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RESEARCH ARTICLE

Detection of dsrAB operon expression in *Desulfotalea psychrophila* cells subjected to simulated Martian conditions of temperature and regolith's sulphate minerals composition

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Abstract

Discoveries of transient liquid water in the Martian polar caps and the presence of liquid lakes and subsurface oceans in icy satellites have increased the interest of scientists in the capabilities of terrestrial extremophiles to grow and remain metabolically active in these extreme environments. The principal goal of this research is to understand the metabolic capacity of the anaerobic psychrophile, *Desulfotalea psychrophila*, cultured at subfreezing temperatures in media containing various concentrations of sulphate minerals. In this regard, our experiments focused on the detection of *D. psychrophila* survival and active metabolism, employing a biochamber that can recreate Martian temperatures. Using standard bacteriological methods for determining growth, combined with molecular and enzymatic determination of sulphate reduction, we have found that *D. psychrophila* is capable to carry out biological processes at temperatures down to −5°C, at concentrations that range from 0.35 to 18 wt% of $MgSO_4$, 0.1 wt% of $CaSO_4$ and 10 to 14 wt% of $FeSO_4$ in which the highest sulphate concentration gradually returned the biosynthetic rate to basal limits, and the lowest temperature decreased bacterial cell division. These chemical salts, whose ions are classified as chaotropes, are known to act by maintaining water molecules in liquid state at subfreezing temperatures and by altering the stability of cellular components. This 'chaotropic effect' could potentially benefit the microbial metabolic activity up to a concentration in which cellular viability is jeopardized. Consequently, our hypothesis is directed towards the detection of metabolic activity as an indirect measurement of the potential influence of these ions in the flexibility/functionality of biological structures that at cold temperatures are highly rigid, compact and partially/non-functional due to water freezing. Studies of this type of microorganism are critical considering the possibility of survival and colonization of psychrophilic sulphate reducers in other planets and icy satellites.

Contents

Introduction

The Martian surface and its subsurface are composed mostly of CO_2 and water ice (Chinnery *et al.*, 2019). However, the presence of hydrated sulphate deposits in locations such as Terra Meridiani, Valles Marineris, the Northern polar cap, Aram Chaos and Juventae Chasma could induce the formation of thin aqueous films of liquid water within the regolith structure (Arvidson *et al.*, 2005; Bibring, 2005; Gendrin *et al.*, 2005; Langevin *et al.*, 2005). Moreover, estimations of the chemical species present in the Martian surface have pointed to the existence of enough Fe, Mg and Ca cations which could easily combine with sulphates (Clark and Van Hart, 1980). Interestingly, McGlynn *et al.* (2012) have estimated that these chemical species could occur in Gusev Crater at concentrations around 5.4 wt%, while in Meridiani Planum, it has been predicted to be at around 5.5 wt%. According to these researchers, $MgSO_4$ and $CaSO_4$ could constitute around 10% of the soil present in Gusev Crater (McGlynn *et al.*, 2012). Therefore, the hypothesis that bacterial life can persist in Martian extreme environments is feasible, at least temporarily in subsurface areas where salty water films, and protection from radiation and extremes of temperatures are provided (Bibring, 2005; Des Marais *et al.*, 2008; Lamarche-Gagnon *et al.*, 2015).

In general, sulphates species such as $MgSO_4$ and $CaSO_4$ have been estimated to form part of the subsurface oceans of icy satellites of the Jovian system (McCord *et al.*, 2001; Zolotov and Shock, 2001; Pappalardo, 2010; Prockter *et al.*, 2010; Lamarche-Gagnon *et al.*, 2015; Schmidt and Manning, 2017; Vance *et al.*, 2018). Furthermore, based on observational studies, Europa's ocean could produce hydrated sulphates of Mg and Ca by means of upwelling in fractures of the icy shell (Fanale *et al.*, 2000; McCord *et al.*, 2001; Zolotov and Shock, 2001; Molyneux *et al.*, 2020). Likewise, in Ganymede's surface, remnants of $MgSO_4$ and $NaSO_4$ brines have been identified (McCord *et al.*, 2001; Molyneux *et al.*, 2020; Pan *et al.*, 2021). Moreover, there is evidence that sulphates could be present in other icy moons of the Saturn system. For example, water pockets located at the lower part of Titan's ice crust as well as cracks of the icy crust have been pointed as suitable environments for microorganisms' colonization (Simakov, 2001).

Extremophile microbes are thought to be the first organisms to colonize Earth and are ultimately responsible for the formation of all ecosystems present in today's World (Pikuta *et al.*, 2007). This phenomenon was accompanied by deep modifications of genetic information that allowed these microbes to adapt to constantly challenging environments. Consequently, our planet ended up acquiring radioresistant, halophilic, barophilic, acidophilic, alkaliphilic, thermophilic and psychrophilic genetic compositions (Pikuta *et al.*, 2007). According to the chronological distribution of these extremophiles on Earth, microorganisms adapted to hot and acidic environments were present in the early stages of its evolution while psychrophilic microbes appeared later due to a temperature drop on the planet's surface (Pikuta *et al.*, 2007). In fact, present extremophiles can be considered relics from the original microorganisms that inhabited these extreme environments (Pikuta *et al.*, 2007).

In the past, it has been stated that little is known about psychrophilic microbes and their adaptations to cold environments (Inniss, 1975; Deming, 2002; Panikov *et al.*, 2006; Poli *et al.*, 2017). Initially, all bacterial studies, regardless of their habitat, were based on the 16S rRNA gene taxonomical clustering which allowed the accurate identification and classification of microbial communities. However, studies of bacterial strains that inhabited cold environments were partially neglected. Nowadays, this line of

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thought has changed due to the implications of global warming and the loss of autochthonous microbial communities from these environments (Deming, 2002).

As expected, a proper definition of psychrophiles was needed to classify microorganisms based on their optimal, minimum and maximal growth temperatures. There were a myriad of misleading definitions that somehow impaired the scientific interest to research true psychrophiles (Hoover and Pikuta, 2010; Moyer and Morita, 2007). Moreover, the term psychrophile was mentioned for the first time by Schmidt-Nielsen while working with microbes that could grow at 0°C (Morita, 1975; Pikuta *et al*., 2007). It was not until 1975 when Morita (1975) described them as organisms that have an optimal growth temperature at or lower than 15°C, a maximal growth temperature at 20°C and a minimal growth temperature at or below 0°C (Inniss, 1975; Morita, 1975; Feller and Gerday, 2003; Hoover and Pikuta, 2010; Moyer and Morita, 2007; Poli *et al*., 2017). As a result of this definition, psychrophilic bacteria can survive and grow in environments which are permanently frozen and at sub-zero temperatures (Steven *et al*., 2006; Piette *et al*., 2011).

Psychrophiles are represented in both Bacteria and Archaea Domains. They can be Gram-positive or Gram-negative, aerobic or anaerobic, heterotrophic or autotrophic (Maccario *et al*., 2014; Poli *et al*., 2017). However, the majority of today's isolates belong to the Gram-negative groups Proteobacteria and Bacteriodetes (Moyer and Morita, 2007). Furthermore, most of them are adapted to more than one environmental stressor. In fact, most psychrophiles have been isolated from marine ecosystems that besides low temperature have high salt concentrations or high pressures (Feller and Gerday, 2003; Moyer and Morita, 2007).

The lowest temperature registered in which bacteria can metabolize is −20°C (Inniss, 1975; Deming, 2002; Panikov *et al*., 2006; Pikuta *et al*., 2007; Moyer and Morita, 2007). Congruently, Lamarche-Gagnon *et al*. (2015) have detected sulphate reduction in in-situ Arctic sediments at this same temperature (Lamarche-Gagnon *et al*., 2015). On the other hand, Mykytczuk *et al*. (2013) have pointed out that there are no reports of bacterial growth below −15°C (Mykytczuk *et al*., 2013), although survival have been reported down to −30°C (*in situ*) and microbial metabolisms have been predicted to occur at temperatures down to −40°C (Pikuta and Hoover, 2003; Price and Sowers, 2004; Moyer and Morita, 2007). Interestingly, under those extreme conditions psychrophilic microbes have developed adaptations that allow them to survive and proliferate. Among them, the most important are induced dormancy (Hoover and Pikuta, 2010), increased unsaturated, short, branched and cyclic fatty acids at the cell membrane level (Inniss, 1975; Feller and Gerday, 2003; Satyanarayana *et al*., 2005; Piette *et al*., 2011; Moyer and Morita, 2007; Poli *et al*., 2017), synthesis of cold shock proteins and cold-acclimation proteins (Deming, 2002; Feller and Gerday, 2003; Pikuta and Hoover, 2003; Satyanarayana *et al*., 2005; Hoover and Pikuta, 2010; Piette *et al*., 2011; Maccario *et al*., 2014; Poli *et al*., 2017), selective gene expression of cold active enzymes (Deming, 2002; Feller and Gerday, 2003; Satyanarayana *et al*., 2005; Piette *et al*., 2011), induction of efficient mechanisms for protein synthesis and protein folding (Feller and Gerday, 2003; Piette *et al*., 2011), synthesis of antifreeze proteins and cryoprotectants (Deming, 2002; Feller and Gerday, 2003; Pikuta and Hoover, 2003; Satyanarayana *et al*., 2005; Hoover and Pikuta, 2010; Moyer and Morita, 2007; Poli *et al*., 2017).

Importantly, psychrophiles can be classified in three different groups according to their metabolic rates: the first one in which the metabolic rate is enough for microbial growth, the second group in which the metabolic rate is exclusive for maintenance and no growth and the last group in which the metabolic rate is exclusive for survival and DNA damage repair mechanisms (Price and Sowers, 2004; Steven *et al*., 2006; Moyer and Morita, 2007).

Additionally, some psychrophilic microbes are assembled within a subdivision of microorganisms known as sulphate reducing prokaryotes (SRP). SRP are a group of microorganisms that are able to subsist and proliferate in different ecosystems such as marine and extreme environments (Teske *et al*., 1996; Karr *et al*., 2005; Kjeldsen *et al*., 2007; Leloup *et al*., 2007; Miletto *et al*., 2011). They can use sulphate anions as terminal electron acceptors in the generation of metabolic energy. In this process, they use an enzyme known as the dissimilatory sulphite reductase (DsrAB) which is

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encoded in the dsrAB operon and intervenes in the last step of sulphate reduction. Furthermore, the dsrAB operon encodes the genes for two other enzymes that are involved in the sulphate reduction process, ATP sulfurylase and adenylyl-sulphate reductase (Karkhoff-Schweizer *et al.*, 1995; Laue *et al.*, 2001; Muller *et al.*, 2015; Thorup *et al.*, 2017; Rüffel *et al.*, 2018). Among this group of bacteria, the Proteobacterium *D. psychrophila* is one of its members that possesses a psychrophilic nature (Rabus *et al.*, 2004; Yadav *et al.*, 2017). Its optimal growth temperature is 10°C and it can grow at temperatures down to −1.8°C (Knoblauch *et al.*, 1999; Rabus *et al.*, 2004). However, as measurements of bacterial growth are dependent on cellular division, no information about the survivability and metabolic activity of this microbe has been described at lower temperatures (or extremes). Simply, because most studies have focused on the quantification of growth by means of the detection of bacterial biomass which only accounts for bacterial strains that are actively dividing within a set of cultural/environmental parameters. Unfortunately, this approach only allows for bacterial quantification, it does not take into consideration bacterial strains whose metabolic energy is used exclusively for maintenance, survival and DNA repair mechanisms instead of growth. Therefore, our study aims to detect the survivability and metabolic activity of this microbe at different temperatures down to −5°C and in the presence of different types and concentrations of sulphate salts using molecular approaches as a proxy to estimate the survival of psychrophilic sulphate reducers in sulphates-rich planets like Mars and icy satellites such as Europa, Ganymede and possibly other satellites from the Jovian and Saturn systems. This type of research is crucial to understand the implications of the presence of these chemical species in these planetary systems and satellites, as it could potentially elucidate whether microbial life and habitability are possible elsewhere in the Universe (Cockell *et al.*, 2016). Furthermore, we are interested to understand the molecular mechanisms involved in the macromolecular flexibility of *D. psychrophila* at suboptimal growth temperatures and in the presence of known chaotropic ions such as Mg^{2+}, Ca^{2+}, Fe^{2+} and Fe^{3+}. These chemical species, which are known to affect the water activity in cold environments, could potentially trigger the formation of aqueous films at the surface or subsurface of Mars and icy satellites. The latter combined with the occurrence of other conditions conducting of life (organics, absence of UV irradiation) provides a platform to consider the existence of microbial life in these environments (Moores and Schuerger, 2012; Cockell *et al.*, 2016).

Materials and methods

Culture conditions

Active cultures of *D. psychrophila* (DSM 12343) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) collection. Furthermore, the medium designated as DSMZ141 was selected to replicate this bacterium since our analyses indicated higher levels of bacterial growth when compared to cultures grown in medium 861a under optimal conditions of temperature and sulphate salts concentrations. Moreover, DSMZ141 maintained its original composition when supplemented with different sulphates as no undesired compounds or secondary reactions were detected in simulations performed with the Geochemist Workbench Professional software release 12 (Bethke *et al.*, 2018). Importantly, DSMZ141 contains a plethora of carbon sources (nutrients and vitamins) which are unlikely to be present in the Martian regolith. However, this medium was used to cultivate our *D. psychrophila* cells since the principal objective of this research is to detect metabolic activity (dependent variable) under a pre-determined set of temperatures and sulphate compounds (our independent variables). Consequently, we excluded the effects of organic materials from our analyses. Despite this, it is important to recognize that planets and icy satellites are constantly bombarded by meteorites containing carbon sources which facilitates the development of complex chemistries (i.e.: tholins, aliphatic and aromatic compounds). Furthermore, recent data from Perseverance and its SHERLOC instrument has detected the presence of organics associated with sulphate salts (Scheller *et al.*, 2022; Ansari, 2023). However, a definite answer about the presence or absence of organic compounds at the Martian surface or subsurface is still elusive. An enquiry that becomes even more challenging as in-situ

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Table 1. *Modified DSMZ141 culture medium (Adopted from DSMZ culture collection) and sulphate concentrations used for temperature positive control (10°C), experiments at −5, 0°C and growth negative controls (sulphate compounds are numbered)*

Component	Quantity	Sulphate compound concentration	
KCl	0.34 g	**1**:$MgSO_4$ 0.35 wt %	$MgSO_4$ 0.35 wt % C-
$MgCl_2$. $6H_2O$	4 g	**2**:$CaSO_4$ 0.1 wt %	$CaSO_4$ 0.1 wt % C-
NH_4Cl	0.25 g	**3**:$MgSO_4$ 10 wt %	$MgSO_4$ 10 wt % C-
$CaCl_2$. $2H_2O$	0.14 g	**4**:$MgSO_4$ 18 wt %	$MgSO_4$ 18 wt % C-
K_2HPO_4	0.14 g	**5**:$FeSO_4$ 10 wt %	$FeSO_4$ 10 wt % C-
NaCL	18 g	**6**:$FeSO_4$ 14 wt %	$FeSO_4$ 14 wt % C-
Trace elements solution[a]	10 ml	**7**:$Fe_2(SO_4)_3$ 10 wt %	$Fe_2(SO_4)_3$ 10 wt % C-
$Fe(NH_4)_2(SO_4)_2 \times 6H_2O$	2.0 ml	**8**:$Fe_2(SO_4)_3$ 20 wt %	$Fe_2(SO_4)_3$ 20 wt % C-
sltn (0.1% w/v)		**9**:$Fe_2(SO_4)_3$ 30 wt %	$Fe_2(SO_4)_3$ 30 wt % C-
Na-acetate[b]	1 g	**10**:$Fe_2(SO_4)_3$ 40 wt %	$Fe_2(SO_4)_3$ 40 wt % C-
Yeast Extract[b]	2 g	**11**:$Fe_2(SO_4)_3$ 48 wt %	$Fe_2(SO_4)_3$ 48 wt % C-
Trypticase peptone[b]	2 g		
Na-resazurin sltn (0.1% w/v)	0.50 ml		
$NaHCO_3$	5 g		
Vit Soltn[c]	10 ml		
Sodium Dithionite[c]	20 mg		
Distilled water	1 l		

C- denotes growth negative controls.

All ingredients were sparged with 80% H_2 and 20% CO_2 gas mixture, except for bicarbonate, vitamins and sodium dithionite which were sparged with N_2 gas. All cultures were kept under 80% H_2 and 20% CO_2 gas mixture during incubation.

[a]Follow instructions from DSMZ141 standard preparation.

[b]This medium contains high concentrations of carbon sources. We have decided to include them in virtue of the fact that many planets and icy satellites are constantly bombarded by meteorites containing complex carbon sources from interstellar space. Also, many icy bodies possess a complex chemistry that produces complex carbon sources such as tholins and other aliphatic and aromatic carbon sources (Titan for example). We have decided to eliminate this confounding variable from our analysis by providing all our cultures with a plethora of carbon sources.

[c]Taken from DSMZ195c.

experiments have failed to determine the exact abundance of organics present in the Martian regolith, sample return missions are still in process, and the existence of carbon sources depends on factors such as resistance to UV decomposition, accretion rate (if any) and rate of UV decomposition (Pizzarello, 2006; Moores and Schuerger, 2012; Pavlov *et al*., 2014; Poch *et al*., 2014; Stalport *et al*., 2019).

DSMZ´s instructions on how to inoculate and cultivate this microorganism under anaerobic conditions were followed. After obtaining fresh cultures of *D. psychrophila* incubated at 10°C, 25 ml serum bottles containing 15 ml of a modified version of DSMZ141 (Na_2S was replaced with sodium dithionite at a concentration of 20 mg l^{-1}) were inoculated at a concentration of 1.5×10^8 cells and incubated at 10°C for 30 days. As *D. psychrophila* duplication time is 27 h (Knoblauch *et al*., 1999), we estimated a concentration of 10^{16} bacterial cells at the end of the 30 days period. These starter cultures were used to inoculate a temperature positive control at 10°C and two different experiments at 0 and −5°C at a concentration of 1.5×10^8 cells in triplicate ($MgSO_4$, was replaced by different sulphate compounds at increasing concentrations, see Table 1).

Simultaneously, growth negative controls (non-inoculated serum bottles containing media) were prepared for all sulphate concentrations at the three temperatures of incubation (making 66 cultures per temperature, 198 cultures total, see Table 1). After inoculation, all cultures were transported on ice and incubated at their respective temperatures for 30 days. Experiments at 0 and −5°C were performed in a biochamber known as Pegasus located at the Keck Lab of the University of Arkansas (Fig. 1), specifics for which have been described in Kral *et al*. (2011). Briefly, Pegasus is a stainless-steel vacuum chamber with dimensions of 76 cm × 51 cm shaped as a horizontal cylinder with three

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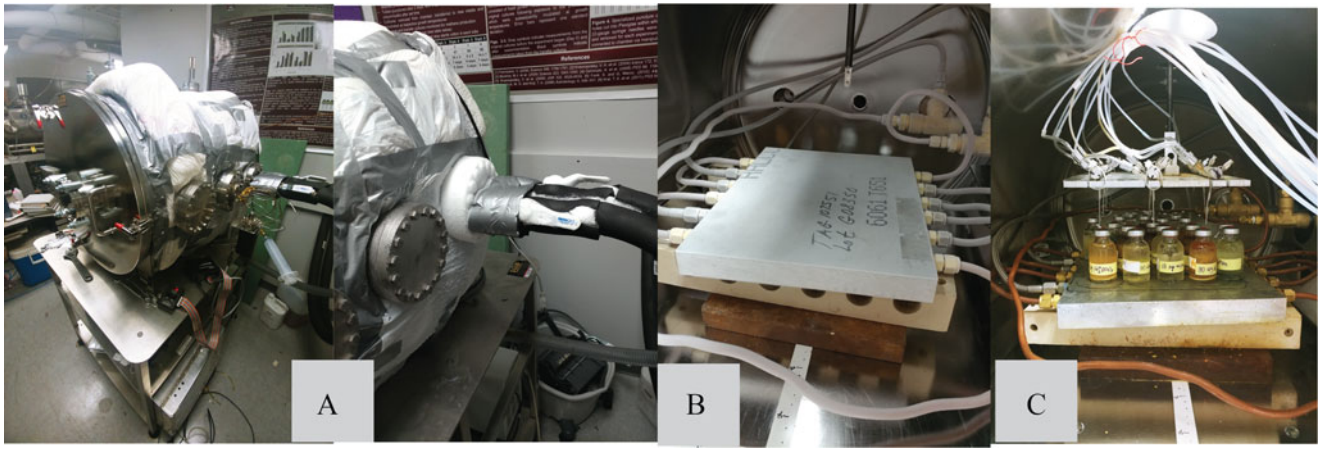

Figure 1. *Pegasus biochamber at the Keck Lab of the University of Arkansas. (a) Biochamber possesses a chiller connected to a (b) cooling plate which is positioned inside of the chamber for (c) cold experiments. The plate is connected to a Lauda Chiller. The Pegasus biochamber was used to incubate temperature positive controls, the experiments at 0 and −5°C and the growth negative controls. Batches of cultures were placed on top of the plate for 30 days. The biochamber was oxygen-evacuated, sealed and CO_2 was bled into the interior to avoid oxygen contamination. A palladium catalyst box was used to eliminate oxygen (if present).*

viewing units (two on the top, and one on the front door). It has a low-pressure component which allows the operator to lower the pressure down to 10^{-7} mbar, it carries all the components required to replace the atmosphere for the desired gases mixtures, and it has an oxygen-scrubbing catalyst box (Kral *et al*., 2011).

In addition to the previous descriptions, a refrigeration component was incorporated by means of a Lauda SmartCool System that uses a heat transfer liquid (silicone oil-based) known as kyro 90 (which supports temperatures down to −90°C). Moreover, the inoculated samples were placed in the interior of the biochamber on top of a metallic hollow plate (platform) which was filled with Kyro 90. The latter was constantly recirculated from the cooling system to the platform using a series of insulated hoses that allowed us to keep the refrigeration in an enclosed system. Furthermore, in all experiments, bacterial growth was assessed by direct observation and comparison with the temperature positive controls and the growth negative controls (non-inoculated cultures). Samples were classified as positive if there was a white precipitate (biomass) formed at the bottom of the serum bottles. Equally important, direct evidence of sulphate reduction was recognized only if this white precipitate turned blackish (deposition/sedimentation of sulphites and sulphides) (Rüffel *et al*., 2018).

Cultures growth

Cultures supplemented with $MgSO_4$ and $CaSO_4$ were evaluated for growth by means of optical density (OD or turbidity) at 600 nm using a Perkin Elmer UV/VIS spectrometer Lambda Bio 20. Aliquots of 1 ml sterile medium (specific for different cultures supplemented with different sulphate concentrations) were used to adjust the equipment. Promptly after, measurements of 1 ml of each sample were performed in triplicate: four cultures and four negative controls at three different temperatures in triplicate make up a total of 72 cultures. Furthermore, 216 measurements were taken by collecting three measurements per bottle. The optical density data generated was analysed by means of pairwise *t*-test at a confidence level of 95% ($\alpha = 0.05$).

As $FeSO_4$ and $Fe_2(SO_4)_3$ samples contained sediments that interfered with proper OD measurements, we assessed the growth of these samples by means of fluorescence using a fluorescence spectrophotometer Hitachi F-7000. Furthermore, we performed a live/dead cells evaluation using the live/dead BacLight kit from Invitrogen. This kit allows for differential staining of live cells (SYTO 9, green-fluorescent nucleic acid stain) and dead cells (propidium iodide, red-fluorescent nucleic acid stain). Moreover, this method takes advantage of the fact that SYTO 9 labels all bacterial cells (with intact or damaged membranes) while propidium iodide only labels cells with damaged membranes. All samples as well as growth negative controls at 10, 0 and −5°C were assessed in triplicate (594 measurements total). After collecting 1 ml of culture in 1.5 ml tubes under anoxic conditions, we centrifuged the samples to aggregate all cells at the bottom of the tube. Subsequently, we washed the cells three times with 1 ml of sterile/anoxic 0.85% NaCl solution. After washing the cells pellet, we resuspended them in 1 ml of sterile/anoxic 0.85% NaCl solution. Immediately after, we mixed the cells suspensions with the fluorescence mix (composed of 3 µl of component A, 3 µl of component B, and 1 ml of sterile/anoxic 0.85% NaCl solution per sample). The equipment was blanked using a preparation containing 1 ml of sterile/anoxic 0.85% NaCl solution (instead of cells suspensions) and 1ml of fluorescence mixture. Results were normalized using fluorescence detected in growth negative controls specific for each supplementation. Data generated was analysed by means of pairwise *t*-test at a confidence level of 95% ($\alpha = 0.05$).

Molecular analysis (DNA and RNA extractions)

Bacterial Genomic DNA from starter cultures was extracted using the MOBIO Microbial DNA Isolation kit. This extraction was performed using proteinase K (20 mg ml^{-1}) and RNase A (10 µg ml^{-1}) incubations steps. Furthermore, DNA concentration and purity were assessed by means of a NanoDrop 2000 Spectrophotometer (ThermoScientific). Once the A260/A280 ratio was 1.8 or

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above, amplifications of the dsrAB operon (2.5 kb) were performed using the universal primers, DSR1FD 5′ – ACTCACTGGAAGCACG-3′ and DSR4RE 5′ – GTGTAACAGTTACCACA-3′. The PCR protocol used was Activation 95°C for 5 min, Denaturation 95°C for 1 min, Annealing 54°C for 1 min, Extension 72°C for 9 min and Final extension 72°C for 10 min, with 30 cycles. Then, an agarose electrophoresis was used to identify the dsrAB amplicon under UV light. Growth negative controls and temperature positive controls as well as positive PCR amplifications were used to assess the purity of the cultures selected for RNA isolation (control at 10°C and experiments at 0 and −5°C).

On the other hand, the dsrAB operon was sequenced using purified PCR products, and the resulting sequence was utilized to design qPCR-MCA (real time polymerase chain reaction-melt curve analysis) primers. Specifically, dsrAB fragments were sequenced by the UAMS DNA sequencing core facility using a 3500xL genetic analyser (Sanger sequencing technology), our purified PCR products as templates (20–50 ng μl^{-1}), and the primers pair DSR1FD/DSR4RE at a 1.6 µM concentration. In addition, dsrAB sequenced fragments were analysed, trimmed, edited, and assembled with Geneious while the qPCR-MCA primers were designed with the IDT software (ThermoFisher). These primers named D.psychroFwd100 (5′-ATCGGTAGCAGGAGTATGACA-3′) and D.psychroRev100 (5′-AAGCCGTGGCAACAAGT-3′) were tested for specificity by amplification of *D. psychrophila* genomic DNA and agarose gel electrophoresis of the PCR products.

RNA was extracted from all replicas (198 samples) of the temperature positive control, the experimental cultures, and the growth negative controls using TRIZOL (Invitrogen). Furthermore, purifications/gDNA digestion were performed using the QIAGEN kit (RNeasy Mini Kit). RNA concentrations were measured using a NanoDrop 2000 Spectrophotometer (ThermoScientific). Samples were kept frozen at −20°C until downstream analyses were performed. cDNAs of samples were synthesized using the High-Capacity cDNA Reverse Transcription kit of Applied Biosystems (AB) which uses random primers to target RNAs. cDNA synthesis was performed using 1 µg of RNA from each individual sample as a template.

Given the fact that most molecular techniques applied to cold environments have been used only for diversity studies (Poli *et al*., 2017), we decided to use qPCR-MCA as a direct method to quantify dsrAB expression. Furthermore, this technique was performed using the primers D.psychroFwd100 and D.psychroRev100 and EvaGreen Dye (Biotium). Temperature positive controls, experimental samples and growth negative controls cDNAs were seeded in 96 well plates for qPCR-MCA in triplicate (594 measurements total). Once plates were seeded with primers, dye and cDNA template, they were subjected to the Melt Curve protocol: 90.0°C for 0:30 s, 90.0°C for 0:15 s, 59.0°C for 0:15 s, 72.0°C for 1 min, 10 cycles; 90.0°C for 0:15 s, 59.0°C for 1 min, 72.0°C for 0:30 s and Plate read (Fluorophore SYBR), 35 cycles; 72.0°C for 3 min, 90.0°C for 0:15 s, 65.0°C for 3 min and Melting curve with the next parameters: 75.0 to 90.0°C with an increment of 0.1°C every 0:05 s and a Plate read (Fluorophore SYBR) at the end of every temperature increment. The real time PCR system used was BIO-RAD CFX96 Real Time System. Standard curves were used to estimate copy numbers per reaction. They were formulated by including co-amplifications of known amounts of *D. psychrophila* DNA in qPCR analyses. Five consecutive dilutions (1:5) were prepared containing 10^9 to 10^6 copies of the dsrAB operon per reaction. Amounts of the targeted dsrAB sequence were obtained by plotting the Ct values onto the standard curve. Finally, differences in expression were analysed by means of an ANOVA and pairwise-*t*-test analysis at a confidence level of 95% ($\alpha = 0.05$).

Methylene blue (MB) analysis

In order to detect sulphides in solution and consequently sulphate reduction, MB analysis of the temperature positive control, all sample replicas and growth negative controls was performed using the MB protocol used by Basic *et al*. (2015) in triplicate (594 measurements) (Cline, 1969; Johnston *et al*., 2005; Basic *et al*., 2015; Thorup *et al*., 2017). By using this protocol, we aimed to detect the presence of dissolved sulphides-sulphur (H_2S, HS^-, S_2^-) in our cultures. Furthermore, 10 µl of each sample were seeded in 96 well plates containing 72 µl of solution A (0.1 mM of diethylenetriaminepentaacetic acid,

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pH = 9.6). Immediately after, 18 µl of solution B was added (contains 17.1 mM of N, N-dimethyl-p-phenylenediamine sulphate and 37 mM $FeCl_3$ in 6 M HCl). Sample preparations were made in triplicates per sulphate concentration and temperature. Subsequently, they were read using the Synergy HT microplate reader from BioTek at a wavelength of 668 nm after 30 min of adding solution B to each well. Results were normalized using absorbance of growth negative controls for each supplementation. Data analysis and significant differences were estimated using an ANOVA and pairwise *t*-test analysis at a 95% confidence level ($\alpha = 0.05$).

Results

D. psychrophila *growth under various sulphate salts*

As a general practice, bacterial growth in liquid medium can be identified by direct observation and further detection of biomass production (turbidity). In our experiments, samples were considered positive for growth (cellular proliferation/regardless of conditions) if there was a thin white precipitate formed at the bottom of the serum bottles containing *D. psychrophila* cells. Furthermore, if samples presented a blackish precipitate or black particles resuspended in the liquid, they were considered positive for sulphate reduction. As it is shown in Fig. 2 (A1 and A2), cultures supplemented with 0.35 wt% of $MgSO_4$ at 0 and 10°C showed evidence of bacterial growth (biomass) along with sulphites/sulphides sedimentation. However, cultures incubated at −5°C showed only minimal growth (A3). Furthermore, samples cultivated under 0.1 wt% of $CaSO_4$ at 0 and 10°C were positive for both biomass production and sulphites/sulphides sedimentation as shown in Fig. 2b (B1 and B2). Like $MgSO_4$ cultures, minimal growth was detected at −5°C (B3).

In Our analysis of turbidity (OD600 nm), we can see that in cultures supplemented with $MgSO_4$ or $CaSO_4$ most growth occurred at 0°C (regardless of sulphate concentration). Importantly, growth at −5°C was minimal at all concentrations (*t*-test, $\alpha = 0.05$) (see Fig. 3).

Furthermore, our live/dead cells analysis showed similar results to our analysis of optical density for $MgSO_4$ and $CaSO_4$ supplementation where the highest bacterial growth occurred at 0°C and the lowest occurred at −5°C (*t*-test, $\alpha = 0.05$) (Fig. 4). Interestingly, supplementations with $MgSO_4$ 10 and 18 wt% at −5°C showed minimal or no indication of active cell division (bacterial growth). Similarly, there was no indication of active growth in cultures supplemented with $FeSO_4$ at −5°C at any of the concentrations tested, and only minimal growth was registered at 10 and 0°C. In addition, $Fe_2(SO_4)_3$ supplementations showed increased growth at 10 wt% under all incubation temperatures, and at increasing concentrations in −5°C cultures. Finally, minimal growth was detected in $Fe_2(SO_4)_3$ supplementations (concentrations 40 and 48 wt%) at 10 and 0°C (Fig. 4).

Molecular analysis

In qPCR-MCA, the cycle quantitation (Ct) value represents the PCR cycle number at which initial fluorescence is detected. This value is inversely proportional to the number of copies of the targeted sequence (cDNA) which is reverse transcribed from the original sample (RNA). Therefore, in our experiments, lower Ct values indicate higher copy numbers of dsrAB transcripts. As all RNAs, in our controls and experiments at low temperatures, were subjected to a reverse transcription process using random primers, the specificity of detection of the dsrAB operon expression relies on the qPCR-MCA primers. Consequently, they were used to assess whether *D. psychrophila* can metabolize at low temperatures in the presence of various concentrations of sulphate compounds. As shown in Figs. 5 and 6, cultures containing $MgSO_4$, and $CaSO_4$ at any of the concentrations and temperatures tested showed high copy numbers of the dsrAB targeted sequence (*t*-test $\alpha = 0.05$). On the other hand, $FeSO_4$ supplementations showed abundant dsrAB copy numbers at any of the concentrations tested, except for the 10 wt% samples incubated at 10°C which showed a downregulation of the dsrAB expression when normalized (see Figs. 5 and 6).

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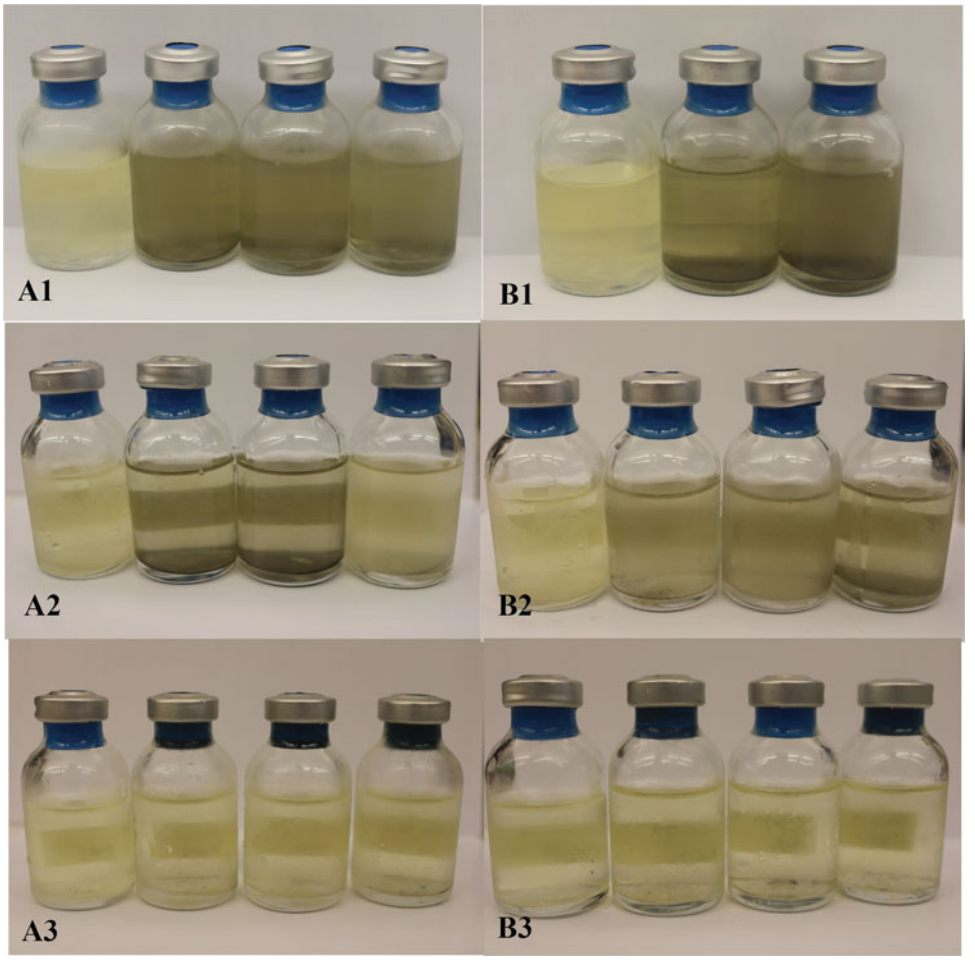

Figure 2. D. psychrophila *growth and sulphide formation – in media supplemented with (a) $MgSO_4$ 0.35 wt% incubated at (A1) 10°C, (A2) 0°C and (A3) −5°C; (b) $CaSO_4$, 0.1 wt % incubated at (B1) 10° C, (B2) 0°C and (B3) −5°C. The first serum bottle (from left to right) in each series is the growth negative control which shows no bacterial growth or sulphide formation. Blackish colour in each serum bottle denotes sulphide formation.*

Furthermore, cultures supplemented with $MgSO_4$ 0.35 wt% showed similar copy numbers at −5°C (subfreezing) and 10°C (optimal growth temperature) while cultures at 0°C were downregulated (see Figs. 5 and 6). Moreover, supplementations with increasing concentrations of MgSO4 (10 and 18 wt%) did not show significant differences among the three temperatures tested (*t*-test, $\alpha = 0.05$) (see Figs. 5 and 6).

Interestingly, high copy numbers were detected at −5°C in cultures supplemented with $CaSO_4$ 0.1 wt% when compared with the temperature positive control at 10°C and the cultures at 0°C (Figs. 5 and 6). Like the $MgSO_4$ samples, all $CaSO_4$ supplementations at the three temperatures tested showed increased copy numbers (*t*-test, $\alpha = 0.05$).

In the samples supplemented with $FeSO_4$ at 10 wt% cells grown at 0 and −5°C had similar detection levels and they were both significantly different than the temperature control at 10°C. Like $MgSO_4$ at increasing concentrations, $FeSO_4$ at 14 wt% did not show differences in copy numbers among the temperatures tested. Given that these experiments are focused solely on the detection of dsrAB transcripts, it is important to note that high copy numbers of this product were present in cultures supplemented with both concentrations of $FeSO_4$ (*t*-test, $\alpha = 0.05$) (see Figs. 5 and 6).

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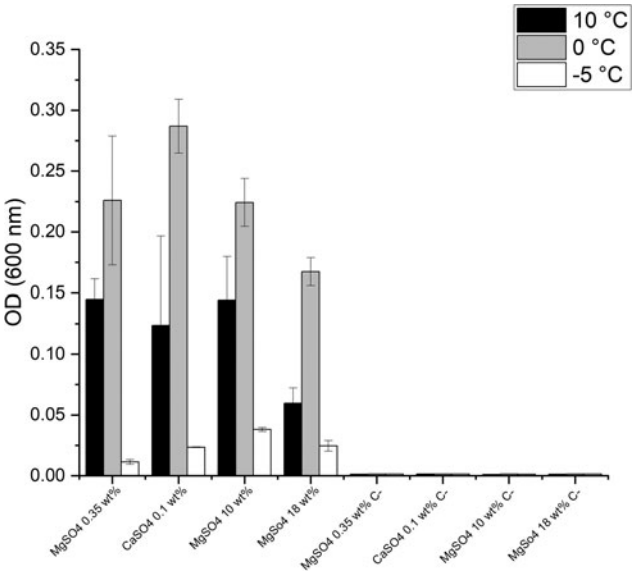

Figure 3. *OD measurements of* D. psychrophila *cultures subjected to $MgSO_4$ and $CaSO_4$ at three different temperatures. (10°C = temperature positive control, 0°C = experimental 1, −5°C = experimental 2, C- = Growth negative controls). N = 216 measurements. 1 ml of each sample was subjected for OD analysis using a Perkin Elmer UV/VIS spectrometer Lambda Bio 20, adjusted to 600 nm, under anoxic conditions. There were 72 samples (24 per temperature) which were analysed in triplicate making 216 measurements.*

Finally, all samples supplemented with $Fe_2(SO_4)_3$ did not show significant differences in copy numbers among the temperatures tested (*t*-test, $\alpha = 0.05$) (see Figs. 5 and 6).

Sulphate reduction (enzymatic activity)

In our analysis of sulphate reduction (MB method), we quantified the absorbance of sulphides in solution as a direct measurement of dsrAB enzymatic activity. Interestingly, we found that cultures supplemented with 0.35 wt% of $MgSO_4$ at −5°C showed the highest sulphate reduction activity, the temperature positive control at 10°C presented the opposite behaviour while the cultures at 0°C exhibited intermediate levels of sulphate reduction (Fig. 7) (*t*-test, $\alpha = 0.05$). Furthermore, cultures supplemented with $MgSO_4$ at increasing concentrations (10 and 18 wt%) showed significant differences in comparison with the growth negative controls. However, cultures at 10°C under $MgSO_4$ 18 wt% showed the highest sulphate reduction (*t*-test, $\alpha = 0.05$) (see Fig. 7). Additionally, cultures grown with $CaSO_4$ showed the highest rate of sulphate reduction at 0°C followed by the cultures at 10 and −5°C both of which had similar sulphate reduction rates (*t*-test, $\alpha = 0.05$). Finally, the data from our MB analysis pointed out the absence of sulphides in solution in any of the $FeSO_4$ supplementations for any of the temperatures tested (*t*-test, $\alpha = 0.05$) while $Fe_2(SO_4)_3$ supplementations showed only minimal sulphate reduction rates at 0 and −5°C (Fig. 7).

Discussion

D. psychrophila *dsrAB copy numbers detection at low temperatures as a model of sulphate brines colonizer*

D. psychrophila is an organism adapted to grow at low temperatures (growth range from 10°C down to −1.8°C) and a sulphate reducer. These two characteristics are paramount to conceive scenarios in

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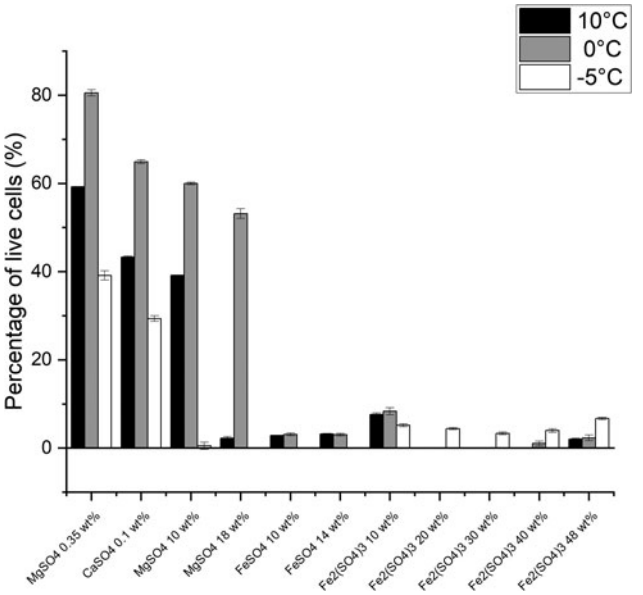

Figure 4. *Percentage of live cells in cultures of* D. psychrophila *subjected to increasing concentrations of sulphate salts at three different temperatures. (10°C = temperature positive control, 0°C = experimental 1, −5°C = experimental 2, C- = Growth negative controls). Samples were assessed by live/dead fluorescence analysis using SYTO 9 and propidium iodide. Samples were normalized using growth negative controls fluorescence. Specifically, SYTO 9 dyes all cells with intact or damaged membranes (viable and non-viable cells), while propidium iodide labels cells with damaged membranes (non-viable), by taking the difference in the bulk fluorescence, this technique allows us to estimate the percentage of cells that are viable in the sample. N = 594 measurements (198 cultures in triplicate).*

which this microbe or similar microbes could potentially colonize other planets, icy body subsurfaces or subsurface oceans. In our experiments we exploited these parameters to an extent that allowed the detection of growth, transcriptional and metabolic activity, at the extremes of the microbes' survival window. Our findings suggest that *D. psychrophila* can synthesize dsrAB mRNAs optimally at lower temperatures than expected (down to −5°C) in cultures supplemented with 0.35 wt% of $MgSO_4$. As it can be seen in Figs. 5 and 6, cultures at −5°C showed no differences in dsrAB copy numbers when compared with the optimal temperature conditions at 10°C (temperature positive control). From a Microbiological standpoint, the ability of this microbe to transcribe at similar rates at −5 and 10°C in pure culture is surprising since the cell proliferation was considerably lower in −5°C cultures (see Figs. 3 and 4). It is possible that the few cells present in these samples were halted in the second or third classification of Price and Sowers in which most energy generated is used exclusively for maintenance and survival rather than for growth (Price and Sowers, 2004). Interestingly, given the function of the DsrAB in the production of ATP, prioritizing maintenance and survival in these cells would end up creating an ATP generating loop in which energy initially available would be used to synthesize more DsrAB and then these enzymatic units would generate more energy in return (ATP synthesis from sulphate reduction). Surprisingly, cultures at 0°C which exhibited the highest growth showed the lowest dsrAB copy numbers. According to these results, a major portion of the cells from these cultures (subclonal population) would prioritize growth rather than energy generation which is evident from the estimations of cell division (OD measurements and percentage of live cells) and the low dsrAB copy numbers detected (compared with the other two cultures in Figs. 3–6). Congruently, cells at 10°C seem to be in an intermediate state in which cells prioritized both microbial growth (high cell division rate) and maintenance (high dsrAB copy numbers).

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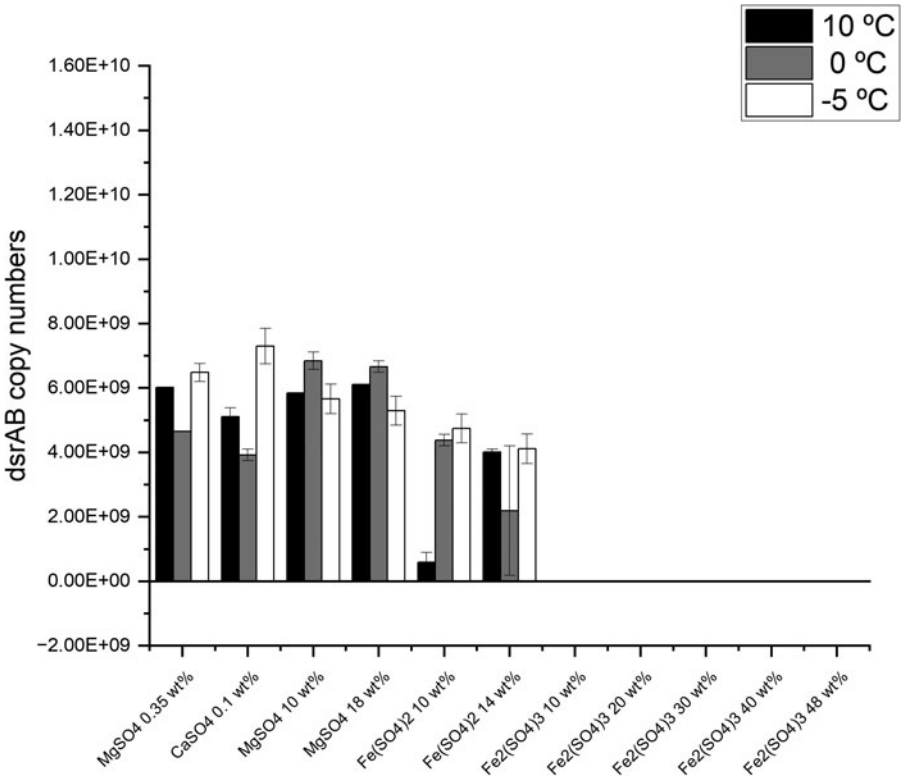

Figure 5. *Detection of Expression of dsrAB operon in samples of* D. psychrophila *cultures grown in different types and concentrations of sulphate compounds classified by dsrAB copy numbers obtained from qPCR-MCA. (Temperature positive control at 10°C, experiment at 0°C and experiment at −5°C). Relative abundance was estimated from standard curves and copy numbers were calculated. The amplification efficiencies of samples and standard curves were between 99.6 and 99.8%. N = 594 measurements (198 samples were measured in triplicate).*

Importantly, the survival capabilities of *D. psychrophila*, and its ability to fine tune its energy requirements and withstand subfreezing temperatures down to −5°C is remarkable since the only other microorganism reported to withstand this temperature in pure culture is the psychrotolerant *Trichococcus patagoniensis* PmagG1^{T}, which was isolated from penguin guano. To add more value to this finding, from the literature we found out that most bacteria reported to survive subfreezing temperatures to date have been reported *in situ* or in mixed environmental communities and not pure cultures (Pikuta *et al*., 2006).

Interestingly, an analogous phenomenon was registered for the *D. psychrophila* cultures subjected to $CaSO_4$ at 0.1 wt%. According to our results, this microbe had high dsrAB copy numbers at −5°C followed by the temperature positive control at 10°C and the experiment at 0°C (See Figs. 5 and 6). Interestingly, like the $MgSO_4$ supplementations, the cell's growth at −5°C was downregulated, the 0°C was upregulated and the 10°C was in between those two levels (see Figs. 3 and 4). As it was mentioned previously, it is possible that *D. psychrophila* cells that were incubated at −5°C coupled most of their metabolic machinery to maintenance functions while portions of the cells incubated at 10 and 0°C redirected it to prioritize both growth and maintenance functions. These findings along with the ones for $MgSO_4$ emphasize the concept coined by Moyer and Morita (2007) in which they stated that psychrophilic microorganisms have adapted to cope with extreme cold environments and the result of that can be measured by the abundant production of mRNA transcripts and energy generating enzymes i.e.:

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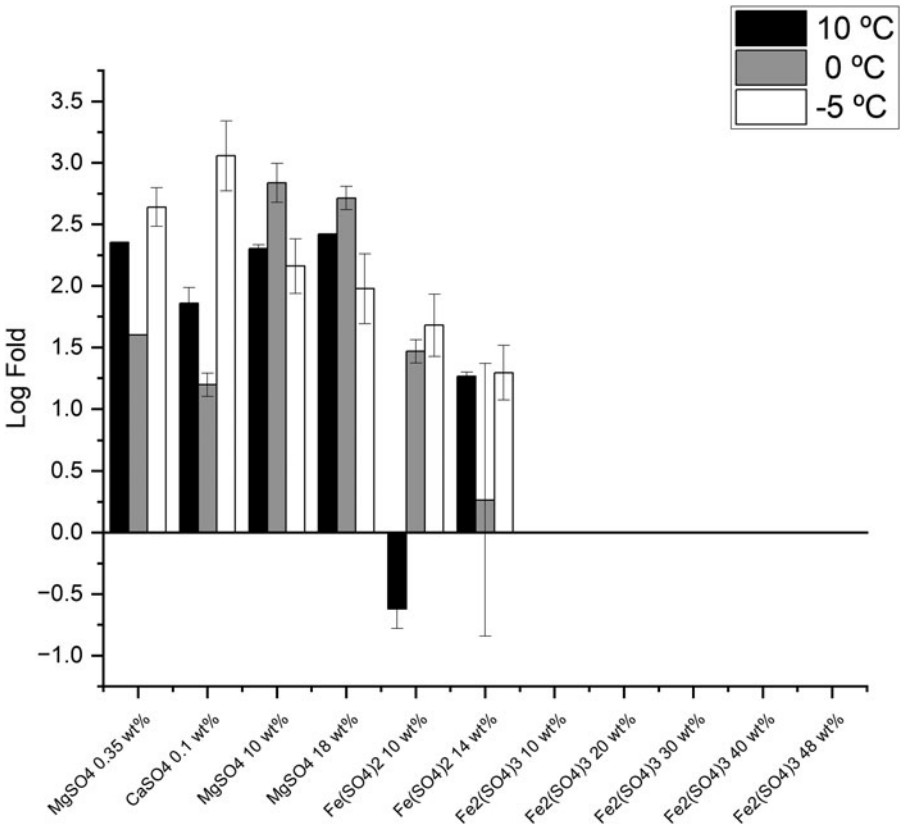

Figure 6. *Log Fold Expression of dsrAB operon in samples of* D. psychrophila *cultures grown in different types and concentrations of sulphate compounds classified by Log Fold of dsrAB transcripts. (Temperature positive control at 10°C, experiment at 0°C and experiment at −5°C). CT values were plotted on standard curves and extrapolated to Log Fold dsrAB expression. The amplification efficiencies of samples and standard curves were between 99.6–99.8%. N = 594 measurements (198 samples were measured in triplicate).*

DsrAB in the case of sulphate reducers. Furthermore, taking into consideration that this microorganism can undergo transcription (biosynthetic process) at lower temperatures than expected under optimal $MgSO_4$ concentration and with a different source of sulphate anions ($CaSO_4$), we can propose that *D. psychrophila* can potentially survive and metabolize rather than proliferate in environments similar to those present in the subsurface of other planets, icy bodies or subsurface oceans where brines or highly concentrated environments containing sulphate anions, and chaotropic cations (such as Mg^{2+} and Ca^{2+}) as well as other environmental conditions conductive of life are present (Inniss, 1975; Morita, 1975; Knoblauch *et al*., 1999; Rabus *et al*., 2004; Moyer and Morita, 2007).

Additionally, experiments at increasing concentrations of $MgSO_4$ (10 and 18 wt%) showed no difference in dsrAB copy numbers when compared by temperature within groups, even though cultures at 0 and 10°C showed the highest cell density (see Figs. 3–6). Interestingly, if we analyse the OD/live/dead results by MgSO4 concentration and temperature tested, we can observe that cellular proliferation decreased as $MgSO_4$ concentrations increased (Figs. 3 and 4). Equally important, cultures at −5°C showed dsrAB copy number levels that were indistinguishable from the other temperatures tested (Figs. 5 and 6). This behaviour is analogous to the behaviour presented in the experiments with $MgSO_4$ at 0.35 wt% and $CaSO_4$ at 0.1 wt% under subfreezing temperatures (−5°C) where cells prioritized maintenance (dsrAB copy numbers were comparable to the ones presented in the temperature

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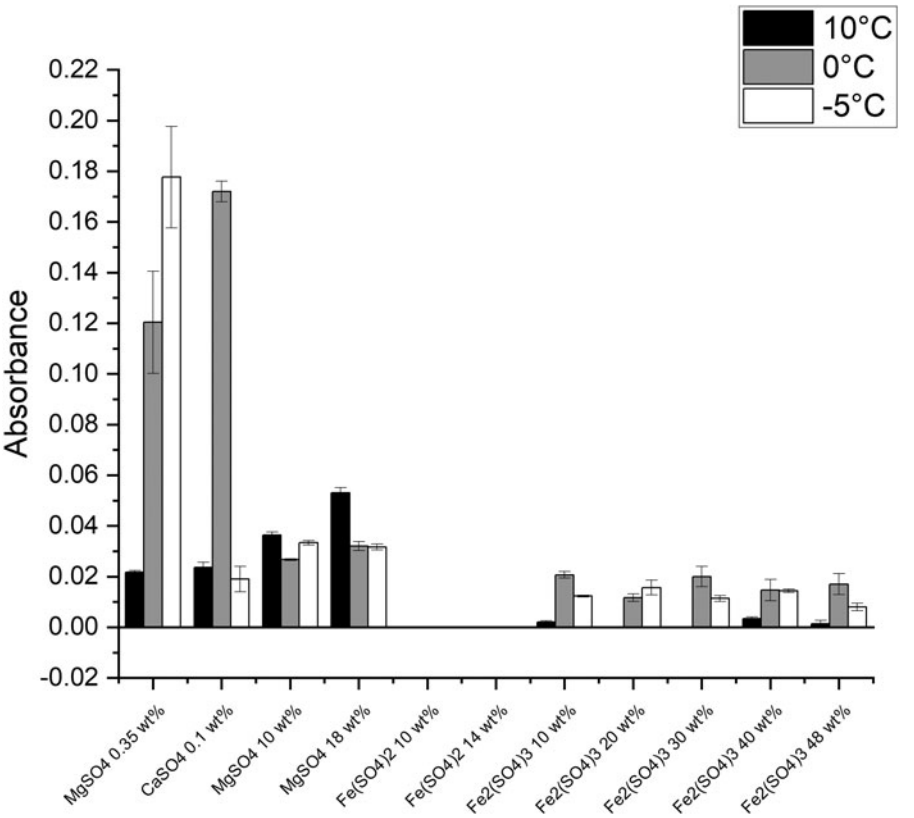

Figure 7. *Sulphides in solution measurements using MB protocol of samples subjected to three different temperatures and different sulphate concentrations. High absorbances indicate increasing concentrations of sulphides in solution (H_2S, HS^-, S_2^-). Note that the highest absorbance was registered at −5°C under $MgSO_4$ 0.35 wt%, followed by the cultures at 0°C. Surprisingly, the highest absorbances were detected with $CaSO_4$ 0.1 wt% at 0°C. C- denotes absorbance from growth negative controls used for normalization. N = 594 measurements (198 samples were measured in triplicate).*

positive control and the 0°C experiment) rather than active growth (no growth or minimal growth). However, it is crucial to understand that all supplementations presented high dsrAB copy numbers (see Figs. 5 and 6). Under these circumstances, we can observe that cells at 10 and 0°C behave in a way that prioritizes both maintenance (increased dsrAB copy numbers) and cellular growth (high cell density) as opposed to the −5°C cultures which coupled their metabolic machinery to mostly maintenance functions. It is imperative to notice that cell viability (growth) decreased with increasing concentrations of $MgSO_4$ (see Figs. 3 and 4). This finding suggests that this mineral possesses a lower and an upper concentration limit in which cell viability is possible. According to the latter, conditions above that upper limit could generate a disadvantageous environment for the cell to survive (osmotic stress). However, our results indicate that this sulphate compound, containing chaotropic magnesium cations, can still induce macromolecular flexibility which allows this microorganism to keep its transcriptional machinery functional at suboptimal temperatures.

As it was stated before, the cultures supplemented with $FeSO_4$ at 10 wt% and incubated at 0 and −5°C presented the highest dsrAB copy numbers in comparison with the positive temperature control at 10°C. However, our results from the live/dead analysis indicated only minimal growth or absence of active cell proliferation in these cultures. A possible explanation for this behaviour could be that cells that were used to inoculate the cultures coped with these conditions, at least temporarily, and synthesized dsrAB mRNAs

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which were then detected in our qPCR-MCA analysis. The same behaviour was observed at increasing concentrations of $FeSO_4$ (14 wt%) in which there were no indications of active cellular growth (see Fig. 4), but the analysis of dsrAB expression revealed the presence of mRNAs (as indicated in Figs. 5 and 6).

This outcome suggests that $FeSO_4$ at any concentration does not offer an advantage for colonization (cell division) at subfreezing temperatures. However, based on the dsrAB copy numbers analysis, this microorganism can transcribe in the presence of ferrous sulphate at subfreezing temperatures which could offer survival (no growth/colonization) advantages. This is crucial because it suggests that *D. psychrophila* can remain metabolically active in environments containing ferrous sulphates at temperatures below 0°C such as those present on other planets, icy satellites and subsurface oceans. As mentioned before, it might be possible that the presence of chaotropic ions such as Fe^{2+} can increase the flexibility of molecules that intervene in basic cellular processes such as transcription, cell membrane maintenance and metabolism.

Finally, in the experiments with ferric sulphate, our results showed no significant differences in dsrAB copy numbers by temperature, within subgroups of $Fe_2(SO_4)_3$ concentrations (See Figs. 5 and 6). However, there were differences in the percentage of live cells present in the cultures subjected to 10 wt% in all the incubation temperatures tested, and at −5°C at increasing concentrations of $Fe_2(SO_4)_3$ which indicates minimal growth (See Fig. 4). It is possible that in these cultures, a few of the cells that were inoculated successfully divided ($Fe_2(SO_4)_3$ 10 wt% at all temperatures), but as the sulphate concentration increased the cells incubated in warmer temperatures resulted compromised. However, active growth at −5°C temperatures suggests that *D. psychrophila* could survive and colonize at least temporarily in environments such as planets or icy bodies with high concentrations of ferric sulphate, but eventually the microbe's metabolic machinery and survival might become impaired.

Comparison of dsrAB transcripts and sulphate reduction under various sulphate conditions

In general, an increase in dsrAB copy number detection should be consistent with an increased rate of sulphate reduction in any given bacterial culture. As more dsrAB transcripts are synthesized, more DsrAB enzymatic units should be available to catalyse the transfer of electrons in this anaerobic respiration process. This expected behaviour was observed in our cultures subjected to $MgSO_4$ 0.35 wt%, in which the samples grown at −5°C presented the highest sulphate reduction rates (enzymatic activity, see Fig. 7) and similar dsrAB copy numbers as the positive temperature control even though their growth was low in comparison with the cultures at 0 and 10°C (See Figs. 3–6). As it was mentioned above, it is possible that these cells prioritized maintenance rather than growth which is evident in the increased enzymatic activity detected in our MB analysis. Furthermore, this behaviour is consistent with the expectation that psychrophilic microbes have developed adaptations to enhance enzymatic capacity under extreme conditions of temperature. Moreover, cells incubated at 0°C, with the highest growth and lowest dsrAB copy numbers (among the three temperatures tested), had an intermediate level in sulphate reduction. A behaviour that is consistent with the concept of metabolic specialization in which a major subpopulation of bacterial cells in our cultures facilitated growth while a minor subpopulation prioritized maintenance (Dubnau and Losick, 2006; Davidson and Surette, 2008; Gefen and Balaban, 2009; Levine *et al*., 2012; Rosenthal *et al*., 2018). Accordingly, cultures at 10°C would facilitate both growth and maintenance. On the other hand, it is also important to take into consideration that our cultures at 0 and 10°C presented significant dsrAB detections (see Figs. 5 and 6), and that an increased sulphate reduction rate could be the result of the adaptability of the DsrAB enzyme to catalyse efficiently at subfreezing temperatures. Furthermore, at increasing concentrations of MgSO4 (10 and 18 wt%), which presented the highest growth at 0°C and lowest at −5°C (see Figs. 3 and 4), showed no significant sulphate reduction differences among the temperatures tested (see Fig. 7). However, these cultures showed significant differences with the growth negative controls which indicate presence of sulphides in solution. Therefore, we can deduce that cultures at 10 and 0°C prioritized

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both growth and maintenance (sulphate reduction detected, high cell proliferation) while cultures at −5°C prioritized maintenance (sulphate reduction detected, low cell proliferation).

In the cultures supplemented with CaSO4 0.1 wt%, we observed that the highest sulphate reduction was registered at 0°C (see Fig. 7) which had the lowest dsrAB detection (See Figs. 5 and 6) and the highest growth (See Figs. 3 and 4). Strikingly, these cells prioritized both growth and maintenance even though their dsrAB copy numbers were the lowest. It is possible that functional cold-adapted DsrAB enzymatic units already synthesized in the inocula cells were efficient in generating energy through the sulphate reduction pathway. Cultures at 10 and −5°C showed similar sulphate reduction rates (See Fig. 7) which suggests that cells incubated at 10°C prioritized both growth and maintenance while the cells at −5°C tilted their metabolic balance towards maintenance (Compare Figs. 3–7). Interestingly, cells at −5°C with the highest dsrAB copy numbers presented low sulphate reduction (minimally active growth, active transcription, active sulphate reduction). This behaviour indicates that cells that survived from the inoculum, initially divided, and then redirected their metabolic machinery to maintenance functions. However, this attenuated sulphate reduction (in comparison with cultures at 0°C) suggests the effects of posttranscriptional/posttranslational modifications or protein folding in which DsrAB enzymatic units were rendered partially non-functional (Feller and Gerday, 2003).

Finally, in the cultures supplemented with $FeSO_4$ and $Fe_2(SO_4)_3$ sulphate reduction was absent or minimal which is not surprising since the transcriptional machinery of these cells was impaired even in the cultures in which evidence of growth was identified. However, another explanation for our MB results relies in the fact that sulphide ions (H_2S) in solution could readily react with ferrous or ferric ions (Fe^{2+} and Fe^{3+}) producing FeS or Fe_2S_3 which could have impaired our capability to detect free H_2S.

In summary, the explanation for the behaviours shown in our results points to the fact that some *D. psychrophila* microbial cells were metabolically active at sub-zero temperatures i.e.: producing high numbers of dsrAB mRNAs and fully active DsrAB enzymes, while some of them were only microbial survivors which utilized sulphates at rates compatible with the metabolic specialization of their clonal subpopulations (Price and Sowers, 2004; Dubnau and Losick, 2006; Steven *et al*., 2006; Davidson and Surette, 2008; Gefen and Balaban, 2009; Levine *et al*., 2012; Rosenthal *et al*., 2018). Putting this into our experiment's context, our data suggest that salts such as $MgSO_4$ and $CaSO_4$ induce an increase in metabolic activity rather than growth below 0°C and at warmer temperatures most clonal subpopulations emphasize both growth and maintenance. Interestingly, in some of the latter, attenuated sulphate reduction suggested the effects of posttranscriptional or posttranslational modifications that rendered non-functional or partially functional enzymes.

Equally important, as it was mentioned before, in cold environments the activity of microorganisms is influenced by the presence of certain solutes. Among these, chaotropic/kosmotropic ions are of great importance due to their ability to disarrange or arrange cellular structures, expand the microbial growth/survival window and lower the freezing point of water which increases the water availability for cellular activities. These factors are of enormous importance in Astrobiological studies. Moreover, as it was demonstrated by Chin *et al*. (2010), the microbial growth/survival window, specifically related to the temperature limit constraint, can be modified by the presence of environmental solutes. In that aspect their results validate our experiments with sulphate compounds and cellular survivability, in which $MgSO_4$ and $CaSO_4$ dissociate in water releasing SO_4^{2-} anions and Mg^{2+} and Ca^{2+} cations. As *D. psychrophila* is a sulphate reducer it uses the sulphate anions as terminal electron acceptors in the generation of metabolic energy while the cations act as chaotropic agents. This is crucial in low temperature environments considering that subfreezing conditions reduce the operational spectrum of macromolecular and cellular systems by increasing the stability of its components: increased hydrogen bonding, hydrophobic effect and electrostatic attraction at the protein, DNA, RNA and lipid bilayer levels (Chin *et al*., 2010; Ball and Hallsworth, 2015; Eardley *et al*., 2019; Timson, 2020). In our experiments this highly structured state of the cells macromolecular machinery was presumably affected by the action of $Mg^{2+}$and Ca^{2+} cations which reduced the rigidity of the cellular components and triggered higher dsrAB expression and sulphate reduction rates at temperatures down to −5°C (See Figs. 5–7). In

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virtue of these observations our research team has proposed that lower temperatures increase molecular rigidity at the macromolecular level and as chaotropic ions disturb macromolecular structures at low temperatures, these substances can potentially fluidize the molecular rigidity of cellular macromolecules triggering stable microbial activity under suboptimal conditions (Zhang and Cremer, 2006; Chin *et al*., 2010; Ball and Hallsworth, 2015; Eardley *et al*., 2019; Timson, 2020). This further suggests that supplementation with chaotropic ions in our experiments did enhance cellular function, which is relevant in terms of Astrobiological interest, and relies in the fact that planets such as Mars and other icy bodies contain chaotropic agents in their regolith (Chin *et al*., 2010). Furthermore, colonization of icy bodies by extremophile bacterial species might be possible due to the fact that some terrestrial microorganisms such as *D. psychrophila* have developed adaptations at the lipids, enzymes and biopolymers level that allow them to cope with most extreme conditions (Deming, 2002; Feller and Gerday, 2003; Joseph *et al*., 2008). Equally important, as the Martian temperature is variable throughout the year and from hour to hour (average of −63°C, lowest of −143°C during winter and highest of 35°C), transient liquid water in the Martian polar caps and permafrost is possible (Pikuta and Hoover, 2003; Pikuta *et al*., 2007; Chevrier and Altheide, 2008). Then, it is only logical to think that microbial colonization or survival could be accomplished at least transiently in thin water films at the brine pockets that form the Martian rock/ice interface or the subsurface oceans in icy satellites from the Jovian and Saturn systems. Moreover, as it has been demonstrated in our experiments *D. psychrophila* is able to metabolize at temperatures within the parameters mentioned above. Also, it can resist relatively high concentrations of salts (sulphate compounds as those present in the Martian soil) and therefore low water availability. Consequently, our results indicate that colonization or survival by bacterial extremophiles is possible, at least under the conditions we have tested in our experiments. Unavoidably, the study of these extremophiles, especially cold-adapted microbes, is of Astrobiological interest due to the possibility to use them as survival models in other planets and icy bodies (Miletto *et al*., 2011; Moyer and Morita, 2007; Poli *et al*., 2017).

Conclusions

In our experiments, *D. psychrophila* was used as a model for adaptability, survivability and proliferation under Martian conditions of temperature (down to −5°C) and soil's mineral composition (increasing concentrations of sulphate salts). Our results at −5°C temperatures indicate that this microbe tends to emphasize metabolic activity rather than growth in the presence of $MgSO_4$ at 0.35 wt% which could be taken as evidence of the survival capabilities of this microbe under suboptimal conditions. Furthermore, the presence of different subclonal populations within individual cultures which prioritized differential growth and maintenance functions at 0 and 10°C highlights the concept of metabolic specialization which is crucial for microorganisms to evaluate their environment, adapt to stressful conditions and decide whether survival (maintenance) or colonization (active cell division and/or maintenance) in harsh environments, such as those present in Mars or icy satellites (Jovian and Saturn satellites), is beneficial or not (Price, 2000; Dubnau and Losick, 2006; Pikuta *et al*., 2007; Davidson and Surette, 2008; Gefen and Balaban, 2009; Levine *et al*., 2012; Rosenthal *et al*., 2018).

Following this same concept of metabolic specialization in which subclonal populations within individual cultures showed evidence of metabolic adaptability, we would like to bring attention to the cultures supplemented with $CaSO_4$ 0.1 wt% at −5°C (prioritized maintenance), $MgSO_4$ at increasing concentrations at 0°C (prioritized both maintenance and growth) and −5°C (prioritized maintenance) which showed attenuated or absent sulphate reduction. The MB results in these cultures suggest the presence of posttranscriptional or posttranslational modifications which rendered non-functional DsrAB enzymatic units. This behaviour comes to no surprise given the fact that we have subjected these cells to conditions of temperatures that are 10 and 15°C below their optimal growth temperature conditions (Dubnau and Losick, 2006; Davidson and Surette, 2008; Gefen and Balaban, 2009; Levine *et al*., 2012; Rosenthal *et al*., 2018).

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Moreover, cultures of *D. psychrophila* at 10 and 14 wt% of $FeSO_4$ showed dsrAB copy numbers at all temperatures, but as it can be observed in our results, the culture conditions were not ideal for active cell proliferation and sulphate reduction. It is possible that the dsrAB transcription detected was associated to temporal survival of the cells from the inoculum which initially transcribed but were eventually inactivated.

Furthermore, cultures supplemented with 10 wt% and increasing concentrations of $Fe_2(SO_4)_3$ showed evidence of growth at all temperatures and −5°C respectively. However, no dsrAB copy numbers and only minimal sulphate reduction were detected. Like the $FeSO_4$ supplementations, it is possible that cells from the inoculum survived temporarily but were inactivated due to the harsh conditions at which these cultures were incubated.

We have proven the ability of *D. psychrophila* cells to activate the dsrAB operon at lower temperatures than expected due to the presence of chaotropic cations. We have suggested that these substances which are known to destabilize macromolecular structures might act as promoters of functionality at lower temperatures in which all macromolecular structures (DNA, RNA, proteins and membranes) tend to achieve a more rigid and inflexible configuration (up to a certain limit in which denaturation is irreversible). Interestingly, from the literature we know that the double helix of DNA adopts a highly stable and supercoiled configuration which can inhibit the transcriptional and translational machinery. In our experimental model, we have suggested that chaotropic agents (sulphate compounds) might act as 'destabilizers' of all the macromolecular structures mentioned above, improving transcription, cellular stability and metabolism at sub-zero temperatures. Currently, our research team is designing better approaches to address these phenomena (Feller and Gerday, 2003; Pikuta and Hoover, 2003; Pikuta *et al*., 2007; Hoover and Pikuta, 2010).

Finally, the presence of transient liquid water in the Martian permafrost and polar caps is theoretically improved by the influence of different minerals and the soil´s particles structures. Among those minerals sulphate compounds, as those used in this study, are abundant and they could potentially maintain water films (acting as an antifreeze/cryoprotectant) which can ultimately trigger microbial survival or colonization in such extreme environments. This is particularly important in our experiments because we have demonstrated that *D. psychrophila* cells can actively metabolize at subfreezing temperatures and in the presence of sulphate salts.

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