfide, and DMSO cultures produced the distinct smell of dimethylsulfide (DMS), indicating reduction of these electron acceptors. In line with these results, genes for the dissimilatory reduction of sulfate and sulfite (*sat, aprBA* and *dsrABCDMKJOPT*; Table S1) and a putative thiosulfate reductase complex (*phsABC*, Table S1) were detected. Ferric iron citrate as electron acceptor supported weak growth, with only few encrusted, misshaped cells visible by microscopy. Whether ferric iron was reduced was not tested, and thus remains unclear. Amorphous MnO_2 was visibly reduced by strain LS5B^T from black to grey. Although OD_{600} could not be reliably quantified due to the presence of the manganese salt precipitates, microscopy indicated abundant growth of cells with normal morphology. Nitrate, nitrite or S^0 did not support growth. No growth was observed with lactate and sulfate in ammonium-free medium, despite the presence of nitrogen fixation genes (*nifDHK*, Table S1).

Table 1. Differential physiological and chemotaxonomic characteristics of strain LS5B^T and related species. All data of strain LS5B^T, the in silico G+C content, 16S rRNA gene identity and amino acid identity (AAI) were determined in this study. Data for *Dp. aestuarii* MSL86^T was obtained from Suzuki et al. (2007), for *Dp. inferna* JS_SRB250Lac^T from Gittel et al. (2010), the *Desulfotalea* spp. from Knoblauch et al. (1999), *Dc. catecholica* NZva20^T from Szewzyk and Pfennig (1987) and Galushko and Kuever (2015), and *Dr. singaporensis* T1^T from Lie et al. (1999). *) longer than 8 months incubation. NA: not applicable. ND: not determined. Intact polar lipid abbreviations: DPG: diphosphatidylglycerol. PA: phosphatidic acid. PE: phosphatidylethanolamines. PG: phosphatidylglycerol.

Species	***Dp. canfieldii***	***Dp. aestuarii***	***Dp. inferna***
Type strain	LS5B^T	MSL86^T	JS_SRB250Lac^T
Isolation source	sulfidic marine sediment	estuarine sediment	tidal sediment
Cell shape	Rod	Rod	Rod
Motility	-	+	-
Cell size (µm)			
Width	0.6 - 0.7	0.7 - 1.2	0.3-0.5
Length	1.4 – 6.0	1.9 - 3.8	1.0-2.0
Genome size (Mb)	7.0	6.1	NA
DNA G+C content (mol%) *in vitro*	ND	54.4	50.3
DNA G+C content mol%) *in silico*	44.3	49.6	NA
Major cellular fatty acids (>10% of total)	$C_{16:1}\omega7$, $C_{16:1}\omega5$, $C_{16:0}$	$C_{16:0}$, $C_{16:1}\omega5$, $C_{17:1}\omega6$	$C_{16:0}$, $C_{16:1}\omega7$, *cyc*-$C_{17:0}$, $C_{18:0}$, $C_{16:1}\omega5$
Polar lipids	PA, PG, DPG, PE, Acyl-PG	ND	ND
Quinones	MK-5(H_2)	MK-8(H_4)	ND
16S rRNA gene identity with strain LS5B^T (%)	NA	95.6	95.3
AAI with strain LS5B^T (%)	NA	69.0	NA
Temp. for growth (°C)			
Range	10 – 30	10 – 40	10-35
Optimum	30	35	28
NaCl for growth (% w/v)			
Range	0.5 - 4.8	0 - 5.0	0.5-5.0
Optimum	1.1 - 2.4	1	2-3
pH for growth			
Range	6.4 - 8.2	6.3 - 8.5	ND
Optimum	7.0 - 7.3	7.5 - 7.6	ND
Electron donors (sulfate reduction)			
H_2/CO_2 + acetate	+	-	+
H_2/CO_2	+	-	+
Acetate	-	-	-
Propionate	-	-	-
Butyrate	-	-	+
Malate	+	-	-
Succinate	+	-	+
Fumarate	+	+	+
Methanol	-	-	ND
Propanol	+	+	+
Butanol	+	+	+
Glycerol	+	+	+
L-glycine	-	-	-
L-alanine	+	-	-
L-serine	-	-	-
Electron acceptors			
Sulfite	+	+	+
Thiosulfate	+	+	-
Nitrate	-	ND	-
DMSO	+	ND	ND

	Dt. psychrophila	***Dt. arctica***	***Dc. catecholica***	***Dr. singaporensis***
	LSv54^T	LSv514^T	NZva20^T	T1^T
	arctic marine sediment	arctic marine sediment	Anoxic bay mud	sulfidic marine mud
	Rod	Rod	Oval to lemon	Rod
	+	-	-	-
	0.6	0.7	1.3-1.8	0.9-1.2
	4.5 - 7.4	1.6 - 2.7	2.2-2.8	1.7-2.3
	3.7	NA	NA	5.0
	46.8	41.8	52.4	50.6
	46.6	NA	NA	50.6
	$C_{16:1}\omega7$, $C_{16:1}\omega5$	$C_{16:1}\omega7$, $C_{16:1}\omega5$	ND	ND
	PE, PG, DPG	PE, PG, DPG	ND	ND
	MK-6(H_2)	MK-6	ND	ND
	95.9	96.3	95.4	93.5
	56.4	NA	NA	59.4
	-1.8 – 19	-1.8 – 26	ND	20-35
	10	18	28	31
	ND	ND	<0.5	ND
	1	1.9 - 2.5	0.1	ND
	ND	ND	ND	6.0-8.2
	7.3 - 7.6	7.2 - 7.9	6.9-7.1	7.4
	+	+	+	-
	-	-	+	-
	-	-	+	-
	-	-	+	+
	-	-	+	+
	+*	-	+	+
	-	-	ND	+
	+	-	+	+
	-	-	+	ND
	+	-	+	+
	+	-	+	+
	-	+	ND	ND
	+	-	ND	ND
	+	-	ND	+
	+	+*	ND	ND
	+	-	+	+
	+	-	+	+
	-	-	+	+
	ND	ND	ND	-

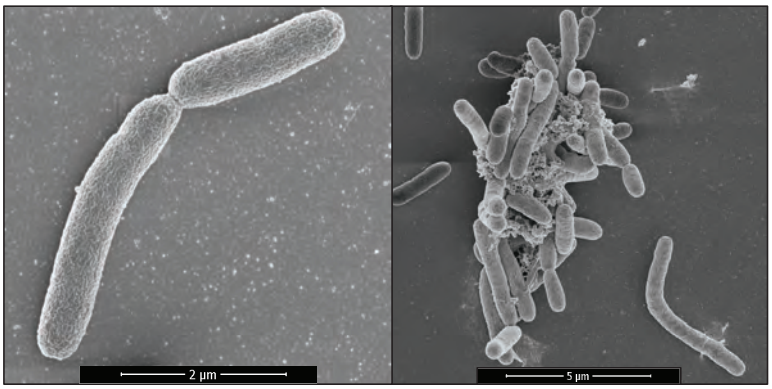


Figure 4. Scanning electron micrographs of strain LS5B^T.

3.4 Phylogeny and chemotaxonomy of strain LS5B^T

Based on the full-length 16S rRNA gene sequence (1,413 bp), strain LS5B^T was classified by the SILVA ACT service as *Desulfobulbaceae*, without affiliation to an established genus. The 16S rRNA gene analysis indicated that *Desulfotalea* spp. and *Dp. aestuarii* MSL86^T (Figure 5A) are closely related to strain LS5B^T, sharing 96% 16S rRNA gene identity (Table S2). Phylogenomics demonstrated a closer affiliation to *Dp. aestuarii* MSL86^T than to *Dt. psychrophila* LSv54^T (Figure 5B). This agrees with the classification of LS5B^T to the genus *Desulfopila* by genome-based classifier GTDB-Tk (Chaumeil et al., 2019). However, the statistical probability that strain LS5B^T belongs to the genus *Desulfopila* based on whole-genome comparison was low (P = 0.33), as calculated by the MiGA server (Rodriguez-R et al., 2018). Strain LS5B^T shared an amino acid identity (AAI) of 69%, 59% and 56% with *Dp. aestuarii*, *Desulforhopalus singaporensis* T1^T and *Dt. psychrophila*, respectively (Table 1, Table S3). Genome-based comparison with *Desulfopila inferna* JS_SRB250Lac^T or *Desulfotalea arctica* LSv514^T was not possible, since their genomes are not available.

The cellular fatty acid (CFA) profile of strain LS5B^T was different from that of *Dp. aestuarii* and was more similar to that of the two *Desulfotalea* spp. (Figure 5C), with $C_{16:1}\omega7$ and $C_{16:1}\omega5$ as most abundant CFAs (Table 1, Table S4). Strain LS5B^T contained a range of intact polar lipids (IPLs, Table 1, Table S4) including the common phospholipids phosphatidylglycerol (PG), phosphatidic acid (PA), phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG, commonly known as cardiolipins). Additionally, the less common acylphosphatidylglycerol (Acyl-PG) was detected. The identification of the acyl-PG lipids was based on the accurate mass of their $[M+NH_4]^+$ ions and their tandem mass spectrometry fragments (Table S5).

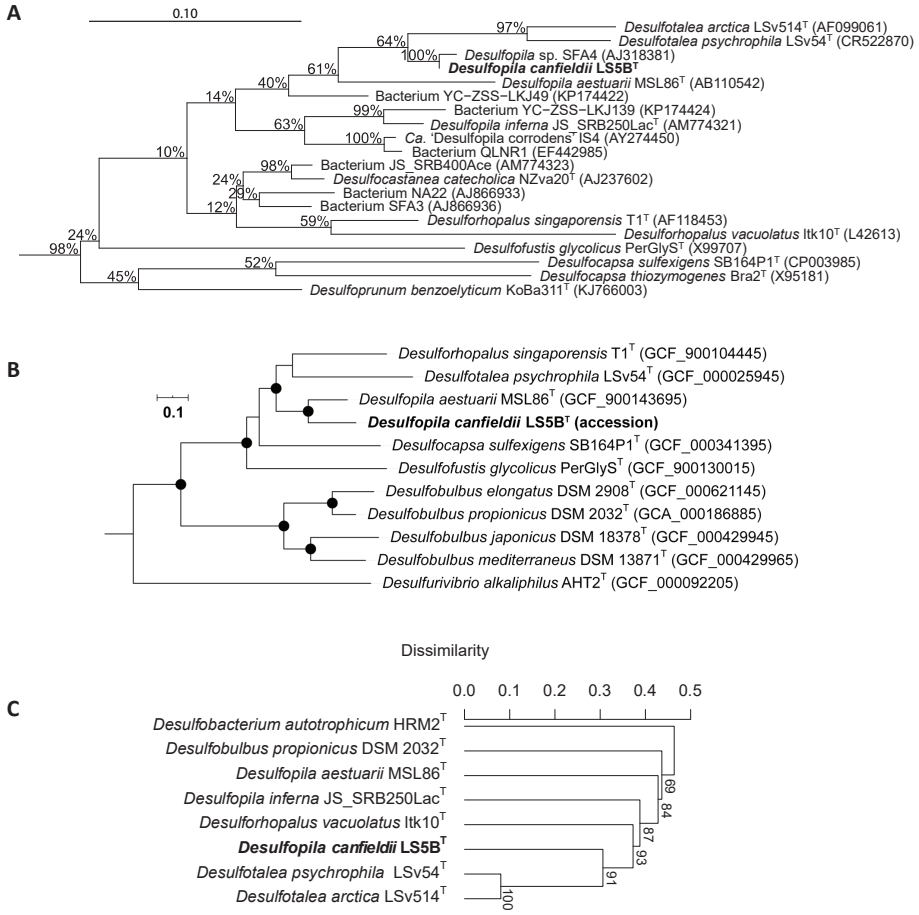


Figure 5. Phylogenetic reconstructions. A) Maximum-likelihood phylogenetic tree of full-length (>1200 bp) 16S rRNA gene sequences of cultured *Desulfobulbaceae* spp. most closely related to strain LS5B^T. The tree was rooted with *Desulfobulbus* spp. and *Desulfobacterium autotrophicum* HRM2^T. Percentages at nodes indicate support values from 100 bootstraps. The scale bar indicates substitutions per site. B) Phylogenomic maximum-likelihood tree constructed from a concatenated alignment of 120 single-copy household genes of all available genomes of described *Desulfobulbaceae* members. Black filled circles indicate that the node is supported by >95% out of 1,000 ultra-fast bootstraps. The tree was rooted with the genome of *Db. autotrophicum* HRM2^T. The scale bar indicates substitutions per site. C) Clustering dendrogram based on cellular fatty acid profiles. Values at nodes are 'approximately unbiased p-values', for which a value higher than 95 indicates strong support, and a value higher than 80 indicates reasonable support (Shimodaira, 2002). Fatty acid profiles of other SRB were obtained from other studies (Knoblauch et al., 1999; Rütters, 2001; Suzuki et al., 2007; Gittel et al., 2010).

4 Discussion

Diverse bacteria were enriched in the sulfate-reducing fatty-acid-degrading cultures, including several well-known SRB genera. *Desulfobacter* spp. are known as SRB that oxidize acetate to CO_2 (Rabus et al., 2013), explaining their emergence in acetate enrichments. Similarly, propionate is known to be a substrate for members of the genus *Desulfobacterium*, and butyrate is a widely used substrate among *Desulfobacteraceae* (Widdel and Bak, 1992). However, the enrichment of *Desulfoconvexum*, *Desulfuromonas* and *Desulfuromusa* spp. in cultures containing acetate and propionate is not in line with known physiology. The only described *Desulfoconvexum* member, *Desulfoconvexum algidum* JHA1^T, is unable to use acetate or propionate as electron donor, although it does oxidize other substrates completely to CO_2 such as formate, butyrate, lactate, ethanol and amino acids (Könneke et al., 2013). *Desulfuromonas* and *Desulfuromusa* spp. are known as sulfur-reducing sulfidogens unable of dissimilatory sulfate reduction (Rabus et al., 2013). Possibly, these bacteria reduced S^0 present in the sediment inoculum or generated from the reducing agent sulfide by residual oxygen or by minor oxygen intrusion during incubation.

Aside from canonical sulfidogens, the enrichments also yielded taxa of which the members are metabolically versatile (*Clostridiales*) or mainly fermentative (*Bacteroidetes*). In contrast to sugars or amino acids, fatty acids are no typical fermentative substrates and can only be fermented in syntrophy with SRB or methanogens (Stams and Plugge, 2009). Polysaccharides and proteins were present endogenously in the organic-rich sediment that was used as inoculum (Karl and Knauer, 1991; Jørgensen et al., 2001), but were apparently not accessible for fermentative bacteria, as no community change or sulfide production was observed in negative control cultures (Van Vliet et al., 2019).

Members of the '*Candidatus* Aegiribacteria' phylum were exclusively enriched with acetate. According to the Genome Taxonomy Database (GTDB; Parks et al., 2017), this group is synonymous to candidate phyla Hyd24-12 and *Fermentibacteria* within the *Fibrobacteres-Chlorobi-Bacteroidetes* superphylum described by Kirkegaard et al. (2016). These bacteria are found in anoxic sediments, hypersaline microbial mats, and digesters (Saad et al., 2017). Three described '*Ca.* Aegiribacteria' genomes from different sources and lineages all indicated a fermentative metabolism (Hamilton et al., 2016; Kirkegaard et al., 2016; Saad et al., 2017). Dissimilatory sulfate reduction genes were not found, nor were they annotated in any of the six '*Ca.* Aegiribacteria' genomes available within the GTDB (AnnoTree; Mendler et al., 2019). The '*Ca.* Aegiribacteria' spp. enriched in our cultures are thus unlikely SRB, and more probable grow fermentatively. Repeated enrichments with acetate and sulfate lead to an abundance of *Desulfobacter* spp., but not of '*Ca.* Aegiribacteria' spp. (data not shown).

Similarly to previous studies on deep or subsurface marine sediments, SRB cultivable with lactate were detected at higher dilutions than with H_2, formate or acetate as substrates (Gittel et al., 2008; Kraft et al., 2013). For the quantification of SRB, cultivation-based techniques are considered obsolete, because they underestimate the actual number of SRB by at least a hundredfold based on comparison of theoretical cellular sulfate reduction rates with those determined for pure cultures (Jørgensen, 1978). Indeed, a most-probable-number calculation using our results indicates maximally $>1.4 \cdot 10^4$ sulfate-reducing cells mL^{-1}, which is much lower than the cell numbers suggested by *dsrA* and *aprA* marker genes analysis of samples of comparable Black Sea sediments (10^7-10^8 cells mL^{-1}; Leloup et al., 2007; Schippers et al., 2012). Similar disparity was largely resolved in other studies by prolonged incubation for ten months, resulting in the enrichment of various *Deltaproteobacteria* (Llobet-Brossa et al., 2002). Extending the incubation period for our dilution series from three months to longer could thus have yielded growth in higher dilutions of more prevalent but more slow-growing sulfidogens.

The dilution cultivation experiment yielded enrichments of the canonical SRB lineages *Desulfobacteraceae*, *Desulfobulbaceae* and *Desulfovibrionaceae*. Our results resemble those of similar previous experiments with marine sediments from Wadden Sea tidal flats and the Benguela Upwelling, yet differ on the genus level due to the absence of enriched *Desulfofrigus, Desulfobacula, Desulfobulbus,* or *Desulfotalea* (Llobet-Brossa et al., 2002; Köpke et al., 2005; Gittel et al., 2008; Kraft et al., 2013). Overall, the enriched communities in parallel dilutions were less similar than the communities in the initial, less diluted enrichments. It is unclear whether the lesser reproducibility among higher dilutions was due to dilution or due to the different conditions applied. We found an enrichment of *Desulfoconvexum* spp. at various conditions, implying a versatile physiology. Whether *Desulfoconvexum*-related SRB also emerged in previous studies is unclear since these studies were reported before the establishment of this genus (Könneke et al., 2013). *Dethiosulfatibacter* spp. overgrew *Desulfoconvexum* spp. in subcultures of lactate/sulfate dilutions. The lack of sulfidogenesis in these cultures indicated fermentative conversion of lactate.

In our experiments, SRB related to *Desulfatiglans*, which made up 8% of the 16S rRNA gene amplicon sequences previously retrieved from the inoculum (Van Vliet et al., 2019), were not enriched. Despite its genus rank, the *Desulfatiglans* lineage encompasses the phylogenetic diversity of a family- or order-rank taxon (Parks et al., 2017; Jochum et al., 2018), and has therefore recently been reclassified as the *Desulfatiglandaceae* family (Waite et al., 2020). Cultivated *Desulfatiglans*-related bacteria are known to degrade aromatic compounds for growth (Schnell et al., 1989; Galushko et al., 1999; Suzuki et al., 2014). This physiology is thought to occur in environmental marine relatives as well (Jochum et al., 2018). It is thus possible that *Desulfatiglans*-related bacteria were not enriched in current and previous cultivation experiments because aromatics were

not used as substrate (Llobet-Brossa et al., 2002; Köpke et al., 2005; Gittel et al., 2008; Kraft et al., 2013). In the Black Sea, most labile organic matter is degraded in the upper 100 meters of the water column (Karl and Knauer, 1991; Jørgensen et al., 2001), which probably causes a higher relative abundance of refractory substrates in 2,100-m-deep sediments which may include compounds containing aromatic structures.

The inclusion of thiosulfate and S^0 in the current study yielded additional novel insights into sulfidogenesis in Black Sea sediments. Disproportionation and sulfur-reducing enrichments supported measurable activity and growth within a similar timeframe as sulfate-reducing fatty acid enrichments, indicating that these processes may occur in deeper Black Sea sediments. The abundance of *Desulfocapsa* spp. in disproportionation enrichments agrees with previous experiments at similar conditions but with different marine and freshwater sediments (Janssen et al., 1996; Finster et al., 1998). However, our enrichments also contained abundant *Desulfobulbaceae* bacteria without clear genus affiliation, indicating this family harbors additional, currently unknown disproportionating members. Further, the *Desulfuromonadales* clade Sva1033 was enriched in conditions with S^0 as electron acceptor and H_2/acetate or formate/acetate as electron donors. Thamdrup et al. (2000) previously enriched *Desulfuromonas*-related bacteria from Black Sea surface sediment (62 m water depth). We found that these were also members of the Sva1033 clade by reanalysis of the 16S rRNA sequences. Growth in these cultures was dependent on reduction of MnO_2 coupled to the oxidation of acetate (Thamdrup et al., 2000), whereas we enriched Sva1033 with S^0 as electron acceptor. The Sva1033 bacteria enriched here were amenable to cultivation, as their 16S rRNA gene sequences were abundant in amplicon datasets of subcultures.

The strain LS5B^T isolated in this study is closely related to strain SFA4 previously isolated from a dilution culture inoculated with tidal mudflat sediment from the East-Frisian Wadden Sea (99.6% 16S rRNA identity; Rütters, 2001). However, a description of strain SFA4 has not been published. Although strain LS5B^T was isolated from one of the highest dilutions supporting growth, an identical OTU sequence was not retrieved from the inoculum, indicating low relative abundance *in situ*. However, coastal sediments often yield abundant sequences related to *Desulfotalea* and *Desulfopila* spp. (Sahm et al., 1999; Gittel et al., 2008; Colin et al., 2013), which are closely related to strains LS5B^T and SFA4.

Although strain LS5B^T could grow on H_2/CO_2 or formate without acetate as carbon source, this did not definitively prove an autotrophic metabolism as yeast extract (0.1 g L^{-1}) was required for growth and could have acted as organic carbon source. However, several other *Desulfobulbaceae* are capable of lithoautotrophic growth, most probably using the reductive acetyl-CoA (Wood-Ljungdahl) pathway for CO_2 fixation (Frederiksen and Finster, 2004; Gittel et al., 2010; Finster et al., 2013). The LS5B^T genome contained the genes of this pathway including the essential acetyl-CoA synthase/CO

dehydrogenase (ACS/CODH) complex genes and two additional CODH genes (Table S1) as typically observed in SRB (Strittmatter et al., 2009), supporting a potential for autotrophy. In model organism *Desulfobacterium autotrophicum* HRM2^T, the acetyl-CoA pathway is reversible and can be used either for CO_2 fixation or for complete oxidation of acetyl-CoA to CO_2 (Schauder et al., 1988). This is thought to be representative for most other SRB using this pathway. Exceptions are *Dp. inferna* JS_SRB250Lac^T (Gittel et al., 2010) and *Desulfosporosinus orientis* strain Singapore 1^T (Klemps et al., 1985), which are facultatively autotrophic SRB for which CODH activity was demonstrated, yet which are incomplete oxidizers producing acetate from organic substrates. A similar phenotype was reported for most *Desulfosporosinus* spp. (Sánchez-Andrea et al., 2015), and here for strain LS5B^T. It is currently unclear why the reductive acetyl-CoA pathway in these organisms is not reversible.

Strain LS5B^T was able to grow in cultures with amorphous MnO_2 as only electron acceptor, implying it can use MnO_2 for respiration. Strain LS5B^T could not reduce S^0, which precludes involvement of an intermediate sulfide-S^0 cycle. The capacity for dissimilatory reduction of manganese and other metals has not been tested for related strains, except for the two described *Desulfotalea* spp. which can use complexed ferric iron as electron acceptor (Knoblauch et al., 1999). The capacity of LS5B^T for dissimilatory MnO_2 reduction is unusual among SRB, but not unique (Sass et al., 2009; Vandieken et al., 2017b). However, SRB are not thought to be key players in heterotrophic dissimilatory MnO_2 reduction in manganese-rich marine sediments (Thamdrup et al., 2000; Vandieken et al., 2012).

Strain LS5B^T could not be unambiguously assigned to an established genus, as its 16S rRNA gene shared 96% identity with both *Desulfotalea* spp. and *Desulfopila aestuarii* MSL86^T. However, phenotypically and genomically strain LS5B^T showed a closer relation to *Dp. aestuarii*. The differences in profiles of utilized electron donors could not be corroborated with differences in the presence of functional genes. Despite the reported inability of *Dp. aestuarii* to use H_2, malate, succinate or L-alanine as electron donors, we could annotate the genes for periplasmic [NiFe] hydrogenase, malate dehydrogenase, fumarate reductase, fumarate hydratase, alanine racemase and D-alanine dehydrogenase, just as in strain LS5B^T. The AAI shared by strain LS5B^T and *Dp. aestuarii* exceeded the genus threshold of 65% proposed by Konstantinidis et al. (2017) with 4%. However, the chemotaxonomic markers represented by CFA profiles and menaquinone type differed significantly. We propose that strain LS5B^T represents a novel species within the genus *Desulfopila*.

Description of *Desulfopila canfieldii* sp. nov. *Desulfopila canfieldii* (can'fiel.di.i. N. L. gen. n., honoring Donald E. Canfield, an American biogeochemist who has made essential contributions to our understanding of the marine sulfur cycle).

The genus description applies, with the following additional features. Cells have a width of 0.6-0.7 μm and a length of 1.4-6.0 μm, and are non-motile. Catalase and oxidase activity tests negative. Yeast extract is required for growth. Can grow on H_2/CO_2 or formate without acetate as added carbon source. Grows with H_2, formate, lactate, pyruvate, malate, succinate, fumarate, ethanol, propanol, butanol, glycerol, L-alanine as electron donor. Compounds that do not support growth are acetate, propionate, butyrate, methanol, L-glycine, L-serine and D-glucose. Can ferment pyruvate, but not lactate. Incompletely oxidizes electron donors to acetate and CO_2. Dissimilatory reduction of sulfate, sulfite, thiosulfate, manganese oxide and dimethylsulfoxide, but not of elemental sulfur, nitrate or nitrite. Does not grow by disproportionation of thiosulfate or elemental sulfur. Growth occurs at 10-30°C, 0.5-4.8 g L^{-1} NaCl, and a pH of 6.4-8.2. Optimal conditions are 30°C, 1.1-2.4 g L^{-1} NaCl and pH 7.0-7.3. The type strain has a genome size of 7.0 Mbp and DNA G+C content of 44.3% (mol%). Major cellular fatty acids are $C_{16:1}\omega7$, $C_{16:1}\omega5$ and $C_{16:0}$. Major intact polar lipid classes are phosphatidic acid, phosphatidylglycerol and diphosphatidylglycerol. The major respiratory quinone is MK-5(H_2).

The type strain is LS5B^T (= DSM 108673^T = KCTC 15796^T), isolated from anoxic Black Sea sediment.

5 Supplementary material

Supplementary Tables S2-S5 have been deposited at 4TU.ResearchData under Digital Object Identifier 10.4121/13317545 and can be accessed with the following link: https://figshare.com/s/efdffafc61ed017b4eb3.

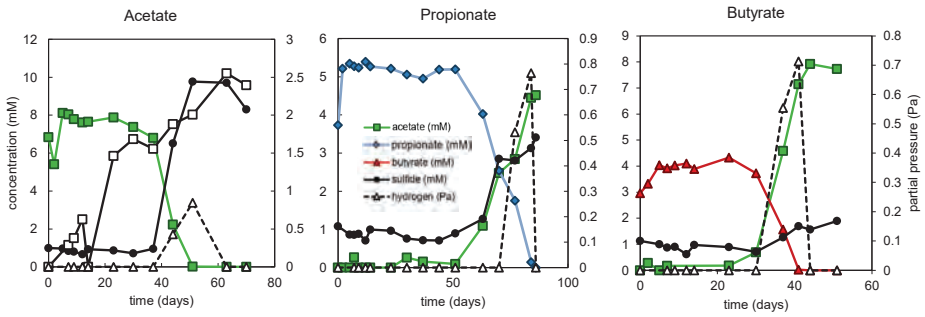


Figure S1. Measured substrates and products in the acetate, propionate and butyrate enrichment cultures. Values shown are average values obtained from duplicate cultures. Closed symbols are read on the left y-axis, open symbols on the right y-axis.

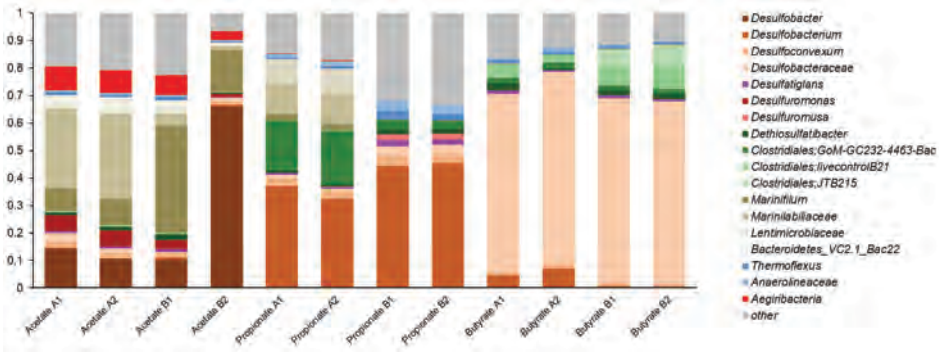


Figure S2. Community analysis of sulfate-reducing enrichment cultures by 16S rRNA gene amplicon sequencing. Sample names consist of the substrate, followed by an 'A' or 'B' indicating biological duplicate culture, and ending with a number '1' or '2' indicating technical duplicate.

Table S1. PCR primers used in this study. Barcodes were barcoded at the 5`-end with a set of 70 different 8-bp long barcodes.

primer	**sequence**
515f	5'-GTGYCAGCMGCCGCGGTAA-3'
806rB	5'-GGACTACNVGGGTWTCTAAT-3'
27F	5'-AGAGTTTGATCMTGGCTCAG-3'
1492R	5'-TACGGYTACCTTGTTACGACTT-3'

Table S2. Functional annotation of genes in the draft genome of strain LS5B^T.

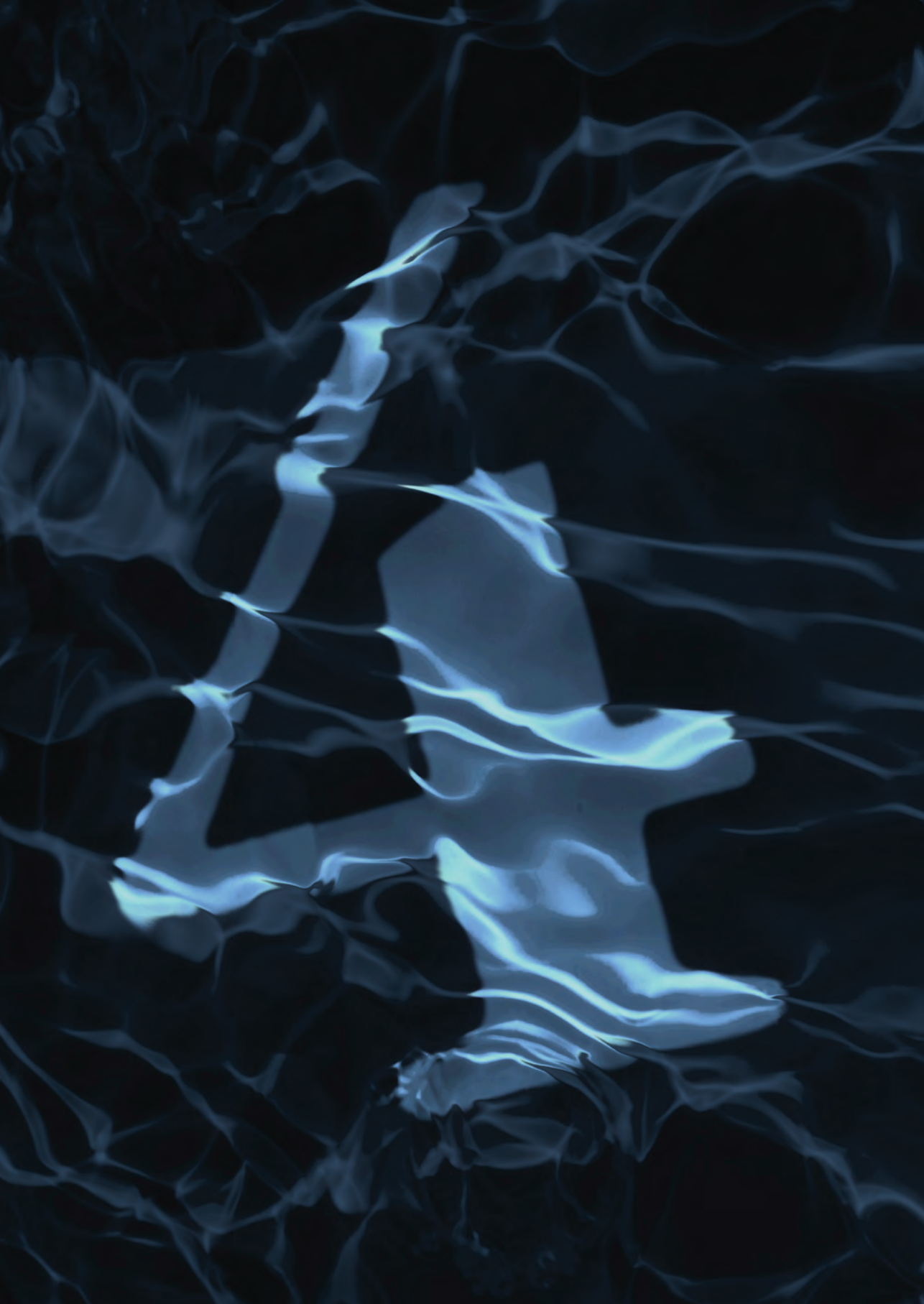
Table S3. 16S rRNA gene identity matrix of cultivated members of the *Desulfobulbaceae* family.

Table S4. Average amino acid identity (AAI) matrix of strain LS5B^T and closely related cultivated *Desulfobulbaceae* spp. for which a genome sequence is available.

Table S5. Cellular fatty acids and intact polar lipids detected in biomass of strain LS5B^T.

Table S6. Acylphosphatidylglycerols (acyl-PGs) detected in strain LS5B^T. Acyl chain carbon numbers and double bonds equivalents show in brackets. Accurate masses from UHPLC HRMS analysis. AEC = assigned elemental composition. mmu = milli mass unit, Δ mmu = (measured mass – calculated mass) x 1000.

Intact polar lipid	$[M+NH_4]^+$	**AEC**	**Δ mmu**	**Dominant fragment(s) in MS^2**
Acyl-PG (16:1,16:1,16:1)	972.7265	$C_{54}H_{103}NO_{11}P^+$	-0.2	547.4721
Acyl-PG (16:1,16:0,16:1)	974.7419	$C_{54}H_{105}NO_{11}P^+$	0.0	549.4878
Acyl-PG (16:1,16:0,16:0)	976.7573	$C_{54}H_{107}NO_{11}P^+$	0.3	549.4876
Acyl-PG (16:1,16:0,18:1)	1002.7722	$C_{56}H_{109}NO_{11}P^+$	1.1	549.4876, 575.5034
Acyl-PG (16:1,16:0,18:0)	1004.7885	$C_{56}H_{111}NO_{11}P^+$	0.5	549.4875, 577.5187



CHAPTER 4

Anaerobic degradation of sulfated polysaccharides by two novel *Kiritimatiellales* strains isolated from Black Sea sediment

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Abstract

The marine environment contains a large diversity of sulfated polysaccharides and other glycopolymers. Saccharolytic microorganisms degrade these compounds through hydrolysis, which includes the hydrolysis of sulfate groups from sugars by sulfatases. Various marine bacteria of the *Planctomycetes-Verrucomicrobia-Chlamydia* (PVC) superphylum have exceptionally high numbers of sulfatase genes associated with the degradation of sulfated polysaccharides. However, thus far no sulfatase-rich marine anaerobes are known. In this study, we aimed to isolate marine anaerobes using sulfated polysaccharides as substrate. Anoxic enrichment cultures were set up with a mineral brackish marine medium, inoculated with anoxic Black Sea sediment sampled at 2100 m water depth water and incubated at 15°C (*in situ* T = 8°C) for several weeks. Community analysis by 16S rRNA gene amplicon sequencing revealed the enrichment of *Kiritimatiellaeota* clade R76-B128 bacteria in the enrichments with the sulfated polysaccharides fucoidan and iota-carrageenan as substrate. We isolated two strains, F1 and F21, which represent a novel family within the order *Kiritimatiellales*. They were capable of growth on various mono-, di- and polysaccharides, including fucoidan. The desulfation of iota-carrageenan by strain F21 was confirmed quantitatively by an increase in free sulfate concentration. Strains F1 and F21 represent the first marine sulfatase-rich anaerobes, encoding more sulfatases (521 and 480, 8.0% and 8.4% of all coding sequences, respectively) than any other microorganism currently known. Specific encoded sulfatase subfamilies could be involved in desulfating fucoidan (S1_15, S1_17 and S1_25) and iota-carrageenan (S1_19). Strains F1 and F21 had a sulfatase gene classification profile more similar to aerobic than anaerobic sulfatase-rich PVC bacteria, including *Kiritimatiella glycovorans*, the only other cultured representative within the *Kiritimatiellaeota*. Both strains encoded a single anaerobic sulfatase-maturating enzyme which could be responsible for post-translational modification of formylglycine-dependent sulfatases. Strains F1 and F21 are potential anaerobic platforms for future studies on sulfatases and their maturation enzymes.

1 Introduction

Polysaccharides present a diversity of functions in different fields of biology. They have a structural role in cell walls of plants (e.g. cellulose and xylan) and fungi (e.g. chitin), as well as in the exoskeleton of arthropods which consist of chitin. Polysaccharides are also the main constituent of peptidoglycan and lipopolysaccharides, which form the cell wall of most prokaryotes. Various polysaccharides (e.g. glycogen, starch, laminarin) are stored as energy reserve by animals, plants and microorganisms. Furthermore, extracellular polysaccharides (exopolysaccharides) are the main constituent of extracellular polymeric substances (EPS; More et al., 2014). Microorganisms excrete EPS to facilitate attachment, aggregation and protection against grazing, desiccation or other stress factors (Wingender et al., 1999).

In the marine environment, many polysaccharides contain sulfate ester groups (Helbert, 2017). The sulfated polysaccharides in the cell walls and extracellular matrix of macroalgae are the best studied, because of their use as gelling or thickening agents. For instance, red algae produce carrageenan, which is broadly used in the food industry (Usov, 2011). Brown and green algae also produce a high diversity of sulfated polysaccharides. This includes fucoidan, which has anticoagulant, antithrombotic and antitumor properties that have extensively been studied because of their potential pharmacological applications (Berteau and Mulloy, 2003; Li et al., 2008a; Pomin and Mourão, 2008; Ale and Meyer, 2013; Kwak, 2014; Atashrazm et al., 2015; Fitton et al., 2015). Sulfated exopolysaccharides are also produced by marine bacteria, microalgae and angiosperms (reviewed by Helbert, 2017). The sulfate groups within sulfated exopolysaccharides form a barrier which can protect against degradation, since their removal requires sulfatase enzymes (Barbeyron et al., 2016a). Additionally, the sulfate groups are thought to mediate aggregation of microorganisms and EPS into 'marine snow' and transparent exopolymer particles (Decho and Gutierrez, 2017).

Saccharolytic microorganisms degrade polysaccharides (Arnosti, 2011), and show different degrees of specialization. In the mammalian gut, for instance, *Fibrobacter succinogenes* and *Ruminococcus albus* can degrade (hemi)cellulose and resistant starch, while *Bacteroides thetaiotaomicron* and *Eubacterium rectale* can grow by degrading starch and other easily degradable polysaccharides (Flint et al., 2008). A clear specialization is found in some haloalkaliphilic anaerobes that exclusively use chitin as growth substrate (Sorokin et al., 2012). In the North Sea, *Bacteroidetes* spp. were identified as specialized microalgal polysaccharide degraders which quickly respond to a diatom bloom (Teeling et al., 2012).

The hydrolysis of polysaccharides is catalyzed by glycoside hydrolases, but may also require other carbohydrate-active enzymes (CAZymes) such as polysaccharide lyases to cleave uronic acid-containing polysaccharide chains, or carbohydrate esterases to

deacetylate substituted saccharides (Cantarel et al., 2009). Additionally, the degradation of sulfated polysaccharides requires the removal of sulfate esters ($ROSO_3^-$) or sulfamates ($RN[H]SO_3^-$), which is catalyzed by sulfatases (Barbeyron et al., 2016a). Sulfatase genes are indeed found in some of the aforementioned marine *Bacteroidetes* spp., such as *Polaribacter* spp. (Xing et al., 2015). However, far higher numbers and diversity of sulfatases are encoded in the genomes of bacteria of the *Planctomyces-Verrucomicrobia-Chlamydia* (PVC) superphylum. The first sulfatase-rich bacterium discovered was the marine organotrophic aerobe *Rhodopirellula baltica* SH1^T for which 110 sulfatase genes were reported (Glöckner et al., 2003). The hypothesis that *R. baltica* is specialized for degrading sulfate polysaccharides *in situ* was reinforced by the finding that it achieves the highest growth rate with the sulfated polysaccharide chondroitin sulfate rather than glucose, and by the finding that it can grow on several sulfated polysaccharides while inducing the expression of specific sulfatases (Hieu et al., 2008; Wegner et al., 2013). Similar numbers of encoded sulfatases were found in the genomes of several marine *Rhodopirellula* species (Wegner et al., 2013), *Blastopirellula marina* DSM 3645^T, *Planctomyces maris* DSM 8797^T and uncultured marine *Planctomycetes* (Woebken et al., 2007). Moreover, PVC phyla other than the *Planctomycetes* also harbour sulfatase-rich marine members: *Verrucomicrobia* (Martinez-Garcia et al., 2012) and *Lentisphaerae* (*Lentisphaera araneosa* HTCC2155^T; Thrash et al., 2010). The only anaerobic sulfatase-rich PVC bacteria known so far are halophilic microorganisms from hypersaline marine environments: *Kiritimatiella glycovorans* L21-Fru-AB^T, the only representative of the phylum *Kiritimatiellaeota* (Spring et al., 2016), and two species of the recently proposed genus *Sedimentisphaera* within the *Planctomycetes* (Spring et al., 2018). However, from the more common marine environment (15-35‰ salinity), no sulfatase-rich facultative or obligate anaerobes are currently known, inside nor outside the PVC superphylum.

In this study, we aimed to isolate novel marine saccharolytic anaerobes capable of growing on sulfated polysaccharides. Sediment sampled from 2100 meters water depth in the Black Sea was used as inoculum. The Black Sea is the largest anoxic basin in the world and a model study site for marine anaerobic microbiology (Kuypers et al., 2003). With the sulfated polysaccharide fucoidan as substrate, we enriched and isolated two strains of sulfatase-rich *Kiritimatiellaeota*. We investigated their taxonomy, physiology, and growth on sulfated polysaccharides in particular. We also studied the quantity, diversity and potential function of encoded sulfatases, and compared their classification with that of ten other aerobic and anaerobic sulfatase-rich PVC bacteria.

2 Materials and methods

2.1 Inoculum source

Sediment samples were collected in February 2016 from station 2 (42°53.992'N, 30°31.036'E, Bulgarian exclusive economic zone) of the 64PE408 research cruise on board the research vessel R/V Pelagia. Sediment cores with a length of 45 cm and a diameter of 10 cm were collected with a multicorer. The sediment cores were immediately brought into a N_2-flushed glovebag (Aldrich® AtmosBag). The upper 10 cm of sediment, consisting of fluff and coccolith ooze, was transferred with sterile syringes to a sterile anoxic 1-liter bottle containing 500 mL anoxic reduced basal medium (see next subsection). Approximately 100 mL of sediment was added resulting in approximately 600 mL of sediment slurry. The slurry bottle was pressurized with N_2 to 0.5 bar overpressure and covered with aluminum foil to protect it from light. The slurry bottle was stored for 40 days at the *in situ* temperature (9°C), and for another 40 days at 15°C. After the first month of storage, 3 mM of sterile sodium sulfide was added to preserve anoxic sulfidic conditions. No change of color of the redox indicator added to the basal medium was observed throughout the sampling and storage process, indicating anoxic conditions were maintained at all times. The pH remained constant at a value of 7.

2.2 Media

A basal bicarbonate-buffered marine medium was designed in this study to match the salinity of Black Sea at 2100 m water depth (22‰ according to Sorokin, 2002). It contained the following final concentrations (g L^{-1}): NaCl, 17.16; KCl, 0.3715; KBr, 0.056; NH_4Cl, 0.155; KH_2PO_4, 0.225; $K_2HPO_4{\cdot}3H_2O$, 0.565; $MgSO_4{\cdot}7H_2O$, 4.211; $MgCl_2{\cdot}6H_2O$, 3.51; $CaCl_2{\cdot}2H_2O$, 0.091, $NaHCO_3$, 4.2; $Na_2S{\cdot}9H_2O$, 0.360, as well as trace elements described by Widdel (2010) and vitamin solution described by Widdel and Bak (1992). All medium components were sterilized by autoclaving, except for $Na_2S{\cdot}9H_2O$ and the vitamin solution, which were filtered-sterilized through a 0.2 μM pore size polyethersulfone filter (Advanced Microdevices, India). The salts $MgSO_4{\cdot}7H_2O$, $MgCl_2{\cdot}6H_2O$, $CaCl_2{\cdot}2H_2O$ and $NaHCO_3$ were added from separately autoclaved stocks to prevent precipitation. All substrates were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), except for fucoidan extracted from *Cladosiphon* spp., which was purchased from Carbosynth (Berkshire, UK).

2.3 Enrichment

Enrichments were carried out in duplicate in 250-mL serum vials containing 100 mL of medium and a 1.5 atm N_2/CO_2 (80:20, v/v) headspace, which were sealed with butyl rubber stoppers (Rubber BV, Hilversum, The Netherlands) and aluminum caps. As substrate, 0.6 g L^{-1} of purified fucoidan from *Fucus vesiculosus* was added from an anoxic, separately autoclaved 30 g L^{-1} stock solution. Enrichment cultures were inoculated with 6 mL of sediment slurry, resulting in an effective final hundred-fold dilution of the sediment. Blank control cultures without substrate but with inoculum were set up in duplicate, as well as a single sterile control culture sterilized by autoclave. Enrichments were incubated for a total of 86 days at 15°C. They were regularly sampled for chemical analyses. Samples for community analysis by 16S rRNA gene amplicon sequencing were taken from the enrichments when the intermediate product acetate reached a concentration of approximately 1 mM (circa 34 days of incubation), as well as from the blank controls after 17 and 77 days of incubation, and from the inoculum. Subcultivation was done as aforementioned in 120-mL or 50-mL serum vials containing 50 or 20 mL of medium respectively, with an inoculum volume of 5% v/v. To investigate the effect of sulfated biopolymers other than fucoidan, three more single enrichments were set up as aforementioned with 0.6 g L^{-1} chondroitin 4-sulfate, iota-carrageenan and mucin as substrates. I-carrageenan was added to the medium before autoclaving. These enrichments were incubated for 56 days, and sampled once for community analysis at the end of the incubation.

2.4 Analytical methods

Concentrations of organic acids, alcohols and monosaccharides were determined by high pressure liquid chromatography using a MetaCarb 67H column (Agilent Technologies, Santa Clara, CA) operated at 45 °C, with 5 mM H_2SO_4 as eluent, and a flow rate of 0.9 mL min^{-1}. Both refractive index (RI) and ultraviolet (UV) detectors were used. As internal standard, 10 mM of dimethylsulfoxide was used. For all measured compounds, the quantification limit was approximately 100 μM. Hydrogen gas partial pressures were measured by gas chromatography using a CompactGC 4.0 (Global Analyser Solutions, The Netherlands) equipped with a Carboxen 1010 pre-column and a Molsieve 5A column operated at 90 °C and a pulsed discharge ionization (PDD) detector operated at 110 °C. Helium was used as carrier gas. After preservation of the sample by addition of 20 mM of $ZnCl_2$, the sulfide concentration was measured using the methylene blue colorimetric assay (Cline, 1969).

2.5 Microbial community analysis

To obtain DNA for microbial community analysis, samples of 2-5 mL were centrifuged at 13,400 *g* for 10 min, supernatant was discarded, and the pellet was resuspended in 250 µL of sterile Milli-Q water. Then, DNA was extracted with the FastDNA Spin Kit for Soil (MP Biomedicals, OH). The FastPrep bead-beater (MP Biomedicals, OH) was used and set up for two bead-beating cycles of 40 s at speed setting 6000 with a break of 2 min. Extracted DNA was cleaned and concentrated with the Zymo DNA Clean & Concentrator kit (Zymo Research, CA). A fragment of the 16S rRNA gene was amplified from the DNA extracts in a two-step polymerase chain reaction (PCR) to generate barcoded 16S rRNA gene amplicons. The first PCR was performed in duplicate reactions of 25 µL containing 1 µL of the extracted DNA as template, HF PCR buffer, 0.2 mM dNTPs, 0.016 U μL^{-1} Phusion Hot Start II DNA polymerase (Thermo Scientific, Waltham, MS), and 0.2 µM of forward primer UniTag1–515f and reverse primer UniTag2-806rB targeting the V4-V5 region of the 16S rRNA gene (Supplementary Table S1). The amplification program consisted of an initial denaturation step at 98°C for 5 min, followed by 30 cycles of denaturation at 98°C for 25 s, annealing at 56°C for 20 s and elongation at 72°C for 20 s, followed by a final extension step at 72°C for 7 min. Duplicate PCR products were pooled, and the length of the amplicons was examined by gel electrophoresis in a 1% w/v agarose gel containing the nucleic acid stain SYBR Safe (Thermo Scientific, Waltham, MS). A negative control PCR without template DNA was included. The second PCR had a volume of 100 µL containing 5 µL of the product of the first PCR as template, HF PCR buffer, 0.2 mM dNTPs, 0.02 U μL^{-1} Phusion Hot Start II DNA polymerase (Thermo Scientific, Waltham, MS) and 500 nM of forward and reverse primer constructed of Unitag1 and Unitag2 sequences, respectively, appended with an 8 bp sample-specific barcode at the 5'-end (Ramiro-Garcia et al., 2016). The amplification program consisted of an initial denaturation step at 98°C for 30 s, followed by 5 cycles of denaturation at 98°C for 10 s, annealing at 52°C for 20 s and elongation at 72°C for 20 s, followed by a final extension step at 72°C for 10 min. PCR products were purified with the HighPrep PCR kit (MagBio Genomics Inc., Gaithersburg, MD). The DNA concentration was quantified with the Qubit dsDNA BR assay kit (Invitrogen, Carlsbad, CA) and a Qubit 2.0 fluorometer (Life Technologies, Darmstadt, Germany). Purified PCR products with different barcodes were pooled in equimolar amounts and sequenced using an Illumina HiSeq 2500 platform (GATC Biotech, Konstanz, Germany) yielding paired end reads of around 300 bp. The samples from fucoidan enrichment B and the negative controls were processed and sequenced in technical duplicate to verify reproducibility (average correlation coefficient: 0.99).

The 16S rRNA gene amplicon sequencing data was analyzed with the pipeline NG-Tax v1.0 (Ramiro-Garcia et al., 2016). Briefly, 16S rRNA gene sequences were clustered into operational taxonomic units (OTUs) with >98.5% sequence similarity. Classification of OTUs by NG-Tax was done using the SILVA Ref NR SSU r132 database (Quast et

al., 2013). To exclude unreliable OTUs represented by only a single read, also known as singletons, the OTU minimum relative abundance was set to 0.01% in the analysis of the complete dataset. Since the lowest number of reads in a sample was 37,071, all OTUs contain at least 3 reads. A more sensitive analysis was done specifically for the inoculum (0.001% OTU minimum relative abundance, 164,113 reads, >1.6 reads per OTU). The demultiplexed Illumina Hiseq reads of the 16S rRNA gene amplicon sequencing were deposited at the European Nucleotide Archive (ENA) under study ERP106613 in fastq format with accession numbers ERR2619103-ERR2619173.

2.6 Isolation

To isolate novel saccharolytic microorganisms, fucoidan enrichment subcultures were used as inoculum for streak and pour plating with solid agar media supplemented with 2.5 mM L-fucose as substrate. For solid media, 1% w/v noble agar was added to the aforementioned medium for streak plates, while 1.5% w/v low-melt agarose (Bio-Rad, CA) was added for pour plates. Phosphate salts were autoclaved separately to increase cultivability, as reported by Tanaka et al. (2014). The plates were incubated in anaerobic jars pressurized with N_2/CO_2 (80:20, v/v) at 15°C in the dark until colonies were observed. Colonies were picked and used as inoculum for 5 mL liquid cultures with fucoidan as substrate. Two more rounds of streak plating and liquid cultivation of picked colonies was performed to ensure purity of the cultures, which was confirmed by 1) full-length 16S rRNA gene analysis and 2) liquid cultivation with 20 mM D-glucose and 0.5 g L^{-1} yeast extract as substrate and inspection of morphology by microscopy. To obtain full-length 16S rRNA gene sequences from isolates, 0.5 mL of liquid culture was centrifuged for 10 min at 13,400 *g*, supernatant was removed, and the pellet was resuspended in 50 µL of sterile demiwater. Of this cell suspension, 2 µL was used as template material for PCR using the primers 27F and 1492R (Supplementary Table S1) as described by Timmers et al. (2015). The PCR product was examined by gel electrophoresis and cleaned as aforementioned. It was then sent to GATC Biotech (Konstanz, Germany) for Sanger sequencing with the 27F and 1492R primers as sequencing primers. The partial sequences were quality trimmed, checked for vector contamination and merged into full-length sequences with DNA Baser version 4.20.0. The resulting sequences were aligned and classified with SINA v1.2.11 (Pruesse et al., 2012) using the SILVA Ref NR SSU r128 database (Yilmaz et al., 2014). The 16S rRNA gene sequences of strains F1 and F21 were deposited to the ENA with respective accession numbers LS482847 and LS453290.

2.7 Phylogenetic reconstruction

Sequence identity calculations and phylogenetic reconstruction of *Kiritimatiellaeota* were performed with ARB version 6.0.2 (Westram et al., 2011) and the SILVA Ref NR SSU r128 database (released September 2016). Only three more sequences have been added to the *Kiritimatiellales* for SILVA Ref NR SSU r132 (released December 2017). Sequence identity calculations were done with the ARB distance matrix using similarity correction. For phylogenetic reconstruction, an initial number of 632 sequences of >1200 bp was selected. The ARB neighbor-joining (Felsenstein correction) and RAxML v7.7.2 maximum-likelihood GTRGAMMA algorithms were applied with of 30% and 50% basepair frequency filters, and from the resulting trees a consensus tree was constructed. A sequence of 820 bp length was added after tree reconstruction with the ARB Parsimony tool.

2.8 Growth tests

The isolated strains F1 and F21 were maintained in 50-mL cultures at 15°C either with 0.6 – 1.2 g L^{-1} fucoidan as substrate, or with 5-10 mM L-fucose as substrate, with a transfer every two months or every two weeks, respectively. Growth was monitored by optical density measurements at 570 or 600 nm wavelength. Growth tests were performed using cultures grown on L-fucose as inoculum, except for polysaccharide growth tests, in which case cultures grown on fucoidan were used as inoculum. The criterion for growth on various substrates by the isolates was an increase in turbidity in two consecutive transfers, together with cell presence as verified by phase-contrast microscopy (Leica DM2000, Leica Microsystems GmbH, Wetzlar, Germany). Substrate tests were done with substrate concentrations of 5 mM of sugars, 20 mM of amino acids and 2 g L^{-1} of polysaccharides, casamino acids, tryptone or yeast extract. The effect of temperature on growth was studied in triplicate 5 mL cultures in Hungate tubes at 4, 10, 15, 20, 25, 30 and 37°C during an incubation period of 50 days. Growth on L-fucose and fucoidan was studied in triplicate 50-mL cultures incubated at 20 °C, since optimal temperature had not yet been determined. Growth of strain F21 on iota-carrageenan was studied in triplicate 50-mL cultures incubated at 25 °C, the optimal temperature. Generation times were calculated from the measured increase in optical density over time, and associated standard errors were calculated by error propagation[6]. L-fucose and fucoidan were quantified colorimetrically with the anthrone method (Loewus, 1952) with L-fucose as standard. Iota-carrageenan was quantified with the same method using iota-carrageenan as standard. Sulfate, sulfite and thiosulfate concentrations were measured by anion chromatography using a Dionex ICS-1000 ion chromatograph (Thermo Fisher Scientific, MS) equipped with an IonPac AS17 column operated at

6 http://www.julianibus.de/

30 °C and a suppressed conductivity detector. The eluent was a KOH solution with a concentration gradient ranging from 1 to 40 mM, which was used at a flow rate of 0.3 mL min^{-1}. As internal standard, 0.5 mM of iodide was used. The total quantity of sulfate present as ester groups in fucoidan and iota-carrageenan was determined experimentally from chemical hydrolysis of 1 g L^{-1} of either polysaccharide in 2 M HCl at 95 °C for 24 hours, and subsequent analysis by anion chromatography as described.

2.9 Scanning electron microscopy

The culture was adhered to poly-L-lysin-coated glass slides (Biocoat, Corning, NY) and incubated for 1h at room temperature. The cells were then fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at room temperature, rinsed 3 times with 0.1 M phosphate buffer (pH 7.4) and post-fixed with 1 % osmium tetroxide for 60 min. Hereafter the cells were dehydrated in a graded alcohol series (10, 30, 50, 70, 80, 96 and 100 %), dried to critic point in 100% ethanol with CO_2 in the Leica EM CPD300 system (Leica Microsystems GmbH, Wetzlar, Germany) and mounted onto aluminum stubs and coated with tungsten. Cells were subsequently studied with a FEI Magellan 400 scanning electron microscope (FEI Company, OR).

2.10 Genome sequencing and analysis

Strain F1 and F21 were grown in 100 mL cultures with 1.6 g L^{-1} fucoidan as substrate at 20 °C for a month. Biomass was collected by centrifugation at 4,700 *g* and 4 °C for 20 min, after which the supernatant was discarded. The pellet was resuspended in sterile phosphate buffer, centrifuged at 13,400 *g* and 4 °C for 10 min, and supernatant was again discarded. This washing step was repeated once. Then, the biomass was flash-frozen in with liquid N_2 and stored at -80°C. Genomic DNA was extracted and sequenced by BaseClear BV (Leiden, The Netherlands) using phenol-chloroform DNA extraction and the Illumina HiSeq2500 and the PacBio Sequel sequencing platforms. Quality and length of the Illumina and PacBio reads was inspected with FastQC version 0.10.1[7]. PacBio reads were trimmed to a maximum length of 10 kbp with Trimmomatic version 0.32 (Bolger et al., 2014). From the trimmed PacBio reads and the paired end Illumina reads, a hybrid assembly was constructed with SPAdes version 3.6.2 (Bankevich et al., 2012) using the '-careful' setting and k-mers 21, 33, 55, 77 and 99. Assembly quality was analyzed with QUAST version 4.2 (Gurevich et al., 2013) and Bandage version 0.8.1 (Wick et al., 2015). Short contigs with aberrant coverage (a difference of a factor two or more) were removed from the assemblies. The resulting draft genomes were checked for completeness, contamination and strain heterogeneity with CheckM (Parks et al.,

7 https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

2015). Average amino acid identity (AAI) between genomes was calculated with the Microbial Genome Atlas web server (version 0.3.6.2; Rodriguez-R et al., 2018) and the aai.rb script from the enveomics collection (Rodriguez-R and Konstantinidis, 2016). Genomes were also classified with GTDB-Tk v0.1.3[8] using Genome Taxonomy Database Release 03-RS86 (Parks et al., 2018). Coding sequence prediction and primary annotation was done with prokka version 1.12 (Seemann, 2014) using standard settings. The predicted protein sequences were additionally analyzed with InterProScan version 5.27-66.0 (Jones et al., 2014) using the TIGRFAM (Haft et al., 2001), HAMAP (Pedruzzi et al., 2015), PROSITE (Sigrist et al., 2012), Pfam (Finn et al., 2016), TMHMM (Krogh et al., 2001) and gram-negative SignalP 4.0 (Petersen et al., 2011) databases. The reads, genome assemblies and prokka annotations of strains F1 and F21 were deposited at the ENA under sample accession numbers SAMEA5207384 and SAMEA5207385.

Hidden Markov Models (HMMs) for the identification of sulfatase protein sequences[9] were constructed as follows: updated PROSITE profiles made by Barbeyron et al. (2016a) were used to query the SwissProt and TrEMBL databases with ScanProsite (de Castro et al., 2006). The hits were clustered with an identity threshold of 50% and representative sequences were downloaded, in order to remove redundant and similar sequences. From the representative sequences, a multiple alignment was made with Clustal Omega (Sievers et al., 2011). The alignments were cropped to the conserved regions in Jalview version 2.10.3 (Waterhouse et al., 2009) and an HMM was made with HMMer v3.1b2 (Eddy, 2009). Trusted bitscore cut-off values were benchmarked by querying various protein databases with HMMER web server v3.1b2 (Finn et al., 2015). The HMM for the identification of family S1 sulfatases was constructed from only the updated PS00523 profile, not from the other S1 profiles made by Barbeyron et al. (2016a). Sulfatase protein sequences were clustered with CD-HIT (Fu et al., 2012) using a 50% identity threshold. The sulfatase gene sequences of strains F1 and F21 were deposited to the ENA with accession numbers LS478118-LS479118.

The sulfatase genes of strains F1 and F21 were compared with those of ten other sulfatase-rich PVC bacteria with publicly available genomes (Table 1). A selection was made consisting of model organism *R. baltica*, three other marine aerobes with the highest numbers of sulfatase genes reported so far (*L. araneosa*, *R. maiorica* and *R. sallentina*), the *Planctomyces* species with the highest number of sulfatase genes reported (*P. brasiliensis*), the closest relative of strains F1 and F21 (*K. glycovorans*) and four additional halophilic anaerobes (*S. cyanobacteriorum*, *S. salicampi* and two *Sedimentisphaerales* strains). Family S1 sulfatase protein sequences were classified into subfamilies by pairwise alignment to the SulfAtlas version 1.0 database (Barbeyron et

8 https://github.com/Ecogenomics/GTDBTk
9 https://github.com/dmvvliet/protein-HMMs

al., 2016a) using DIAMOND (Buchfink et al., 2014). Similarity indices and clustering patterns were calculated from the sulfatase gene classification profiles in R with the packages 'vegan' version 2.5-2[10] and APE (Paradis et al., 2004). Conserved residues of family S1 sulfatase protein sequences were inspected with Jalview.

Table 1. Publicly available genomes analyzed for sulfatases

Microorganism	**Genome accession number**
Kiritimatiella glycovorans L21-Fru-AB	CP010904
Lentisphaera araneosa HTCC2155	ABCK00000000
Planctomyces brasiliensis DSM5305	CP002546
Rhodopirellula baltica SH1	BX119912
Rhodopirellula maiorica SM1	ANOG00000000
Rhodopirellula sallentina SM41	ANOH00000000
Sedimentisphaera cyanobacteriorum L21-RPul-D3	CP019633
Sedimentisphaera salicampi ST-PulAB-D4	CP021023
Sedimentisphaerales strain SM-Chi-D1	CP019646
Sedimentisphaerales strain ST-NAGAB-D1	CP019791

3 Results

3.1 Enrichment activity and microbial community

The degradation of fucoidan in enrichment cultures was apparent through the formation of transient degradation products hydrogen, acetate and propionate (Figure 1). The acetate and propionate formed from fucoidan were degraded through sulfate reduction, producing sulfide (91% electron recovery). A similar hydrogen partial pressure (4 Pa) was found in one of the two blank control cultures between 27 and 34 days of incubation (Supplementary Figure S1), indicating the degradation of organic matter within the inoculum. However, no acetate, propionate or sulfide production was measured in the blank control cultures within 100 days of incubation (Supplementary Figure S1, S2). Additionally, the microbial communities detected in the blank control enrichments were similar to that of the inoculum (Supplementary Table S2, average correlation

10 https://CRAN.R-project.org/package=vegan

coefficient: 0.863), except for the community of one of the two enrichments after 77 days of incubation (average correlation coefficient: 0.159), which showed enrichment of *Sulfurimonas* (35% relative abundance). This taxon was not detected in fucoidan enrichments.

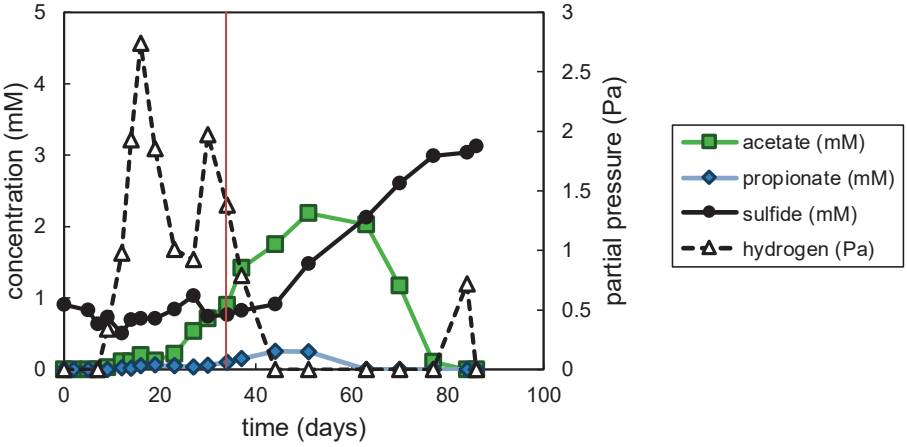


Figure 1. Measured products of polysaccharide degradation in the fucoidan enrichments. Values shown are average values obtained from duplicate enrichments. The red vertical line indicates time point of sampling for the microbial community analysis . Closed symbols are read on the left y-axis, open symbols on the right y-axis.

The fucoidan enrichments were dominated by sequences classified as *Kiritimatiellaceae* R76-B128, *Marinilabiaceae* and *Draconibacterium* (Figure 2). The sulfated biopolymers mucin and iota-carrageenan resulted in similarly strong enrichment of the R76-B128 clade, but with chondroitin 4-sulfate mainly *Marinilabiaceae* were enriched (Figure 2). Additionally, a sister clade named MSBL3 made up 2.6% of all reads from the iota-carrageenan enrichment, and was aside from this enrichment only found in the fucoidan enrichment samples with relative abundances below 0.22%. The R76-B128 clade comprised 0.024% of the sequences retrieved from the inoculum (Supplementary Table S2).

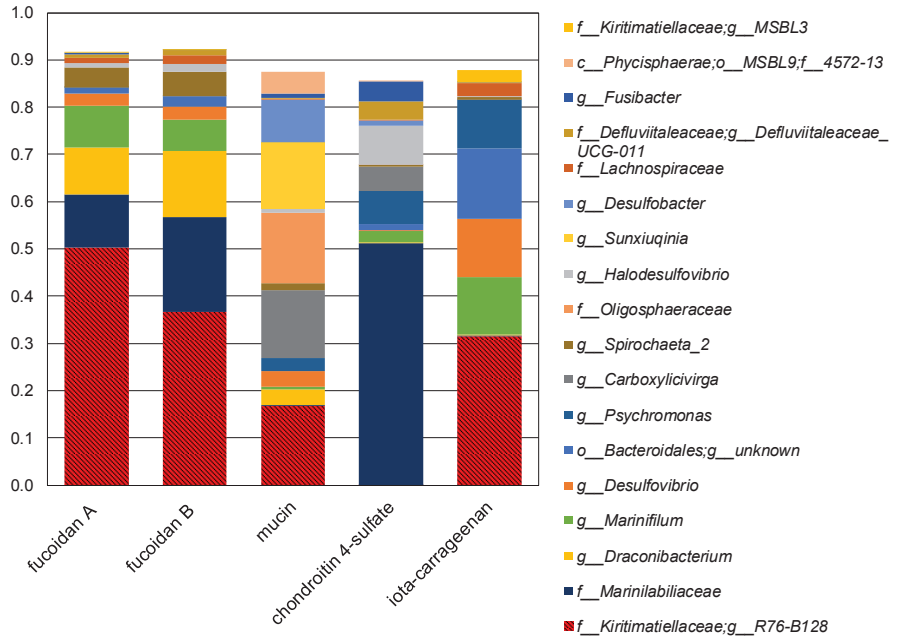


Figure 2. Taxonomic composition of 16S rRNA gene amplicon sequences obtained from enrichments. The shown taxa have >2.5% relative abundance in at least one single sample. Duplicate enrichments are indicated by suffixes 'A' and 'B'. Legend prefixes: g, genus; f, family; o, order; c, class; p, phylum.

3.2 Clade R76-B128 isolates

The R76-B128 clade falls within the recently proposed *Kiritimatiellaeota* phylum, formerly *Verrucomicrobia* subdivision five (Spring et al., 2016). Because of the phylogenetic novelty of the R76-B128 clade, subcultures of the fucoidan enrichments were selected as focus for isolation of microorganisms. We isolated six pure strains of R76-B128 bacteria, of which the 16S rRNA gene sequences clustered into two groups of three strains each, with an intragroup sequence identity >99.15% and 100%. Two strains named F1 and F21 were selected as representatives for these two groups.

3.2.1 Phylogeny

Strains F1 and F21 shared 94.1% 16S rRNA gene identity. *Kiritimatiella glycovorans* L21-Fru-AB^{T}, the only described species of the phylum *Kiritimatiellaeota* (Spring et al., 2016), shares 84.0% and 83.4% 16S rRNA gene sequence identity with strains F1 and F21, respectively. Following the 16S rRNA-based taxonomic identity thresholds for genus and family level of respectively 94.5% and 86.5% (Yarza et al., 2014), the isolated

strains thus would represent two different novel genera belonging a novel family within the *Kiritimatiellales* order. This is in disagreement with the current SILVA taxonomy (SILVA Ref NR SSU r132), which places the R76-B128 clade – including strains F1 and F21 – within the *Kiritimatiellaceae* family. Moreover, the R76-B128 clade has previously been described as an order-level lineage (Yilmaz et al., 2015; Spring et al., 2016) which included *K. glycovorans* (Spring et al., 2016).

To resolve these disagreements, we did further phylogenetic and taxonomic investigation. Phylogenetic reconstruction based on currently available 16S rRNA genes revealed the *Kiritimatiellales* as monophyletic clade with two monophyletic subclades: the genus *Kiritimatiella* and a subclade composed of the R76-B128 and MSBL3 clades (Figure 3). The sequences of the *Kiritimatiellales* order showed a minimum and median identity of 81.6% and 90.6%. The minimum is below the 82.0% order threshold identity (Yarza et al., 2014), but the median identity is somewhat higher than the 89.2% order median identity. Order rank thus seems appropriate for this clade. For the R76-B128 clade we found 86.6% minimum identity and 93.1% median identity, and for the MSBL3 clade we found 88.2% minimum identity and 92.7% median identity. These values agree best with family rank for both clades (87.7% minimum identity, 92.3% median identity; Yarza et al., 2014). Moreover, the genomes of strains F1 and F21 share 46% AAI with that of *K. glycovorans,* supporting a novel family within the NCBI taxonomy (P=0.08; Rodriguez-R et al., 2018). According to the newly proposed genome-based GTDB taxonomy, the isolates were also classified to a novel family within the *Kiritimatiellales* order (family UBA1859; Parks et al., 2018). Together, these analyses indicate that the R76-B128 clade is not a subclade of the *Kiritimatiellaceae* family as in the SILVA taxonomy, but represents a novel family within the *Kiritimatiellales*, and that the *Kiritimatiellaceae* family encompasses only the *Kiritimatiella* genus.

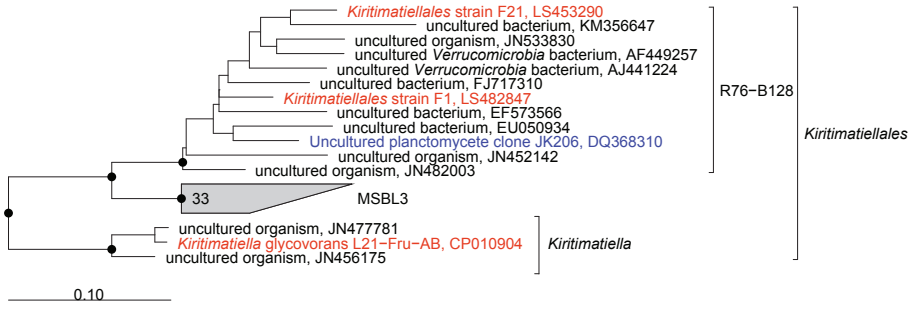


Figure 3. Phylogenetic consensus tree of 16S rRNA gene sequences of the *Kiritimatiellales*. Sequences of cultured organisms are depicted in red, an environmental sequence from a clone library from the Black Sea (Kirkpatrick et al., 2006) is depicted in blue. Dots indicate phylogenetic groups that were conserved in maximum-likelihood and neighbor-joining algorithms with basepair frequency filters of 30 and 50%. The tree was rooted with sequences of other *Kiritimatiellaeota*. Scale bar 0.10 changes per nucleotide position.

3.2.2 Physiology

Strains F1 and F21 were strict anaerobes, being unable to grow aerobically or in non-reduced medium from which the reducing agent sulfide was omitted. Strain F1 showed growth between 10 and 30 °C, with an optimum temperature of 25 °C. Strain F21 showed growth between 4 and 25 °C, with optimum temperature at 25 °C. In addition to the purified *F. vesiculosus* fucoidan used for enrichment, fucoidans extracted from *Cladosiphon* spp., *Macrocystis pyrifera* and *Undaria pinnatifada* also supported growth of strain F1. Both strains showed growth on the sugars D-cellobiose, D-fructose, D-galactose, D-glucose, D-lactose, D-maltose, D-sucrose, D-trehalose, D-xylose, L-rhamnose and N-acetylglucosamine, and on D-glucuronic acid and chondroitin 4-sulfate. No growth for either strain was observed with the sugars D-glucosamine, D-ribose, L-sorbose, raffinose, nor with the sugar derivatives D-gluconic acid, dulcitol, myo-inositol, sorbitol, nor with the amino acids L-alanine, L-cysteine, L-glutamate, L-glycine or L-isoleucine, and nor with laminarin, casamino acids, tryptone or yeast extract. Of the two strains, only strain F21 was able to grow on iota-carrageenan, D-galacturonic acid, D-mannitol or D-mannose, while only strain F1 could grow with D-tagatose or L-arabinose.

When grown on fucoidan, cells of strains F1 and F21 have a coccoid shape with an approximate diameter of 0.5 μm (Figure 4A, B). When grown on other substrates such as L-fucose, glucose or iota-carrageenan, cells commonly join in pairs as diplococci, and increase in diameter to an average nearing 1.0 μm (Figure 4C), with occasional cells reaching up to 2.0 μm diameter. Extracellular wiry structures were observed for both strains grown on fucoidan (Figure 4B), L-fucose (Figure 4C) and iota-carrageenan. No motility or spore formation was observed under any of the physiological conditions tested.

Growth of strain F1 on L-fucose and fucoidan led to the formation of the fermentation products hydrogen, acetate, ethanol and minor amounts of succinate and lactate with respectively 80% and 79% electron recovery (Supplementary Figure S3). In strain F21, 1,2-propanediol was an additional main fermentation product and electron recovery was 88% (Supplementary Figure S3). Growth on fucoidan was much slower than on L-fucose, with generation times of 220 h (±30 h SE) and 12.4 h (±2.7 h SE) respectively for strain F1 growing at 20°C. Strain F1 was grown on a starting concentration of fucoidan equivalent to 3.7 mM L-fucose (± 0.16 mM SE) and showed 44% (± 5.7% SE) degradation after 29 days and 64% (± 4.9% SE) after 120 days of incubation (Figure 5). Similarly, strain F21 showed 65% (± 3.1% SE) degradation after 125 days of incubation (data not shown).

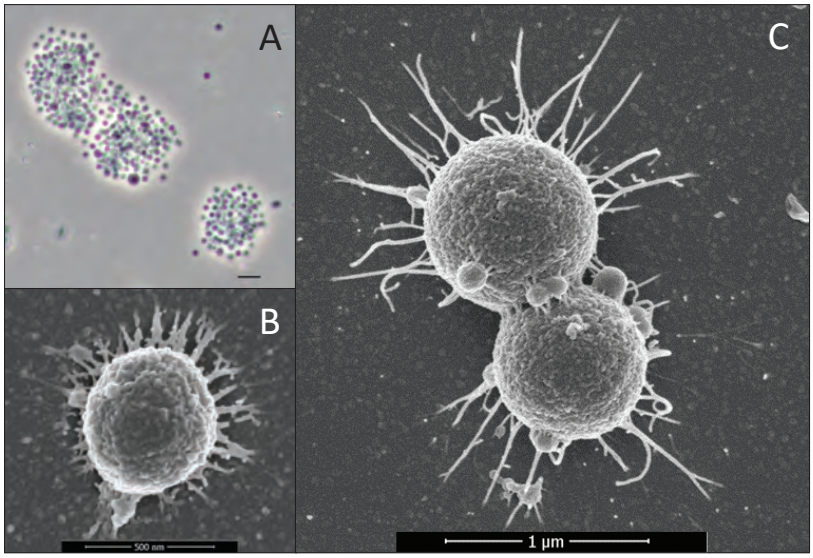


Figure 4. Micrographs of strains F1 and F21. A) Phase-contrast micrograph of aggregated strain F1 cells grown on fucoidan as substrate. The scale bar represents 2 µm. B) Scanning electron micrograph of strain F1 cells grown on fucoidan as substrate. C) Scanning electron micrograph of strain F21 cells grown on L-fucose as substrate.

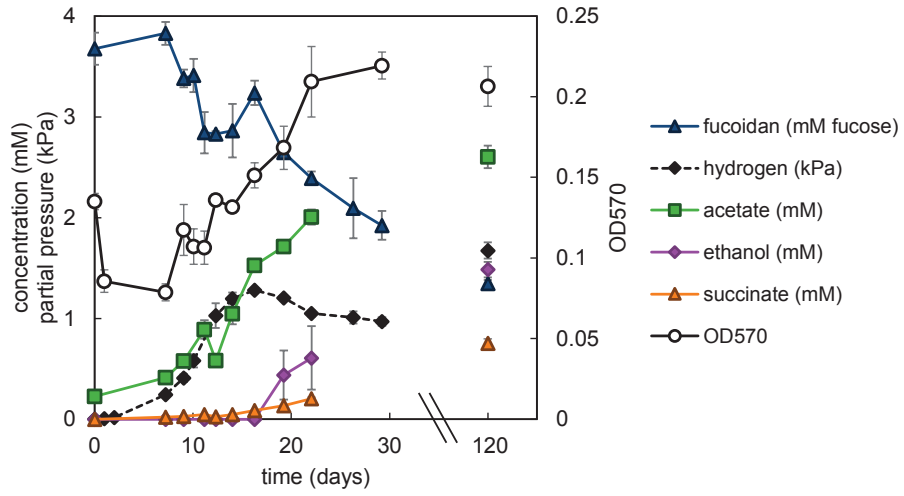


Figure 5. Strain F1 with 1.33 g L^{-1} *F. vesiculosus* fucoidan incubated at 20 °C. Values are the average of triplicate cultures. Error bars depict standard deviation. Open symbols should be read on the right y-axis.

3.2.3 Desulfation

As of yet, we have been unable to reproducibly quantify sulfate from fucoidan-grown cultures. However, we were able to do so for cultures of strain F21 growing on iota-carrageenan (Figure 6). These cultures produced acetate, ethanol, hydrogen and minor amounts of lactate and formate. The concentration of the iota-carrageenan monosaccharide constituents galactose and 2,3-anhydrogalactose could not be measured with the anthrone method, since they have different absorbance coefficients and their ratio varies (Yaphe, 1960). Assuming all non-sulfate weight of iota-carrageenan is composed of galactose and 2,3-anhydrogalactose in equal ratio, we found an electron recovery of only 47% (±7.2% SE). This likely reflects our assumption is incorrect, implying the iota-carrageenan contained impurities, such as often found for algal polysaccharides (Fitton et al., 2015). Sulfate recovery was 88% (±12.0% SE) assuming complete desulfation, and 101% (±13.8% SE) when corrected for the observed incomplete degradation of iota-carrageenan.

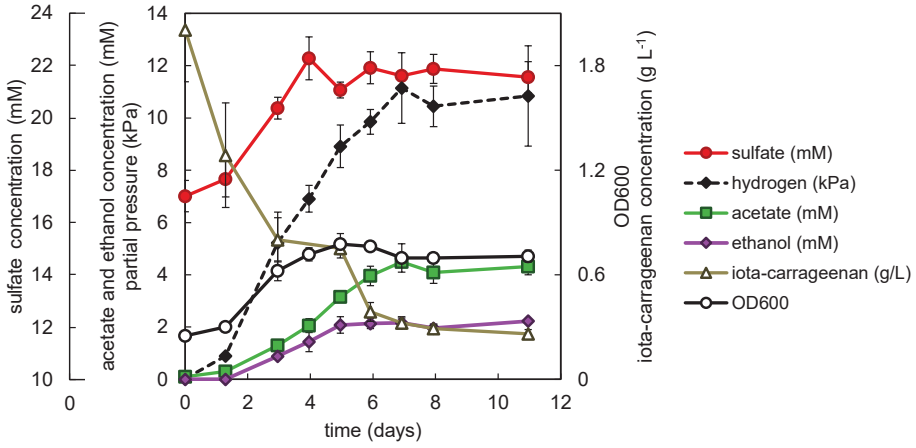


Figure 6. Strain F21 with 2 g L^{-1} iota-carrageenan incubated at 25 °C. Values are the average of triplicate cultures. Error bars depict standard deviation. Open symbols should be read on the right y-axis. Sulfate (mM) should be read on the outer left y-axis, other closed symbols on the main left y-axis.

3.3 Analysis of sulfatase genes

The draft genomes of strain F1 and F21 consisted of 5 and 6 contigs with a total length of 8.6 Mbp and 7.4 Mbp respectively. CheckM assessed the genomes to be near-complete (94.8% and 94.1% completeness). In both genomes a low level of contamination was reported (2.73%), which occurs for the majority of all pure culture genomes (Parks et

al., 2015). Probably, this is caused by duplication of single-marker genes, rather than the presence of foreign DNA.

The genomes of strains F1 and F21 contained exceptionally high numbers of sulfatase genes according to the prokka annotations (>400). We investigated different methods of sulfatase annotation. For instance, in the genome of strain F1, 531 genes were annotated as sulfatase genes by Prokka, 548 by Pfam profiles PF00884 and PF14707, 367 by PROSITE profiles PS00149 and PS00523, and 521 by our new HMMs. The Prokka and HMM annotation methods had considerable overlap, sharing 516 annotated genes. The HMM annotation method was chosen as most reliable, because the HMMs are based on recently and carefully designed detection profiles (Barbeyron et al., 2016a). The HMM method resulted in 521 sulfatase genes for strain F1 comprising 8.0% of all genes, and 480 sulfatase genes for strain F21 comprising 8.4% of all genes. Sulfatase genes formed 351 and 305 protein clusters in strains F1 and F21, respectively, of which 148 protein clusters were shared between the two strains. Most encoded sulfatases contained a signal peptide for export (354 and 339 respectively) to the periplasmic or extracellular space. Of these, respectively 280 and 266 were predicted to be soluble and respectively 74 and 69 to be membrane-anchored. Of the remainder, respectively 141 and 106 were predicted to be soluble cytoplasmic proteins, and respectively 26 and 35 to be membrane-anchored cytoplasmic proteins.

A sulfatase reannotation with the HMM method was performed for the ten other sulfatase-rich PVC bacteria to allow for a better comparison with strains F1 and F21. Strains F1 and F21 have the highest number as well as the highest relative abundance of sulfatase genes out of the 12 compared bacteria (Figure 7). Classification of all sulfatase genes into (sub)families revealed that strain F1 and F21 additionally have the highest number of encoded sulfatase (sub)families (Figure 7).

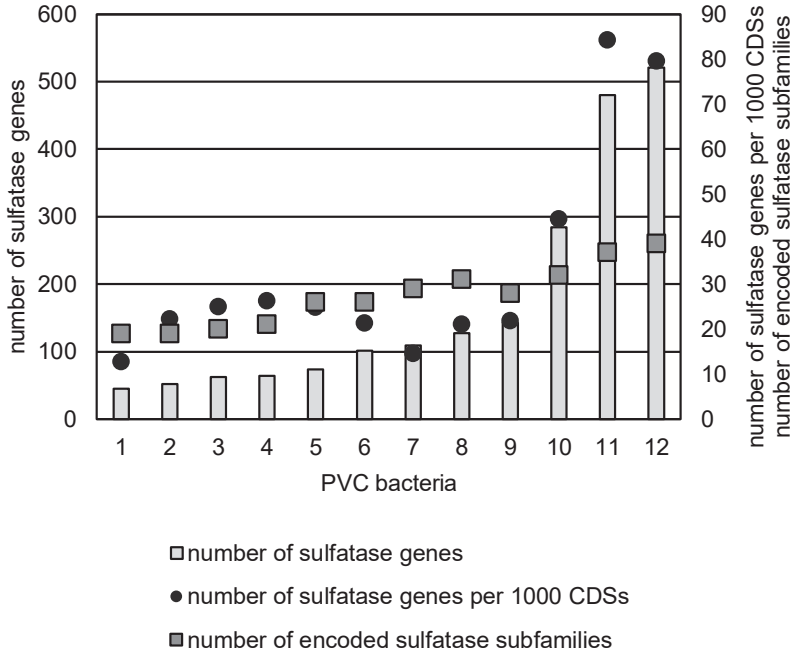


Figure 7. Abundance of sulfatase genes and subfamilies in sulfatase-rich PVC bacteria, including strains F1 and F21: 1. *Sedimentisphaerales* strain ST-NAGAB-D1; 2 . *Sedimentisphaera cyanobacteriorum* L21-RPul-D3; 3. *Kiritimatiella glycovorans* L21-Fru-AB; 4. *Sedimentisphaera salicampi* ST-PulAB-D4; 5. *Sedimentisphaerales* strain SM-Chi-D1; 6. *Planctomyces brasiliensis* DSM5305; 7. *Rhodopirellula baltica* SH1; 8. *Rhodopirellula maiorica* SM1; 9. *Rhodopirellula sallentina* SM41; 10. *Lentisphaera araneosa* HTCC2155; 11. *Kiritimatiellales* strain F21; 12. *Kiritimatiellales* strain F1.

The encoded sulfatases were mainly classified as family S1 sulfatases (Figure 8). This is the largest sulfatase family, and the only family known to contain carbohydrate sulfatases (Barbeyron et al., 2016a). S1 sulfatases are also known as formylglycine-dependent sulfatases (FGly-sulfatases), since they have a formylglycine as catalytic residue which is generated from a cysteine or serine residue by post-translational modification (Dierks et al., 1998). Out of the 2040 bona fide encoded FGly-sulfatases in our analysis, only 5 contained serine in the active site, of which two were found in *K. glycovorans*, one in strain F21, but none in strain F1. Strains F1 and F21 share a relatively similar sulfatase classification profile and cluster together with *Lentisphaera araneosa*, *Planctomyces brasiliensis* and the three *Rhodopirellula* species (Figure 8, Supplementary Figure S4).

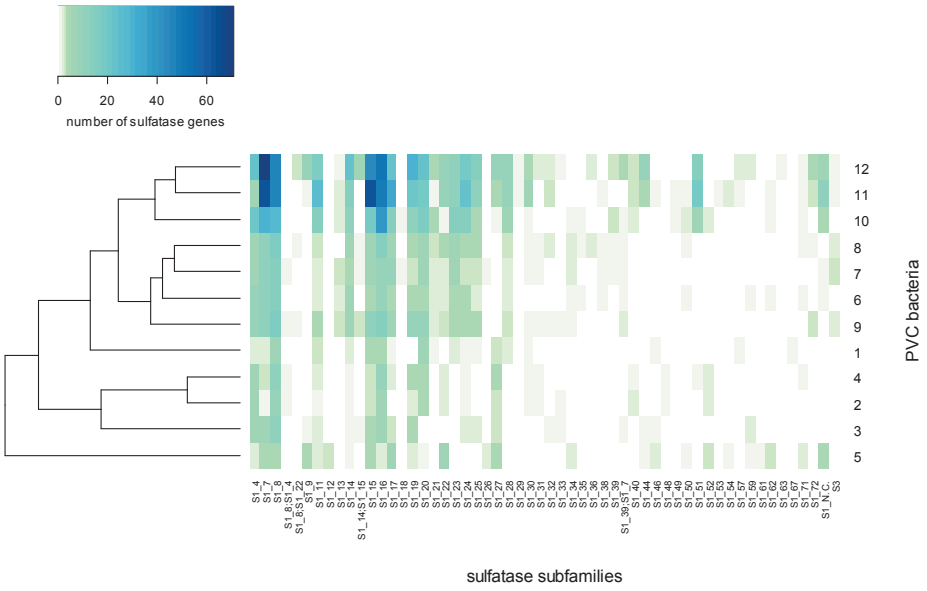


Figure 8. Heatmap of the number of sulfatase genes per sulfatase subfamily per genome with Bray-Curtis clustering. The numbering of sulfatase-rich PVC bacteria on the Y-axis label corresponds to the numbering in Figure 7: 1. *Sedimentisphaerales* strain ST-NAGAB-D1; 2. *Sedimentisphaera cyanobacteriorum* L21-RPul-D3; 3. *Kiritimatiella glycovorans* L21-Fru-AB; 4. *Sedimentisphaera salicampi* ST-PulAB-D4; 5. *Sedimentisphaerales* strain SM-Chi-D1; 6. *Planctomyces brasiliensis* DSM5305; 7. *Rhodopirellula baltica* SH1; 8. *Rhodopirellula maiorica* SM1; 9. *Rhodopirellula sallentina* SM41; 10. *Lentisphaera araneosa* HTCC2155; 11. *Kiritimatiellales* strain F21; 12. *Kiritimatiellales* strain F1.

The post-translational modification of cysteine or serine to formylglycine can be catalyzed by formylglycine-generating enzyme (FGE) or by anaerobic sulfatase-maturating enzyme (anSME; Bojarová and Williams, 2008). Strain F1 and F21 have 20 and 11 genes with one or more FGE domains (PF03781) respectively, but both have only a single anSME gene.

4 Discussion

Using traditional culturing techniques, we successfully enriched and isolated novel saccharolytic marine anaerobes. Strains F1 and F21 showed to be capable of growth of sulfated polysaccharides. Moreover, these *Kiritimatiellales* clade R76-B128 isolates represent a novel family, thus showing a high degree of phylogenetic novelty. Strains F1 and F21 are the first sulfatase-rich anaerobes isolated from a marine environment (15-35‰ salinity). Strikingly, they harbor more sulfatase genes and more encoded sulfatase (sub)families than any other currently known organism (Figure 7). Even if we only consider the numbers of sulfatase protein clusters (351 and 305 respectively), thereby ignoring potentially non-functional duplicated genes, these numbers exceed the total sulfatase gene counts of other known organisms.

Few microorganisms are known to grow on fucoidan. Another marine PVC bacterium (*Opitutales* sp.) has previously been isolated with *Cladosiphon* fucoidan as substrate (Sakai et al., 2003), but this isolate was aerobic and its genome has not been sequenced yet. The marine facultative anaerobe *Vibrio* strain N-5 (Furukawa et al., 1992) and the marine aerobe *Zobellia galactanivorans* (Barbeyron et al., 2016b) can also grow on different types of fucoidan. However, while the aerobic PVC model organism *R. baltica* can grow on carrageenans and chondroitin 4-sulfate, it cannot grow on *F. vesiculosus* fucoidan (Wegner et al., 2013). Also the anaerobic *K. glycovorans* (Spring et al., 2016), *S. cyanobacteriorum* and *S. salicampi* (Spring et al., 2018) are not able to grow on *F. vesiculosus* fucoidan. However, even among high-sulfatase PVC bacteria (e.g. *L. araneosa*; Cho et al., 2004), it is not a common practice to test for growth on fucoidan. Additionally, fucoidan from different sources share a backbone of alpha-1,3-linked and sometimes also alpha-1,4-linked L-fucose, but can vary strongly in other structural features such as substitution with sulfate, acetate or sugars (Berteau and Mulloy, 2003; Ale et al., 2011). As a consequence, microorganisms may selectively grow on only specific types of fucoidan (Barbeyron et al., 2016b). Therefore, it is difficult to assess how rare this trait is.

Strains F1 and F21 seem well-adapted for growth on sulfated polysaccharides, as reflected by their capability to grow on different types of fucoidan, chondroitin 4-sulfate, and – only by strain F21 – iota-carrageenan, as well as on various mono- and disaccharides. Additionally, the enrichment of the R76-B128 clade on mucin and iota-carrageenan (17% and 32% respectively, Figure 2) support that this clade is competitively successful growing on sulfated biopolymers other than fucoidan. Sequences of the R76-B128 clade were also detected in the sediment slurry used as inoculum (0.024%, Supplementary Table S2). Additionally, 16S rRNA gene sequences of this clade were detected in anoxic column water samples from the Black Sea collected at 250 m depth in 2013 (0.36%; L. Villanueva, personal communication; see Egger et al. [2016] for sampling methods). The majority of the organic matter produced by micro- and macroalgae in surface waters

is degraded at depths more than 200 m (Karl and Knauer, 1991), implying that algal fucoidan is not present at the depths at which the R76-B128 clade was detected. Instead, it seems that the main available substrates for saccharolytic microorganisms such as strains F1 and F21 are exopolysaccharides and cell wall constituents produced locally by other anaerobic microorganisms, as was also proposed for *K. glycovorans* (Spring et al., 2016). Marine microbial communities are likely to produce exopolysaccharides that are sulfated (Helbert, 2017) and heterogeneous in structure (Sutherland, 2001). *F. vesiculosus* fucoidan shares these properties, since it is also sulfated and is heterogeneous with respect to glycosidic linkage, sulfate ester substitution position and variable substitution with acetate (Berteau and Mulloy, 2003) and other sugars (Ale and Meyer, 2013; Fitton et al., 2015) such as xylose (Chevolot et al., 2001).

Marine microorganisms form the biggest reservoir of sulfatase diversity (Barbeyron et al., 2016a), owing to the high diversity of sulfated biopolymers in the marine environment (Helbert, 2017). Sulfatases can be classified into four families, based on protein sequence homology (Barbeyron et al., 2016a; Helbert, 2017). The largest of these, the S1 family encompassing all FGly-sulfatases, is split phylogenetically into 73 subfamilies which were proposed to be substrate-specific (Barbeyron et al., 2016a). However, this proposition was based on studies on 42 predominantly mammalian sulfatases within only 13 subfamilies (S1_1 to S1_12 and S1_19), leaving the large diversity of microbial sulfatases and the majority of the S1 subfamilies still to be studied.

Most sulfatases of strains F1 and F21 were predicted to be exported, suggesting most sulfatase are active in the periplasm or extracellularly. Sulfatase subfamilies S1_7, S1_8, S1_15 and S1_16 were most abundant in strains F1 and F21 (Figure 8). Subfamily S1_8 contains a human N-sulfoglucosamine sulfamidase (Scott et al., 1995) and a sulfamidase from *Pedobacter heparinus* ATCC13125^{T} (Myette et al., 2009), both active towards the N-sulfated glycosaminoglycans heparin and heparan sulfate. Subfamily S1_7 contains a murine (Daniele et al., 1993) and a human (Wilson et al., 1990) sulfatase active towards iduronate, a constituent of heparin/heparan sulfate, as well as an endo-kappa-carrageenan sulfatase from *Pseudoalteromonas atlantica* T6c (Préchoux et al., 2013). In the genomes of strains F1 and F21, we observed S1_7 sulfatase genes containing domains of glycosyl hydrolase family 10 (PDESU_06089, SCARR_04194) and family 32 (SCARR_04167). These genes probably constitute gene fusions of sulfatase and glycosyl hydrolase genes, as observed before in *Polaribacter* strain Hel1_33_49 (PHEL49_1323; Xing et al., 2015) and two marine *Planctomycetes* strains (Kim et al., 2016). Glycosyl hydrolase families 10 and 32 are active towards xylan and fructan, respectively. This substrate specificity may also apply to the fused S1_7 sulfatases. Thus, the sulfatase subfamily S1_7 could have a broad substrate specificity towards sulfated polysaccharides with various compositions. Sulfatase fusion genes were detected within subfamilies S1_15 and S1_16, of which no sulfatases have been studied yet. A S1_15 sulfatase gene in strain F1 (PDESU_05015) contains an alpha-L-fucosidase domain

(glycoside hydrolase family 29), and can thus possibly desulfate fucoidan. Such an alpha-L-fucosidase/sulfatase fusion gene is also encoded by *Rhodopirellula rubra* strain SWK7 (RRSWK_03833). Further, a S1_16 sulfatase gene in strain F21 (SCARR_05639) was annotated as amylopullulanase. Thus, strains F1 and F21 encode sulfatases that could hydrolyze sulfamate groups in heparin-like polysaccharides, and that could hydrolyze sulfate esters bound to various carbohydrate structures.

Iota-carrageenan is a heteropolysaccharide composed of a repeating disaccharide of galactose 4-sulfate and 3,6-anhydrogalactose 2-sulfate (Usov, 2011). We quantitatively demonstrated the desulfation of iota-carrageenan by strain F21 (Figure 6), confirming the activity of iota-carrageenan sulfatases. Both isolates encode sulfatases of subfamily S1_19 which contains a characterized iota-carrageenan sulfatase from *P. atlantica* T6c (Préchoux et al., 2013). Both strains encode these sulfatases, but only strain F21 is able to grow on iota-carrageenan. However, strain F1 is able to grow on the main constituent of carrageenans, galactose, implying that its inability has an origin in the hydrolytic genes. While we were not able to quantitatively demonstrate desulfation of fucoidan, the degradation of fucoidan involves the removal of sulfate esters from the fucose backbone. Recently, the first two fucoidan sulfatases with known sequences were reported and assigned to subfamilies S1_17 and S1_25 (Silchenko et al., 2018). Both these subfamilies are encoded by strains F1 and F21, and may thus be involved in fucoidan degradation. The abundantly encoded S1_15 subfamily may also be involved, since it contains the aforementioned alpha-L-fucosidase/sulfatase fusion gene (PDESU_05015) found in strain F1. However, these three subfamilies are also encoded – although in lower numbers – by the non-fucoidan degraders *K. glycovorans* and *R. baltica*, raising questions on the diversity of exact substrate specificities within sulfatase subfamilies.

The sulfatase classification profile of strains F1 and F21 is more similar to that of several marine aerobic PVC bacteria than to that of several anaerobic PVC bacteria from hypersaline microbial mats, including their closest relative *K. glycovorans* (Figure 8). Although the selection of genomes in this study was small, this result suggests that the type of sulfatases encoded by a PVC bacterium is not determined by its relation to oxygen or by phylogeny, but rather by the habitat of the bacterium (marine/hypersaline microbial mat). Similar conclusions were drawn with regard to both the sulfatase and CAZyme profiles of marine heterotrophic bacteria, although phylogeny at the phylum level was also found to be a significant factor (Barbeyron et al., 2016b). Possibly, the habitat factor represents the type of substrate available.

The formylglycine that forms the catalytic residue of FGly-sulfatases can be generated from cysteine by oxygen-dependent FGEs (Bojarová and Williams, 2008) and from cysteine or serine by oxygen-independent anSMEs (Berteau et al., 2006). Barbeyron et al. (2016a) constructed a sulfatase gene database and found 78% of all FGly-sulfatase

genes to contain a cysteine as precursor for formylglycine, and 22% to contain a serine instead. Even though our dataset included sulfatase genes from seven sulfatase-rich PVC anaerobes, only 5 out of 2040 encoded sulfatases contained a serine as precursor. In contrast, the sequences from our dataset and those of Barbeyron and colleagues showed closely matching degrees of conservation for other residues involved in the catalytic site or in calcium coordination (following *Pseudomonas aeruginosa* PAO1 AtsA residue numbering: Arg55, Gly61, Asp13, Asp14, Asp317, Asn318, His211 and Lys375).

Strains F1 and F21 both harbored a single anSME. In the gut anaerobe *Bacteroides thetaiotaomicron,* a single anSME could post-translationally modify multiple sulfatases (Benjdia et al., 2011). Also in *R. baltica,* only one of the six encoded FGEs is expressed during growth on sulfated polysaccharides (Wegner et al., 2013). Paradoxically, several putative FGEs were also encoded by strains F1 and F21, contrasting with their strict anaerobic nature. The oxygen dependency of FGEs has been concluded from research on mammalian FGEs (Bojarová and Williams, 2008; Appel and Bertozzi, 2014), and was also confirmed for prokaryotic FGEs from *Mycobacterium tuberculosis* and *Streptomyces coelicolor* (Carlson et al., 2008). Therefore, we speculate that the putative FGEs encoded by our strain are inactive in their anoxic habitat or have an alternate function.

5 Conclusion

Novel *Kiritimatiellaeota* of subclade R76-B128 were enriched on the sulfated polysaccharides fucoidan and iota-carrageenan, and on the sulfated glycoprotein mucin. Strains F1 and F21 were isolated from fucoidan enrichments, and were found to represent a novel family within the order *Kiritimatiellales*. Both strains were capable of using various mono-, di- and polysaccharides as substrates for anaerobic growth, including fucoidan. These strains represent the first sulfatase-rich anaerobes isolated from a marine environment. Sulfatase activity was confirmed by quantitative demonstration of sulfate group hydrolysis from iota-carrageenan by strain F21. Analysis of sulfatase genes showed that strain F1 and F21 harbor the highest number and relative abundance of encoded sulfatases of all currently known organisms. These results imply that the isolates are well-adapted for the degradation of heterogeneous sulfated polysaccharides. Specifically, the encoded sulfatase subfamilies S1_15, S1_17 and S1_25 could play a role in the degradation of fucoidan. Other abundantly encoded sulfatase subfamilies may be active towards sulfate esters and sulfamates bound to a variety of polysaccharide structures. The sulfatase gene classification profile of strains F1 and F21 showed more similarity to that of aerobic than anaerobic sulfatase-rich PVC bacteria, including their closest relative *K. glycovorans*. In line with previous research (Barbeyron et al., 2016b),

results indicate habitat as main determinant for sulfatase profile. Strains F1 and F21 both encoded a single anSME, but paradoxically also multiple putative FGEs, which are thought to be oxygen-dependent. Future expression studies and analysis of operons will yield insight into the degradation mechanism of sulfated polysaccharides by these strains, including the role the encoded sulfatases and their maturation enzymes.

6 Supplementary material

Supplementary tables and figures can be accessed at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00253/full#supplementary-material.

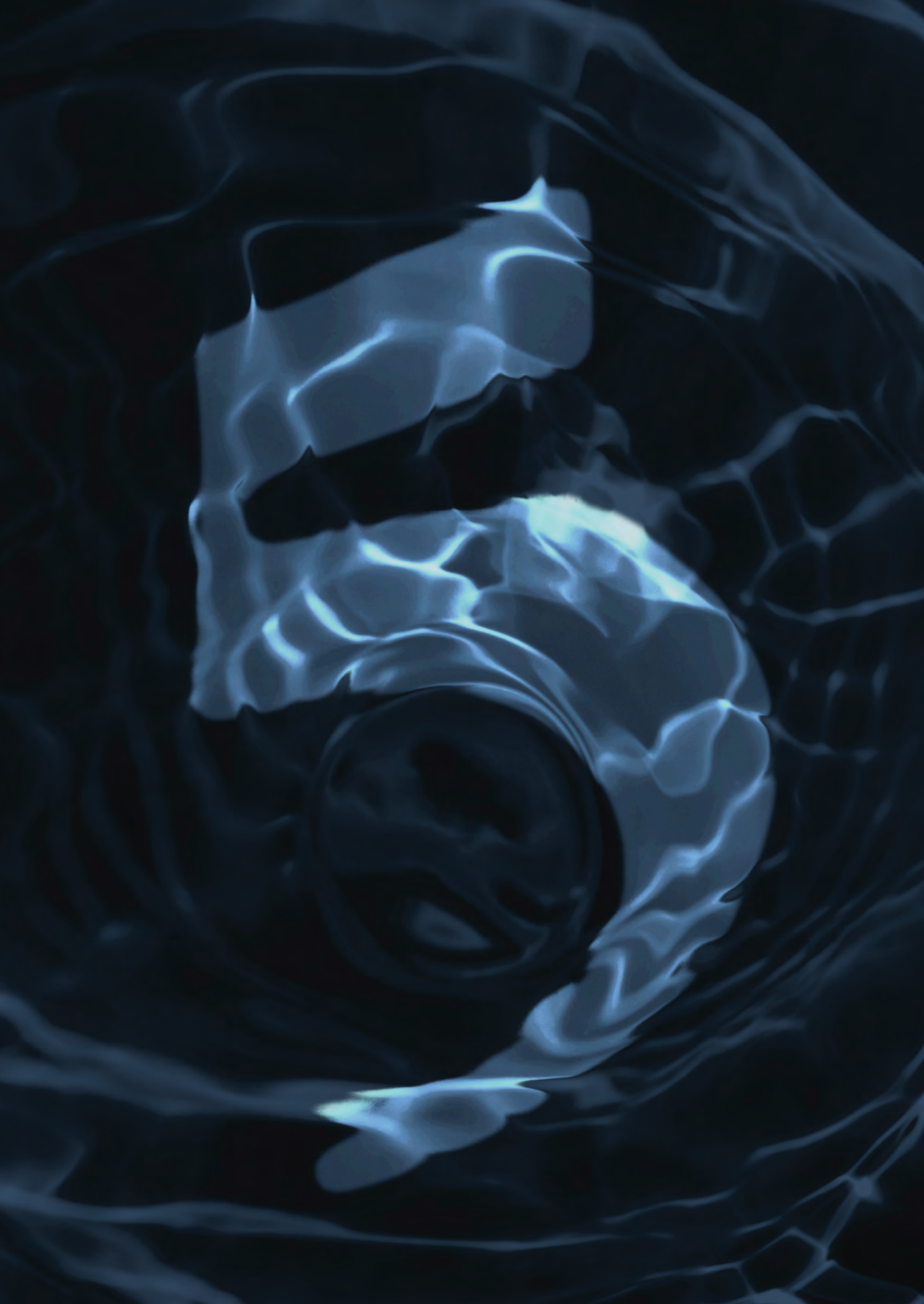
Table S1. Primers used in this study.

Figure S1. Measured compounds in the negative control enrichment cultures. Values shown are the average values obtained from duplicate cultures. Closed symbols are read on the left y-axis, open symbols on the right y-axis.

Figure S2. Measured compounds in the killed control enrichment culture. Closed symbols are read on the left y-axis, open symbols on the right y-axis.

Figure S3. Electron recoveries calculated from triplicate cultures grown at 20°C.

Figure S4. Heatmap of the number of sulfatase genes per sulfatase subfamily per genome, with Jaccard clustering. Labelling as in Figure 7, ordered by total number of sulfatase genes.



CHAPTER 5

Pontiella desulfatans gen. nov., sp. nov., and *Pontiella sulfatireligans* sp. nov., two marine anaerobes of the *Pontiellaceae* fam. nov. producing sulfated glycosaminoglycan-like exopolymers

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Abstract

Recently, we isolated two marine strains, F1^T and F21^T, which together with *Kiritimatiella glycovorans* L21-Fru-AB^T are the only pure cultures of the phylum *Kiritimatiellaeota*. Here, we present an in-depth genome-guided characterization of both isolates with emphasis on their exopolysaccharide synthesis. The strains only grew fermentatively on simple carbohydrates and sulfated polysaccharides. Strains F1^T, F21^T and *K. glycovorans* reduced elemental sulfur, ferric citrate and anthraquinone-2,6-disulfonate during anaerobic growth on sugars. Both strains produced exopolysaccharides during stationary phase, probably with intracellularly stored glycogen as energy and carbon source. Exopolysaccharides included N-sulfated polysaccharides probably containing hexosamines and thus resembling glycosaminoglycans. This implies that the isolates can both degrade and produce sulfated polysaccharides. Both strains encoded an unprecedently high number of glycoside hydrolase genes (422 and 388, respectively), including prevalent alpha-L-fucosidase genes, which may be necessary for degrading complex sulfated polysaccharides such as fucoidan. Strain F21^T encoded three putative glycosaminoglycan sulfotransferases and a putative sulfate glycosaminoglycan biosynthesis gene cluster. Based on phylogenetic and chemotaxonomic analyses, we propose the taxa *Pontiella desulfatans* F1^T gen. nov., sp. nov. and *Pontiella sulfatireligans* F21^T sp. nov. as representatives of the *Pontiellaceae* fam. nov. within the order *Kiritimatiellales*.

1 Introduction

Sulfated polysaccharides are diverse and widespread. In animals, sulfated polysaccharides are present as sulfated glycan side-chains of mucin (Jin et al., 2017), and as sulfated glycosaminoglycans (mucopolysaccharides) such as chondroitin sulfate, an important component of cartilage (Meyer et al., 1956). Sulfated polysaccharides are prevalent in marine environments, where they are produced in high quantities by macroalgae, microalgae and bacteria (Helbert, 2017). This class of compounds forms an important substrate for marine microorganisms due to their prevalence. Degradation of sulfated polysaccharides involves the removal of sulfate groups by sulfatases (Barbeyron et al., 2016a). Sulfatase genes are present in high numbers in the genomes of some marine bacteria of the *Planctomycetes-Verrucomicrobia-Chlamydiae* (PVC) superphylum such as *Rhodopirellula baltica* SH1^T (Glöckner et al., 2003) and *Lentisphaera araneosa* HTCC2155^T (Thrash et al., 2010), supporting the idea that PVC bacteria are likely to be key degraders of sulfated polysaccharides in the marine environment. Anaerobic strains of sulfatase-rich PVC bacteria have rarely been studied thus far, despite the importance of anoxic marine sediments in the mineralization process (Arndt et al., 2013).

In a previous study, we isolated two anaerobic marine bacteria from anoxic Black Sea sediment, strains F1^T and F21^T, which grew on sulfated polysaccharides and belonged to the *Kiritimatiellaeota* phylum (Van Vliet et al., 2019). This clade was formerly known as *Verrucomicrobia* subdivision 5 (Spring et al., 2016), and is still regarded as subdivision in the Genome Taxonomy Database (Parks et al., 2018). *Kiritimatiellaeota* are widespread and abundant in anoxic environments such as the intestinal tract of vertebrate animals (Frey et al., 2006; Steelman et al., 2012) and marine or hypersaline sediments (Cardman et al., 2014; Spring et al., 2016). Our marine isolates, strains F1^T and F21^T, were found to represent a novel family-level *Kiritimatiellaeota* lineage (Van Vliet et al., 2019) previously marked as ubiquitous yet uncultured (R76-B128; Yilmaz et al., 2015). Currently, the only described species of the *Kiritimatiellaeota* phylum is *Kiritimatiella glycovorans* L21-Fru-AB^T, a moderate halophile isolated from a hypersaline microbial mat (Spring et al., 2016). Like several PVC bacteria described before (Glöckner et al., 2003; Derrien et al., 2004; Thrash et al., 2010; Wegner et al., 2013), *K. glycovorans* was proposed to degrade sulfated polysaccharides *in situ* based on the presence of glycoside hydrolase and sulfatase genes in the genome, although stable growth could not be achieved *in vitro* (Spring et al., 2016). In contrast, strains F1^T and F21^T were able to grow on complex sulfated polysaccharides, such as fucoidan, and notably encoded exceptionally high numbers of sulfatases (521 and 480, respectively; Van Vliet et al., 2019). Here, we present a comprehensive characterization of both strains. Important physiological traits are revealed such as their ability to produce sulfated glycosaminoglycan-like exopolymers, a feature not yet described for bacterial pure cultures.

2 Materials and methods

2.1 Strains, growth conditions and substrates

Strains F1^T and F21^T were deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany) under the respective accession numbers DSM 106878^T and DSM 106829^T, and at the Korean Collection for Type Cultures (KCTC; Jeongeup-si, South Korea) under the respective accession numbers KCTC 15641^T and KCTC 15642^T. *K. glycovorans* L21-Fru-AB^T was ordered from the DSMZ (Braunschweig, Germany). Strains F1^T and F21^T were cultured in a basal anoxic bicarbonate-buffered marine medium described previously (Van Vliet et al., 2019) containing 25 g L^{-1} NaCl for optimum salinity for growth, with 10 mM L-fucose as the substrate, unless mentioned otherwise. *K. glycovorans* was cultured in the same basal medium except for containing 60 g L^{-1} NaCl, with 5 mM D-glucose as the substrate. All cultures were incubated statically at 25 °C in the dark, unless mentioned otherwise. Substrates were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless mentioned otherwise. Polysaccharide substrates were obtained from various distributors (Table 1).

Table 1. Polysaccharide substrates used in this study, their source or type, distributor and lot number, if mentioned on the packaging. "NR" stands for "not reported".

Polysaccharide	Source/Type	Distributor	Lot Number
alginic acid	NR	Thermo Fisher Scientific (Waltham, MA, US)	NR
arabinan	sugar beet	Megazyme (Bray, Ireland)	80902b
cellulose	microgranular, CC41	Whatman (Maidstone, UK)	1441024
chitin	shrimp shells	Sigma-Aldrich (St. Louis, MO, US)	SLBL2694V
chitosan	shrimp shells	Sigma-Aldrich (St. Louis, MO, US)	BCBQ3414V
chondroitin sulfate	bovine trachea	Sigma-Aldrich (St. Louis, MO, US)	NR
laminarin	*Eisenia bicyclis*	abcr (Karlsruhe, Germany)	1025869
pectin	apple	Sigma-Aldrich (St. Louis, MO, US)	BCBK7271V
pullulan	*Aureobasidium pullulans*	Sigma-Aldrich (St. Louis, MO, US)	NR
starch	soluble	Sigma-Aldrich (St. Louis, MO, US)	SLBL2691V
xanthan gum	*Xanthomonas campestris*	Sigma-Aldrich (St. Louis, MO, US)	100M0218V
xylan	beechwood	Sigma-Aldrich (St. Louis, MO, US)	107H1209
κ-carrageenan	NR	Sigma-Aldrich (St. Louis, MO, US)	BCBR6980V
ι-carrageenan	NR	Sigma-Aldrich (St. Louis, MO, US)	SLBJ7874V

2.2 Genome annotation and visualization

Genome sequencing, assembly and annotation with Prokka and InterProScan 5 was described previously (Van Vliet et al., 2019). Additionally, genes for the production of secondary metabolites (ectoine, pigments, potential antibiotics) were annotated with antiSMASH version 5.0 (Blin et al., 2019). Peptidase genes were annotated by alignment with the MEROPS scan v12.0 database (Rawlings et al., 2018) using DIAMOND (Buchfink et al., 2014). CAZymes were annotated with the dbCAN2 web server (HMMdb v7; Zhang et al., 2018). Only hidden Markov model (HMM) matches were considered, and these were manually curated to exclude false positives. Genomes were visualized with CGView Server (Grant and Stothard, 2008). The visualization of inter-genome homology was based on a comparison of predicted proteins with a translated genome using blastx with an E-value cutoff of 10^{-5} and an identity cutoff of 30%. In general, encoded pathways for the degradation of substrates were explored through annotation with RAST v2.0 (Overbeek et al., 2014) and analysis and visualization with Pathway Tools v23.0 (Karp et al., 2010) and the MetaCyc database (Caspi et al., 2018). Cellular localization was predicted with SignalP (Petersen et al., 2011) and PSORTb v3.0.2 (Yu et al., 2010). Homologs of fucose degradation genes and glycosaminoglycan sulfotransferase genes were found by reciprocal matching of coding sequences. Blastp matches with protein sequences from literature were queried against the UniProtKB/Swiss-Prot database (The UniProt Consortium, 2019), and bitscores were compared to test reciprocity. Genome assemblies and Prokka annotations are available at the European Nucleotide Archive (ENA) through the sample accession numbers SAMEA5207384 and SAMEA5207385 for strain F1^T and strain F21^T, respectively. The 16S rRNA gene sequences can be found with the respective accession numbers LS482847 and LS453290.

2.3 Physiological tests

Analysis of respiratory quinones was carried out by the Identification Service and Dr. Brian Tindall of the DSMZ (Braunschweig, Germany). Catalase activity was tested by applying drops of 15% *v*/*v* hydrogen peroxide onto a pellet of active biomass obtained through centrifugation of 5 mL of a liquid culture (4700 × *g*, 10 min). Oxidase activity was tested with diagnostic oxidase strips (Merck, Darmstadt, Germany). Gram staining was performed according to standard protocols and confirmed by applying a drop of 1 M NaOH solution onto a cell pellet, which leads to slimy wire formation within 10 s for Gram-negative cells. Cell size was deduced from phase contrast micrographs and scanning electron micrographs obtained as previously reported (Van Vliet et al., 2019). Growth in liquid culture was monitored through measuring optical density at 600 nm. The effect of salinity on growth was tested in triplicate 5 mL cultures in Hungate tubes with the NaCl concentration in the medium modified to 0%, 0.5%, 1%, 1.5%, 1.7%,

1.9%. 2.3%, 2.7%, 3.1%, 3.5%, 4.3%, 5%, 5.7% and 6.3%. The effect of pH on growth was also tested in triplicate 5 mL cultures buffered with 50 mM MES (pH 5, 5.5), PIPES (pH 6, 6.5, 7, 7.5) or Tris (pH 8, 8.5, 9). At pH values of 8.5 and 9, precipitation occurred. This could be avoided by a tenfold reduction in the added quantities of $CaCl_2$, $MgSO_4$ and $MgCl_2$, but this inhibited growth in positive control cultures with a pH of 7. Therefore, absence of growth at pH > 8.5 is possibly due to the lack of Ca and/or Mg instead of pH effect. For ectoine analysis, strain F21^T was grown at the highest tolerated NaCl concentration (5% *w*/*v*). Ectoine was extracted by resuspending a cell pellet in 70% *v*/*v* ethanol and bead-beating the sample with mixed zirconia/silica beads of 2.5 and 0.5 mm diameter and a FastPrep bead-beater (MP Biomedicals, OH, USA) twice for 20 s at a speed setting of 6.0. Ectoine concentration was measured by high-pressure liquid chromatography (HPLC) using a Thermo Scientific Accela 600 HPLC equipped with an Agilent Polaris 3 NH2 column (100 × 4.6 mm) and a UV detector. The system was operated at 30 °C and 0.8 mL min^{-1} elution. The eluent was an isocratic mix of 75% *v*/*v* acetonitrile and 25% *v*/*v* Milli-Q water. Substrate tests for polysaccharides, organic acids, alcohols and H_2/CO_2 (80:20 *v*/*v*) were performed as described previously (Van Vliet et al., 2019).

2.4 Reduction of external electron acceptors

The reduction of electron acceptors was tested in duplicate cultures with the following electron acceptors (concentration in mM): nitrate, 10; nitrite, 2; sulfite, 2; thiosulfate, 10; chemically produced elemental sulfur, 50; colloidal elemental sulfur, 50; biologically produced elemental sulfur (THIOPAQ®, Paques, Balk, The Netherlands), 50; dimethylsulfoxide (DMSO), 20; ferric citrate, 20; fumarate, 20; manganese oxide (MnO_2), 10; MnO_2/anthraquinone-2,6-disulfonate (AQDS), 10/0.1. Amorphous MnO_2 was prepared from $KMnO_4$ and $MnCl_2$ as described by Burdige and Nealson (1985). Previously described methods were applied to measure nitrate and thiosulfate concentrations through anion chromatography, H_2 partial pressures through gas chromatography (GC), concentrations of organic acids and alcohols through HPLC and dissolved sulfide concentration through the methylene blue colorimetric assay (Van Vliet et al., 2019). Dissolved dimethylsulfide produced by the reduction of DMSO was analyzed using a Thermo Scientific Accela 600 HPLC equipped with an Agilent Poroshell 120 EC-C18 column (4.6 × 250 mm; 4 μm) and a UV detector. The system was operated at 30 °C and 1 mL min^{-1} elution. The eluent was 0.1% *w*/*v* formic acid with a linear increase of 15% to 80% acetonitrile over the course of 12 min, followed by 80% acetonitrile for 5 min. Ferric and ferrous iron concentrations were quantified with the ferrozine assay (Stookey, 1970). To measure Mn(II) produced by the reduction of MnO_2, the formaldoxime assay was used (Brewer and Spencer, 1971; Armstrong et al., 1979) after acidification of the samples to pH 1.5 with HCl.

2.5 Oxygen gradient cultures

To prepare oxygen gradient cultures, autoclaved Hungate tubes sealed with cotton plugs were filled with 10 mL anoxic basal medium containing 1% *w*/*v* SeaPlaque low-melting agarose (Lonza, Basel, Switzerland) and 5 mM L-fucose, and left to solidify. The final pH was 7.8. Media were inoculated with 5% *v*/*v* liquid culture and mixed well before dispensing into Hungate tubes. Phosphate salts were added from separately autoclaved anoxic stock solutions (Tanaka et al., 2014). Oxidation of the medium was inferred from the color of the redox indicator resorufin, the product of the irreversible reduction of resazurin. Amplification, sequencing and analysis of full-length 16S rRNA gene sequences from culture samples was performed as described previously (Van Vliet et al., 2019), with the purpose of verifying culture purity.

2.6 Energy storage compound analysis

Cells were fixed for transmission electron microscopy (TEM) according to an adaptation of the protocol of Wittmann et al. (2014), as performed by Spring et al. (2016). Shortly, biomass pellets were fixed in a fixative solution (5% *w*/*v* formaldehyde, 2% *w*/*v* glutaraldehyde) on ice for one hour, washed twice with washing buffer (0.1 M cacodylate), resuspended in 100 µL of phosphate-buffered gelatin and left to solidify for 20 min at 4 °C. Then, 500 µL of fixative solution was applied and the samples were incubated for 15 min at room temperature. The solid samples were cut into small pieces of around 0.2 mm^3, fixed with fixation solution for another 30 min at room temperature and washed six times with washing buffer. Samples were then fixed with 1% *w*/*v* osmium tetroxide for 1 h at room temperature and washed three times with Milli-Q water. Then, samples were dehydrated with a graded series of acetone (10%, 30%, 50%, 70%, 90%, 100%) and embedded in Spurr epoxy resin as previously described (Spurr, 1969). Ultrathin sections were cut with a Leica EM UC7 ultramicrotome (Leica, Wetzlar, Germany), and poststained using uranyl acetate and lead citrate. The sections were then examined on a JEOL JEM-1400 series 120kV TEM (JEOL, Tokyo, Japan). Cells were delicate, as many cells looked disintegrated, whereas *Desulfovibrio desulfuricans* G11 cells processed in parallel appeared mostly intact. Following the protocol for polyphosphate staining in bacterial cells by Havemeyer (2013), cell pellets were stained with a 4′,6-diamidino-2-phenylindole (DAPI) solution of 1 µg mL^{-1}, incubated for 30 min at room temperature, washed with phosphate-buffered saline and inspected with a BX41 fluorescence microscope (Olympus, Tokyo, Japan) equipped with an X-Cite Series 120Q metal-halide fluorescence lamp (Excelitas, Waltham, MA, USA), a 330–385 nm excitation filter and a 510–550 nm emission filter. As positive control, the staining of DNA by DAPI was inspected with the same excitation filter and a long-pass 420 nm emission filter.

2.7 Extracellular polymeric substances analysis

For the analysis of exopolysaccharides, resazurin was omitted from the basal medium. The biomass was centrifuged at 10,000 × *g* for 20 min. The extracellular polymeric substances (EPS) in the supernatant were precipitated by the addition of cold absolute ethanol to a final concentration of 50% (*v*/*v*). The precipitate was collected by centrifugation (10,000 × *g*, 30 min), washed three times in absolute ethanol and lyophilized. Fourier-transform infrared spectroscopy (FTIR) spectra of the extracted EPS were recorded on an FTIR Spectrometer (PerkinElmer, Waltham, MA, USA) with a wavenumber range from 550 to 4000 cm^{-1}. The lyophilized EPS was analyzed with scanning electron microscopy energy-dispersive X-ray (SEM-EDX) using a Philips XL 30 SEM (Philips, Amsterdam, The Netherlands). Prior to the SEM-EDX analysis, samples were metallized with gold and palladium. The sulfated polysaccharide content in the lyophilized EPS was measured with the Blyscan™ assay (Biocolor, Carrickfergus, UK) following the manufacturer's instructions. In brief, the sample (1.5 mg) was digested by papain extraction reagent overnight at 65 °C. After centrifugation at 10,000 × *g* for 10 min, the supernatant was collected. Total sulfated polysaccharides were quantified by using the Blyscan™ dye reagent containing 1,9-dimethylmethylene blue (DMMB) with bovine tracheal chondroitin 4-sulfate as the standard. In addition, the ratio of O- and N-sulfation of the sulfated polysaccharides was determined with the nitrous acid cleavage method. Nitrous acid reacts with N-sulfated hexosamine, cleaving off the sulfate ester group (Bienkowski and Conrad, 1985). The difference between the total sulfated sites and the amount of O-sulfated sites after nitrous acid cleavage was used to determine the relative amount of N-sulfated hexosamine.

2.8 Phylogenetic reconstruction

In May 2019, *Kiritimatiellales* genomes were retrieved for reconstructing phylogeny. Microbial Genomes Atlas (MiGA; Rodriguez-R et al., 2018) was used to query NCBI genomes, the metagenome-assembled genomes (MAGs) from Parks et al. (2018) and the Tara Oceans MAGs from Delmont et al. (2018). NCBI genomes were also searched manually by taxonomic description. All selected genomes were >50% complete and <6% contaminated, with contamination occurring only in genomes with >80% completeness, as determined with CheckM v1.0.5 (Parks et al., 2015). GTDB-Tk v0.2.2 was used to identify genes and generate a trimmed concatenated alignment (Chaumeil et al., 2019), which was trimmed further with TrimAI v1.3 (Capella-Gutiérrez et al., 2009) using the "—gappyout" setting to a length of 4451 amino acid positions. Maximum-likelihood phylogeny was calculated with IQ-TREE v1.6.10 (Nguyen et al., 2015) using the LG+C30+F+G8 evolutionary model as selected by ModelFinder (Kalyaanamoorthy et al., 2017) from various possibilities. Branch support was determined with 1000 SH-like approximate likelihood ratio tests (Guindon et al., 2010) and 1000 ultrafast bootstraps

(Hoang et al., 2018). The tree was inspected with FigTree v1.4.2[11]. Average amino acid identity (AAI) between genomes was calculated with the enveomics aai.rb script (Rodriguez-R and Konstantinidis, 2016) using blastp.

2.9 Lipid and cellular fatty acid analysis

For the analysis of intact polar lipids (IPLs) and cellular fatty acids (CFAs), triplicate cultures of strains F1^T and F21^T and a culture of *K. glycovorans* were grown to early stationary phase at 20 °C with glucose (10 mM) as the substrate. The biomass was harvested by centrifuging at 10,000 × *g*, washed twice with 1.7% sterile saline solution and freeze-dried. In order to obtain CFAs, the freeze-dried biomass was hydrolyzed and derivatized as described previously (Bale et al., 2019a). Fatty acid methyl ester (FAME) quantification was carried out on an Agilent 7890B GC (Agilent, Santa Clara, CA, USA) with an Agilent CP Sil-5 silica column (25 × 0.32 mm) with gases, flow rate and oven temperature as described previously (Bale et al., 2019a). FAME identification was carried out on an Agilent 7890A GC coupled to an Agilent 5975C VL MSD mass spectrometer (MS) operated at 70 eV, with a mass range *m/z* 50–800 and 3 scans per second with the same column and oven settings as for the quantification. FAMEs were identified based on literature data and library mass spectra. Double bond positions were determined using dimethyldisulfide derivatization of the FAMEs as described previously (Bale et al., 2019a). IPLs were extracted from the freeze-dried biomass using a modified Bligh–Dyer procedure and analyzed through ultra-high pressure liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS) as described by Bale et al. (2019a). IPLs were quantified in terms of their MS peak area response. As different IPLs show different response behavior, the relative abundance of the peak area does not necessarily reflect the actual relative abundance of the different IPLs. However, this method allows for a comparison between the strains analyzed in this study.

3 Results

3.1 Phenotypic characterization

Strains F1^T and F21^T were neutrophilic and mesophilic cocci, stained Gram-negative and had a substrate range restricted to carbohydrates, similar to *K. glycovorans* (Table 2).

11 https://github.com/rambaut/figtree

However, unlike *K. glycovorans*, strains F1^T and F21^T were not oligotrophic and only slightly halophilic as defined by Ollivier et al. (1994). Ammonium was required as nitrogen source, despite the presence of nitrogenase genes in the genomes (*nifDHK*, Table S1). The strains were capable of sulfate assimilation as demonstrated by sustained growth in sulfide-free cultures using ferrous iron as the reducing agent. This was in line with the presence of assimilatory sulfate reduction and sulfate transporter genes (*cysDNCHIJKM* and *sulP*, Table S1). Both strains lacked catalase activity despite encoding a catalase gene in their genomes (*katG*, Table S1). Strain F1^T showed no oxidase activity, whereas strain F21^T tested positive for oxidase. Yeast extract did not enhance the growth of the strains, and instead even slowed down the growth of strain F21^T (data not shown). Only strain F21^T showed psychrotolerance, as it was able to grow at temperatures as low as 0 °C. Additionally, strain F21^T was able to grow at higher salinity than strain F1^T (5.0% versus 3.1%, Figure S1). Strain F21^T produced ectoine (1 mg L^{-1} in a culture with an OD_{600} of 0.5), in line with the presence of the full ectoine biosynthesis pathway (*ectABC*, SCARR_04141-4143, Figure 1), which might explain its higher salt tolerance. Unlike strain F21^T cultures, cultures of strain F1^T had a yellow color. This may be due to the formation of lycopene, other carotenoids and/or aryl polyenes, as genes involved in their synthesis (*ctrBDQ*, *carA2*, *fabBFG*; Table S1) were encoded in the genome of strain F1^T but not in that of strain F21^T.

Table 2. Differential traits of strain F1^T, F21^T and *Kiritimatiella glycovorans* L21-Fru-AB^T. Abbreviations: MK, menaquinone; CL, cardiolipin; LCL, lysocardiolipin; MGDG, monogalactosyl diglyceride; PG, phosphatidylglycerol; PG-Gly, phosphatidylglycerohexose; +, positive; +/−, unstable, ceasing growth after the first transfer; −, negative; NDA, no data available. Major cellular fatty acids (CFAs), intact polar lipids (IPLs), quinones and fermentation products are reported in order of abundance. The CFA and IPL data for *K. glycovorans* were generated during this study, other data for *K. glycovorans* were obtained from Spring et al. (2016). * Data from Van Vliet et al. (2019).

Species	***P. desulfatans***	***P. sulfatireligans***	***K. glycovorans***
Type Strain	**F1^T**	**F21^T**	**L21-Fru-AB^T**
Isolation source	Anoxic marine sediment *	Anoxic marine sediment *	Hypersaline microbial mat
Cell diameter (µm)	0.5–1.2 *	0.5–1.0 *	1.0–2.0
Genome size (Mbp)	8.6 *	7.4 *	3.0
DNA G+C content (mol%)	56.0	54.6	63.3
Quinones	MK-7, MK-6, MK-8	MK-9, MK-8, MK-6, MK-7	none
Major CFAs (>5% of total)	$C_{18:0}$, *i*-$C_{12:0}$, *i*-$C_{14:0}$, *i*-$_{C18:}$0	$C_{18:0}$, *i*-$C_{12:0}$, *i*-$C_{18:0}$, *i*-$C_{14:0}$, *i*-$C_{16:0}$	*i*-$C_{14:0}$, $C_{18:0}$, *i*-$C_{18:0}$
Major IPLs	PG, LCL, CL, MGDG	PG, CL, MGDG, LCL	PG, CL, MGDG, PG-Gly, LCL
Oxidase activity	−	+	−

Species	***P. desulfatans***	***P. sulfatireligans***	***K. glycovorans***
Type Strain	**F1^T**	**F21^T**	**L21-Fru-AB^T**
Temp. for growth (°C)			
Range	10–30 *	0–25	20–40
Optimum	25 *	25 *	28
NaCl conc. for growth ($g\ L^{-1}$)			
Range	10–31	10–50	20–180
Optimum	23	23	60–70
pH for growth			
Range	6.5–8.5	6.0–8.5	6.5–8.0
Optimum	7.5	7.5	7.5
Substrate utilization			
Chondroitin sulfate	+ *	+ *	-
Fucoidan	+ *	+ *	+/-
Iota-carrageenan	– *	+ *	+/-
Arabinose	+ *	– *	–
Cellobiose	+ *	+ *	–
Fructose	+ *	+ *	–
Fucose	+ *	+ *	–
Galactose	+ *	+ *	+/–
Galacturonate	– *	+ *	NDA
Lactose	+ *	+ *	–
Maltose	+ *	+ *	–
Mannitol	– *	+ *	–
Mannose	– *	+ *	+
Rhamnose	+ *	+ *	+/–
Sucrose	+ *	+ *	–
Tagatose	+ *	- *	NDA
Trehalose	+ *	+ *	–
Major fermentation products from L-fucose	Acetate, H_2, ethanol, lactate *	Acetate, ethanol, H_2, 1,2-propanediol *	–
Major non-gaseous fermentation products from D-glucose	Acetate, ethanol, lactate	Acetate, ethanol, lactate	Ethanol, acetate

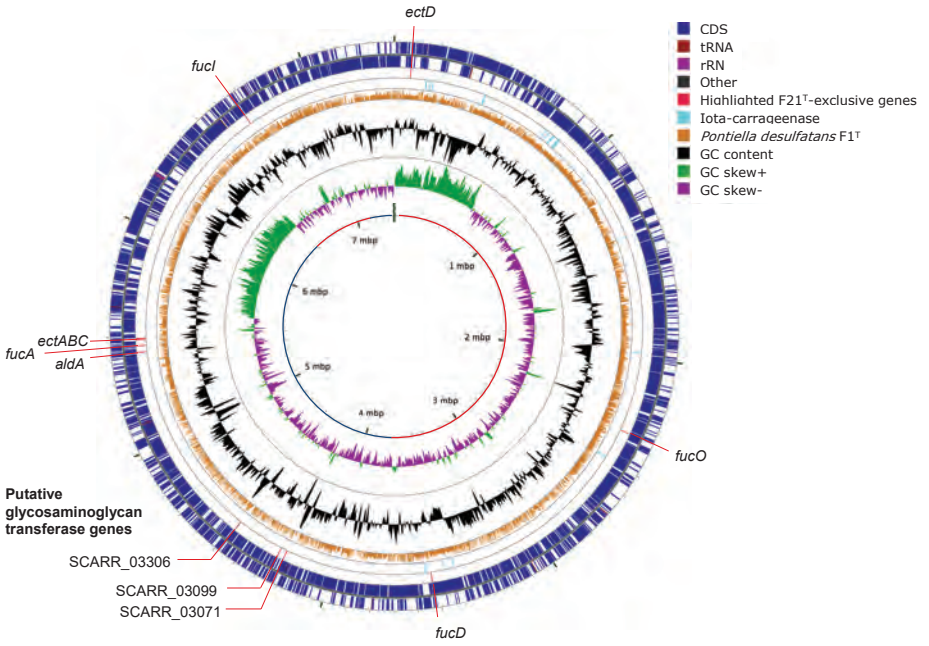


Figure 1. Circular visualization of the 7.4 Mbp strain F21^T genome. The inner circle shows the length of the genome in Mbp and distinguishes the contigs by an alternating red and blue color. Blastx hits with predicted proteins of strain F1^T are drawn with a height proportional to the amino acid identity percentage (0–100%). The outer ring shows coding sequences with a forward orientation, whereas the last-to-outer ring shows coding sequences with a backward orientation. Genes: *aldA*, lactaldehyde dehydrogenase; *ectABCD*, ectoine and 5-hydroxyectoine synthesis; *fucA*, L-fuculose phosphate aldolase; *fucD*, L-fuconate dehydratase; *fucI*, L-fucose isomerase; *fucO*, lactaldehyde reductase.

3.2 Substrate utilization and genetic capacity

Strains F1^T and F21^T showed growth on simple carbohydrates and sulfated polysaccharides (Table 2, Table S2). The sulfated polysaccharides supporting growth included four types of fucoidan formed by different macroalgae (Van Vliet et al., 2019). The strains did not grow on casamino acids, tryptone, yeast extract, L-alanine, L-aspartate, L-cysteine, L-glutamate or L-glycine (Van Vliet et al., 2019), despite encoding respectively 83 and 70 peptidases, as well as amino acid transporters and amino acid degradation pathways. The strains did not show growth on H_2/CO_2, pyruvate, lactate, formate, acetate, propionate, butyrate, citrate, fumarate, malate, succinate, glycerol, methanol, ethanol, propanol, butanol or 1,2-propanediol. Growth on simple carbohydrates was in most cases consistent with the presence of degradation pathways and substrate transport genes in the genome (Table S2). However, the predicted ability to grow on D-mannose (strain F1^T) and D-sorbitol (both strains) was

not confirmed *in vitro*. Conversely, strain F1^T previously showed growth on D-tagatose and D-trehalose, but known genes for their degradation were not identified. Moreover, dedicated transporters for D-xylose, D-galacturonate and D-glucuronate were not identified, yet these compounds were utilized by both strains. Lastly, some genes of the fucose degradation pathway could not be identified (L-fuculokinase, *fucK*, both strains; lactaldehyde dehydrogenase, *aldA*, strain F1^T).

The strains did not grow on any of the tested non-sulfated polysaccharides, such as agar, alginate, arabinan, cellulose, chitin, chitosan, laminarin, pectin, pullulan, starch, xanthan gum or xylan. Seemingly in contrast with this phenotype, exceptionally high numbers of carbohydrate-active enzymes (CAZymes) were encoded by strains F1^T and F21^T (540 and 514, respectively; Figure S2; Table S3). Most of these were glycoside hydrolase (GH) genes amounting to 422 and 388 genes, respectively accounting for 6.4% and 6.8% of all genes. Both strains encoded 59 different GH families. The most abundant GH genes were α-L-fucosidase genes (GH29, GH95, GH141) and genes of a polyspecific family (GH2). The strains also encoded fucoidanases (GH107), acetyl esterases (CE1-11), methyl esterases (CE15), chondroitin lyases (PL8) and a diversity of other GHs (Table S3). Strain F21^T possessed five α-2-*O*-methyl-L-fucosidase genes (GH139; Ndeh et al., 2017), versus none in strain F1^T. Strain F1^T encoded 56 α-L-rhamnosidase genes (GH28, GH78, GH106), whereas strain F21^T encoded only five. Their inability to degrade chitin, chitosan, alginate and pectin is in line with the absence of genes encoding the required hydrolytic enzymes (Table S3). However, they did have the genetic potential to degrade agar, arabinan, cellulose, laminarin, pullulan, starch and xylan. Taking agar as an example, agarose degradation to D-galactose and 3,6-anhydro-L-galactose requires β-agarase (GH50, 86), α-1,3-L-neoagarooligosaccharide hydrolase (GH117) and neoagarobiose hydrolase (GH117). Genes encoding enzymes of the according GH families were present in the genomes of our strains (Table S1), yet no growth on these substrates was observed.

3.3 Reduction of external electron acceptors during anaerobic growth on sugars

Our two strains F1^T and F21^T as well as *K. glycovorans* reduced elemental sulfur, ferric citrate and fumarate when grown on their respective sugar substrates (Figure 2). In addition, dimethysulfoxide was reduced by strain F1^T (0.5 mM) and *K. glycovorans* (not quantified). Thiosulfate and nitrate were not reduced by any of the strains tested. The presence of sulfite or nitrite (2 mM) inhibited fermentative growth. While amorphous MnO_2 was detectably reduced only by *K. glycovorans*, the addition of the electron shuttle and humic acid analogue AQDS (0.1 mM) stimulated MnO_2 reduction in all the tested strains, particularly in strain F21^T. Cultures of strain F21^T produced 4.9 mM of Mn(II) and about 75% less H_2 than controls, equivalent to 14.9 kPa partial pressure or

6.1 aqueous mM difference. They produced 2.9 mM 1,2-propanediol versus 3.9 mM in controls. However, acetate production was unchanged at 7.2–7.3 mM. No growth was observed in transfer cultures with H_2 as the electron donor and AQDS/MnO_2 as the electron acceptors.

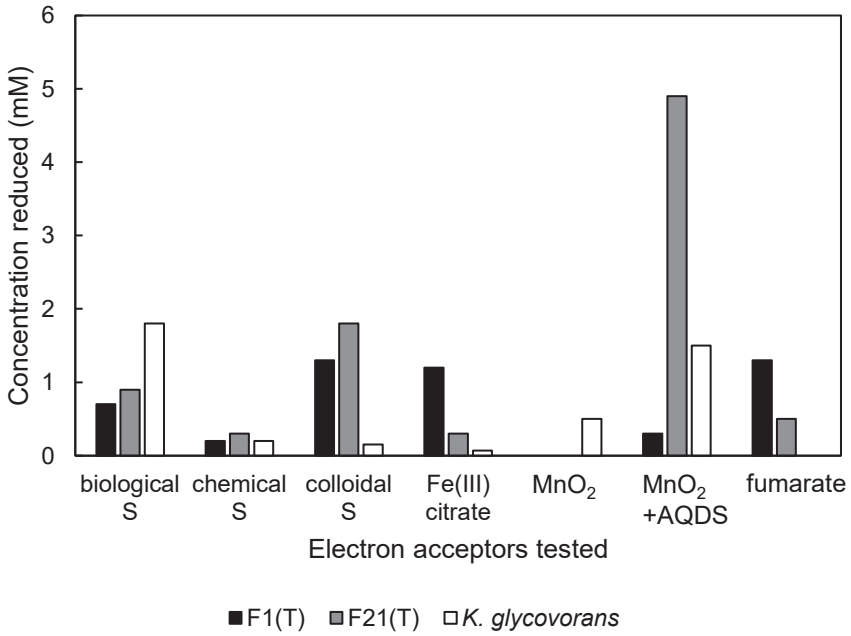


Figure 2. Concentration of external electron acceptor reduced during growth on sugars. Abbreviations: S, elemental sulfur; Fe(III), ferric iron; MnO_2, manganese oxide; AQDS, anthraquinone-2,6-disulfonate.

3.4 Response to different redox conditions and oxygen

The strains grew in media with various reducing agents, such as cysteine (4 mM; E'^0 = −0.22 V) or ferrous iron (2 mM; E'^0 = 0 V). They also grew at higher redox potentials in the presence of ferric citrate (E'^0 = 0.37 V) and amorphous MnO_2 (E'^0 = 0.47 V). Strains $F1^T$ and $F21^T$ did not grow in oxic media (Van Vliet et al., 2019). Strain $F1^T$ was also incapable of growth in non-reduced liquid medium. In contrast, strain $F21^T$ could grow in non-reduced medium and reduce it whilst doing so, although this ability was not completely reproducible among replicates. In oxygen gradient cultures, the strains grew only in the reduced zone (Figure 3A,B). Contaminations were ruled out by microscopical inspection of cell morphology and 16S rRNA gene amplicon sequencing. The reduced zone was larger in inoculated cultures than in uninoculated negative

controls (Figure 3C). Its size remained stable for longer than a week, in contrast to the negative controls in which the diffusion of oxygen into the medium was visible as the oxidized zone enlarged over time (Figure 3D).

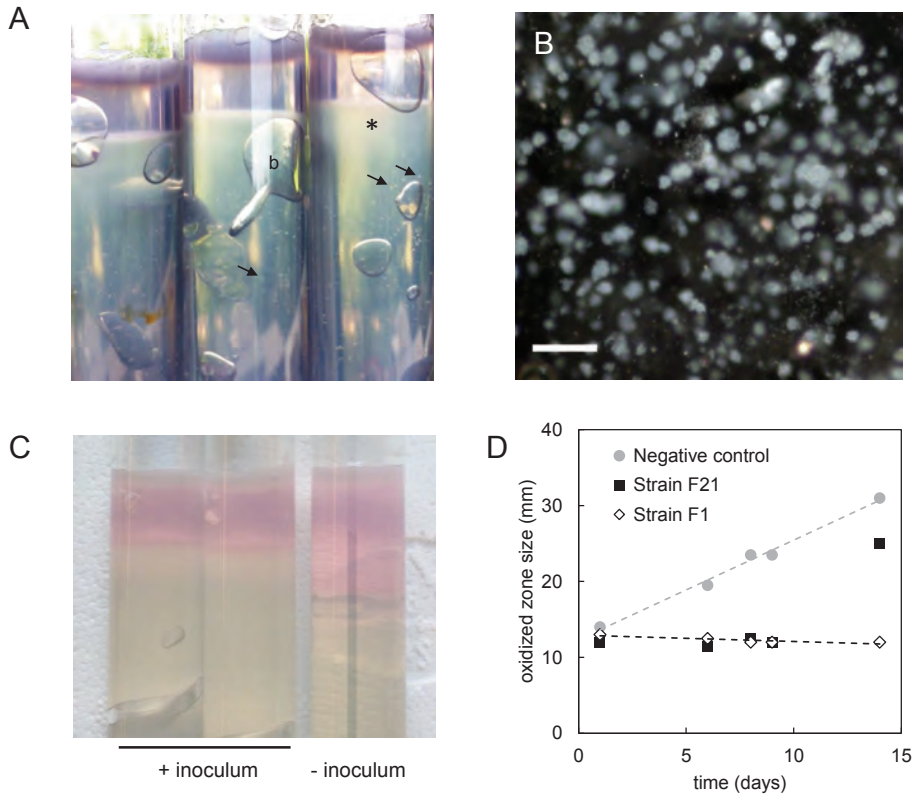


Figure 3. Growth of strains F1^T and F21^T in oxygen gradient cultures. (A) Cysteine-reduced cultures of strain F1^T after 19 days of incubation. Indicated are visible colonies (arrows), gas bubbles (b) and diffuse growth at the oxidized/reduced interface (*). (B) Micrograph showing the growth of microcolonies at the turbid oxidized/reduced interface. The scale bar represents a length of 100 μM. (C) Sulfide-reduced cultures after 8 days of incubation, of which two were inoculated with strain F21^T (left) and one was left uninoculated as the negative control (right). (D) Size of the pink oxidized zone versus incubation time in cultures reduced with sulfide. The negative controls were uninoculated. Plotted values are averages of two replicate cultures, which behaved reproducibly.

3.5 Formation of energy reserve materials

In stationary phase, all fucose or glucose (up to 10 mM) was consumed by strains F1^T and F21^T. The somewhat low electron recovery (strain F1^T: 80%, strain F21^T: 88%), not taking biomass into account (Van Vliet et al., 2019), led us to hypothesize that the bacteria might form energy reserve materials. Microorganisms may store energy in granules of glycogen, polyphosphate and polyhydroxyalkanoates. Strains F1^T and F21^T showed the genetic potential for producing and using glycogen (*glgABCPX*, Table S1) and polyphosphate (*ppk, ppx*; Table S1), but not polyhydroxyalkanoates. Transmission electron microscopy of strain F1^T cells in exponential phase confirmed the presence of intracellular storage polymer granules (Figure 4), which appear electron-light since they do not stain with the applied osmium tetroxide or uranyl acetate (Khadem et al., 2012; Damrow et al., 2016; Rubin-Blum et al., 2019). A polyphosphate staining was negative for cells in exponential growth, indicating glycogen was probably the only energy storage compound formed in the conditions tested.

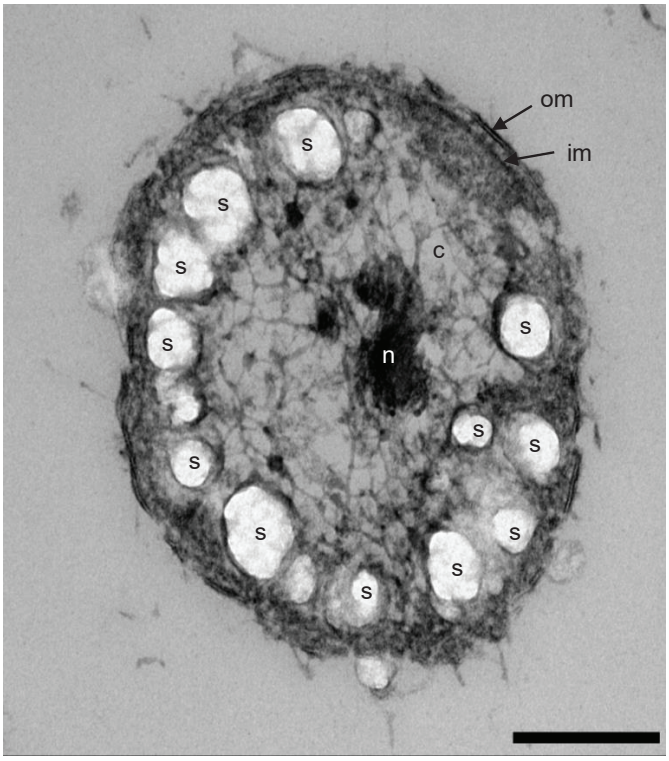


Figure 4. Transmission electron micrograph of a positively stained thin section of a *P. desulfatans* F1^T cell in late exponential phase, grown with glucose as the substrate. The scale bar corresponds to 200 nm. Arrows mark the outer membrane (om) and inner membrane (im). Further indicated are cytoplasm (c), nucleoid (n) and storage polymer granules (s).

3.6 Production of sulfated glycosaminoglycan-like exopolymers in stationary phase

In stationary phase after growth on glucose or fucose, an increase in the viscosity of cultures was observed. Since the cells were intact under microscopical observation, we hypothesized the increased viscosity was not due to cell lysis but due to the production and release of extracellular polymeric substances (EPS). The spent medium supernatant contained sugars (approximately 50 μM), as determined with the anthrone assay with L-fucose as the standard. The presence of carbohydrate-based polymers in the supernatant was confirmed with Fourier-transform infrared spectroscopy (FTIR). The FTIR analysis indicated the presence of carbohydrates (a broad band at 3000–3600 cm^{-1}, a strong band with the peak at 1080 cm^{-1}) and sulfate substitutions (a shoulder band at 1230 cm^{-1}), which was confirmed by scanning electron microscopy energy-dispersive X-ray analysis (File S1). The total sulfated polysaccharides in the EPS of strain $F1^T$ and $F21^T$ were 9 ± 1 and 11 ± 2 mg/g, respectively, based on the reaction with the 1,9-dimethylmethylene blue dye. Both pools of sulfated polysaccharide were primarily N-sulfated rather than O-sulfated, with 75% and 80% N-sulfation, respectively. A specific subclass of sulfated polysaccharides are sulfated glycosaminoglycans, defined as having a backbone of a hexosamine-containing repeating disaccharide, sulfated by a sulfotransferase (DeAngelis, 2002b). Strain $F21^T$ encoded three sulfotransferase genes with similarity to known glycosaminoglycan sulfotransferase genes (SCARR_03071, SCARR_03099 and SCARR_3306; Figure 1, Table S4). One of these putative glycosaminoglycan sulfotransferase genes (SCARR_03099) was located in a gene cluster containing eight potential hexosaminyltransferases (GT2, GT4) and a predicted dTDP-4-amino-4,6-dideoxy-D-glucose transaminase (Figure 5).

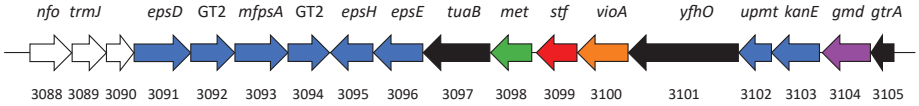


Figure 5. Putative sulfated glycosaminoglycan biosynthesis gene cluster in strain $F21^T$. Locus tag numbers without the "SCARR_" prefix are indicated below the genes. Genes encoding glycosyltransferases are marked blue, auxiliary proteins marked black, methyltransferases marked green, sulfotransferases marked red, transaminases marked orange, dehydratases marked purple and genes of other or unknown function are marked white. Genes with the prefix *eps* are homologs with putative EPS biosynthesis glycosyltransferases from *Bacillus subtilis* strain 168. Abbreviations from left to right: *nfo*, apurinic endonuclease; *trmJ*, tRNA methyltransferase; *epsD*, GT4; *mfpsA*, mannosylfructose-phosphate synthase (GT4); *epsH*, GT2; *epsE*, GT2; *tuaB*, teichuronic acid biosynthesis protein; *met*, methyltransferase; *stf*, sulfotransferase; *vioA*, dTDP-4-amino-4,6-dideoxy-D-glucose transaminase; *yfhO*, bacterial membrane protein; *upmt*, undecaprenyl-phosphate mannosyltransferase (GT2); *kanE*, **α**-D-kanosaminyltransferase (GT4); *gmd*, GDP-mannose 4,6-dehydratase; *gtrA*, GtrA-like protein.

3.7 Phylogenomics and chemotaxonomy of the order *Kiritimatiellales*

In a concatenated single-copy gene phylogeny constructed with *Kiritimatiellales* genomes, strains F1^T and F21^T were placed in a monophyletic clade together with metagenome-assembled genomes (MAGs) from anoxic and oxic marine locations (Figure 6). The amino acid identity (AAI) of this clade with *K. glycovorans* was 44–50% (Table S5), close to the conservatively proposed AAI family-level threshold of 45% (Konstantinidis et al., 2017). In contrast, the intra-clade AAI was >53%. The clade was congruent to a family-level clade (UBA1859) within the Genome Taxonomy Database (GTDB; Parks et al., 2018). Strain F1^T and F21^T shared an AAI of 73%, exceeding the 65% genus threshold (Konstantinidis et al., 2017). Their digital DNA–DNA hybridization value was 24.5%, well below the species threshold of 70% (Auch et al., 2010).

Unlike *K. glycovorans*, strains F1^T and F21^T synthesized menaquinones (Table 2). Both strains produced MK-6, MK-7 and MK-8. Additionally, only strain F21^T produced MK-9, which also was the dominant menaquinone for this strain (55%). *K. glycovorans* and the isolates could also be distinguished by their cellular fatty acid (CFA) and intact polar lipid (IPLF) profiles (Table 2). While all three microorganisms produced *i*-$C_{14:0}$, *i*-$C_{18:0}$ and $C_{18:0}$ as major CFAs, *i*-$C_{14:0}$ was dominant in *K. glycovorans* (42%), whereas $C_{18:0}$ was the most abundant CFA in strains F1^T and F21^T (40% and 35%, respectively). Additionally, the isolates contained a major fraction of *i*-$C_{12:0}$. Only strain F21^T contained *i*-$C_{16:0}$ as a major CFA. A detailed overview of CFAs can be found in Table S6. The major IPL classes observed in both strains F1^T and F21^T were phosphatidylglycerol (PG), monogalactosyldiacylglycerol (MGDG), cardiolipins and lyso-cardiolipins (Table 2). *K. glycovorans* had a similar IPL distribution, but in addition to PG, MGDG and the cardiolipins, two phosphoglycolipids were detected, confirming the previously reported detection of a phosphoglycolipid (Spring et al., 2016). There were also low contributions from two unknown polar lipid components (Table S6). The phosphoglycolipids were further identified as phosphatidylglycerohexose (PG-Gly) based on a comparison of the tandem mass spectrometry fragmentation with published spectra (Giordano et al., 2007; Bale et al., 2019b) and based on the accurate mass of the PG-Gly lipids detected (Table S7).

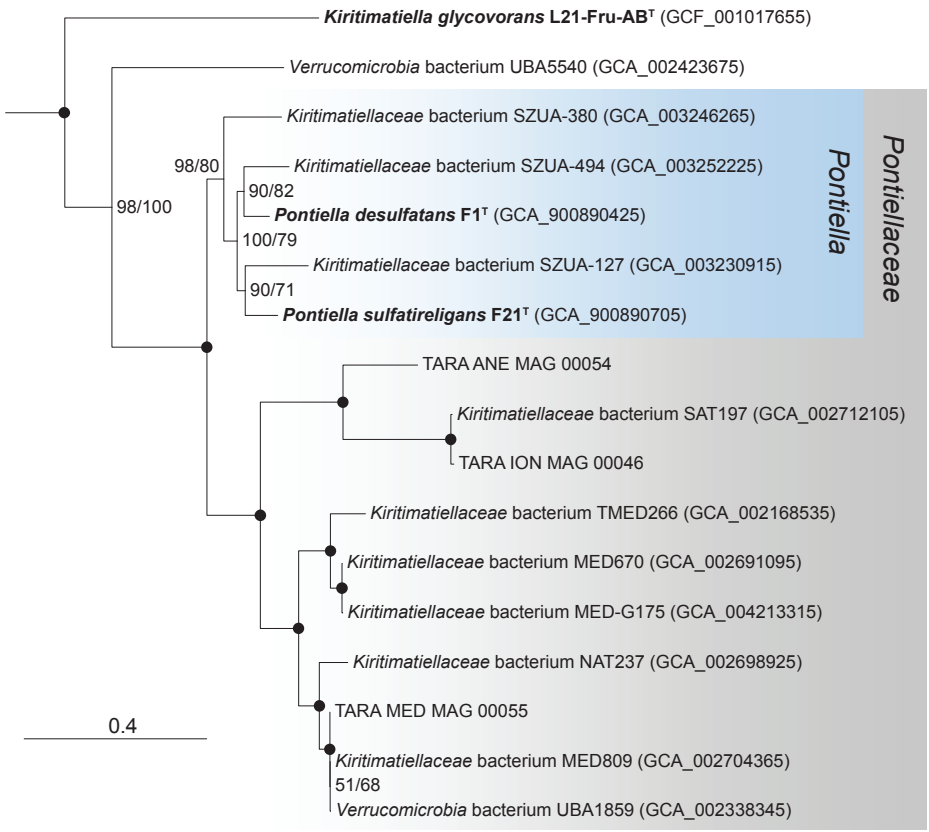


Figure 6. Maximum-likelihood phylogenetic tree of the members of the order *Kiritimatiellales* constructed from concatenated alignments of single-copy genes. Two *Kiritimatiellaeota* genomes outside of the *Kiritimatiellales* order (GTDB order UBA8416) were taken as outgroup and were omitted from the figure. Branch support is indicated with SH-like approximate likelihood ratio test values and ultra-fast bootstraps values, in that order. Black circles indicate support values of 100/100. The scale bar indicates substitutions per site. All cultured members are highlighted with bold font. The proposed novel genus and family are shaded in blue and grey, respectively. Since the UBA5540 and the SAT197 metagenome-assembled genomes (MAGs) share only 48% amino acid identity (AAI), we tentatively excluded MAG UBA5540 from the proposed novel family. MAG UBA5540 represents the uncultivated MSBL3 cluster based on the classification of its 16S rRNA gene using Silva ACT (Pruesse et al., 2012), and thus may represent an additional novel family within the *Kiritimatiellales*. NCBI accession numbers are indicated between parentheses, except for TARA oceans MAGs from Delmont et al. (2018) which can be accessed from https://doi.org/10.6084/m9.figshare.4902923.

4 Discussion

The various types of fucoidan are known for their heterogeneous compositions and complex structures (Ale and Meyer, 2013). The ability of strains F1^T and F21^T to grow on different types of fucoidan is thus consistent with the expansive CAZyme gene repertoires presented here and the reported sulfatase gene repertoires (Van Vliet et al., 2019). However, it should be noted that although the degradation of fucoidan by bacteria has been shown to involve fucosidases (Dong et al., 2017), fucoidanases (Colin et al., 2006; Descamps et al., 2006; Silchenko et al., 2013), deacetylases (Ohshiro et al., 2012) and sulfatases (Silchenko et al., 2018), there is currently no model of the exact enzymatic mechanism by which bacteria break down fucoidan into monomers. Various polysaccharides were tested as the substrate in this study, but only the sulfated ones were used by the isolated strains. However, the sulfated and non-sulfated polysaccharides tested also differ in backbone composition, implying other factors than sulfation could lead to the observed substrate profiles. To test the effect of polysaccharide sulfation on utilization by strains F1^T and F21^T, the test should include non-sulfated fucoidan, carrageenan and chondroitin. Unfortunately, such compounds are not available commercially.

Strains F1^T and F21^T encoded the highest numbers of sulfatases, 521 and 480 sulfatases, respectively, reported for any described microorganism so far (Van Vliet et al., 2019). These numbers are exceptionally high, which can be best illustrated by a comparison with other bacteria that are known to contain a high number of sulfatase genes, such as *L. araneosa* HTCC2155^T (284 sulfatase genes) and *R. baltica* SH1^T (109 sulfatase genes). The research presented here has revealed similarly exceptional numbers of glycoside hydrolase genes (422 and 388, respectively). These exceed the numbers of GH genes predicted in *Bacteroidetes* spp.—which are regarded as important biopolymer degraders in marine and other environments (Martinez-Garcia et al., 2012; Fernández-Gomez et al., 2013; Barbeyron et al., 2016b)—such as *Bacteroides ovatus* ATCC 8483^T (324; El Kaoutari et al., 2013), *B. intestinalis* DSM 17393^T (319; El Kaoutari et al., 2013), *B. thetaiotaomicron* VPI-5482^T (286; Lombard et al., 2014) and the marine *Zobellia galactanivorans* Dsij^T (141; Barbeyron et al., 2016b). The highest number of GH genes reported in the PVC superphylum is 261, encoded by *Victivallis vadensis* ATCC BAA-548^T (El Kaoutari et al., 2013). Similar GH gene richness has only been found in fungi such as *Fusarium oxysporum* (396; Zhao et al., 2013).

Both strains encoded putative carrageenan sulfatases (Van Vliet et al., 2019) and potential kappa-carrageenases (polyspecific family GH16), but only strain F21^T encoded iota-carrageenases (GH82, Figure 1). In accordance, both strains were able to grow on kappa-carrageenan, but only F21^T grew on iota-carrageenan. The abundance of rhamnosidase genes in strain F1^T, numbering 56, suggests its substrate range may include rhamnans. The GH gene profile of strain F1^T was similar to that of *Verrucomicrobia*

MAGs from a freshwater humic bog (He et al., 2017), implying the presence of similar compounds serving as substrates. Since strains F1^T and F21^T did not grow on amino acids or peptides, the many encoded peptidases may have a role in accessing the glycan chains of proteoglycans/glycoproteins through degradation of the peptide chains.

The ability to reduce external electron acceptors such as fumarate, elemental sulfur and ferric iron during fermentative growth on sugars is not unique to the three *Kiritimatiellales* spp. tested in this study. As an example, the reduction of elemental sulfur has also been observed for thermophilic archaea (Rabus et al., 2013), several *Planctomycetes* (Elshahed et al., 2007; Slobodkina et al., 2015; Slobodkina et al., 2016) and the firmicute *Lucifera butyrica* (Sánchez-Andrea et al., 2018), without a noticeable effect on the growth rate or yield. The *Kiritimatiellales* strains possessed no genes encoding proteins that could facilitate anaerobic respiration of sulfur compounds, such as polysulfide reductase or other types of molybdoenzymes (Duval et al., 2008), dissimilatory (bi)sulfite reductase pathway proteins or other reductive proteins listed by Wasmund et al. (2017). Fumarate reduction was consistent with the presence of succinate dehydrogenase/fumarate reductase genes (*sdhABC*, Table S1). Nitrite inhibited growth, probably due to toxicity. However, the presence of cytochrome *c* nitrite reductase genes (*nrfAH*, Table S1) in the genomes of strains F1^T and F21^T suggests that the dissimilatory reduction of nitrite could occur at lower non-toxic concentrations of nitrite. AQDS is known to be reduced by lactic acid bacteria growing fermentatively, although not by *Escherichia coli* (Benz et al., 1998). The reduction of metals during fermentative growth has been reported for members from various phyla (Lovley, 2013; Vandieken et al., 2017a). The reduction of AQDS and metals can proceed through periplasmic or outer membrane c-type cytochromes that deliver electrons from quinones to these electron acceptors (Mehta et al., 2005; Voordeckers et al., 2010). Although strains F1^T and F21^T did produce menaquinones and did encode multiple c-type cytochromes predicted to be localized in the periplasm or extracellularly (Table S1), *K. glycovorans* lacks both quinones and c-type cytochrome genes (Spring et al., 2016) yet still reduced AQDS and metals. It is thus unclear how and why these *Kiritimatiellales* reduce elemental sulfur, DMSO, AQDS and metals.

Strains F1^T and F21^T were shown to be intolerant to oxygen, contrasting the aerotolerance of *K. glycovorans*, which can grow fermentatively in oxic medium (Spring et al., 2016). The strains showed resilient growth in anoxic cultures with heightened redox potential due to the addition of amorphous MnO_2. Although the redox potential was not measured, the pink color of the redox indicator resorufin implied a redox potential higher than −0.02 to −0.03 V (Veldkamp, 1970). It is thus unclear if the inhibition of growth in non-reduced liquid cultures was due to the presence of trace oxygen, or due to a too high redox potential. Curiously, the oxygen gradient cultures showed a reduction of part of the oxidized zone and the prevention of oxidation over time (Figure 3). This could be explained by the reduction of oxygen, but also by a lowering of the redox potential

by reduced fermentation products such as H_2. As discussed in the previous paragraph, the strains are able to reduce external electron acceptors. The behavior in the oxygen gradient cultures could therefore be due to the reduction of such electron acceptors present in the medium, such as flavins, thiols or elemental sulfur (produced by sulfide oxidation), in turn causing the chemical reduction of oxygen. Such an oxygen reduction mechanism was demonstrated for the anaerobic, non-aerotolerant gut bacterium *Faecalibacterium prausnitzii* strain A2–165 (Khan et al., 2012). Alternatively, the strains could reduce microaerobic levels of oxygen directly through the activity of cytochrome *bd* terminal oxidase (*cydAB*, Table S1) and/or cytochrome cbb_3 oxidase (*ccoNOP*, Table S1), as previously proposed for the gut anaerobe *Akkermansia muciniphila* Muc^T (Ouwerkerk et al., 2016). Both oxidases have high affinity for oxygen and allow the conservation of energy through generating a proton motive force, although only cytochrome cbb_3 oxidase is proton-pumping (Pitcher and Watmough, 2004; Borisov et al., 2011). Strain F21^T showed oxidase activity when grown anaerobically, indicating a constitutive expression of at least one of these oxidase systems. Possibly, this constitutive expression enabled strain F21^T to grow in non-reduced liquid medium. Since the results from the oxygen gradient cultures are inconclusive, in-depth research as conducted for *F. prausnitzii* and *A. muciniphila* (Khan et al., 2012; Ouwerkerk et al., 2016) is required to investigate whether oxygen is reduced, and if so, what mechanism is responsible. However, the results presented here—in combination with the multiple encoded putatively oxygen-dependent sulfatase maturation enzymes reported previously (Van Vliet et al., 2019)—show that strains F1^T and F21^T could be adapted to proliferate under low oxygen concentrations.

Strains F1^T and F21^T produced EPS in the stationary phase, which is unusual but has also been reported for *L. araneosa* (Cho et al., 2004) and some other marine bacteria (Delbarre-Ladrat et al., 2014). From our chemical and genomic analyses, we conclude that the EPS contained sulfated polysaccharides containing N-sulfated hexosamines, thus resembling sulfated glycosaminoglycans. Although the applied 1,9-dimethylmethylene blue assay is not specific for glycosaminoglycans and interacts with various sulfated polysaccharides (Aquino et al., 2005; Troeberg et al., 2012), the high degree of N-sulfation in the sulfated polysaccharides detected here indicates the presence of sulfated hexosamines, such as found in the sulfated glycosaminoglycans heparin and heparan sulfate (DeAngelis, 2002b). This is supported by the high number of predicted hexosaminyltransferases in the putative sulfated glycosaminoglycan biosynthesis gene cluster of strain F21^T (Figure 5). The detection of sulfated glycosaminoglycan-like compounds in the EPS of strains F1^T and F21^T is of fundamental microbiological importance. Sulfated glycosaminoglycans such as heparin/heparan sulfate and chondroitin sulfate are important components of animal tissues. Some prokaryotes are known to produce non-sulfated glycosaminoglycans (DeAngelis, 2002a; Widner et al., 2005) and sulfated exopolysaccharides (Delbarre-Ladrat et al., 2014), but little information concerning prokaryotes producing sulfated

glycosaminoglycan-like polymers is available. Recently, sulfated glycosaminoglycan-like compounds have been detected in the extracellular matrix of granular sludge (Bourven et al., 2015; Felz et al., 2020) and anammox granules (Boleij et al., 2020), but it remains unclear which of the members of these microbial communities produced these compounds. The biosynthesis of sulfated glycosaminoglycans requires the sulfation of oligo- or polysaccharides, carried out by sulfotransferases (Kusche-Gullberg and Kjellén, 2003). While thoroughly studied in eukaryotes, sulfotransferases active towards glycosaminoglycans are currently not known to be encoded by prokaryotes. The identification of three putative glycosaminoglycan sulfotransferase genes and a putative sulfated glycosaminoglycan biosynthesis gene cluster (Figure 5) in strain F21^T corroborates the detection of sulfated glycosaminoglycan-like exopolymers. Additional research is needed to determine the structure and composition of the detected sulfated glycosaminoglycan-like exopolymers, and to identify the enzymes that synthesize them.

Our chemotaxonomic investigations revealed the presence of PG-Gly lipids in *K. glycovorans*. These have been found previously in other halophiles, namely halophilic *Halomonas* bacteria (Giordano et al., 2007) and extremely haloalkaliphilic *Natronobiforma cellulositropha* archaeal strains (Bale et al., 2019b). The production of PG-Gly lipids by *K. glycovorans* reinforces the association with halophiles, and lends further support to the hypothesis of Giordano et al. (2007) that PG-Gly lipids enhance the osmotic stability of the cellular membrane by increased steric protection through hydrogen bonding with lipid glycosyl headgroups. Our phylogenetic and chemotaxonomic results support the establishment of the novel taxonomic family *Pontiellaceae* fam. nov. These results are consistent with previous analyses, largely based on 16S rRNA genes (Van Vliet et al., 2019). Although these analyses have indicated that strains F1^T and F21^T represent two different genera, we now propose them as novel species of the genus *Pontiella* gen. nov. based on the phenotypic similarity and whole-genome comparison.

Description of *Pontiellaceae* fam. nov. *Pontiellaceae* (Pon.ti.el.la.ce'ae. L. fem. dim. n. *Pontiella*, type genus of the family; suff. *-aceae*, ending to denote a family; L. fem. dim. pl. n. *Pontiellaceae*, the *Pontiella* family). Members of this family stain Gram-negative, and are found mainly in marine environments. The *Pontiellaceae* family corresponds phylogenetically to the R76-B128 clade as defined in the SILVA SSU r132 database and the UBA1859 family within GTDB taxonomy. It encompasses the type genus *Pontiella*, which contains two described species.

Description of *Pontiella* gen. nov. *Pontiella* (Pon.ti.el'la. Gr. masc. adj. *pontios*, from the sea; L. fem. dim. n. *Pontiella*, she from the [Black] sea, referring to the origin of the type species). Stain Gram-negative. Non-motile and non-spore forming coccoid cells, which divide through binary fission. They produce menaquinones. Major cellular fatty acids are *i*-$C_{12:0}$, *i*-$C_{14:0}$, $C_{18:0}$ and *i*-$C_{18:0}$. Major intact polar lipid classes are

phosphatidylglycerol, monogalactosyldiacylglycerol, cardiolipins and lyso-cardiolipins. Catalase activity is negative. Obligately anaerobic, mesophilic and neutrophilic. No yeast extract is required for growth, but at least 10 g L^{-1} NaCl is required. Growth occurs with simple carbohydrates and sulfated polysaccharides as the substrate. No dissimilatory reduction of nitrate or thiosulfate. Reduction of elemental sulfur, ferric citrate, fumarate and anthraquinone-2,6-disulfonate during fermentation. Sulfate is assimilated as a sulfur source when growing on glucose or fucose. Not aerotolerant. Produce and excrete exopolysaccharides, including sulfated glycosaminoglycan-like compounds. The type species is *Pontiella desulfatans*.

Description of *Pontiella desulfatans* sp. nov. *Pontiella desulfatans* (de.sul.fa'tans. L. pref. *de*, off; N.L. masc. n. *sulfas –atis*, sulfate; N.L. part. adj. *desulfatans*, removing sulfate, referring to sulfate ester substitutions in polysaccharides). The genus description applies, with the following additional features. Cells have a diameter of 0.5–1.2 μm. Growth occurs at 10–30 °C, 10–31 g L^{-1} NaCl and a pH of 6.5–8.5. Optimal conditions are 25 °C, 23 g L^{-1} NaCl and pH 7.5. The following substrates are utilized: D-fructose, D-galactose, D-glucose, D-tagatose, D-trehalose, D-xylose, L-arabinose, L-fucose, L-rhamnose, D-cellobiose, D-lactose, D-maltose, D-sucrose, N-acetylglucosamine, D-glucuronate, kappa-carrageenan, chondroitin sulfate and fucoidan from *Cladosiphon* spp., *Fucus vesiculosus*, *Macrocystis pyrifera* and *Undaria pinnatifida*. The following compounds are not utilized: D-mannose, D-ribose, L-sorbose, raffinose, D-glucosamine, D-galacturonate, D-gluconate, D-galactitol, D-mannitol, D-sorbitol, myo-inositol, agar, arabinan, cellulose, laminarin, pullulan, starch, xanthan gum, xylan, chitin, chitosan, alginate, pectin, iota-carrageenan, casamino acids, tryptone, yeast extract, L-alanine, L-cysteine, L-glutamate, L-glycine, L-isoleucine, acetate, benzoate, butanol, butyrate, citrate, ethanol, formate, fumarate, glycerol, lactate, malate, methanol, propanol, propionate, pyruvate, succinate and H_2/CO_2. The main non-gaseous fermentation products from L-fucose are acetate and ethanol. The dominant menaquinone is MK-7. The type strain has a genome size of 8.6 Mbp and DNA G+C content of 56.0% (mol/mol). The type strain is F1^T (= DSM 106878^T = KCTC 15641^T), isolated from anoxic Black Sea sediment.

Description of *Pontiella sulfatireligans* sp. nov. *Pontiella sulfatireligans* (sul.fa'ti.re.li.gans. N.L. masc. n. *sulfas –atis*, sulfate; L. v. *religare*, to bind back, fasten up; N. L. part. adj. *sulfatireligans*, binding back sulfate, referring to sulfate ester substitutions in exopolymers). The genus description applies, with the following additional features. Cells have a diameter of 0.5–1.0 μm. Growth occurs at 0–25 °C, 10–50 g L^{-1} NaCl and a pH of 6.0–8.5. Optimal conditions are 25 °C, 23 g L^{-1} NaCl and pH 7.5. The following substrates are utilized: D-fructose, D-galactose, D-glucose, D-mannose, D-trehalose, D-xylose, L-fucose, L-rhamnose, D-cellobiose, D-lactose, D-maltose, D-sucrose, N-acetylglucosamine, D-galacturonate, D-glucuronate, D-mannitol, kappa-carrageenan, iota-carrageenan, chondroitin sulfate and fucoidan from *Cladosiphon*

spp., *Fucus vesiculosus*, *Macrocystis pyrifera* and *Undaria pinnatifida*. The following compounds are not utilized: D-ribose, D-tagatose, L-arabinose, L-sorbose, raffinose, D-glucosamine, D-gluconate, D-galactitol, D-sorbitol, myo-inositol, agar, arabinan, cellulose, laminarin, pullulan, starch, xanthan gum, xylan, chitin, chitosan, alginate, pectin, Casamino acids, tryptone, yeast extract, L-alanine, L-cysteine, L-glutamate, L-glycine, L-isoleucine, acetate, benzoate, butanol, butyrate, citrate, ethanol, formate, fumarate, glycerol, lactate, malate, methanol, propanol, propionate, pyruvate, succinate and H_2/CO_2. The main non-gaseous fermentation products from L-fucose are acetate, ethanol and 1,2-propanediol. The cellular fatty acid *i*-$C_{16:0}$ is produced in addition to the major cellular fatty acids in the genus description. The dominant menaquinone is MK-9. Ectoine is produced. The type strain has a genome size of 7.4 Mbp and DNA G+C content of 54.6% (mol/mol). The type strain is F21^T (= DSM 106829^T = KCTC 15642^T), isolated from anoxic Black Sea sediment.

5 Supplementary material

The following are available online at https://www.mdpi.com/2076-2607/8/6/920/s1.

File S1: FTIR and SEM-EDX results of EPS from strains F1^T and F21^T.

Figure S1: The effect of salinity on growth of strain F1^T and F21^T.

Figure S2: Circular visualization of the 8.7 Mbp strain F1^T genome.

Table S1: Annotation of selected genes in strains F1^T and F21^T including a complete list of all CAZyme genes.

Table S2: Utilization of saccharide and peptide substrates and presence of the corresponding degradation pathway genes and transporter genes in the genomes of strains F1^T and F21^T.

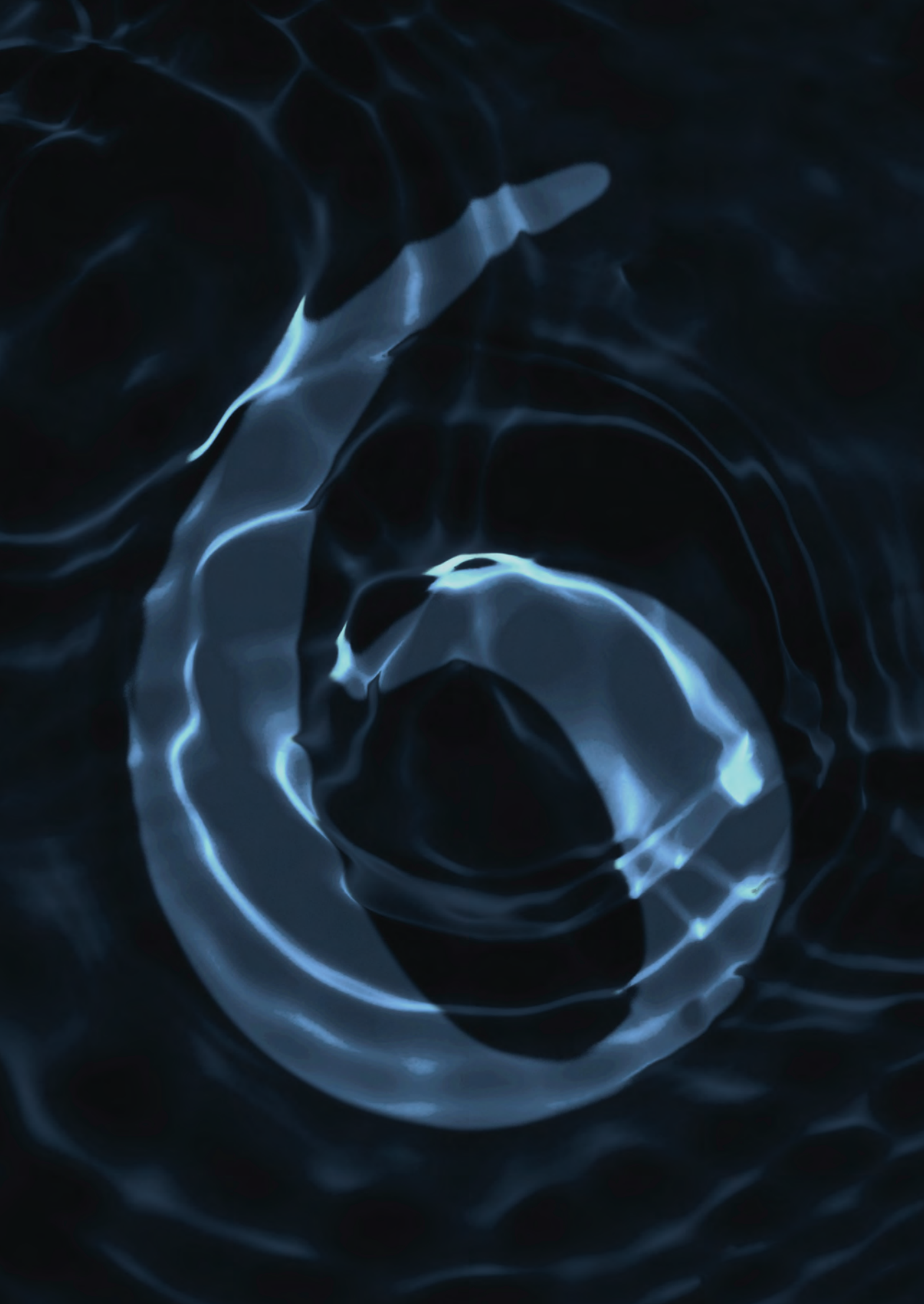
Table S3: Number of CAZyme genes detected in strains F1^T and F21^T per CAZyme (sub)family.

Table S4: Sulfotransferase genes encoded by strain F21^T with a PF13469 sulfotransferase domain and their BlastP matches with studied sulfotransferases.

Table S5: Amino acid identity matrix of *Kiritimatiellales* genomes.

Table S6: CFAs and IPLs detected in strains F1^T and F21^T and *K. glycovorans.*

Table S7: Accurate masses of the two detected IPLs with a phosphatidylglycerohexose (PG-Gly) head group.



CHAPTER 6

Transcriptome analysis of fucoidan and L-fucose degradation by *Pontiella desulfatans* F1^{T}

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Abstract

Fucoidans are sulfated polysaccharides with a complex, branched molecular structure found in brown macroalgae. Their degradation by marine microorganisms is thought to involve various glycoside hydrolases, deacetylases and sulfatases, but an exact pathway or mechanism of microbial degradation has not been resolved. Here, we examined the gene expression of the marine anaerobe *Pontiella desulfatans* $F1^T$ during growth on fucoidan from *Fucus vesiculosus* and on its monomer L-fucose with transcriptomics to obtain insight into the pathways involved. A high number of sulfatase and predominantly exo-acting glycoside hydrolase genes were upregulated, similar to recent observations with a fucoidan-degrading aerobe. Most are organized in fucoidan-specific polysaccharide utilization loci (PULs) additionally containing regulatory elements. We propose a so-called 'selfish' mode of fucoidan depolymerization involving uptake of oligosaccharides, as the PULs also encoded putative surface glycan-binding proteins, extracellular endo-fucanases, and potential TonB-dependent oligosaccharide transporters. Furthermore, fucoidan and L-fucose induced expression of L-fucose permease and isomerase genes, and of an 11-gene operon not known to be involved in studied L-fucose degradation pathways, and lacking bacterial microcompartments. Analysis of syntenic operons of *Kiritimatiellaeota*, *Spirochaetes*, *Bacteroidetes* and *Clostridia* spp. revealed conserved genes encoding sugar alcohol dehydrogenase, NAD(P)H-dependent short-chain dehydrogenase, phosphatase, and 2-oxoacid dehydrogenase complex. We postulate that these enzymes are involved in a novel L-fucose degradation pathway.

1 Introduction

Polysaccharides constitute a large part of the carbon cycled in the marine environment, yet their degradation by microorganisms remains a poorly understood aspect of the carbon cycle (Arnosti et al., 2021). Marine polysaccharides can have complex structures, owing to the many possible types of carbohydrate monomers, glycosidic linkages, branching, and substitutions with methyl, acetyl or sulfate groups. To depolymerize polysaccharides microorganisms require a set of depolymerizing enzymes (Kabisch et al., 2014; Reisky et al., 2019). Important are carbohydrate-active enzymes (CAZymes) encompassing glycoside hydrolases (GHs) and polysaccharide lyases (PLs) which break glycosidic linkages, and carbohydrate esterases (CEs) which hydrolyze methyl or acetyl substitutions (Lombard et al., 2014). Sulfatases, which hydrolyze sulfate substitutions (Barbeyron et al., 2016a), are often also essential, as sulfate substitutions are common in marine polysaccharides (Helbert, 2017).

The complexity of polysaccharides is correlated with the number of enzymes required for depolymerization. Highly complex, branched polysaccharides such as rhamnogalacturonan II, ulvan and fucoidan require as much as 100 different depolymerization enzymes (Ndeh et al., 2017; Reisky et al., 2019; Sichert et al., 2020). Fucoidans are found in the cell walls of brown macroalgae (Deniaud-Bouët et al., 2017) and have been used as model compound for recalcitrant polysaccharides in marine biogeochemical research (Arnosti, 2011). Fucoidans are sulfated, branched polysaccharides with an alpha-1,3/1,4-linked L-fucose backbone and variable other substitutions (Chevolot et al., 2001; Berteau and Mulloy, 2003). Few bacterial isolates are known to degrade fucoidan, and they can only do so partially (Van Vliet et al., 2019; Sichert et al., 2020), consistent with high complexity and recalcitrance. Despite characterization of exo-fucosidases, endo-fucanases, sulfatases and a deacetylase acting on fucoidans (Berteau et al., 2002; Katayama et al., 2004; Nagao et al., 2017; Silchenko et al., 2017; Nagao et al., 2018; Silchenko et al., 2018) and a recent expression study of fucoidan degrader '*Lentimonas*' strain CC4 (Sichert et al., 2020), the exact pathway and mechanism of fucoidan depolymerization are not completely known.

Initially, polysaccharides were thought to be depolymerized mainly extracellularly by secreted enzymes. This idea was corroborated by the insolubility of many terrestrial polysaccharides (Warren, 1996) and the apparent size limitation of bacterial passive diffusion channels, which did not allow diffusion of molecules larger than a trisaccharide (Weiss et al., 1991), although exceptions were known such as maltodextrin uptake the LamB maltodextrin porin (Nikaido and Vaara, 1985). A second, so-called 'selfish' mechanism was discovered in Gram-negative bacteria, comprised of initial depolymerization of polysaccharides into oligosaccharides by extracellular endo-acting enzymes, active transport of oligosaccharides into the periplasm by TonB-dependent transporters (TBDTs) within the outer membrane, and completion of depolymerization

by periplasmic exo-acting enzymes yielding monomers (Cho and Salyers, 2001; Martens et al., 2009). This mechanism has the benefit of preventing loss of depolymerized substrate to scavenging bacteria that have no depolymerization activity (Cuskin et al., 2015). The selfish mechanism is prevalent among Gram-negative bacteria in marine waters (Reintjes et al., 2017), consistent with the large pool of dissolved polysaccharides and the high competition for substrate among microorganisms (Arnosti et al., 2021).

In members of the *Bacteroidetes* phylum, in which the selfish mechanism was discovered, polysaccharide utilization enzymes are encoded in polysaccharide utilization loci (PULs), which are colocalized, coregulated genes that detect, import and depolymerize polysaccharides (Grondin et al., 2017; Schwalm and Groisman, 2017). The PUL archetype is the starch utilization system (Sus) of the model microorganism *Bacteroides thetaiotaomicron* (Martens et al., 2009). Canonical PULs from *Bacteroidetes* all encode – besides CAZymes and regulatory elements – SusD-like surface glycan-binding proteins (SGBPs) and SusC-like TBDTs that bind polysaccharides to the cell surface and actively import it into the periplasm (Grondin et al., 2017). However, non-canonical PULs have been found in other phyla, and do not necessarily include *susCD* genes (Grondin et al., 2017; Ausland et al., 2020). PULs can act on sulfated polysaccharides, and accordingly contain sulfatase genes (Xing et al., 2015; Reisky et al., 2019).

Pontiella desulfatans F1^T is an anaerobic member of the *Kiritimatiellaeota* phylum within the *Planctomycetes-Verrucomicrobia-Chlamydiae* (PVC) superphylum (Van Vliet et al., 2020). *P. desulfatans* F1^T and several other PVC bacteria are thought to be specialized in degrading complex sulfated polysaccharides, owing to their extensive repertoires of genes encoding sulfatases and other depolymerizing enzymes (Glöckner et al., 2003; Thrash et al., 2010; Van Vliet et al., 2019). *P. desulfatans* F1^T has an extraordinarily high number of genes encoding sulfatases (n = 521; Van Vliet et al., 2019) and glycoside hydrolases (n = 422; Van Vliet et al., 2020), many of which were predicted to act on fucoidans. Yet, a complete pathway for the degradation of L-fucose could not be found in the genome (Van Vliet et al., 2020). Here, we report an expression study on *P. desulfatans* F1^T growing on fucoidan from *Fucus vesiculosus* and on its monomer L-fucose, with D-glucose as control condition and chondroitin sulfate as example of simpler sulfated polysaccharide, with the aim to obtain insight into the enzymatic basis for fucoidan depolymerization and L-fucose degradation. Based on the recent study on another PVC bacterium by Sichert et al. (2020), we expected upregulation of a high number of seemingly redundant enzymes organized in PULs, and a predominance of exo-acting enzymes supporting an external fucoidan depolymerization mechanism. However, our results suggest a selfish mechanism of fucoidan degradation.

2 Materials and methods

2.1 Growth conditions and substrates

Pontiella desulfatans F1^T was cultured in a basal anoxic bicarbonate-buffered marine medium described previously (Van Vliet et al., 2019) at 25 g L^{-1} NaCl and 25 °C for optimal growth. Cultures were incubated statically and in the dark. The monosaccharide substrates fucose and glucose were added at a concentration of 10 mM. The sulfated polysaccharide substrates chondroitin 4-sulfate from bovine trachea and fucoidan from *Fucus vesiculosus* were added at a concentration of 1 g L^{-1}. All substrates were obtained from Sigma-Aldrich (St. Louis, MO, USA). Growth was monitored by optical density measurements at 600 nm wavelength (OD_{600}). Additionally, growth in fucoidan-containing cultures was monitored with protein concentration measurements using the Bradford assay. Shortly, pelleted biomass was resuspended in 1M NaOH and hydrolyzed at 100 °C for 15 min. The pH was neutralized with 1M HCl, sample was mixed 1:1 v/v with Bradford reagent and the absorbance at 595 nm wavelength was recorded. Bovine serum albumin was used as calibration standard. For growth curves, *P. desulfatans* F1^T was cultured in quadruplicate in 50 mL cultures within 120-mL serum vials with a 1.5 atm N_2/CO_2 (80:20 v/v) headspace and sealed with butyl rubber stoppers (Rubber BV, Hilversum, Netherlands) and aluminum caps. Concentrations of monosaccharides, alcohols and organic acids were measured through high-pressure liquid chromatography using a Shimadzu LC-2030C (Kyoto, Japan) equipped with refractive index and ultraviolet detectors and a Shodex SH1821 column (8.0 x 300 mm; 6 µm; Showa Denko K.K., Tokyo, Japan) operated at 45 °**C** with 5 mM H_2SO_4 as eluent at a flow rate of 0.8 mL min^{-1}. Dimethylsulfoxide was used as internal standard. H_2 partial pressures were measured with gas chromatography and sulfate concentrations were measured by ion chromatography using previously described methods (Van Vliet et al., 2019). Dissolved sulfide concentrations were measured with the methylene blue assay (Cline, 1969). Growth rates were estimated by fitting OD_{600} or protein levels to a modified Gompertz model (Zwietering et al., 1990).

2.2 RNA extraction and sequencing

For RNA extractions, *P. desulfatans* F1^T biomass was grown in triplicate 100 mL cultures in 250-mL serum vials. Biomass was harvested in exponential phase. Cultures were mixed with 150 mL of ice-cooled fresh anoxic medium to quickly reduce transcriptional activity. Subsequently, cultures were kept on ice and centrifuged at 10,000 x *g* for 10 min at 4 °C in a Sorvall Legend XTR centrifuge (Thermo Fisher Scientific, Waltham, MA, USA). The supernatant was discarded, and pelleted biomass was washed with ice-cooled sterile PBS and centrifuged again. After discarding the supernatant, the

pelleted biomass was snap-frozen with liquid nitrogen and stored at -80 °C until further processing. Cells were lyzed and DNA and RNA were extracted with the MasterPure™ Gram Positive DNA Purification Kit (Epicentre, Madison, WI, USA) used according to the manufacturer's instructions. β-mercaptoethanol was added to the lysis solution to inactivate RNases. After protein precipitation, the protein-free supernatant was mixed with an equal volume of isopropanol and incubated on ice for 10 min to precipitate the DNA and RNA. RNA was purified with the RNeasy Mini Kit of Qiagen (Hilden, Germany) according to manufacturer's instructions, including a DNase treatment step performed with RNase-free recombinant DNase I from Roche (Basel, Switzerland). The resulting concentrations of RNA and DNA were measured with the Qubit™ Broad-Range Assay Kits for DNA and RNA (Thermo Fisher Scientific, Waltham, MA, USA). For samples with >10% DNA contamination, the DNase treatment was repeated. RNA integrity was assessed with the Qsep100™ system (BiOptic, New Taipei City, Taiwan). Purified RNA extracts were stored at -80 °C until further processing. Depletion of rRNA and sequencing was performed by Novogene (Beijing, China). The NovaSeq 6000 Sequencing System (Illumina, San Diego, CA, USA) generated paired-end reads of 150 bp. Sequencing data was deposited at the European Nucleotide Archive (ENA) under study PRJEB24761 and samples ERS5373009-ERS5373022.

2.3 Transcriptomics analysis

Quality and length of the reads was analyzed with FastQC[12] version 0.11.2 and MultiQC version 1.7 (Ewels et al., 2016). Adapter sequences and low-quality sequences were trimmed with Trimmomatic version 0.36.5 (Bolger et al., 2014) using a 4-bp sliding window of an average quality of >20 and a minimum read length of 100 bp. Trimmed reads were mapped to the draft genome sequence of *P. desulfatans* F1^T (accession number GCA_900890425.1) with BWA-MEM version 0.7.17.1 (Li, 2013). Mapping quality statistics were generated with the SAMtools flagstat function (Li et al., 2009) and showed >95% properly mapped reads in all samples. Read counts per gene were compiled from mapped reads with *featureCounts* (Liao et al., 2013) based on the previously published Prokka-based coding sequence prediction of the *P. desulfatans* F1^T draft genome (Van Vliet et al., 2019). Expression data was normalized and statistically analyzed for differential expression with DESeq2 version 1.28.1 (Love et al., 2014). Shortly, DESeq2 tests for differential expression by fitting the normalized counts per gene to a generalized linear model using the negative binomial distribution, also known as the gamma-Poisson distribution, and then performing a Wald significance test comparing test conditions to the control condition. Expression levels are reported here in average Transcripts Per Million (TPM) per condition, which was calculated by dividing the normalized counts by the corresponding gene length in kbp, dividing the

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resulting normalized counts per kbp by their own sum per sample, multiplying the result by 1,000,000 to yield TPM values, and taking the average per condition.

Results were visualized in R version 4.0.2 (R Core Team, 2020). Z-scores per gene were calculated from normalized counts transformed to a $\log_2$-scale (DESeq2 'rlog' values) using the R 'scale' function, which calculates the mean normalized count value, subtracts it from the other normalized counts, and divides the result by the standard deviation. Methods for automated and curated annotation of the genes have been described previously (Van Vliet et al., 2019; Van Vliet et al., 2020). Additionally, hydrogenase genes were classified with HydDB (Søndergaard et al., 2016), subcellular localization of proteins was predicted with PSORTb v3.0.2 (Yu et al., 2010), signal peptides were predicted with SignalP 5.0 (Almagro Armenteros et al., 2019), PRED-TAT (Bagos et al., 2010) and SecretomeP 2.0 (Bendtsen et al., 2005), and outer membrane localization of beta-barrel proteins was predicted with PRED-TMBB2 (Tsirigos et al., 2016). Synteny was determined through curated blastp queries and genome inspection with Artemis v18.0 (Carver et al., 2008).

3 Results

3.1 Growth curves

P. desulfatans F1^T produced H_2, acetate, ethanol, succinate and traces of lactate during growth on D-glucose, L-fucose, chondroitin sulfate and fucoidan (Figures 1A-D). The doubling times were approximated at 10.4 h, 12.9 h, 59 h and 55 h, respectively. Electron recovery was approximately 60% on D-glucose and 70% on L-fucose, biomass excluded. The free sulfate concentration showed a small but statistically significant increase in chondroitin sulfate and fucoidan cultures ($P<0.05$, paired T-test; Figures 1C-D), further supported by a positive linear correlation between time and free sulfate concentration with an R^2 of 0.65 and 0.88, respectively. In contrast, the sulfate concentration in D-glucose and L-fucose cultures did not show any change (data not shown). Since the increase in OD_{600} in fucoidan cultures was smaller than in other conditions and microscopy showed aggregation of cells, which potentially interferes with OD_{600} measurements, growth was also monitored with protein measurements. OD_{600} and protein concentrations showed a similar increase over time, although with the steepest increase in protein concentration was observed at a somewhat later time point than in the OD_{600} data (Figure 1E).

3.2 Polysaccharide depolymerization

Transcriptomics replicates showed high similarity, demonstrating reproducible expression (Figure S1, S2). 46 sulfatase genes were expressed [>50 Transcripts Per Million (TPM)] during growth on D-glucose (Table S1a), indicating constitutive expression. Most constitutive sulfatase genes were lowly expressed (50-100 TPM), eight were medium expressed (100-250 TPM), and one was highly expressed (>250 TPM). 84 CAZyme genes were constitutively expressed (Table S1b). Of the 34 medium or highly expressed constitutive CAZyme genes, 22 were glycosyltransferases. They were predicted to be cytoplasmic and involved in biosynthesis of peptidoglycan (GT28), lipopolysaccharide (GT9, GT19, GT26, GT30, GT81), and glycogen (GT5, GT35). Three constitutive GHs were predicted to be involved in sucrose or glycogen degradation/synthesis, three were annotated as galactosidases (GH2, GH35) and one as fucosidase (GH29, PDESU_05472). Among the 50 lowly expressed constitutive CAZyme genes, there was a higher fraction of putatively catabolic GHs, among which ten fucosidases (GH29, GH95, GH141), as well as a heparinase polysaccharide lyase (PL0).

A total of 492 genes were substantially upregulated (log_2-fold change >2, adjusted P-value <0.01) in the L-fucose, fucoidan or chondroitin sulfate conditions in comparison with the D-glucose control condition (Table S1c). This included 96 genes predicted to encode CAZymes/sulfatases. 26 CAZymes/sulfatases were induced by L-fucose, 42 were induced only by fucoidan, and 27 only by chondroitin sulfate. Most expression levels of the substantially upregulated CAZymes/sulfatases passed our threshold of expression (81 out of 96, >50 TPM), and even of medium expression (73, >100 TPM). 85 out of the 96 substantially upregulated CAZymes/sulfatases were located in genomic loci which were coregulated, enriched in CAZymes/sulfatases, and containing or bordering regulatory elements (Table S1d). These features are key characteristics of polysaccharide utilization loci (PULs) across bacterial phyla (Grondin et al., 2017). We identified 15 distinct PULs, based on the presence of at least two substantially upregulated CAZymes/sulfatases within a coregulated 'block'. PULs were scattered across the genome of *P. desulfatans* F1^T (Figure 2). Together, they represented 4.8% of the total number of genes, 10.6% of all CAZymes and 8.8% of all sulfatase genes. We also found 13 sets of two-component system genes colocalized with CAZymes/sulfatases that were not significantly upregulated (adjusted P-value >0.01, data not shown), possibly representing inactive additional PULs. Active PULs were often localized in CAZyme/sulfatase-rich genomic regions (Figure 2). Some CAZyme/sulfatase-rich genomic regions lacked the presence of PULs, notably the CAZyme/sulfatase islands between 2.8 and 3.2 Mbp and between 6.4 and 6.8 Mbp with an aberrant GC content.

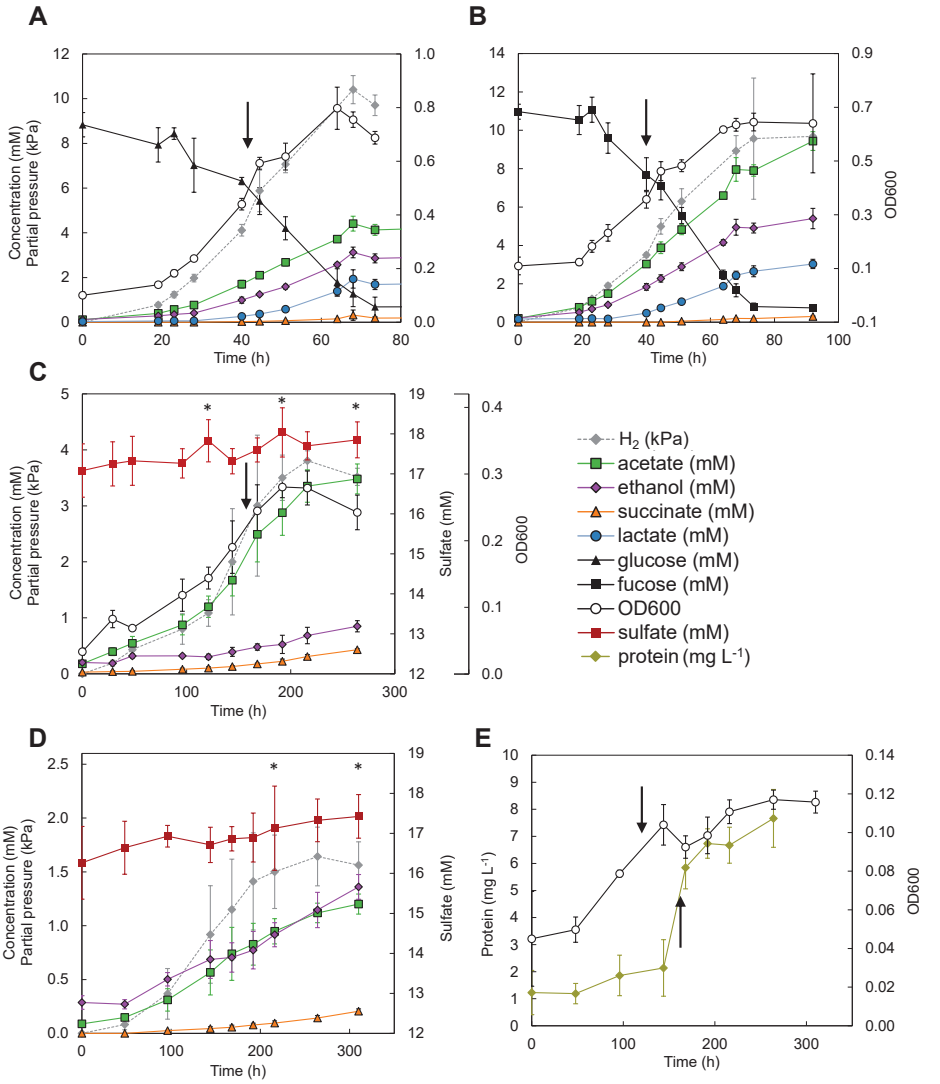


Figure 1. Growth curves of *P. desulfatans* F1^T with (A) D-glucose, (B) L-fucose, (C) chondroitin sulfate or (D, E) fucoidan as substrate. Black arrows indicate the average OD_{600} or protein concentration at which subsequent cultures were harvested for RNA extraction. Asterisks indicate the increase in sulfate concentration compared to t=0 is significant (P<0.05, paired T-test).

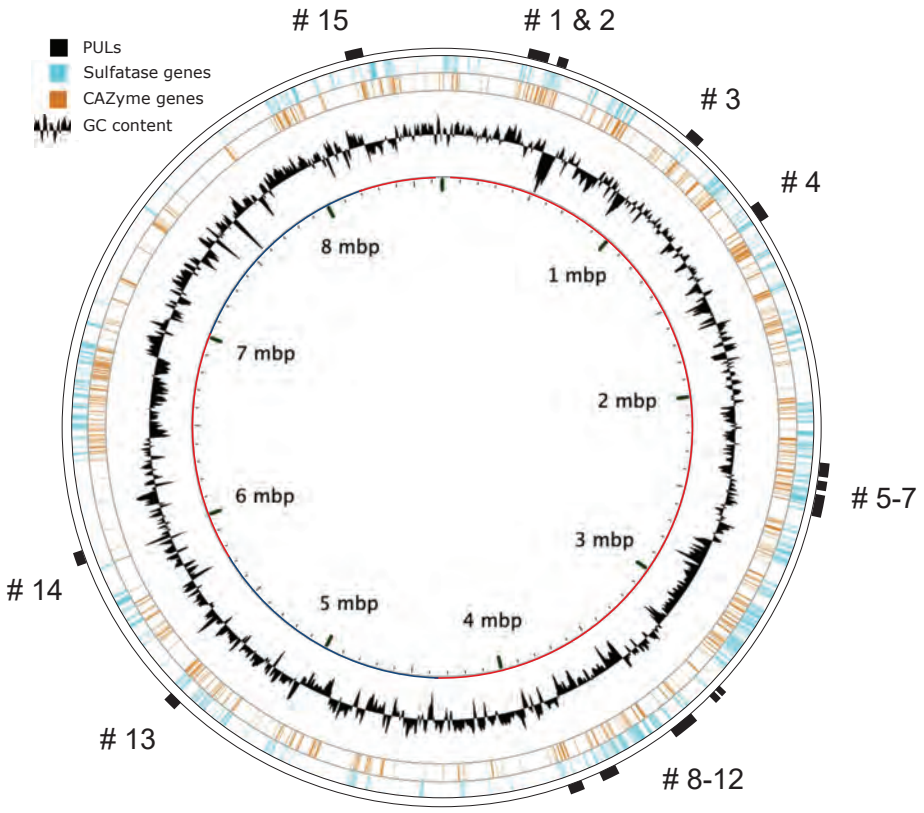


Figure 2. Polysaccharide utilization loci (PULs) mapped on the 8.6 Mbp *Pontiella desulfatans* F1^{T} genome. The inner circle shows the length of the genome in Mbp and distinguishes the contigs by alternating red and blue color. Adapted from Van Vliet et al. (2020).

All PULs contained or bordered a regulatory element, except for PUL 2. Putative surface glycan-binding protein (SGBP) genes without homology to *susD* were present in five of the PULs, whereas neither *susC* homologs nor other TBDT genes could be detected in PULs (Table S1d). Eight PULs were upregulated only by fucoidan (PULs 1, 2, 5, 6, 7, 9, 13, 14), and three were upregulated only on chondroitin sulfate (PULs 8, 11, 12; Figure 3). PULs 3, 4 and 10 were upregulated with all three tested substrates, and PUL 15 was upregulated by L-fucose and chondroitin sulfate, but not fucoidan. The median expression level was high in five PULs, medium in seven PULs, low in two PULs, and below the expression threshold in PUL 15 (Figure 3).

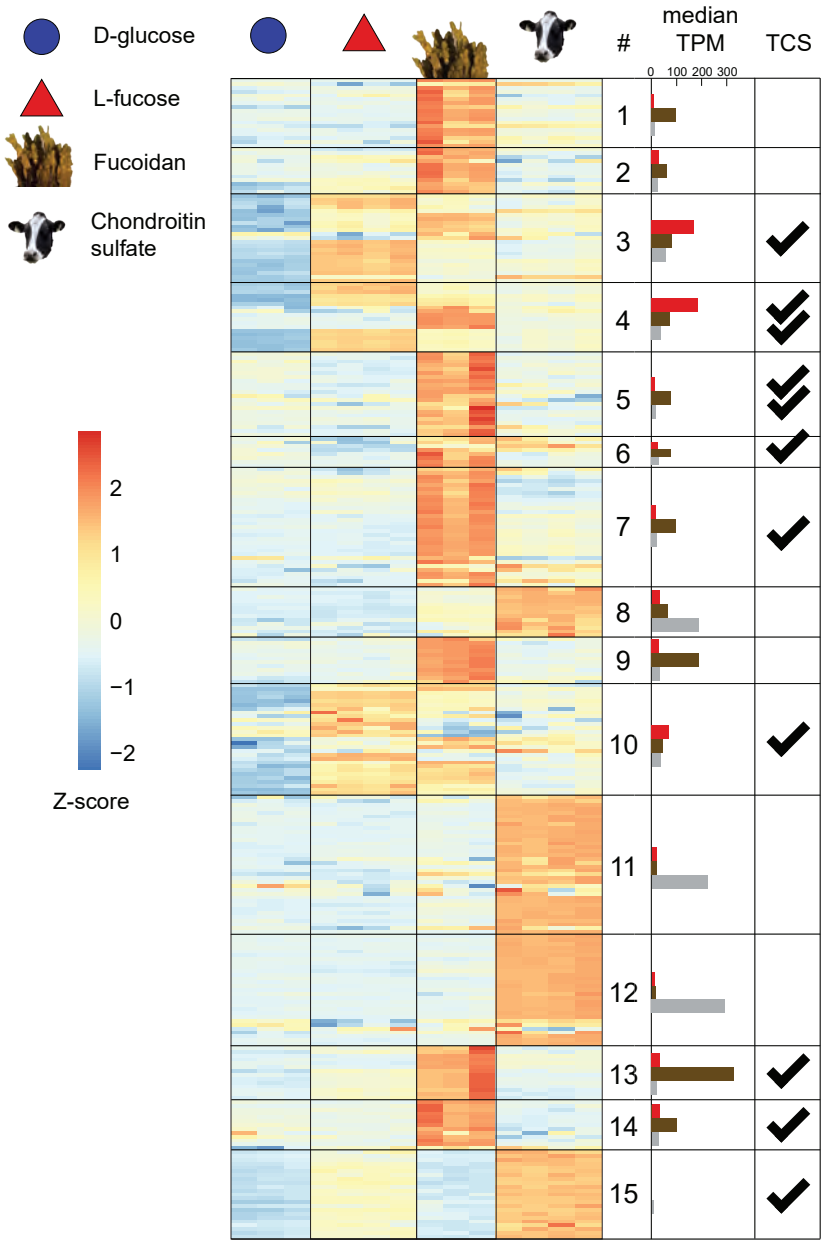


Figure 3. Heatmap of the standardized expression of the genes within polysaccharide utilization locus (PULs) per sample. Z-score represents the number of standard deviations by which an expression value deviates from the mean expression value across all samples within all conditions. PULs are numbered 1 to 15 and correspond to the numbering in Figure 2. Bar charts indicate the median average TPM of PULs per test condition, with red representing L-fucose, brown representing fucoidan and gray representing chondroitin sulfate. Abbreviations: TCS, two-component system; TPM, Transcripts Per Million.

Together, the PULs encompassed 299 genes. These included 60 CAZymes of 17 different families, 47 sulfatases of 14 different subfamilies, six peptidases, but also 93 genes without predicted function (Table S1d). The most represented were putative exo-fucosidases (n = 21; GH29, GH95, GH141) and putative fucoidan sulfatases (n = 21; S1 subfamilies 15-17 and 25). Putative chondroitin lyase genes (n = 6, PL8_2) were present only in chondroitin sulfate-specific PULs. Some non-PUL substantially upregulated genes were of (sub)families not present among the PUL genes, such as a putative ulvan lyase (PL25, PDESU_02003) upregulated on fucoidan, and a putative 4,5-hexuronate-2-O-sulfate sulfatase (S1_9, PDESU_04122) upregulated and highly expressed on chondroitin sulfate (Table S1c). There was little overlap between constitutively expressed and upregulated CAZymes/sulfatases. Of the 46 constitutively expressed sulfatases, six were within PULs (Table S1a). Four constitutively expressed sulfatases were also substantially upregulated, of which two are within PULs. A single constitutively expressed CAZyme showed substantial differential expression (PDESU_01702, GH29 fucosidase), and coincidentally was located within a PUL (Table S1b).

Several systems for the secretion of enzymes were expressed: the Sec preprotein-translocation pathway (*secYEG*, *secA*), the twin-arginine translocation (TAT) pathway (*tatBCA*), the type II secretion system (*gsp* genes), multiple translocation and assembly modules of the type V secretion system (*bamAB*) also known as the 'autotransporter' pathway (Selkrig et al., 2012), and finally the type IX secretion system (*epsH*, PDESU_03180) also known as the 'exosortase' system (Haft et al., 2006). Some of the *gsp* genes were upregulated when grown with fucoidan. Most sulfatases, CAZymes (excluding glycosyltransferases) and PUL proteins were predicted to be secreted (95%, 84% and 76% respectively; Table S1). This included 7 periplasmic proteins, 106 outer membrane or extracellular proteins, and 113 secreted proteins that could be periplasmic, outer membrane or extracellular, but of which the specific localization could not be confidently predicted. The putatively cytoplasmic proteins (16%) were mostly composed of regulatory proteins and transposases.

As regulatory element, most PULs contained one or two two-component systems (Figure 3) composed of adjacent sensor histidine kinase and LuxR transcriptional activation protein family genes. Other putative regulatory elements were genes encoding for LacI protein, sigma factor and anti-anti-sigma factor, RhaR/AraC family protein, IclR protein, KdgR regulator, arabinose/xylose regulatory protein and Hpt protein. PULs also contained genes potentially involved in monosaccharide uptake and degradation. PUL 3 contained a fucose permease gene (*fucP*, PDESU_00788). PUL 8 contained tripartite ATP-independent periplasmic transporter genes, as well a D-xylose dehydrogenase gene (*xdh*, PDESU_02281), and a KduI/IolB family ketol-isomerase gene (PDESU_02286). PUL 9 contained a DeoC/LacD family aldolase (PDESU_02302), and the *dhaKL* genes of the dihydroxyacetone kinase complex. PUL

10 contained an arylsulfotransferase gene (PDESU_02421), a xylulose kinase gene (*xylB*, PDESU_02423), an enoyl reductase gene (PDESU_02424) and a deoxyribose-phosphate aldolase gene (*deoC*, PDESU_02426). PULs 11, 12, 14 and 15 contained sodium:solute symporter genes (Table S1d).

P. desulfatans F1^T encoded and expressed TonB-dependent transporters (TBDTs, Table S1e). Active transport by TBDTs is driven by the proton motive force consumed by the associated inner membrane TonB-ExbBD complex, of which several were also encoded and expressed. In a *tonB-exbBD* gene cluster (PDESU_04392-04296), the *tonB* gene was medium expressed, whereas the *exbBD* genes were highly expressed and fucoidan-upregulated. Three predicted TBDTs were expressed: two putative vitamin B12 transporters (*btuB*, PDESU_03486 & 05385) and one TBDT with unknown function that was lowly expressed, but somewhat upregulated with fucoidan and chondroitin (PDESU_05885). TBDTs are a subclass of beta-barrel 'porin' proteins, which normally serve as passive diffusion channels for small solutes including monosaccharides (Zafar and Saier, 2018). Three other porin genes were also fucoidan-upregulated (Table S1e): a medium-expressed maltoporin gene (*lamB,* PDESU_02860), a highly expressed type 2 putative beta-barrel porin gene (*bbp2*, PDESU_04084) colocalized with *exbBD* genes, and a substantially upregulated and highly expressed putative *ompF* porin gene (PDESU_06156). Moreover, *tolB* genes were present in the *tonB-exbBD* gene cluster and in PULs 7 and 10 (Table S1d).

To function, sulfatases require post-translational modification of a cysteine or serine in the active site to a formylglycine (Dierks et al., 1998). This is catalyzed by anaerobic sulfatase-maturating enzyme (anSME) or by the oxygen-dependent formylglycine-generating enzyme (FGE; Bojarová and Williams, 2008). *P. desulfatans* F1^T expressed its only anSME gene (PDESU_04110) at medium to high levels. Of the 20 putative FGE genes, one was constitutively expressed at a tenfold higher level (PDESU_03366), and others at similar levels (PDESU_00199, 004 85, 04176, 04597, 04658) as the anSME gene. Two additional FGE genes were substantially upregulated by fucoidan (PDESU_02298, 04642), one of which was located in PUL 9 and highly expressed (PDESU_02298).

3.3 L-fucose degradation

Three pathways for the breakdown of L-fucose have been described: the canonical phosphorylative pathway (Ghalambor and Heath, 1962; Heath and Ghalambor, 1962; Gunn et al., 1994), the non-phosphorylative diketohydrolase pathway (Yew et al., 2006; Hobbs et al., 2013) and the non-phosphorylative aldolase pathway (Wolf et al., 2016). The first two pathways both have the toxic L-lactaldehyde as intermediate. None of the three pathways was predicted to be complete through genome annotation (Figure 4,

Table S2). Of the canonical phosphorylative pathway, the genes for L-fucose permease (*fucP*), L-fucose mutarotase (*fucU*, PDESU_02900) and L-fucose isomerase (*fucI*, PDESU_02811) were identified by automated annotation. A homolog of the L-fuculose phosphate aldolase (*fucA*, PDESU_05629) gene from *Methanocaldococcus jannaschii* (Choi et al., 1998) was manually identified. Although L-fuculokinase (*fucK*) was not encoded, this function could also be fulfilled by L-rhamnulokinase (PDESU_1491), as observed *in vitro* for other bacteria (Chiu and Feingold, 1964; Fessner et al., 1992; Wen et al., 2016). However, a L-lactaldehyde dehydrogenase (*aldA*) or lactaldehyde reductase (*fucO*) gene was not found, as previously reported (Van Vliet et al., 2020). The absence of *fucO* was expected, as *P. desulfatans* F1^T does not produce 1,2-propanediol as fermentation product. L-fucose can also be degraded through the two variants of the non-phosphorylative pathway. Homologous genes were identified for every step, but apart from the L-fuconate dehydratase (*fucD*, PDESU_06372) gene all with low similarity and ambiguous reciprocity.

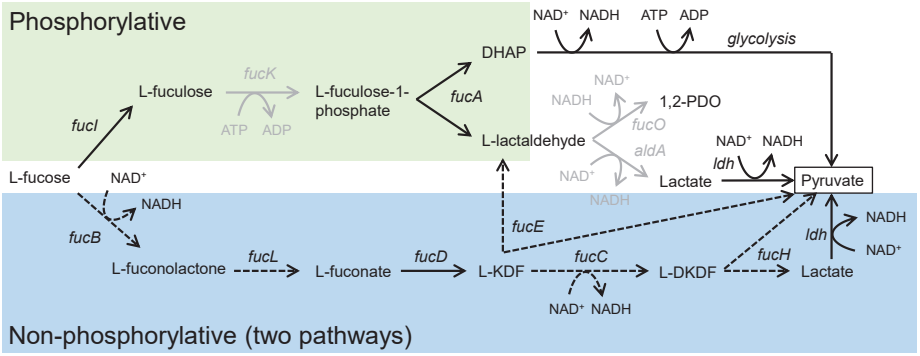


Figure 4. Annotation of genes of known L-fucose degradation pathways in *P. desulfatans* F1^T. The top route shows the phosphorylative pathway, with characteristic steps shaded green. The bottom route shows the two non-phosphorylative pathways, with unique steps shaded blue. Solid black lines indicate the presence of homologous genes. Dashed lines indicate weak homology. Grey lines indicate no homologous genes were identified. Abbreviations: DHAP, dihydroxyacetone phosphate; L-DKDF, 2,4-diketo-3-deoxy-L-fuconate; L-KDF, 2-keto-3-deoxy-L-fuconate; 1,2-PDO, 1,2-propanediol. Genes: fucI, L-fucose isomerase; fucK, L-fuculokinase; fucA, L-fuculose-1-phosphate aldolase; fucO, L-lactaldehyde reductase; aldA, L-lactaldehyde dehydrogenase; ldh, lactate dehydrogenase; fucB, L-fucose dehydrogenase; fucL, L-fuconolactonase; fucD, L-fuconate dehydratase; fucE, 2-keto-3-deoxy-L-fuconate aldolase; fucC, 2-keto-3-deoxy-L-fuconate 4-dehydrogenase; fucH, 2,4-diketo-3-deoxy-L-fuconate hydrolase.

Two of the five *fucP* transporter genes – one of which located in PUL 3 – and the *fucU* gene were expressed and upregulated, specifically on chondroitin sulfate (Table S3a). The *fucI* gene was strongly upregulated and highly expressed only in the L-fucose and fucoidan conditions. The putative *fucA* gene was lowly and constitutively expressed. Of the putative non-phosphorylative genes, none were upregulated on L-fucose,

except for the putative *fucC* gene (PDESU_02424), which was located in PUL 10 and was medium expressed on L-fucose and fucoidan. In contrast, an operon of 11 genes with mostly sugar-degrading predicted functions was strongly upregulated and highly expressed in the L-fucose and fucoidan conditions (PDESU_01489-01499, 272-4904 TPM, log_2-fold change 1.4-5.8, Table S3b). It encoded a phosphohexomutase (PDESU_01489), transketolase (*tkt*, PDESU_01490), L-rhamnulokinase (*rhaB*, PDESU_01491), two phosphatases (PDESU_01492, 01495), a sugar alcohol dehydrogenase (PDESU_01493), a NAD(P)H-dependent short-chain dehydrogenase (PDESU_01494), and a 2-oxoacid dehydrogenase complex with similarity to the pyruvate and acetoin dehydrogenase complexes (E1-3, PDESU_01496-01498). Except for transketolase, which is also part of the highly expressed non-oxidative PPP branch, all genes of this putative operon were not or lowly expressed in the D-glucose and chondroitin sulfate conditions. All translated enzymes were predicted to be cytoplasmic, and did not contain any bacterial microcompartment domains.

We found similar operons in other *Kiritimatiellaeota* bacteria, but also in fermentative bacteria of various other phyla (Figure 5). *Pontiella sulfatireligans* F21^T possessed the entire operon as found in *P. desulfatans* F1^T. The HAD hydrolase family phosphatase gene (PDESU_01492) was present only in the two *Pontiella* spp. and not in the other analyzed bacterial genomes, although *Kiritimatiellaeota* bacterium *Tichowtungia aerotolerans* S-5007^T did encode a pyrophosphatase gene downstream, and the spirochaete *Marispirochaeta aestuarii* JC444^T encoded a HAD hydrolase gene upstream. *Pontiellaceae* bacterium TMED266 encoded a *fucI* gene upstream, but lacked 2-oxoacid dehydrogenase E3, *tkt* and phosphohexomutase genes. *T. aerotolerans* encoded *rhaB, tkt* and phosphohexomutase genes elsewhere scattered in the genome. *M. aestuarii* similarly encoded *rhaB* and *tkt* elsewhere in the genome, but lacked a homolog of the phosphohexomutase gene. *Thermoclostridium stercorarium* DSM 8532^T encoded a reordered version of the operon followed by a peptidase gene (*ykfC*), encoded a transketolase gene elsewhere but lacked the phosphohexomutase gene. In the bacteroidete *Sunxiuqinia elliptica* DQHS4^T, the operon was split into two gene clusters separated by eight other genes with mostly unknown functions, and followed by a fructose-6-phosphate aldolase (*fsa*) gene. Again, *rhaB* and *tkt* were encoded elsewhere on the genome, while the phosphohexomutase gene was missing. Notably, in this bacterium the short-chain dehydrogenase gene additionally contained an aldolase class II domain with potential L-fuculose-phosphate aldolase activity. Syntenic operons were not found in the genomes of *Kiritimatiella glycovorans* L21-Fru-AB^T or of the fucoidan-degrading bacteria '*Lentimonas*' strain CC4, *Zobellia galactanivorans* Dsij^T or *Wenyingzhuangia fucanilytica* CZ1127^T.

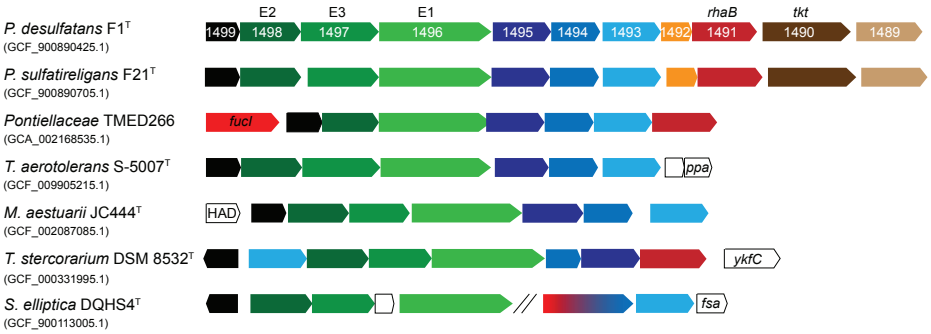


Figure 5. Gene clusters with synteny with the putative L-fucose degradation operon of *P. desulfatans* F1^T. Locus tags are indicated by the numbers within the *P. desulfatans* F1^T genes (PDESU_0xxxx). Colors indicate homology, except for black genes which have a regulatory function but are not necessarily homologs, and the red-to-blue gradient gene of S. elliptica representing a gene containing both class II aldolase and short-chain dehydrogenase domains. Genes: E1, thiamin pyrophosphate-dependent dehydrogenase; E2, dihydrolipoamide acetyltransferase; E3, dihydrolipoyl dehydrogenase; HAD, HAD-family hydrolase; fucI, L-fucose isomerase; fsa, fructose-6-phosphate aldolase; ppa, inorganic pyrophosphatase; rhaB, L-rhamnulokinase; tkt, transketolase; ykfC, peptidase.

3.4 D-glucuronate and N-acetyl-D-galactosamine degradation

Chondroitin sulfate is composed of D-glucuronate and N-acetylgalactosamine. Genes for D-glucuronate degradation were previously reported to be present in *P. desulfatans* F1^T (Van Vliet et al., 2020). The genes encoding D-glucuronate isomerase, D-mannonate dehydrogenase, D-mannonate dehydratase and 2-dehydro-3-deoxygluconokinase are present in a putative operon (PDESU_03529-03541, Table S3c) upregulated and highly expressed on chondroitin sulfate. In another location in the genome were another upregulated 2-dehydro-3-deoxygluconokinase gene (PDESU_05181) and a highly expressed 2-dehydro-3-deoxyphosphogluconate aldolase gene (PDESU_05182), leading to the production of D-glyceraldehyde 3-phosphate and pyruvate, thus forming the link to glycolysis. Several genes for N-acetyl-D-galactosamine transport and degradation are also colocalized in a putative operon (PDESU_04332-04340), upregulated and highly expressed (Table S3d). The operon encodes a sodium:sugar symporter, two N-acetylhexosamine kinases, a deacetylase, and a hexosamine-6-phosphate deaminase/isomerase, together yielding D-tagatose-6-phosphate. Several kinases (e.g. PDESU_02898) could be responsible for the subsequent phosphorylation of D-tagatose-6-phosphate to D-tagatose-1,6-bisphosphate. Finally, a highly upregulated and expressed D-tagatose-1,6-bisphosphate aldolase gene (PDESU_02896) likely forms the final link to glycolysis.

3.5 Central carbon metabolism

The central carbon metabolism pathways expressed by *P. desulfatans* F1^T are summarized here, but described and discussed in more detail in Supplementary Material S1. The glycolysis/gluconeogenesis pathway was highly expressed in all conditions, with no substantial differential expression between test conditions and D-glucose (Table S3e). There was a high level of functional redundancy, with expression of both the canonical ATP-dependent glycolysis enzymes as well as less common PP_i-dependent enzymes such as pyrophosphate (PP_i)-fructose-6-phosphate-1-kinase (*pfp*, PDESU_05674) and pyruvate dikinase (*ppdk*, PDESU_00626). The close relative *K. glycovorans* L21-Fru-AB^T was predicted to rely solely on PP_i-dependent glycolysis based on genome analysis (Spring et al., 2016). Unusually high expression levels of GDP-dependent PEP carboxykinase (*pck*) and Na^+-transporting oxaloacetate decarboxylase (*oadABC*) were observed (Table S3e). Furthermore, *P. desulfatans* F1^T expressed D-lactate dehydrogenase for producing lactate from pyruvate, other fermentation genes for the conversion of acetyl-CoA to acetate (phosphate acetyltransferase, *pta*, PDESU_04375; acetate kinase, *ackA*, PDESU_04376) and ethanol (aldehyde-alcohol dehydrogenase, *adhE*, PDESU_03407), and various hydrogenase complexes for generating the fermentation product H_2 (Table S3f).

An incomplete oxidative tricarboxylic acid (TCA) cycle was expressed (Table S3g), producing the amino acid precursors oxaloacetate and 2-oxoglutarate and the fermentation product succinate. On fucoidan, several genes of the TCA cycle were upregulated, including *fumAB* genes, consistent with a higher succinate production. The oxidative branch of the pentose phosphate pathway (PPP), which converts D-glucose 6-phosphate into the five-carbon D-ribose 5-phosphate and $NADP^+$ into NADPH, was constitutively expressed at a low to medium level. The non-oxidative branch of the PPP was not canonical, lacking a transaldolase of which the functionality is most likely replaced by one of the four expressed 6-phosphofructokinases in combination with fructose-1,6-bisphosphatase aldolase as recently demonstrated for *Pseudoclostridium thermosuccinogenes* (Koendjbiharie et al., 2020). The non-oxidative PPP branch was constitutively highly expressed.

4 Discussion

The current study identified eleven polysaccharide utilization loci (PULs) that were upregulated during growth of *Pontiella desulfatans* F1^T on fucoidan. These included eight fucoidan-specific PULs which contained the majority of the fucoidan-upregulated CAZyme/sulfatase genes. Currently, the only other reports of fucoidan-specific PULs are two confirmed PULs in the '*Lentimonas*' strain CC4 chromosomal DNA - along with a larger megaplasmid encoding many fucoidan depolymerization genes - (Sichert et al., 2020), and three putative fucoidan PULs in the fucoidan-degrading bacteroidetes *Zobellia galactanivorans* Dsij^T (Barbeyron et al., 2016b) and *Wenyingzhuangia fucanilytica* CZ1127^T (Silchenko et al., 2018). The identification of PULs constrained the number of predicted CAZymes/sulfatases potentially involved in fucoidan degradation by *P. desulfatans* F1^T from over 900 genes to 75 genes, but also revealed 47 secreted proteins without predicted function for which a direct role in fucoidan depolymerization is presumable.

The predicted functionality of the constrained CAZymes/sulfatases was similar to that of fucoidan-upregulated proteins of '*Lentimonas*' strain CC4, specifically the predominance of genes encoding seemingly redundant fucosidases (n = 18; GH29, GH95, GH141), which presumably are exo-acting (Berteau et al., 2002; Katayama et al., 2004; Ndeh et al., 2017). Other resemblances were the upregulation of CE7 genes with homology to a fucoidan deacetylase gene (Nagao et al., 2017), GH36, GH97 and GH117 genes (n = 9) potentially serving to remove uronic acid substitutions, and of specific sulfatase subfamilies (n = 25; S1 subfamilies 15-17, 22, 23, 25, 28). However, *P. desulfatans* F1^T additionally showed upregulation of putative cell-surface-bound SGBPs (carbohydrate-binding module families 6 and 62, n = 5), CE10 deacetylase genes (n = 4) and sulfatase subfamily S1_14 genes (n = 8). Sulfatase subfamilies S1_17 and S1_25 are known to contain exo-2*O* and exo-3*O* fucoidan sulfatases (Silchenko et al., 2018). Our results indicate the seven other fucoidan-upregulated subfamilies, which lack characterized enzymes, also act on fucoidan structures.

The expression studies in *P. desulfatans* F1^T (this study) and in '*Lentimonas*' strain CC4 (Sichert et al., 2020) demonstrate that PULs are present in PVC bacteria. Previous genome analysis already indicated PULs in genomes of freshwater *Verrucomicrobia* spp. (He et al., 2017) and the planctomycete *Rhodopirellula* strain SKW7 (Ficko-Blean et al., 2017). PULs in phyla other than the *Bacteroidetes* phylum often lack identifiable canonical PUL elements such as surface glycan-binding proteins (SGBPs; Thomas et al., 2012; Dejean et al., 2013; Neumann et al., 2015). This was also observed for PULs in '*Lentimonas*' strain CC4, which lacked identifiable SGBPs and – except for one carrageenan-specific PUL – TonB-dependent transporters (TBDTs; Sichert et al., 2020). In contrast, the PULs identified in *P. desulfatans* F1^T did contain putative SGBPs, but lacked TBDTs.

PULs can be complemented by genes located elsewhere, as previously reported for endo-acting GH genes (Attia et al., 2016) and regulatory elements (Despres et al., 2016). A single potential oligosaccharide-uptake TBDT was identified outside of PULs in *P. desulfatans* F1^T (PDESU_05885), but only lowly expressed and not strongly upregulated with fucoidan, providing only weak evidence for its possible role. It is more plausible that the stronger fucoidan-upregulated, medium-to-high expressed putative porin genes (*lamB*, *bbp2*, *ompF*) are responsible for uptake of mono- or oligosaccharides. LamB proteins are thought to be specific for passive oligosaccharide transport, taking up malto-oligosaccharides in *Escherichia coli* (Boos and Shuman, 1998). The *bbp2* gene was colocalized with highly expressed *exbBD* genes, suggesting an active mode of transport. Importantly, a role could also be in store for very highly expressed and strongly fucoidan-upregulated PUL genes encoding beta-barrel outer membrane proteins without predicted function (PDESU_00272, 01766, 01770; 778-3414 TPM). The additional expression of *tolB* genes and their presence within PULs is notable, as TolB proteins – typically involved in cellular stability – can interact with and may aid in the assembly of trimeric porins such as LamB and OmpF (Rigal et al., 1997).

Fucoidan depolymerization by '*Lentimonas*' strain CC4 was proposed to proceed primarily through extracellular exo-cleavage, also of the backbone, partly based on the scarcity of identifiable endo-acting fucanases in the proteome (Sichert et al., 2020). Initial activity of exo-enzymes is indeed likely needed for fucoidan depolymerization in general, as endo-fucanases are usually unable to cleave fucoidan without prior treatment (Silchenko et al., 2017; Schultz-Johansen et al., 2018; Shen et al., 2020). *P. desulfatans* F1^T indeed encoded seven exo-fucosidases within fucoidan-specific PULs that were predicted to be extracellular or outer membrane-bound, but also 11 periplasmic or non-specifically secreted exo-fucosidases. Characterized endo-α-1,4-L-fucanases are all part of the GH107 family (Colin et al., 2006; Silchenko et al., 2017; Schultz-Johansen et al., 2018; Zueva et al., 2020). Expressed GH107 genes were indeed relatively scarce in '*Lentimonas*' strain CC4 (n = 3; Sichert et al., 2020) and *P. desulfatans* F1^T (n = 2). Nevertheless, a crucial role cannot be dismissed, as many physiologically essential genes have only one copy per genome. This was supported by the high expression of an extracellular GH107 enzyme in *P. desulfatans* F1^T during growth on fucoidan (PDESU_04029, 529 TPM).

The involvement of currently unknown endo-fucanases is not precluded by these expression studies. Recently, a biochemical characterization of fucoidan-upregulated genes of *W. fucanilytica* CZ1127^T with unknown function led to the discovery of the first endo-α-1,3-fucanase and establishment of the GH168 family (Shen et al., 2020). Through a blastp homology search (bitscore cut-off = 60), we identified four GH168 genes within *P. desulfatans* F1^T PULs. Three additional GH168 genes were colocalized and coregulated with a periplasmic GH29 fucosidase gene, and bordering a *gntR* family regulatory gene, thus presenting a sixteenth small PUL (PDESU_01600-01604).

Although their expression on fucoidan was below threshold or low, all were fucoidan-upregulated (log_2-fold change 1.4-3.9). Additionally, several PUL genes without predicted function were highly expressed (484-2901 TPM), substantially fucoidan-upregulated (log_2-fold change 5.3-8.9) and predicted to encode extracellular proteins. These would thus be prime candidates for novel, potentially endo-acting fucoidan depolymerization enzymes.

We have shown that fucoidan degradation by *P. desulfatans* F1^T likely involves surface-bound SGBPs, extracellular endo-fucanases, extracellular as well as periplasmic exo-fucosidases and sulfatases, and possibly also involves TBDTs. Therefore, we propose a selfish mode of fucoidan depolymerization rather than an external one (Figure 6). Selfish uptake of fucoidan has been observed with microorganisms in ocean surface waters through incubations with fluorescently labelled fucoidan (Reintjes et al., 2019). In general, selfish degradation is thought to be promoted by polysaccharide complexity (Arnosti et al., 2021), which is a hallmark of fucoidan.

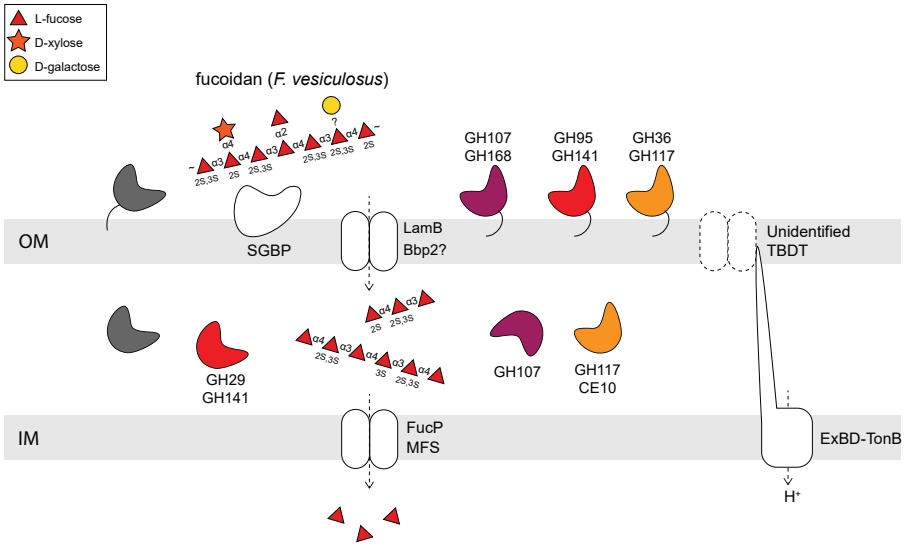


Figure 6. Global model of the mechanism of fucoidan depolymerization in *P. desulfatans* F1^T. The molecular structure of fucoidan from *F. vesiculosus* was based on that presented by Sichert et al. (2020). Uronic acid substitutions additionally known to be present, but are not depicted here. Fucoidan is incompletely degraded by pure cultures. The structure of the residual fucoidan is currently unknown, and therefore not depicted here. The fate of D-xylose, D-galactose and uronic acid substitutions is not discussed and not depicted. Enzyme colors: dark gray, sulfatase; red, exo-fucosidase; burgundy, endo-fucanase; orange, other CAZyme. Abbreviations: OM, outer membrane; IM, inner membrane; MFS, major facilitator superfamily transporter.

Our results suggest that *P. desulfatans* F1^T does not degrade L-fucose through known pathways. Little evidence for the described phosphorylative and non-phosphorylative L-fucose degradation pathways was obtained, as most genes were either not detected (*fucK, aldA*) or not differentially expressed on L-fucose (putative *fucABDEHL* genes). Furthermore, L-fucose degradation genes were only observed in gene clusters in other bacteria (Yew et al., 2006; Stahl et al., 2011; Hobbs et al., 2013; Petit et al., 2013; Erbilgin et al., 2014; Wolf et al., 2016), with exception of *fucP, fucU* and *fucI* (Sichert et al., 2020), whereas the putative canonical genes in *P. desulfatans* F1^T were scattered throughout the genome. Thus, we postulate that L-fucose is degraded through an alternative pathway, involving the genes within the putative sugar-degrading operon strongly upregulated by L-fucose and fucoidan.

We hypothesize that L-fucose is first transported into the cytoplasm by one of the two upregulated FucP symporters, then isomerized to L-fuculose by FucI, followed by phosphorylation by RhaB, similar to the canonical phosphorylative pathway. However, we can only speculate on how L-fuculose-1-phosphate would be processed further, and what the roles of the other genes in the putative L-fucose degradation operon are. Transketolase and phosphohexomutase may not play essential roles, as these were not conserved in the operon in bacteria outside of the *Pontiella* genus (Figure 5). Other microorganisms that grow well on L-fucose or L-rhamnose utilize bacterial microcompartments to compartmentalize the toxic intermediate L-lactaldehyde (Petit et al., 2013; Erbilgin et al., 2014; Sichert et al., 2020). The absence of bacterial microcompartment domains or proteins in *P. desulfatans* F1^T implies L-lactaldehyde is not an intermediate in its L-fucose degradation pathway.

5 Conclusion

Through transcriptomics we identified several fucoidan-specific polysaccharide utilization loci (PULs) in *P. desulfatans* F1^T, which are probably involved in fucoidan depolymerization. Fucoidan depolymerization enzymes had similar numbers and predicted functionality as those of the recently studied PVC bacterium '*Lentimonas*' strain CC4. However, we propose a selfish mode of fucoidan degradation, based on the expression of surface glycan-binding proteins, extracellular endo-fucanases, periplasmic exo-fucosidases, and the potential involvement of TonB-dependent transporters. We hypothesize that novel endo-fucanases and TonB-dependent transporters are present among several highly expressed PUL genes with secreted products without predicted functions. Moreover, we found evidence for a novel yet unexplored phosphorylative L-fucose degradation pathway that does not involve bacterial microcompartements.

6 Supplementary material

Supplementary Material S1 and Tables S1-S3 have been deposited at 4TU.ResearchData under digital object identifier (DOI) 10.4121/13317587 and can be accessed with the following link: https://figshare.com/s/857f09f1d10726a44f13.

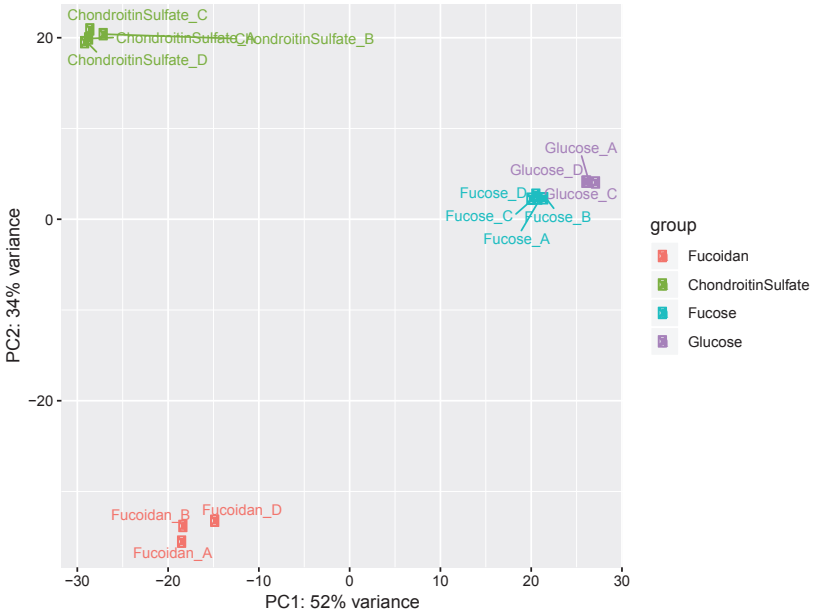


Figure S1. Principal component analysis (PCA) plot constructed from normalized expression values of individual samples.

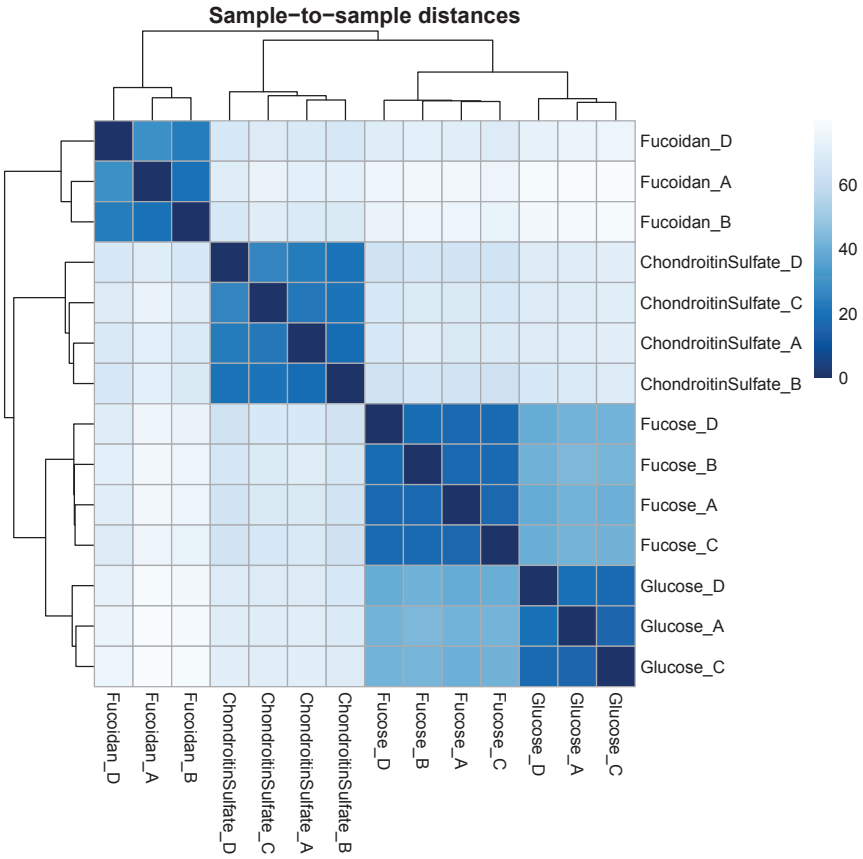


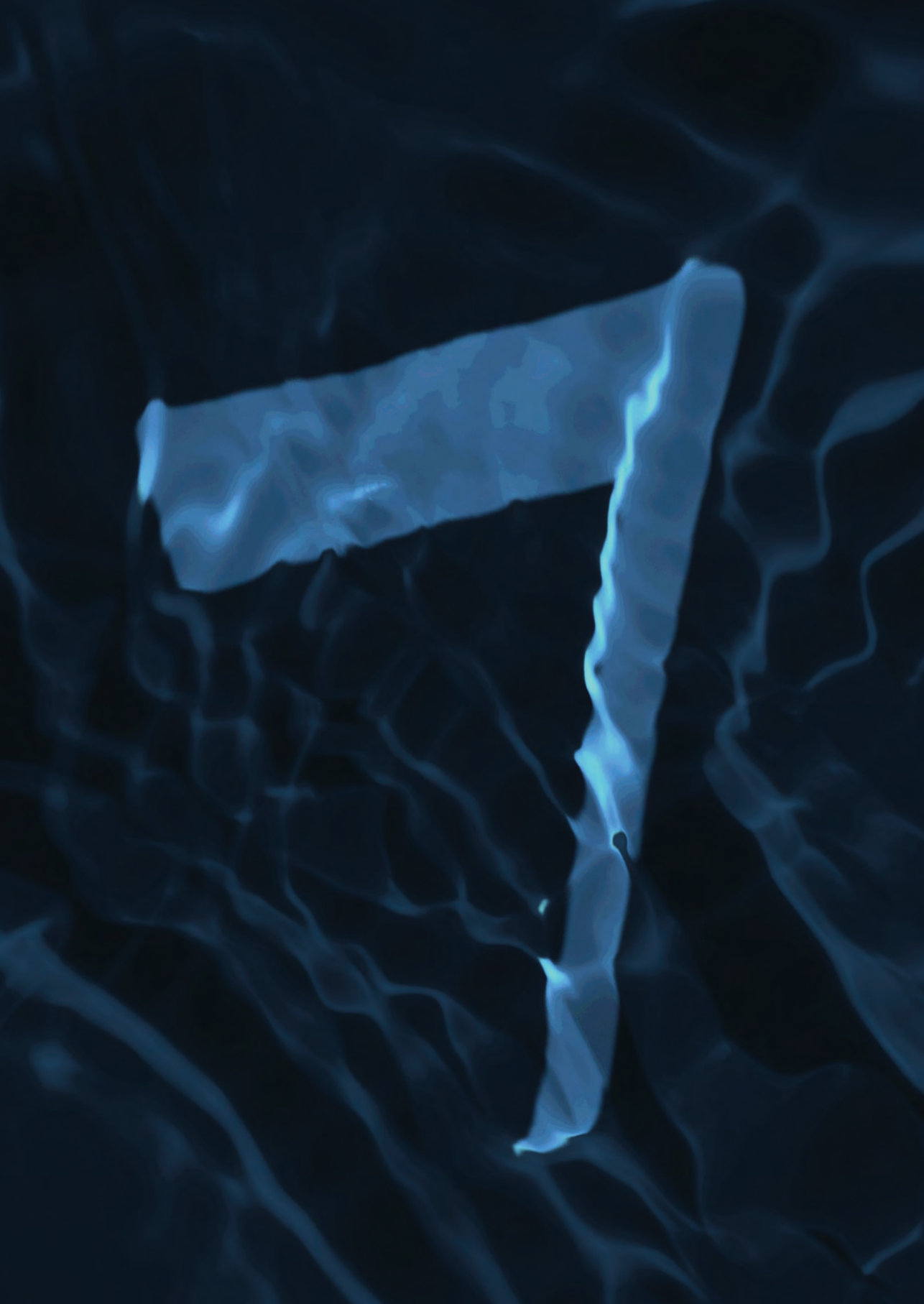
Figure S2. Euclidian distance matrix constructed from normalized expression values of individual samples.

Supplementary Material S1. Results and discussion on expression of the central carbon metabolism of *Pontiella desulfatans* F1^T.

Table S1. Annotation and expression of a) sulfatase genes, b) CAZyme genes, c) substantially upregulated genes, d) polysaccharide utilization loci genes, and e) genes encoding TonB-ExbBD, TonB-dependent transporters and other beta-barrel porins.

Table S2. Genes with homology to canonical L-fucose degradation genes.

Table S3. Annotation and expression of genes involved in a) canonical L-fucose degradation pathways, b) the putative novel L-fucose degradation pathway, c) D-glucuronate degradation, d) N-acetylgalactosamine (GalNAc), e) glycolysis and/or gluconeogenesis, f) production of fermentation products, g) the tricarboxylic acid (TCA) cycle.



CHAPTER 7

General discussion

The aim of this PhD thesis was to advance our knowledge on microorganisms involved in the marine sulfur cycle, specifically of the Black Sea. This was done through a combination of classical anaerobic cultivation and modern DNA- and RNA-based omics methods. Much of this work was explorative and descriptive, starting with a broad metagenomics-based exploration of the microbial community associated with the Black Sea sulfur cycle in the water column, continuing with enrichment, isolation and characterization of isolates from sediment, and finalizing with a more hypothesis-driven transcriptomics analysis of the genetic expression of *Pontiella desulfatans* F1^T during growth on fucoidan.

1 A (meta)genomic view on diversity and phylogeny

Genomics has revolutionized our view on microbiological diversity and phylogeny. Thanks to advances in sequencing technology and bioinformatic tools during the last decade, obtaining draft genomes from pure cultures has become affordable and straightforward. Genomes have substantial scientific value. In recognition of these facts, the International Committee on Systematics of Prokaryotes requires since 2018 that new species descriptions are accompanied by a publicly available genome sequence. The technological advances have also greatly facilitated obtaining metagenome-assembled genomes (MAGs) of uncultivated microorganisms. In **chapter 2**, we used genome-resolved metagenomics to investigate the diversity and taxonomic identity of sulfur-cycling microorganisms in the Black Sea and other dysoxic marine waters. Metagenomics is more reliable than PCR-based techniques as they do not suffer from primer bias, and thus provides a more comprehensive view on microbial diversity (Hug et al., 2016). Metagenomics can also be used for quantification, with the caveats of proper normalization and DNA extraction bias (Nayfach and Pollard, 2016).

1.1 Uncultivated taxonomy

Phylogenetic analyses of MAGs has shown that a large proportion of microbes has not been cultured yet. Despite increasing cultivation efforts (Thrash, 2019), isolation and description of novel bacterial species continues at a more or less constant rate (600-800 y^{-1}; Overmann et al., 2017). Furthermore, cultivation is biased, as isolates are predominantly from four well-known bacterial phyla (*Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*; Overmann et al., 2017). Publicly available MAGs are

nowadays generated at much higher numbers, sometimes with thousands per study (Anantharaman et al., 2016; Parks et al., 2017; Pasolli et al., 2019; Almeida et al., 2021). MAGs are less biased towards specific phyla. They currently contain genotypic and phylogenetic information for thousands of uncultivated, undescribed species across many phyla (Parks et al., 2020).

One of the biggest current challenges in environmental microbiology is how to systematically name all this newfound microbial diversity based on genomes rather than pure cultures. This has been extensively debated in literature (Konstantinidis et al., 2017; 2018; Oren and Garrity, 2018; Overmann et al., 2019; Rossello-Mora et al., 2020). Currently, cultivated representatives deposited in culture collections are the cornerstone (i.e. type material) of taxonomy, as dictated by the International Code of Nomenclature of Prokaryotes (Parker et al., 2019). There are no guidelines for uncultivated taxa, which has resulted in unregulated and confusing naming practices. As example, the SAR406 clade is synonymous with Marine Group A, candidate phylum *Marinimicrobia* (Rinke et al., 2013), and recently also *Marinisomatota*[13]. As described in **chapter 4**, the extent of the *Kiritimatiellaeota* R76-B128 clade was also unclear. The existing '*Candidatus*' status for uncultivated taxa requires phenotypic data (Parker et al., 2019), has until recently lacked any nomenclatural review (Oren et al., 2020), and does thus not accommodate the current need. In practice, '*Candidatus*' status is useful for naturally enriched microbes such as symbionts, or for cultivable microbes that can be enriched but not isolated such as anammox bacteria, but not for uncultivated taxa in a more strict sense.

An official proposal to modify the Code to allow taxa based on genomes (Whitman, 2016) was rejected due to various concerns, including possible ramifications for clinical microbiology (Sutcliffe et al., 2020). Therefore, the creation of a new 'Uncultivated Code' has been initiated, which can count on broad support (Murray et al., 2020). A comprehensive genome-based standardized taxonomic framework called the Genome Taxonomy Database (GTDB) has already been developed (Parks et al., 2018; Chaumeil et al., 2019; Parks et al., 2020). Since the GTDB has quickly become a standard in environmental microbiology studies involving MAGs, I expect this resource and its methodology will form the groundwork for future uncultivated taxonomy. This will include the replacement of rigid, universal identity thresholds by taxon-specific thresholds based on normalized evolutionary divergence. In **chapter 2**, we decided to propose uncultivated taxa with genomes as type material based on preliminary guidelines (Konstantinidis et al., 2017; Chuvochina et al., 2019) in lieu of a new Code.

13 https://gtdb.ecogenomic.org/

1.2 Reclassifications

Genomics has also offered a better insight into phylogeny of cultivated microorganisms than previously possible through phenotype. This has led to numerous reclassifications. Reclassifications are necessary to straighten out the taxonomic framework, and to make sure that taxa of equal rank contain similar levels of phylogenetic diversity. Although they are essential, reclassifications can make taxonomy confusing. A recent PhD thesis proposition from our laboratory aptly referred to taxonomy as "*a necessary evil*" (Koendjbiharie, 2020). Overextended genera such as *Clostridium* (Collins et al., 1994), *Desulfotomaculum* (Watanabe et al., 2018) or *Desulfovibrio* (Waite et al., 2020) have been split into several new genera. The genus *Desulfopila* may also be split in the future, as *Desulfopila inferna* and '*Candidatus* Desulfopila corrodens' share only 94.1-94.2% 16S rRNA gene identity with type species *Desulfopila aestuarii* (**chapter 3**), less than the 94.5% threshold (Yarza et al., 2014). The genome of *Dp. inferna* should be sequenced for better phylogenetic insight. Moving on the phylum level, the long-standing phylum *Proteobacteria* has been revealed to be polyphyletic (Yarza et al., 2014; Hug et al., 2016). This has lead to the reclassification of its classes *Epsilonproteobacteria* and *Deltaproteobacteria* into several new phyla, including *Campylobacterota* and *Desulfobacterota* (Waite et al., 2017; 2018; Waite et al., 2020). Due to the recent nature of the *Deltaproteobacteria* taxonomic overhaul, this PhD thesis still relies on the now-obsolete *Deltaproteobacteria* taxonomy, unless explicitly mentioned otherwise.

1.3 *Kiritimatiellaeota*

Despite improved phylogenetic tools, guidelines and peer-review, taxonomy is still subject to errors and disagreements just as other fields of biology. From the phylogenetic analysis of the lineages within the *Kiritimatiellales* order in **chapter 4** and **chapter 5**, we concluded the MSBL3 and R76-B128 clades to represent novel families, and named the latter *Pontiellaceae*. However, a more recent publication concluded that these two clades should have the rank of orders rather than families, and together form the novel class *Tichowtungiia* within the phylum *Kiritimatiellaeota* (Mu et al., 2020). These conclusions are thus in conflict, yet they were made based on very similar results. Both cases present agreeing data from similar 16S rRNA gene identity analyses, and both continue with a comparison to benchmark identity values from (Yarza et al., 2014), as was previously also done for the proposal of the phylum *Kiritimatiellaeota* (Spring et al., 2016). However, Mu et al. (2020) erroneously interpreted intra-clade benchmark identity values as threshold identity values for pairwise comparisons, causing inflated conclusions. Unfortunately, the authors did not compare their conclusions to those drawn in **chapter 4 and 5**. Future taxonomic publications will have to resolve this unfortunate issue.

2 Metabolism of inorganic-sulfur-cycling microorganisms

Insight into the pathways mediating dissimilatory reduction (Dsr) and sulfur oxidation (rDsr, Sox, Hdr-like) has matured in the last decade (e.g. Götz et al., 2019; Löffler et al., 2020). However, for other relevant metabolic capabilities of marine sulfur-cycling microbes, such as disproportionation of S^0, the pathways are not so well understood.

2.1 Biochemical blind spots

As discussed in **chapter 2 and 3**, disproportionation of S^0 probably represents a significant flux in the sulfur cycle of dysoxic marine waters and sediments respectively. Yet, few cultured microorganisms are known to grow by disproportionation of S^0, and the metabolic pathway(s) for this type of energy metabolism remain unresolved. The lack of marker genes makes it difficult to evaluate the diversity of S^0-disproportionating marine microbes with for instance omics techniques. However, novel S^0-disproportionating bacteria are discovered through cultivation at an increasing rate, with cable bacteria (Müller et al., 2020) and a *Nitrospirae* sp. (Umezawa et al., 2020) as most recent examples. Comparative genomics and expression studies have yielded different candidate pathways (Thorup et al., 2017; Florentino et al., 2019). Testing these requires genetic or biochemical experiments, for instance involving knockout mutants of S^0-disproportionating bacteria, or heterologous expression in the genetically accessible model sulfate-reducing bacterium *Desulfovibrio vulgaris*.

The acetyl-CoA (Wood-Ljungdahl) pathway is commonly thought to be reversible in sulfate-reducing bacteria (SRB), allowing both biosynthesis of acetyl-CoA from CO_2 as well as degradation of acetyl-CoA into CO_2. However, as discussed in **chapter 3**, some SRB such as *Desulfosporosinus orientis, Dp. inferna* and probably also *Desulfopila canfieldii* are able to use the reductive but not the oxidative acetyl-CoA pathway. The acetyl-CoA pathway is thus not intrinsically reversible in SRB. This may be explained by small modifications in key enzymes such as CO dehydrogenase between the two directions, supported by a study on marine model SRB *Desulfobacterium autotrophicum* (Schauder et al., 1988). It could also be caused by an inflexible reductive redox level of the electron carriers in the cell, as the reactions acetyl-CoA pathway generally operate close to thermodynamic equilibrium (Schuchmann and Müller, 2014). *Ds. orientis*, *Dp. inferna* and *Db. autotrophicum* are suitable model organisms for testing these hypotheses, as they can grow lithoautotrophically in mineral medium, and CO dehydrogenase activity has been previously demonstrated.

2.2 Omics methodology

To answer the question "what do the microbes do?", the use of metagenomics as stand-alone approach has considerable limitations and caveats. Metagenomics-based predictions of microbial metabolism are hypothetical, and limited by our – still incomplete – fundamental understanding of the metabolisms of cultivated microorganisms. Moreover, MAGs are commonly a composite of multiple strains of a single species. MAGs furthermore vary in terms of completeness and contamination, which may not be accurately estimated by standard quality analysis tools (Meziti et al., 2021).

In **chapter 2** we used metagenomics to identify and name a considerable number of microbes, of which the involvement in the Black Sea sulfur cycle ranged from possible to highly probable. We predicted some aspects of their metabolism, based on 1) the detection of functional marker genes and 2) an estimation of their relative abundance throughout different depths and conditions in the water column, following a normalization across samples using single-copy genes. However, these metabolic predictions may have been affected by the quality (completeness and contamination) of our MAGs. Firstly, the MetaBAT tool used for binning MAGs compares unfavorably to newer tools (Kang et al., 2019). Also, although MAGs were screened and filtered based on their quality with the standard tool CheckM, they were not manually cleaned as recommended by (Meziti et al., 2021) and done for other Black Sea MAGs from the same dataset (Villanueva et al., 2021). Future in-depth omics investigations into a subset of the sulfur-cycling bacteria from **chapter 2** should first test our findings with newer methodology and manual curation of MAGs.

Metatranscriptomics and metaproteomics could have been used to further constrain metabolic predictions by determining the expression levels of functional genes, but have considerable methodological limitations. RNA abundance is easily biased during sampling of deeper waters or sediments (Feike et al., 2011; Torres-Beltrán et al., 2019), metaproteomics often has limited analytical depth (Hawley et al., 2014), and both are quite costly. Still, if properly applied these techniques could provide valuable insight into the metabolic potential and flexibility of the sulfur-cycling bacteria from **chapter 2**. Such a genome-resolved approach is particularly needed to study nitrogen fixation in dysoxic marine waters and sediment (Christiansen and Loescher, 2019).

3 Cultivation of marine sulfur-cycling microorganisms

Compared to omics, cultivation of axenic cultures and incubation of microcosms more reliable and unequivocal evidence of microbial physiology and morphology. However, when combined in a single study, cultivation or incubation and omics yield far more scientific insight into physiology than they independently would, regardless of whether the focus lies on the role of microbes *in situ* (Callbeck et al., 2019; Suominen et al., 2019) or on investigating metabolism *in vitro* (Spring et al., 2016; Shah et al., 2019). Illustrating this point, **Chapters 3, 4, 5 and 6** combined classical anaerobic cultivation methods with modern omics methodology to yield insight into the genetic background of the observed physiological properties, and helps formulating interesting research question and hypotheses for future studies.

3.1 Traditional versus novel methods

Although microbiology is less dependent on cultivation nowadays than it was in the 20th century, having a microbe in culture still holds the key to comprehensive physiological investigations necessary to discover novel properties. As discussed, few of the total known microbial diversity has been brought into culture. A recent study estimated that on average only 13-14% of the cells in marine sediment or seawater belonged to cultivated genera or species (Lloyd et al., 2018). Although progress in cultivation is gradual, some milestone anaerobes have been cultivated from marine sediments in recent years. '*Candidatus* Prometheoarchaeum syntrophicum' is the first reported cultivated archaeon of the Asgard superphylum from which eukaryotes emerged (Imachi et al., 2020). Further, *Atribacter laminatus* is the first described cultivated member of the phylum *Atribacteria* (Katayama et al., 2020), a ubiquitous and abundant lineage in anoxic marine sediments (Nobu et al., 2016). Both these marine anaerobes showed novel cell structures which were unexpected and could not be predicted from genomic information. Similarly, the discovery of sulfated glycosaminoglycan-like exopolymers produced by *Pontiella* species in **chapter 4** was unexpected and cultivation-based.

Traditional cultivation methods still hold much potential for investigating uncultivated or poorly cultivated lineages. '*Ca.* P. syntrophicum', *A. laminatus* and the *Pontiella* species were cultivated with traditional methods such as enrichment culture, dilution-to-extinction and solid agar media. Traditional methods also enabled a recent major cultivation effort for little-cultivated *Planctomycetes* phylum (Wiegand et al., 2020). However, often considerable patience is required. It took over one year to isolate the relatively fast-growing *Pontiella* bacteria, but it took twelve years to obtain an axenic *'Ca.* P. syntrophicum' co-culture (Imachi et al., 2020). Appropriate scale of the cultivation

experiment should also be considered, as some microbes may have low probability of being isolated despite use of the appropriate medium and conditions (Thrash, 2019; Henson et al., 2020). Possibly, innovative cultivation methods such as 'reverse genomics' or microfluidics will overcome these time and scale limitations. Cultivation may become centralized in high-throughput specialized laboratories offering these services to other labs (Carini, 2019). I will not elaborate on such methods here, as several comprehensive reviews on this topic were published recently (Gutleben et al., 2018; Lewis et al., 2020; Thrash, 2021).

3.2 Targets for cultivation

Various uncultivated groups of bacteria have been identified as potentially important to the marine sulfur cycle (Table 1), in previous research as well as in this thesis. Some archaea found in marine sediments may also have the capacity for sulfur oxidation or reduction (Baker et al., 2021). The cultivation of these uncultivated microorganisms in axenic culture may be possible using currently available methods, for which I will offer some advice here. In the case of (strict) anaerobes, maintaining anoxic conditions in the inoculum is crucial. Sediment does not oxidize quickly during sampling and can be stored for years as anoxic slurry. However, column water is quick to oxidize both during sampling and storage, and the microbes therein may be reliant on a steady influx of energy sources or electron acceptors. Therefore, incubation or inoculation should be timely, and is best performed on-board during research cruises (Callbeck et al., 2019; Suominen et al., 2019).

The enrichment method selects for rapid growth. As such, it is necessary to apply conditions that prevent opportunistic, copiotrophic, frequently isolated microbes. This can be achieved by mimicking *in situ* conditions, for instance using batch cultures with natural filter-sterilized seawater as medium incubated at relatively low temperatures (Grote et al., 2012; Callbeck et al., 2019), or in a bioreactor simulating steady inflow of electron donors and acceptors, which may especially be suitable for enrichment of sulfur-cycling *Nitrospirae* (Arshad et al., 2017). Simpler possibilities include using antibiotics (Spring et al., 2016) or selective substrates. In the Black Sea, incubation at low temperatures and the use of polysaccharide substrates enriched novel *Kiritimatiellaeota* spp. from sediment (**chapter 4**) and members of the uncultivated phylum *Cloacimonetes* from euxinic column water (Subhash Yadav, personal communication). Proteinaceous substrates may facilitate future cultivation of potentially sulfur-cycling *Marinimicrobia* (Bertagnolli et al., 2017; Suominen et al., 2019). Similarly, aromatic substrates are likely key substrates for marine *Desulfatiglandales* (Jochum et al., 2018), although this metabolic aspect was not investigated for *Desulfacyla* MAGs in **chapter 2**.

Table 1. Uncultivated microbial lineages with relevance for the marine sulfur cycle. *) *Desulfatiglandales sensu* Waite et al. 2020. Includes [U]*Desulfacyla*. *Desulfatiglandales* spp. have been cultivated, but not from pristine marine sediment.

Group	**Environments (marine)**	**Hypothetical metabolism**	**Hypothetical sulfur metabolism**
[U]*Thiopontia* (BS-GSO2)	euxinic basins	chemolithoautotrophy	sulfur & organosulfur oxidation
Gammaproteobacteria EOSA-II	OMZs	chemolithoautotrophy	sulfur oxidation
Sulfurimonas GD17	euxinic basins	chemolithoautotrophy	sulfur oxidation
'*Ca.* Hyrcanianaceae'	deep sea, OMZs, suboxic zones	chemolithoheterotrophy, methylotrophy	sulfur oxidation
Candidate phylum SAR324	deep sea, OMZs, suboxic zones	mixotrophy	sulfur & organosulfur oxidation
sulfur-oxidizing *Nitrospinae*	OMZs	chemolithoautotrophy	sulfur oxidation
sulfur-cycling *Nitrospirae*	Black Sea suboxic & euxinic zone	unclear	unclear
sulfur-oxidizing *Desulfobacterales*	OMZs	unclear	sulfur oxidation
Desulfobacterales Sva0081	sediments	chemolithotrophy	sulfate reduction
*Desulfatiglandales**	euxinic basins, sediments	organoheterotrophy, degrading aromatics	sulfate reduction
[U]*Desulfatifera*, [U]*Desulfobia*, [U]*Desulfatibia*, [U]*Desulfaltia*	Black Sea suboxic & euxinic zone	organoheterotrophy	sulfate/sulfur reduction, disproportionation
[U]*Desulfolinea*	Black Sea suboxic & euxinic zone	unclear	sulfate/sulfur reduction
Candidate phylum AAMBMS-125-24	euxinic basins, OMZs, sediments	organoheterotrophy	sulfate reduction
'*Ca.* Marinimicrobia'	OMZs, surface sediment	organoheterotrophy, oligotrophy	sulfur oxidation/ reduction
'*Ca.* Aegiribacteria' (Fermentibacteria)	ubiquitous	organoheterotrophy, fermentation	associated with SRB

Isolation is traditionally achieved with solid media, or if not successful, dilution-to-extinction in liquid media. The success of solid media may be increased by trying various gelling agents (Rygaard et al., 2017; Kitzinger et al., 2018) or by reducing peroxides through autoclaving phosphates separately (Tanaka et al., 2014). The latter was applied for isolating novel *Pontiella* spp. (**chapter 4**), but it was not tested whether this was crucial. The dilution-to-extinction method using liquid media often initially yields enrichments rather than axenic cultures, as in **chapter 3**, and usually needs to be repeated for isolation

purposes. Isolation of SUP05 bacterium '*Candidatus* Thioglobus autotrophicus' was achieved through dilution-to-extinction in natural medium supplemented with only thiosulfate (Shah et al., 2017). Such an approach may be successful for isolation of more SUP05 members and other abundant chemolithoautotrophic sulfur-oxidizing groups such as *[U]Thiopontia* (BS-GSO2) or the *Sulfurimonas* GD17 clade from euxinic basins. Natural media may also be effective for isolating uncultivated SRB (Vester and Ingvorsen, 1998).

Some microbes cannot be obtained in axenic culture with current methods. Some are readily enriched but resist isolation (anammox bacteria, sulfur-cycling *Nitrospirae*, *Aegiribacteria*), possibly due to requiring unknown forms of interaction with other microbes (Kaeberlein et al., 2002). Some microbes – specifically some anaerobes in subsurface sediments – are simply not culturable due to extremely low growth rates (Jørgensen and Boetius, 2007). Alternatively to cultivation, short incubation experiments with environmental samples can be another route to insights into the sulfur metabolism of microbes. Unfortunately, powerful methods such as stable isotope probing (SIP) or nanoscale secondary ion mass spectrometry (NanoSIMS) are not applicable as dissimilatory and assimilatory sulfur metabolism are not coupled. However, incubation experiments in which column water samples were spiked with sulfur compounds and/or electron acceptors have yielded promising insights into the *in situ* metabolism of specific sulfur-cycling microbes (Labrenz et al., 2005; Jost et al., 2010).

4 Use and biosynthesis of sulfated polysaccharides by *Pontiella* spp.

Three chapters of this thesis were devoted to describing novel *Pontiella* spp. and investigating how they anaerobically degrade sulfated polysaccharides, specifically fucoidan. At the start of this thesis project, a handful of aerobic marine bacteria were known to grow on fucoidan (Furukawa et al., 1992; Sakai et al., 2003; Ohshiro et al., 2012; Silchenko et al., 2013; Barbeyron et al., 2016b). Some enzymes were known to act on fucoidan, all glycoside hydrolases (Berteau et al., 2002; Katayama et al., 2004; Colin et al., 2006). Several additional enzymes were characterized in the last five years, including bacterial glycoside hydrolases (Silchenko et al., 2017; Nagao et al., 2018; Shen et al., 2020), sulfatases (Silchenko et al., 2018) and deacetylases (Nagao et al., 2017). However, it remained unclear how and where in a Gram-negative bacterial cell these enzymes are combined to form a fucoidan depolymerization pathway. By now, some

insight into these questions has been obtained by studying fucoidan degradation and concomitant gene expression in the aerobic *'Lentimonas'* strain CC4 (Sichert et al., 2020) and the anaerobic *Pontiella desulfatans* F1^T (**chapter 4, 5 and 6**). These studies have examined the role of known families of fucoidan-depolymerizing enzymes *in vivo*. However, they have also made visible how much is still not known; many seemingly redundant genes as well as altogether unknown genes were expressed, the proposed mechanisms for fucoidan depolymerization are hypothetical and not very specific, and subsequent L-fucose degradation in *P. desulfatans* F1^T probably proceeds through a novel unresolved pathway.

4.1 Fucoidan degradation pathway

There are many experimental possibilities for further elucidation of the mechanism behind *Fucus vesiculosus* fucoidan depolymerization by *P. desulfatans* F1^T. Better quantitative analysis of fucoidan and its degradation products during *P. desulfatans* F1^T growth could be achieved by using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) in addition to the other types of chromatography used. Whether the degradation mechanism is selfish, or at least partly selfish, could be tested using incubation with fluorescently labelled fucoidan and subsequent inspection by super-resolution fluorescence microscopy (Reintjes et al., 2017; Reintjes et al., 2019). This powerful technique also remains to be applied for fucoidan depolymerization by '*Lentimonas*' strain CC4. To unequivocally reveal how fucoidan degradation proceeds in either organism, the localization and role of individual proteins needs to be addressed. A comprehensive approach to determine protein localization would be proteomics analysis of different subcellular fractions (Wöhlbrand et al., 2013). Determining the function of proteins generally requires *in vitro* biochemical characterization (e.g. Reisky et al., 2019; Shen et al., 2020). However, it would be worthwhile to investigate the genetic accessibility of *P. desulfatans* F1^T as well as '*Lentimonas*' strain CC4, as experiments with knockout strains could shed light on the *in vivo* function. Knockout mutants of *P. desulfatans* F1^T would also be instrumental for studying the novel L-fucose degradation pathway.

In general, whether and how bacteria of the *Planctomycetes-Verrucomicrobia-Chlamydiae* (PVC) superphylum take up oligosaccharides into their periplasm is poorly investigated. *Planctomycetes* bacteria have relatively few genes encoding potential oligosaccharide uptake proteins such as porins and TonB proteins (Speth et al., 2012; Paparoditis et al., 2014; Andrei et al., 2019). However, some *Planctomycetes* are capable of uptake of the intact polysaccharide dextran in an enlarged periplasmic space, possibly through uncharacterized crateriform structures in the outer membrane (Boedeker et al., 2017), thus presenting an alternative mechanism of selfish polysaccharide degradation. In contrast, *Verrucomicrobia* members generally encode a TonB protein and many porins

(Speth et al., 2012), and some are known to take up oligosaccharides. *Akkermansia muciniphila* is thought to take up oligosaccharides originating from mucin degradation through a carbohydrate-specific porin (Ottman et al., 2017). Diverse marine *Verrucomicrobia* members bind or take up fluorescently-labelled xylan and laminarin (Martinez-Garcia et al., 2012), suggesting a selfish degradation mechanism. '*Lentimonas*' strain CC4 was not reported to encode or express potential fucoidan TonB-dependent transporters (TBDTs), consistent with external depolymerization (Sichert et al., 2020). However, it did encode a putative carrageenan TBDT, and abundantly expressed a porin with unknown function, which was presumed to be involved in nitrogen uptake (Sichert et al., 2020). Sichert et al. detected extracellular L-fucose during fucoidan degradation, but no oligosaccharides, and interpreted this to support complete extracellular depolymerization by exo-acting enzymes. However, I argue this could alternatively be explained by a selfish mechanism involving high-affinity active uptake of oligosaccharides, followed by complete depolymerization in the periplasm into L-fucose, which could then freely diffuse from periplasm to extracellular space through general diffusion channels. These hypotheses should be addressed experimentally through the aforementioned methods.

4.2 Brown seaweed and fucoidan biorefinery

Brown seaweeds such as *Fucus vesiculosus* have the potential of becoming a valuable resource for a sustainable, circular and biobased economy. They currently represent 30-40% of the global seaweed production at over 10 million tonnes wet weight per year (Ferdouse et al., 2018), and are mainly used as source of the hydrocolloid alginate and as animal feed. However, brown seaweed biomass has the potential to be produced at a manifold greater scale through farming, and to be processed into various biobased products with many more applications through biorefinery (Hreggviðsson et al., 2020). The biomass is rich in protein and polysaccharides, which both have different uses. However, the cell walls pose a challenge for the separation of protein and polysaccharide under mild conditions and for the further processing of polysaccharides, largely due to the structural complexity and heterogeneity of the cell wall polysaccharides such as fucoidan (Hreggviðsson et al., 2020; Pliego-Cortés et al., 2020). Enzyme treatment is an advantageous approach, yet suitable enzymes are not on the market (Terme et al., 2020). Currently commercially available enzymes are terrestrial in origin, and therefore efficient in degrading terrestrial but not marine polysaccharides. Marine saccharolytic microorganisms – such as *P. desulfatans* F1^{T} – are a valuable source of potentially suitable enzymes (Lange et al., 2020). To achieve sufficient depolymerization of fucoidan to enable further processing, application of enzyme mixes or active bacterial cells may be an interesting approach, as fucoidan degradation seems to require a large number of excreted CAZymes and sulfatases (**chapter 6**; Sichert et al., 2020). However, using single enzymes yields a more controllable process, and may also have sufficient activity

towards fucoidan. Promising examples are endo-fucanase from *Formosa algae* KMM 3553 (Silchenko et al., 2013; Silchenko et al., 2017) and fucoidan deacetylase from *Luteolibacter algae* H18 (Nagao et al., 2017).

The enzymes produced by *P. desulfatans* F1^T and *'Lentimonas'* strain CC4 may have additional uses in science and medicine. Fucoidan has anticoagulant, antithrombotic and antitumor properties with potential for various clinical uses (Fitton et al., 2015; Hsu and Hwang, 2019). These bioactive properties are dependent on the structure of fucoidan, including the degree and position of sulfation (Ale et al., 2011). Fucoidan oligosaccharides are most desirable as these result in higher absorption efficiency to human target cells (Chen et al., 2017a) and may also have use as prebiotic (Sardari and Nordberg Karlsson, 2018). These oligosaccharides could be best be generated through an mild enzymatic rather than a harsh chemical treatment. Therefore, there is a need for a much larger suite of well-characterized fucoidanases and fucoidan sulfatases. Furthermore, these enzymes could be used as part of a method to detect fucoidan in the environment. Distinguishing and quantifying polysaccharides such as fucoidan in environmental samples is challenging through analytical chemistry (Arnosti et al., 2021). Enzyme-based carbohydrate microarrays could circumvent this issue, as already demonstrated for the polysaccharide laminarin in marine water samples with the use of laminarin-specific glycoside hydrolases (Becker et al., 2017). An analogous microarray could be made for fucoidan to elucidate its importance in the marine carbon cycle. Experiments with monoclonal antibodies have already demonstrated the production of recalcitrant fucoidan-like carbohydrates by diatom microalgae in the North Sea (Sichert, 2020), suggesting the prevalence and biogeochemical importance of fucoidan in the marine environment is larger than currently appreciated.

4.3 Production and application of sulfated glycosaminoglycans

The exopolymers and associated biosynthetic enzymes produced by the *Pontiella* spp. may also have an application. Like fucoidan, sulfated glycosaminoglycans have a variety of medically relevant properties, such as anticoagulation of blood, inhibition of tumor growth and metastasis, and control of inflammatory processes (Köwitsch et al., 2018). The main bottleneck in their application lies in their predominantly animal origin, which causes compositional variation between batches, involves laborious downstream purification, and leads to risk of contamination with infectious agents or structurally similar compounds, which has caused allergic reactions and even deaths of patients (Guerrini et al., 2008; Liu et al., 2009). These limitations have prompted the search for alternative production approaches (Badri et al., 2018). Recently, the successful *in vivo* sulfation of glycosaminoglycans by a microbial production platform has been reported for the first time (Badri et al., 2021). Badri and colleagues engineered *Escherichia coli*

to produce chondroitin sulfate by accumulating the precursor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) and expressing a rationally designed human chondroitin sulfotransferase. However, effective sulfation still required inhibition of chondroitin secretion and a temperature drop from 37 °C to 16-20 °C, which is problematic for down-stream processing and scale-up. Addressing these issues requires a deeper understanding of sulfated polysaccharide biosynthesis and secretion in marine bacteria (Delbarre-Ladrat et al., 2014), with a potential key role for the biosynthesis of sulfated glycosaminoglycan-like exopolymers by *Pontiella sulfatireligans* F21^T and the associated putative glycosaminoglycan sulfotransferases (**chapter 5**).

5 Conclusion

The research presented here has yielded a deeper understanding of the diversity and potential metabolism of putative sulfur-cycling microorganisms in dysoxic marine waters of the Black Sea and other marine systems. The recent developments in sequencing technology have enabled us to view more of the astonishing level of microbial diversity in the environment, including the marine one. Genomic data may even allow us to systematically describe and communicate this newfound diversity, once a proper taxonomic framework is agreed upon. In this thesis, a little head start was made in this direction. However, discovering microbial physiological diversity requires more than omics data, as microbial physiology consists of complex emergent properties of which only part can be deduced from omics data. Cultivation remains the most powerful, yet is often biased or time- and resource-intensive. As listed in this discussion, many environmentally important lineages of sulfur-cycling marine bacteria remain to be cultivated. I hope the anaerobic sulfur cultivation explorations, achievements and lessons in this thesis inform and inspire future experiments, and will reduce time and resource requirements.

Our cultivation-based explorations have yielded several novel sulfur-cycling marine anaerobes with interesting metabolic and physiological properties. The inexplicable inability of *Desulfopila canfieldii* LS5B^T to use the acetyl-CoA pathway oxidatively warrants further research, although other SRB may be better study platforms. The *Pontiella* spp. showed more unique properties, such as anaerobic fucoidan degradation, tremendous numbers of CAZyme and sulfatase genes, production of sulfated glycosaminoglycan-like exopolymers, and a putative novel L-fucose degradation pathway. These exploration- and characterization-based findings are microbiologically valuable in a fundamental and environmental sense. They may also form a seedling for future applications in medicine and in using brown seaweed biomass for a sustainable biobased economy.

Appendices

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Summary

Marine microorganisms are drivers of elemental cycles, and thus have global biogeochemical importance. Many marine environments have dysoxic (<1 μM O_2) or anoxic (<10 nM O_2) conditions. Here, sulfur-cycling microorganisms such as sulfur-oxidizing and sulfate-reducing bacteria (SOB, SRB) play key roles. The largely anoxic Black Sea is a model ecosystem for studying sulfur-cycling microbes. Nonetheless, there is only rudimentary insight into the diversity and metabolism of SRB, sulfur-reducing/disproportionating microorganisms, or microorganisms that degrade the sulfated polysaccharides ubiquitously available in marine environments. Therefore, we set out to advance our understanding of these sulfur-cycling microbes in the Black Sea.

We started off with a critical review of the literature on sulfur-cycling microbes in dysoxic marine waters, combined with genome-resolved metagenomics to obtain an improved view on the diversity of putative SOB and SRB in the Black Sea water column, both described in **chapter 2**. We chose to taxonomically name several of these uncultured putative SOB and SRB based on phylogenetic, metabolic and habitat profiling analyses, providing taxonomic 'handles' with specific criteria for future studies.

We sampled Black Sea sediment from 2,100 meters water depth, at the same site where metagenomics samples were taken, as inoculum for anaerobic cultivation. In **chapter 3** we report enrichment of diverse bacteria in sulfate- and sulfur-reducing cultures, including *Aegiribacteria* spp. with unknown metabolism and novel sulfur-reducing *Desulfuromonadales* Sva1033 bacteria. Furthermore, we isolated and characterized *Desulfopila canfieldii* sp. nov., which showed to be capable of dissimilatory sulfate, sulfite, thiosulfate and manganese oxide reduction. *Desulfopila* spp. encode the acetyl-CoA pathway, which is presumed bidirectional. However, they appear to use this pathway exclusively in the reductive direction from CO_2 to acetyl-CoA, and not in the oxidative direction, as they are incomplete oxidizers. This is a fundamentally relevant avenue for future research on SRB.

We also enriched and isolated novel anaerobes that degrade sulfated polysaccharides, as described in **chapter 4**. We used commercially available *Fucus vesiculosus* fucoidan as substrate, which is a structurally complex sulfated polysaccharide with an α-1,3/α-1,4-linked L-fucose backbone. **Chapter 5** presents a genome-guided phenotypic and phylogenetic characterization of the isolates belonging to the *Kiritimatiellaeota* phylum, which we proposed to name *Pontiella desulfatans* $F1^T$ gen. nov., sp. nov. and *Pontiella sulfatireligans* $F21^T$ sp. nov. These bacteria grew only fermentatively on monosaccharides or the sulfated polysaccharides fucoidan, chondroitin sulfate, and iota-carrageenan. Further, they produced N-sulfated glycosaminoglycan-like exopolysaccharides during stationary phase, which are known to be produced by eukaryotes but not bacteria.

A putative biosynthesis gene cluster and sulfotransferase genes were identified in *P. sulfatireligans* F21^T.

The *Pontiella* spp. encoded unprecedented numbers of sulfatase genes (521 and 480, respectively) and glycoside hydrolase genes (422 and 388, respectively). We observed an increase in free sulfate during growth on iota-carrageenan in **chapter 4** and on fucoidan in **chapter 6**, demonstrating sulfatase-mediated desulfation. In **chapter 6** we looked deeper into the enzymatic mechanism of fucoidan and L-fucose degradation by *P. desulfatans* F1^T by analysis of gene expression with transcriptomics, and prediction of the subcellular localization of proteins. This revealed high numbers of sulfatase and exo-acting glycoside hydrolase genes were induced by fucoidan. These were organized in polysaccharide utilization loci (PULs), indicating a so-called 'selfish' mechanism of fucoidan depolymerization involving uptake of oligosaccharides, rather than complete extracellular depolymerization and subsequent uptake of monomers. Furthermore, L-fucose degradation was probably catalyzed by a novel pathway encoded by an 11-gene operon, which was strongly induced by L-fucose and fucoidan, and showed widespread synteny among fermentative anaerobes.

The field of environmental microbiology has been transformed owing to the recent developments in sequencing technology and bioinformatic tools, or 'omics'. In **chapter 7** I discuss the presented research in the context of these developments. Although omics has robustly revolutionized our view on microbial diversity, cultivation remains the workhorse for discovery and elucidation of microbial (sulfur) metabolism with omics as powerful complement. This thesis has showcased the effectiveness of combining the two methods. Our findings may even have relevance for biotechnological applications of sulfated polysaccharides. Efforts to cultivate the many uncultured sulfur-cycling marine microorganisms should thus be prioritized.

Samenvatting

Mariene micro-organismen zijn de motor van elementaire kringlopen en zijn daarom van mondiaal biogeochemisch belang. Veel mariene omgevingen hebben dysoxische (<1 μM O_2) of anoxische (<10 nM O_2) omstandigheden. Daar spelen micro-organismen met een rol in de zwavelkringloop zoals zwaveloxiderende en sulfaatreducerende bacteriën (ZOB, SRB) een sleutelrol. De grotendeels anoxische Zwarte Zee is een modelecosysteem voor het bestuderen van zulke zwavelkringloop-microben. Desalniettemin is er slechts een rudimentair inzicht in de diversiteit en het metabolisme van SRB, zwavelreducerende/-disproportionerende micro-organismen of micro-organismen die de gesulfateerde polysacchariden afbreken die alom aanwezig zijn in mariene omgevingen. Daarom hebben wij ons erop gericht de kennis van de zwavelkringloop-microben in de Zwarte Zee te vergroten.

We zijn begonnen met een kritische bespreking van de literatuur over zwavelkringloop-microben in dysoxische zeewateren, gecombineerd met 'metagenomics' op genoomniveau om een beter zicht te krijgen op de diversiteit van vermeende ZOB en SRB in de waterkolom van de Zwarte Zee, zoals beschreven in **hoofdstuk 2**. We hebben ervoor gekozen om verschillende van deze niet-gekweekte vermeende ZOB en SRB een taxonomische rang en naam te geven op basis van fylogenetische, metabole en habitatprofileringanalyses, die taxonomische 'handvatten' bieden met specifieke criteria voor toekomstige studies.

We hebben sediment bemonsterd uit de Zwarte Zee op een waterdiepte van 2.100 meter, op dezelfde coördinaten waar metagenomics-monsters werden genomen, als inoculum voor anaerobe kweek. In **hoofdstuk 3** rapporteren we de verrijking van diverse bacteriën in sulfaat- en zwavelreducerende culturen, waaronder *Aegiribacteria* ssp. met onbekend metabolisme en nieuwe zwavelreducerende *Desulfuromonadales* Sva1033 bacteriën. Verder hebben we *Desulfopila canfieldii* sp. nov. geïsoleerd en gekarakteriseerd. Deze bacterie bleek in staat te zijn tot dissimilerende sulfaat-, sulfiet-, thiosulfaat- en mangaanoxide-reductie. *Desulfopila* spp. coderen de acetyl-CoA-route in hun genoom, die wordt verondersteld intrinsiek omkeerbaar te zijn. Ze lijken deze route echter uitsluitend in de reductieve richting van CO_2 naar acetyl-CoA te gebruiken, en niet in de oxidatieve richting, aangezien het onvolledige oxideerders zijn. Dit is een fundamenteel relevant onderwerp voor toekomstig onderzoek naar SRB.

We hebben ook nieuwe anaëroben die gesulfateerde polysacchariden afbreken verrijkt en geïsoleerd, zoals beschreven in **hoofdstuk 4**. Hiervoor gebruikten we commercieel verkrijgbaar *Fucus vesiculosus* fucoidan als substraat, een structureel complexe, gesulfateerde polysaccharide met een ruggengraat van α-1,3/α-1,4-gebonden L-fucose. **Hoofdstuk 5** presenteert een genoom-geleide fenotypische en fylogenetische karakterisering van de isolaten, die behoren tot het *Kiritimatiellaeota* phylum en die

we hebben voorgesteld om *Pontiella desulfatans* F1^T gen. nov., sp. nov. en *Pontiella sulfatireligans* F21^T sp. nov. te noemen. Deze bacteriën groeiden alleen fermentatief op monosacchariden of de gesulfateerde polysacchariden fucoidan, chondroïtinesulfaat en jota-carrageen. Verder produceerden ze N-gesulfateerde glycosaminoglycaanachtige exopolysacchariden tijdens de stationaire fase, waarvan bekend is dat ze worden geproduceerd door eukaryoten maar niet door bacteriën. Een vermeend biosynthese-gencluster en sulfotransferasegenen werden geïdentificeerd in *P. sulfatireligans* F21^T.

De *Pontiella* spp. bezaten ongekende aantallen sulfatase-genen (respectievelijk 521 en 480) en glycoside-hydrolase-genen (respectievelijk 422 en 388). We zagen een toename van vrij sulfaat tijdens de groei op jota-carrageen in **hoofdstuk 4** en op fucoidan in **hoofdstuk 6**, wat desulfatie door sulfatases aantoont. In **hoofdstuk 6** gingen we dieper in op het enzymatische mechanisme van de afbraak van fucoidan en L-fucose door *P. desulfatans* F1^T door middel van analyse van genexpressie met transcriptomics, en voorspelling van de subcellulaire lokalisatie van eiwitten. Dit liet zien dat er hoge aantallen genen voor sulfatases en exo-werkende glycoside hydrolases werden geïnduceerd door fucoidan. Deze waren georganiseerd in polysaccharide-gebruiks-loci (PGL), wat duidt op een zogenaamd 'zelfzuchtig' mechanisme van fucoidan-depolymerisatie met opname van oligosacchariden, in plaats van volledige extracellulaire depolymerisatie en daaropvolgende opname van monomeren. Daarnaast werd de afbraak van L-fucose waarschijnlijk gekatalyseerd door een nieuwe metabole route die wordt gecodeerd door een operon bestaande uit een elftal genen, dat sterk werd geïnduceerd door L-fucose en fucoidan, en dat een wijdverspreide syntenie vertoonde tussen fermentatieve anaëroben.

Het veld van de omgevingsmicrobiologie is getransformeerd als gevolg van de recente ontwikkelingen op het gebied van 'sequencing'-technologie en bio-informatica-programma's, oftewel 'omics'. In **hoofdstuk 7** bespreek ik het gepresenteerde onderzoek in de context van deze ontwikkelingen. Hoewel omics onze kijk op microbiële diversiteit drastisch heeft veranderd, blijft het kweken van microben het werkpaard voor de ontdekking en opheldering van microbiële (zwavel)metabolismen met omics als krachtig complement. Dit proefschrift heeft de effectiviteit van het combineren van beide methoden aangetoond. Onze bevindingen zouden zelfs relevant kunnen blijken voor biotechnologische toepassingen van gesulfateerde polysacchariden. Pogingen om de vele niet-gekweekte mariene zwavelkringloop-microben te kweken, moeten daarom prioriteit krijgen.

Краткое содержание

Морские микроорганизмы играют существенную роль в круговороте различных элементов и поэтому имеют глобальное биогеохимическое значение. Во многих морских средах присутствуют дисоксические (<1 мкМ O_2) или анаэробные (<10 нМ O_2) условия. Здесь ключевую роль играют те микроорганизмы, которые участвуют в цикле серы, такие как сероокисляющие и сульфатредуцирующие бактерии (СОБ, СРБ). Почти что бескислородное Черное море является модельной экосистемой для изучения микроорганизмов обеспечивающих биогеохимический цикл серы. Тем не менее, существует лишь элементарное понимание разнообразия и метаболизма СРБ, то есть микроорганизмов восстанавливающих серу или приводящих к её диспропорционированию, а также микроорганизмов, которые разлагают сульфатированные полисахариды, повсеместно доступные в морской среде. Поэтому мы решили провести исследование, чтобы углубить наше понимание круговорота серы в Черном море.

Мы начали с критического обзора литературы по микробам, обеспечивающим кругооборот серы в дисоксических морских водах, в сочетании с геномно-разрешенным метагеномным исследованьем, чтобы получить улучшенное представление о разнообразии предполагаемых СОБ и СРБ в водной толще Черного моря, как описано в **главе 2**. Мы решили таксономически определить несколько из этих некультивируемых предполагаемых СОБ и СРБ на основе филогенетического-метаболического анализа и анализа профиля среды обитания, предоставив таксономические критерии для будущих исследований.

В качестве инокулята для анаэробного культивирования, мы взяли образцы донных отсадков Черного моря на глубине 2100 метров, на том же участке, где был взят материал для анализа метагенома. В **главе 3** мы описываем обогащение различных бактерий в сульфат- и сероредуцирующих культурах, включая *Aegiribacteria* spp. с неизвестным метаболизмом и новыми сероредуцирующими бактериями *Desulfuromonadales* Sva1033. Кроме того, мы изолировали и охарактеризовали *Desulfopila canfieldii* sp. nov., которые показали способность к диссимиляционному восстановлению сульфата, сульфита, тиосульфата и оксида марганца. *Desulfopila* spp. кодируют путь ацетил-КоА, который считается обратимым. Однако они используют этот путь исключительно в восстановительном направлении от CO_2 до ацетил-КоА, а не в окислительном направлении, поскольку эти бактерии являются неполными окислителями. Это принципиально важное направление для будущих исследований СРБ.

Мы также обогатили и выделили новые анаэробы, которые разлагают сульфатированные полисахариды, как описано в **главе 4**. Мы использовали коммерчески доступный фукоидан из Fucus vesiculosus в качестве субстрата,

который представляет собой структурно сложный сульфатированный полисахарид с α-1,3/α-1,4-связью на основе L-фукозы. В **главе 5** представлена геномная фенотипическая и филогенетическая характеристика изолятов принадлежащих к типу *Kiritimatiellaeota*, которые мы предложили назвать Pontiella desulfatans F1^T gen. nov., sp. nov. и *Pontiella sulfatireligans* F21^T sp. nov. Эти бактерии ферментативно росли только на моносахаридах или сульфатированных полисахаридах на основе фукоидана, хондроитинсульфате и йота-каррагинане. Кроме того, они продуцировали N-сульфатированные гликозаминогликано-подобные экзополисахариды во время стационарной фазы, которые, как известно, вырабатываются эукариотами, но не бактериями. Предполагаемый кластер генов биосинтеза и гены сульфотрансферазы были идентифицированы в *P. sulfatireligans* F21^T.

Pontiella spp. кодирует беспрецедентное количество генов сульфатаз (521 и 480 соответственно) и генов гликозил-гидролаз (422 и 388 соответственно). Мы наблюдали увеличение свободного сульфата во время роста на йота-каррагенане в **главе 4** и на фукоидане в **главе 6**, демонстрируя десульфатацию опосредованную сульфатазой. В **главе 6** мы более подробно рассмотрели ферментативный механизм деградации фукоидана и L-фукозы под действием *P. desulfatans* F1^T путем анализа экспрессии генов с помощью транскриптомики и предсказания субклеточной локализации белков. Это выявило большое количество генов сульфатаз и экзо-действующих гидролаз гликозидов, индуцированных фукоиданом. Они были организованы в локусы утилизации полисахаридов (ЛУП), что указывает на так называемый «эгоистичный» механизм деполимеризации фукоидана, включающий усвоение олигосахаридов, а не полную внеклеточную деполимеризацию и последующее усвоение мономеров. Кроме того, деградация L-фукозы, вероятно, катализируется новым путем, кодируемым опероном из 11 генов, который сильно индуцируется L-фукозой и фукоиданом и демонстрирует широко распространенную синтению среди ферментативных анаэробов.

Область экологической микробиологии была преобразована в результате недавних разработок в области технологии секвенирования и биоинформатических инструментов, или «омик». В **главе 7** я обсуждаю представленные исследования в контексте этих разработок. Хотя омики радикально изменили наш взгляд на микробное разнообразие, культивирование остается основным средством для открытия и выяснения микробного (серного) метаболизма, а омики - мощным дополнением. Настоящая диссертация продемонстрировала эффективность сочетания этих двух дисциплин. Наши результаты могут иметь отношение даже к биотехнологическим применениям сульфатированных полисахаридов. Таким образом, следует уделять первоочередное внимание усилиям по культивированию многих некультивируемых морских микроорганизмов, участвующих в круговороте серы.

List of publications

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About the author

Daan Marius van Vliet was born on the 19th of November 1991 in Leiden, the Netherlands. He attended secondary education at the Stedelijk Gymnasium Leiden in 2010. He started the bachelor study Biotechnology at Wageningen University that same year. In 2013 he continued with the master study Biotechnology, specializing in environmental microbiology. This included an internship at the AkzoNobel Laboratory of Biodegradation and Ecotoxicology in Arnhem on microbial biodegradation of surfactants, and two theses: one at the Wageningen Laboratory of Microbiology on cultivation of sulfate-reducing bacteria, and a thesis at the Max Planck Institute for Marine Microbiology in Bremen, Germany, on profiling microbial communities found at the underseas Chapopote asphalt volcano. These projects sparked a fondness for research.

In late 2015, Daan started his PhD project at the Wageningen Laboratory of Microbiology in the Microbial Physiology group, to study the (mostly) anaerobic microorganisms driving the sulfur cycle in the Black Sea. The result is the thesis you are reading. His current occupation is assistant researcher biocatalysis at Wageningen Food & Biobased Research. Aside from research, Daan enjoys rock climbing, travelling, learning languages, (board)gaming, brewing and consuming fermented beverages, and music.

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Selection of Oral Presentations

- *Enrichment of polysaccharide-degrading anaerobes from Black Sea sediment*. Annual meeting of the Microbial Ecology division of the Koninklijke Nederlandse Vereniging voor Microbiologie, 4 November 2016, Nijmegen, The Netherlands
- *Novel marine anaerobes from the Black Sea: sulfated-polysaccharide-degrading Kiritimatiellaeota*. Symposium Novel Anaerobes, 10 November 2017, Braga, Portugal
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