environmental microbiology reports

Environmental Microbiology Reports (2021) **13**(6), 899–910 doi:10.1111/1758-2229.13012

Brief Report

The physiology and metabolic properties of a novel, low-abundance *Psychrilyobacter* species isolated from the anoxic Black Sea shed light on its ecological role

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Summary

Members of the *Psychrilyobacter* spp. of the phylum *Fusobacteria* have been recently suggested to be amongst the most significant primary degraders of the detrital organic matter in sulfidic marine habitats, despite representing only a small proportion (<0.1%) of the microbial community. In this study, we have isolated a previously uncultured *Psychrilyobacter* species (strains SD5^T and BL5; *Psychrilyobacter piezotolerans* sp. nov.) from the sulfidic waters (i.e., 2000 m depth) of the Black Sea and investigated its physiology and genomic capability in order to better understand potential ecological adaptation strategies. *P. piezotolerans* utilized a broad range of organic substituents (carbohydrates and proteins) and, remarkably, grew at sulfide concentrations up to 32 mM. These flexible physiological properties were supported by the presence of the respective metabolic pathways in the genomes of both strains. Growth at varying hydrostatic pressure (0.1–50 MPa) was sustained by modifying its membrane lipid composition. Thus, we have isolated a novel member of the 'rare biosphere', which endures the extreme conditions and may play a significant role in the degradation of detrital organic matter sinking into the sulfidic waters of the Black Sea.

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Introduction

The existence of the 'rare biosphere' (Sogin *et al*., 2006) still remains an incompletely understood phenomenon in microbial ecology, partly due to the limited knowledge of the physiology of the microbes present in low abundance. As an example, free-living *Psychrilyobacter* spp. of the phylum *Fusobacteria* have been recently reported to be present in extremely low abundance (i.e., representing <0.1% of the microbial community) in subarctic marine sediments but have been proven to be amongst the most important degraders (i.e., 10% of the total) of the protein component of the detrital organic matter of this system (Müller *et al*., 2018; Pelikan *et al*., 2021) and also in anoxic tidal flat sediments (Graue *et al*., 2012). *Psychrilyobacter* spp. 16S rRNA gene sequences have also reported to be under detection level in the subsurface waters of the Gulf of Mexico but became abundant (~40%) after the Deepwater Horizon oil spill, probably due to the potential capacity of the members of this genus for hydrocarbon degradation under anoxic conditions (Gutierrez *et al*., 2016). Some of these studies applied methods beyond 16S rRNA gene amplicon sequencing, e.g., ecophysiological analyses based on stable-isotope probing and metagenomics. Nevertheless, little is known about the physiology and adaptation strategies of these members of the *Fusobacteria* phylum as part of the 'rare biosphere' in marine habitats.

In this study, we have examined the distribution and abundance of fusobacterial members in the Black Sea water column. The Black Sea is the largest permanent anoxic basin on Earth, with a maximum depth of approximately 2200 m. The Black Sea deep waters (>2000 m) are characterized recalcitrant nutrient conditions (i.e., the presence of difficult to degrade sulfurized dissolved organic matters; Gomez-Saez *et al*., 2021), elevated hydrostatic pressure and high sulfide concentration (400 μM; Volkov and Neretin, 2007), conditions that are expected to harbour microorganisms adapted to deal with those conditions (e.g., Vetriani *et al*., 2003; Wakeham

et al., 2003, 2007; Lin *et al*., 2006; Leloup *et al*., 2007; Henkel *et al*., 2019; Yadav *et al*., 2020; Suominen *et al*., 2021a, 2021b). Here, we have successfully enriched members of the genus *Psychrilyobacter* (up to 58% of the total microbial community) of the phylum *Fusobacteria* and isolated two representative strains (*Psychrilyobacter* sp. strains SD5^T and BL5) from the deep sulfidic waters of the Black Sea (i.e., 2000 m depth). To elucidate their role in the sulfidic waters of the Black Sea, we investigated the genome sequences of the isolated strains and tested their physiology and adaptation strategies by mimicking the *in situ* physicochemical conditions (i.e., elevated hydrostatic pressure, high sulfide concentration). Our study demonstrates the successful enrichment and isolation of a novel member (*Psychrilyobacter piezotolerans*) of the 'rare biosphere' and sheds light onto its potential ecological relevance in sulfidic marine habitats.

Results and discussion

Distribution and ecology of Fusobacteria in the Black Sea water column

Overall, 16S rRNA gene amplicon sequencing analysis revealed that *Fusobacteria*-affiliated sequences were present in low abundance in the Black Sea water column, representing <0.01% of all 16S rRNA gene sequences identified throughout the water column (Fig. 1C). Estimated fusobacterial 16S rRNA gene copy numbers ranged between 0.4 and 5.0 $\times$ 10^3 l^{-1} in the Black Sea water column in the 50–2000 m depth range, with the lowest estimated abundances at 50 m (Fig. 1B). The highest estimated abundance of both *Fusobacteria* and *Psychrilyobacter* 16S rRNA gene sequences was detected at 120–150 m depth within the chemocline. This zone could be most favourable for these microorganisms due to the suboxic-anoxic conditions, availability of various nutrients [e.g., NH_4^+ (~14 μM), PO_4^{3-} (~5.5 μM; Fig. 1A)], presence of dissolved organic carbon (~200 μM; Suominen *et al*., 2021b), and relatively low hydrostatic pressure (1.2–1.5 MPa). Phylogenetic analysis, based on the 16S rRNA gene amplicon sequences attributed to *Fusobacteria* recovered from the Black Sea water column, revealed that they represent seven putative clades within this phylum (Fig. 1C). Sequences of most of these operational taxonomic units (OTUs) [(i.e., those falling in clade V (SD5^T, BL5 and closely related sequences) and VI (*Leptotrichiaceae*-related sequences)] were distributed throughout the water column (Fig. 1B). However, 16S rRNA gene sequences of a few taxa within the phylum *Fusobacteria*, like *Cetobacterium* (clade III), were only detected at 2000 m. Sequences affiliated to *Psychrilyobacter*, the most abundant group within *Fusobacteria* (i.e., representing 60%–100% of all fusobacterial 16S rRNA gene sequences; Fig. 1B), were encountered in waters ranging from 50 to 2000 m. The presence of the *Psychrilyobacter* 16S rRNA gene sequences in the oxic zone (50 m; O_2 concentration 121 μM) is intriguing, since members of the phylum *Fusobacteria* have been described to be strict anaerobes (Janssen and Liesack, 1995; Brune *et al*., 2002; Hofstad, 2006; Zhao *et al*., 2009).

Enrichment and isolation of Psychrilyobacter spp.

The 16S rRNA gene composition of enrichments grown in media BS1, BS2 and BS3 (containing cellulose and tryptone as nutrient sources) revealed that sequences affiliated to the *Psychrilyobacter* genus represented 32%–58% of the total microbial community (Supplementary Fig. S1) with a high density of rod to oval shaped cells (Fig. 2). Repeated streaking resulted in the isolation of two bacterial strains, designated SD5^T (type strain, T, characterized based on a polyphasic taxonomic approach) and BL5. As *Psychrilyobacter* 16S rRNA gene sequences had been detected in the oxic and suboxic zones of the Black Sea (Fig. 1B), the isolated strains were tested for growth under aerobic and microaerophilic conditions but turned out to be strict anaerobes. Both strains were non-spore-forming bacteria and showed negative reaction for oxidase and catalase activities, in agreement with their anaerobic lifestyle. Their 16S rRNA gene sequences were identical and they shared 100% similarity with 16S rRNA gene amplicon sequences of *Psychrilyobacter* (considering the ca. 300 bp fragment used for the 16S rRNA gene amplicon analysis) obtained from various depth of the Black Sea waters (Fig. 1).

General genomic features and phylogenetic affiliation of strains SD5^T and BL5

Whole genome sequencing of the strains SD5^T and BL5 yielded genomes of 3 358 809 bp and 3 344 081 bp in length, respectively, which were almost complete (98.88%) and free of contamination (Supplementary Table S1). The G + C mol% of the SD5^T and BL5 was 33.85% and 33.83%, respectively. No CRISPR repeats, signatures of viral infection (Sorek *et al*., 2008), were identified. Viral lysis of bacteria depends on the encounter possibilities, which are fewer for low abundant microbial taxa (Wilcox and Fuhrman, 1994). Hence, the absence of CRISPR repeats indicates that *Psychrilyobacter* spp. are probably not affected by viral predation due to their extremely low abundance in the Black Sea water column.

The phylogenomic analysis based on the concatenation of 49 marker genes (Fig. 3; see supplementary

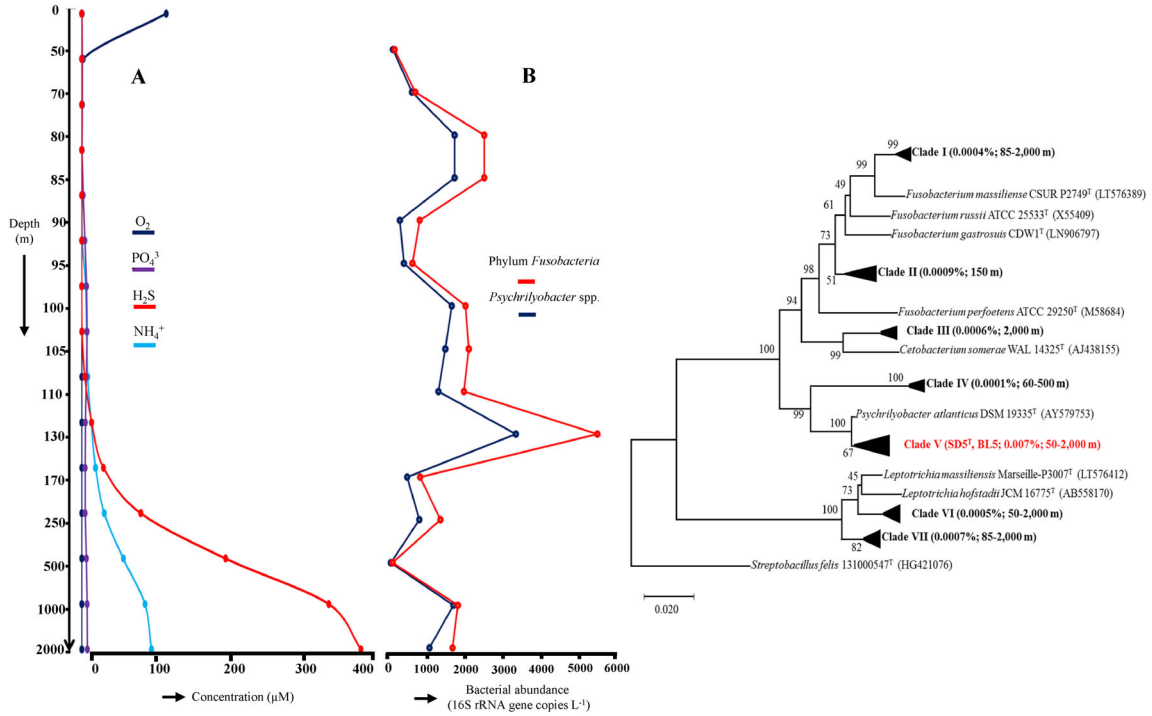

Fig 1. Fusobacteria in the Black Sea. A. Water column concentration profiles of oxygen, PO_4^{3-}, HS^- and NH_4^+ in the centre of the gyre of the western basin (42 53.78' N 30 40.72′) of the Black Sea (data from the study by Sollai *et al*., 2019; Suominen *et al*., 2021a). B. Estimated abundance (expressed as the number of 16S rRNA gene copies l^{-1}) of the members of the phylum *Fusobacterial* and *Psychrilyobacter* spp. in the Black Sea water column. Data were obtained by sampling with Niskin bottles, filtration, DNA extraction, 16S rRNA gene amplicon sequencing and quantitative PCR, both using the same universal bacterial primers (see Yadav *et al*., 2020; Villanueva *et al*., 2021). Note that the depth axis in (A) and (B) is not to scale. C. Phylogenetic tree based on partial (~304 bp) 16S rRNA gene sequences obtained from SPM of the Black Sea water at various depth (50–2000 m) and enrichment cultures. For reference, the full-length 16S rRNA gene sequences of various type strains of the fusobacteria and those of the isolated strains SD5^T and BL5 were used in the construction of the tree. The phylogenetic analysis shows the presence of seven clades within the phylum *Fusobacteria* in the Black Sea waters. The relative abundance of sequences affiliated with each clade (in % of all recovered 16S rRNA sequences) and their depth distribution is listed in parentheses. The sequences of the two isolated strains SD5^T, and BL5 and most of those obtained from the enrichment cultures were affiliated with clade V. The tree was constructed by the neighbour-joining method and rooted by using the *Streptobacillus felis* 131000547^T (HG421076) 16S rRNA gene sequence as an outgroup. Numbers at nodes represent bootstrap values (based on 1000 resamplings). The length of the bar indicates two nucleotide substitutions per 100 nucleotides.

information for methods) showed that both strains clustered with *Psychrilyobacter atlanticus* DSM 19335^T. In addition, they showed 99.5% 16S rRNA gene sequence similarity with *P. atlanticus* DSM 19335^T. However, average nucleotide identity (ANI) and digital DNA–DNA hybridization (DDH) between *P. atlanticus* DSM 19335^T and strains SD5^T and BL5 were only 86.6% and 32.9%, respectively (Supplementary Table S3), suggesting that both strains belong to a novel species in the genus *Psychrilyobacter* of the phylum *Fusobacteria*.

Members of the phylum *Fusobacteria* are adapted to two contrasting lifestyles, i.e., free-living and host associated. Our genome-based phylogeny separates these two types to some extent (Fig. 3): strains SD5^T and BL5, which are adapted to a free-living lifestyle, are more closely related to other fusobacteria with a free-living lifestyle. However, previous 16S rRNA gene-based studies indicated that their closest phylogenetic neighbours are associated with various marine organisms that do possess a host-associated lifestyle (Palmer *et al*., 1994; Fernandez-Piquer *et al*., 2012; Nelson *et al*., 2013; Bik *et al*., 2016; Eisenberg *et al*., 2016; Aronson *et al*., 2017; Friel *et al*., 2020; Lai *et al*., 2020). For example, *Psychrilyobacter* spp. are reported as the most abundant bacteria in pacific oysters and gut of the Antarctic seals (Fernandez-Piquer *et al*., 2012; Nelson *et al*., 2013). Indeed, the size of the genomes of strains SD5^T and BL5 is larger (ca. 3.35 Mb; Supplementary Table S1) than that of their host-associated phylogenetic neighbours (ca. 2.3 Mb). They also possessed relatively higher genomic G + C content, a greater gene repertoire and a higher number of tRNAs (Supplementary Table S1). Such genomic properties are often possessed by free-living bacteria in comparison to their host-associated stages (Moran and Wernegreen, 2000; Klasson and Andersson, 2004; Moran and Plague, 2004; Wernegreen, 2005; Moya *et al*., 2008; Merhej *et al*., 2009) supporting their free-living lifestyle. Moreover, it is still possible to

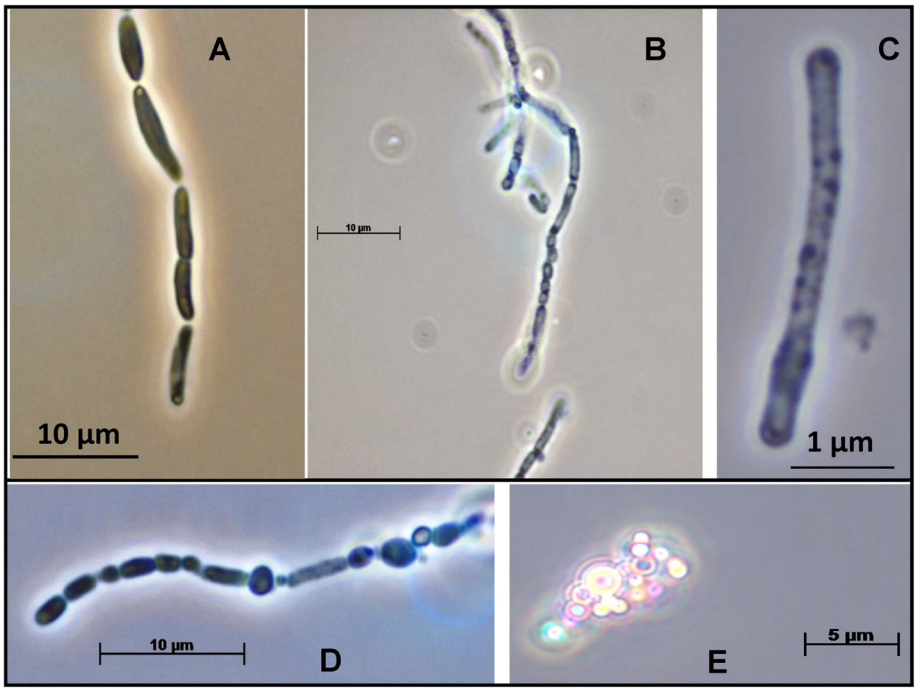

Fig 2. Cell morphology of strain SD5^T grown at optimal growth conditions (A), at 11 mM (B, C, D) and (E) 32 mM of sodium sulfide concentration (C) Sulfur granules are observed under phase contrast-microscope after 3 days of growth.

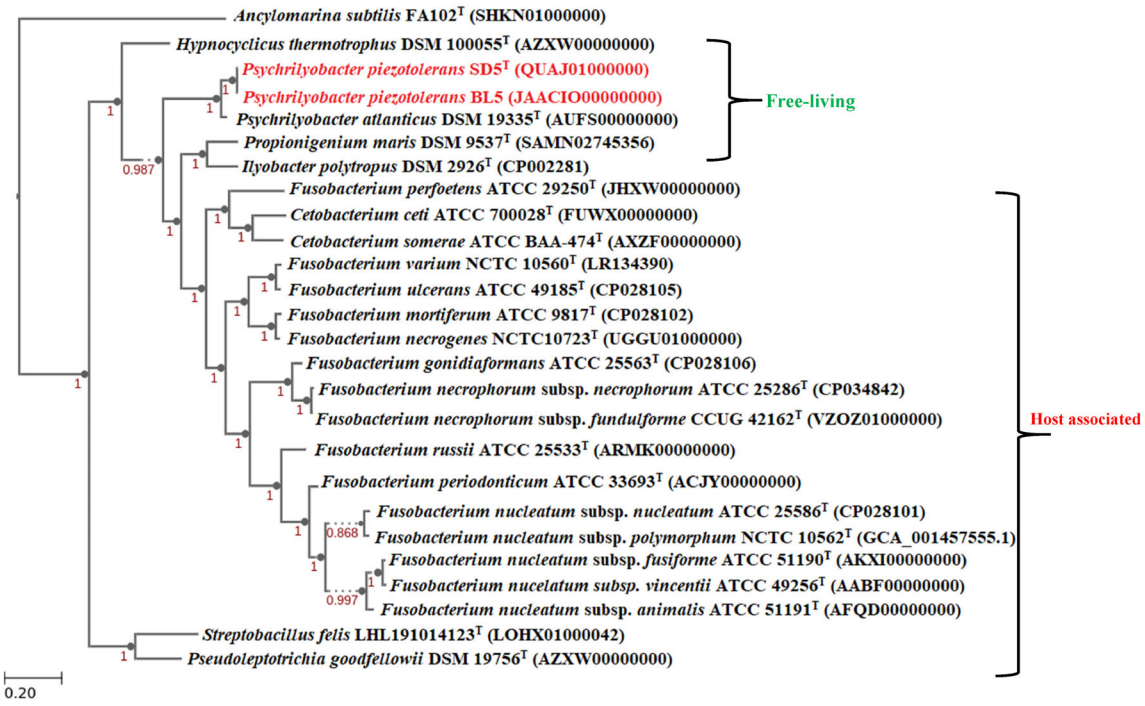

Fig 3. Phylogenomic tree of strains SD5^T and BL5, and their nearest phylogenetic neighbors affiliated with the phylum *Fusobacteria*. The tree was built by the maximum likelihood method using the Species TreeBuilder v.2.1.10 based on highly conserved concatenated protein sequences of 49 core, universal genes defined by COG (Clusters of Orthologous Groups) listed on the KBase server (Arkin *et al.*, 2018). The tree was rooted by using *Ancylomarina* subtilis FA 102^T (SHKN01000000) as an outgroup. The genome sequence accession numbers are shown between parentheses. Numbers at nodes represent bootstrap values (1 refers to 1000). The length of the bar indicates two nucleotide substitutions per 10 nucleotides.

observe genomic traits of their potential host-associated ancestor, such as putative virulence factors (Supplementary Fig. S2) and the isoprenoid MEP/DOXP pathway, which has been previously related to pathogenicity (Sarowska *et al*., 2019) and intracellular survival (Begley *et al*., 2004; Shin *et al*., 2006), respectively. Hence, we assume that these strains also have the potential to colonize higher organisms in a host-associated lifestyle.

Physiology

Strains SD5^T and BL5 hydrolyzed complex polysaccharides such as cellulose, chitin, salicin and starch, and grew anaerobically by fermenting a wide range of various carbon sources (Supplementary Table S4). Amongst all carbon sources tested for growth, pyruvate was utilized preferably by both strains (Supplementary Table S4). They hydrolyzed proteins and grew well by fermenting various amino acids, i.e., threonine, lysine, glutamate, and aspartate (Supplementary Table S4). The range of compounds that can support growth of the novel isolates is very broad which includes various organic substituents derived from biogenic remains (Supplementary Table S4). Their growth was also improved by acetate which indicates its utilization as carbon and energy source. Considering both the physiology and genomic signatures, it is likely that their capacity to hydrolyze and ferment a wide range of carbohydrates and amino acids allows them to use the detrital organic matter sinking through the sulfidic waters (Diercks and Asper, 1997). This is supported by their high abundance in the nutrient-rich medium mimicking the *in situ* physicochemical conditions (Supplementary Fig. S2). Our results further support that *Psychrilyobacter* spp. could be important detrital organic matter degraders along with other anaerobic bacteria in sulfidic marine sediments (Graue *et al*., 2012; Müller *et al*., 2018; Pelikan *et al*., 2021).

We tested our strains for growth at sulfide concentration ranging from 0.1 to 40 mM. Interestingly, both strains were able to grow at sulfide concentrations up to 32 mM at pH 7.0, which is 80-fold the natural concentration found in the Black Sea deep waters (i.e., 400 μM). To the best of our knowledge, growth at such high sulfide concentration has not been reported for any organisms. At lower (<4 mM) sulfide concentration, the cells appeared as chains of rods (0.6–0.7 × 4.0–6.0 μm), but at higher (>4 mM) sulfide concentration, few cells appeared spherical with 0.8–0.9 μm in diameter (Figs. 2A–D). They also showed the presence of sulfur granules in the cell (e.g., Fig. 2C). At 32 mM sulfide concentration, both strains grew in chains of brightly shining cells because of the presence of refractive sulfur granules (Fig. 2E). Growth of both strains was relatively slower in the presence of higher (>7 mM) sulfide concentration and the doubling time extended from 2 to 3 h at optimal conditions up to 5–7 h (Fig. 4A).

Strains SD5^T and BL5 were able to grow up to 50 MPa of hydrostatic pressure under strict anaerobic conditions. However, growth slowed down at elevated hydrostatic pressure (>20 MPa). The addition of glutamate (0.01%) to the growth medium slightly increased growth at elevated hydrostatic pressure (30 MPa; Fig. 4). This is expected since glutamate functions as one of the most prominent compatible solutes under stress conditions (Csonka, 1989). Glutamate has been previously reported as a 'piezolite' in *Desulfovibrio hydrothermalis*, where it significantly improved the growth at elevated hydrostatic pressure (Amrani *et al*., 2014). In addition, glutamate allows enzymes to function efficiently (Brown, 1990; Walker and van der Donk, 2016), eventually protecting macromolecular structures to maintain metabolic functions at elevated hydrostatic pressure (Welsh, 2000; Bhaganna *et al*., 2010).

We also investigated the effect of elevated hydrostatic pressure on the composition of the cell membrane core lipid (CL) and intact polar lipid (IPL) composition of strain SD5^T. Overall, the core lipid distribution was similar between the strains grown at 0.1 and 20 MPa but changed significantly for the strain grown at 30 MPa. In the cultures grown at 0.1 and 20 MPa, the relative abundance of unsaturated fatty acids (not including hydroxy fatty acids) was 30% and 35%, respectively (Table 1), while at 30 MPa, this had increased to 56%. Similarly, earlier studies have also reported a prominent correlation between growth at elevated hydrostatic pressure and increase of the unsaturated fatty acid content (Allen *et al*., 1999, Grossi *et al*., 2010, Yadav *et al*., 2020). Another observed change in the core lipid distribution was that the abundance of hydroxy fatty acids, probably derived from Lipid A in the lipopolysaccharides (LPS) layer; it decreased with increasing pressure (Table 1). The total hydroxy fatty acids decreased from 18% to 14% to 10% as growth pressure increased from 0.1 to 20 to 30 MPa. Likewise, a decrease in the relative abundance of hydroxy fatty acids has been observed in *Profundimonas piezophila* grown at elevated hydrostatic pressure (Cao *et al*., 2014). Alterations in the relative abundance of hydroxy fatty acids are expected to change cell membrane properties by affecting the polarity of core lipids (Nichols *et al*., 2004). However, there are no detailed studies available related with the physical role of hydroxy fatty acids in the adaptation of cellular membranes under elevated hydrostatic pressure. An increase in hydroxy fatty acids has been observed in psychrophilic bacteria upon cold adaptation (Bale *et al*., 2019). Consequently, it could be hypothesized that a decrease in the relative abundance of hydroxy acids could be induce by elevated hydrostatic pressure, while an increase in the

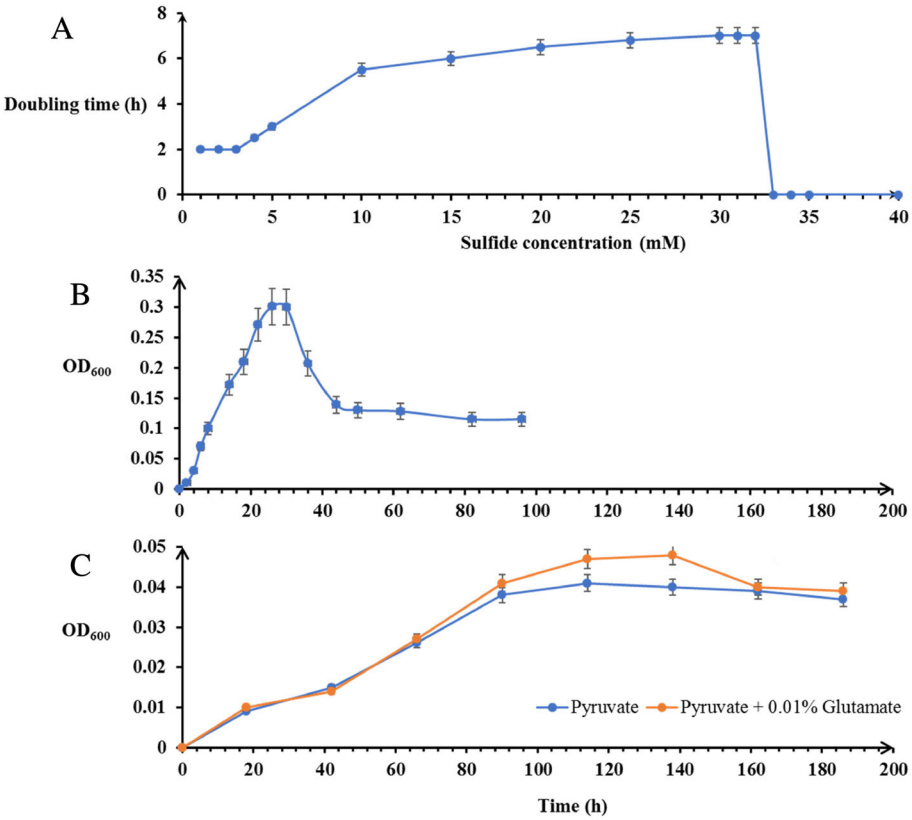

Fig 4. Doubling time of strain SD5^T grown at various sulfide concentrations (A) and growth at optimal conditions (B; 23°C; 0.1 MPa, and pyruvate as carbon source) and (C) at 30 MPa with and without the addition of glutamate (other conditions unchanged). Key: OD_{600} = optical density measured at 600 nm. Error bars refer to the standard error.

membrane unsaturated fatty acids is an adaptation to conditions in the strains under study here. This change agrees with the fact that polyunsaturated fatty acids chain implement a more expanded conformation with lower melting temperatures than their saturated counterparts allowing the cell membrane flexibility at higher hydrostatic pressure (Hazel and Williams, 1990).

A notable change of the IPL head group composition with increasing pressure was observed between 20 MPa and 30 MPa (Table 2). Of the IPL headgroups, the largest change was in the relative abundance of phosphatidylglycerol (PG), which was 19% and 24% at 0.1 and 20 MPa, respectively, but 51% at 30 MPa (Table 2). The largest concomitant decrease was in the cardiolipins (including lyso-cardiolipins): from 38% and 45% at 0.1 and 20 MPa, respectively, to 22% at 30 MPa (Table 2). This shift may be an adaptive process relating to membrane fluidity at elevated hydrostatic pressure. To date, there are no other reports of such specific membrane adaption under high hydrostatic pressure in piezophilic bacteria. The nitrogen-containing headgroup PC also decreased in relative abundance (Supplementary Table S2) with increasing pressure (17.8%, 6.0% and 7.6% at 0.1, 20 and 30 MPa, respectively). An increase in PG relative to PE with increasing pressure has been previously observed in the piezo-sensitive bacterium, *Vibrio* sp. strain LT25 (Mangelsdorf *et al*., 2005) and in *Desulfovibrio indonesiensis* strain P23 at certain temperatures (Fichtel *et al*., 2015). Conversely, PG was reported to decrease relative to PE with increasing pressure, for the piezophilic bacterial strains 16C1 and 2D2 (Yano *et al*., 1998).

Metabolism based on genomic analysis

Genomes of both strains contained genes related to aerotolerance (i.e., superoxide reductase, ruberythrin and thioredoxin reductase; Fig. 5), which might be an adaptation to survive in oxic and suboxic zone of the Black Sea. They utilized a wide range of carbon and nitrogen sources, which is supported by the presence of various genes in the genomes (Fig. 5; Supplementary Table S2). Genes encoding enzymes involved in glucose fermentation, i.e., production of acetate, ethanol, lactate, formate, hydrogen and carbon dioxide (Fig. 5; Supplementary Table S2), and acetate utilization were identified. The

Table 1. Core lipid composition of strains SD5^T, BL5 and *Psychrilyobacter atlanticus* JCM 14977^T released by base hydrolysis.

		BL5		SD5^T		
		0.1 MPa	*P. atlanticus* JCM 14977^T	0.1 MPa	20 MPa	30 MPa
CLs	$C_{11:0}$	-	0.5	-	-	
	$C_{12:0}$	1.1	0.6	1.4	1.4	
	$C_{12:1}$	-	0.2	-	-	-
	$C_{13:0}$	0.3	0.2	0.4	0.5	0.6
	$C_{14:1}$ ω6C	-	-	-	-	0.9
	$C_{14:1}$ ω 7C	0.6	-	0.6	0.9	
	$C_{14:1}$ ω 9C	0.9	-	1.1	0.9	0.7
	$C_{14:0}$	10.9	7.5	10.6	8.7	6.4
	$C_{15:1}$ ω7C	0.1	-	0.1	0.2	8.1
	$C_{15:1}$ ω9C	2.2	-	2.2	4.7	0.5
	$C_{15:0}$	1.4	2.2	1.5	2.0	2.9
	$C_{16:1}$ ω9C	23.0	23.1	23.1	22.9	36.7
	$C_{16:1}$ ω11C	0.5	-	0.2	0.4	1.1
	$C_{16:0}$	36.0	41.5	36.7	34.2	18.4
	$C_{17:1}$ ω9C	0.5	0.9	0.4	1.4	1.4
	$C_{17:1}$ ω11C	0.5	1.2	0.5	1.4	3.3
	$C_{17:0}$	1.2	2.5	1.1	2.9	1.9
	$C_{18:1}$ ω9C	0.4	0.5	0.2	0.4	2.0
	$C_{18:1}$ ω11C	1.3	1.9	0.9	1.6	1.5
	$C_{18:1}$ ω13C	-	-	0.3	-	
	$C_{18:0}$	1.5	1.6	0.9	2.0	2.7
	Σ saturated FAs	52.4	56.6	52.6	51.7	32.9
	Σ unsaturated FAs	30.0	27.8	29.6	34.8	56.2
Hydroxy FAs	β-OH $C_{10:0}$	0.5	0.4	0.6	0.5	-
	β-OH $C_{11:0}$	0.5	0.5	0.5	0.6	0.2
	β-OH $C_{12:1}$	0.4	-	0.7	0.4	0.4
	β-OH $C_{12:0}$	5.3	3.6	5.7	4.1	1.7
	β-OH $C_{13:0}$	0.5	0.6	0.6	0.6	0.8
	β-OH $C_{14:0}$	3.0	1.8	2.5	1.7	2.3
	β-OH $C_{15:0}$	0.4	0.9	0.4	0.5	0.8
	β-OH $C_{16:1}$	0.2	-	0.3	0.2	0.8
	β-OH $C_{16:0}$	4.9	5.1	4.8	3.5	2.3
	β-OH $C_{17:0}$	0.1	0.4	0.1	0.3	0.4
	β-OH $C_{18:0}$	0.1	0.8	0.1	0.1	-
	3-Methoxy $C_{11:0}$	0.1	-	1.1	0.1	-
	3-Methoxy $C_{12:0}$	1.1	-	0.8	1.0	-
	Di-hydroxy FAs	0.6	-		0.3	-
	Σ hydroxy FAs	17.7	14.1	18.2	13.9	9.7

CLs = core lipids.

presence of seven putative [FeFe]-hydrogenase genes involved in the generation of H_2 during carbohydrate and protein fermentations in various microorganisms were also annotated (Greening *et al*., 2016; Wolf *et al*., 2016). This is further supported by the presence of pyruvate-formate lyase and pyruvate-ferredoxin oxidoreductase genes in the genomes (Fig. 5; Supplementary Table S2).

The two known genes for sulfide oxidation, i.e., sulfide-quinone-oxidoreductase (*sqr*) and flavocytochrome *c* sulfide dehydrogenase were not detected in the genomes. However, sulfide oxidation may be catalyzed by some unclassified flavoproteins encoded by the genomes. We could detect seven gene copies coding for rhodanese (EC 2.8.1.1; Supplementary Table S2), which are involved in sulfur trafficking and oxidation in bacteria (Dahl, 2017; Koch and Dahl, 2018). Rhodanese cleaves the S—S bond present in thiosulfate, producing sulfur and sulfite (Dahl, 2017; Koch and Dahl, 2018). A complete set of genes encoding the enzymes involved in the heterodisulfide reductase (HDR) system (Fig. 5; Supplementary Table S2), acting as an elemental sulfur oxidation enzyme in the cytoplasmic space of bacteria and archaea (Wang *et al*., 2019), was also identified. Their presence suggests the involvement in sulfur metabolism and potentially in the alleviation of sulfidic toxicity in the *Psychrilyobacter* spp. at elevated sulfide concentrations.

We also observed the presence of the genes encoding enzymes in the glutamine synthetase (GS) and glutamate synthase (GOGAT) pathway, and glutamate and glutamine transporters in the genomes of the strains SD5^T and BL5 (Fig. 5; Supplementary Table S2). These genes have been reported to aid in the osmoregulation and survival mechanisms in piezophilic bacteria (Csonka, 1989; Kang and Hwang, 2018). Therefore, the GS/GOGAT

Table 2. The relative abundance of the intact polar lipids, grouped by headgroup.

Polar head group	Acyl moieties of abundant species	SD5^T (0.1 MPa)	SD5^T (20 MPa)	SD5^T (30 MPa)	BL5
PEs	$C_{16:0}$, $C_{12:0}$ $C_{16:1}$, $C_{14:0}$ $C_{16:1}$, $C_{16:0}$ $C_{16:0}$, $C_{16:0}$	14.7	15.9	15.1	13.8
PGs	$C_{16:0}$, $C_{15:1}$ $C_{16:1}$, $C_{14:0}$ $C_{16:1}$, $C_{16:0}$ $C_{16:1}$, $C_{16:1}$	18.6	24.4	50.8	18.4
PCs	$C_{16:1}$, $C_{18:1}$ $C_{16:1}$, $C_{16:0}$ $C_{16:1}$, $C_{16:1}$	17.8	6.0	7.6	18.2
Cardiolipins	$C_{16:0}$, $C_{16:1}$, $C_{16:0}$, $C_{12:0}$ $C_{16:0}$, $C_{16:1}$, $C_{16:1}$, $C_{14:0}$ $C_{16:0}$, $C_{16:1}$, $C_{16:0}$, $C_{15:1}$ $C_{16:0}$, $C_{16:1}$, $C_{16:1}$, $C_{16:0}$	23.7	28.6	18.7	21.2
Lyso PEs	$C_{16:0}$	11.2	9.0	4.2	11.7
Lyso PCs	$C_{16:0}$ $C_{18:0}$	-	-	-	3.8
Lyso cardiolipins	$C_{16:0}$, $C_{16:1}$, $C_{16:0}$ $C_{16:0}$, $C_{16:1}$, $C_{16:1}$	13.9	16.2	3.6	12.9

PEs, phosphatidylethanolamine; PGs, phosphatidylglycerol; PCs, phosphatidylcholine. The acyl moieties of the most abundant species of each polar head group are given.

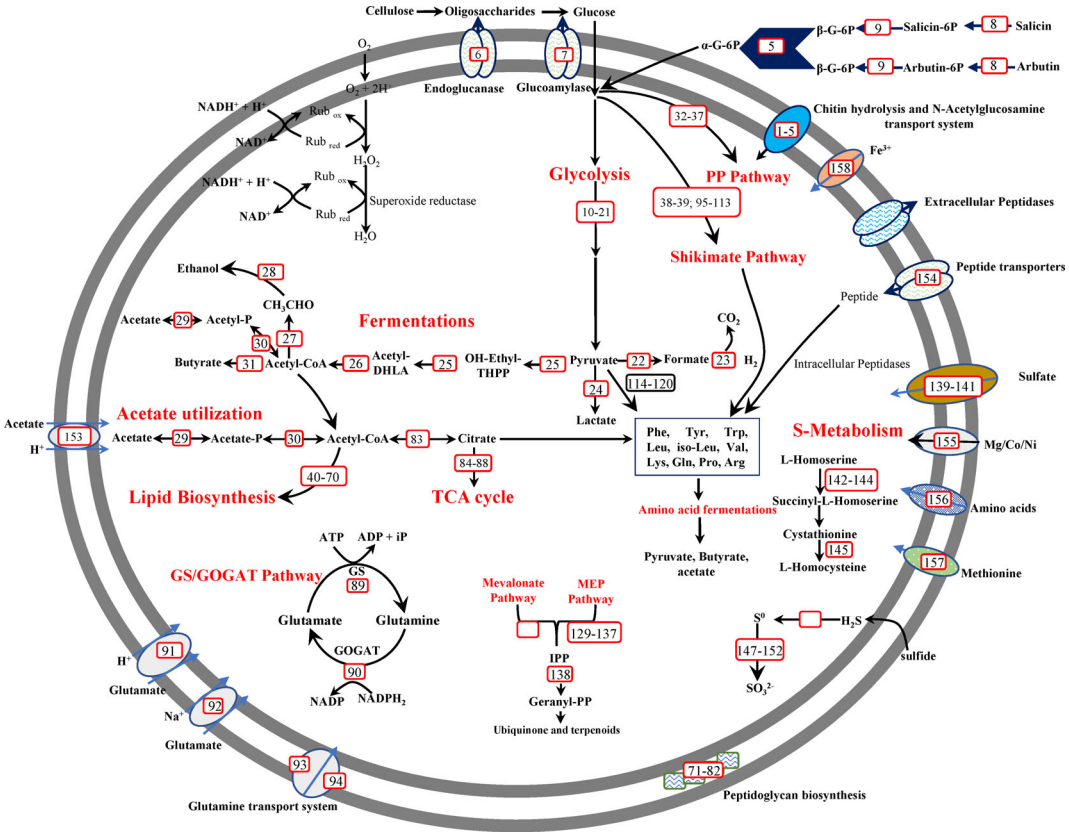

Fig 5. Reconstruction of the central metabolic pathway of strain SD5^T and BL5 based on different physiological analyses and by the presence of various genes identified in the genome sequence. Empty boxes indicate missing genes. IPP, isopentenyl pyrophosphate; PP Pathway, pentose phosphate pathway; geranyl-PP, geranyl pyrophosphate; GS, glutamine synthetase; glutamate synthase (GOGAT). Numbers identify genes according to the key in Supplementary Table S2.

pathway and glutamate transporters (Fig. 5) might be involved in the cell internalization of extracellular glutamate and other solutes in *Psychrilyobacter* strains to survive at elevated hydrostatic pressures in the deep sulfidic waters of the Black Sea.

All genes involved in 2-*C*-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (MEP/DOXP) pathway of isoprenoid biosynthesis were identified in the genomes of the strains SD5^T and BL5 (Fig. 5; Supplementary Table S1-S2). We could only detect the phosphomevalonate decarboxylase coding gene of the mevalonate pathway for isoprenoid biosynthesis. Nevertheless, growth of both strains in the medium amended with either/both fosmidomycin [a pathway inhibitor of 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway] and simvastatin (a pathway inhibitor of mevalonate pathway) suggested that both MEP and mevalonate isoprenoid biosynthetic pathways were functional. Hence, the evidence for the presence and potential functionality of both isoprenoid biosynthetic pathways in these strains is enigmatic and requires further study to validate their role.

Conclusions

Psychrilyobacter spp. typically belong to the 'rare biosphere' in marine environments but incubation studies have identified them to be major degraders of organic matter in sulfidic marine sediments. Here, we isolated two representative strains of *Psychrilyobacter*, which are adapted to both elevated hydrostatic pressure and the high sulfide concentrations of the Black Sea deep water column. Their ability to use a variety of organic substituents and adaptations to various environmental stressors help them to endure with the conditions of the Black Sea. This demonstrates the versatile metabolic potential of a member of the 'rare biosphere', which harbours adaptable strategies to deal with various environmental stressors. However, despite these versatile traits, *Psychrilyobacter* spp. remains a quantitatively minor member of the microbial community of the Black Sea for unknown reasons. Incubation studies may therefore provide a quantitatively unrealistic view of the microbes involved in anaerobic organic matter degradation. Overall, our study evidently suggests that a collective approach, i.e., the stimulations by organic rich substances, enrichments and isolation in conjunction with culture-independent methods, are required to unravel the ecological relevance of free-living *Fusobacteria* in sulfidic marine habitats.

Description of Psychrilyobacter piezotolerans sp. nov.

Psychrilyobacter piezotolerans (pie.zo.to'le.rans. Gr. v. piezo to press; L. part. adj. tolerans tolerating, N.L. part. adj. piezotolerans tolerating high hydrostatic pressure).

Properties. Cells are motile and rod (0.6–0.7 $\times$ 3–6 μm) shaped. Cell shape changes from rod to spherical shape (diameter, 1.1–1.2) under higher sulfide concentration (>4 mM). Cells stain Gram-negative and are obligate anaerobes. Growth occurs between pH 6.5 and 8.8 (optimum 7.0–8.0). Tolerates up to 5.5% NaCl with optimum growth at 2%–3%. Optimum growth occurs at 20–25°C (range 4–35°C). Glutamate supports the growth at higher hydrostatic pressure. Pyruvate is preferred carbon source for the growth. Yeast extract is required for the growth. Dominant fatty acids (>10%) are $C_{16:0}$, $C_{16:1}$ ω9C and $C_{14:0}$. Fatty acids in moderate abundance (4%–10%) are β—OH $C_{12:0}$ and β—OH $C_{16:0}$. Phosphatidylethanolamine (PE), phosphatidylglycerol (PG), cardiolipins and lyso-PE are major polar lipids. G + C content is 33.8%. Type strain is SD5^T (JCM 32482^T = KCTC 15663^T).

Author contributions

S.Y., L.V. and J.S.S.D. designed the study. S.Y. performed most of the laboratory work and data analysis; M.K. and N.B. performed the fatty acid and intact polar lipid analysis; S.Y., L.V. and J.S.S.D. wrote the manuscript.

Acknowledgements

The authors are thankful to Marianne Baas for helping in fermentation product (gas phase) analysis, to Sanne Vreugdenhil and Maartje Brouwer for their support with the molecular genetic analyses, and to Denise Dorhout for lipid analysis. The authors would like to thank the cruise leader, captain, crew, and scientific participants of Black Sea expedition-2017 (64PE418) on board of the *R/V* Pelagia for sampling and technical support. This research was supported by the SIAM Gravitation Grant (024.002.002) from the Dutch Ministry of Education, Culture and Science (OCW) to J.S.S.D. and L.V. J.S.S.D. and N.B. received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (Grant Agreement No. 694569) funded to JSSD.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Relative abundance (in % of total bacterial and archaeal 16S rRNA gene sequences) of the *Psychrilyobacter* spp. and the composition of the rest of the microbial community in the enrichments using the growth media BS1, BS2 and BS3.

Fig. S2. Putative virulence related regions identified in the genomes of strains $SD5^T$ and BL5

Appendix S1: Supporting Information