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Examining the Expression Patterns of Desulfotalea psychrophila dsrAB Operon and pit at Subfreezing Temperatures and Different Concentrations of Sulfate Salts as a Model for Mars and Icy Worlds Survival and Colonization

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Examining the Expression Patterns of *Desulfotalea psychrophila* dsrAB Operon and pit at Subfreezing Temperatures and Different Concentrations of Sulfate Salts as a Model for Mars and Icy Worlds Survival and Colonization.

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ABSTRACT

Since ancient times, Humanity has been fascinated with the idea of what lies beyond the borders of our planet. Fortunately, the combined efforts of many nations have made it possible to send unmanned spacecraft to orbit planets located close to Earth. These missions have the principal goal to collect data that could help us understand the basic environmental conditions that persist on those planets, or for evidence of past or present life. Equally important, landers and rovers have been successfully deployed to start the in-situ exploration of many planets of the Solar System. Among them, Mars has been extensively studied due to its closeness with Earth, and because it is located within the habitable zone. Many hypotheses about the presence of current microbial life in this planet have been formulated. However, a definite answer is still elusive. In this research, we have tested the ability of a psychrophilic bacterium, *Desulfotalea psychrophila* (*D. psychrophila*), to survive and proliferate at subfreezing temperatures and at increasing concentrations of sulfate minerals known to be present in the Martian regolith and icy satellites from the Jovian and Saturn Systems. We have found that *D. psychrophila* cells can survive and proliferate, at least temporarily, at temperatures down to -5 °C and -10 °C in which sulfate compounds, specially $MgSO_4$ and $CaSO_4$, induced a combined effect of chaotropicity and physical protection against mechanical damage of the cellular membrane allowing these bacterial cells to metabolize at suboptimal temperatures. Furthermore, our studies have shown evidence of the importance of metabolic specialization in which bacterial cells from the same clonal population can react differently by prioritizing maintenance, growth, or both when challenged with temperatures below their optimal growth temperature. This type of investigation is relevant to the field of Astrobiology because it facilitates the identification of the lower boundaries of cold temperatures that could permit the development of metabolic processes in planets or satellites other than our own.

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DEDICATION

I would like to dedicate this dissertation to my Mom, María Isabel, my brother José Luis, and my Grandma Isolina, for all the love and support they gave me while physically present in this World.

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CHAPTER I

1. Introduction/Literature Review

1.1 Astrobiology

Astrobiology is an interdisciplinary field that studies the origin of life, its evolution, and the possibility of its existence beyond the borders of Earth. Furthermore, the fundamental question whether there exists (or existed) life in other planets, satellites, or galaxies is still elusive. This comes to no surprise since there is no clear scientific evidence that suggests life, how we know it, is possible beyond Earth. However, the fact that humanity have not being able to detect life in other planets does not necessarily mean that the rest of the Universe is devoid of life. The opposite is equally truth, we cannot assume that there are innumerable worlds sustaining life-forms across the Universe. In the absence of absolute certainty about this predicament, it is possible to hypothesize the existence of planets conveying similar biological systems as the ones present on our planet, although this assumption should be taken cautiously since life-forms present in other planets or solar systems could be inherently different from terrestrial organisms if their environmental conditions are drastically distinct from the ones encountered on Earth. The latter reflects the biological premise that life-forms subjected to different environmental stressors would evolve differently. Therefore, the search for life in the Universe started by looking at planets conveying similar environmental conditions such as those present on Earth since an absolute truth is that life prevailed in this planet since its origin 3.5 billion of years ago, and it is only logical to search for what we know to be truth. Moreover, the Astrobiological community, following basic principles of Earth's Biology, determined that life itself requires the presence of water for its origin and long-term sustainability. As a result of this, the concept of "habitable zone", which is no more

than the distance between a planet, moon or asteroid to their hot star required for water to remain liquid in their surface, was formulated to guide Astrobiologists in the search of planets, satellites or asteroids that are conductive of life (see Figure 1) (Cottin et al., 2017; Vance et al., 2016, Leger, Pirre & Marceau, 1993).

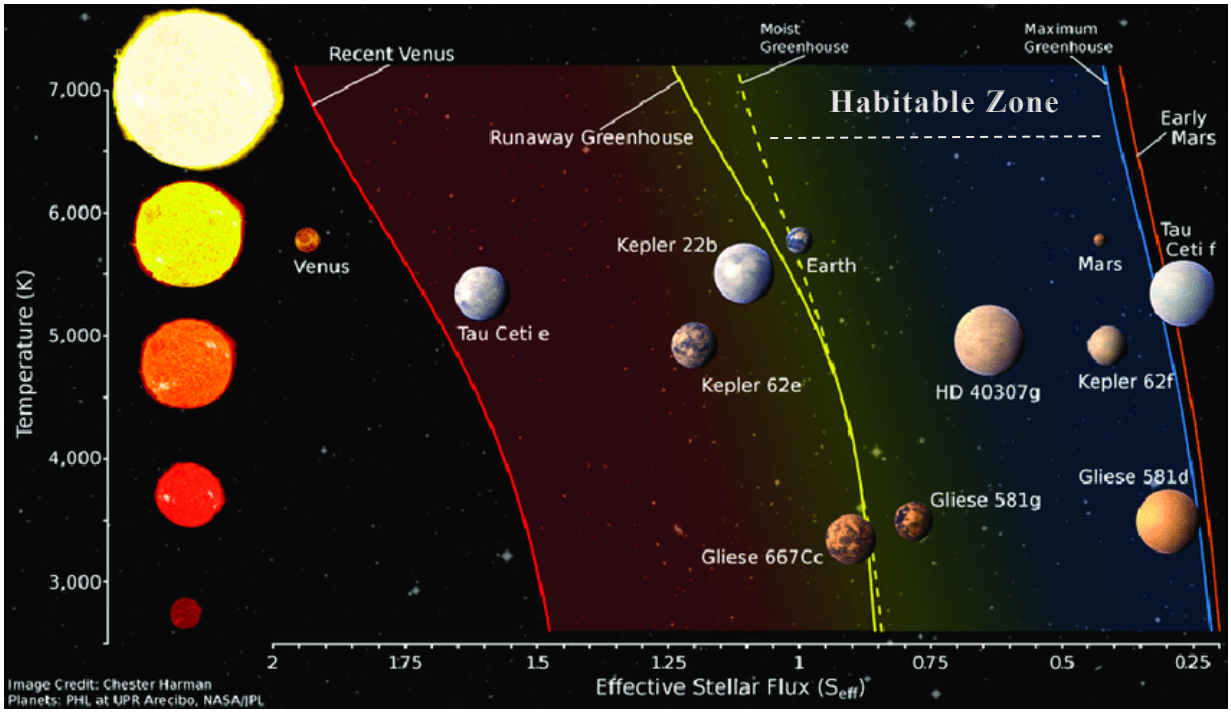

Figure 1. Depiction of habitable zones. This image shows the boundaries of temperature in relation to the relative closeness of planets to their hot star. Modified from: Kasting et al., (2014).

1.2 Planetary Protection

The fluid concept of planetary protection is defined as the guiding principles used to preserve pristine environments in Solar System bodies from terrestrial contaminants (live organisms). A notion that was originally called "forward contamination prevention". As expected, this same concept applies to the reverse process known as "backward contamination protection" which encompasses the protection of terrestrial environments from life-organisms that could potentially travel in return missions from other Solar System bodies. Interestingly, the planetary protection concept is carved within the 1967 United Nations Treaty on Principles Governing the Activities of States in the Exploration and Use of Outer Space (General Assembly of the United Nations, 1967, Article IX) which principal purpose is to regulate the planning and functioning of interplanetary missions. In the rigorous process to update this policy, national and international space agencies as well as scientific organizations recommend guidelines about the current state of space exploration to the Committee on Space Research (COSPAR). The latter, which is an interdisciplinary committee of the International Council of Science, consults with the United Nations, and if there is consensus about the recommendations relevance, they proceed to incorporate them into the COSPAR Planetary Protection Policy. Furthermore, specific outbound missions are categorized based on the mission target (planet, moon, or asteroid) and whether the spacecraft is a lander, an orbiter, or a flyby. Taking these into consideration, missions are then organized in four planetary protection categories (Categories I-IV) which are assigned based on the likelihood of the target body to sustain or if it has sustained potential microbial life (based on scientific proof of chemical evolution). In one side of the spectrum, category one encompasses target bodies which are known to have a low probability of supporting/supported microbial life, while in the opposite, category IV, applies to missions in which the targeted planet has been proven to have sustained life or if it is capable of sustaining life. Then, based on the assigned category,

the COSPAR planetary protection policy mandates specific protocols for spacecraft's cleaning and bioload-reduction. Importantly, all inbound missions that are planned to return samples to Earh are assigned category V which are the most strict set of regulations in the COSPAR policy (Berry et al., 2010; Hedman & Kminek, 2022; Rettberg et al., 2016).

1.3 Mars

Mars has been defined as one of the planets that could have supported (or could possibly support) life. Therefore, following the guidelines for Planetary Protection, this planet has been classified as category IV. In addition, COSPAR has established a special classification for Mars exploration, the so called "Mars Special Regions" (COSPAR 2015). Furthermore, a Mars special region is defined as a region in which terrestrial organisms can proliferate (replicate) or a region where there is evidence of a high potential for the presence of current or extant Martian life. This classification is determined based on three crucial aspects: water activity (0.5 – 1.0), temperature (-25°C with no upper limit), and timescale (500 years) (National Academies of Sciences, Engineering and Medicine, 2015).

Mars environmental conditions are extreme, especially in its surface where any organism would be exposed to high radiation (UV, cosmic and mineral radiation), extremely low temperatures, low water activity (due to its absence or if present, due to its solid state), extreme low pressures, oxidant soils, and low/absent carbon sources available for metabolic activity. However, in subsurface regions, cells and organic material could be protected from the harmful effects of UV radiation, and extreme variations of temperature. Equally important, in subsurface regions, films of liquid water could be available for cellular functions which increases the chances for cell survival, metabolic activity and possibly proliferation (Berry et al., 2010; Crisler et al., 2012; Moores & Schuerger, 2012; Pavlov et al., 2014; Poch et al., 2014, Gilichinsky et al., 1995).

1.3.1 Liquid water and temperature

Liquid water is necessary for any organism to subsist and its existence in different biological systems depends on the water activity (aw) which is regulated by environmental conditions. Furthermore, aw is defined as the thermodynamic availability of water molecules in reference to pure water under constant pressure and temperature. This scale goes from aw =1.0 for pure water, and it decreases as chemical species are added to a particular solution or if the temperature is reduced. As Mars is permanently frozen, with an average global temperature of -61 °C, water molecules for cellular activity are almost absent in the surface (extremely low water activity). Furthermore, the triple point of water is 0.01 °C (273.16 K) under 6.12 mbar (612 Pa) at Earth's surface. As expected, most water on the Martian counterpart (6 mbar/600 Pa of pressure and temperatures below 0 °C) is either in solid or vapor state, only allowing phase transitions from ice to gas or viceversa (See Figure 2). As described by Christensen et al. (2003) and Titus et al. (2003), most of the water present in the Martian surface, subsurface and the polar caps is stable only in solid state (in mixtures of CO_2 and water ice). However, it is important to understand that temperatures in this planet are constantly fluctuating as it was revealed by the Viking landers which detected temperatures around -10 °C and increases of up to 20 °C during austral summer. A phenomenon that could increase the aw for cellular functions in regions of the planet that have pressures above the surface pressure (i.e. subsurface environments) (Berry et al., 2010; Millot et al., 2021). Moreover, many scientific groups have presented evidence that suggests that certain regions of Mars could potentially harbor transient liquid water as a result of sublimation of solid water and CO_2 ice, subsequent water vapor deposition, and deliquescence. Interestingly, deep subsurface regions have been predicted to be the most stable candidates for presence of liquid

water in Mars (Chinnery et al., 2019; Christensen et al., 2003; Crisler et al., 2012; Dundas et al., 2017; Hecht, 2002; Ojha et al., 2015; Titus et al., 2003).

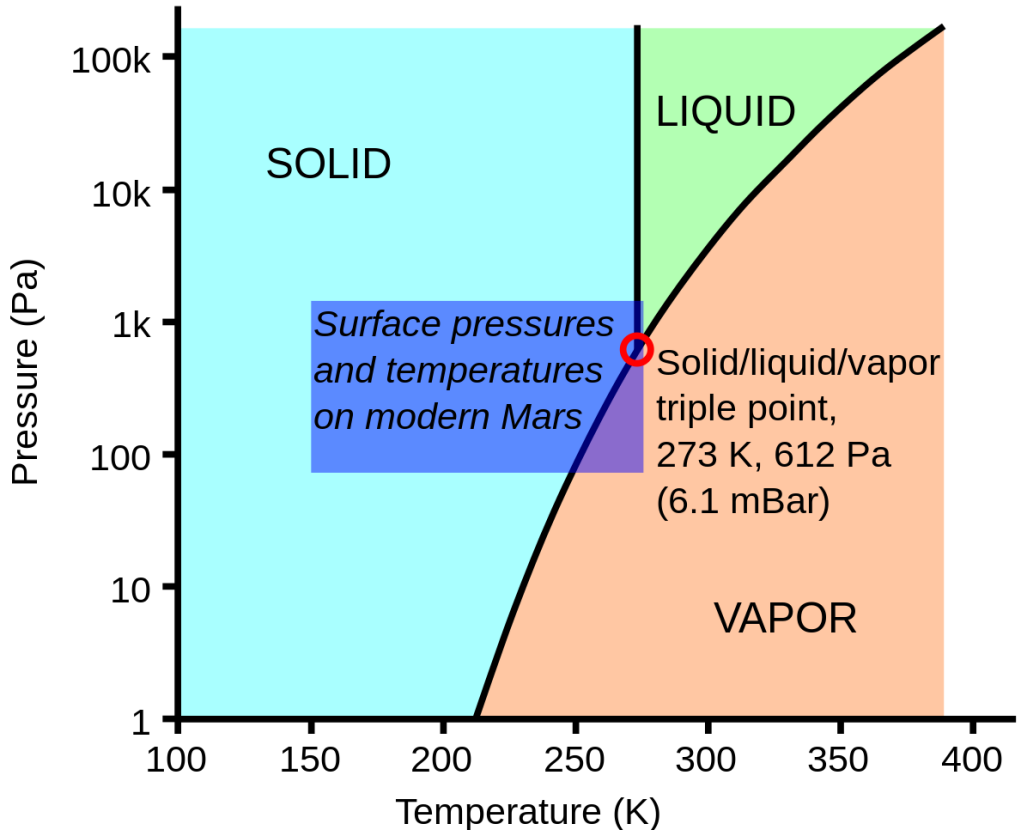

Figure 2. Triple point of water calculated on Earth's surface. At 273.16 K and 612 Pa water phases coexist in solid, liquid and vapor states. On the Martian surface environments with pressures of 6 mbar (600 Pa) water is expected to remain in solid or vapor states. Therefore, pure water would transition to vapor state. However, salty solutions do not freeze or evaporate as fast as pure water and liquid water films could remain stable temporarily. Image obtained from: Daniel Hobley, CC BY-SA 3.0 <https://creativecommons.org/licenses/by-sa/3.0>, via Wikimedia Commons. https://commons.wikimedia.org/wiki/File:Phase_diagram_of_water_under_martian_conditions.svg.

In addition, data from the Mars Odyssey thermal emission imaging system (THEMIS) and the Mars Global Surveyor thermal emission spectrometer (TES) have shown variations in the temperature of the interior of Korolev Crater. This crater is believed to be formed by water ice or ice rich regolith and it is estimated that water vapor can condense and be deposited in its surface

(acting as a trap for water ice and water vapor). More importantly, using spectrophotometric data from the Mars Reconnaissance Orbiter (MRO), hydrated salts have been tentatively detected in association with brines in regions of the Martian surface presenting streaks of low reflectance (decrease albedo) with downslope directionality known as recurring slope lineae (RSL) (see Figure 3). According to this group of researchers, these regions are formed due to current water activity when temperatures range from -23 °C to 27 °C. Furthermore, the chances of water activity occurrences increases if we take into consideration that regions where gullies and RSLs have been detected possess temperatures close to the triple point of water. This phenomenon in conjunction with the presence of high salinity (i.e.: increased sulfate salts concentrations) can decrease the freezing point of water allowing water molecules to remain in liquid form at a lower temperature. Interestingly, the major constituent of the Martian atmosphere is CO_2 while water is present in low concentrations. This dynamic indicates a low partial pressure of water. However, in a static atmosphere (enclosed system protected from the Martian atmosphere as a whole), the sublimation is dependent on the diffusion of water molecules from the vapor layer that is in close contact with ice. In these enclosed systems (such as those formed by dust burial in gullies) the partial pressure of water can increase drastically allowing for more water vapor molecules to accumulate (increasing the partial pressure of H_2O to the level of the surrounding pressure). This phase transition from solid to gas and probably transient liquid water would be consistent with the observation that liquid water in small quantities is required for common Martian's regolith processes such as avalanches or mass movements. Surprisingly, it has been pointed out that this phase transition can happen at the soil pore spaces level in which the surface tension of a layer of quasi-liquid that surround the soil grains can act as a barrier that allows for an increase in the partial pressure of water favoring the phase transition (Audouard et al., 2014; Crisler et al., 2012;

Hecht, 2002; Ojha et al., 2015; Schorghofer et al., 2002). Interestingly, these findings have been debated since other groups of scientists have attributed the RSLs formation to the effect of bright dust removal and deposition or due to the effect of granular flow (Dundas et al., 2017; Vincendon et al., 2019). However, it has been mentioned that in all cases RSLs require a water phase transition to occur (Schorghofer et al., 2002). Curiously, this type of debates reflects the fact that Astrobiology is a relatively new Science, and a great majority of the available data have its inherent flaws mostly due to differences in techniques, and depth of analyses.

Another piece of information comes from the presence of bromine in the regolith which indicates mobilization of salts due to the action of thin films of liquid water in ancient Mars (Bish et al., 2013; McGlynn et al., 2012; McSween et al., 2010; Schorghofer et al., 2002). It was proposed that this thin layer of liquid water could be the result of sublimation of deposited frost and its subsequent condensation in cold traps over geological times which will allow the mobilization of ions in salts to the actual concentrations of bromine. However, there is no definite evidence that this phenomenon operates in today's Martian soils (Clark and Van Hart, 1980, Gellert, 2004; Yen et al., 2005, 2013).

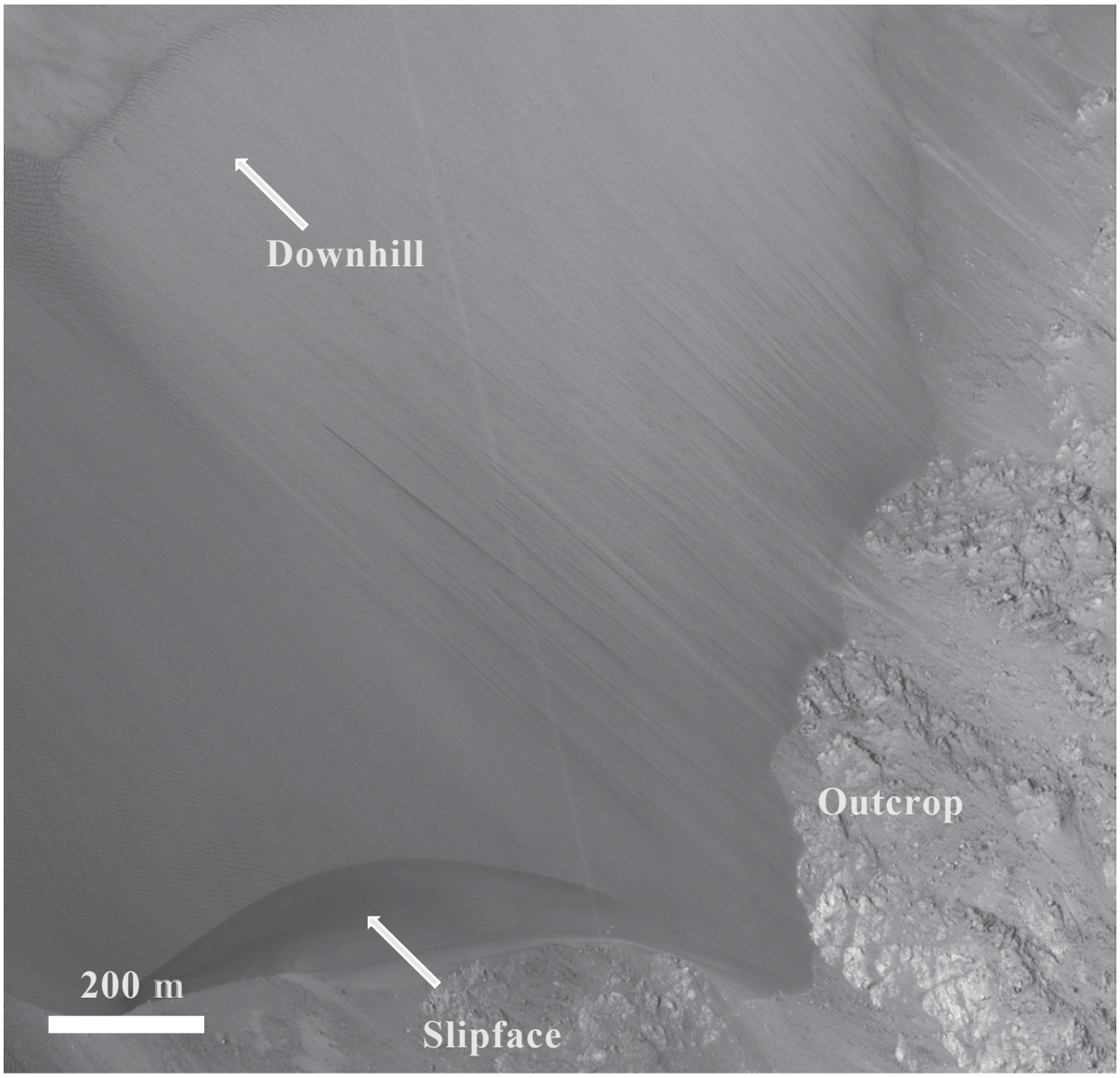

Figure 3. Image of the merger between a climbing dune and a recurrent slope lineae (RSL). As it can be seen in the image the dune sand is stopped by the outcrop (upslope) and there is formation of a slipface. Slope lineae can be seen in downslope directionality. Extracted from Dundas et al., (2017), and it was provided to them by the NASA/JPL/University of Arizona.

Thus, one thing is for certain, hypersaline Mg^{2+}/ Ca^{2+} rich paleolakes existed in Ancient Mars especially since recent radar data suggest the presence of a large body of hydrated salts/brines in the subglacial regions of the Martian southern polar cap (La Cono et al., 2019).

On the other hand, there have been discrepancies in the calculations of water content (ice) from orbit and in-situ measurements. For instance, OMEGA (orbit observations) have identified that the water content at the Northern plains of Gale Crater are approximately 4-5 wt% while Curiosity showed a range from 1.5-3 wt%. Furthermore, at the Phoenix landing site OMEGA calculated approximately 9-11 wt% water availability while comparisons with the in-situ experiments have situated it at about 2 wt%. This reduction in water content can be explained based on the differences in the techniques used to calculate this variable. Specifically, OMEGA performed estimations only of few microns depth while in-situ experiments were performed at centimeters depth. Also, in-situ experiments of the regolith's samples were executed using sieved material (<150 μm) while OMEGA measurements were performed from orbit (Audouard et al., 2014).

In general, half of the evidence presented suggests that liquid water could be present in the form of transient films while the other half disproves it. However, as this research is not directed towards the approval or disapproval of water availability in Martian environments, we have assumed that limited water activity is present possibly in films at the Martian's regolith subsurface where microbial organisms could potentially utilize it for cellular processes.

1.3.2 UV effects on carbon sources and life-forms

The topic about the persistence of organic materials in the Martian regolith and its subsurface is still an ongoing debate since the first calculations generated from data collected in 1976 at the Viking 2 landing site only revealed the presence of small concentrations of CO_2 (0.07 wt%). Furthermore, these experiments were performed by combustion of soil samples at 500 °C and detection by gas chromatography-mass spectrometry (GCMS). At the time, the scientific community was surprised since it was assumed that the Martian regolith should contained at least traces of more complex organics which are known to be provided by materials from the interstellar

space (impact of meteorites, asteroids and comets containing carbonaceous materials). Approximately 15 years later, data collected from the Viking 2 landing site as well as Viking 1 were re-evaluated by incorporating the presence of perchlorates (detected by the Phoenix lander in 2008). This chemical which is known to oxidize organic compounds at temperatures above 200 °C would have been the reason of the absence of organics detection in the Viking 1 and Viking 2 landing sites. An in-depth analysis of the data revealed that the samples taken at these sites contained approximately 0.11-0.32 wt% of calcium, magnesium or iron oxalate minerals which accounts for 150-170 ppm of organic carbon, and chlorinated hydrocarbons (chloromethane). Additionally, the Phoenix lander tentatively detected the presence of calcium carbonate (calcite) at a concentration that ranged from 3 to 5 wt%, and possibly toluene, benzene and thiopenes. Equally important, it has been pointed out that iron and magnesium oxalates decomposition with iron and magnesium carbonates could have been present in the analyses of Rocknest sand shadow and Sheepbed mudstone (Gale Crater, studied by the Curiosity Rover) which could have also contributed with the formation of chlorobenzene and other chlorinated aromatics (Applin et al., 2015; Moores & Schuerger, 2012; Stalport et al., 2019).

Nowadays, it is well known that the Martian surface is constantly bombarded by meteorites, dust particles, and comets containing organic material (such as aromatic hydrocarbons, aminoacids, carboxylic acids and even nucleobases) which leads to the formation of carbon deposits in the surface and subsurface of the Martian regolith. However, these deposits are constantly modified by the action of cosmic, galactic, and UV radiation (3.6 W/m^2) which decompose them at a pace that allows organic compounds to remain stable only momentarily. From these three types of radiation, UV has the largest decomposition and biocidal effects in organic material and life forms respectively (Chinnery et al., 2019; Moores & Schuerger, 2012; Mumma & Charnley, 2011;

Parnell et al., 2007; Pavlov et al., 2014; Pizzarello, 2006; Poch et al., 2014). However, it is crucial to understand that the process of organic matter decomposition is dependent on the thickness of the deposit and there is evidence that suggests the presence of resistance to carbon photodecomposition which allows material organic to remain stable for a few months. Furthermore, it has been predicted that carbon species present in the Martian surface (seeded from interplanetary dust particles) can potentially reach an equilibrium between UV decomposition and surface deposition which could eventually lead to the accretion of carbon-bearing materials at the Martian surface (a process that has been tested in adenine and mellitic acid deposits). Ultimately, these carbon materials would be released into the atmosphere as methane. However, if these compounds form a photo-resistant (UV refractory) layer surrounding the organic matter deposits, they could persist in the Martian surface for longer periods of time. This finding along with mixing events of the carbonaceous material with the regolith and the fact that UV radiation can only penetrate at depths down to 500 μm could potentially prevent the total decomposition of organic material available for cellular uptake and drastically reduce the biocidal effects on life-forms at the Martian's regolith subsurface. As our predictions of survivability and proliferation are directed towards this particular niche, it is important to mention that ideally cells will not be affected by the effects of UV radiation (carbon depletion, free radicals or volatile oxidants) (Berry et al., 2010; Moores & Schuerger, 2012; Pavlov et al., 2014; Poch et al., 2014; Stalport et al., 2019).

1.3.3 Martian atmosphere and pressure

Mars atmosphere is composed primarily of CO_2 and traces of other components such as O_2, H_2O, Ar, CO, and N_2 (Rafkin & Banfield, 2020). This combination of gases spread across its atmosphere creating an average global pressure of 6.5 mbar which fluctuates from 6.55 to 7.4 mbar (Kamata et al., 2015; Kömle et al., 2018; Taylor et al., 2010). Furthermore, it develops partially due to gravity which retains the heaviest gases and partially due to the absence of a magnetic field which allows most lighter gases to escape to space. More importantly, the absence of a magnetic field leaves the planet atmosphere, its surface and life-forms (if present) exposed to the eroding effects of space weather, energized particles bombardment (solar winds), UV, and cosmic radiation (Bogdanov & Vaisberg, 1975; Gringauz, 1976). Therefore, it has been established that the proliferation of bacterial cells in the Martian surface is almost impossible to achieve and only radiation resistant dormant stages could survive (i.e: *Deinococcus radiodurans*) (Bauermeister et al., 2011; Schirmack et al., 2016; Weiss et al., 2002). However, the Martian's regolith subsurface could potentially shield most life-forms from the effects of these harmful influences. In general, low pressure is not considered a stringent environmental condition that could limit the viability of certain life-forms especially when compared with the drastic effects of desiccation or UV radiation. However, there is evidence that low pressure can still affect the viability and proliferation of microbial cells down to 25 mbar. This biocidal effect resulted exponential if combined with other factors such as high salinity (Berry et al., 2010; Mickol & Kral, 2017; Schwendner et al., 2020).

1.3.4 Sulfate compounds in the Martian regolith

The Martian regolith, its surface and its chemical composition have been studied for almost half a century. These studies have been crucial to understand the past and present environmental conditions of the planet which can be inferred from the composition and properties of the soil present there today. Martian soils have been characterized as principally formed by basaltic components, in which igneous materials dominate. They are supplemented with a volcaniclastic additive of enriched chloride and sulfur, a meteorite fraction high in nickel, and a magnetic dust fraction formed by pyroxene (Ca, Mg and Fe), olivine (Mg, Fe, and other elements), carbonate, sulfate and silicates (Bell et al., 2000; McGlynn et al., 2012). Furthermore, these soils have a high content of iron (haematite), glass, plagioclase feldspar (Na and Ca), oxides, pigeonite and augite (Bish et al., 2013; McGlynn et al., 2012; McSween et al., 2010; Rogers & Aharonson, 2008; Yen et al., 2005).

The first mission to successfully generate valuable information that could be used to understand today's Martian environmental conditions was the Viking mission in 1975. This mission which was composed of two orbiters and two landers generated substantial information about the general characteristic of the planet as well as the elemental composition of the Martian regolith (Soffen, 1977). Furthermore, the Viking landers using their X-ray fluorescence spectrometer (XRFS) showed that all soils had iron contents ranging from 15 wt% to 19 wt% (Bell et al., 2000; Yen et al., 2005). Moreover, sulfur was estimated to occur at an average concentration of 3.5 wt% (Rieder et al., 1997), although other authors have reported concentrations as high as 7.9 wt% (Bell et al., 2000). Additionally, sulfur-containing minerals such as sulfates have been predicted to occur at concentrations that range from 8 wt% to 15 wt% (Clark and Van Hart, 1980). According to XRFS data analyses, there are copious amounts of Fe (12.7 wt%), Ca (4 wt%), Al (3 wt%) or Mg cations

(5 wt%) that could be combined with sulfate anions. From these ions, Al and Fe can be easily excluded since they hydrolyze to form acidic forms leaving $MgSO_4$ and $CaSO_4$ as the major candidates to represent the sulfate minerals present in the Martian soil. From these two compounds, $MgSO_4$ is highly soluble while $CaSO_4$ is relatively insoluble (products of ~ 10^{-4}) (Clark et al., 2005; Clark and Van Hart, 1980; Prestel et al., 1979; Tosca et al., 2008). Consequently, as pointed out in the studies of Clark and Van Hart (1980), Martian brines have been hypothesized to occur in the presence of $MgSO_4$ as the dominant sulfate in solution (Clark and Van Hart, 1980).

Two decades later, NASA launched the Mars Global Surveyor (1996), which principal objective was to study and map the surface of the planet. It successfully recovered information about the planet's atmosphere, temperature, pressure, meteorological conditions, and status of the polar caps (Albee et al., 2001). In the same year, the lander known as Mars Pathfinder touched the surface of the red Planet. This lander deployed a rover known as Sojourner, which principal goal was to retrieve data about the soil along with information about the atmosphere and magnetic properties of the Planet. Interestingly, Sojourner was the first rover ever deployed in the surface of another planet and laid out crucial information needed for the operation of other rovers (Greeley et al., 1999; Landis & Jenkins, 2000; Team, 1997). Alpha Particle X-Ray Spectrometer (APXS) data generated from the Sojourner showed little variability among different samples of soils and with the overall elemental composition retrieved by the Viking mission (except for S, Cl and Si). In contrast to the Viking Mission, the sulfur content was estimated at an average around 5.4 wt%, a variation that could be the result of the different landing sites used for both missions (Bell et al., 2000).

Using previous information from the Viking project, the Mars Global Surveyor, and the Sojourner, the Mars Exploration Rovers (MER: Spirit and Opportunity) were launched in 2003. Both rovers

were extremely successful in operations and data recovery. Surpassing their life expectations, Spirit remained active up to 2010 when it got stuck in a sand trap, and Opportunity lost signal in 2018. However, a plethora of data regarding the soil composition was generated as an effort to understand the mineralogy present in the surface and immediate subsurface of the red planet. Moreover, different models were developed to reconcile the spectra generated by these rovers' instruments and the composition of the Martian soil. Based on three different technologies used by Spirit and Opportunity, APXS, miniature thermal emission spectrometry (mini-TES) and Mossbauer spectrometry, scientists were able to model the composition of the Martian soil and have identified the presence of sulfate-rich deposits which are stable at surface conditions of pressure, UV radiation, and temperature. For instance, Opportunity identified sulfate-rich outcrops in its landing site (Rogers & Aharonson, 2008). It is unclear how these deposits were originated since they require water for their formation. Alternative events such as volcanic outgassing has been proposed as a possible explanation for the formation of magnesium, calcium, and iron sulfates-rich deposits in which acidic (sulfuric acid) rain could have altered olivine rocks. Interestingly, these sulfate outcrops are estimated to possess concentrations that range from 22-32 wt% of magnesium and calcium while ferrous and ferric sulfates could have concentrations up to 38wt% (Applin et al., 2015; Bibring, 2005; Bish et al., 2013; Clark and Van Hart, 1980; Gellert, 2004; McGlynn et al., 2012; McSween et al., 2010; Yen et al., 2005, 2013).

Based on studies by Rogers and Aharonson (2008) in which they summarized orbit studies with low-albedo and low-sulfur sands from Meridiani Planum, sulfates abundances were placed at vol% concentrations ranging from 5% to 12% and based on their analyses with the mini-TES and TES (from orbit) at vol% concentrations of 10% and 8% respectively. Interestingly, these authors used a spectral library derived from the TES data (Mars Global Surveyor) and a least-squares fit model

to estimate the presence of sulfate minerals. From their results they found four phases within the sulfate group corresponding to anhydrite, gypsum, natrojarosite and kieresite which are minerals composed by $CaSO_4$ and $MgSO_4$. From there, using various models, they concluded that the derived abundance of sulfates (mini-TES) was in a range between 6-8 wt%, calcium at about 5-15 wt% and magnesium at 7-11 wt%. Furthermore, these authors agree with the concept that today's Martian sulfate composition could be the result of accumulation of H_2SO_4(S) due to volcanic outgassing which would eventually give origin to the non-distinct sulfate composition detected in Meridiani Planum and possibly in other parts of the Martian regolith. However, they have also mentioned that some other events such as derivation from the local outcrop or transport from a distant source could have originated these sulfate deposits (Hynek et al., 2015; McSween et al., 2010; Rogers & Aharonson, 2008).

In addition, McSween et al. (2010) used a similar approach model to identify the chemical composition of the Gusev soil. From their analyses with mini-TES spectra, they concluded that sulfur is present at a concentration of 7 wt%. Furthermore, after calculation of the minerals abundance, sulfates were estimated at about 11.3 wt% and from APXS elemental analyses magnesium was calculated at concentrations of 9.03 wt% and calcium at 5.38 wt% (McSween et al., 2010). Similarly, Gellert (2004) calculated the maximum concentrations of sulfur at 7.38 wt%, Na at 3.8 wt%, Mg at 9.7 wt%, Ca at 6.63 wt%, and Fe at 16.3 wt% (Gellert, 2004). Moreover, McGlynn et al. (2012) estimated that sulfates in both sites Gusev Crater and Meridiani Planum oscillate between 5.4 to 5.5 wt% assuming that these sulfates are coupled with calcium or magnesium. Significantly, some of their samples registered APXS values of SO_3 up to 32 wt% which classifies these soils as high in sulfur. In general, these authors have estimated that magnesium or calcium sulfates represent 10%-20% of the Martian soils. They pointed out that this

high presence of sulfur containing alteration products (sulfates) might be the result of olivine dissolution in acid-sulfur, an idea shared by many scientific teams as shown above (McGlynn et al., 2012; McSween et al., 2010). Besides these focal points, magnesium and calcium sulfates associated with hydrated-silicates and/or ferric oxides have been mapped in different regions of the Martian's regolith (Mar's Express OMEGA data). Interestingly, these sulfate deposits have been speculated to extend to subsurface areas of the planet (Bibring, 2005).

On the other hand, one of the most recent rovers sent to Mars, Curiosity (MER) preliminary data have shown sulfur concentrations of 5.18 wt% (using APXS) in Gale Crater. Regardless of the differences in sulfur content from the analyses shown above, one thing is for certain, the Martian soil contains higher concentrations of sulfur, iron and chloride in comparison with terrestrial continental soils and lunar mare fines (Clark et al., 1982). Furthermore, some cations that were identified were Na^{+} with a concentration of 2.22 wt%, Mg^{2+} with a concentration of 6.53 wt%, Ca^{2+} with a concentration of 7.38 wt% and $Fe^{2+/3+}$ with a concentration of 21.0 wt%. Interestingly, this data is similar to the basaltic soils encountered by Spirit in Gusev Crater (Bish et al., 2013; Gellert, 2004; Yen et al., 2013). However, even with all the information shown above targeting the chemical composition of the Martian soils, their primary and alteration phases have not been accurately identified (McGlynn et al., 2012; McSween et al., 2010).

Generally, most Martian soils samples showed little variability in their chemical composition. As it was demonstrated in the analyses of soils from Chryse Planitia (Viking 1), Utopia Planitia (Viking 2), Ares Vallis (Sojourner), and Gusev Crater (Spirit) (Gellert, 2004). Therefore, the existence of a universal Martian regolith has been hypothesized given the chemical similarities between these different landing sites. Significantly, this homogeneous regolith would be the result of weathering and alteration processes that acted and keep acting on the parental rock material

(Bell et al., 2000; Clark et al., 1982; Yen et al., 2005). This apparent homogeneity could suggest the presence of an aeolian transport system that could blend the chemical components of the soil and distribute them at a global scale (Clark et al., 1982; McSween et al., 2010). However, some other mechanisms that could explain this phenomenon have been proposed. For example, McGlynn et al. (2012) have examined various physical mechanisms (physical/hydrodynamic sorting, impact, mineral fractionation, and mobility from active dunes) using data from MER (AXPS, MB, and mini-TES) in addition to microscopic evaluation of soil textures (high-resolution Microscopic Imager). Surprisingly, these authors concluded that impact gardening, physical/hydrodynamic sorting and limited chemical weathering are crucial determinants in the composition of the Martian soils (McGlynn et al., 2012; McSween et al., 2010).

Additionally, it is well known that sulfate minerals are poor depressors of the freezing point of water. It has been proven that below -10 °C these salts cannot avoid the formation of ice. However, Martian soils also contain NaCl, a moderate depressor of the freezing point of water (at -21 °C). Therefore, the presence of these salts could render stable brines at those temperatures (through deliquescence and hydration). As it was demonstrated by the Rover environmental station (Curiosity Rover) water vapor from the atmosphere could be absorbed onto the Martian regolith at Gale crater under saturation conditions (regolith acts as a cold trap below 0 °C forming frost). Interestingly, these frosts could potentially form transient films of water in liquid state which could persist for approximately 4.5 hours at temperatures equal or above 0 °C (Clark and Van Hart, 1980). More importantly, a system in which Na^+, K^+, Ca^{2+}, and Mg^{2+} were at specific concentrations could depress the freezing point of water down to -63 °C (Brass, 1980). However, as the Martian atmosphere is so low (H_2O vapor pressure of 0.1 to 1μbar) brines in the surface of Mars are theoretically non-existent. Nonetheless, the formation of stable brines, in close contact

with ice, at the Martian subsurface have been speculated (Audouard et al., 2014; Clark, 1978; Clark 1979; Brass, 1980; Hecht, 2002; Vakkada Ramachandran et al., 2021).

Interestingly, based on geochemical limits, solubility, and reactivity, it has been postulated that the mineral composition of Martian soils is dominated by $MgSO_4$, Na_2SO_4, NaCl, $MgCO_3$ and $CaCO_3$. More importantly, it has been speculated that brines with this composition would depress the freezing point of water down to a range of -21 °C to -65 °C , but only if sulfates have precipitated and these brines could be isolated from soluble sulfates (subsurface reservoirs of ice) (Clark and Van Hart, 1981; Bell et al., 2000). Furthermore, it has been predicted that the Martian soil porosity is crucial for the formation of brines. In a model explained in Crisler et al. (2012), it has been suggested that soil pore spaces which are known to store ice are crucial in the formation of brines. In this model the soil's pore void would progressively fill with water vapor due to the sublimation of ice. This phenomenon would induce the formation of cold traps which would increase the concentration of minerals in the ice phase. Furthermore, this increase in concentration would eventually melt the ice and trigger the formation of a stable brine. As proposed by La Cono et al. (2019), if brines or subglacial lakes were to be stable under Martian conditions today, they would be dominated by Mg^{2+} and Ca^{2+} ions (athalassohaline) and perchlorates in comparison with Earth dominated brines/lakes which are thalassohaline (dominated by NaCl). As $CaSO_4$ and any other iron sulfate are relatively insoluble below pH 3 it would only allow the formation of brines containing Mg^{2+} and SO_4^{2-} ions (Crisler et al., 2012; La Cono et al., 2019).

1.3.5 Microbes in Mars and sulfate reducing prokaryotes

Nowadays, there is no evidence that suggest that microbes can proliferate in the Martian surface. However, in subsurface regions, bacterial cells could tentatively proliferate and metabolize by taking advantage of the presence of water films created due to the regolith's porosity, and the presence of high salinity (provided by different minerals). To understand the possibility of microbial proliferation in brines formed in other planets, first we must understand the type of organisms that can actively proliferate in terrestrial analogs. This information will allow us to identify the microbial physiological limits for extreme environments on Earth which could be extrapolated to Martian and other icy bodies environments (Foster et al., 2010; Fox-Powell & Cockell, 2018). These two premises bring us to the actual subject of our investigation, Sulfate reducing Prokaryotes (SRP).

SRP are a diverse chemolithotrophic group of microorganisms that play a crucial role in the carbon and sulfur cycles (see Figures 4, and 5). Their importance in both cycles come from the fact that they have been identified as the causal agents for 50% of the marine sediments carbon mineralization. Furthermore, they thrive in anaerobic environments such as the intestinal tract of warm-blooded animals, wetland soils, ponds (saline and hypersaline), marine, salt marsh, and estuarine sediments, riverbeds, and lakebeds (saline and hypersaline). Moreover, SRP generate energy by degrading organic compounds in a type of anerobic respiration known as sulfate reduction. In this process, they utilize sulfates or other oxidized sulfur compounds as terminal electron acceptors. On Earth, most sulfur compounds are present in the form of sulfates, sulfides, and elemental sulfur. SRP intervene in the sulfur cycle by anaerobically reducing sulfates to sulfides (as can be seen in Figure 5). The opposite reaction is achieved by sulfur chemolithotrophs (aerobically) and purple and green bacteria (anaerobically) (see Figure 5). Moreover, SRP are

phylogenetically diverse having members in both the Bacteria and Archaea Domains (see Figure 6). Furthermore, sulfate reducing bacteria (SRB), the object of this research, are represented by close to 40 bacterial Genera from which the majority belongs to the Proteobacteria (δ-subclass) and the Firmicutes Phylum (Bacillus-Clostridium group) and various thermophilic bacteria (Gram-negative *Thermodesulfovibrio* Spp., *Thermodesulfobacterium* Spp., and *Thermodesulfobium* Spp.). These bacteria can use various substrates for sulfate reduction, among them pyruvate, fumarate, ethanol, lactate, benzoate, ethanol, malate, butyrate, succinate, H_2, and some other aromatic compounds have been extensively documented. Moreover, these substrates are oxidized, incompletely to acetate or completely to CO_2 and H_2S (see Figures 9, and 10). Interestingly, they have been used in bioremediation research and in the mineral recovery industry given their complex physiological profile which allows them to metabolize complex hydrocarbons, and toxic metals (Barton & Fauque, 2009; Castro et al., 2000; Kushkevych, Hýžová, et al., 2021; Leloup et al., 2005; Muyzer & Stams, 2008; Pester, 2012; Plugge et al., 2011; Schulze & Mooney, 1994). Species of SRB can be thermophilic, psychrophilic, halophilic, strict anaerobes, microaerophilic, sporulating, or non-sporulating. In soils with variable concentrations of oxygen, most anaerobic SRB are present as dormant/resistant forms. Interestingly, in this type of habitat, spore-formers such as *Desulfotomaculum* Spp. have been detected. However, there have been reports of the occurrence of non-sporulating species such as *Desulfovibrio* Spp. and *Desulfobulbus* Spp. Moreover, in soil environments with low concentrations of oxygen (i.e., freshwater, marine mud, and rice fields) members of the *Desulfobotulus* Spp., *Desulfobulbus* Spp., and *Desulfovibrio* have been identified. Furthermore, SRB can be present in freshwater habitats, where lower concentrations of sulfates make them compete with methanogens for acetate and H_2. Some examples of SRB present in this type of environments are *Desulfovibrio* Spp. and

Desulfotomaculum Spp. Moreover, in wastewater environments where sulfate concentrations are higher than freshwater, *Desulfobulbus* Spp. and close to six other genera have been detected. Additionally, in marine environments, where sulfate concentrations are non-limiting, SRB tend to outcompete methanogens. Surprisingly, in anoxic sediments, SRB and methanogens coexist in a dynamic interaction that allow them to establish a syntrophic relationship in which H_2 molecules produced by SRB are metabolized further by methanogens (acetoclastic SRB-Hydrogenotropic methanogens). Interestingly, SRB have been identified in hypersaline environments (i.e., Dead Sea, salt ponds and the Great Salt Lake) and in geothermal environments where temperatures oscillate above 54 °C. Notably, members of the *Desulfotomaculum* Spp. group have been detected in this type of habitat. Finally, some species of *Desulfovibrio* Spp. have been isolated from warm-blooded animals including humans. They have been characterized as commensals, although recent data have associated SRB with inflammatory bowel disease (Barton & Fauque, 2009; Barton & Hamilton, 2007; Dordević et al., 2021; Kushkevych, Dordević, et al., 2021; Kushkevych, Hýžová, et al., 2021; McInerney & Bryant, 1981; Muyzer & Stams, 2008; Okabe et al., 1999; Ollivier et al., 2016; Ouattara & Jacq, n.d.; Pester, 2012; Plugge et al., 2011; Postgate, 1979; Rabus et al., 2006; Widdel et al., 1992).

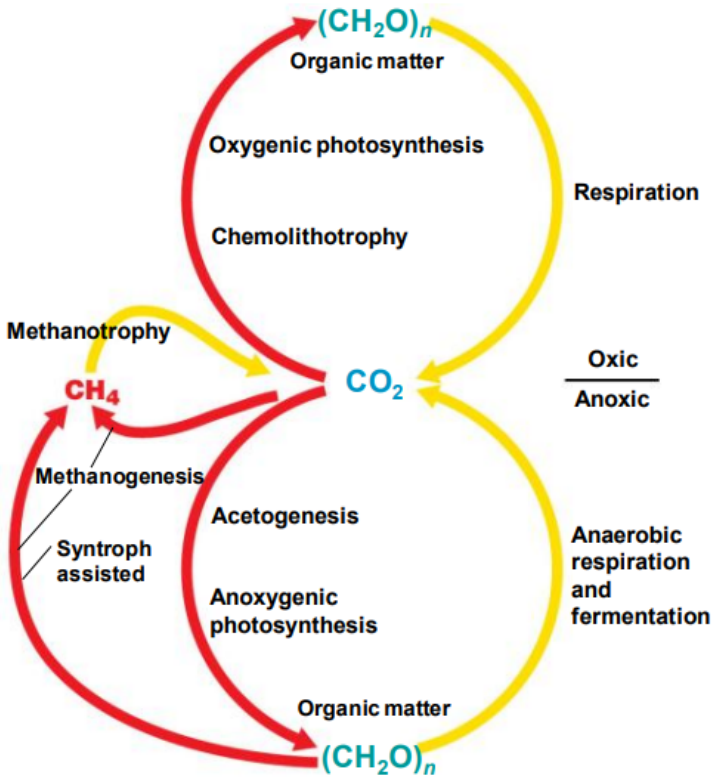

Figure 4. Carbon cycle. SRB use organic matter and through sulfate reduction form CO_2. Extracted from: Madigan et al., (2015).

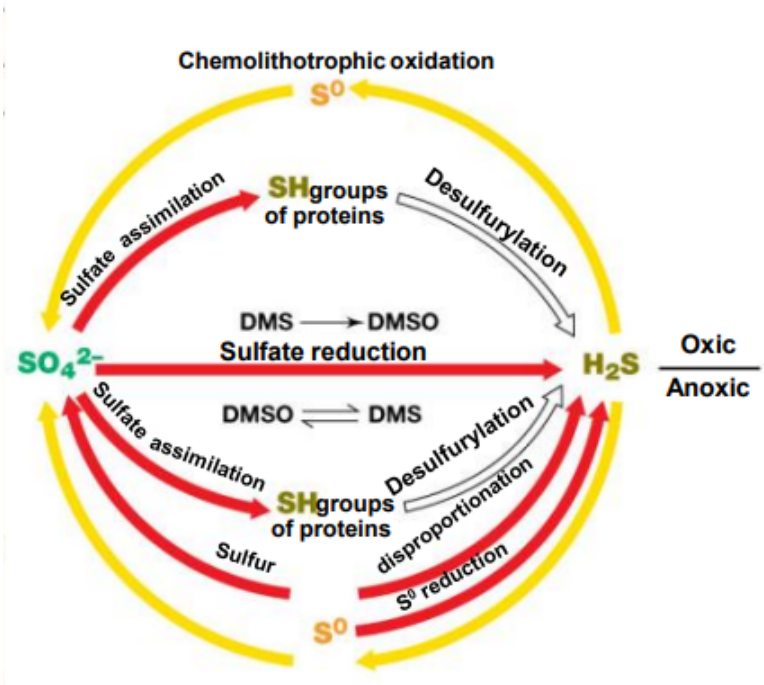

Figure 5. Sulfur cycle. SRB use $SO4^{2-}$ as terminal electron acceptors and reduce them to H_2S (sulfide). Extracted from: Madigan et al., (2015).

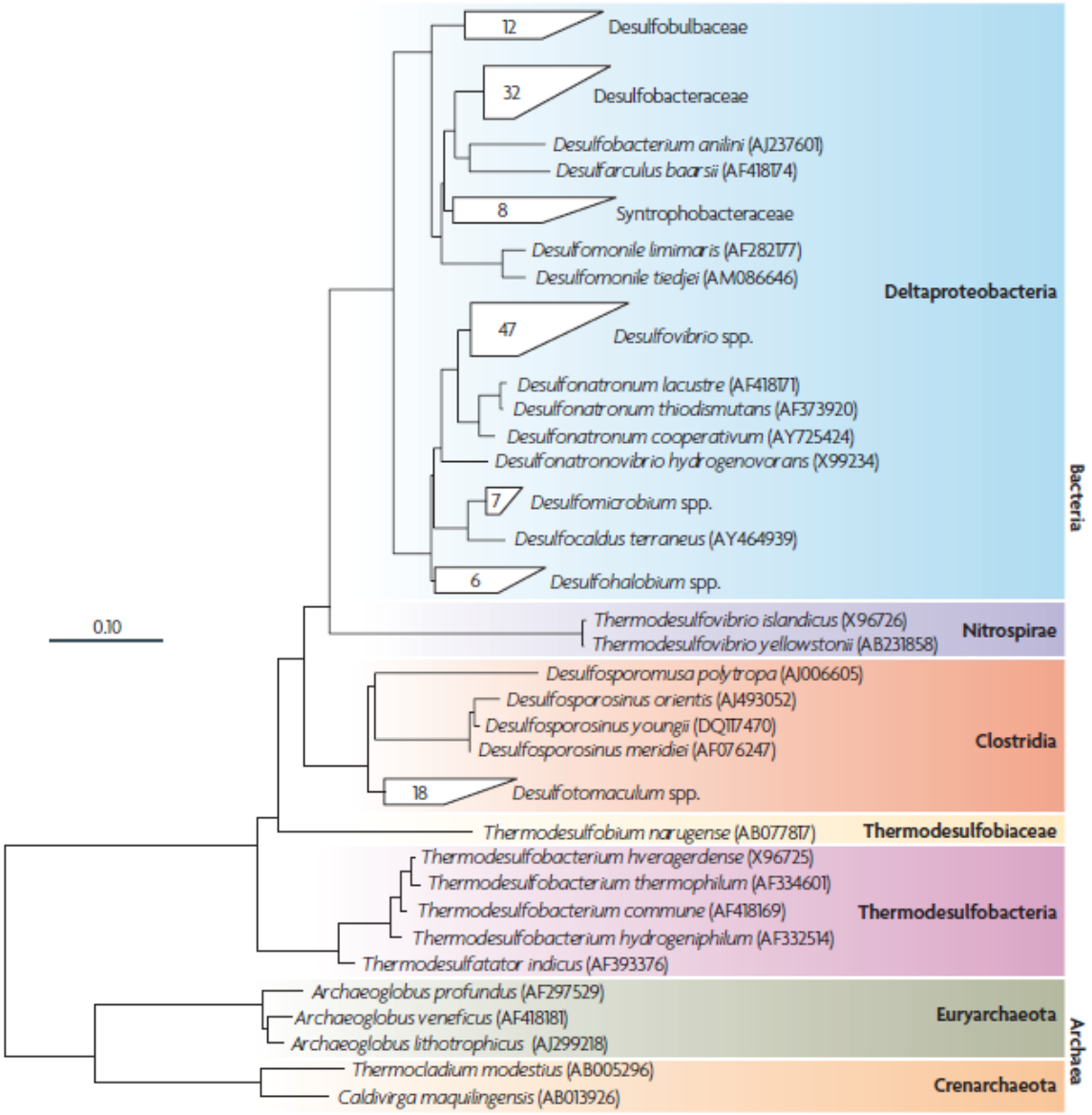

Figure 4 | **Phylogenetic tree based on nearly complete 16S ribosomal RNA (rRNA) sequences of described sulphate-reducing bacterial species.** The sequences were obtained from the SILVA small subunit (SSU) rRNA database (version 03 08 22)[149] and the tree was created using ARB software[150] (see Further information). Note the seven phylogenetic lineages of sulphate-reducing bacteria, two in the Archaea and five in the Bacteria. The number within the collapsed clusters indicates the number of different species within a particular group. The scale bar indicates 10% sequence difference.

Figure 6. Phylogenetic tree of SRP based on 16S rRNA gene for species classification. Extracted from: Muyzer & Stams (2008).

1.3.5.1 *Desulfotalea psychrophila*

D. psychrophila, the object of our research, is phylogenetically classified as an SRB member of the Desulfobulbaceae Family which is clustered within the Proteobacteria (δ-subclass) in the Proteobacteria Phylum (see Figure 6). It is an anaerobic psychrophilic microbe which optimal growth temperature oscillates at approximately 10 °C – 18 °C and pH 7.3-7.6. However, growth at temperatures down to -1.8 °C has been documented. It was isolated from Arctic marine sediments from the coast of Svalbard. It is a rod-shaped Gram-negative bacterium of sizes 0.6 μm wide and 4.5-7.4 μm long that can develop motility in aged cultures (See Figure 21 in appendix). It can grow optimally in salinity concentrations of 1% NaCl and 0.034-0.7% $MgCl_2$ although it was initially isolated at salinity concentrations of 2.5% NaCl and 1.1% of $MgCl_2$. Its duplication time has been calculated to be approximately 27 hours when grown on lactate and optimal growth temperature. Interestingly, upon first isolation and regrowth 10% of inoculum had to be used for passes since it had an unpredictable and prolonged lag phase if smaller inocula were used. Furthermore, it has been documented that this microorganism can grow in H_2-acetate, malate, pyruvate, formate, fumarate, lactate, ethanol, propanol, butanol, alanine, glycine, serine, and alanine (See Table 4 in appendix). Interestingly, when *D. psychrophila* cells are grown on lactate it oxidizes it to acetate (incomplete oxidation). It can use alternative electron acceptor such as sulfite, ferric (III) citrate, and thiosulfate. Furthermore, its fatty acids composition is formed by even-numbered monounsaturated acids (16:1c9 and 16:1c11). It also contains other lipids typical of organisms growing in subfreezing polar conditions such as, phosphatidylglycerol, diphosphatidylglycerol, and phosphatidylethanolamine (Knoblauch et al., 1999).

1.3.5.2 Genome

D. psychrophila LSv54 genome is composed of a circular chromosome of size 3523383 bp which encodes approximately 3118 open reading frames (genes) (See Figure 7). Furthermore, this microbe also possesses two plasmids of sizes 121586 bp and 14663 bp. Interestingly, *D. psychrophila* contains genes for the tricarboxylic acid cycle, TAT system (transport and secretion of proteins), cytochromes C_{553}, C_3 and ncc, nine cold-shock proteins along with cold-shock inducible proteins, approximately 30 two-component regulatory systems and 42 members of the ATP-binding subunits of ABC transporters. Moreover, it possesses seven 16S-23S-5S operons and all 20 aminoacids tRNAs (besides a selenocysteine tRNA). The G+C content of the circular chromosome is 46.8% while the G+C content is relatively lower in the large plasmid (43.6%) and small plasmid (27.5%). Furthermore, the large plasmid contains genes for conjugation functions, a reverse transcriptase (maturase), and a pyruvate dehydrogenase complex while the small plasmid contains a replication protein, a primase, and a TrsK/TraK homologue for DNA transfer. Furthermore, the circular chromosome encodes the genes for 22 proteins which are involved in flagellar organization, and it possesses 85 genes thought to be associated with vitamins synthesis. Notably, it is believed that low temperatures do not affect mRNAs stabilities in *D. psychrophila* cells. Similarly, at the protein level, they have evolved mechanisms that allow them to have more flexibility. For instance, their elongation factors have shown reduced salt bridges between protein domains, increased interactions with their solvents (increase in charge and polarity), loops (with reduced number of proline residues), and increased interactions in the protein's core. Another adaptation to cold environments is the presence of dihydrouridine incorporation in its tRNAs which allow them to maintain flexibility at low temperatures. Furthermore, the genome of *D. psychrophila* contains genes for the β-keto-acyl-CoA synthase I which is involved in the elongation of unsaturated fatty acids which confers resistance to cold environments. Interestingly,

it has been mentioned that about 80% of the fatty acids present in the membrane of this microbe are unsaturated (Rabus et al., 2004).

1.3.5.3 dsrAB operon and DsrAB (Dissimilatory sulfite reductase)

In *D. psychrophila* circular chromosome the dsrAB operon which encodes the DsrAB is located between positions 897386 and 899853 (see Figure 8). This operon which is highly conserved among SRB has a size of approximately 2.5 kb encompassing two of the crucial components of the sulfate reduction process known as dsrA and dsrB. Furthermore, there is a third component known as dsrC which encodes the DsrC polypeptide which is known to be the subunit involved in the contact between the DsrAB enzyme and the sulfur anions. More importantly, as dsr genes are highly conserved among sulfate reducers, they have been used to phylogenetically classified most SRB (along with the 16srRNA gene). A total of 13 Families, within the Bacteria Domain, have been classified, and it has been deduced that this enzyme evolved from transfer events between members of the Archaeal and Bacterial Domains. Specifically, it is believed that dsr genes were transferred from members of the archaeal *Moorella* Spp Genus from which horizontal and vertical divergence occurred among different Genera of SRB. As mentioned above, the dsrAB operon encodes the dissimilatory sulfite reductase which is formed by a pair of $\alpha_2\beta_2$ subunits containing sirohemes and Fe_4S_4 sulfur clusters. These subunits possess a total molecular weight of 180 – 220 kDa. Finally, DsrA secondary structures are composed of 15 helices which are separated by 6 β-strands while DsrB proteins are composed of 13 helices and 11 β-strands separated by loops (Karkhoff-Schweizer et al., 1995, Klein et al., 2001, Laue et al., 2001, Leloup et al., 2005, Zverlov et al., 2005, Ghosh & Bagchi, 2015, Muller et al., 2015, Muyzer & Stams, 2008, Karkhoff-Schweizer, Bruschi & Voordouw, 1993).

In our studies we used the *D. psychrophila*'s LsV54 Genome (Genbank accession number: NC_006138) (Figure 7) to first isolate most of the dsrA and dsrB genes using the universal primers DSR1FD and DSR4RE which rendered an amplicon size of 1.9 kb. This insert was sequenced to corroborate the nucleotides composition and then it was used to design qPCR-MCA primers to target the dsrB subunit. The primers generated were: D.psychroFwd100 (5'-ATCGGTAGCAGGAGTATGACA-3') and D.psychroRev100 (5'-AAGCCGTGGCAACAAGT-3') which amplify an sequence of 100 bp between positions 899055-899154 (See Figures 9, and Figure 22 in appendix).

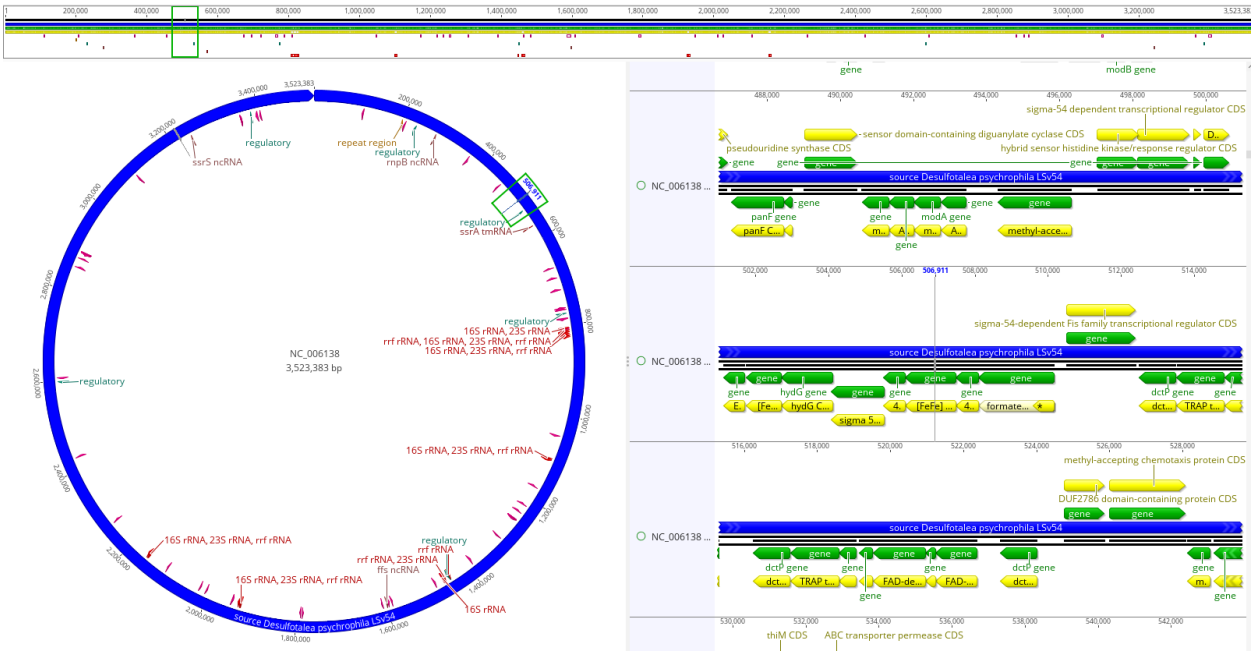

Figure 7. *D. psychrophila*'s Genome (Genbank accession number: NC_006138). This template was used to design all primers for qPCR-melting curve analyses. NCBI microbial genomes database was used to locate specific proteins and their locations in the genome. Image obtained from Geneious software.

Figure 8. *D. psychrophila*'s Genome (Genbank accession number: NC_006138) dsrAB operon. On the right, we can see the dsrAB operon (dsrA and dsrB subunits) which has an amplicon size of 2.5 kb and it is situated in positions 897386 and 899853. Portions of dsrA and dsrB were extracted using DSR1FD and DSR4RE universal primers (positions 897533-899480) which has an amplicon size of 1.9 kb. This amplicon was used to design qPCR-MCA primers D.psychroFwd100 (5'-ATCGGTAGCAGGAGTATGACA-3') and D.psychroRev100 (5'-AAGCCGTGGCAACAAGT-3') which targets the dsrB subunit (positions 899055-899154).

1.3.5.4 Sulfate reduction

Sulfate reduction in SRP is driven by two types of metabolic pathways. The assimilatory sulfate reduction pathway and the dissimilatory sulfate reduction pathway (Figure 9). In the assimilatory sulfate reduction pathway, sulfate reduction is performed incompletely just to generate enough energy to synthesize cellular materials (H_2S is not generated in high quantities), while in the dissimilatory sulfate reduction pathway, sulfate is completely reduced to H_2S (high concentrations of sulfide are generated). In both pathways, sulfate is transported to the interior of the cell by a H^+ dependent symporter known as sulP, along with an alternative ABC transporter known as cysPTA which transports thiosulfate (Figure 10). Furthermore, the assimilatory pathway starts when the enzyme ATP-sulfurylase generates adenosine-5'-phosphosulfate (APS) and pyrophosphate (PP) by catalyzing a reaction which involves ATP and sulfate. Furthermore, APS is phosphorylated at

its carbon 3 by an enzyme known as APS-kinase (adenylyl sulfate kinase) to form 3'-phosphoadenosine 5'-phosphosulfate (PAPS) and ADP. Finally, PAPS is reduced to 3',5'-diphosphoadenosine (PAP) and sulfite using reduced triphosphorydine nucleotide (thioredoxin or TPNH) as the electron donor and catalyzed by the PAPS-reductase. Interestingly, some bacterial strains use the assimilatory sulfate reduction pathway to further reduce sulfite to sulfide and cysteine by means of the assimilatory sulfite reductase and the O-acetylserine sulfhydrolase respectively (See Figure 9). On the other hand, the dissimilatory sulfate reduction pathway uses ATP-sulfurylase to generate APS from ATP and sulfate. Furthermore, APS is reduced by means of the APS reductase to form sulfite and adenosine monophosphate (AMP), and finally sulfite is reduced to sulfide by means of the dissimilatory sulfite reductase (See Figure 9). Moreover, ATP is generated by means of the oxidation of organic compounds. As it can be seen in Figure 10, aminoacids are assimilated into the TCA cycle by their biotransformation into pyruvate and further decarboxylation into acetylCoA, lactate is transformed into pyruvate, acetate enters the TCA cycle as acetylCoA, alcohols are transformed into pyruvate or acetylCoA, and succinate, fumarate, and malate enter the TCA cycle directly. All of these pathways will eventually generate NADH molecules which will generate a proton motive force due to electron transfer in the electron transport chain and further ATP generation by the F1/F0-ATPase (See Figure 10) (Peck, 1961, Rabus et al., 2004).

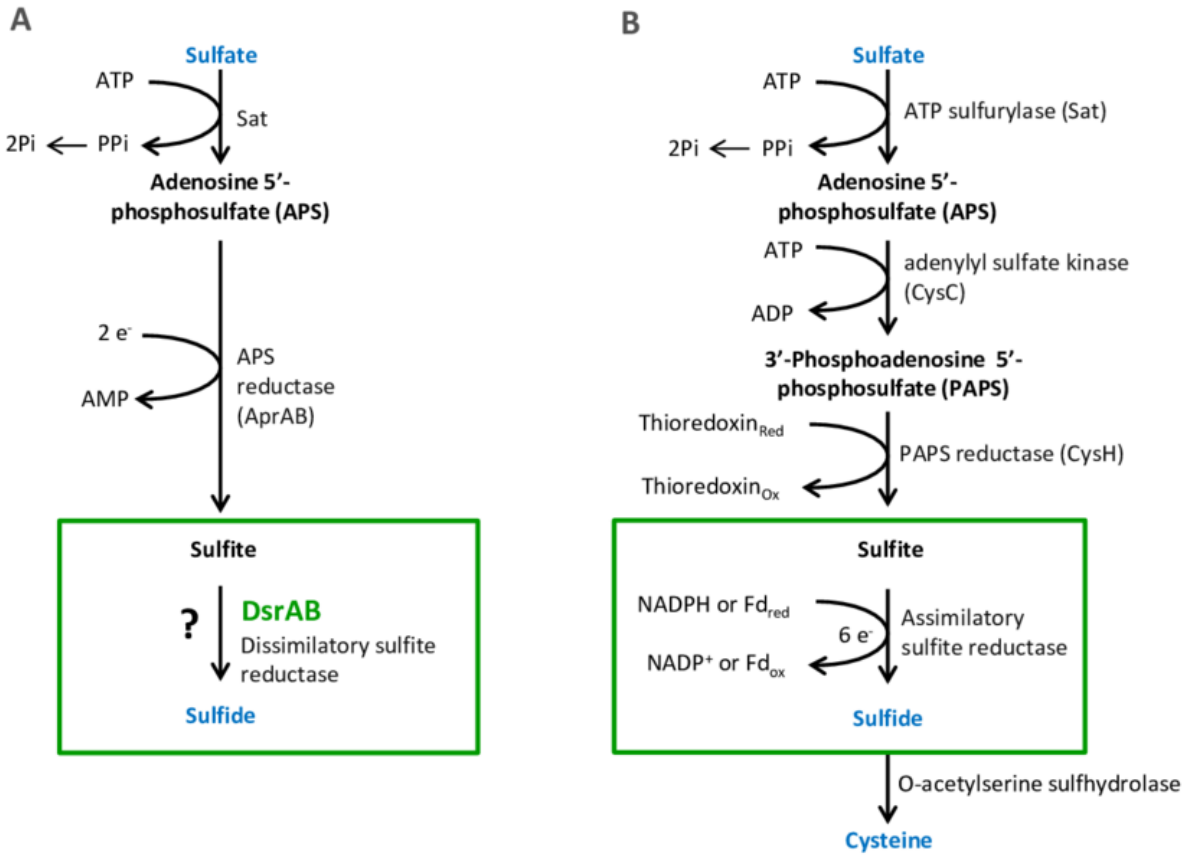

Figure 9. Sulfate reduction pathways. In A) Dissimilatory sulfate reduction pathway and B) Assimilatory sulfate reduction pathway, both performed by SRP. Image extracted from: A. A. Santos et al., (2015).

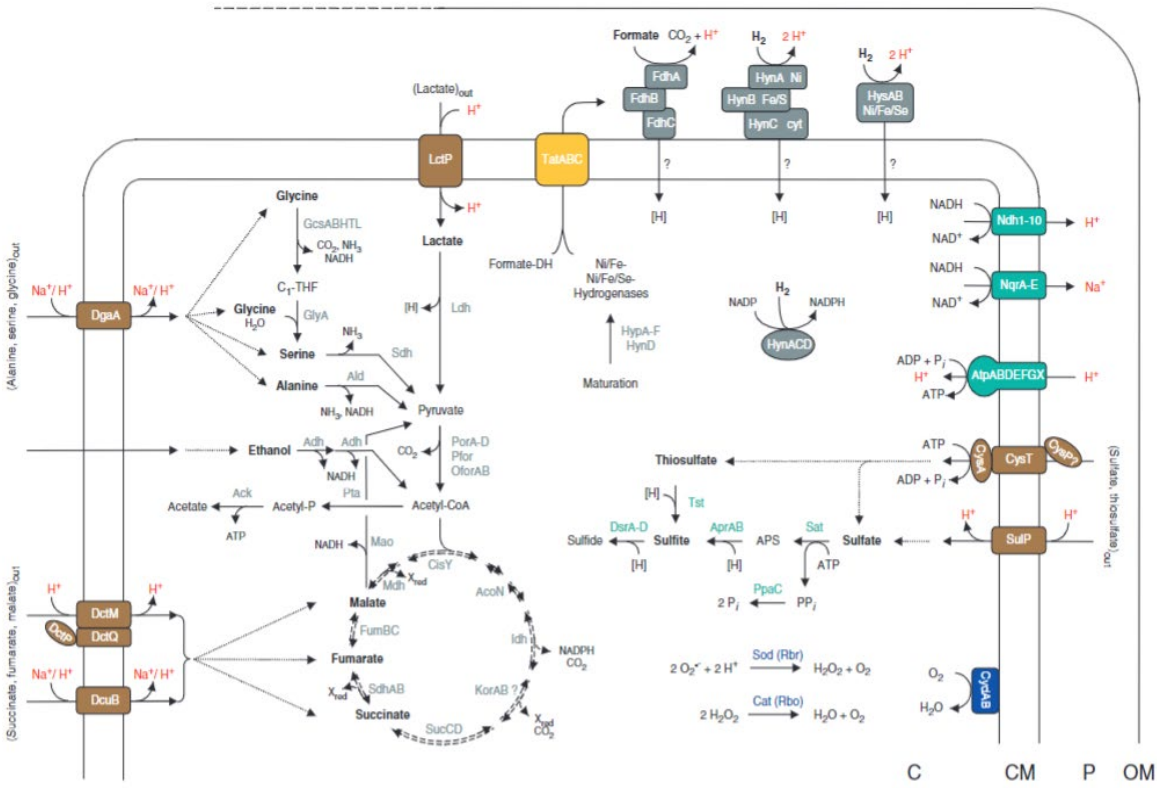

Figure 10. Scheme of carbon sources oxidation, sulfate reduction and ATP synthesis in *D. psychrophila* cells. Transporters of raw materials are also shown. Figure was extracted from Rabus et al., (2004) publication.

Finally, the field of Astrobiology has advanced exponentially in the last 60 years, from the experiments of Miller and Urey about early Earth's prebiotic life to the current ability to send robotic devices to other planetary bodies (Miller, 1958; Leger, Pirre & Marceau, 1993). However, in virtue of the increasing interest in the exploration of planets where extreme low temperatures dominate and as there is evidence that points out to the presence of sulfate compounds in these icy worlds, our research team have hypothesized that *D. psychrophila* cells could maintain metabolic activity at subfreezing temperatures in environments bearing increasing concentrations of those minerals. Moreover, we have suggested that the ability of *D. psychrophila* cells to metabolize at

these temperatures could be the result of chaotropic effects provided by Mg^{2+}, Ca^{2+}, Fe^{2+} and Fe^{3+} ions in solution which could aid in the maintenance of the cell's membrane fluidity. This topic is relevant to the field of Astrobiology since charting the mechanisms that psychrophilic bacteria use to sustain metabolic activity at subfreezing temperatures is crucial to understand the possible mechanisms that microbes could use to survive in other planetary bodies. More importantly, this investigation has implications associated to the formulation of the COSPAR planetary protection policy as well as the classification of Mars special regions, both of which are used to regulate interplanetary travel.

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CHAPTER II

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Title: Detection of dsrAB operon expression in *D. psychrophila* cells subjected to simulated Martian conditions of temperature and regolith's sulfate minerals composition.

1. Abstract

The 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 extremophile microorganisms 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 sulfate 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 and pressures. Using standard bacteriological methods for determining growth, combined with molecular and enzymatic determination of sulfate reduction, we have found that *D. psychrophila* is capable to carry out biological processes at temperatures down to -5 ºC, at concentrations that range from 0.35 wt% to 18 wt% of $MgSO_4$, 0.1 wt% of $CaSO_4$, and 10 wt% to 14 wt% of $FeSO_4$ in which the highest sulfate 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 proteins, DNA, RNA, membranes, and other biological structures. In cold environments, 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 sulfate reducers in other planets and icy satellites within and out of the solar system.

2. 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 sulfate 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 water within the regolith structure. Moreover, estimations of the chemical species present in the Martian surface have pointed out to the existence of enough Fe, Mg and Ca cations which could easily combine with sulfates (Clark and Van Hart, 1980). Interestingly, McGlynn et al. (2012) has estimated that these chemical species could occur in Gusev Crater at concentrations around 5.4 wt%, while in Meridiani Planum, it has been estimated to be at around 5.5 wt%. Strikingly, $MgSO_4$ and $CaSO_4$ constitute around 10% of the Martian soil. Therefore, the hypothesis that bacterial life could persist in Martian extreme environments is feasible, at least temporarily in subsurface areas where water films, and protection from radiation and extremes of temperatures are provided (Arvidson et al., 2005; Bibring, 2005; Clark and Van Hart, 1981; Gendrin et al., 2005; Langevin et al., 2005; McGlynn et al., 2012).

In general, sulfates 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; Pappalardo, 2010;

Prockter et al., 2010; Schmidt & Manning, 2017; Vance et al., 2018; Zolotov & Shock, 2001). Furthermore, based on observational studies, Europa's ocean could produce hydrated sulfates of Mg and Ca by means of upwelling in fractures of the icy shell. Likewise, in Ganymede's surface, remnants of $MgSO_4$ and $NaSO_4$ brines, possibly sustained by Ganymede's subsurface ocean, have been identified (McCord et al., 2001; Molyneux et al., 2020; Pan et al., 2021). There is also evidence that sulfates could be present in other icy moons of the Saturn system. Furthermore, water pockets located at the lower part of Titan's ice crust as well as cracks of the icy crust where brines could develop have been pointed out as suitable environments for microorganisms' colonization (Simakov, 2001).

Extremophile microbes are thought to be the first organisms to colonize Earth and are responsible for the formation of all ecosystems present in today's World. This phenomenon was accompanied by deep modifications of genetic information that allowed these microbes to adapt to constant challenging environments. As a consequence of this, our planet ended up acquiring radioresistant, halophilic, barophilic, acidophilic, alkaliphilic, thermophilic and psychrophilic genetic compositions. According to the chronological distribution of these extremophiles on Earth, it is known that 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. 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 (Deming, 2002; Inniss, 1975; Panikov, Flanagan, Oechel, Mastepanov, and Christensen, 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 thought have changed due to the implications of global warming and the loss of autochthonous microbial communities from these environments (Deming, 2002).

As it is 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 & Pikuta, 2010; Moyer & Morita, 2017). Moreover, the term psychrophile was mentioned for the first time by Schmidt-Nielsen while working with microbes that could grow at 0º 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 ºC, a maximal growth temperature at 20 ºC, and a minimal growth temperature at or below 0 ºC (Feller & Gerday, 2003; Hoover & Pikuta, 2010; Inniss, 1975; Morita, 1975; Moyer & Morita, 2017; 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 (Piette et al., 2011; Steven et al., 2006).

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 & Morita, 2017). 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 & Gerday, 2003; Moyer & Morita, 2017).

The lowest temperature registered in which bacteria can metabolize is -20 ºC (doubling time of 160 days) (Deming, 2002; Inniss, 1975; Moyer & Morita, 2017; Panikov et al., 2006; Pikuta et al., 2007). However, Satyanarayana et al., (2005) and Steven et al., (2006) suggested that microbial life is limited to temperatures down to -12 ºC. According to them, beyond that temperature metabolic activity is restricted. Similarly, Mykytczuk et al. (2013) have pointed out that there are no reports of bacterial growth below -15 ºC (Mykytczuk et al., 2013), although survival have been reported down to -30 ºC (in situ) and microbial metabolisms have been predicted to occur at temperatures down to -40 ºC (Moyer & Morita, 2017; Pikuta & Hoover, 2003; Price & Sowers, 2004). However, sulfate reduction on Earth has only been detected down to -20 °C (Lamarche-Gagnon et al., 2015). 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, increased unsaturated, short, branched and cyclic fatty acids at the cell membrane level, synthesis of cold shock proteins and cold-acclimation proteins, selective gene expression of cold active enzymes, induction of efficient mechanisms for protein synthesis and protein folding, synthesis of antifreeze proteins, and cryoprotectants, etc. (Deming, 2002; Feller & Gerday, 2003, 2003; Hoover & Pikuta, 2010; Inniss, 1975; Maccario et al., 2014, 2014; Moyer & Morita, 2017; Piette et al., 2011, 2011; Pikuta et al., 2007; Pikuta & Hoover, 2003; Poli et al., 2017; Satyanarayana et al., 2005).

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 (Moyer & Morita, 2017; Price & Sowers, 2004; Steven et al., 2006).

Additionally, some psychrophilic microbes are assembled within a subdivision of microorganisms known as sulfate 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 (Karr et al., 2005; Kjeldsen et al., 2007; Leloup et al., 2007; Miletto et al., 2011; Teske et al., 1996). They can use sulfate anions as terminal electron acceptors in the generation of metabolic energy. In this process, they use an enzyme known as the dissimilatory sulfite reductase (DsrAB) which is encoded in the dsrAB operon and intervenes in the last step of sulfate reduction. Furthermore, the dsrAB operon encodes the genes for two other enzymes that are involved in the sulfate reduction process, ATP sulfurylase and adenylyl-sulfate reductase (Karkhoff-Schweizer et al., 1995; Laue et al., 2001; Muller et al., 2015; Rüffel et al., 2018; Thorup et al., 2017). Among this group of bacteria, *D. psychrophila,* a Proteobacterium, is distinctive due to its psychrophilic nature (Rabus et al., 2004a; Yadav et al., 2017). *D. psychrophila* has an optimal growth temperature of 10 ºC and it can grow at temperatures down to -1.8 ºC (Knoblauch et al., 1999; Rabus et al., 2004a). 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 the scope of bacterial biomass (growth) measurements only accounts for bacterial strains that are actively dividing within a set of cultural/environmental parameters (First group of Price and Sowers classification). Therefore, our study aims to detect the survivability and metabolic activity of this microbe at different temperatures down to -5 ºC and in the presence of different types and concentrations of sulfate salts using molecular approaches as a proxy to estimate the survival of psychrophilic sulfate reducers in sulfates-rich planets such as Mars and icy satellites such as Europa, Ganymede, and possibly other satellites from the Jovian and Saturn systems. 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+}.

3. Materials and Methods

3.1 Culture conditions

Active cultures of *D. psychrophila* (DSM 12343) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) collection. The medium used to replicate this organism was DSMZ141. 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 ºC, 25 mL serum bottles containing a modified version of DSMZ141 (Na_2S was replaced with sodium dithionate at a concentration of 20mg/L) were inoculated and incubated at 10 ºC for 30 days. These starter cultures were used to inoculate a temperature positive control at 10 ºC and two different experiments at 0 ºC and -5 ºC in triplicates ($MgSO_4$, was replaced by different sulfate compounds at increasing concentrations, see Table 1). Simultaneously, growth negative controls (non-inoculated serum bottles containing media) were prepared for all sulfate concentrations at the three temperatures of incubation. After inoculation, all cultures were transferred on ice and incubated at their respective temperatures for 30 days. Experiments at 0 ºC and -5 ºC were performed in a biochamber known as Pegasus located at the Keck Lab of the University of Arkansas (Figure 27 in appendix) which specifics have been described by 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 ºC). 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 a separated 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 sulfate reduction was recognized only if this white precipitate turned blackish (deposition/sedimentation of sulfites and sulfides) (Rüffel et al., 2018).

3.2 Cultures growth

Cultures 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. These measurements were performed in triplicates, after blanking the equipment with 1 mL of sterile medium (specific for different cultures supplemented with different sulfate concentrations). Readings of 1 mL of sterile medium (specific for each supplementation) were used to ensure that the spectrometer was ready for samples measurement. The optical density data generated was analyzed by means of pairwise t-test at a confidence level of 95% (α=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 °C, 0 °C and -5 °C were assessed in triplicates. 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 1mL 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 analyzed by means of pairwise t-test at a confidence level of 95% (α=0.05).

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

Component	Quantity	Sulfate 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	10 mL	**7**:$Fe_2(SO_4)_3$ 10 wt %	$Fe_2(SO_4)_3$ 10 wt % C-
$Fe(NH_4)_2(SO_4)_2$ x $6H_2O$	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	1 g	**10**:$Fe_2(SO_4)_3$ 40 wt %	$Fe_2(SO_4)_3$ 40 wt % C-
**Yeast Extract	2 g	**11**:$Fe_2(SO_4)_3$ 48 wt %	$Fe_2(SO_4)_3$ 48 wt % C-
**Trypticase peptone	2 g		
Na-resazurin sltn (0.1 % w/v)	0.50 mL		
$NaHCO_3$	5g		
***Vit Soltn	10 mL		
***Sodium Dithionite	20 mg		
Distilled water	1 L		

*Follow instructions from DSMZ141 standard preparation

C- denotes growth negative controls

**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 the interstellar space. Also, many icy bodies possess a complex chemistry that produce 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.
***Taken from DSMZ195c

3.3 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) and RNase A (10 µg/mL) incubations steps. Furthermore, DNA concentration and purity were assessed by means of a NanoDrop 2000 Spectrophotometer (ThermoScientific). Once these parameters were ideal, amplifications of the dsrAB operon (2.5 kb) were performed using the universal primers, DSR1FD 5' – ACTCACTGGAAGCACG- 3' and DSR4RE 5' – GTGTAACAGTTACCACA- 3´ (see Figure 8). The PCR protocol used was Activation 95º C for 5 min, Denaturation 95º C for 1 min, Annealing 54º C for 1 min, Extension 72º C for 9 min and Final extension 72º 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 ºC and experiments at 0 ºC and -5 ºC). Furthermore, positive PCR products were used to sequence the dsrAB operon and further qPCR-MCA (real time polymerase chain reaction-melt curve analysis) primers design. Moreover, dsrAB sequences were analyzed, 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 (See Figure 22 in appendix).

RNA was extracted from all replicas 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 ºC until downstream analysis 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.

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 triplicates. Once plates were seeded with primers, dye and cDNA template, they were subjected to the Melt Curve protocol: 90.0º C for 0:30 sec, 90.0º C for 0:15 sec, 59.0 ºC for 0:15 sec, 72.0 ºC for 1 min, 10 cycles; 90.0 ºC for 0:15 sec, 59.0 ºC for 1 min, 72.0 ºC for 0:30 sec and Plate read (Fluorophore SYBR), 35 cycles; 72.0 ºC for 3 min, 90.0 ºC for 0:15 sec, 65.0 ºC for 3 min and Melting curve with the next parameters: 75.0 ºC to 90.0 ºC with an increment of 0.1 ºC every 0:05 sec 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 per reaction. Amounts of the targeted dsrAB sequence were obtained by plotting the Ct values onto the standard curve. Finally, differences in expression were analyzed by means of an ANOVA and pairwise-t-test analysis at a confidence level of 95% (α=0.05).

3.4 Methylene blue (MB) Analysis

In order to detect sulfides in solution and consequently sulfate reduction, MB analysis of the temperature positive control, all samples replicas and growth negative controls was performed using the MB protocol used by Basic et al. (2015) (Basic et al., 2015; Cline, 1969; Johnston et al., 2005; Thorup et al., 2017). By using this protocol, we aimed to detect the presence of dissolved sulfides-sulfur (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, pH =9.6). Immediately after, 18 µL of solution B was added (contains 17.1 mM of N,N-dimethyl-p-phenylenediamine sulfate and 37 mM $FeCl_3$ in 6 M HCl). Samples preparations were made in triplicates per sulfate concentration and temperature. Subsequently, they were read using the Synergy HT microplate reader from BioTek at a wavelength of 668 nm after 30 minutes 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 (α=0.05).

4. Results

4.1 *D. psychrophila* growth under various sulfate 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 sulfate reduction. As it is shown in Figure 11A, cultures supplemented with 0.35 wt% of $MgSO_4$ at 0 ºC and 10 ºC showed evidence of bacterial growth (biomass) along with sulfites/sulfides sedimentation. Furthermore, samples cultivated under 0.1 wt% of $CaSO_4$ at 0º C and 10 ºC were positive for both biomass production and sulfites/sulfides sedimentation (Figure 11B). However, as it is shown in Figure 11C, some other cultures did not present evidence of bacterial growth and/or sulfites/sulfides precipitation.

In Our analysis of turbidity (OD600nm), we can see that in cultures supplemented with $MgSO_4$ or $CaSO_4$ most growth occurred at temperatures of 0 °C or 10 °C (at all concentrations). However, at increasing concentrations of $MgSO_4$ ($MgSO_4$ 10 wt% and 18 wt%) cells were able to duplicate better at 0 °C temperatures rather than 10 °C. Importantly, growth at -5 °C was minimal at all concentrations (t-test, α=0.05) (see Figure 12).

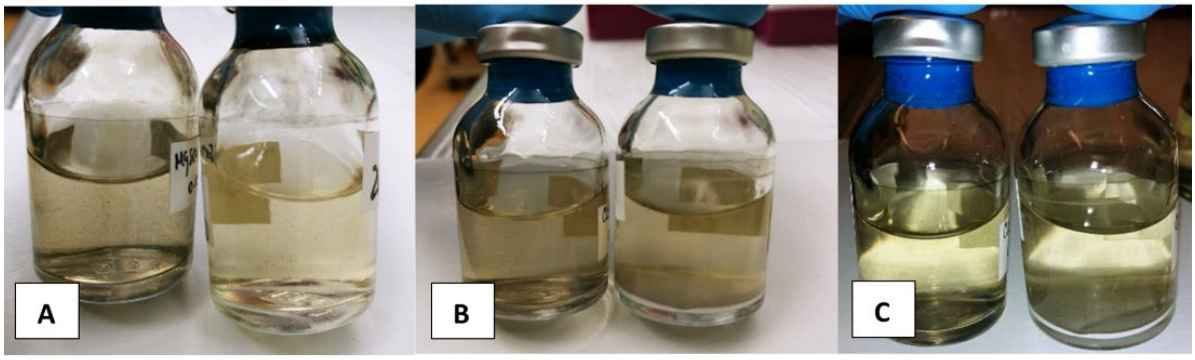

Figure 11. *D. psychrophila* growth and sulfide formation in medium supplemented with: (A) $MgSO_4$, (B) $CaSO_4$, (C) $MgSO_4$ and $CaSO_4$ with no bacterial growth.

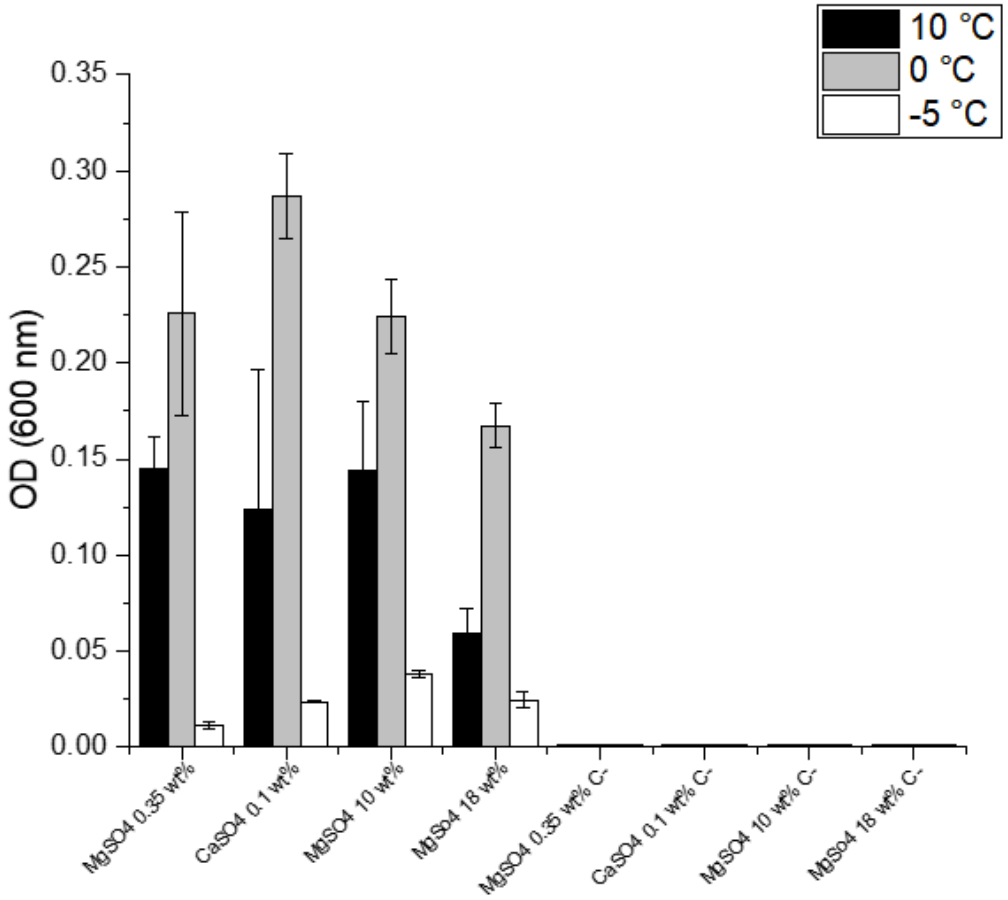

Figure 12. OD measurements of *D. psychrophila* cultures subjected to $MgSO_4$ and $CaSO_4$ at three different temperatures. (10 °C=temperature positive control, 0 °C=experimental 1, -5 °C=experimental 2, C- = Growth negative controls). N = 72.

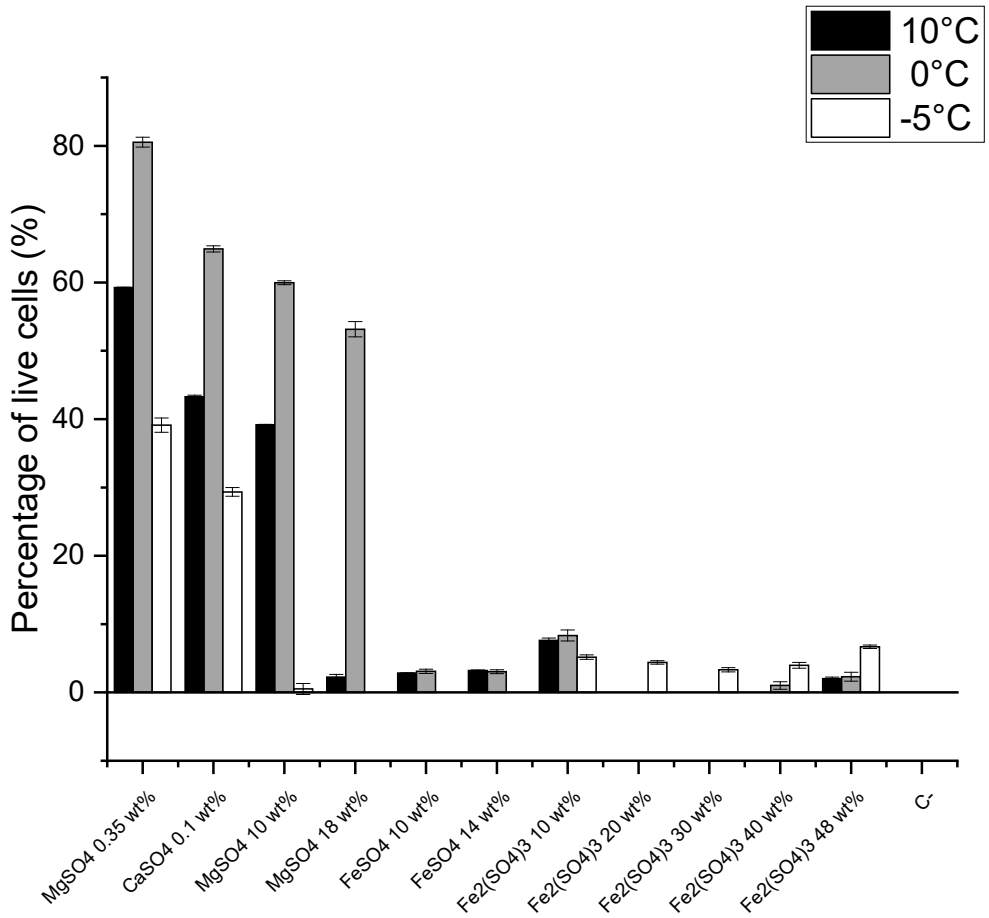

Figure 13. Percentage of live cells in cultures of *D. psychrophila* subjected to increasing concentrations of sulfate salts at three different temperatures. (10 °C=temperature positive control, 0 °C=experimental 1, -5 °C=experimental 2, C- = Growth negative controls). Samples were assessed by live/dead fluorescence analysis using SYTO 9 and propidium iodide. Samples were normalized using growth negative controls fluorescence. N = 198.

Furthermore, our live/dead cells analysis showed similar results than our analysis of optical density for $MgSO_4$ and $CaSO_4$ supplementations where the highest bacterial growth occurred at 0 °C and the lowest occurred at -5 °C (t-test, α=0.05) (Figure 13). Interestingly, supplementations with $MgSO_4$ 10 wt% 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 °C 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 wt% and 48 wt%) at 10 °C and 0 °C (Figure 13).

4.2 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. Therefore, they were used to assess whether *D. psychrophila* can metabolize at low temperatures in the presence of various concentrations of sulfate compounds. Furthermore, as there was no evidence of active growth or sulfate reduction in cultures supplemented with $Fe_2(SO_4)_3$ at 20 wt% and 30 wt% under 10 °C incubations (Figure 13, Figure 15, and Table 2), their Ct values were used to establish a cutoff value for Ct detection. As it is shown in Figure 14 and Table 2, cultures containing $MgSO_4$, $CaSO_4$ and $FeSO_4$ at any of the concentrations and temperatures tested showed higher copy numbers of the targeted sequence (t-test α=0.05).

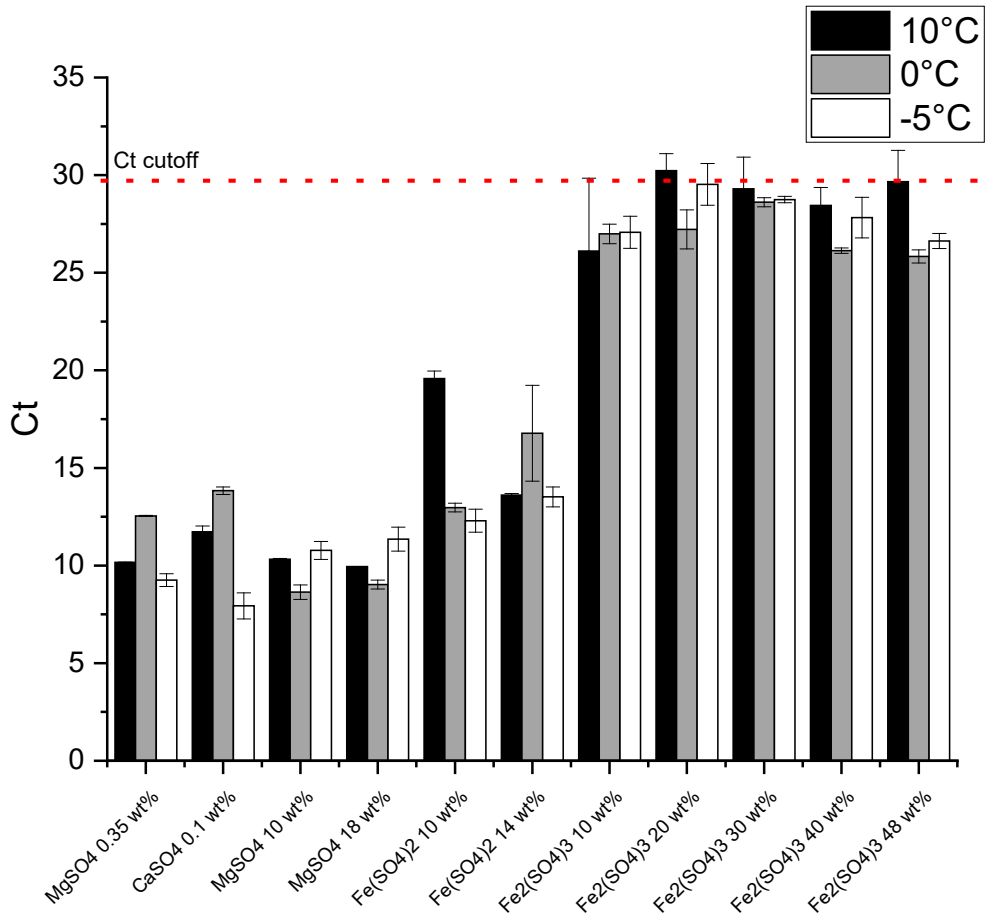

Figure 14. Detection of Expression of dsrAB operon in samples of *D. psychrophila* cultures grown in different types and concentrations of sulfate compounds classified by Ct values and temperatures. (temperature positive control at 10 ºC, experiment at 0 ºC, and experiment at -5 ºC). Lower Ct values indicate higher detection of RNAs synthesized from the dsrAB operon. Ct cut off for detection was estimated based on $Fe_2(SO_4)_3$ gradient at 10 °C (Ct =29.7). N = 198.

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 Fig 14 and Table 2). Moreover, supplementations with increasing concentrations of MgSO4 (10 wt% and 18 wt%) did not show significant differences among the three temperatures tested (t-test, α=0.05) (Figure 14, and Table 2).

Table 2. Copy numbers per reaction estimated from standard curve. Results include temperature positive control (10 °C), and experiments at -5 ºC, and 0 ºC. N = 198

Sulfate concentration	Copy numbers/reaction		
	10º C	0º C	-5º C
$MgSO_4$ 0.35 wt%	6.02 x10^9	4.65 x10^9	6.50 x10^9
$CaSO_4$ 0.1 wt%	5.11 x10^9	3.92 x10^9	7.30 x10^9
$MgSO_4$ 10 wt%	5.84 x10^9	6.84 x10^9	5.70 x10^9
$MgSO_4$ 18 wt%	6.11 x10^9	6.67 x10^9	5.30 x10^9
$Fe(SO_4)_2$ 10 wt%	5.84 x10^8	4.38 x10^9	4.74 x10^9
$Fe(SO_4)_2$ 14 wt%	4.01 x10^9	2.19 x10^9	4.11 x10^9
$Fe_2(SO_4)_3$ 10 wt%	Not detected	Not detected	Not detected
$Fe_2(SO_4)_3$ 20 wt%	Not detected	Not detected	Not detected
$Fe_2(SO_4)_3$ 30 wt%	Not detected	Not detected	Not detected
$Fe_2(SO_4)_3$ 40 wt%	Not detected	Not detected	Not detected
$Fe_2(SO_4)_3$ 48 wt%	Not detected	Not detected	Not detected

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 (Figure 14 and Table 2). Like the $MgSO_4$ samples, all $CaSO_4$ supplementations at the three temperatures tested showed increased copy numbers when compared with the Ct cutoff value (t-test, α=0.05).

In the samples supplemented with $FeSO_4$ at 10 wt% cells grown at 0 °C 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. Nevertheless, if we take into consideration the Ct cutoff value, we can observe that there was high dsrAB copy numbers at both concentrations and at all temperatures tested (t-test, α=0.05) (see Figure 14 and Table 2).

Finally, all samples supplemented with $Fe_2(SO_4)_3$ did not show significant differences in copy numbers among the temperatures tested (t-test, α=0.05) (see Figure 14 and Table 2).

4.3 Sulfate reduction (Enzymatic activity)

In our analysis of sulfate reduction (MB method), we quantified the absorbance of sulfides 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 sulfate reduction activity, the temperature positive control at 10 °C presented the opposite behavior while the cultures at 0 °C exhibited intermediate levels of sulfate reduction (Fig 15) (t-test, α=0.05). Furthermore, cultures supplemented with $MgSO_4$ at increasing concentrations (10 wt% 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 sulfate reduction (t-test, α=0.05) (see Fig 15). Additionally, cultures grown with $CaSO_4$ showed the highest rate of sulfate reduction at 0 °C followed by the cultures at 10 °C and -5 °C both of which had similar sulfate reduction rates (t-test, α=0.05). Finally, the data from our MB analysis pointed out to the absence of sulfides in solution in any of the $FeSO_4$ supplementations for any of the temperatures tested (t-test, α=0.05) while $Fe_2(SO_4)_3$ supplementations showed only minimal sulfate reduction rates at 0 °C and -5 °C (Figure 15).

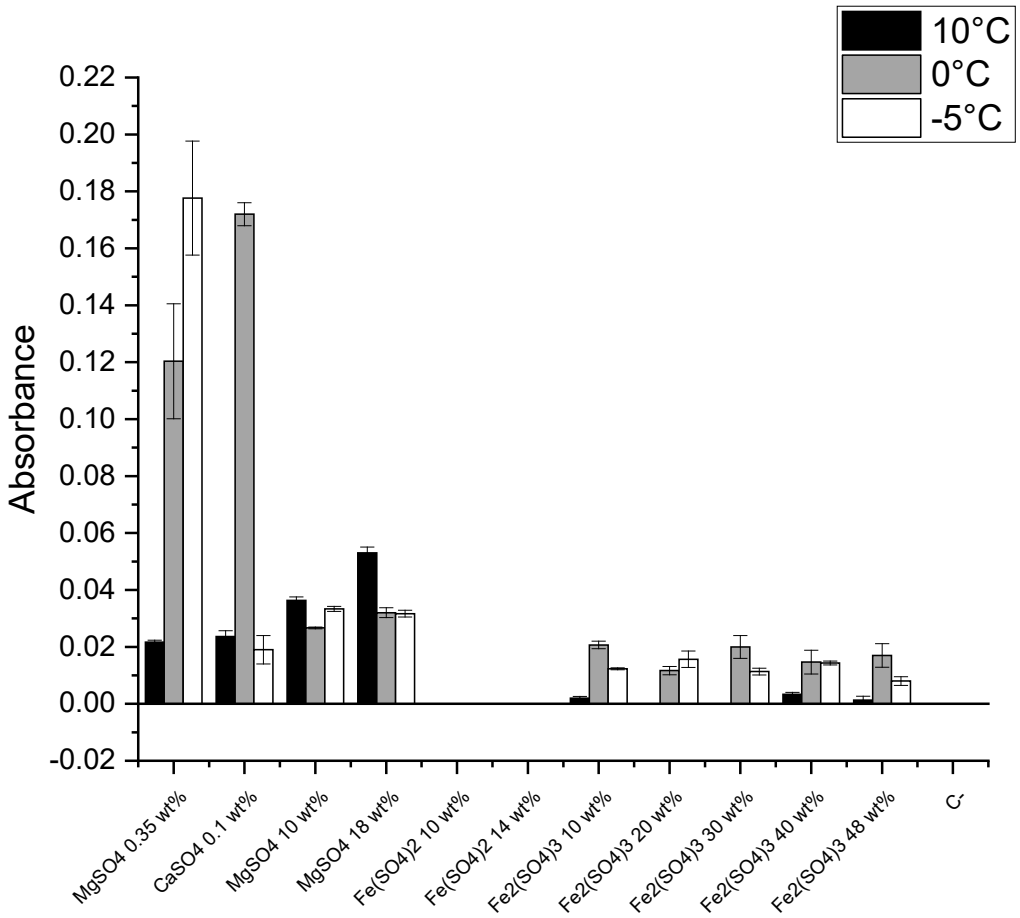

Figure 15. Sulfides in solution measurements using MB protocol of samples subjected to three different temperatures and different sulfate concentrations. High absorbances indicate increasing concentrations of sulfides in solution. Note that highest absorbances were registered at -5 ºC under $MgSO_4$ 0.35 wt%, followed by the cultures at 0 ºC. Surprisingly, highest absorbances were detected with $CaSO_4$ 0.1 wt% at 0 ºC. C- denotes absorbance from growth negative controls used for normalization. N = 198.

5. Discussion

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

D. psychrophila is an organism adapted to grow at low temperatures (growth range from 10 ºC down to -1.8 ºC) and a sulfate reducer. These two characteristics are paramount to conceive scenarios in which this microbe or similar microbes could potentially colonize other planets, icy bodies 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 -5º C) in cultures supplemented with 0.35 wt% of $MgSO_4$. As it can be seen in Figure 14 and Table 2, 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 the Microbiological standpoint, the ability of this microbe to transcribe at similar rates at -5 ºC and 10 °C in pure culture is surprising since the cell proliferation was considerably low in -5 °C cultures (see Figure 12, and 13). 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 & 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 sulfate 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 Figures 12, 13, 14 and Table 2). 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). Importantly, the survival capabilities of *D. psychrophila*, 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 has higher dsrAB copy numbers at -5 ºC followed by the temperature positive control at 10º C and the experiment at 0 ºC (See Figure 14 and Table 2). 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 Fig 12, and 13). 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 °C 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 et al. (2017) 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.: DsrAB in the case of sulfate 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 sulfate 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 sulfate anions, and chaotropic cations (such as Mg^{2+} and Ca^{2+}) as well as other environmental conditions conductive of life are present (Morita, 1975; Inniss, 1975; Knoubach et al., 1999; Rabus et al., 2014; Moyer & Morita, 2017).

Furthermore, 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 °C and 10 °C showed the highest cell density (see Figure 12, 13, 14 and Table 2). Interestingly, if we analyze the OD/live/dead results by MgSO4 concentration and temperature tested, we can observe that cellular proliferation decreased as $MgSO_4$ concentrations increased (Figure 12, and 13). Equally important, cultures at -5 °C showed dsrAB copy number levels that were indistinguishable from the other temperatures tested (Figure 14 and Table 2). This behavior is analogous to the behavior 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 positive control and the 0 ºC 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 Fig 14 and Table 2). Under these circumstances, we can observe that cells at 10 °C 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 Figures 12, and 13). 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 sulfate 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 °C 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 behavior could be that cells that were used to inoculate the cultures coped with these conditions, at least temporarily, and synthesized dsrAB mRNAs which were then detected in our qPCR-MCA analysis. The same behavior was observed at increasing concentrations of $FeSO_4$ (14 wt%) in which there were no indications of active cellular growth (see Figure 13), but the analysis of dsrAB expression revealed the presence of mRNAs (as indicated per Ct cutoff value in Figure 14 and Table 2).

This outcome suggests that $FeSO_4$ at any concentration do 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 sulfate 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 sulfates at temperatures below 0 °C such as those present in 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 sulfate, our results showed no significant differences in dsrAB copy numbers by temperature, within subgroups of $Fe_2(SO_4)_3$ concentrations or with the Ct cutoff value (See Figure 14 and Table 2). 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 Figure 13). 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 sulfate concentration increased the cells incubated in warmer temperatures resulted compromised. However, active growth at -5 ºC temperatures suggests that *D. psychrophila* could survive and colonize at least temporarily environments such as planets or icy bodies with high concentrations of ferric sulfate, but eventually the microbe's metabolic machinery and survival might result impaired.

5.2 Comparison of dsrAB transcripts and sulfate reduction under various sulfate conditions.

In general, an increased dsrAB copy numbers detection should be consistent with an increased rate of sulfate reduction in any given bacterial culture. As more dsrAB transcripts are synthesized, more DsrAB enzymatic units should be available to catalyze the transfer of electrons in this anaerobic respiration process. This expected behavior was observed in our cultures subjected to $MgSO_4$ 0.35 wt%, in which the samples grown at -5 °C presented the highest sulfate reduction rates (enzymatic activity, see Figure 15) and similar dsrAB copy numbers as the positive temperature control even though their growth was low in comparison with the cultures at 0 °C and 10 °C (See Figure 12, 13, 14 and Table 2). 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 behavior 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 sulfate reduction. A behavior 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 (Davidson & Surette, 2008; Dubnau & Losick, 2006; Gefen & 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 °C and 10 °C presented significant dsrAB detections when compared with the Ct cutoff value (see Figure 14 and Table 2), and that an increased sulfate reduction rate could be the result of the adaptability of the DsrAB to catalyze efficiently at subfreezing temperatures. Furthermore, at increasing concentrations of MgSO4 (10 wt% and 18

wt%), which presented the highest growth at 0 °C and lowest at -5 °C (see Figure 12, and 13), showed no significant sulfate reduction differences among the temperatures tested (see Figure 15). However, these cultures showed significant differences with the growth negative controls which indicate presence of sulfides in solution. Therefore, we can deduce that cultures at 10 °C and 0 °C prioritized both growth and maintenance (sulfate reduction detected, high cell proliferation) while cultures at -5 °C prioritized maintenance (sulfate reduction detected, low cell proliferation).

In the cultures supplemented with CaSO4 0.1 wt%, we can observe that the highest sulfate reduction was registered at 0 °C (see Figure 15) which had the lowest dsrAB detection (See Figure 14 and Table 2) and the highest growth (See Figure 12, and 13). 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 sulfate reduction pathway. Cultures at 10 °C and -5 °C showed similar sulfate reduction rates (See Figure 15) 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 Figures 12, 13, 14, 15 and Table 2). Interestingly, cells at -5 °C with the highest dsrAB copy numbers presented low sulfate reduction (minimal active growth, active transcription, active sulfate reduction). This behavior indicates that cells that survived from the inoculum, initially divided, and then redirected their metabolic machinery to maintenance functions. However, this attenuated sulfate reduction (in comparison with cultures at 0 º C) suggests the effects of posttranscriptional/posttranslational modifications or protein folding in which DsrAB enzymatic units were rendered partially non-functional.

Finally, in the cultures supplemented with $FeSO_4$ and $Fe_2(SO_4)_3$ sulfate 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 sulfide 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 behaviors shown in our results points out 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 sulfates at rates compatible with the metabolic specialization of their clonal subpopulations (Davidson & Surette, 2008; Dubnau & Losick, 2006; Gefen & Balaban, 2009; Levine et al., 2012; Price & Sowers, 2004; Rosenthal et al., 2018; Steven et al., 2006). 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 sulfate 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 sulfate 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 sulfate reducer it uses the sulfate 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. In our experiments this highly structured state of the cells macromolecular machinery was presumably interrupted by the action of $Mg^{2+}$and Ca^{2+} cations which reduced the rigidity of the cellular components and triggered higher dsrAB expression and sulfate reduction rates at temperatures down to -5º C (See Figures 14, 15 and Table 2). In 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 interrupt the molecular rigidity of cellular macromolecules triggering stable microbial activity under suboptimal conditions. This further suggests that supplementation with chaotropic ions in our experiments did enhance cellular function, which relevance in terms of Astrobiological interest, 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 & 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 ºC,

lowest of -143 ºC during winter and highest of 35 ºC), transient liquid water in the Martian polar caps and permafrost is possible (Chevrier & Altheide, 2008; Pikuta et al., 2007; Pikuta & Hoover, 2003). 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 (sulfate 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 & Morita, 2017; Poli et al., 2017).

6. Conclusions

In our experiments, *D. psychrophila* was used as a model for adaptability, survivability, and proliferation under Martian conditions of temperature (down to -5 ºC) and soil's mineral composition (increasing concentrations of sulfate salts). Our results at -5 ºC temperatures indicate that this microbe tend 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 ºC and 10 ºC 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 (Davidson & Surette, 2008; Dubnau & Losick, 2006; Gefen & Balaban, 2009; Levine et al., 2012; Pikuta et al., 2007; Price, 2000; 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 $CaSO_4$ 0.1wt% at -5 ºC (prioritized maintenance), $MgSO_4$ at increasing concentrations at 0 ºC (prioritized both maintenance and growth) and -5 ºC (prioritized maintenance) which showed attenuated or absent sulfate reduction. The MB results in these cultures suggest the presence of posttranscriptional or posttranslational modifications which rendered non-functional DsrAB enzymatic units. This behavior comes to no surprise given the fact that we have subjected these cells to conditions of temperatures that are 10 °C and 15 °C below

their optimal growth temperature conditions (Davidson & Surette, 2008; Dubnau & Losick, 2006; Gefen & Balaban, 2009; Levine et al., 2012; Rosenthal et al., 2018).

Moreover, cultures of *D. psychrophila* at 10 and 14 wt% of $FeSO_4$ showed dsrAB copy numbers at all temperatures (in comparison with the Ct cutoff value), but as it can be observed in our results, the culture conditions were not ideal for active cell proliferation and sulfate 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 sulfate 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 stability/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 (sulfate 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 & Gerday, 2003; Hoover & Pikuta, 2010; Pikuta et al., 2007; Pikuta & Hoover, 2003).

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 sulfate 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 sulfate salts.

7. References

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CHAPTER III

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Title: Studies of the expression of pit and sulfate reduction at subfreezing temperatures in cultures of *Desulfotalea psychrophila* supplemented with increasing concentrations of $MgSO_4$ and $CaSO_4$.

1. Abstract

In our Solar System, the four biggest moons of Jupiter were first observed by Galileo Galilei in 1610. Forty-five years later (1655), Titan, one of the moons of the Saturn System was discovered by Christiaan Huygens, and more than a century after (1789), Enceladus, another moon of the Saturn System, was discovered by William Herschel. Nowadays, after approximately 434 years of the first discovery, our knowledge about these satellites have increased drastically. Moreover, this progress in scientific knowledge comes to no surprise since theoretical postulations about the possibility of the existence of salty subsurface oceans, in at least five of these moons, has fueled the interest of Astrobiologists to determine if there is a possibility for current or pre-biotic types of life. Furthermore, in our studies of Mars habitability, a planet that can be also considered an extremely cold Celestial Body, we were able to detect expression of the dsrAB operon and sulfate reduction at subfreezing temperatures, presumably facilitated by chaotropic effects of sulfate salts as those present in today's Martian regolith. As two of those compounds have been theorized to be present in the surface and subsurface oceans of icy satellites, our research team decided to test the integrity of the cellular membrane of *D. psychrophila* cells cultured with $MgSO_4$ and $CaSO_4$ supplementations by means of the expression of the inorganic phosphate transporter (pit). Furthermore, this investigation was structured as a model to understand the biological processes

that govern cold environments as those possibly present below the external shell of icy moons of the Jovian and Saturn Systems and to determine if chaotropic effects can in fact aid in the survival and proliferation of psychrophilic sulfate reducers at subfreezing temperatures. Our findings have revealed that $MgSO_4$ at concentrations below 10 wt% induced a chaotropic effect that provided *D. Psychrophila* cells with limited resistance to mechanical damage of the cell membrane by ice crystals formation, while $CaSO_4$ presumably provided physical protection against cold temperatures facilitating active sulfate reduction at suboptimal temperature conditions.

2. Introduction/Literature review

In the Solar System several satellites orbiting Saturn and Jupiter have been denominated "icy worlds" as to reference the condition that many of them possess an external icy shell of variable thickness. As it is expected in Celestial Bodies which are distant from their system hot star, low temperatures experienced at the satellite's surfaces are responsible for the formation of the outer shell. Interestingly, in many of these moons the existence of subsurface oceans has been proposed, mostly due to observational data collected with the Hubble Space Telescope, and the Cassini and Galileo spacecrafts. Furthermore, the information obtained by these pieces of technology is then used to model the internal thermal and structural composition of the satellites and their subsurface oceans (Kamata et al., 2015). The Jovian satellites as well as the Saturn moons tentatively have all the basic components required for the formation and support of life (i.e.: water, raw materials, and chemical energy). However, most of these moons are not classified within the habitable zone (as is the case for Mars) due to the presence of extreme environmental conditions not conducting of life. However, microniches where environmental conditions are less extreme and conductive of life can occur (Vance et al., 2016).

The first indication of the presence of subsurface oceans in the icy satellites of the Galilean system resulted from analyses of the Galileo spacecraft when studying the induced magnetic field in Europa and Callisto, a phenomenon associated with the inner magnetosphere of Jupiter. As a result of the data analyses, it was speculated that layers of significant electrical conductivity were present in the interior of these moons. Therefore, it was estimated that Europa could have a subsurface ocean while Callisto could have an ocean located between two convecting layers given its incomplete differentiation. Furthermore, it was also speculated that Ganymede could possess a subsurface ocean since this moon is completely differentiated and it has an intrinsic magnetic field (see Figure 16) (Khurana et al., 1998; Tobie et al., 2014).

On the other hand, the first evidence of the presence of a subsurface ocean in Titan was determined by means of experimental simulations using radio science data collected with the Cassini spacecraft (flybys T11, T22, T33, T45, and T68). These simulations allowed the prediction of a subsurface ammonia rich ocean sitting underneath a layer of ice from which methane emissions are released into the atmosphere due to the presence of clathrates in the icy shell. Furthermore, this model predicted by Lunine and Stevenson (1987) has been updated by Fortes et al. (2007) who suggested an ammonium hydrosulfate ocean which could supply methane into the atmosphere via volcanism and outgassing (Fortes et al., 2007; Lunine & Stevenson, 1987; Rappaport et al., 2008). Moreover, a subsurface ocean in Enceladus was proposed for the first time when data from the Cassini's close flybys of Enceladus's south pole detected evidence of a plume of gas and dust containing icy particles and water vapor. Many mechanisms for the origin of these ejections were suggested (large impactors, geysers, volcanism, etc.). However, there is still an ongoing debate about the geological source (see Figure 16) (McKay et al., 2008; Porco et al., 2006; Spencer et al., 2006).

Furthermore, it is crucial to take into consideration that in the study of icy satellites, and the possibilities of presence of life based on raw materials detection, the latter can be classified based on the location of their origin as endogenous or exogenous. Endogenous materials are formed due to autochthonous physical chemistry processes, while exogenous materials are originated in outer space and further deposited in the surface of the moon. The relative relevance of both types of materials is based on their ability to facilitate the understanding of the dynamics that operate in these moons. For instance, materials produced endogenically, if present at the surface, can give us insights into the mechanisms that occur in their interior (transport and exchange), while exogenic materials, if they remain in the atmosphere, surface, or interior, possess relevance if they are transported to the moon's interior (Dalton, 2010). Finally, for the purposes of our investigation, we have used sulfate minerals as supplementations, as they have been estimated to be present in cracks of the icy shell or dissolved in the subsurface oceans of these moons (Schmidt & Manning, 2017; Vance et al., 2018).

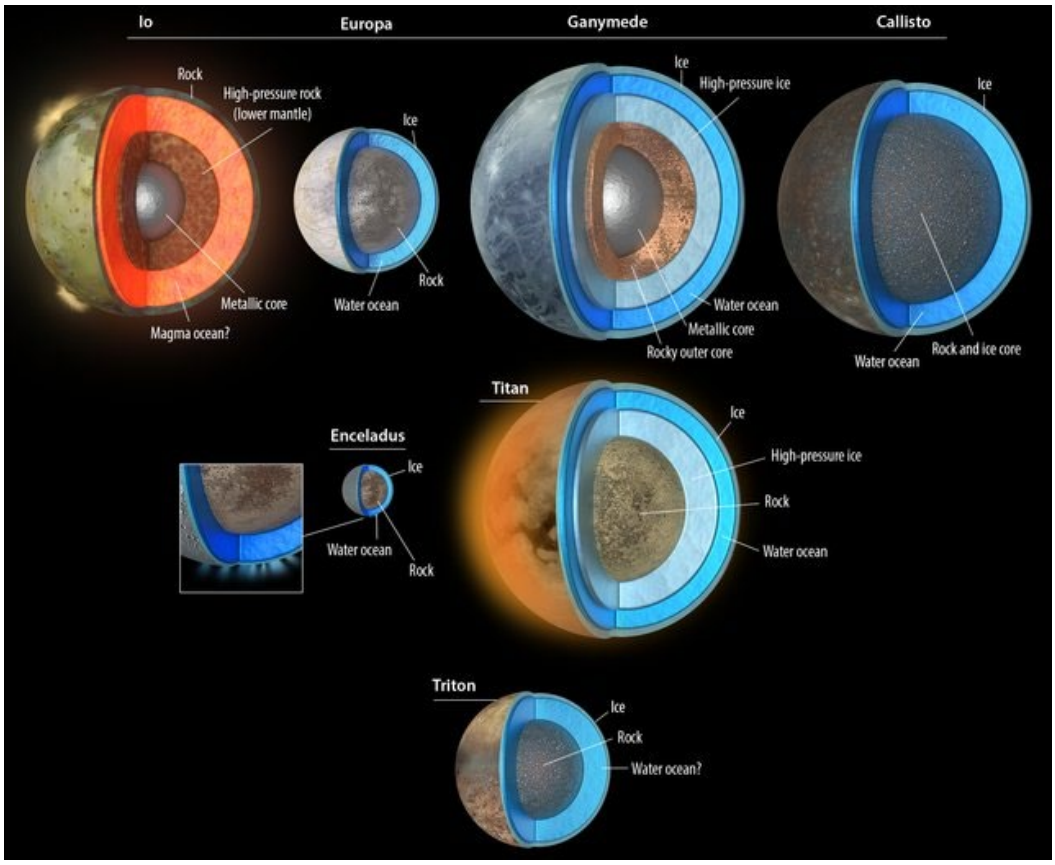

Figure 16. Depiction of icy satellites of the Jovian and Saturn Systems. Image extracted from de Kleer et al., (2019).

2.1 Jupiter (Jovian/Galilean) satellites

From the approximate 80 satellites present in the Jovian system, three moons have caught the scientific interest as possible locations for Astrobiological studies since water ice has been characterized as one of their principal components. Even more interesting, hydrated minerals are estimated to be present in high quantities due to the leaching of the rock which is in close contact with the subsurface ocean (McCord et al., 2001).

2.1.1 Europa

Europa possesses an iron-nickel core completely differentiated, and its regolith is composed mostly of silicate rock and water ice. Its surface temperatures oscillate between -221.15 °C and -163.15 °C with a global average of -173.15 °C. Furthermore, Europa's surface pressure has been estimated at around 10^{-12} bar, and its atmosphere is composed of a thin layer of O_2 (formed from radiolysis of H_2O vapor due to electron bombardment from Jupiter's magnetosphere). According to models of its internal structure, this moon is formed by a conductive icy shell, followed by a convective ice layer where tidal dissipation reaches its maximum, and immediately after, a subsurface ocean which is in direct contact with the rocky mantle. This is crucial to our understanding of this moon since organics compounds mixed in the subsurface ocean have been predicted to occur (Ashkenazy, 2019; Castillo et al., 2000; Hall et al., 1995; W. B. Moore & Schubert, 2000, 2003; Ojakangas, 1989; Pappalardo, 2010; Somogyi et al., 2016; Tobie et al., 2005; Wahr et al., 2006; Zolotov & Shock, 2001).

Additionally, it has been hypothesized that lenticulae and chaos regions are the result of upwellings of molten ice in areas around hot plumes (maximal heating) which could be associated to tidal heating influence on the icy shell. Just the presence of chaos regions has been pointed out as indicative of the presence of a subsurface ocean since they suggest the occurrence of encased lakes (Tobie, 2003; Tobie et al., 2005).

There is evidence of volcanism, radioactive decay, and hydrothermal vents along with serpentinization of the rock. Importantly, Europa also contains hydrated $MgSO_4$ and $CaSO_4$ in its surface and possibly Na_2SO_4 which could have been originated in the subsurface ocean and retained at the surface during upwelling in fractures of the icy shell. Therefore, it has been hypothesized that the subsurface and the surface of the moon have had contact, perhaps through

pull-apart bands or cracks (Kargel et al., 2000; Marion et al., 2003; McCord et al., 2001; Pappalardo, 2010; Prockter et al., 2010; Zolotov & Shock, 2001).

2.1.2 Callisto

Callisto has been characterized as one of the most cratered surfaces in the Solar System. Its surface temperatures oscillate between -193 °C and -108 °C with a global average of -139 °C. Callisto's atmosphere is composed of CO_2, H_2 and O_2 which amounts to a surface pressure of 0.75 μPa (7.4 x 10^{-12} atm). This satellite possesses a small undifferentiated silicate core, an icy crust formed by a layer of "dirty material" and an icy mantle "clean ice zone". Its regolith is formed by equal amounts of rock and water ice, magnesium and iron-bearing silicates, ammonia, carbon dioxide (trapped after impact craters and ejecta), sulfur dioxide, and organic compounds (tholins). It has been hypothesized (based on magnetometer data from Galileo) that this moon possesses a salty liquid subsurface ocean which in models has been placed between two solid convecting regions of ice I and ice V (Khurana et al., 1998; Pappalardo, 2010; Prockter et al., 2010). Interestingly, from these analyses, it has been suggested that accretional and radiogenic heating are not enough to separate the ice and rock inside Callisto, leaving it in an incomplete state of differentiation (W. B. Moore & Schubert, 2003; Somogyi et al., 2016).

Additionally, this moon is not heavily affected by Jupiter´s magnetosphere and it is not tidally heated since it is not influenced by the Laplace Resonance of Jupiter´s moons (even though it does not have an intrinsic magnetic field). Moreover, this satellite has been categorized as the least likely to harbor life since the subsurface ocean is not in direct contact with the mantle as it happens with the other satellites of the Jovian system. Notably, there is no evidence of plate tectonics activity, volcanism, or any type of evidence of communication between the surface and the subsurface of the moon. Interestingly, it is not entirely understood whether there is or not

cryovolcanic resurfacing (Bagenal, Dowling & McKinnon, 2004; W. B. Moore & Schubert, 2003; Prockter et al., 2010).

2.1.3 Ganymede

Ganymede, the largest moon of Jupiter, has temperatures that oscillate between -203 °C and -121 °C with a global average of -129 °C. Its atmosphere is composed of a thin layer of O_2, O, and ozone, as well as H_2 and water vapor which accounts for a surface pressure of 0.2-1.2 μPa. This satellite possesses a core formed by two components: an internal solid iron core and an external liquid core of iron, nickel, and sulfides (Tobie et al., 2005). Furthermore, Galileo spacecraft's data indicates that Ganymede is differentiated, and it possesses an internal magnetic field (Bagenal, Dowling & McKinnon, 2004). Its regolith is formed by silicate rock, and it has a salty subsurface ocean which is located between two layers of ice, an internal layer in contact with the mantle and an external layer formed by water ice (icy crust) (Pappalardo, 2010; Tobie et al., 2005). Interestingly, using data from the Galileo's near-infrared mapping spectrometer (NIMS) hydrated $MgSO_4$ and Na_2SO_4 have been identified. It is speculated that these hydrated salts found in the surface could represent frozen remnants (frozen deposits) of brines that were sustained by Ganymede's subsurface ocean at some time (Bagenal, Dowling & McKinnon, 2004; McCord et al., 2001; Molyneux et al., 2020; Pan et al., 2021). Furthermore, some other compounds that have been detected are CO_2 in clusters, hydrogen sulfate, sulfur dioxide, cyanogen, volatile ammonia, and various organic compounds of distinct carbon lengths. Interestingly, these carbon compounds have been attributed to radiolysis on the surface of the moon or due to asteroidal and cometary material (Bagenal, Dowling & McKinnon, 2004). Finally, Ganymede possesses three sources of internal heat, radioactive decay, tidal heating and differentiation, although only minimal tectonic

activity and cryovolcanism have been detected (Kalousová & Sotin, 2018; McCord et al., 2001; Molyneux et al., 2020; Somogyi et al., 2016; Tobie et al., 2005).

2.2 Saturn satellites

From the 83 satellites present in the Saturn system two of them, Enceladus and Titan, have captivated the attention of the scientific community for their unique characteristics and chemistry.

2.2.1 Enceladus

Enceladus temperature ranges from -240° C to -128° C with an average temperature of -222 °C (Cruikshank et al., 2005). This moon's atmosphere is composed principally of water vapor, nitrogen, carbon dioxide and methane. Moreover, simple organics such as propane, acetylene, formaldehyde as well as complex organics such as benzene and macromolecular organics of up to 15 carbons have been detected in its atmosphere and regolith respectively (Cruikshank et al., 2005). It possesses a subsurface ocean extending from the south pole to around 50° south latitude which was detected using gravity measurements. Furthermore, it was estimated that the water ocean thickness could be placed at around 10 km and that it is surrounded by an ice layer of around 30 to 40 km (Cruikshank et al., 2005; Taubner et al., 2014). Enceladus is formed by a silicate core, with ongoing radioactive decay, which is in contact with the subsurface ocean (Khawaja et al., 2019). In addition, it is believed that the sources of heat of this moon are dominated by a mix of active plate tectonics, tidal heating, solar influence, and radioactive decay which can generate enough energy to explain the activity of water rich plumes, hydrothermal vents, and cryovolcanism (Cruikshank et al., 2005; Khawaja et al., 2019; Prockter et al., 2010). Interestingly, cryovolcanic activity pushes detectable amounts of liquid, ice, water vapor, sodium chloride, and other volatiles into space in the form of potent ejections from the interior of the satellite (plumes). Moreover, it has been hypothesized that these plumes are originated from materials of the subsurface ocean in

a process in which its contents penetrate the icy crust (warm cracks). From there, they are ejected into space and incorporated into Saturn's E ring, or fall back onto the moon's surface. (Khawaja et al., 2019). Interestingly, Cassini's data pointed out to the presence of organic species in the plumes' contents and complex organics in the ice grains present in Saturn's E ring (thought to be low-mass amines, carbonyls, and aromatics). Therefore, it has been hypothesized that a layer of organics concentrates immediately below the moon's icy crust from where they are ejected into space by the plumes' action (Khawaja et al., 2019; Somogyi et al., 2016). Equally important, hydrothermal vents, at the bottom of the subsurface ocean, as well as serpentinization reactions at the boundary between the liquid water subsurface ocean and the silicate core, could produce hydrogen and support abiotic and biotic synthesis of organics. Interestingly, H_2 could be used along with dissolved CO_2 by methanogenic bacteria (if present) for metabolic activity (Cruikshank et al., 2005; Khawaja et al., 2019; Taubner et al., 2014).

2.2.2 Titan

Titan, one of the Saturn's moons has been characterized by having a thick atmosphere which allows it to trigger unique interactions with the surface (Prockter et al., 2010). Its atmosphere is formed mostly of nitrogen, methane, hydrogen, carbon monoxide, propane, cyanoacetylene, ethylene, ethane, acetylene, hydrogen cyanide, carbon dioxide, and water vapor. Furthermore, the surface pressure is estimated to be around 146.7 kPa (1.45 atm) and its global temperature is approximately -179.5 °C. Moreover, Titan's core is composed of water-bearing silicate rock surrounded by various layers of ice and a subsurface water ocean. It has been estimate that the outer icy shell is approximately 40 km thick while the subsurface ocean amounts to less than 100 km (Tobie et al., 2005, 2014)). Interestingly, this moon is the only other world, besides Earth, to harbor superficial lakes (Cable et al., 2012; Coustenis & Taylor, 2008; Iess et al., 2012; J. M.

Moore & Pappalardo, 2011; Stofan et al., 2007; Vuitton et al., 2019). It has methane-ethane clouds and a subsurface water ocean which are thought to be the principal sources in the formation of superficial lakes. Moreover, water-ice forms the crust of the moon and there have been estimations of the potential presence of cryovolcanoes. Most importantly, based on the observations of the Cassini-Huygens mission (and even before that), many models have been developed to explain the formation of hydrocarbons in its atmosphere. These models take into consideration processes such as photolysis and radiolysis of methane and N_2 in the upper atmosphere which facilitates the polymerization of hydrocarbons and nitriles down to the troposphere. These organics of variable chemical composition could be potentially transported, in the form of aerosols, to the surface of the moon and form deposits (adsorbed or aggregates) of organic materials known as tholins (Cours et al., 2020; Hörst et al., 2012; Prockter et al., 2010; Somogyi et al., 2016). As shown in Cassini's data, most of this moon's surface is formed by organic material. Unfortunately, tholins generated in simulated experiments using different techniques tend to form organics of different composition. Therefore, the definite chemical repertoire of tholins in Titan is still unknown. However, molecules such as ethane, propane, propene, butane, butene, benzene, toluene, naphtalene, HCN, tetrazine, butenenitrile, aldehydes, acetylene, diacetylene, cyanoacetylene, acrylonitrile, propionitrile, alkynes, ketones, ammonia, primary amines, vinyl cyanides, polycyclic aromatic hydrocarbons, and hydrogenated amorphous carbons have been identified. Interestingly, even prebiotic molecules (aminoacids and nucleobases) have been predicted to occur (Cours et al., 2020; Hörst et al., 2012). Furthermore, as models have hypothesized a subsurface water ocean, tholins (if present) could be utilized as substrates by a great deal of microorganisms if they can survive the extreme environmental conditions of Titan and similar icy moons (Pudritz et al., 2007; Stoker et al., 1990; Tobie et al., 2005). It has been postulated that living systems would use metabolic pathways related

to methanogenesis, nitrate/nitrite/dinitrogen reduction and/or sulfate reduction. Interestingly, water pockets at the lower part of Titan's ice crust have been classified as conductive of microbial life since they contain high concentrations of sulfates, and organics from the atmosphere (recirculated if in contact with the subsurface ocean through cryovolcanic activity, or through icy caves-cryovolcanism duo). The same occurrence would be possible if brines could develop at cracks of the icy crust which will act as a method for transporting organics vertically into the subsurface ocean and viceversa. Another phenomenon that could exist in this moon is the presence of hydrothermal vents in which the synthesis of organic compounds can occur and supply the upper layers of the subsurface ocean (Simakov, 2001). Finally, it has been predicted that co-crystals formed by organic compounds could form energy rich deposits that would be readily available for utilization by organisms (if present) near the surface or in the interior of Titan (Cable et al., 2021). Interestingly, it has been postulated that these organic molecules could represent the base of the food chain in an evolving biosphere (Cable et al., 2012, 2021; Cassidy et al., 2010; Coustenis & Taylor, 2008; de Vanssay et al., 1998; Kalousová & Sotin, 2018; McKay, 1996; Pudritz et al., 2007; Simakov, 2001; Somogyi et al., 2016; Stoker et al., 1990; Vuitton et al., 2019; Yung et al., 1984).

2.3 Chaotropicity and microbial growth

The term chaotrope has been defined as the property of certain ions to destabilize proteins and membranes or decrease the ordered structure of water molecules. Both of which involve the breaking of non-covalent bonds by interacting directly with the structures/molecules in solution (Ball & Hallsworth, 2015; Eardley et al., 2019; Zhang & Cremer, 2006). As an extension of the previous definition, kosmotropes are defined as ions that stabilize non-covalent bonds in proteins, and membranes. Furthermore, the relative behavior of specific ions in solution can be deduced

from the Hofmeister series, a scale of ions chaotropicity and kosmotropicity which was formulated by Hofmeister in his studies about protein denaturation (Hofmeister series: $K^+ > Na^+ > Mg^{2+} > Ca^{2+}$; $SO_4^{2-} > HPO_4^{2-} > Cl^- > NO_3^- > Br^- > ClO_3^- > I^- > ClO^-_4$). As it can be inferred from the Hofmeister series, Mg^{2+} and Ca^{2+} ions are known to behave as chaotropes in solution (Bhaganna et al., 2016; Crisler et al., 2012; Kunz et al., 2004; Oren & Hallsworth, 2014; Rangel et al., 2015; Yakimov et al., 2015; Zajc et al., 2014).

As mentioned above, chaotropic species can affect the structure of membranes, proteins, and other biological macromolecules by disrupting their non-covalent interactions and unfolding their supramolecular arrangements (Eardley et al., 2019; Hallsworth, 2016; Timson, 2020). Thus, as chaotropic substances have a tremendous impact on the stability of macromolecular structures, they have been suggested as the determining factors that define the biotic limits for metabolic activity which is crucial for the survival of microorganisms in extreme environments. In fact, there are numerous reports of microbial communities from saturated hypersaline environments that suggest chaotropicity as the determinant factor for colonization. Interestingly, since many extremophile microorganisms have shown to be able to function in environments containing chaotropic salts, a different classification of extremophile microbes “chaophiles” has been suggested. Furthermore, it is well known that subfreezing environments promote the increase of non-covalent interactions, a phenomenon that rigidifies supramolecular structures. Notably, it is believed that under these conditions, chaotropic ions could enhance macromolecular flexibility and therefore increase metabolic rates by fluidization of the plasma membrane which could counteract the negative effects of low-temperature stress and expand the survival window. Interestingly, as chaotropic chemicals can be present in planets or satellites other than our own, they represent factors that should be considered in studies of survivability and active metabolism

in extraterrestrial environments (Ball & Hallsworth, 2015; Bhaganna et al., 2016; Hallsworth, 1998, Hallsworth et al., 2003; 2007; 2016, Chin et al., 2010; Cray et al., 2013; dC Rubin et al., 2017; de Lima Alves et al., 2015; La Cono et al., 2019; Oren, 2013; Rummel et al., 2014; P. M. Santos et al., 2004; Yakimov et al., 2015).

Finally, most terrestrial extremophile isolates that can sustain metabolic activity at high concentrations of $MgSO_4$ and low temperatures are mesophiles. Furthermore, many of them have been isolated from natural environments on Earth that possess high divalent:monovalent ion ratios which make them ideal candidates for extraterrestrial analogs containing sulfate brines. However, there is no evidence of psychrophilic bacterial isolates from these environments (Fox-Powell & Cockell, 2018; Pontefract et al., 2017).

2.4 Inorganic phosphate transporter (pit)

Phosphate is an important macronutrient in Prokaryotic systems. It is used to biosynthesize ATP, nucleotides, phospholipids and in intracellular communication processes. Furthermore, there are two systems used in the uptake of inorganic phosphate, which occurrence in the membrane depends on the availability of this nutrient in the environment. The high affinity system which is synthesized de-novo when phosphate is limiting and the low affinity system which is steadily synthesize in conditions where phosphate is available in excess. Furthermore, the high affinity system is composed of a periplasmic protein-dependent phosphate-specific transport system (Pst) which is only synthesized under starvation conditions and belongs to the Superfamily ABC transporters (Banerjee & Chakraborti, 2000). Moreover, Pst is composed of four different membrane-associated proteins. Among them, PstC and PstA are transmembrane proteins that form a translocase which spans the cellular membrane from the cytoplasmic side to the intracellular side. The other two components, PstB and PstS, are cytoplasmic and periplasmic respectively.

PstB, which is also known as the ATP-binding component, hydrolyzes ATP and allows the use of this energy to power the transport process while PstS is the binding site for inorganic phosphate. Additionally, there is a regulatory complex or repression complex which regulates the pst operon. This regulatory complex is formed by the phosphate-specific ABC transporter, the two-component regulatory system PhoB/PhoR and PhoU, an additional protein which has been described in Gram negative bacteria (*Escherichia coli*) (Banerjee & Chakraborti, 2000; Duerre, 2005; Martín & Liras, 2021; Neznansky et al., 2014; Nikata et al., 1996; Santos-Beneit, 2015; Spira et al., 2010).

Furthermore, the low-affinity system is formed by members of the inorganic phosphate transporter family (Pit). In contrast with the high-affinity uptake systems, Pit is a symporter which uses divalent cations (Ca^{2+}, Mg^{2+}, Mn^{2+}, or Co^{2+}) to cotransport inorganic phosphate. Furthermore, in the Gram-negative model (*Escherichia coli*), two Pit transport systems have been described, PitA and PitB from which pitA is expressed constitutively in high or low concentrations of phosphate, while pitB is expressed only in low concentrations of this nutrient. Interestingly, other versions of the Pit family have been identified as systems that cotransport phosphates and Na^{+} ions. Moreover, they have been classified as members of the pfam01384 Pho4 symporters group (closely related to Pi-repressible Pi:Na^{+} symporter of the fungi *Neurospora crassa*). Finally, these symporters are known to be constitutively expressed in conditions where phosphate and Na^{+} are not limiting and they act as viral receptors for gamma viruses by means of non-covalent interactions (Duerre, 2005; Hoffer et al., 2001; Martín & Liras, 2021; Santos-Beneit, 2015; Versaw & Metzenberg, 1995).

2.5 Cold shock protein (csp)

Cold temperatures are known to be stressors of biological systems. Furthermore, they affect the membrane fluidity, and essential processes such as transcription, translation, and DNA replication in many organisms. Among them, bacteria overcome cold stressors and their effects by regulating the saturation of their membrane phospholipids, which confers the plasma membrane with more flexibility. Another strategy that bacteria use to resist the adverse effects of cold temperatures is the synthesis of cold-shock proteins. Moreover, they are present in Eukaryotic and Prokaryotic organisms, and are responsible to preserve the basic physiological and cellular functions when cells are exposed to abrupt temperature drops. They are synthesized as a controlled process known as "cold shock response" that protects the cells from the detrimental effects of cold shock. Interestingly, these proteins are widespread among bacteria and are known to be crucial for their survival during cold-shock acclimation. CspA, the first cold-shock protein discovered, was isolated from *E. coli*. Shortly after its discovery, homologs of this protein were detected in various groups of bacteria (psychrophilic, psychrotrophic, mesophilic and thermophilic). Some cold-shock proteins are known to act as DNA/RNA chaperones (prevent formation of secondary structures) while some others allow for protein folding/re-folding of cold-damaged proteins. Interestingly, it has been hypothesized that csp genes were originated as the result of genetic duplications which in the case of the cspA family rendered a complete series of environmental stress-dependent genes. The expression of cold-shock proteins happens at high rates during the acclimation stage. Interestingly, the CspA family in *E. coli* seems to be highly regulated at the transcriptional level by its 5'-UTR where a sequence of 11 bases constitute the "cold-box" and at the translation level by means of its mRNA portion known as the downstream box which forms the translation preinitiation complex (aids in the initiation of translation). Interestingly, ribosomes have been pointed out as responsible for sensing heat or cold (physiological sensor) during the shock phase

and preferentially becoming non-functional to all mRNAs except for those that encode cold-shock proteins. After the acclimation phase, cold-unadapted ribosomes acquire cold-shock ribosomal factors which permits the synthesis of all other cellular mRNAs. In mesophiles, cold-shock proteins are synthesized immediately after exposure to low temperatures and remain overexpressed only temporarily, while cold-acclimation proteins are continuously synthesized when bacterial strains grow under permanent low temperatures and their overexpression remains for hours. On the other hand, microbes adapted to cold extremes continuously synthesize both cold-shock and cold-acclimation proteins in a non-temporary fashion (for as long as the microbe can metabolize). Cold-shock proteins such as CspA and CspB are formed by 5 antiparallel β-strands with two RNA-binding domains located in β-2 and β-3 strands respectively in which the interaction of RNA and the protein occur through 7 aromatic residues located at the surface of the protein. This interaction locks the RNA in place (bases and aromatic residues interaction) which further induces its RNA-chaperone function by preventing the formation of RNA secondary structures (intermolecular base complementarity) or interactions with other molecules (intramolecular interaction) (Phadtare et al., 1999).

In our studies, we were interested to test the integrity of *D. psychrophila*' s cellular membrane at subfreezing temperatures (-5 °C and -10 °C), as it is known that these conditions induce the formation of ice crystals that could mechanically disrupt this supramolecular structure. We addressed this by measuring the expression of pit, a structural and functional component of the cell membrane, in cultures of *D. psychrophila* supplemented with increasing concentrations of $MgSO_4$ and $CaSO_4$. As Mg^{2+} and Ca^{2+} ions are known chaotropes, we were also interested to understand whether these ions could potentially provide an advantage for metabolic activity by

reducing non-covalent interactions of the plasma membrane and preserving its fluidity which would allow to maintain sulfate reduction rates at subfreezing conditions.

3. Materials and Methods

3.1 Cultures

Active cultures containing *D. psychrophila* cells were purchased from the DSMZ-German collection of microorganisms and cell cultures. These active cultures were seeded and incubated at 10 °C on 15 mL serum bottles containing sterile DSMZ141 medium for 30 days using 1.5 mL of the active cultures as inoculum. These starter cultures were used to inoculate a positive temperature control (untreated group incubated at 10 °C), and two experiments at -5 °C and -10 °C in triplicates. In these experiments, the DSMZ medium formula was modified by replacing 3.45g (0.35 wt%) of $MgSO_4$ with increasing concentrations of $MgSO_4$ (ranging from 0.35 wt% to 25 wt%) and $CaSO_4$ (ranging from 0.1 wt% to 25 wt%) (See Table 3). In short, 15 mL of anoxic modified medium was dispensed in 15 mL serum bottles that were pre-evacuated with N_2 (g) and kept on an anaerobic glove. Furthermore, they were sealed and clamped with rubber stoppers and aluminum seals before autoclave sterilization. After sterilization, serum bottles containing the supplemented media were cooled down in an anaerobic hood overnight. Each serum bottle was inoculated with 1.5 mL of the starter cultures using sterile 5 mL syringes and 20G needles. Growth negative controls (non-inoculated) were included for all samples. Finally, cultures were incubated at 10 °C, -5 °C or -10 °C for a period of 30 or 90 days.

Table 3. Modified DSMZ141 culture medium (Adopted from DSMZ culture collection) and sulfate concentrations used for temperature positive control (10 °C), experiments at -5 ºC, -10 ºC, and growth negative controls (sulfate compounds are numbered).

Component	Quantity	Sulfate compound concentration + C-	
KCl	0.34 g	1:$MgSO_4$ 0.35 wt %	$MgSO_4$ 0.35 wt % C-
$MgCl_2$. $6H_2O$	4 g	2:$MgSO_4$ 1 wt %	$MgSO_4$ 1 wt % C-
NH_4Cl	0.25 g	3:$MgSO_4$ 5 wt %	$MgSO_4$ 5 wt % C-
$CaCl_2$. $2H_2O$	0.14 g	4:$MgSO_4$ 10 wt %	$MgSO_4$ 10 wt % C-
K_2HPO_4	0.14 g	5:$MgSO_4$ 15 wt %	$MgSO_4$ 15 wt % C-
NaCL	18 g	6:$MgSO_4$ 20 wt %	$MgSO_4$ 20wt % C-
*Trace elements solution	10 mL	7:$MgSO_4$ 25 wt %	$MgSO_4$ 25 wt % C-
$Fe(NH_4)_2(SO_4)_2$ x $6H_2O$	2.0 mL	8:$CaSO_4$ 0.1 wt%	$CaSO_4$ 0.1 wt% C-
sltn (0.1 % w/v)		9: $CaSO_4$ 1 wt%	$CaSO_4$ 1 wt% C-
**Na-acetate	1 g	10: $CaSO_4$ 5 wt%	$CaSO_4$ 5 wt% C-
**Yeast Extract	2 g	11: $CaSO_4$ 10 wt%	$CaSO_4$ 10 wt% C-
**Trypticase peptone	2 g	12: $CaSO_4$ 15 wt%	$CaSO_4$ 15 wt% C-
Na-resazurin sltn (0.1 % w/v)	0.50 mL	13: $CaSO_4$ 20 wt%	$CaSO_4$ 20 wt% C-
$NaHCO_3$	5g	14: $CaSO_4$ 25 wt%	$CaSO_4$ 25 wt% C-
***Vit Soltn	10 mL		
***Sodium Dithionite	20 mg		
Distilled water	1L		

*Follow instructions from DSMZ141 standard preparation

C- indicates growth negative controls

**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 the interstellar space. Also, many icy bodies possess a complex chemistry that produce 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.

***Taken from DSMZ195c

3.2 Assessment of cultures growth

Growth was assessed visually by detecting sulfate reduction and changes in the aspect of the medium. As cultures containing $CaSO_4$ had high turbidity due to increasing concentrations of this mineral, assessment of growth was measured indirectly by detecting sulfides-sulfur (H_2S, HS^-, S_2) in solution using the methylene blue protocol (Basic et al., 2015; Cline, 1969; Johnston et al., 2005; Thorup et al., 2017). At the 30- or 90-days mark, 100 µL of each sample was mixed in UV Grade polymethylmethacrylate cuvettes pre-seeded with 720 µL of solution A (0.1 mM of diethylenetriaminepentaacetic acid, pH =9.6). After mixing the sample with solution A, 180 µL of solution B (17.1 mM of N,N-dimethyl-p-phenylenediamine sulfate and 37 mM $FeCl_3$ in 6 M HCl) was added and mixed. Reactions were left to rest for 30 minutes. After stabilization, absorbances were measured in triplicates using a Perkin Elmer UV/VIS spectrometer Lambda Bio 20 at a wavelength of 668 nm. Data per time breaks and temperatures of incubation were analyzed using a pairwise t-test at a confidence level of 95% (α=0.05).

3.3 Molecular analysis

3.3.1 pit and csp primer designs

pit was searched in the *D. psychrophila* genome accession number NC_006138.1 using the NCBI microbial genomes database. CDS searches resulted in accession number WP_011189869.1. Our results indicated that pit was located between positions 2988445 and 2989710 in the microbes' genome and it corresponded to a sequence of 1265 bp. Furthermore, CDS for this gene were extracted and processed further using the Geneious software which allowed us to have a clearer image of its location and size. Primers for pit were designed using the IDT software of ThermoFisher. pit primers specificity was corroborated by aligning them with the bacterium's

genome using Geneious and Blast. Finally, the best suitable primer pair was chosen. This primer pair align with *D. psychrophila* genome in positions 2988787 and 2988886 spanning a region of 100 bp (See Figure 25 in appendix). Primer sequences generated with the IDT software (ThermoFisher) were: 5'- TCCACCACCCACTCCATTAT-3' and 5'- TGGCAACAACACTGCCTATT-3'.

The same process was used to generate primers for the csp gene (our reference gene) which is located in positions 390661 and 390861 with a size of 201 bp. Primers for csp were designed to align in positions 390665 and 390764 spanning a region of 100 bp (See Figure 23 in appendix). Primer sequences generated with the IDT software (ThermoFisher) were: 5'- CTGAAGGAACTGTGAAGTGGT-3' and 5'- GCATTGATGCTGGTGTGATG-3'

Both primers were standardized for optimal annealing temperature by running gradient PCRs at 54 °C with 5 °C increments and further analyzing the band amplifications in agarose gel electrophoresis (See Figures 24, and 26 in appendix). Furthermore, optimal amplification for both primers were registered at an annealing temperature of 56 °C.

3.3.2 RNA extraction and cDNA synthesis

RNA was extracted from all replicas of each individual set of experiments and controls using TRIZOL (Invitrogen) and performing overnight precipitations in isopropanol at -20 °C. Purifications and genomic DNA digestions were processed using the RNeasy Mini kit of QIAGEN following the manufacturer's instructions for purifications in silica membranes. Finally, cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription kit of Applied Biosystems (AB) following the manufacturer's instructions for targeting total RNAs.

3.3.3 qPCR-Melt curve analysis (qPCR-MCA) for pit and csp

qPCR-MCA were performed using EvaGreen Dye (Biotium) and cDNAs from all samples an replicas in triplicates following the EvaGreen manufacturer's instructions and the following protocol: 90.0º C for 0:30 sec, 90.0º C for 0:15 sec, 56.0 ºC for 0:15 sec, 72.0 ºC for 1 min, 10 cycles; 90.0 ºC for 0:15 sec, 56.0 ºC for 1 min, 72.0 ºC for 0:30 sec and Plate read (Fluorophore SYBR), 35 cycles; 72.0 ºC for 3 min, 90.0 ºC for 0:15 sec, 65.0 ºC for 3 min and Melting curve with the next parameters: 75.0 ºC to 90.0 ºC with an increment of 0.1 ºC every 0:05 sec 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. Differences in expression between the temperature positive control (untreated samples at 10 °C) and the experiments at -5 °C and -10 °C experiments were analyzed by means of $2^{-(\Delta\Delta T)}$ method using csp as our reference gene (Livak & Schmittgen, 2001). Statistical differences were performed using an ANOVA and pairwise-t-test analysis at a confidence level of 95% (α=0.05).

4. Results

In the cultures supplemented with $MgSO_4$ for 30 days, we can observe that there was a significant increase in pit expression when the incubation temperature was lowered from 10 °C down to -10 °C. As we can see in Figure 17, there were no significant differences at increasing concentrations for the -5 °C group while the -10 °C group showed a 2-fold increase in cultures supplemented with 1% MgSO4. Furthermore, supplementations with 0.35 wt%, 5wt% and 10 wt% $MgSO_4$ presented consistent 1-fold increases. As expected, this effect gradually faded at concentrations above 10 wt% (Figure 17).

Similarly, cultures supplemented with $CaSO_4$ showed a gradual increase up to 2-fold in pit expression when the temperatures were lowered from 10 °C to -5 °C and then to -10 °C. However, as we can see in Figure 17, there were not significant expression differences among the -5 °C samples at increasing concentrations (when present), while in the -10 °C group, cultures supplementations with 0.1 wt%, 5 wt%, 15 wt%, and 25 wt% showed similar pit expression with increments up to 1.5-fold. Interestingly, cultures supplemented with 1 wt%, 10 wt%, and 20 wt% presented the highest increments in pit expression (2-fold).

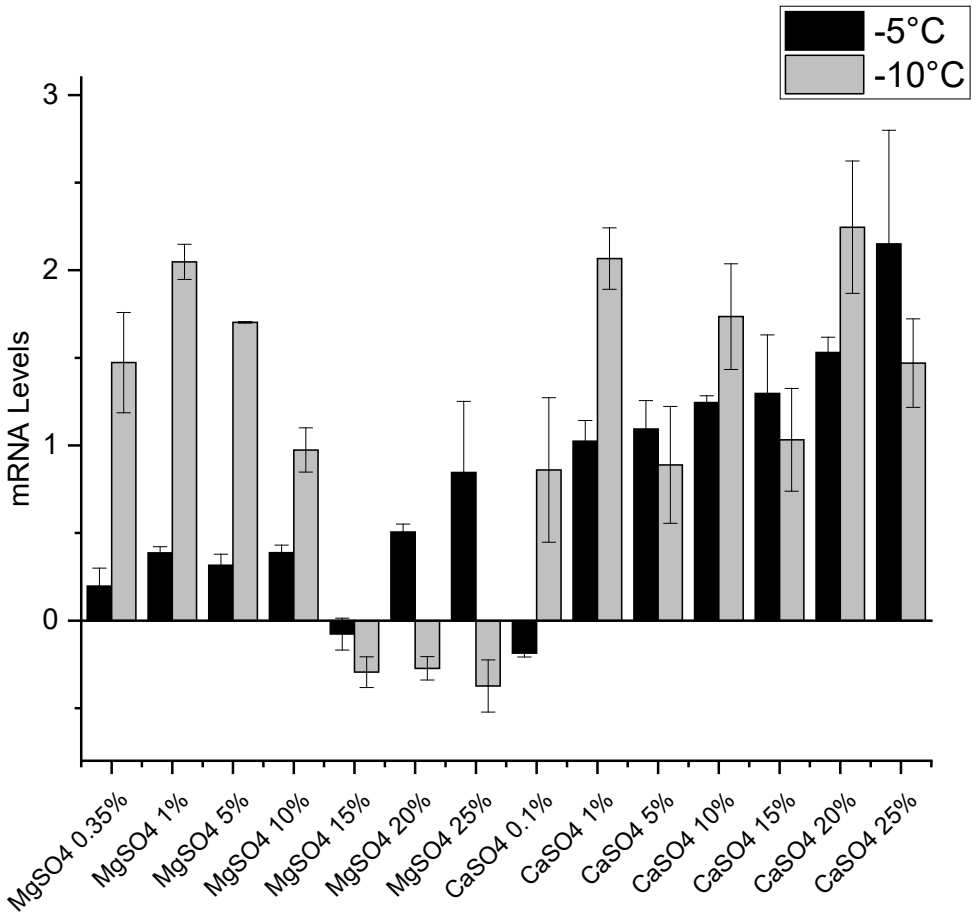

Figure 17. mRNA Level expression of pit in cultures subjected to incubations at -5 °C and -10 °C for 30 days. Ct results of pit expression were analyzed using csp as the reference gene and the temperature positive control (untreated). Figure shows Log10 results from samples processed with $2^{\wedge-(ddCt)}$ method for samples incubated at increasing concentrations of $MgSO_4$ and $CaSO_4$. Growth negative controls were not included as no amplification for these samples were registered. N =252.

Similar to our experiments for 30 days, cultures incubated with $MgSO_4$ for 90 days showed increase expression of pit when the temperatures were dropped from 10 °C to -5 °C. Interestingly, this group showed a 1-fold increase expression of pit at concentrations below 1 wt% which is higher than the expression registered with the same supplementations in our experiments for 30 days. In fact, almost all supplementations at -5 °C showed increase pit expression when compared

with shorter incubations except for the supplementations at 25 wt% (compare Figures 17, and 18). Consistent with our experiments for 30 days, this increase in expression gradually faded as concentrations increase until it is not detected at concentrations above 20 wt%. Surprisingly, cultures at -10 °C showed a strong attenuation of pit expression (less than 1-fold) when incubated for longer times (compare Figures 17, and 18). Consistent with the latter, our cultures supplemented with $CaSO_4$ at both temperatures showed attenuated pit expression when experiments were incubated for longer periods of time except for supplementations at 0.1 wt% at -5° C incubations and at 10 wt%, and 25 wt% in cultures incubated at -10°C which seem to increase or be consistent with the experiments incubated for 30 days (compare Figures 17, and 18).

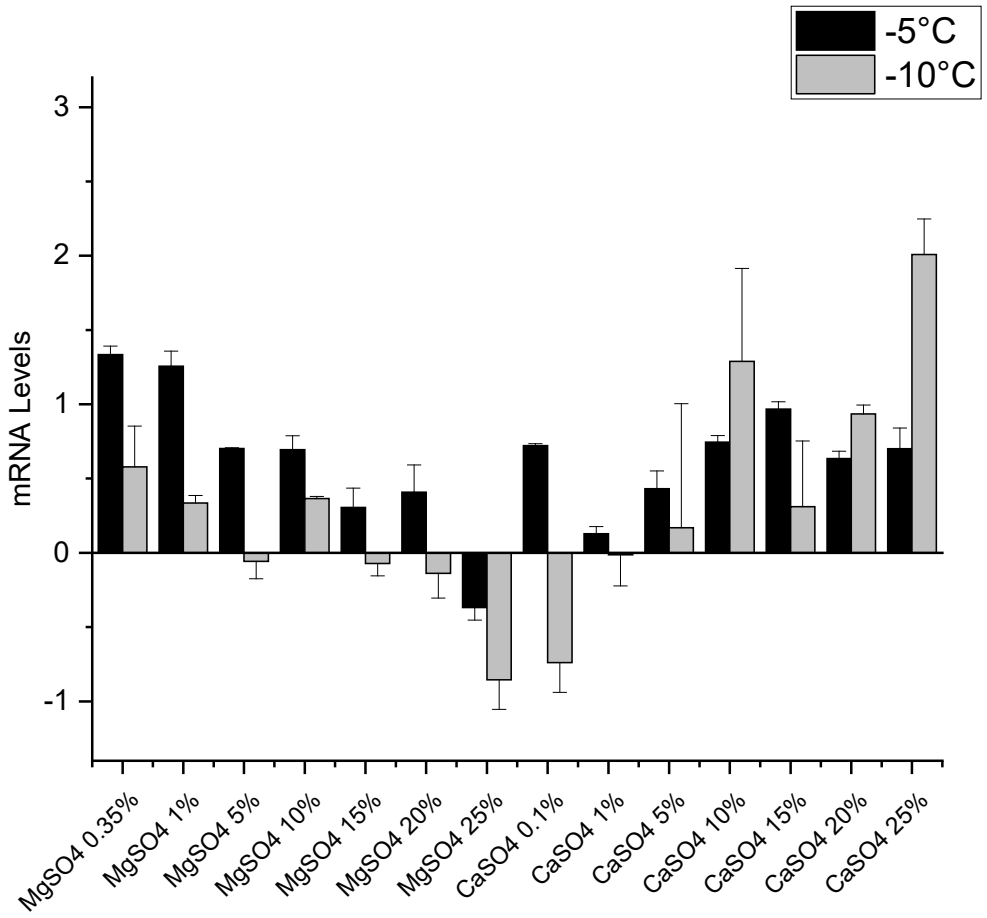

Figure 18. mRNA Level expression of pit in cultures subjected to incubations at -5 °C and -10 °C for 90 days. Ct results of pit expression were analyzed using csp as the reference gene and the temperature positive control (untreated). Figure shows Log10 results from samples processed with $2^{\wedge-(ddCt)}$ method for samples incubated at increasing concentrations of $MgSO_4$ and $CaSO_4$. Growth negative controls were not included as no amplification for these samples were registered. N = 252.

In our detection of sulfides in solution in cultures incubated for 30 days, we can observe that sulfate reduction rates were comparable to the temperature positive control at all concentrations below 10 wt% in supplementations with $MgSO_4$ at both temperatures tested. However, in the -5 °C samples supplementations with 1 wt% showed significant differences with the temperature positive control, while at -10 °C supplementations with 1 wt% to 25 wt% presented increased sulfate reduction rates in comparison with the temperature positive control. Furthermore, at increasing

concentrations of this compound there was a decrease in sulfides detection in the temperature positive control (10 °C) and in the -5 °C group. Surprisingly, as seen in Figure 19, concentrations above 1 wt% of $MgSO_4$ facilitated higher sulfate reduction rates in cultures incubated at -10°C. As expected, this increase was gradually reduced since sulfate reduction rates plummeted at concentrations above 15 wt% (Figure 19).

On the other hand, sulfate reduction in cultures supplemented with $CaSO_4$ were detected in all concentrations and in all temperatures tested. In general, the -5 °C group presented the highest sulfate reduction rates while the temperature positive control showed inconsistencies along the mineral gradient. Finally, sulfate reduction rates at -10 °C were similar across all concentrations except in supplementations with 5 wt% and 20 wt% where the sulfate reduction rates were higher than the temperature positive control (Figure 19).

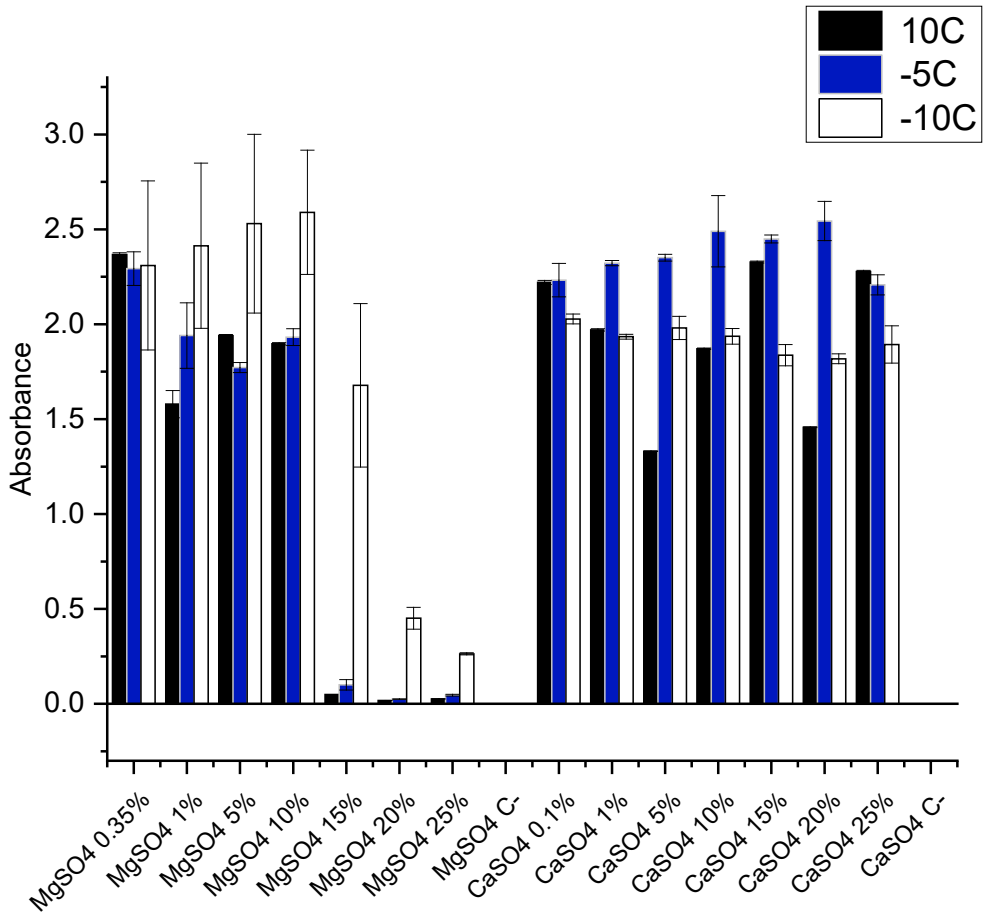

Figure 19. Sulfate reduction in cultures supplemented with increasing concentrations of $MgSO_4$ and $CaSO_4$ and incubated for 30 days. Figure shows the absorbance obtained after applying the methylene blue protocol which allows us to detect sulfides-sulfur in solution. Growth negative controls were averaged and are shown as $MgSO_4$ C- or $CaSO_4$ C-. N = 252.

Furthermore, our analyses of sulfate reduction for 90 days showed attenuated sulfate reduction in the -5 °C group in both minerals tested. In general, all sulfate reduction processes at -10 °C were strongly reduced when samples supplemented with $MgSO_4$ were incubated for longer times (compare Figures 19, and 20). Moreover, the same phenomenon was registered in our cultures with $CaSO_4$ which showed a decrease in sulfate reduction at all concentrations except for supplementations at 0.1 wt%, 5 wt%, and 15 wt% (compare Figures 19, and 20). Interestingly,

sulfate reduction rates at 10 °C were kept practically steady at all $MgSO_4$ and $CaSO_4$ concentrations for short and long incubation times (compare Figures 19, and 20).

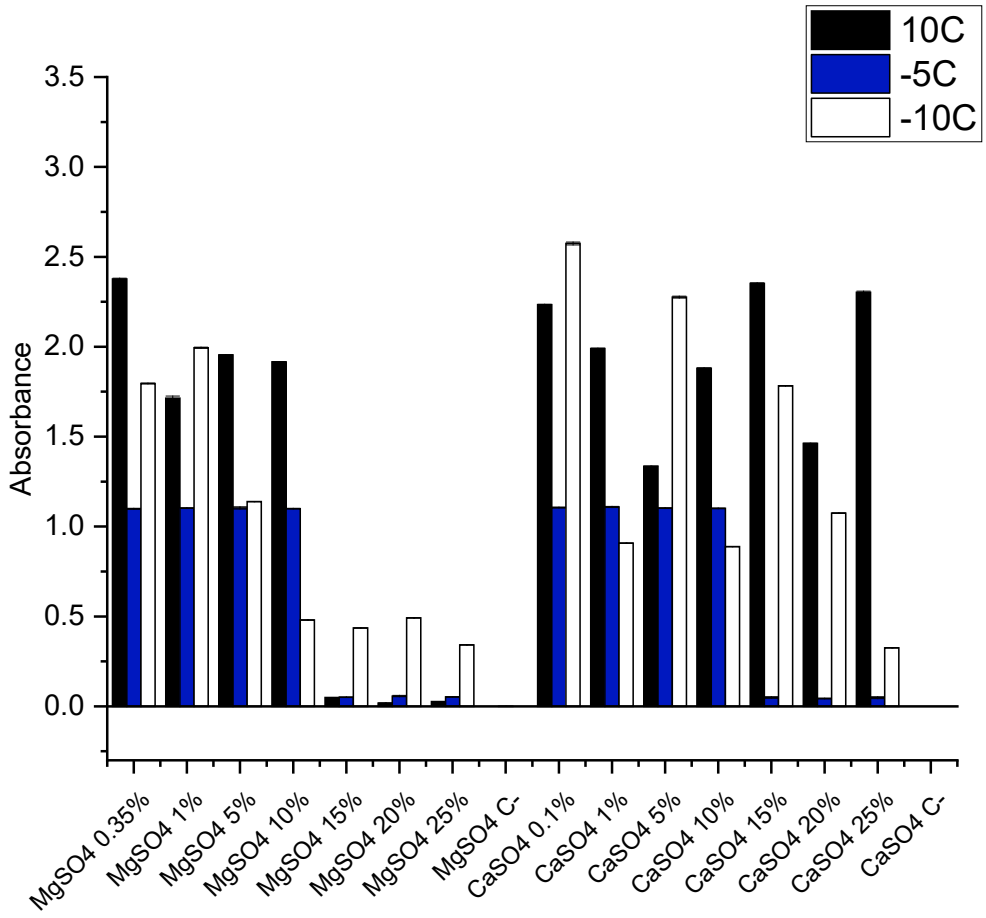

Figure 20. Sulfate reduction in cultures supplemented with increasing concentrations of $MgSO_4$ and $CaSO_4$ and incubated for 90 days. Figure shows the absorbance obtained after applying the methylene blue protocol which allows us to detect sulfides-sulfur in solution. Growth negative controls were averaged and are shown as $MgSO_4$ C- or $CaSO_4$ C-. N = 252.

5. Discussion

Just in our Solar System, six out of eight planets possess environments were freezing conditions prevail. Even more exciting, most of their satellites also possess permanently frozen environments. Therefore, if life were to exist in these locations, it would be crucial to understand the mechanisms that microorganisms could use to withstand extremes of temperatures. In our experiments, we were able to detect sulfate reduction down to -10 °C in cultures cultivated for 30 days under increasing concentrations of $MgSO_4$ and $CaSO_4$. Furthermore, our data suggests the occurrence of mechanical damage of the cell membrane due to ice crystals formation which was possibly counteracted by upregulating the biosynthesis of Pit, a structural and functional component of the cell membrane, and by reducing non-covalent interactions of the plasma membrane due to the action of Ca^{2+} and Mg^{2+} ions in solution. This phenomenon known as chaotropic effect increases the flexibility of the membrane in cold temperatures where increased non-covalent interactions induce inflexibility of its components affecting its transport functions. Moreover, these ions enhanced the plasma membrane's functionality by directly destabilizing non-covalent interactions or by inducing the biosynthesis of unsaturated fatty acids which will allow the sulfate reduction process to operate normally (Ingram, 1981). We also found that this chaotropic effect is short-lived since sulfate reduction was strongly attenuated in cultures exposed to -10 °C for 90 days. However, it is remarkable that these cells remained metabolically active at this low temperature. Similar to our experiments, Chin et al. (2010) detected chaotropic effects in cultures of fungi incubated at 1.7 °C (active cells) and down to -80 °C (spores) while Crisler et al. (2012) found that bacterial isolates from the Great Salt Plains could tolerate temperatures down to -70 °C in freeze-thaw cycles experiments. Moreover, Chin et al. (2010) data suggested that cell viability and active metabolism were enhanced due to the presence of these agents. Interestingly, the authors mentioned that even though the exact mechanism that allowed these microorganisms to tolerate subfreezing

temperatures is still elusive, it might be possible that the total effect of chaotropic ions is indistinguishable to the cell (regardless of its association to a particular taxa). Furthermore, they suggested that chaotropic ions could in fact counteract the effects of subfreezing temperatures where intense macromolecular interactions occur. Notably, as it was stated in Gilichinsky et al. (1995) microbes from terrestrial permafrost can remain metabolically active at -10 °C. Therefore, if this phenomenon happens on Earth, it is not impossible to think that this same occurrence could happen in the Martian regolith where water pockets or water films firmly associated with the regolith's minerals have been speculated to exist. Moreover, as similar water pockets have been theorized to be present in Titan's icy shell and other icy satellites, the possibility of having microenvironments where life conductive conditions can be present are not that distant from the imagination (Chin et al., 2010; Cray et al., 2015; Crisler et al., 2012; Gilichinsky et al., 1995; Stevenson, Hamill, Medina, et al., 2017).

5.1 $MgSO_4$ brines

In general, our experiments at subfreezing temperatures showed an increase in pit expression above 1-fold and up to 2-fold when samples were incubated at -10 °C for 30 days under 0.35 wt% to 10 wt% of $MgSO_4$ concentrations (Figure 17). This increased expression could be related to the fact that $MgSO_4$ can reduce the freezing point of water only down to -4 °C (Toner et al., 2014). As our experiments were performed at temperatures lower than this value, ice crystals and the expansion of the cell volume could have mechanically disrupted the cellular membrane (Mindock et al., 2001). Therefore, our pit expression observations could be the result of the increasing need of the cell to replace structural components of the membrane to keep up with its metabolic demand. However, the fact that these cells can find strategies to survive and metabolize at these low temperatures could indicate a chaotropic effect provided by Mg^{2+} ions resuspended in the culture

medium which indirectly aided in the maintenance of the membrane's fluidity allowing sulfate reduction to occur normally (see Figures 17, and 19). As we know from the literature, cold temperatures induce an increased rigidity of the plasma membrane affecting its transport functions (increased non-covalent interactions). Cold-adapted microbes cope with this environmental challenge by increasing the unsaturated fatty acids present on their membranes or by synthesizing compatible solutes (Cray et al., 2013; Fox-Powell & Cockell, 2018; Hallsworth et al., 2003; Hassan et al., 2020; Ingram, 1982; Lever, 2016; Steven et al., 2006; Stevenson, Hamill, Medina, et al., 2017; Zajc et al., 2014). However, in our experiments at subfreezing temperatures, the presence of chaotropic ions could have assisted in the maintenance of the membrane's fluidity by disrupting non-covalent interactions in this macromolecular structure which is known to rigidify at below 0 °C temperatures. Therefore, in our experiments an increase in pit expression and subsequent detection of sulfate reduction might indicate mechanical damage to the cell membrane that is being continuously repaired to keep up with the cell's metabolic demand while the chaotropic effect provided by Mg^{2+} and Ca^{2+} ions in solution could have increased the membrane's fluidity and therefore its functionality. Analogous to our results Hallsworth et al. (2003) found that under chaotropic conditions cells of *Pseudomonas putida* upregulated the production of a protein of the outer membrane (H1) related to transport and folding of other proteins (Bhaganna et al., 2016; Hallsworth et al., 2003). Similarly, the studies of Chin et al. (2010) or Crisler et al. (2012) mentioned above also corroborate our findings in which microbial species were viable under subfreezing temperatures in the presence of chaotropic agents (Chin et al., 2010; Crisler et al., 2012). Importantly, cultures supplemented with high concentrations of $MgSO_4$ (above 10 wt%) and incubated at -10 °C for 30 days showed a downregulation in pit expression and their sulfate reduction rates gradually faded as we increased the concentration of this compound. This

occurrence could be related with osmotic stress, toxicity, and cellular death induced by high salinity concentrations since the effects of chaotropicity and osmotic stress on cellular functions are not mutually exclusive (compare Figures 17, and 19) (Eardley et al., 2019; Mcgenity & Oren, 2012; Stevenson, Hamill, Dijksterhuis, et al., 2017).

Furthermore, at -5 ºC incubations, the pit expression was lower than 0.5-fold (Figure 17) for concentrations below 10% which suggests the absence of mechanical damage to the cell membrane and that these cells are not drastically affected by these subfreezing growth conditions since their sulfate reduction rates were analogous to the ones presented for the temperature positive control at 10 ºC (Figure 19). It is also important to note that cultures at high concentrations (above 15 wt% and specially 20 wt%, and 25 wt%) showed attenuated fold increases in pit expression which could be related to the fact that these cells were under osmotic stress due to high concentrations of $MgSO_4$ which could be corroborated due to the absence of sulfate reduction (compare Figure 17, and 19).

Furthermore, the increased mRNA levels registered for our -10 ºC experiments at increasing concentrations of $MgSO_4$, did not last longer as we can see that in cultures incubated for 90 days the expression of pit was absent or drastically reduced at concentrations below 10 wt% (Figure 18). Interestingly, this could indicate two possible scenarios, one in which the cells adapted to cope with this temperature or another one in which cells died due to prolonged exposure to freezing conditions. We are inclined to think that the last scenario is more logical since the sulfate reduction rates were also reduced (Figure 20). Interestingly, cultures at high concentrations (above 15 wt%) did not show pit expression increases (Figure 18). However, prolonged times facilitated basal sulfate reduction detections (Figure 20). It is possible that these cells were still viable, but they were only surviving those harsh conditions of extreme temperatures and high salinity.

Interestingly, cultures incubated at -5 ºC under $MgSO_4$ for 90 days showed an increased expression of pit in comparison with the 30 days incubations (compare Figures 17, and 18). This increase in pit expression suggests cell membrane mechanical damage that was delayed since our experiments at -5 ºC were close to the eutectic point of $MgSO_4$ brines (-4 ºC). Accordingly, at -5 ºC ice crystals would gradually form allowing microbial cells to survive and metabolize for short periods of time. However, prolonged exposition to this subfreezing temperature could have promoted cellular death due to mechanical lysis by ice crystals. Probably, these cells might be in a survival state that would end in cellular death if longer incubations times would have been used. This idea comes from the fact that sulfate reduction rates were reduced, but they were not totally absent (Figure 20).

5.2 $CaSO_4$ brines

On the other hand, our analyses of mRNA expression with $CaSO_4$ at -5 ºC and -10 ºC for 30 days showed inconsistencies in pit expression between specific concentrations, but in general there was increased mRNA levels in all samples except for $CaSO_4$ at its lowest concentration under -5 ºC incubations. As mentioned above, increased pit expression could indicate mechanical damage to the cell membrane at both temperatures. Analogous to our experiments with $MgSO_4$, chaotropic effects due to Ca^{2+} suspended in the medium could have induced a reduction of non-covalent interactions in the cell membrane which will allow these microbes to sustain metabolic rates even when the negative effects of extremely cold temperatures were present (see sulfate reduction rates in Figure 19). However, it is also important to take into consideration that $CaSO_4$ solubility in water is relatively low (0.26g/100 mL at 25 °C) and probably lower at our experimental conditions. This occurrence would render a limited availability of Ca^{2+} ions to promote non-covalent bonds breakage on the cell membrane of *D. psychrophila*. Furthermore, as we can see in Figure 17, all cultures experienced mechanical damage to their cell membranes (increased pit expression at both

temperatures tested) but given the limited chaotropic properties of these brines and taking into consideration their increased sulfate reduction rates (Figure 19) the only reasonable explanation is that $CaSO_4$ sediments and matrixes formed during freezing conditions offered a temporal protective effect from extreme temperatures similar to the phenomenon that happens in Earth's evaporites with cryptoendolithic or endoevaporitic microbial communities (Canfield et al., 2004; Douglas & Yang, 2002; Rhind et al., 2014). These microhabitats which are commonly form by $CaSO_4$ precipitation from evaporated seawater or brines are present in lakes, volcanic environments, and hydrothermal waters (Hughes & Lawley, 2003; Reiss et al., 2021). In our experiments, $CaSO_4$ in saturation precipitated to the bottom of the serum bottles (-5 °C experiments) or expanded in the bulk of water (-10 °C experiments) since its solubility is low at subfreezing temperatures (see Figure 34-41). This phenomenon allowed the survival of *D. psychrophila* cells in our cultures, similar to cryptoendolithic communities in evaporites, by living between and upon the grains of precipitated minerals (Dong et al., 2007; Douglas & Yang, 2002; Mancinelli, 2005; Reiss et al., 2021; Tang et al., 2014). This in fact could have been the cause of the increased sulfate reduction rates registered in our experiments in the presence of limited Ca^{2+} chaotropic effects. As it was explained by Dong et al. (2007) the study of terrestrial evaporites as analogs to Martian environments and other icy bodies started with the discovery of cryptoendolithic communities in the Antarctic desert. Furthermore, in lieu of the fact that most evaporites present in the Antarctic desert are formed by sandstone (absent in Martian environments), it was suggested that a better analog would be the hyperarid Atacama Desert which contains $CaSO_4$ deposits. Furthermore, these cryptoendolithic communities are formed by primary producers (normally phototrophic organisms) and associated heterotrophic organisms. Interestingly, the latter is composed of a great variety of microbes which includes members of the

Alpha, Beta and Delta-Proteobacteria, Actinobacteria, and Acidobacteria. Evidently, *Desulfotalea psychrophila* could adopt a cryptoendolithic behavior since this microbe was first isolated from permanently cold marine sediments of the Artic ocean (Svalbard) and it is also a member of the Delta-Proteobacteria (Dong et al., 2007; McKay & Friedmann, 1985; Navarro-González et al., 2003; Rabus et al., 2004b; Wierzchos et al., 2011; Ziolkowski et al., 2013). As suggested by Dong et al. (2007), subsurface environments within gypsum evaporites (increasing accumulation of sediments) could provide a suitable environment for microbes to survive and metabolize since they could potentially protect themselves from negative environmental conditions by using the sedimented minerals' pores as a protective layer. More importantly, it has been suggested that in natural habitats when environmental conditions are extreme, microorganisms could potentially migrate to the interior of rocks and sedimentary material further increasing their protective capabilities. As *D. psychrophila* is a motile bacterium, it is possible that cells migrated from the column of water to the sediment strata formed due to $CaSO_4$ precipitation where they established stable microniches (Billi et al., 2011; Casero et al., 2021; Crits-Christoph et al., 2016; Dong et al., 2007; Douglas & Yang, 2002; Gómez et al., 2012; Hughes & Lawley, 2003; Jorgevillar et al., 2006; Rhind et al., 2014; Rothschild, 1990). Moreover, it has been suggested that given the harsh conditions present in today's Mars or icy bodies, if life were to exist (or existed), it would be formed by microorganisms able to colonize the physical space in pores of $CaSO_4$ deposits or rocks containing $CaSO_4$ (Edwards, 2010; Gómez et al., 2012; Hughes & Lawley, 2003; Mckay, 1997; Wierzchos et al., 2006, 2011; Ziolkowski et al., 2013). However, this physical barrier would render only temporal protection as we can see that in our experiments with $CaSO_4$ at -5 ºC incubations for 90 days, pit expression as well as sulfate reduction rates plummeted (see Figures 18, and 20). This finding resembles our results with $MgSO_4$ for 90 days incubations at -10 °C in which

downregulation of pit expression and a decrease in sulfate reduction rates might indicate that cells present in these cultures are on survival mode or are gradually perishing. However, cultures at 0.1wt%, and 5 wt% of $CaSO_4$ incubated at -10 °C showed sulfate reduction rates comparable to the temperature positive control at 10 °C which would indicate that these cultures were able to establish persistent microcommunities within the sediments protective matrix (Figure 20). Similar to phenomena that is predicted to occur in the Martian subsurface and in the warm diapirs of Europa, the presence of thin aqueous films of liquid water surrounding minerals deposits could boost metabolic activity down to -40 °C, a phenomenon that has been studied in microbial communities inside ice and permafrost on Earth (Grasby et al., 2003; Price & Sowers, 2004).

6. Conclusions

In the analysis of the expression of pit at temperatures down to -10 °C for 30 days, we were able to observe increases of up to 2-fold expression of this cell membrane structural component as a response to mechanical damage by formation of ice crystals in cultures supplemented with 0.35 wt% to 10 wt% of $MgSO_4$ concentrations (like Hallsworth et al., 2003 studies). Interestingly, these cells were successfully metabolizing since the sulfate reduction rates were comparable to the positive control at 10 °C. This increased sulfate reduction could indicate the possible aid of chaotropic effects of Mg^{2+} ions which probably destabilized the membrane allowing it to remain functional at those harsh conditions. On the other hand, it is also possible that Pit, which is involved in the transport of inorganic phosphate (used in phosphorylation activities such as ATP synthesis, intracellular communication, etc.), was upregulated as a direct consequence of chaotropicity as it occurred in Bhaganna et al. (2016) studies who found that proteins related to energy metabolism (ATP synthesis) were upregulated in the presence of chaotropic agents (Bhaganna et al., 2016). However, it is important to understand that based on the biological context, chaotropic substances

have shown to provide protective functions. Therefore, we suggest that in our experiments chaotropicity facilitated sulfate reduction by counteracting the effects of subfreezing temperatures (mechanical damage to the cell membrane due to ice crystals formation). These findings are relevant to the field of Astrobiolgy since chaotropic agents as those used in this study are present in today's Martian regolith (cold traps or brines in the subsurface) as well as in the subsurface oceans of icy moons, especially Europa, Ganymede and Titan. Therefore, our findings have implications for the survival and proliferation of bacterial cells in these planetary bodies which is of relevance for Planetary protection policy making (Chin et al., 2010; Cray et al., 2013; Crisler et al., 2012; Gilichinsky et al., 1995; Greenberg, 2010; Lingam & Loeb, 2019; Lipps & Rieboldt, 2005; Marion et al., 2003; Martin & McMinn, 2018; Stevenson, Hamill, Medina, et al., 2017).

This research has let us gather some more knowledge of the behavior of microbes under $MgSO_4$ type brines which have not been extensively studied as its counterpart NaCl type brines which are the most abundant on Earth. Analogous to our results, there have been reports of microbes which remain viable at high concentrations of $MgSO_4$ or $CaSO_4$, but only if Na^+ or Cl^- ions are present at considerable quantities (highest limit: Mg^{2+} = 2.5 M and NaCl 2M). However, in our experiments (max concentration of Mg^{2+} or Ca^{2+} = 1M, and NaCl 0.3M) this halotolerance was challenged at lower concentrations (Crisler et al., 2012; Javor, 1984; Markovitz, 1961; Markovitz & Sylvan, 1962; Mcgenity & Oren, 2012; Mullakhanbhai & Larsen, 1975; Oren, 1983; Oren et al., 1995; Yakimov et al., 2015). Interestingly, in these studies it was proposed that organisms adapted to high salinity environments have kept the ability to interact with chaotropic ions especially at their membrane proteins level which is crucial in our experiments in which we have shown that environments with high concentration of $MgSO_4$ and subfreezing temperatures upregulated the expression of pit in an effort to repair potential damage to the cell membrane due

to ice crystals formation and allowed *D. psychrophila* cells to perform sulfate reduction at similar rates as the temperature positive controls. Also these chaotropic agents could have upregulated the biosynthesis of unsaturated fatty acids in *D. psychrophila cells* acting as a cold stressor and protecting these cells from extreme temperatures (Fox-Powell & Cockell, 2018; Oren et al., 2005). This type of study is crucial to predict the type of behaviors we could find in brines formed in other planetary bodies and it has shown that the relationships between chaotropicity and bacterial survival/growth are complex. Thus, further research is necessary to understand these relationships in outer planetary bodies and on Earth (Fox-Powell & Cockell, 2018; Lever, 2016; Zajc et al., 2014).

Furthermore, we were able to determine that this chaotropic effect is only temporary as our cultures incubated at -10 °C for 90 days showed a drastic reduction in pit expression and sulfate reduction rates. Moreover, as our results for 30 days showed an increase damage to the cell membrane due to ice crystals, it is possible that a prolonged exposure to subfreezing conditions rendered an environment in which the rate of mechanical damage was faster than the ability of the microbe to repair its cellular membrane and avoid cellular death. On the other hand, it is possible that these cells were entering stationary phase typical in batch cultures since sulfate reduction was not totally absent. It is important to also take into consideration that these cells were subjected to more than one stressor (subfreezing temperatures, high concentrations of $MgSO_4$, strong osmotic and chaotropic effects) which can drastically affect bacterial metabolism during prolonged exposures (Crisler et al., 2012).

Interestingly, we were able to observe that *D. psychrophila* cells subjected to $MgSO_4$ concentrations below 10 wt% at -5° C for 30 days did not show evidence of mechanical damage to their cell membranes or any negative effect due to freezing temperatures in their sulfate

reduction process which corroborates our findings from chapter II of this dissertation where we were able to detect dsrAB expression and sulfate reduction in cells growing at -5 °C for a space of 30 days.

Moreover, we were able to identify a delayed effect of cell membrane integrity damage in our experiments at -5 °C for 90 days as cells growing in these conditions showed an increase in pit expression which was associated with a decrease in sulfate reduction. This finding agrees with our experiments at -5 °C for 30 days in which initially we did not detect damage to the membrane or attenuated sulfate reduction, but as time progressed for 60 days more, cells in these cultures accumulated more damage to their membranes (upregulated pit expression) which could not be recovered and thus induced cellular death triggered a decrease in sulfate reduction.

Furthermore, we were able to detect mechanical damage to the cell membrane of *D. psychrophila cells* due to ice crystals formation in cultures supplemented with $CaSO_4$ at all concentrations and incubated at both temperatures for 30 days (except for samples at 0.1 wt% in the -5 °C experiments). Analogous to our experiments with $MgSO_4$, this increased expression of pit could be related to mechanical damage of the cell membrane and its continuous repair. Moreover, it is possible that chaotropic effects of Ca^{2+} ions in solution, at least at permissible concentrations due to its solubility, enhanced sulfate reduction metabolism in *D. psychrophila* cells by reducing non-covalent interactions in the cell membrane and preserving its functionality at subfreezing temperatures since these conditions are known to increase non-covalent interactions and inflexibility of the plasma membrane. However, it is also possible, that this increase of pit expression is only related to damage to the cell membrane and its continuous repair while *D. psychrophila* cells adopted a cryptoendolithic type of life by surviving and metabolizing within the $CaSO_4$ mineral grains precipitated (sediments and matrixes) formed due to its low solubility in

water. Furthermore, this physical barrier could have served as a protective layer against the extreme temperatures used in our experiments (Billi et al., 2011; Casero et al., 2021; Crits-Christoph et al., 2016; Dong et al., 2007; Douglas & Yang, 2002; Gómez et al., 2012; Hughes & Lawley, 2003; Jorgevillar et al., 2006; Rhind et al., 2014; Rothschild, 1990).

Interestingly, after 90 days of incubation at both temperatures and at all $CaSO_4$ concentrations tested, pit expression was strongly attenuated which could be related to cold-induced cellular death as the cells were not able to cope with the ice crystals damage to their cell membranes. This finding was corroborated in our cultures at -5 °C which showed attenuated sulfate reduction rates. However, as we were able to observe, cultures incubated at -10 °C under 0.1wt% and 5wt% maintained their sulfate reduction at comparable rates to the positive temperature control at 10 °C which could indicate that these cultures were able to survive within the matrixed/pores of $CaSO_4$ sediments adopting a cryptoendolithic type of life (Canfield et al., 2004; Douglas & Yang, 2002; Rhind et al., 2014).

7. References

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CHAPTER IV

Conclusions

The exploration of our Solar System has been an intriguing topic for humanity since ancient times. Starting with the discovery of the five planets (Mercury, Mars, Venus, Jupiter, and Saturn) by means of simple observation of the sky (naked eye) and the movement of the stars, followed by the discovery of Uranus and Neptune with the invention of the telescope. Undoubtedly, the scientific community has been interested to explore these planets in search of potential presence of other life-forms or habitable worlds. This interest catalyzed the advance in space exploration exponentially to present times in which highly technological devices are constantly orbiting other planets or have landed in their surfaces. Moreover, the first contact that was established with the Red planet occurred approximately 58 years ago when the Mariner 4 flyby took the first images of another planet. In addition to this extraordinary step in robotic space exploration, humanity was able to send the first man-crewed spacecraft to the Moon approximately 53 years ago when Neil Armstrong set foot for the first time in this satellite (1969). As a result of these incredible advances in space exploration, concerns about the pristine conditions of other planets and the protection of our own gave origin to a set of guidelines that would regulate the planning and functioning of interplanetary missions. This set of guidelines, known as the COSPAR planetary protection policy, takes into consideration the possibility of contaminating other planets with terrestrial microorganisms and the possibility of contaminating our planet with extraterrestrial life-forms (if existing). Therefore, research about the capabilities of terrestrial microbes to survive and even colonize other planets 'environments, conductive of life, are crucial to determine the ways in which humanity could explore these interesting niches. In our studies, we tested the ability of a psychrophilic bacterium, *D. psychrophila*, to survive, proliferate, and metabolize at subfreezing temperatures in cultures supplemented with increasing concentrations of sulfate compounds as

those present in today´s Martian regolith. Interestingly, we found that this microbe can withstand temperatures down to -5 ºC in the presence of $MgSO_4$ at 0.35 wt% to 18 wt% concentrations. Under these conditions, *D. psychrophila* cells tend to prioritize sulfate reduction (metabolic activity), rather than microbial growth. An occurrence that can be related to the survival capabilities of this microorganism under these suboptimal temperature conditions. Furthermore, our experiments indicated the occurrence of metabolic specialization which allowed subclonal populations of *D. psychrophila* cells to behave dynamically under different environmental stressors such as subfreezing temperatures and high concentrations of sulfate compounds. This is interesting because it shows the different strategies that extremophile microorganisms could adopt to withstand harsh conditions such as those present temporarily in the Martian subsurface. Furthermore, we were able to detect the effects of subfreezing temperatures in the metabolic machinery of *D. psychrophila* cells in cultures supplemented with $CaSO_4$ 0.1 wt% (at -5 ºC), and $MgSO_4$ at increasing concentrations (0 ºC and -5 ºC) in which dsrAB operon transcripts were presumably altered by posttranscriptional or posttranslational modifications rendering non-functional DsrAB enzymatic units (attenuated sulfate reduction). Moreover, we were able to detect expression of the dsrAB operon in samples incubated with $FeSO_4$ supplementations. However, these cultures presented minimal microbial growth and absent sulfate reduction, at all temperatures tested, which indicates that *D. psychrophila* cells could potentially survive temporarily in conditions where high concentrations of $FeSO_4$ are present, but eventually the cellular viability would be compromised. A similar phenomenon was observed in the $Fe_2(SO_4)_3$ cultures where minimal microbial growth occurred (10 wt% at all temperatures tested and at -5 ºC under increasing concentrations), but no dsrAB transcripts or sulfate reduction were detected. Analogous

to the cultures supplemented with $FeSO_4$, it is possible that cells subjected to these conditions survived, at least temporarily, but they eventually perished.

In addition, we tested the ability of *D. psychrophila* cells to sustain metabolic activity at temperatures down to -5 °C and -10 °C in concentration gradients of $MgSO_4$ and $CaSO_4$ salts for 30 and 90 days. In these experiments we assessed the integrity of the cell membrane as a proxy to understand if chaotropic ions could counteract the effects of subfreezing temperatures by maintaining the fluidity of the cell membrane and allowing these bacterial cells to perform sulfate reduction processes. These experiments were targeted to test the ability of *D. psychrophila* cells to survive in extreme environments such as the subsurface oceans of Europa, Ganymede, and Titan, but in reality, it could be applied to any other icy body in which subfreezing temperatures dominate. Interestingly, in our analyses of pit expression, we found that cultures supplemented with $MgSO_4$ at 0.35 wt% to 10 wt% presented increased damage of the cell membrane due to ice crystals formation at temperatures down to -10 ºC. However, increased sulfate reduction in these cultures indicated the possible effect of chaotropic Mg^{2+} ions in solution which could have induced the reduction of non-covalent interactions in the plasma membrane preserving its fluidity. Furthermore, we were able to determine that this chaotropic effect is only temporal since our experiments for prolonged times (90 days) showed attenuated sulfate reduction rates. Interestingly, it was suggested that a possible explanation for this phenomenon is related to the fact that prolonged exposures to -10 ºC temperatures could induce more damage to the cell membrane and therefore outcompete the ability of *D. psychrophila* cells to repair their cell membrane.

In general, we were able to detect limited chaotropic effects of Ca^{2+} ions in solution which would preserve the membrane fluidity at -5 °C and -10 °C allowing *D. psychrophila* cells to perform sulfate reduction processes. However, according to our experiments, this chaotropic effect was

only minor since the solubility of $CaSO_4$ under our experimental conditions was minimal. Interestingly, it was suggested that *D. psychrophila* cells could adopt a cryptoendolithic type of life since evidence of stable microniches (sulfides accumulations within the $CaSO_4$ sediments) was detected. According to this assumption, microbial cells would protect themselves from the harmful subfreezing conditions by establishing stable microcommunities within the $CaSO_4$ mineral grains (sedimentary in -5 °C and in matrices in -10 °C incubations). Interestingly, this physical barrier allowed the establishment of persistent microbial communities within the sediments which were able to withstand the effects of subfreezing temperatures for prolonged experimental times ($CaSO_4$ at 0.1 wt% and 5 wt% under -10 °C incubations for 90 days).

Finally, this piece of research has allowed us to understand the possible mechanisms used by microbial cells to survive under subfreezing temperatures in $MgSO_4$ and $CaSO_4$ types of brines which is of relevance for Astrobiological studies since both compounds have been identified in the Martian regolith, and in the subsurface oceans and icy shell surfaces of satellites from the Jovian and the Saturn systems. Finally, it is crucial to mention that the temperatures tested in this research have been detected in the Martian surface. However, it is well known that in satellites of the Jovian and Saturn Systems colder temperatures dominate. Despite this, it is believed that warmer temperatures could be present under the icy shell, but this idea has not been proven yet. In any of these cases, the same mechanisms used to survive extreme temperatures by *D. psychrophila* can be used by strains of cold-adapted bacteria with higher survival capabilities. Therefore, the information presented in this investigation could be extrapolated to those other extreme microbes.

CHAPTER V

Appendix

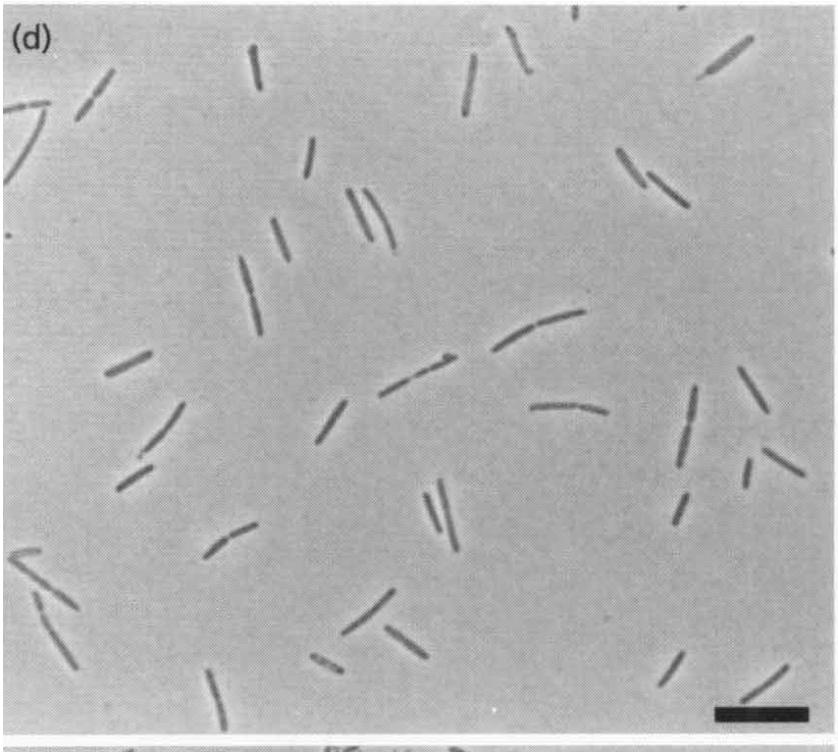

Figure 21. Phase-contrast photomicrograph of *D. psychrophila* cells. Image extracted from: Knoblauch et al., 1999.

Table 4. *D. psychrophila* general characteristics, carbon sources and electron acceptors. Extracted from: Knoublach et al., 1999

Characteristic	*Desulfofrigus oceanense* ASv26^T	*Desulfofrigus fragile* LSv21^T	*Desulfofaba gelida* PSv29^T	*Desulfotalea psychrophila* LSv54^T	*Desulfotalea arctica* LSv514^T
Cell size (μm):					
Width	2·1	0·8	3·1	0·6	0·7
Length	4·2–6·1	3·2–4·2	5·4–6·2	4·5–7·4	1·6–2·7
pH optimum	7·0–7·5	7·0–7·4	7·1–7·6	7·3–7·6	7·2–7·9
Optimum salt requirement (%):					
NaCl	1·5–2·5	1·0–2·5	1·4–2·5	1·0	1·9–2·5
$MgCl_2.6H_2O$	0·003–2·0	0·3–2·0	0·015–2·5	0·03–0·7	0·3–1·4
Temperature optimum/range (°C)	10/−1·8–16	18/−1·8–27	7/−1·8–10	10/−1·8–19	18/−1·8–26
Growth rate (h^{-1})/doubling time (h) at optimum temperature	0·0041/169	0·036/19	0·0048/144	0·026/27	0·021/33
Electron donors (mM):*					
Formate (20)	+ +	+	+	+ +	+ +
Acetate (10)	+ +	−	−	−	−
Propionate (15)	−	−	+ +	−	−
Butyrate (5)	+ +	+	+	−	−
Valerate (5)	+	−	−	−	−
Caproate (3)	−	+ +	−	−	−
Caprate (2)	−	+ +	−	−	−
Palmitate (2)	−	+ −	−	−	−
Lactate (10)	+ +	+ +	+ +	+ +	+ +
Pyruvate (10)	+	+ +	+	+ +†	+ +
Malate (10)	+ +	+ +	+	+ −	−
Succinate (10)	−	−	+	−	−
Fumarate (10)	−	+	+	+ +	−
Ethanol (10)	+ +	+ +	+ +	+ +	+ +
Propanol (10)	+ +	+ +	+ +	+ +	−
Butanol (10)	+ +	+ +	+ +	+ +	−
Glycerol (10)	+ −	+ +	+ −	−	+
Glycine (10)	+ −	−	+ −	+	−
Alanine (10)	−	+	+	+	−
Serine (10)	+ −	+	−	+	+ −
H_2/CO_2 + acetate (2)	+ −	−	−	+ +	+ +
Electron acceptors (mM):‡					
Sulfate (28)	+	+	+	+	+
Thiosulfate (10)	+	−	+	+	−
Sulfite (2)	+	−	+	+	−
Sulfur	−	−	−	−	−§
Iron(III) citrate (30)	+	+	−	+	+
Iron(III) oxyhydroxide	−	−	−	−	−‖
Fermentable compounds (mM):					
Pyruvate (10)	+	+	+	+	+
Malate (10)	+	+	−	−	−
Lactate (10)	+	−	−	−	−
Fumarate (10)	−	−	+	+	−
Polar lipids¶	PE, PG	PE, PG	PE, PG	PE, PG, DPG	PE, PG, DPG
Major menaquinones	MK-9	MK-9	MK-8	MK-$6H_2$	MK-6
G+C content (mol %)	52·8	52·1	52·5	46·8	41·8

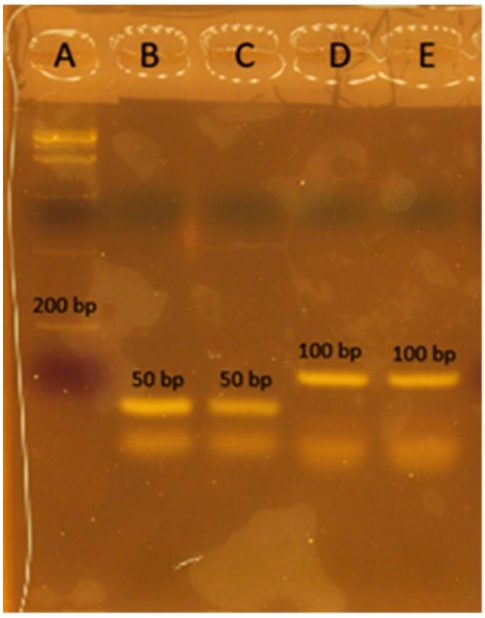

Figure 22. PCR amplification of *D. psychrophila* genomic DNA using primers designed for qPCR-MCA. (A) 1 kb ladder with a 200 bp band labeled. (B and C) 50 bp bands obtained from amplification with primers D.psychroFwd50 and D.psychroRev50. (D and E) 100 bp bands obtained from amplification with D.psychroFwd100 and D.psychroRev100.

Figure 23. csp (cold-shock protein) in *D. psychrophila* genome used to design qPCR-MCA primers in positions 390665 and 390764 spanning a region of 100 bp. csp-FWD and csp-REV 5'- CTGAAGGAACTGTGAAGTGGT-3' and 5'- GCATTGATGCTGGTGTGATG-3'.

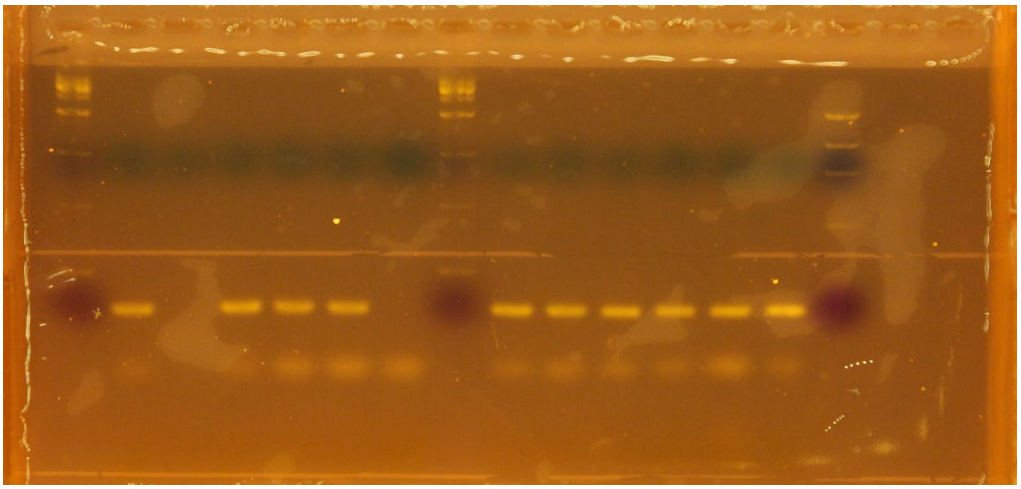

Figure 24. PCR amplification of *D. psychrophila* genomic DNA using primers designed for qPCR-MCA of csp. All bands presented are the result of a gradient test with csp primers using genomic DNA and ran in a gradient PCR thermocycler with a temperature of annealing of 54°C and 5°C increases. Band products have a size of 100 bp.

Figure 25. pit (Inorganic phosphate transporter) in *D. psychrophila* genome used to design qPCR-MCA primers. This primer pair align in positions 2988787 and 2988886 spanning a region

of 100 bp. piT-FWD 5'- TCCACCACCCACTCCATTAT-3' and piT-REV 5'-TGGCAACAACACTGCCTATT-3'.

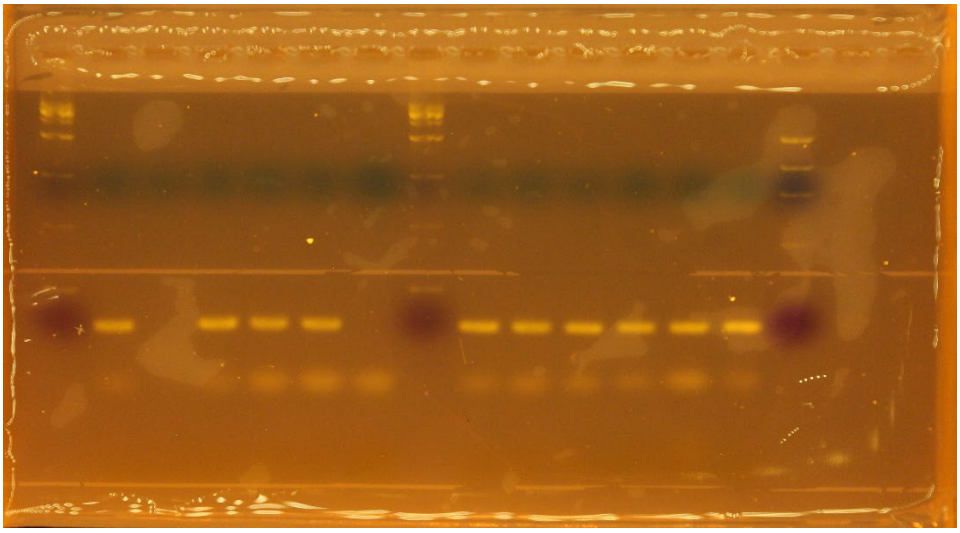

Figure 26. PCR amplification of *D. psychrophila* genomic DNA using primers designed for qPCR-MCA of pit. All bands presented are the result of a gradient test with pit primers using genomic DNA and ran in a gradient PCR thermocycler with a temperature of annealing of 54°C and 5°C increases. Band products have a size of 100 bp.

Figure 27. Pegasus biochamber at the Keck Lab of the University of Arkansas. This chamber possesses a chiller connected to a cooling plate which is positioned inside of the chamber for cold experiments. Plate is connected to a Lauda Chiller.

Figure 28. Cultures incubated at 10 °C for 30 days and supplemented with $MgSO_4$. From left to right: Growth negative control, 0.35 wt%, 1 wt%, 5 wt%, 10 wt%, 15 wt%, 20 wt%, and 25 wt% supplementations with $MgSO_4$.

Figure 29. Cultures incubated at 10 °C for 90 days and supplemented with $MgSO_4$. From left to right: Growth negative control, 0.35 wt%, 1 wt%, 5 wt%, 10 wt%, 15 wt%, 20 wt%, and 25 wt% supplementations with $MgSO_4$.

Figure 30. Cultures incubated at -5 °C for 30 days and supplemented with $MgSO_4$. From left to right: Growth negative control, 0.35 wt%, 1 wt%, 5 wt%, 10 wt%, 15 wt%, 20 wt%, and 25 wt% supplementations with $MgSO_4$.

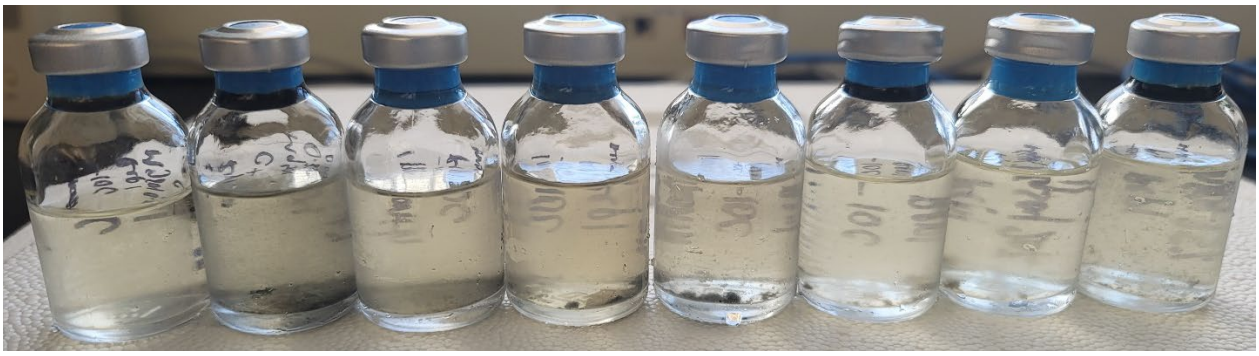

Figure 31. Cultures incubated at -5 °C for 90 days and supplemented with $MgSO_4$. From left to right: Growth negative control, 0.35 wt%, 1 wt%, 5 wt%, 10 wt%, 15 wt%, 20 wt%, and 25 wt% supplementations with $MgSO_4$.

Figure 32. Cultures incubated at -10 °C for 30 days and supplemented with $MgSO_4$. From left to right: Growth negative control, 0.35 wt%, 1 wt%, 5 wt%, 10 wt%, 15 wt%, 20 wt%, and 25 wt% supplementations with $MgSO_4$.

Figure 33. Cultures incubated at -10 °C for 90 days and supplemented with $MgSO_4$. From left to right: Growth negative control, 0.35 wt%, 1 wt%, 5 wt%, 10 wt%, 15 wt%, 20 wt%, and 25 wt% supplementations with $MgSO_4$.

Figure 34. Cultures incubated at 10 °C for 30 days and supplemented with $CaSO_4$. From left to right: Growth negative control, 0.35 wt%, 1 wt%, 5 wt%, 10 wt%, 15 wt%, 20 wt%, and 25 wt% supplementations with $CaSO_4$.

Figure 35. Cultures incubated at 10 °C for 90 days and supplemented with $CaSO_4$. From left to right: Growth negative control, 0.35 wt%, 1 wt%, 5 wt%, 10 wt%, 15 wt%, 20 wt%, and 25 wt% supplementations with $CaSO_4$.

Figure 36. Cultures incubated at -5 °C for 30 days and supplemented with $CaSO_4$. From left to right: Growth negative control, 0.35 wt%, 1 wt%, 5 wt%, 10 wt%, 15 wt%, 20 wt%, and 25 wt% supplementations with $CaSO_4$.

Figure 37. Cultures incubated at -5 °C for 90 days and supplemented with $CaSO_4$. From left to right: Growth negative control, 0.35 wt%, 1 wt%, 5 wt%, 10 wt%, 15 wt%, 20 wt%, and 25 wt% supplementations with $CaSO_4$.

Figure 38. Cultures incubated at -10 °C for 30 days and supplemented with $CaSO_4$. From left to right: Growth negative control, 0.35 wt%, 1 wt%, 5 wt%, 10 wt%, 15 wt%, 20 wt%, and 25 wt% supplementations with $CaSO_4$.

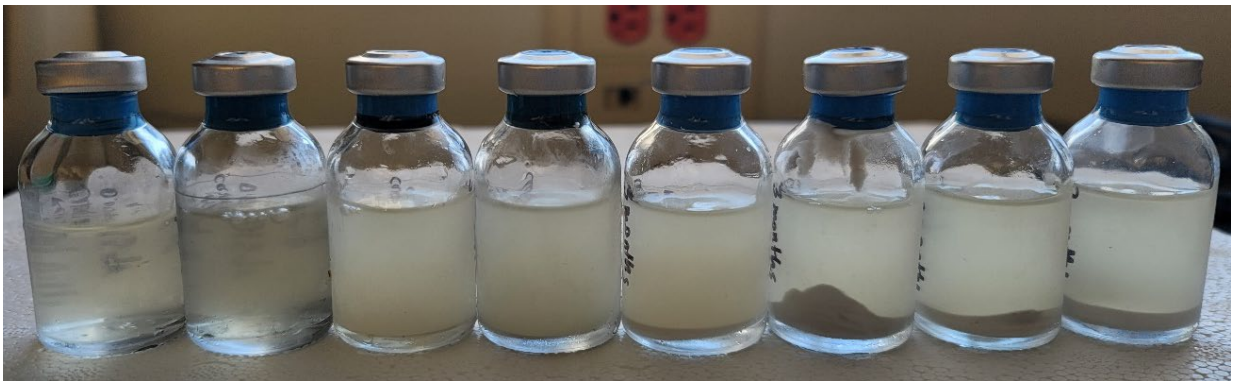

Figure 39. Cultures incubated at -10 °C for 90 days and supplemented with $CaSO_4$. From left to right: Growth negative control, 0.35 wt%, 1 wt%, 5 wt%, 10 wt%, 15 wt%, 20 wt%, and 25 wt% supplementations with $CaSO_4$.

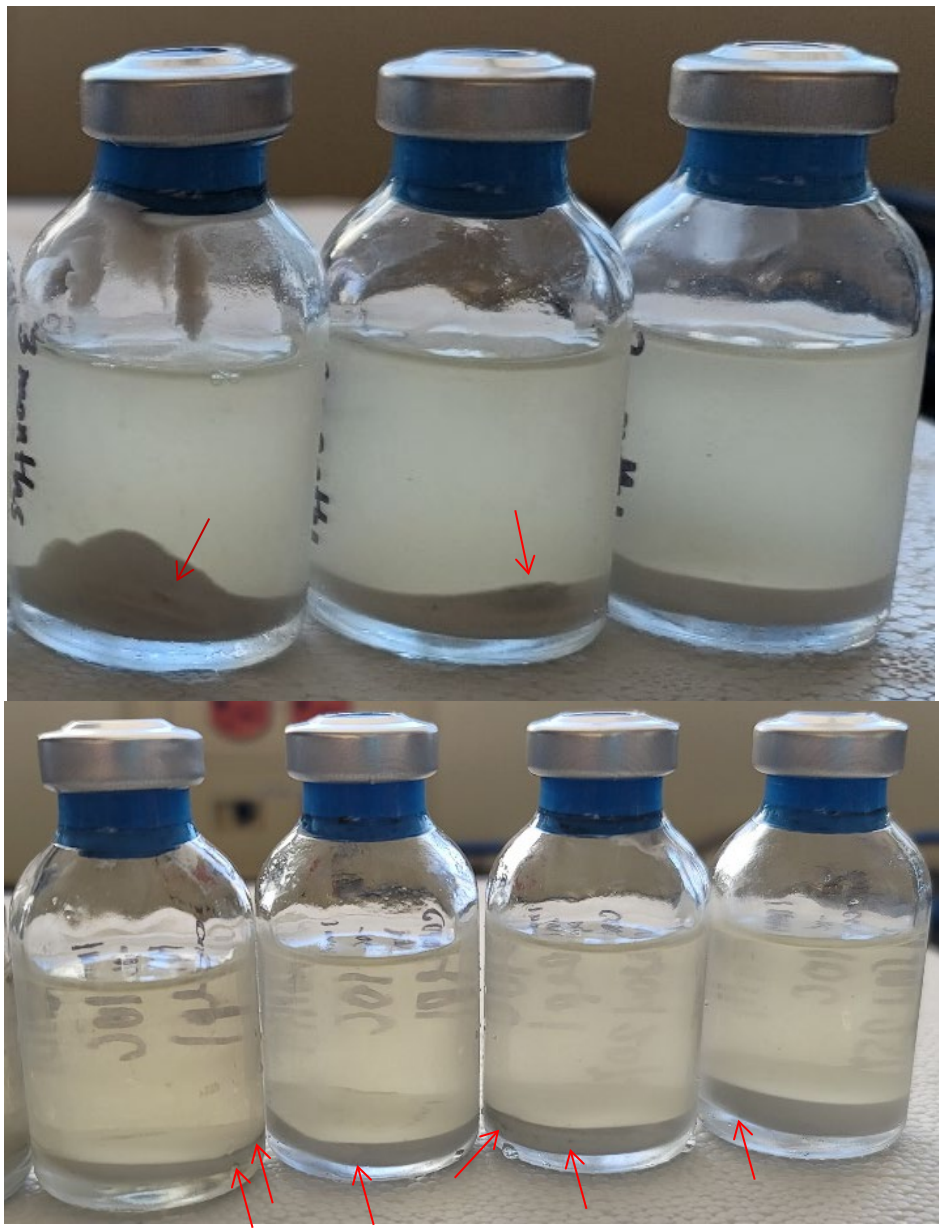

Figure 40. Cultures of *D. psychrophila* supplemented with CaSO4 at -10 °C for 90 days showing cryptoendolithic microniches. Arrows represent focal points of growth of *D. psychrophila* cells as cryptoendolithic communities.

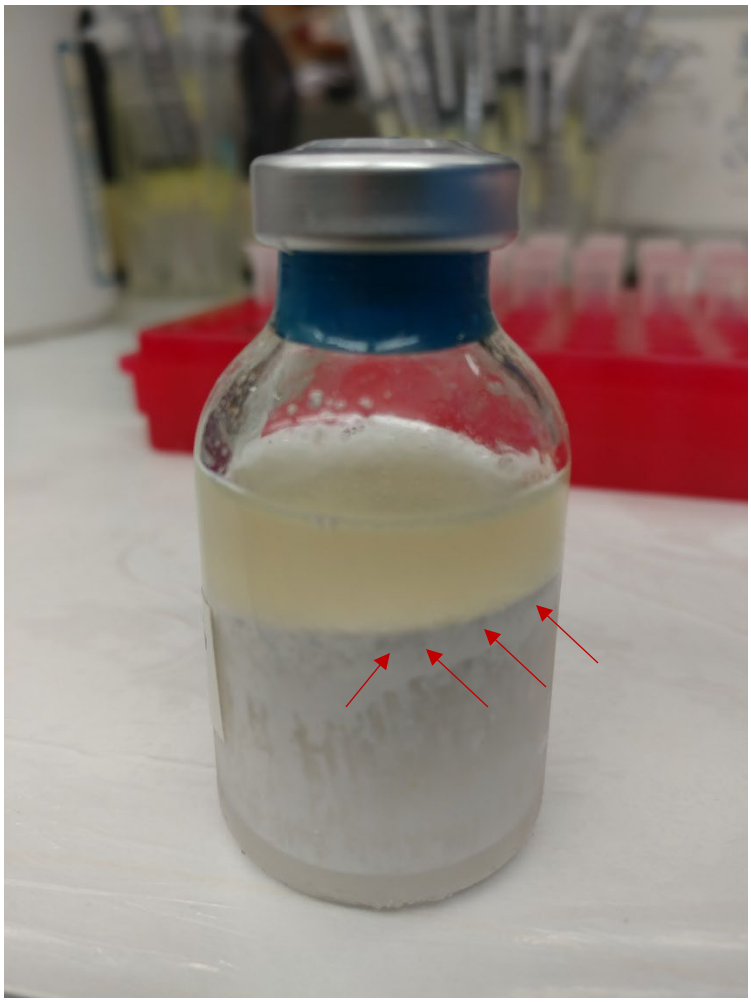

Figure 41. Partially frozen sample of cultures at -10 °C supplemented with $CaSO_4$ at 25 wt%. This image shows the matrices of sulfate compounds generated due to freezing conditions where $CaSO_4$ expanded in the serum bottles containing *D. psychrophila cells*. Arrows show evidence of sulfate reduction from cryptoendolithic microcommunities generated within the $CaSO_4$ matrix.