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Detection of Survival and Proliferation of Sulfate Reducers Under Simulated Martian Atmospheric and Soil Conditions

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Detection of Survival and Proliferation of Sulfate Reducers Under Simulated Martian Atmospheric and Soil Conditions

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Abstract

Numerous studies have tried to determine the survivability and proliferation of microorganisms under simulated Martian conditions. Furthermore, most of them have been focused on the ability of these microbes to cope with high brines' salt (NaCl) concentrations inherent of the Martian surface. However, there are not studies related to the ability of bacteria to survive on subsurface environments that have increasing concentrations of sulfate compounds. For this research, a group of microorganisms known as sulfate-reducing bacteria or simply sulfate reducers were chosen due to their ability to use sulfate compounds as terminal electron acceptors to produce metabolic energy, their tolerance to low temperatures (psychrophilic microbes) and their anaerobic metabolism. Moreover, the principal purpose of this study was to determine the ability of sulfate reducers to carry active metabolism under conditions similar to those present on Mars subsurface (low temperature, high concentration of sulfate compounds, anoxic atmosphere-95% carbon dioxide, low nutrients availability, among others). Furthermore, we cultivated strains of *Desulfotalea psychrophila, Desulfuromusa ferrireducens and Desulfotomaculum arcticum* using different concentrations of minerals. The latter ($CaSO_4$, $MgSO_4$, $FeSO_4$ and $Fe_2(SO_4)_3$) are normally found as part of the Martian subsurface components and they can act as terminal electron acceptors in sulfate respiration. Moreover, PCR amplifications of the 16S rDNA gene and the dsrAB genes were performed in order to determine the growth and survivability of the three microorganisms tested. Finally, we were able to determine that they were metabolically active at the different types and mineral concentrations under study.

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I. Introduction

Nowadays, the ability of microbes to grow, replicate and proliferate under Martian surface environmental conditions is unknown. The latter involve low temperatures, low pressure, high oxidant soils, ultraviolet (UV), cosmic, and mineral irradiations, high desiccation rate, low water activity, anoxic atmosphere and high minerals content. Moreover, current studies have focused on the detection of survival of dormant spores, vegetative cells and terrestrial contamination under surficial Martian conditions. However, there are not studies that have evaluated the same parameter on subsurface environments or determined the ability of sulfate/sulfite reducers to undergo metabolic activity under Martian physicochemical/environmental conditions. As it was suggested by Berry et al., (2010) microorganisms could potentially survive on the subsurface and the Martian surface UV soil protected areas due to the possible presence of habitable niches. Therefore, the presence of life in Mars environments should be analyzed not only in terms of detection, but also in terms of microbial survival, replication and proliferation (Berry, Jenkins, & Schuerger, 2010).

In the other hand, it has been demonstrated that formation of transient liquid water on the Martian subsurface is also possible. Furthermore, its presence in subglacial-type environments is increased by the presence of high mineral concentrations. The most abundant minerals on the Martian surface/subsurface are composed of a sulfate component. The latter can be used by a group of microorganisms known as sulfate reducing-bacteria to generate energy for replication and proliferation. Basically, these microbes can use hydrogen (H_2) as an electron donor, carbon dioxide (CO_2) as a carbon source and sulfate compounds as terminal electron acceptors due to their metabolic versatility in the production of energy (Berry et al., 2010; Crisler, Newville, Chen, Clark, & Schneegurt, 2012; Des Marais et al., 2008; KARKHOFF-SCHWEIZER, BRUSCHI, & VOORDOUW, 1993a; Ollivier, Caumette, Garcia, & Mah, 1994; Skidmore, Foght, & Sharp, 2000; Squyres et al., 2006).

Another feature of sulfate reducers is the presence of a highly conserved region of DNA known as the dsrAB operon (dsr genes) which encodes the genes that are required for the formation of the dissimilatory sulfite reductase (DsrAB). This enzyme intervenes in the reduction of sulfate compounds specifically in the last reaction of sulfite reduction to sulfide (Karkhoff-Schweizer, Huber, & Voordouw, 1995a, 1995b;

Klein et al., 2001a; Laue, Friedrich, Ruff, & Cook, 2001; Muller, Kjeldsen, Rattei, Pester, & Loy, 2015; Zverlov et al., 2005).

In this research, we used a combination of culture and Molecular Biology techniques to detect the survival, proliferation and metabolic activity of three different bacterial strains that are considered "psychrophilic/mesophilic anaerobic and chemoorganotrophic/autotrophic microbes" under an anoxic atmosphere (H_2/CO_2) and increasing concentrations of different sulfate compounds. Furthermore, we detected the ability of these microorganisms to use hydrogen as an electron donor, carbon dioxide as a carbon source and sulfate compounds as terminal electron acceptors by detecting these microbes' survival using molecular techniques such as PCR amplification of the 16 S rDNA gene and the dsrAB operon.

A. Hypothesis and Research Objectives

Hypothesis:

We hypothesize that three strains of sulfate-reducing bacteria: *Desulfotalea psychrophila*, *Desulfuromusa ferrireducens* and *Desulfotomaculum arcticum,* are able to undergo survival and proliferation under Martian simulated atmospheres and soil composition (using H_2, CO_2 and different sulfate compounds as electron donor, carbon source and terminal electron acceptors respectively). We hypothesize that their survivability and especially their proliferation under the Martian conditions tested are directly proportional to the expression of the dsrAB operon which encodes the dissimilatory sulfite reductase responsible for anaerobic sulfate reduction.

Objective 1:

Contrast growth patterns of *D. psychrophila, D. ferrireducens and D. arcticum* at different culture conditions: in a complex medium in which the electron donor and carbon source are represented by complex molecules such as lactate and yeast extract versus a minimal medium (simulating Martian atmospheric conditions) in which the electron donor and the carbon source are H_2 and CO_2.

Objective 2:

Evaluate the ability of *D. psychrophila, D. ferrireducens and D. arcticum* to use different types of sulfate compounds at increasing concentrations as terminal electron acceptors (simulating Martian soil conditions) in the generation of metabolic energy through anaerobic sulfate reduction in minimal medium (simulating Martian atmospheric conditions).

Objective 3:

Detect the survival of these microorganisms under Martian atmospheric and soil conditions by amplifying the 16S rDNA gene and the dsrAB genes as indicators of active microbial growth.

B. Literature Review

B.1 Mars Explorations and Discoveries

Astrobiology explorations address three principal topics: the origin of life and its evolution, the presence of life in other planets and the future of terrestrial life on Earth and space. Furthermore, Des Marais et al., (2008) defined the term habitability as the ability of a planet to sustain life that is originated there or to sustain life that is carried there by other means. Moreover, the authors also emphasized the environmental conditions that the planet needs to have in order to sustain life. Among them, the presence of liquid water is the most important because it triggers the assembly of energetic and organic molecules of increased complexity. The latter are necessary for the maintenance of metabolism and ultimately life (Des Marais et al., 2008).

Life on Earth has evolved from biogeochemical processes that resulted from interactions of the crust with the atmosphere and oceans (biogeochemical component). Furthermore, these constant processes were developed within organized microbial ecosystems (biological component) subjected to constant changes in environmental conditions. The understanding of the interactions between the biogeochemical component and the biological component that originated today's Earth complex ecosystems will help us to determine the future and past states of terrestrial environments. Furthermore, it is fascinating how terrestrial life has developed different mechanisms that allow individual microorganisms to survive under difficult/extreme environments. These mechanisms have permitted their survival and adaptation to environments with extreme low temperatures, high desiccation rates, exposition to irradiation, low nutrients availability among other physical and chemical conditions. However, the evolution of life on Earth represents just one of the multiple pathways that could originate life. Thus, the origin of it in other planets remains unresolved. Nevertheless, the understanding of the mechanisms implied by these microbes to survive and adapt to extreme conditions will allow us to understand the basic mechanisms that trigger the formation of life and how it evolved not only on Earth, but also in other planets. Moreover, the presence of life in other planets will ultimately depend on the ability of microorganisms to use extraterrestrial resources and their adaptation/evolution to those environments (Des Marais et al., 2008).

One of the principal goals of Mars explorations is the discovery of organic compounds that can relate to present or past life. Although the principal compound used to determine the presence of life in other planets is water, some other markers have been used for the same purpose. Moreover, these measurable indicators are known as biosignatures or signatures of life which indicate the presence of biological processes. Some examples of the latter are the presence of complex physical and chemical structures, inorganic minerals (produced by life forms), carbonaceous debris, organic matter, chiral molecules, isotropic fractionation of carbon, utilization of energy, production of biomass and waste compounds. However, these biosignatures should be rigorously interpreted because some of them are the result of planets' non-biological mimics (created by non-biological processes/abiotic or inanimate processes) (Des Marais et al., 2008; Parnell et al., 2007).

For almost four decades, the National Aeronautics and Space Administration (NASA) has been sending spacecrafts and robotic devices to the Red Planet in order to recover information about the physicochemical conditions, presence of life and biosignatures of this planet (Crisp et al., 2003). The first NASA's operations on Mars were based on basic flyby manoeuvres which have the principal goal to collect simple pictures of the planet. Later, the development of new technology made possible the study of the Martian atmosphere using orbiters and finally, with the great advances in robotic of this century, most International agencies started the use of landers and rovers to study the surface's physical properties and chemical composition (Circi, Ortore, Bunkheila, & Ulivieri, 2012).

The first orbiters launched to Mars were the Mariner 3 and Mariner 4 in 1964. Mariner 3 failed on its mission to reach the Martian atmosphere while the second one successfully orbited it in1965. It was the first time in which the NASA got images of the Martian surface. Subsequently, in 1971 this same agency sent the Mariners 8 and 9. The first one failed on its mission while the second one reached the Martian orbit. As a result of this hard work, the orbiter captured close to 7000 images that were useful in determining the possible presence of water and flood events that modelled the surface of this planet (Naderi, McCleese, & Jordan, 2006).

Later, the Viking landers and orbiters were launched (1976). Furthermore, the orbiters were designed to patrol the Martian atmosphere looking for the best descend locations for the landers. Once the best

positions for landing were determined, the Viking Landers 1 and 2 were positioned. Basically, they crossed the Martian atmosphere and soft landed into the surface of the Red Planet. Therefore, the Viking landers were the first robotic devices that successfully landed on the Martian surface (1992) (M. P. Golombek et al., 1997; Matthew P. Golombek, 1997; Naderi et al., 2006)

Twenty years later, another rover was delivered into the Martian surface by the NASA's Mars Pathfinder mission (1997). The rover named Sojourner recollected information about the rocks composition, specifically the rock known as "Barnacle Bill". The principal objective of this rover was to accurately confirm the information collected previously by orbiters. Furthermore, this rover detected areas with high amounts of silica (indicator of past thermal activity). Moreover, the Sojourner analyses about soils' dynamic identified the presence of "conglomerates" (formed by the pushing of components from different types of soils) which suggested the presence of water (past catastrophic floods) (M. P. Golombek et al., 1997; Matthew P. Golombek, 1997).

NASA's orbiters – Mars Odyssey (2001), Mars Reconnaissance (2005) and MAVEN (2013)- have contributed with a high quantity of information about the atmospheric conditions, presence of water and biosignatures in this planet. Furthermore, the Mars Global Surveyor (launched in 1997) and the Mars Odyssey determined the geological composition of the rovers (Opportunity and Spirit) landing sites and atmospheric characteristics of the Red Planet. These rovers, Opportunity (launched on 2003, operating for 14th years) and Spirit (launched in 2003 and replaced by Curiosity in 2012) have contributed with information about the physical and chemical conditions in the surface of the Red planet such as soils' conditions and composition (minerals), but their principal goal was to identify present or past forms of life and evidence of water (Crisp et al., 2003; Dooling, 2017; Maki et al., 2003; Witze, 2016). As it was stated before, the clear identification of this compound along with the presence of life in the Red Planet is still inconclusive (Berry et al., 2010). For this reason, Exobiologists and Astrobiologists had relied on the use of Martian simulated experiments. The latter are developed on Earth and they recreate the Martian harsh environmental conditions to determine the possibility of survival and proliferation of bacterial cells and other organisms. The interest to identify actual or past microbial life on Mars increased soon after the rovers Spirit and Opportunity recollected evidence of a liquid past environment (ancient body of salty

water). (Arvidson et al., 2014; Crisp et al., 2003; Gasda et al., 2015; Maki et al., 2003; Mars Exploration Rover, 2017; Naderi et al., 2006; Witze, 2016). Moreover, other space agencies such as the European Space Agency, United Arab Emirates, India and China have been interested in the exploration of the Red Planet especially after the discovery of the Mar's Reconnaissance Orbiter which described the presence of transient liquid water (and not only evidence of past water presence) under the Martian surface (subsurface-regolith interface) and methane (CH_4) emissions (Witze, 2016). Therefore, as it is mentioned in Valdivia-Silva, Karouia, Navarro-Gonzalez, & Mckay, (2016), Mars is one of the planets that contains possible habitable niches for the proliferation of extraterrestrial microbes (Valdivia-Silva et al., 2016).

B.2 Mars Physicochemical/Environmental conditions

The principal physicochemical characteristics of Mars' environments are high mineral (salinity) content, presence of heavy metals perchlorates, oxidants (volatiles and in the soil), extreme desiccation, low water activity, extreme low temperatures and high rate of irradiation (UV, solar particle events, galactic cosmic rays, mineral irradiation). These conditions limit the survival, proliferation and development of any form of life at the Martian surface. However, the subsurface of the planet can potentially offer a habitable environment for microbial proliferation. Furthermore, the possible presence of microbial life on the Martian subsurface is even more palpable if we take into consideration the existence of terrestrial microorganisms that can survive and proliferate under extreme environments (such as those associated with the Earth's subsurface). It has been suggested that similar organisms (with the same capabilities) can proliferate in planets that have extreme physicochemical conditions as the Red Planet (A. Schuerger & Nicholson, 2006; B.C. Clark et al., 2005; Benton C Clark, 1993; Berry et al., 2010; Clark, 1998; Crisler et al., 2012; Des Marais et al., 2008; McEwen et al., 2011; Valdivia-Silva et al., 2016; Wänke, Brückner, Dreibus, Rieder, & Ryabchikov, 2001).

Furthermore, it has been shown that environmental factors such as low temperature, low pressure and high CO_2 content are bacteriostatic and not bactericidal for some species of bacteria (endospore formers). However, interactive effects of these environmental factors can inhibit the growth and germination of different bacterial strains. Moreover, the effects of these factors are always dependent on the bacterial specie (A. Schuerger & Nicholson, 2006).

B.2.1 UV Irradiation

The UV irradiation at the Martian surface is approximately 3.6 W/m^2 (wavelength of 200 to 280 nm) for a day period of 8 hours (Berry et al., 2010). Although, the solar UV is lower than that experienced on our planet (due to a greater distance between the Red planet and the Sun), it is the most important type of radiation that affects life on Martian environments. This phenomenon can be explained based on the absence of a dipole field which could deviate the direct exposition to UV (as in the case of Earth). Despite, it only penetrates 500 μm in the Martian soil, it can destroy organic molecules, cells and spores within minutes to hours. Nevertheless, UV biocidal effects diminish proportionally to increasing soil depths. In addition, cosmic radiation and solar energy particles can affect all other UV-resistant organic molecules producing free radicals which increase the oxidizing potential of the Martian soils (aromatic molecules for example). Thus, microbial life and any kind of organic material cannot persist at the Martian surface. However, they can potentially survive and proliferate at deeper strata where cosmic radiation, UV irradiation and solar energy particles cannot affect them. In the other hand, we need to take into consideration mineral radiation which normally does not affect vegetative cells or spores as a result of direct exposition, but it can affect them by cumulative effects (spores' DNA damage previous to germination or DNA damage in vegetative cells) (Berry et al., 2010; Parnell et al., 2007; Velasco, Usero, Jiménez, Aguirre, & Vázquez, 2015).

Furthermore, the UV light irradiation at the soil's surface generates volatile oxidants that can eventually diffuse into deeper strata. However, studies on the production of these compounds (such as $H_2O_2^-$, OH^-, O_2^-) and their influence on the survival of microorganisms under Martian conditions have suggested that they do not represent a source of biocidal activity (Berry et al., 2010).

B.2.2 Low Water Availability (Low Water Activity)

The low temperatures present on the Red Planet prevent the formation of high amounts of liquid water (extreme low water activity). This phenomenon increases the desiccation state of the Martian surface and subsurface. As a consequence of these environmental conditions, most forms of life cannot survive and proliferate (Berry et al., 2010).

However, orbiters and rovers have collected evidence of presence of water or transient liquid water. Furthermore, two specific sites of the Red Planet that have been extremely studied are Gusev Crater and Meridiani Planum. These locations were selected due to their distinctive geological features. In 2004, Spirit landed on Gusev crater and it identified the presence of rocks that were basically composed of olivine-bearing basalts (high chromium contents). Moreover, a softness comparison between these rocks and basaltic rocks from Earth revealed the occurrence of thin layers of water and antient water floods (Arvidson et al., 2006; Mars Exploration Rovers, 2013). These findings were confirmed by the Spirit's identification of hematite and sulfates (indicators of the presence of past water activity, specifically in Columbia Hills). The second site, Meridiani Planum, was explored by the rover Opportunity in 2004. It found an environment similar to those exposed to shallow water on Earth. Opportunity identified the presence of hematite and abundant deposits of jarosite and sulfates, which indicated the presence of past water on Mars surface and/or below it. The principal goals of both rovers were to study the mineral composition of Mars and look for evidence of actual or past presence of water. These goals were accomplished by both of them (Berry et al., 2010; Crisler et al., 2012; Des Marais et al., 2008; Kuchynka et al., 2014; Mars Exploration Rovers, 2013; Squyres et al., 2006).

Nowadays, it is known that the surface and shallow subsurfaces of Mars were once partially covered by liquid water which was probably sustained by aquifers and atmospheric precipitation. Furthermore, model simulations of the Martian surface have suggested the presence of a groundwater reservoir that probably have cooperated to maintain subsurface life forms (evidence also supported by CH_4 gas emissions that were detected at the Martian atmosphere) (Des Marais et al., 2008).

Also, it has been suggested the possible presence of transient liquid water at the surface of the Martian polar caps (surface melting). These transient watercourses could be potentially accumulated at Martian subglacial microenvironments. Furthermore, its terrestrial counterparts have been identified as parallel models for Martian cold environments in which bacterial growth and survival could be accomplished due to the protective effects that this type of environments can offer (protection from low temperatures and UV irradiation). Moreover, the thermal protection effect could eventually produce transitory basal-melting of

the polar caps generating more liquid water that further increases the chances of development of life (Skidmore et al., 2000).

Furthermore, it is known by modelling and simulations that the layer of ice that surrounds the surface of the planet does not reach the water's melting temperature under any condition. This event occurs due to high dryness of the surface which allows the ice to sublimate without melting. Nevertheless, the presence of high concentration of minerals on the soil's regolith can potentially drive the process of melting (Mellon and Phillips, 2001).

The melting point of ice water is 0º C. Furthermore, the combined actions of the Sun´s heat, soil´s heating distribution and the presence of high concentrations of minerals in the Martian soils could increase the sublimation rate of ice, increase the minerals' concentrations and lower the water´s melting temperature leading to the stable presence of transient liquid water. Although, this scenario is chemically and physically possible, the melting point is normally decreased just a few degrees. However, the structure of the Martian soil pore spaces in the regolith triggers the contact between particles of ice or between particles of ice and particles of soil. This phenomenon induces the formation of thin films of liquid water at temperatures under the water´s melting point. This event has been demonstrated multiple times in studies based on the behavior of water molecules at subfreezing temperatures (Crisler et al., 2012; Jakosky, Nealson, Bakermans, Ley, & Mellon, 2003).

Although the presence of transient liquid water is a remarkable discovery, the production of high amounts of it can be harmful rather than beneficial for the proliferation of microorganisms in soil environments due to their high oxidant states. Furthermore, Valdivia-Silva et al., (2016) found that soils with an increased oxidant activity were correlated with low or absent bacterial populations. Moreover, they suggested that low water content in environments with low oxidant activity can be beneficial, but extremely harmful in environments with high oxidant activity (Valdivia-Silva et al., 2016).

B.2.3 Mineral Content

The presence of brines with high concentration of minerals seems to be the result of water evaporation of antient dry lakes (Komatsu, 2012). This phenomenon resulted in the formation of hydrated minerals

deposits that could/can be potentially colonized by microbial communities. Furthermore, sulfate rich veins were located at the Matijevic formation and the Copper Cliff breccias. These sulfate rich veins are formed principally of calcium sulfate ($CaSO_4$) and they are present in the form of gypsum which is the hydrated form of calcium sulfate (evidence of past water activity-Curiosity findings at the veins of the west region of Cape York) (Arvidson et al., 2014). In concordance with this discovery, the principal goal of the NASA´s most recent landed rover Curiosity is to identify evidence of salty habitable past environments in the Red Planet (such as gullies, layered salt deposits or hydrated salts, among others) (Gasda et al., 2015; Naderi et al., 2006).

Furthermore, as it was reported by the Mars Exploration rover Opportunity in 2004, the Martian surface contains high concentrations of siliciclastics and sulfates. This last group has compounds such as magnesium sulfate (MgSO4), calcium sulfate (CaSO4) and jarosite which can be used as terminal electron acceptors in the production of energy by sulfate reducers (Berry et al., 2010; Crisler et al., 2012; Des Marais et al., 2008; Squyres et al., 2006).

As it was stated before, the presence of a high mineral content (without taking into consideration which type of salt) can depress the freezing point of water and make it available on liquid form at low temperatures. This phenomenon increases the water activity and it influences the bacterial resistance to desiccation (Berry et al., 2010). Furthermore, the presence of sulfates in Martian-like brines could potentially be more favorable for the development of life in comparison with brines that have higher chloride's content (NaCl for example on Earth-like brines). This event can be explained taking into consideration that sulfate-like brines show an increased water activity in comparison with Earth-like brines (Crisler et al., 2012; Marion, Fritsen, Eicken, & Payne, 2003).

In any case, the effects of a high mineral content environment over bacterial cells survival, metabolism and proliferation are different for different bacterial species (It is a specie specific stressor), which means that two different strains of bacteria have a different reaction to a high mineral content environment (Berry et al., 2010).

B.2.4 Low Nutrients

As it is mentioned in Parnell et al., (2007), the Red Planet receives organic material from space in the form of dust particles, cometary matter and meteorites. These reservoirs of organic material contain a variety of organic compounds that include aminoacids, carboxylic acids and aromatic structures (at the Martian surface they are degraded by UV irradiation). However, the most interesting carbon molecules present in Mars atmosphere are CO_2 and CH_4, which can be associated to the presence of active metabolism of methanogenic microbes/sulfate reducers or to a series of abiotic processes. Furthermore, the CH_4 half live is short and its presence in the Martian atmosphere is constant. The latter indicates its probable biologic replenishment after atmospheric short residence (Des Marais et al., 2008; Parnell et al., 2007).

In addition, it is important to mention that the inability to find organic compounds at the Martian surface or subsurface does not necessarily mean they are absent. As it is mentioned in Parnell et al., (2007), organic molecules might be converted directly to CO_2 due to extremely oxidant conditions of the soil, but some intermediates might be originated and accumulated over time at the Mars regolith (Parnell et al., 2007).

Furthermore, as it is mentioned in Parnell et al., (2007), the search of organic molecules at the Martian subsurface can eventually lead to the finding of biota that could show metabolic activity due to the stability of liquid water at those depths and the inability of UV irradiation and oxidizing soil's conditions to decompose organic matter (Parnell et al., 2007).

B.2.5 Extreme low temperatures

The typical temperature in the Red Planet ranges from 20 (during austral summer) to -50º C (day/night diurnal period). Although, the Viking landers never experienced temperatures lower than -10º C (daytime), the average global temperature of Mars is of -61º C (Berry et al., 2010). However, the composition of the Martian dust affects directly the composition, behavior and evolution of the planet's atmosphere which can result in an increase in temperatures in both the atmosphere and the surface of the planet. This phenomenon can increase the probability of microbial survival and proliferation (Lemmon et al., 2015).

Although, the ability of microorganisms to proliferate and be metabolically active (active uptake and processing of nutrients) at subfreezing temperatures (-10º C) has been demonstrated, microorganisms adapted to cold environments (temperatures from -17º C to -20º C) have a slow metabolism, which can be proved by their slow growth rate on cultures (Carpenter, Lin, & Capone, 2000; Marion et al., 2003; Priscu et al, 1998; Rivkina, Friedmann, McKay, & Gilichinsky, 2000).

B.2.6 Low Pressure

Under normal conditions, the Mars global pressure is approximately 5.17 torr while the surface's pressure is approximately 7 torr (Berry et al., 2010; Gasda et al., 2015). Although, normally this variable does not affect microbial growth, as it is mentioned in Berry et al., (2010), it has shown to slightly reduce bacterial populations of spore formers and non-spore formers (Berry et al., 2010). The same findings were obtained in a study of replication/germination of *Bacillus* species and their endospores under simulated Martian conditions. Furthermore, they found out that vegetative cells were more resistant than spore forms to reduced pressures (active replication) (A. Schuerger & Nicholson, 2006). Thus, Martian low pressure is another environmental variable that can affect the development of life (Gasda et al., 2015).

B.2.7 Martian Atmosphere

The Martian atmosphere´s gas composition is composed mostly of CO_2 (95%). The rest 5% of its components is represented by nitrogen (N_2), argon (Ar) , oxygen (O_2) and water vapor (H_2O) (Gasda et al., 2015).

B.3 Sulfate/Sulfite Reducers

There are some natural environments on Earth that resemble certain characteristics of the Red Planet. Locations with high minerals content such as Hot and Basque Lakes (Pacific Northwest), Guerrero Negro salterns, The Great Salt Plains (Oklahoma), the Orca Basin (Mexico), the dry Valleys of Antarctica or Don Juan Pond, the Artic Polar Desserts, Mono Lake, Lake Magadi, the Atacama Desert, the Dead Sea and Yellowstone National Park represent the natural habitat of extreme microorganisms that could potentially survive and proliferate under Martian conditions (Javor, 1984; Kilmer et al., 2014; Marion et al., 2003; Navarro-Gonzalez et al., 2003; Valdivia-Silva et al., 2016). However, it is necessary to take into

consideration that the brines present on Earth are different from those present in Mars. In the latter, the predominant mineral specie is magnesium sulfate ($MgSO_4$) while on Earth it is sodium chloride (NaCl) (Berry et al., 2010; Crisler et al., 2012; A. Roychoudhury, 2004). Furthermore, other terrestrial habitats have combinations of environmental conditions that are similar to those present on Mars environments. Terrestrial habitats with low temperatures and brines with high minerals content (for example in Artic glaciers, Lake Vostok in Antarctica, Precambrian shields of Canada and Finland, the Witwatersrand Basin in South Africa) have been used to demonstrate and evaluate the ability of some microorganisms to survive and replicate under extreme conditions. These studies have found the presence of different cell morphologies and even dividing cells (metabolically active) that are not necessarily extremophile microbes (Sherwood Lollar et al., 2007; Skidmore et al., 2000).

In concordance with the previous statement, numerous studies have concluded that a few common microbes (such as human bacteria, sporulating and non-sporulating microorganisms, salt-tolerants, anoxigenic bacteria, etc.) are viable under simulated Martian conditions. Moreover, they were able to resist factors such as low temperature, low pressure, high UV irradiation, low water availability and anoxic gas composition (Crisler et al., 2012; A. C. Schuerger, Mancinelli, Kern, Rothschild, & McKay, 2003; A. Schuerger & Nicholson, 2006; Skidmore et al., 2000). This finding suggests that some microbes present on Earth environments could potentially undergo active metabolism, survive and proliferate under real Martian environments. Therefore, if terrestrial common and extreme microorganisms can resist those conditions, it might be possible that microbes of Martian origin (present in Mars) had been able to survive and proliferate, but we have not been able to detect them. (B.C. Clark et al., 2005; Benton C Clark, 1993; Clark, 1998; Crisler et al., 2012; McEwen et al., 2011; A. Schuerger & Nicholson, 2006; Valdivia-Silva et al., 2016; Wänke et al., 2001).

Also, it is important to mention that any microorganism of terrestrial origin that could survive in Mars should be able to adapt to low concentrations of carbon sources. In concordance with this last comment, subsurface Earth environments have provided the required conditions that trigger the development of autotrophic forms of life. Furthermore, some of them are represented by autotrophic/chemoorganotrophic terrestrial bacteria that undergo active metabolism using H_2 and CO_2 as their only sources of carbon an

energy (both gases present on Mars atmosphere) (Aullo, Ranchou-Peyruse, Ollivier, & Magot, 2013; A. Roychoudhury, 2004).

As we already know, water in liquid form could be present in the deep subsurface of the Red Planet. Basically, warmer temperatures from the Planet's core and the Sun's radiation can potentially melt the ice that surrounds the rock allowing the production of geochemical energy (reactions between water and the rock). Added to this, Mars's surface and subsurface's minerals content is composed of high concentrations of sulfate compounds (magnesium, calcium and iron). Thus, the presence of these salts and the possibility of generation of melted water could potentially create high sulfate content brines (Berry et al., 2010; Crisler et al., 2012). Therefore, the potential formed liquid water along with the soil's high minerals content and the available energy can be used by terrestrial dormant microorganisms that might be colonizing these areas. However, Mar's subsurface explorations have not been successful due to limited technology that can lead us to determine if this hypothesis (determined by modelling) is accurate (Crisler et al., 2012; Jakosky et al., 2003; Mellon and Phillips, 2001; A. Roychoudhury, 2004; Sherwood Lollar et al., 2007; Shock, 1997; Varnes, Jakosky, & McCollom, 2003).

Moreover, sulfate reducers are a clear example of bacteria that can undergo survival and metabolic activity under extreme conditions. These microorganisms use sulfate/sulfite as the principal electron acceptors in their anaerobic metabolism (Ollivier et al., 1994). Although, these bacteria are difficult to enrich in synthetic media because it renders an extremely low number of cells, sulfate reducers are known for their versatility in the use of different nutritional sources and metabolic pathways to generate energy. The latter suggest the existence of different Genera with a unique enzymatic complexity (different types of enzymes responsible for sulfate reduction – different dissimilatory sulfite reductases) (Brandt, Vester, Jensen, & Ingvorsen, 2001; KARKHOFF-SCHWEIZER et al., 1993a; A. N. Roychoudhury & McCormick, 2006).

Moreover, as it is cited on Skidmore et al., (2000), psychrophilic sulfate reducers are one of the types of microorganisms that can proliferate under Martian conditions due to their ability to persist in environments with limited carbon sources and low temperatures. Therefore, as it was indicated in this study and in our research, subsurface and/or sedimentary microenvironments can offer a suitable scenario for the

development of microbial metabolic activity due to its protective effect (subglacial environments) and the presence of specific electron donors and terminal electron acceptors (Skidmore et al., 2000).

Although, sulfate reducers can tolerate high salinities (high sodium chloride-NaCl concentrations), this condition is not required for growth. Most microbes acquire resistance to high mineral concentrations by either accumulating salts in their cytoplasm and modifying their proteins to an acidic structure (adaptive mechanism in low substrate environments) or by producing organic compounds that can osmoregulate and compensate intracellular concentrations (in environments where substrate availability is not limited). Although energetically expensive, most Archaea and anaerobic fermenting Eubacteria have adopted this last mechanism producing and/or taking (from their environment) organic compounds such as glycine and betaine (Oren, 2008; Porter, Roychoudhury, & Cowan, 2007).

Most sulfate reducers identified are slight (Optimal growth: 2 - 5 % NaCl) or moderate (Optimal growth: 5 - 20% NaCl) halophiles (as mentioned in Porter et al., 2007). Unfortunately, most studies have focused on their biologic response to increasing concentrations of NaCl and just a few of them have focused on the microbial response to increasing concentrations of the final electron acceptor: sulfate/sulfite compounds. Thus, the sulfate reducers used in this research were chosen because they represent known sulfate reducers, halophilic, anaerobic and facultative members of the Bacteria Domain. Furthermore, due to their ability to resist high concentrations of minerals and low temperature, they can potentially survive and undergo active metabolism at conditions present in other planets (Porter et al., 2007). Moreover, as it is mentioned in Sørensen, Canfield & Oren (2004) and in Roychoudhury (2004), the understanding of the mechanisms implied in the survival of these bacteria could potentially increase our understanding of the evolution of life on Earth and the limiting environmental factors that affect it in other planets (A. Roychoudhury, 2004; Sorensen, Canfield, & Oren, 2004).

B.3.1 *Desulfotalea psychrophila*

D. psychrophila is a member of the Bacteria Domain, Phylum Proteobacteria, Class Deltaproteobacteria, Order Desulfobacterales, Family Desulfobulbaceae, Genus Desulfotalea (extracted from My RDP Genome *Desulfotalea psychrophila* LSv54). It is a Gram negative non-spore former rod-shaped flagellated chemoorganotrophic microorganism (respiratory and fermentative metabolism) that was first

isolated from cold marine sediments (Coast of Svalbard). Furthermore, it is a strict anaerobe with a duplication time of 27 hours at optimal temperature (10-18º C) although it can survive below 0º C. This microorganism can break down sulfur due to the presence of dsr genes. Moreover, it has a genome of 3,523,383 bp (circular chromosome), two circular plasmids (121,586 bp and 14,663 bp) which constitute 3118 predicted genes. In addition, they can use a wide variety of carbon sources/electron donors such as acetate, propionate, glycine, propanol, butanol, alanine, serine, pyruvate, fumarate, malate, ethanol, butyrate, lactate and hydrogen. Furthermore, they can use a variety of terminal electron acceptors such as sulfate, sulfite, thiosulfate and ferric citrate. Moreover, this microbe does not need vitamins. Its optimal pH range is 7.2-7.9 and it requires NaCl (1%) and $MgCl_2$ for growth (Knoblauch, Sahm & Jorgensen, 1999; Rabus et al., 2004).

B.3.2 *Desulfuromusa ferrireducens*

D. ferrireducens is a member of the Bacteria Domain, Phylum Proteobacteria, Class Deltaproteobacteria, Order Desulfuromonadales, Family Desulfuromonadaceae, Genus Desulfuromusa (extracted from the Encyclopedia of Life). It is a Gram negative strict anaerobe rod-shaped (0.7-1 x 3-5 μm) flagellated (monopolar lophotrichous) bacterium that was isolated from marine sediments in the west coast of Svalbard. Furthermore, it is a psychrophilic microbe (it can survive at temperatures of -2º C) although its optimal temperature range is 14-17º C (it cannot grow above 23º C). Moreover, this microbe can use acetate, lactate, fumarate, formate, succinate, pyruvate, ethanol, propanol, butanol, propionate, proline and hydrogen as electron donors and Fe^{3+}, fumarate, sulfur and Mn^{4+} as electron acceptors (Greene, 2014; Vandieken, 2006b).

B.3.3 *Desulfotomaculum arcticum*

As it is mentioned in Aullo, Ranchou-Peyruse, Ollivier & Magot (2013), Desulfotomaculum species are phylogenetically clustered in the Phylum: Firmicutes, Class Clostridia, Order Clostridiales, Family Peptococcaceae, Genus Desulfotomaculum. They are Gram positive rods and sulfate reducers that have obtained the genes that encode the dissimilatory sulfite reductase through a process of lateral gene transfer (Aullo et al., 2013; Zverlov et al., 2005). Furthermore, these microbes can survive in extreme environments (especially those associated with deep subsurface) due to their ability to produce

resistance structures (endospores, which are normally round or oval and positioned central to terminal in bacterial cell) and autotrophic growth (they can use H_2 and CO_2 to produce energy and sulfide). They are mesophilic to thermophilic anaerobic bacteria that can reduce sulfate to sulfide (using it as the terminal electron acceptor in anaerobic respiration). Moreover, they can use other electron acceptors such as elemental sulfur, thiosulfate, sulfite, and/or metals such as manganese (IV), iron (III), chromium (VI) or uranium (V). In addition, they can oxidize organic acids, glucose, formate, propionate, fructose, pyruvate, malate, fumarate, succinate, lactate, aromatic hydrocarbons, butyrate, ethanol, methanol, propanol, butanol, aminoacids (proline, alanine and glycine) and hydrogen. Finally, this microorganism tends to live in anoxic environments with low sulfate concentration. However, it grows better in environments where sulfate is not a limiting factor (Aullo et al., 2013; Vandieken, 2006a).

D. arcticum was isolated from sediments of the west coast of Svalbard. It can use H_2 as a source of energy and it grows optimally at 42º C (Vandieken et al., 2006). One of the principal features that allowed the survival of this type of microbe in extreme environments is the production of endospores. This characteristic allows bacterial strains to resist changing/unfavorable temperatures and redox conditions, nutrients deprivation, etc. (Aullo et al., 2013; Krieg et al., 2011).

B.4 Sulfate Reduction Process

Microbial sulfate reduction has been targeted as one of the principal mechanisms that needs to be studied by the field of Astrobiology. This interest in sulfate reduction increased when habitable sulfate-rich sediments were discovered at Meridiani Planum (Parnell et al., 2007). Furthermore, these niches can be exploited by sulfate reducers which generate metabolic energy through the action of the DsrAB. This enzyme catalyzes the anaerobic reduction of sulfite to sulfide (last step of the dissimilatory sulfate reduction) using sulfate, sulfite or organosulfonates as terminal electron acceptors (Karkhoff-Schweizer et al., 1995a; Laue et al., 2001; Muller et al., 2015).

Besides the DsrAB, other enzymes intervene in the reduction of sulfate. Furthermore, the ATP sulfurylase catalyzes the reaction from sulfate to adenosine-5-phosphosulfate and the adenylyl-sulfate reductase catalyzes the reaction of adenosine-5-phosphosulfate to sulfite (Muller et al., 2015).

As it is mentioned in Porter et al (2007), the enzymatic process of sulfate reduction occurs in the cytoplasm of these microorganisms specifically in the inner leaflet of the cell membrane. In fact, sulfate reducers use active ABC transporters to deliver the negatively charged sulfate anion across the cell membrane (Crisler et al., 2012; Porter et al., 2007). Moreover, under high salinity concentrations, these microbes maintain a cell membrane gradient (using a Na+/H+ antiporter) that facilitates the transport of sulfate through their electroneutral $Na+/SO_4^{2-}$ symporter. Together, they globally expel Na+ and introduce SO_4^{2-}. However, in low water activity environments, they introduce and accumulate K+ in their cytoplasm or produce osmoprotectants (low-molecular weight organic compounds) (Galinski & Truper, 1994; Kreke & Cypionka, 1994; Porter et al., 2007). Thus, the reduction of sulfate by microorganisms is a process that occurs in a wide range of salinities due to the bacterial cell membranes inherent features (Porter et al., 2007). However, it is also known that sulfate reduction rates are higher in environments with an excess of sulfate compounds and low carbon sources (Porter et al., 2007). In this regard, this type of microorganisms spend a high amount of metabolic energy to maintain homeostasis (Crisler et al., 2012; Oren, 1999).

Thus, the behavior and metabolic activity of different microorganisms in brines with high content of sulfates can be measured through the detection of sulfate reduction. The latter depends on the microbial composition and their adaptations/mechanisms to survive under extreme environments (Porter et al., 2007). As it was stated before, Porter et al., (2007) found out that environments with low carbon content and high sulfate content, as those present in sediments of coastal pans, increase the rate of sulfate reduction in bacterial communities adapted to live in high salinity environments. Furthermore, these can be the principal factors that could potentially increase the chances of bacterial survival and metabolic processing under Mars conditions (low carbon sources and high sulfate concentrations). Furthermore, they mentioned that sulfate reducers that produce osmoprotectants under high NaCl content environments, such as *Desulfovibrio vulgaris,* tend to upregulate the expression and translation of F-type ATPases which normally suggests an increase in sulfate reduction. Basically, this upregulation represents the bacterial cell response to the energy required for the transport of ions to the extracellular medium and osmoprotectants from it. However, it is important to take into consideration that these microbes are present in environments with low carbon sources, which indicates low energy production. Furthermore,

their presence in high salt content environments does not favor their proliferation because they need to use extra energy to osmoregulate. All of these factors added to a low water activity (as present in Mars environments) make it even more difficult for these cells to survive or duplicate (Mukhopadhyay et al., 2006; Porter et al., 2007). However, microbial sulfate reduction under Martian environments is possible. Furthermore, it has been suggested that it might be the result of bacterial consortia with a complex metabolic network. As it is known nowadays, the presence of CH_4 as a biosignature of metabolic activity, has been detected at the Martian atmosphere. In this regard, it has been hypothesized that sulfate reducers and methanogens coexist and cooperate to produce metabolic energy. This hypothesis has been elaborated by comparisons with the occurrence of the same phenomenon on Earth subsurface microenvironments. Moreover, it was demonstrated by Scholten et al., (2005) on their studies of sulfate reducers and methanogens in a terrestrial environment (meromictic soda lake). They attributed this phenomenon to a process known as sulfate-dependent CH_4 oxidation or SDMO in which anaerobic oxidation of methane is coupled to sulfate reduction (methanogens and sulfate reducers coexisting) (Muller et al., 2015; Scholten, Joye, Hollibaugh, & Murrell, 2005).

B.5 dsrAB operon and DsrAB Protein

Archaea and Bacteria (anaerobes) are the only microorganisms that reduce sulfate to sulfide (using sulfate or sulfite as terminal electron acceptors) to produce metabolic energy by means of the DsrAB. As it is mentioned in Klein et al., (2001), the genes encoding this enzyme are highly conserved among bacterial sulfate reducers. Furthermore, they are clustered in an operon that weights approximately 1.9 kb (Figure 8). The latter is composed of multiple subunits, but the most important are dsrA and dsrB. In addition, there is another sequence portion of the operon known as the dsrD which encodes a small polypeptide of unknown function (rich in lysine residues). Moreover, the two genetical subcomponents dsrA and dsrB emerged from a common ancestor followed by a vertical evolution among highly divergent prokaryotes. This characteristic is important in the molecular identification of these microorganisms because it can be used to group together different species of sulfate reducers that are non-related phylogenetically (belong to different bacterial divisions). Therefore, the simple use of specific primers that PCR amplify a DNA fragment contained within the dsrAB genes (in any of its two subcomponents dsrA or

dsrB genes) is a helpful tool that can be used to determine the presence of sulfate reducers in a bacterial culture. In addition to vertical evolution, multiple lateral gene transfer events among the Bacteria and Archaea Domains have been documented. Nevertheless, these genes remained conserved through evolution within sulfate reducing bacteria (Karkhoff-Schweizer et al., 1995b; Klein et al., 2001b; Laue et al., 2001; Zverlov et al., 2005). Based on phylogenetic analysis, thirteen bacterial Families that carry the dsrAB genes have been classified. However, most microbes of this group have not been cultivated and/or identified/characterized (Ghosh & Bagchi, 2015; Muller et al., 2015).

It is universally acknowledged that the dsrAB operon encodes 15 different proteins, but the principal ones involved in the oxidation-reduction of sulfur compounds are DsrA and DsrB. However, it is also important to mention that DsrC is the portion of the complex that establishes direct contact with the sulfur anions in the oxidative and reductive pathways (Ghosh & Bagchi, 2015). Under normal conditions, the dsrAB genes are translated into a functional protein with two different polypeptidic subunits $\alpha_2\beta_2$ (no frameshift or nonsense mutations) with an approximate molecular weight of 180 - 220 kDa (α subunit: 50 kDA and β subunit: 40 kDa). These subunits contain two sirohemes and approximately four sulfur clusters (Fe_4S_4 type). However, some possible changes of the N-terminal dsrB portion of the dsrAB genes (beta subunits) in a few bacterial strains, as *Thermodesulfobacterium commune, T. mobile* and *Desulfovibrio desulfuricans*, are responsible for the modification of the siroheme-(Fe_4S_4) binding sites which allows the binding of four siroheme cofactors per $\alpha_2\beta_2$ molecule, in contrast with the typical sulfate reducers which have two binding sites (KARKHOFF-SCHWEIZER, BRUSCHI, & VOORDOUW, 1993b; Karkhoff-Schweizer et al., 1995a; Klein et al., 2001a; Laue et al., 2001; Muller et al., 2015).

Nowadays, it is known that the DsrAB evolved primarily as a reductive enzyme. This phenomenon was clarified by Muller et al., (2015) in their analysis of dsrAB sequences and DsrAB proteins, in which their phylogenetic trees associated the dsrAB genes of the bacterial strain *Moorella thermoacetica* and the reductive archaeal type DsrAB enzyme as the most probable common ancestor that originated the dissimilatory sulfite reductase (Muller et al., 2015).

Until today, the mechanisms associated with the sulfur anion oxidation and reduction by the dsrAB operon are not fully understood. However, the sequence of aminoacids (most of them of basic nature)

involved in the interaction with the sulfur anions (to be reduced or oxidized) are conserved in microbes that have the dsrAB Operon (Ghosh & Bagchi, 2015).

As it is mentioned on Ghosh & Bagchi (2015), the DsrA is a helical protein composed of 15 helices separated by 6 β strands, while the DsrB is an αβ protein composed of 13 helices and 11 β strands connected by loops. Furthermore, in their studies they found out that the secondary structures of the DsrAB proteins, for both the oxidizing and reducing bacteria, are similar with the exception that the DsrA component of the reducing bacterial strains has two extra helices which are absent at the oxidizing bacterial strain (Ghosh & Bagchi, 2015).

As both sulfur oxidizing and reducing bacteria have the same dsrAB operon (with slight differences), the DsrAB protein has similar composition. Therefore, both types of bacteria have binding sites for sulfate, sulfite, sulfide and thiosulfate. Furthermore, the portion of the DsrAB complex that binds sulfur compounds in oxidizing bacteria is composed of positively charged aminoacids (which helps in the interaction with negatively charged sulfur compounds such as thiosulfate). However, some differences have been found for the sulfide binding site. The latter has uncharged polar aminoacids instead of positively charged aminoacids. This phenomenon is overcome by the high surface charge density of sulfide which induces its binding to the DsrAB protein. In the other hand, the binding site for sulfate and sulfite in reducing bacteria is composed mostly of hydrophobic aminoacids which makes it difficult for these sulfur compounds to bind. However, they interact strongly with their binding sites due to presence of positively charged aminoacids (in comparison with thiosulfate or sulfide binding sites in the reducing bacteria). In general, oxidizing bacteria binding sites interact better with thiosulfate and sulfide because their binding sites have more positively charged aminoacids in comparison with those present in the binding sites of sulfate and sulfite. The same is true for reducing bacteria binding sites. They have more positively charged aminoacids in the binding sites for sulfate and sulfite in comparison with the binding sites of thiosulfate and sulfide (Ghosh & Bagchi, 2015).

In addition, the dsrB gene of some sulfate reducers can be expressed as a fusion protein of dsrB and dsrD (i.e. *Bilophila wadsworthia*, which is uncappable of sulfate reduction, but it is able to reduce sulfite). In this microorganism, the dsrA and dsrB encode nucleotides that are translated into 438 aminoacid

residues/49kDa (α subunit) and 483 aminoacid residues/53kDa (β subunit + γ subunit, from which 11kDa corresponds to the γ subunit). Furthermore, the presence of a promoter sequence upstream of the dsrA gene translational start site (approximately 123 bp) and a terminator sequence downstream of the dsrB stop codon suggest that even though the dsrB encodes a fusion protein, the whole operon is transcribed as a single unit (Laue et al., 2001).

It is interesting that the dsrAB operon along with the 16S rDNA gene have been used to determine phylogenetic relationships among different species of sulfate reducers and the evolutionary traits of this microbial group. Moreover, the authors found out that vertical-divergence (for example in *Desulfobacula toluolica* or *Desulfobacter latus*) and lateral gene transfers (between non-phylogenetic related bacterial strains for example *Desulfobacterium anilini* with *Desulfotomaculum* species and even more distant between Bacteria and Archaea) have occurred (Zverlov et al., 2005). As it was expected, none of the bacterial strains that received these genes through lateral transfer clustered with species that received them by vertical transfer. This finding suggests that the dsrAB common ancestor has not been described yet or it is non-existent nowadays (Muller et al., 2015; Zverlov et al., 2005). All these findings support the idea that the dissimilatory sulfite reductases are ancient enzymes that have been affected by multiple evolutive pressures (Muller et al., 2015; Zverlov et al., 2005). In fact, they have been selected through three principal mechanisms: divergence by speciation (vertical gene transfer), functional diversification and lateral gene transfer (horizontal gene transfer) (Muller et al., 2015). Consequently, the dsrAB gene cluster has been used as a phylogenetic marker to determine the presence of sulfate reducers across different environments (Muller et al., 2015).

II. Materials and Methods

D. psychrophila, D. arcticum and *D. ferrireducens* obtained from the Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen were inoculated in two different types of medium: A complex medium (Table Nº 1-Lactate medium) in which the terminal electron acceptor ($MgSO_4$) was replaced for Na_2SO_4 and a minimal medium (Table Nº 2) in which the final electron acceptor (Na_2SO_4) was replaced for different types of sulfate compounds at increasing concentrations (Table Nº 10 and Table Nº 11).

A. Complex culture medium

All the components of the complex culture medium were weighed separately (Table Nº 1). Subsequently, they were mixed in a beaker containing 987 mL of distilled water under stirring (the terminal electron acceptor $MgSO_4$ was replaced for Na_2SO_4). Once all the components were mixed properly, the medium was aliquoted into test tubes (4 experimental test tubes and 1 growth negative control per microbe, 9 mL of medium per test tube). Then, they were subjected to a gas flow of 100% N_2 until reaching anoxigenic conditions. As soon as the latter was achieved, the test tubes were sealed with rubber stoppers. Subsequently, the gas head spaces of the test tubes were oxygen evacuated using a 100% N_2 gas delivery system (formed by a 100% N_2 gas tank connected through plastic hoses to syringes and needles). Furthermore, the medium was autoclaved at 121º C for 15 min. The inoculations were performed on ice and under an anoxigenic atmosphere. Moreover, each strain was inoculated in 4 test tubes by means of 2 mL sterile syringes and needles. Once all test tubes were inoculated, each strain was incubated close to its optimal temperature (*D. psychrophila* and *D. ferrireducens* at 4º C and *D. arcticum* at 42º C).

B. Martian conditions Minimal culture medium

All different solutions (Tables Nº 2, 3, 4, 5, 6, 7, 8 and 9) were prepared separately. First, every component was weighed and mixed in stock solutions. Subsequently, smaller proportions were aliquoted into smaller vials. The latter were subjected to a H_2/CO_2 (80%:20%) gas flow until reaching anoxigenic conditions. Immediately, these vials were sealed with rubber stoppers and the gas head space of each

one was replaced with H_2/CO_2 (80%:20%) using a delivery system (formed by an 80%:20% H_2/CO_2 gas tank connected through plastic hoses to syringes and needles). All solutions, except vitamins-containing solutions (Table Nº 6, 7 and 8) were autoclaved at 121º C for 15 min. Moreover, vitamins-containing solutions were sterilized by filtration using microdisc filters of 0.2 µm under an anoxigenic atmosphere. Once all solutions were sterilized and cooled down, we proceeded to use the anoxigenic chamber to mix specific volumes of the sterilized solutions (Trace elements, selenite-tungstate, $NaHCO_3$, vitamins, Na_2S and sulfate compounds-Tables Nº 3 to 10) into sealed bottles containing 50 mL of anoxic basal medium for sulfate reducers (Table Nº 2). As it is shown in Table Nº 11 the sulfate compound (terminal electron acceptor) Na_2SO_4 was replaced in some cultures for increasing concentrations of different sulfate compounds (Table Nº 10). Furthermore, the cultures A2, A4 and A6 (Table Nº 11) were used to contrast growth patterns of the three microbes tested in the minimal medium (using H_2, CO_2 and Na_2SO_4) versus the complex medium (using lactate, yeast extract and Na_2SO_4). Likewise, the same cultures A2, A4 and A6 were used as positive controls for the experiments with increasing concentrations of different sulfate compounds (Table Nº 11).

All procedures and inoculations (Table Nº 11) were performed on ice and under an anoxigenic atmosphere. Every 2 weeks the head space of every culture was replaced with fresh H_2/CO_2 gas mixture.

C. DNA Extractions

Genomic DNA of every culture was extracted using the MOBIO Microbial DNA Isolation kit. We introduced some modifications in order to increase the genomic DNA concentration and purity. Furthermore, we used proteinase K (20 mg/mL, VWR) and RNase A (10µg/mL, Akron Biotech) following the manufacturers' instructions.

D. PCR with 16 S rDNA primers and dsrAB primers

We used the primers 27F and 16S R1 in order to amplify a portion of the bacterial 16 S rDNA gene. In addition, we used the primers specific for the dsrAB operon of *D. psychrophila:* DSR1FD and DSR4RE, the dsrAB operon general primers for *D. ferrireducens*: DSR1F and DSR4R and finally for *D. arcticum,* we used the primers dsrA_FWD and dsrA_REV which amplify a portion of the dsrA gene.

The 16 S rDNA gene primers have the sequence

1. 27 F 5'- AGAGTTTGATCMTGGCTCAG - 3'
2. 16S R1 5' - GGYTACCTTGTTACGACTT – 3' (a small modification of 1492R primer)

Amplicon size: approximately 1465 bp

The dsrAB primers used for *D. psychrophila* amplifications were

1. DSR1FD 5' – ACTCACTGGAAGCACG- 3'
2. DSR4RE 5' – GTGTAACAGTTACCACA- 3'

Amplicon size: approximately 1900 bp (Figure 8)

The dsrAB primers used for *D. ferrireducens* amplifications were

1. DSR1F 5'- ACSCACTGGAAGCACG- 3'
2. DSR4R 5'- GTGTAGCAGTTACCGCA- 3'

Amplicon size: approximately 1900 bp (Figure 8)

The dsrA primers used for *D. arcticum* amplifications have the sequence

1. dsrA_FWD 5'- TTATCGATCTGTGCCCTT-3'
2. dsrA_REV 5'- TTCTGCCTTCTTCCATCC-3'

Amplicon size: approximately 285 bp (Figure 9)

Furthermore, we used the PCR mixture described in Table Nº 12 with the enzyme Gold Taq 2X MM (GoTaq Green). All reactions were prepared on ice. Moreover, PCR tubes containing the reaction mixture described in Table Nº 12 were subjected to DNA amplification using the thermocycler and the programs described in Tables Nº 13, 14 and 15 according to the set of primers and microorganisms tested. The thermocycler used was the model Primus 96 plus of MWG-BIOTECH (Greensboro).

E. Agarose Gel Electrophoresis

The amplifications of the 16S rDNA gene of the three microbes and the dsrAB operon of *D. psychrophila* and *D. ferrireducens* were run in 0.9% regular agarose gels. They were prepared by solubilizing 0.36 g of regular agarose in 40 mL of TAE (1X) under heating conditions. Once agarose crystals were solubilized and the temperature of the mixtures was close to 45º C, 4 µL of SYBR Safe DNA gel stain (Invitrogen) was added. Subsequently, the gels were poured in gel electrophoresis molds. Then, we let them solidify for 20 min and finally, we placed the gels in electrophoresis cells containing the running buffer (TAE 1x).

The PCR products of the dsrA gene of *D. arcticum* were run in 2.5 % low melting agarose gels. The latter were prepared by solubilizing 1.0 g of low melting agarose in 40 mL of TAE (1X) under heating conditions. The rest of the preparation was performed as it was explained for the 16S rDNA gene and the dsrAB operon PCR products.

Furthermore, we loaded 5-10 µL of the PCR products and 3 µL of each of the ladders (1Kb Gibco or 1X ROCHE and 25 bp DNA step Promega). Subsequently, we run the samples at 100 V for approximately 30 min (16 S rDNA and dsrAB operon) or 50 V for approximately 45 min (dsrA of *D. arcticum*). Once the run was done, the gels containing separated bands were visualized and analyzed under an UV transilluminator (ULTRA.LUM).

III. Results

The 16S rDNA gene band amplification, which indicates bacterial presence/proliferation, was absent in the cultures prepared with complex components for the three sulfate reducers tested in this research: *D. psychrophila, D. ferrireducens and D. arcticum* (Table Nº 1, Figure 1). However, the 16S rDNA gene amplification from the minimal cultures prepared with H_2, CO_2, Na_2SO_4 and different types and concentrations of sulfate compounds showed evidence of active growing (Figure 2-lanes Nº 12 and 13, Figure 4-lanes Nº 3, 6, 7 and 11 and Figure 6-lanes Nº 3, 4, 5, 6, 7, 8 and 13). The latter indicates their incredible plasticity and adaptation to environments with low nutrients and increased concentrations of sulfate compounds. Moreover, these microbes were able to carry active metabolism under conditions similar to those experienced in Mars atmosphere (presence of CO_2 and H_2).

As we can observe in Figure 2 (lanes 12 and 13), cultures of *D. psychrophila* showed evidence of 16S rDNA amplifications in the presence of two different concentrations of $Fe_2(SO_4)_3$ (40 and 48 wt %). However, the bands (faint bands) suggested its growing was relatively low. In addition, an interesting phenomenon registered is the absence of growing in the presence of Na_2SO_4 (lane 3, Figure 2) which is the most common terminal electron acceptor used for the cultivation of sulfate reducers under laboratory conditions. As it is shown in Figure 3, *D. psychrophila* cultures did not showed amplification of the dsrAB operon using the specific primers DSR1FD and DSR4RE (Figure 3).

Furthermore, in Figure 4, *D. ferrireducens* showed evidence of 16S rDNA amplification in three different types and concentrations of sulfate compounds (lanes 6, 7 and 11) from which lane 6 presented the best amplification (Figure 4, lane 6, *D. ferrireducens* in $MgSO_4$ 18 wt %). In addition, as it was expected this microbe was able to grow using Na_2SO_4 as terminal electron acceptor (lane 3, Figure 4) although the band of the 16S rDNA gene amplification was relatively faint in comparison with the one present in $MgSO_4$ 18 wt %. Moreover, there was active growing in the presence of two different iron sulfate compounds, $FeSO_4$ and $Fe_2(SO_4)_3$, at concentrations of 10 and 30 wt % respectively. However, the amplification of the dsrAB operon using the general primers for detection of sulfate reducers (DSR1F and DSR4R) was absent (Figure 5).

Finally, in Figure 6, *D. arcticum* showed evidence of amplification of the 16S rDNA gene in five different sulfate compounds. The latter suggested its broad metabolic plasticity spectrum. As we can observe, it was able to grow in Na_2SO_4 (as it was expected), $CaSO_4$ (0.1 wt %) which presented one of the best amplifications and $MgSO_4$ (10 and 18 wt %). Furthermore, its best growing was registered with $FeSO_4$ at 10 wt % although it was also detected at 14 wt % (at a lower intensity). Moreover, growth was also detected with $Fe_2(SO_4)_3$ 48 wt %, but this amplification resulted in a faint band. Congruent with our expectations, the amplification of the dsrA portion of the dsrAB operon of *D. arcticum* using the primers drsA_FWD and drsA_REV was positive in the same samples that resulted positive for the amplifications of the 16S rDNA gene. Interestingly, the bands for samples in the lanes 3 (Na_2SO_4), lane 5 ($MgSO_4$ 10 wt %) and lane 6 ($MgSO_4$ 18 wt %) showed a better amplification of the dsrAB operon in comparison with the amplifications of the 16S rDNA gene. However, lane 8 ($FeSO_4$ 14 wt %) showed a faint band for both types of analysis while lane 13 ($Fe_2(SO_4)_3$ 48 wt %) which showed a faint band on the 16S rDNA amplification totally disappeared in the dsrAB analysis (Figure 7).

IV. Discussion

Desulfotalea psychrophila, Desulfuromusa ferrireducens and Desulfotomaculum arcticum in complex medium (16S rDNA amplifications).

The proliferation analysis of *D. psychrophila, D. ferrireducens and D. arcticum* in the presence of complex substrates such as lactate and yeast extract (Table Nº 1) pointed out their inability to degrade/utilize complex molecules in our experiments (Connon & Giovannoni, 2002; Russell et al., 1990; Vartoukian, Palmer, & Wade, 2010). The latter was demonstrated with the amplification of the 16S rDNA gene which showed no evidence of active growing (Figure 1). However, amplifications of this gene were registered in the minimal medium which was composed of H_2 and CO_2 as electron donor and carbon source (*D. ferrireducens* in Figure 4-lane 3 and *D. arcticum* in Figure 6-lane 3). Nevertheless, it has been reported that the three microbes tested can use lactate as their electron donor and carbon source (Aullo et al., 2013; Greene, 2014; Knoblauch, Sahm & Jorgensen, 1999; Rabus et al., 2004; Vandieken, 2006a, 2006b). A possible explanation for our inability to amplify the 16S rDNA gene in complex medium comes from the fact that these microbes are adapted to metabolize small molecules as substrates (adaptation to low nutrients environments) instead of complex molecules such as lactate or yeast extract. This finding suggested their capability to metabolize small molecules such as H_2 and CO_2 faster than complex molecules such as lactate (Aullo et al., 2013; Connon & Giovannoni, 2002; Russell et al., 1990; Vartoukian et al., 2010). Interestingly, *D. psychrophila* did not show evidence of active growing in any of the media used (Figure 1-lane 2, and Figure 2-lane 3) which indicated its inability to metabolize complex or small molecules in our experiments. However, a possible explanation for this phenomenon is that this psychrophilic microbe has a high duplication time. In addition, it was subjected to temperatures (4º C) below its optimal growth temperature (10 – 18º C) because it has been reported that they can carry out active metabolism below 0º C (Greene, 2014; Knoblauch, Sahm & Jorgensen, 1999; Rabus et al., 2004; Vandieken, 2006b). Therefore, our experimental conditions (lower temperature than optimal) probably rendered an increased duplication time and a slower growth rate. As a result, it might be possible that the DNA extractions were performed before there were enough cells to render a good quality DNA template for downstream PCR amplifications.

In summary, this part of this research pointed out that the proliferation of *D. psychrophila* was affected by a combinatory effect of the type of nutrients present in culture (lactate and yeast extract in comparison with simple nutrients such as H_2 and CO_2) and temperature (lower than optimal) while for *D. ferrireducens* and *D. arcticum* the substrate type (lactate and yeast extract in comparison with simple nutrients such as H_2 and CO_2) affected its growth (Connon & Giovannoni, 2002; Russell et al., 1990; Vartoukian et al., 2010).

***Desulfotalea psychrophila, Desulfuromusa ferrireducens and Desulfotomaculum arcticum* in minimal medium with different types and concentrations of sulfate compounds (16S rDNA and dsrAB amplifications).**

The low presence of the 16S rDNA gene amplification product in cultures from the minimal medium with *D. psychrophila* and *D. ferrireducens* and the high products of amplification of *D. arcticum* (Tables 2 to 9, Figures 2, 4 and 6) suggested their ability to utilize small molecules to undergo active metabolism (Connon & Giovannoni, 2002; Russell et al., 1990; Vartoukian et al., 2010). This feature is one of the principal requirements to proliferate under Martian environments in which the substrates' availability is relatively low (Des Marais et al., 2008; Parnell et al., 2007). Furthermore, the microorganisms' failure to degrade complex molecules illustrated a possible mechanism of adaptation to environments with low nutrients. Moreover, their active growing under this condition pointed out their inherent advantage to utilize gaseous molecules such as H_2 (electron donor) in the production of energy and CO_2 as their source of carbon for biomass production (Aullo et al., 2013; A. Roychoudhury, 2004; Skidmore et al., 2000).

Furthermore, as we can observe in Figure 2, *D. psychrophila* in minimal medium showed a relatively low amplification of the 16S rDNA gene (lanes 12 and 13, Figure 2). The terminal electron acceptor tested in these lanes was $Fe_2(SO_4)_3$ 40 wt % and 48 wt % respectively. Although it might seem that this microbe was growing in a high concentration of $Fe_2(SO_4)_3$, the reality is that this mineral is almost insoluble in water. Therefore, the concentration of sulfate anions available to use as terminal electron acceptors can be close to minimal due to solubility constraints at 4º C. However, it is maximal if we take into consideration that an increased concentration of the mineral will maximize the number of anions that can

be available for sulfate reduction. Nevertheless, the presence of these amplification products indicated the competence of this psychrophilic strain to survive and proliferate in environments that contain sulfate compounds. Moreover, it used H_2 as the electron donor in anaerobic sulfate reduction and CO_2 as the only source of carbon. In the other hand, we expected to detect microbial growth at lower concentrations of sulfate compounds. A possible explanation for this phenomenon is the inherent effect of low temperatures in cellular processes and nutrients uptake. As it is mentioned in Pomeroy & Wiebe (2001), low temperatures (near limiting temperatures) affect a variety of cell processes that reduce the growth rate of any microorganism as well as its ability to access different carbon sources (Pomeroy & Wiebe, 2001; Russell et al., 1990). Furthermore, in our experiments *D. psychrophila* was subjected to temperatures (4º C) below its optimal growth temperature (10-18 ºC) which could affect/decelerate the uptake of nutrients and the terminal electron acceptors. This scenario turns out to be more restraining for bacterial proliferation if we take into consideration that the only nutrients present (H_2 and CO_2) were gaseous molecules and their diffusion rate into water molecules (culture medium) is decreased at lower temperatures (Callister, 2010; Carpenter, Lin, & Capone, 2000; Jähne, Heinz, & Dietrich, 1987; Marion et al., 2003; Priscu et al, 1998; Rivkina, Friedmann, McKay, & Gilichinsky, 2000; Tan, 2014).

Interestingly, it seemed that the microbe's proliferation was inhibited at lower concentrations of minerals. This feature is congruent with the discoveries of Aullo et al., (2013) who indicated that sulfate reduction is higher in cold environments where sulfate sources are not limiting (Aullo et al., 2013; Mukhopadhyay et al., 2006; Porter et al., 2007; Skidmore et al., 2000).

Although the proliferation of *D. psychrophila* was detected through the 16S rDNA amplifications, the dsrAB operon PCRs that targeted the whole dsrAB operon (primers DSR1FD/DSR4RE) showed no evidence of active growing. This phenomenon was expected as the two bands generated with the 16S rDNA gene amplification were relatively faint. Furthermore, this outcome can be explained based on the suggestion of Aullo et al., (2013) and Scholten et al., (2005) who mentioned that cultivation techniques employed in the growing of sulfate reducers adapted to anoxic environments are normally tedious and most of the cases unsuccessful in comparison with cultivation techniques used for faster growing bacterial species (Aullo et al., 2013; Carpenter, Lin, & Capone, 2000; Marion et al., 2003; Priscu et al,

1998; Rivkina, Friedmann, McKay, & Gilichinsky, 2000; Scholten et al., 2005). The latter pointed out the inability of this microbe to proliferate in a high number of cells (due to the same temperature and substrates' availability constraints mentioned above) which is translated in our inability to extract a good quality DNA template for the PCRs of the 16S rDNA or the dsrAB operon. Furthermore, the same phenomenon occurred with the dsrAB operon amplifications of *D. ferrireducens* using the primers DSR1F/DSR4R. However, we were able to detect its proliferation through 16S rDNA amplifications in different types and concentrations of sulfate compounds $MgSO_4$ 18 wt % (solubility of 26.9 wt % at 0º C), $FeSO_4$ 10 wt % (solubility of 15.6 wt % at 0º C) and $Fe_2(SO_4)_3$ 30 wt % (almost insoluble in water) (Figure 4). Interestingly this microbe was able to proliferate in the apparent high concentration of $Fe_2(SO_4)_3$ as it was registered for *D. psychrophila* (Figure 2). As a matter of fact, the same explanation can be extended to the proliferation of *D. ferrireducens* at that high concentration. Furthermore, it showed a 16S rDNA amplification product at $MgSO_4$ 18 wt % in which the 100% of the sulfate anions were in solution and available for anaerobic reduction (solubility higher than 26.9 wt %, at 4º C). This is congruent with the discoveries of Crisler et al., (2012) which find out that sulfate reducers can grow at MgSO4 concentrations of 24 wt % (Crisler et al., 2012). The same explanation can be extended to the 16S rDNA amplification product of this microbe in $FeSO_4$ 10 wt % in which 100 % of the sulfate anions were in solution (solubility higher than 15.6 wt % at, 4º C). In any case, these low amplification products of the 16S rDNA gene and the absence of them with the dsrAB operon again suggested the need of a better template for DNA downstream PCR amplifications (better DNA extraction or a culture with higher biomass/bacterial cells production) (Brandt et al., 2001; KARKHOFF-SCHWEIZER et al., 1993a; A. N. Roychoudhury & McCormick, 2006).

As it was mentioned for the 16S rDNA amplifications of these microbes in complex medium, another explanation for our inability to detect the presence of bacterial active growing (through dsrAB amplifications) in minimal medium is that these microbes might be replicating at a slower rate due to the low availability of the electron donor and carbon source (H_2 and CO_2 decreased diffusion due to low temperature) although both have been reported in the literature as normal substrates for these microbes' proliferation. If this is the case, the detection of its presence with conventional molecular techniques such as PCR amplifications will render unsuccessful results. In other words, it might be possible that the

number of vegetative cells present at the moment of the DNA extraction was too low to render a good quantity and quality DNA template. As it was suggested by Schuerger & Nicholson (2006) more sensitive techniques should be used to determine the presence of active bacterial metabolism (Brandt et al., 2001; KARKHOFF-SCHWEIZER et al., 1993a; A. N. Roychoudhury & McCormick, 2006; A. Schuerger & Nicholson, 2006).

In the other hand, *D. arcticum* exhibited impressive metabolic capabilities. As it was shown in Figure 6, 7 and 10, this microbe was able to survive, proliferate and undergo anaerobic sulfate reduction under simulated Martian atmospheric conditions. Furthermore, it used three of the four terminal electron acceptors tested in this research ($CaSO_4$ 0.1 wt %, $MgSO_4$ 10 wt % and 18 wt %, $FeSO_4$ 10 wt % and a low rate was registered for $FeSO_4$ 14 wt %; all of these mineral species had a sulfate anion 100 % solubility at 42º C). However, the 16S rDNA and dsrA patterns of amplifications suggested a possible constraint in its metabolic activity at higher sulfate compounds concentrations. Moreover, a decrease/arrest on its metabolic activity/growth is obvious at sulfate salt concentrations higher than 14 wt % (for FeSO4). The latter indicated an inability to cope with increased mineral concentrations and its failure to maintain cellular homeostasis through mechanisms of osmoregulation (Mukhopadhyay et al., 2006; Porter et al., 2007). However, the presence of a weak amplification product of the 16S rDNA at $Fe_2(SO_4)_3$ 48 wt % again illustrates the same phenomenon that occurred with *D. psychrophila* in which this mineral had an apparent high concentration, but its solubility in water indicated that the sulfate anions available for sulfate reduction are actually really low (see Figure 3 and Figure 6). This data suggested that it might be possible for *D. arcticum* to proliferate in Martian microenvironments that are rich in sulfate compounds such as $CaSO_4$, $MgSO_4$ and $FeSO_4$. However, other observations pointed out this scenario is unrealistic. First, the high UV irradiation is bactericidal for the vegetative form of this microbe while the endospore form will eventually degenerate due to cumulative DNA damage. Second, the extreme low temperatures will decrease its metabolism due to its requirement of warmer temperatures to carry out active growth and metabolic activity (it is a mesophilic/moderate thermophilic microbe). However, its survival under Martian subsurface physicochemical conditions is possible. The latter turns to be more logic, if we take into consideration that this microorganism is an endospore former and the UV light does not affect biological forms more than 500 µM in the soil's depth. Furthermore, these conditions provide it

with a niche that increases the chances of survivability (Arvidson et al., 2014; Berry et al., 2010; Brandt et al., 2001; Crisler et al., 2012; Des Marais et al., 2008; KARKHOFF-SCHWEIZER et al., 1993a; Parnell et al., 2007; A. N. Roychoudhury & McCormick, 2006; Squyres et al., 2006; Velasco et al., 2015). In the other hand, the microbe's proliferation in subsurface environments is extremely affected by low temperatures similar as it is affected in the Martian surface (it will render extreme low/absent metabolic activity). Therefore, temperature is the principal constraint for the microbe's proliferation even though the electron donor and the carbon source are not limiting factors (abundant CO_2 almost 95 % and traces of H_2 from UV photodissociation of transient liquid water) (Bhattacharyya, 2016).

Finally, some difficulties associated to this study were the extraction of representative samples of DNA and subsequent amplifications of the dsrAB genes. Furthermore, the same problems were experienced by Scholten et al., (2005) in their studies of sulfate reducers in a meromictic soda lake (Scholten et al., 2005). This inability to extract DNA comes from the fact that in sedimentary environments, such as those present in Mars, the concentration of cells is normally low. Therefore, low concentrations of nucleic acids are recovered after DNA extractions. Nevertheless, this disadvantage has been overcome through the modification of various extraction techniques that include the use of freeze-thawing cycles, mechanical lysis, specific buffers and specific chemical lysis procedures. Also, it has been reported that microorganisms adapted to harsh environments (such as those present at the Earth´s subsurface) develop structures that interfere with the mechanical lysis steps or with the DNA extraction in general (different composition of cell membrane and cell wall) (Alain et al., 2011; Valdivia-Silva et al., 2016).

V. Conclusions

In this research work we can conclude that:

1. We were able to determine that microbes adapted to low nutrients environments (in which the electron donors and carbon sources are limiting) can metabolize small substrates' molecules such as H_2 and CO_2 faster than complex molecules such as lactate and yeast extract. This finding is the result of our analysis/detection of the 16S rDNA gene amplification in cultures that were prepared with complex (no amplification) and with minimal substrates (low/detectable amplifications).
2. Active growth of *D. psychrophila* and *D. ferrireducens* (psychrophilic microbes) was detected at Martian atmospheric (H_2 and CO_2 content) and soil conditions (different types and concentrations of sulfate compounds) through the amplification of the 16S rDNA gene. Although the amplification process was constrained by low quality DNA templates due to low availability of cells, we were able to detect the growth of *D. psychrophila* at two concentrations of $Fe_2(SO_4)_3$ (40 and 48 wt %) and *D. ferrireducens* with three types of sulfate compounds $MgSO_4$, $FeSO_4$ and $Fe_2(SO_4)_3$ (18, 10 and 30 wt % respectively). These findings pointed out the incredible metabolic capabilities inherent of these microbes.
3. We were able to determine that in order to amplify the dsrAB operon in psychrophilic microbes, better DNA quality should be extracted. However, the latter depends on the microbial growth present in each culture. In our case, different conditions such as low nutrients availability and diffusion, low temperatures, and increasing concentrations of minerals (sulfate compounds) constrained the microbial growth. Consequently, amplifications of the dsrAB operon in these strains were inconclusive.
4. *D. arcticum* was the only microbe of this study that showed high metabolic and survival capabilities. Furthermore, we detected active growing with the four sulfate compounds tested $CaSO_4$, $MgSO_4$, $FeSO_4$ and $Fe_2(SO_4)_3$ according to 16S rDNA amplifications and in three of them $CaSO_4$, $MgSO_4$ and $FeSO_4$ according to dsrA amplifications. Moreover, we were able to detect its presence at sulfate concentrations of 48 wt % (similar *to D. psychrophila*) which indicates their

impressive osmoregulation mechanisms. However, the proliferation of this microbe under surface and subsurface microenvironments in the Red Planet is unrealistic due to its requirement for warmer temperatures.

5. The detection of active growth in each of these cultures through amplifications of the 16S rDNA gene and the dsrA component of the dsrAB operon suggested the active expression of the dissimilatory sulfate reductase (DsrAB). Moreover, this study is just the beginning of future studies in which we have planned to measure the expression of the DsrAB.

VI. References

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VII. Appendix

Table 1. List of components of complex culture medium (Lactate medium adopted from Butlin, Adams & Thomas, 1949 and Postgate, 1963).

Component	Quantity
60% sodium lactate	4 mL
Yeast extract	1.0 g
Ascorbic acid	0.1 g
*Magnesium sulfate ($MgSO_4. 7H_2O$)	0.2 g
K_2HPO_4	0.01 g
$Fe(SO_4)_2 (NH_4)_2. 6H_2O$	0.2 g
NaCl	10.0 g
dH_2O	987 mL
pH	7.3

*Terminal electron acceptor was replaced for Na_2SO_4 (0.2 g/L).

Table 2. Basal medium for sulfate reducers (Minimal medium adopted from Widdel and Bak, 1992).

Component	Quantity
NaCl	1.0 g
$MgCl_2. 6H_2O$	0.4 g
$CaCl_2. 2H_2O$	0.1 g
*Na_2SO4	4.0 g
NH_4Cl	0.25 g
KH_2PO_4	0.2 g
KCl	0.5 g
Trace elements solution	1.0 mL
Selenite-tungstate solution	1.0 mL
$NaHCO_3$ solution	30.0 mL
Vitamin mix solution	1.0 mL
Thiamine solution	1.0 mL
Vitamin B12 solution	1.0 mL

Table 2. Basal medium for sulfate reducers (Minimal medium adopted from Widdel and Bak, 1992) (Cont.).

Na_2S solution	7.5 mL
Resazurin soltn. (0.1% w/v)	0.50 mL
dH_2O	1000 mL
ph	7.0-7.3

*Terminal electron acceptor was replaced for different concentrations of sulfate compounds.

Table 3. Trace Elements solution

Component	Quantity
HCl (25% = 7.7 M)	12.5 mL (100 mM)
$FeSO_4. 7H_2O$	2100 mg (7.5 mM)
H_3BO_3	30 mg (0.5 mM)
$MnCl_2. 4H_2O$	100 mg (0.5 mM)
$CoCl_2. 6H_2O$	190 mg (0.8 mM)
$NiCl_2. 6H_2O$	24 mg (0.1 mM)
$CuCl_2. 2H_2O$	2 mg (0.01 mM)
$ZnSO_4. 7H_2O$	144 mg (0.5 mM)
$Na_2MoO_4. 2H_2O$	36 mg (0.15 mM)
dH_2O	987 mL

Table 4. Selenite-Tungstate solution

Component	Quantity
NaOH	0.4 g (10mM)
$Na_2SeO_3. 5H_2O$	6 mg (0.02 mM)
$Na_2Wo_4. 2H_2O$	8 mg (0.02 mM)
dH_2O	1000 mL

Table 5. $NaHCO_3$ solution

Component	Quantity
$NaHCO_3$	84 g
dH_2O	10000 mL

Table 6. Vitamin Mix solution

Component	Quantity
Biotin	2 mg
Folic acid	2 mg
Pyridoxine-HCl	10 mg
Thiamine-HCl. $2H_2O$	5.0 mg
Riboflavin	5.0 mg
Nicotinic acid	5.0 mg
D-Ca pantothenate	5.0 mg
Vitamin B-12	0.10 mg
p-aminobenzoic acid	5.0 mg
Lipoic acid	5.0 mg
dH_2O	1000 mL

Table 7. Thiamine solution

Component	Quantity
Thiamine chloride dihydrochloride	10 mg
25 mM Sodium Phosphate pH 3.4	100 mL

Table 8. Vitamin B12 solution

Component	Quantity
Cyanocobalamine	5 mg
dH_2O	100 mL

Table 9. Na_2S solution

Component	Quantity
$Na_2S.\ 9H_2O$ (0.20 M)	48 g
dH_2O	1000 mL

Table 10. Sulfate compounds (Replacement of terminal electron acceptor on Basal medium for sulfate reducers).

Sulfate compound	Concentration
$CaSO_4$	0.1 wt %
$MgSO_4$	10 wt %
$MgSO_4$	18 wt %
$Fe(SO_4)_2$	10 wt %
$Fe(SO_4)_2$	14 wt %
$Fe_2(SO_4)_3$	10 wt %
$Fe_2(SO_4)_3$	20 wt %
$Fe_2(SO_4)_3$	30 wt %
$Fe_2(SO_4)_3$	40 wt %
$Fe_2(SO_4)_3$	48 wt %

Table 11. Experimental Design for inoculation of cultures under Martian conditions. H_2/CO_2 carbon source and different sulfate compounds as terminal electron acceptors.

Control ID	Experimental ID	Microorganism	Sulfate Compound	pH	Temperature
A1	A2	*D. psychrophila*	Na_2SO_4	7.0-7.3	4º C
A3	A4	*D. ferrireducens*	Na_2SO_4	7.0-7.3	4º C
A5	A6	*D. arcticum*	Na_2SO_4	7.0-7.3	42º C
B1	B2	*D. psychrophila*	$CaSO_4$ 0.1 wt %	7.0-7.3	4º C
	C1	*D. ferrireducens*	$CaSO_4$ 0.1 wt %	7.0-7.3	4º C
	C2	*D. arcticum*	$CaSO_4$ 0.1 wt %	7.0-7.3	42º C
B3	B4	*D. psychrophila*	$MgSO_4$ 10 wt %	7.0-7.3	4º C
	C3	*D. ferrireducens*	$MgSO_4$ 10 wt %	7.0-7.3	4º C
	C4	*D. arcticum*	$MgSO_4$ 10 wt %	7.0-7.3	42º C
B5	B6	*D. psychrophila*	$MgSO_4$ 18 wt %	7.0-7.3	4º C
	C5	*D. ferrireducens*	$MgSO_4$ 18 wt %	7.0-7.3	4º C
	C6	*D. arcticum*	$MgSO_4$ 18 wt %	7.0-7.3	42º C
B7	B8	*D. psychrophila*	$FeSO_4$ 10 wt %	7.0-7.3	4º C
	C7	*D. ferrireducens*	$FeSO_4$ 10 wt %	7.0-7.3	4º C
	C8	*D. arcticum*	$FeSO_4$ 10 wt %	7.0-7.3	42º C
B9	B10	*D. psychrophila*	$FeSO_4$ 14 wt %	7.0-7.3	4º C
	C9	*D. ferrireducens*	$FeSO_4$ 14 wt %	7.0-7.3	4º C
	C10	*D. arcticum*	$FeSO_4$ 14 wt %	7.0-7.3	42º C
B11	B12	*D. psychrophila*	$Fe_2(SO_4)_3$ 10 wt %	7.0-7.3	4º C
	C11	*D. ferrireducens*	$Fe_2(SO_4)_3$ 10 wt %	7.0-7.3	4º C
	C12	*D. arcticum*	$Fe_2(SO_4)_3$ 10 wt %	7.0-7.3	42º C
B13	B14	*D. psychrophila*	$Fe_2(SO_4)_3$ 20 wt %	7.0-7.3	4º C
	C13	*D. ferrireducens*	$Fe_2(SO_4)_3$ 20 wt %	7.0-7.3	4º C
	C14	*D. arcticum*	$Fe_2(SO_4)_3$ 20 wt %	7.0-7.3	42º C
B15	B16	*D. psychrophila*	$Fe_2(SO_4)_3$ 30 wt %	7.0-7.3	4º C

Table 11. Experimental Design for inoculation of cultures under Martian conditions. H_2/CO_2 carbon source and different sulfate compounds as terminal electron acceptors (Cont.).

	C15	*D. ferrireducens*	$Fe_2(SO_4)_3$ 30 wt %	7.0-7.3	4º C
	C16	*D. arcticum*	$Fe_2(SO_4)_3$ 30 wt %	7.0-7.3	42º C
B17	B18	*D. psychrophila*	$Fe_2(SO_4)_3$ 40 wt %	7.0-7.3	4º C
	C17	*D. ferrireducens*	$Fe_2(SO_4)_3$ 40 wt %	7.0-7.3	4º C
	C18	*D. arcticum*	$Fe_2(SO_4)_3$ 40 wt %	7.0-7.3	42º C
B19	B20	*D. psychrophila*	$Fe_2(SO_4)_3$ 48 wt %	7.0-7.3	4º C
	C19	*D. ferrireducens*	$Fe_2(SO_4)_3$ 48 wt %	7.0-7.3	4º C
	C20	*D. arcticum*	$Fe_2(SO_4)_3$ 48 wt %	7.0-7.3	42º C

Control= Non-inoculated medium

Table 12. PCR Mixture

Reagent	Volume
Molecular H_2O	6.5 µL
Master Mix (Enzyme)	12.5 µL
Forward Primer	1 µL (4 µM)
Reverse Primer	1 µL (4 µM)
Template	4 µL
Total Volume Rxn.	25 µL

Table 13. Thermocycler program for 16 S rDNA gene

Steps	Temperature	Time
Activation	95º C	5 min
Denaturation	95º C	1 min (Open 30 cycles)
Annealing	52º C	1 min
Extension	72º C	9 min (Close 30 cycles)
Final Extension/Elongation	72º C	10 min

Table 14. Thermocycler program for *D. psychrophila* (DSR1FD and DSR4RE) and *D. ferrireducens* (Primers: DSR1F and DSR4R).

Steps	Temperature	Time
Activation	95º C	5 min.
Denaturation	95º C	1 min (Open 30 cycles)
Annealing	54º C	1 min
Extension	72º C	9 min (the 30 cycles)
Final Extension/Elongation	72º C	10 min.

Table 15. Thermocycler program for *D. arcticum* (Primers: dsrA_FWD and dsrA_REV).

Steps	Temperature	Time
Activation	95º C	5 min
Denaturation	95º C	1 min (Open 30 cycles)
Annealing	52º C	1 min
Extension	72º C	9 min (Close 30 cycles)
Final Extension/Elongation	72º C	10 min

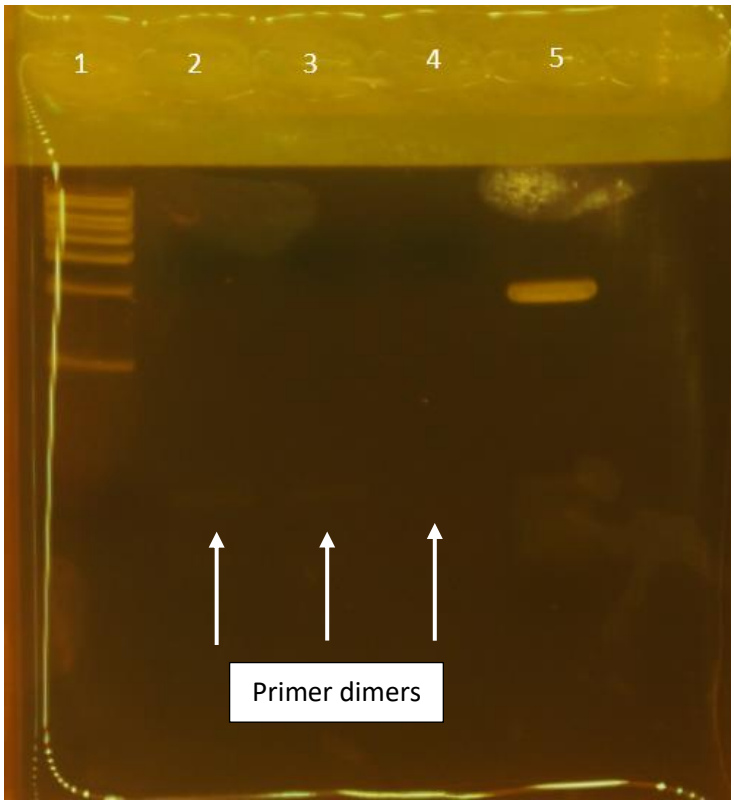

Figure 1. 16S rDNA gene amplification of cultures prepared in complex medium. The PCR amplification was made with the primers 27F and 16S R1 which render an amplicon size of approximately 1465 bp. These samples were run in a regular agarose gel (0.9 %) submerged in TAE (1X) at 100 volts. Furthermore, the lanes represent samples, **1**: Ladder 1X ROCHE, **2**: *D. psychrophila* amplification, **3**: *D. ferrireducens* amplification, **4***: D arcticum* amplification, **5**: 25 bp step ladder.

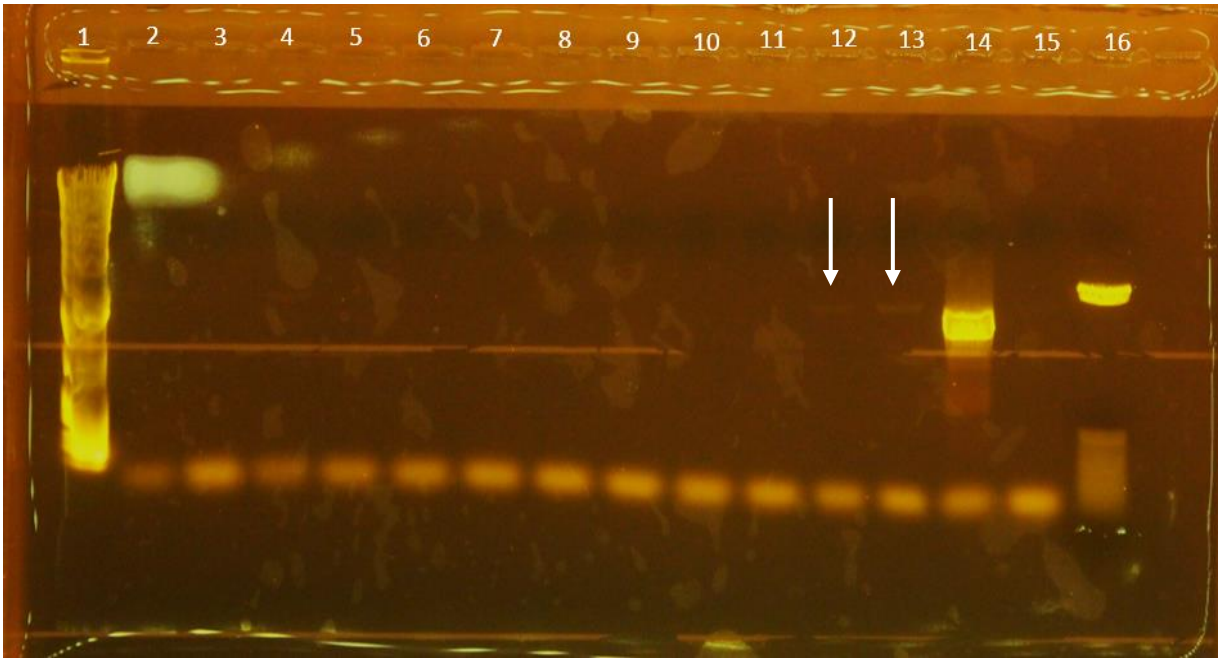

Figure 2. Amplification products of bacterial small ribosomal subunit gene (16S rDNA) of *D. psychrophila.* The primers used were 27F and 16S R1 which render an amplicon of approximately 1465 bp. The samples were run in a regular agarose gel (0.9%) submerged in TAE (1X) at 100 volts. Furthermore, the lanes represent samples, **1**: Ladder 1Kb; **2**: A5, medium negative control; **3**: A6, growth control with Na_2SO_4; **4**: C2, $CaSO_4$ 0.1 wt %; **5**: C4, $MgSO_4$ 10 wt %; **6**: C6, $MgSO_4$ 18 wt %; **7**: C8, $FeSO_4$ 10 wt %; **8**: C10, $FeSO_4$ 14 wt %; **9**: C12, $Fe_2(SO_4)_3$ 10 wt %; **10**: C14, $Fe_2(SO_4)_3$ 20 wt %; **11**: C16, $Fe_2(SO_4)_3$ 30 wt %; **12**: C18, $Fe_2(SO_4)_3$ 40 wt %; **13**: C20, $Fe_2(SO_4)_3$ 48 wt %; **14**: PCR positive control, **15**: PCR negative control, **16**: 25 bp step ladder.

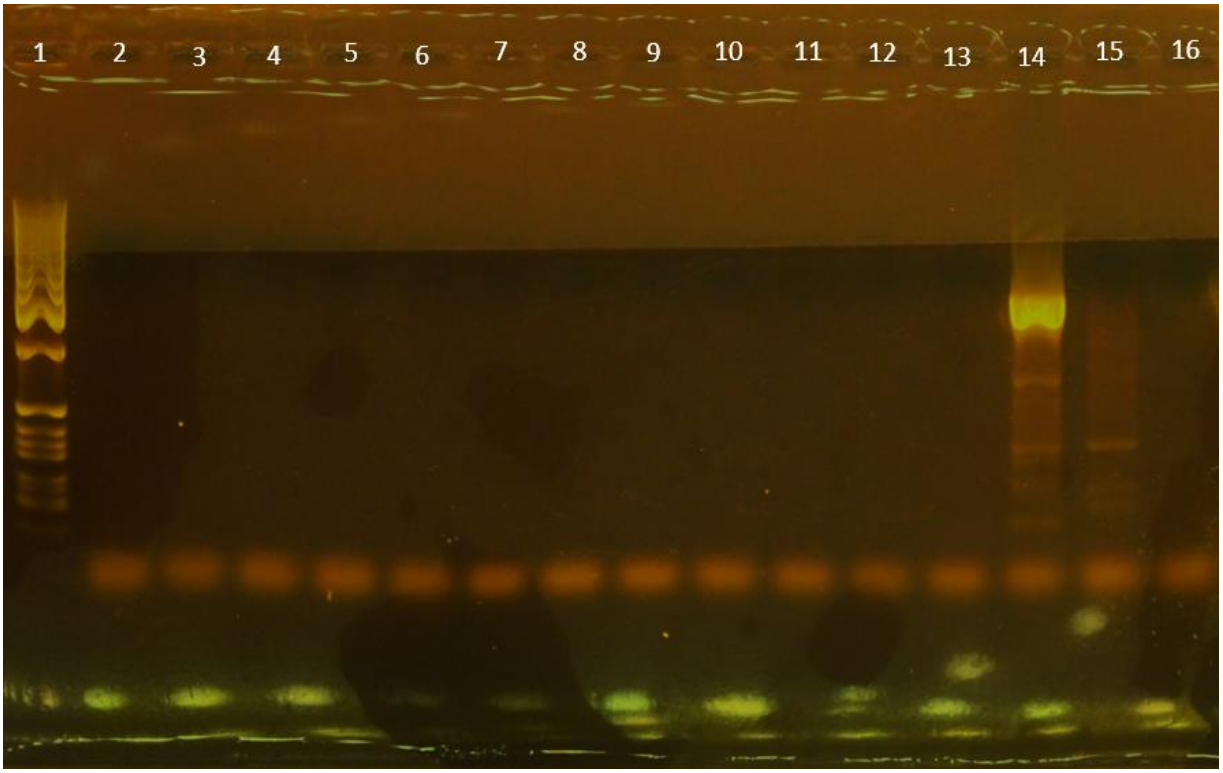

Figure 3. Gel electrophoresis of dsrAB PCR products from *D. psychrophila* subjected to different sulfate compounds. The primers used were DSR1FD and DSR4RE which render an amplicon of approximately 1.9 Kb. The samples were run in a regular agarose gel (0.9%) submerged in TAE (1X) at 100 volts. Furthermore, the lanes represent samples, **1**: Ladder 1X (ROCHE); **2**: A5, medium negative control; **3**: A6, growth control with Na_2SO_4; **4**: C2, $CaSO_4$ 0.1 wt %; **5**: C4, $MgSO_4$ 10 wt %; **6**: C6, $MgSO_4$ 18 wt %; **7**: C8, $FeSO_4$ 10 wt %; **8**: C10, $FeSO_4$ 14 wt %; **9**: C12, $Fe_2(SO_4)_3$ 10 wt %; **10**: C14, $Fe_2(SO_4)_3$ 20 wt %; **11**: C16, $Fe_2(SO_4)_3$ 30 wt %; **12**: C18, $Fe_2(SO_4)_3$ 40 wt %; **13**: C20, $Fe_2(SO_4)_3$ 48 wt %; **14**: PCR positive control, **15**: PCR positive control, **16**: PCR negative control.

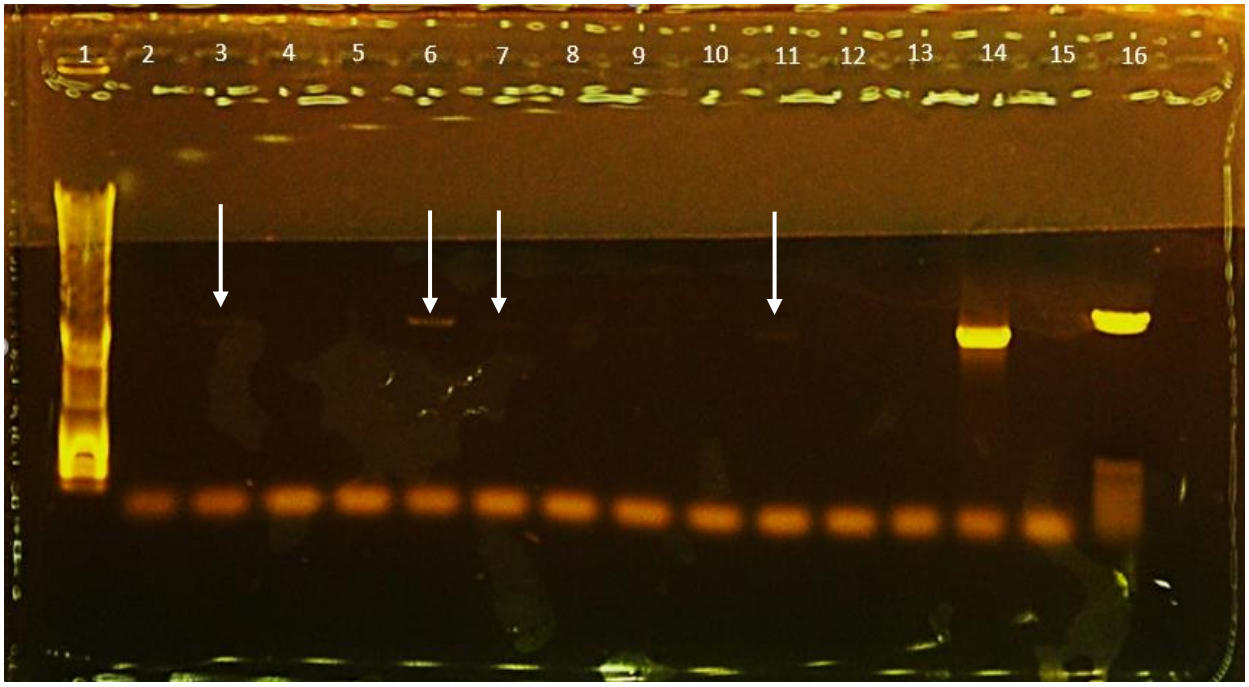

Figure 4. Amplification products of bacterial small ribosomal subunit gene (16S rDNA) of *D. ferrireducens.* The primers used were 27F and 16S R1 which render an amplicon of approximately 1465 bp. The samples were run in a regular agarose gel (0.9%) submerged in TAE (1X) at 100 volts. Furthermore, the lanes represent samples, **1**: Ladder 1Kb; **2**: A5, medium negative control; **3**: A6, growth control with Na_2SO_4; **4**: C2, $CaSO_4$ 0.1 wt %; **5**: C4, $MgSO_4$ 10 wt %; **6**: C6, $MgSO_4$ 18 wt %; **7**: C8, $FeSO_4$ 10 wt %; **8**: C10, $FeSO_4$ 14 wt %; **9**: C12, $Fe_2(SO_4)_3$ 10 wt %; **10**: C14, $Fe_2(SO_4)_3$ 20 wt %; **11**: C16, $Fe_2(SO_4)_3$ 30 wt %; **12**: C18, $Fe_2(SO_4)_3$ 40 wt %; **13**: C20, $Fe_2(SO_4)_3$ 48 wt %; **14**: PCR positive control, **15**: PCR negative control, **16**: 25 bp step ladder.

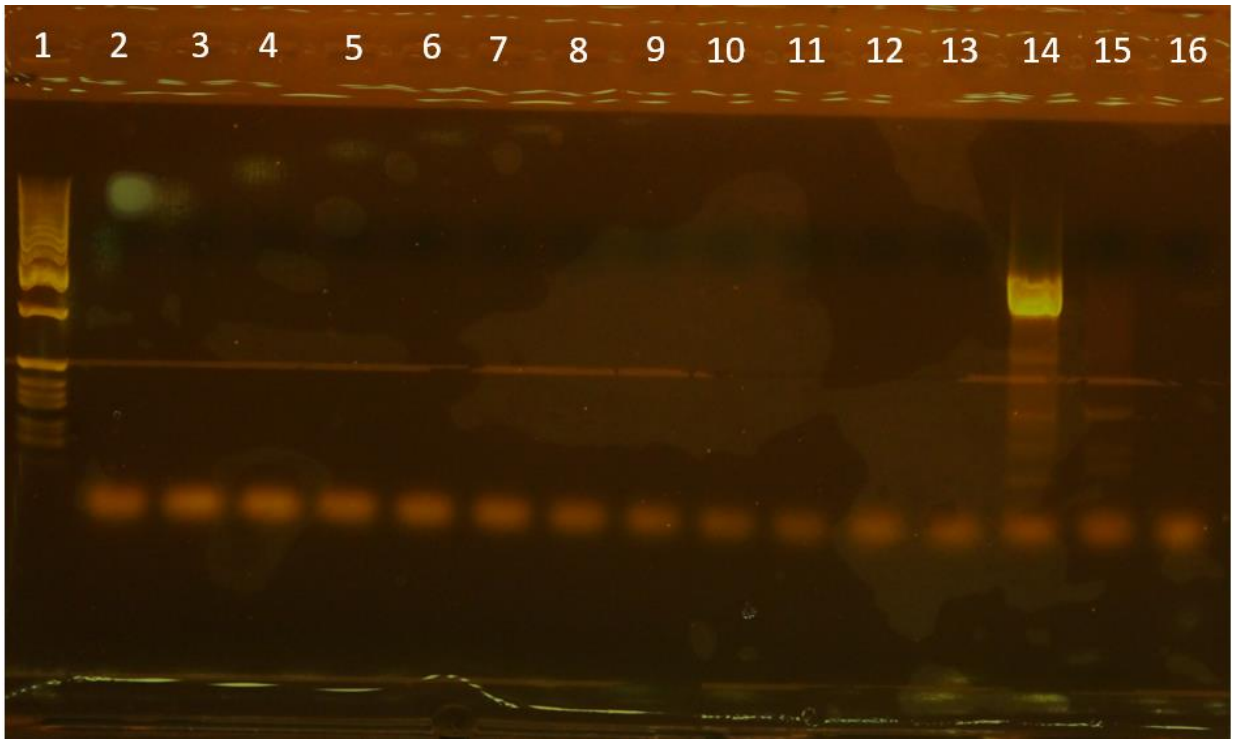

Figure 5. Gel electrophoresis of dsrAB PCR products from *D. ferrireducens* subjected to different sulfate compounds. The primers used were DSR1F and DSR4R which render an amplicon of approximately 1.9 Kb. The samples were run in a regular agarose gel (0.9%) submerged in TAE (1X) at 100 volts. Furthermore, the lanes represent samples, **1**: Ladder 1X ROCHE; **2**: A5, medium negative control; **3**: A6, growth control with Na_2SO_4; **4**: C2, $CaSO_4$ 0.1 wt %; **5**: C4, $MgSO_4$ 10 wt %; **6**: C6, $MgSO_4$ 18 wt %; **7**: C8, $FeSO_4$10 wt %; **8**: C10, $FeSO_4$ 14 wt %; **9**: C12, $Fe_2(SO_4)_3$ 10 wt %; **10**: C14, $Fe_2(SO_4)_3$ 20 wt %; **11**: C16, $Fe_2(SO_4)_3$ 30 wt %; **12**: C18, $Fe_2(SO_4)_3$ 40 wt %; **13**: C20, $Fe_2(SO_4)_3$ 48 wt %; **14**: PCR positive control, **15**: PCR positive control, **16**: PCR negative control.

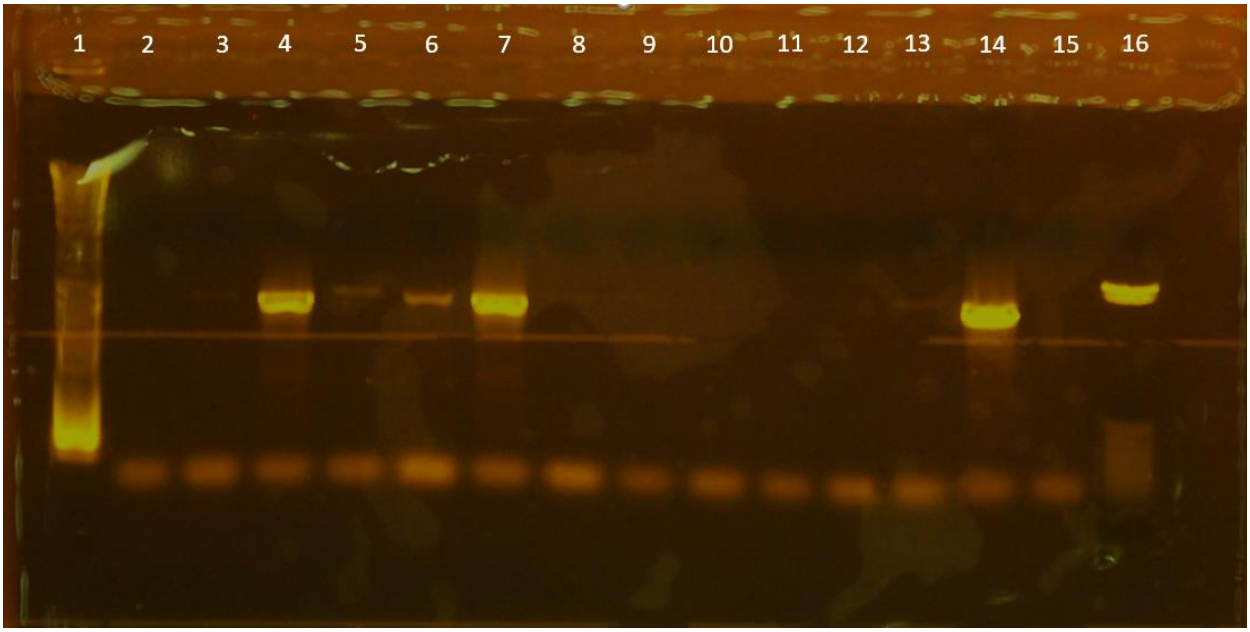

Figure 6. Amplification products of bacterial small ribosomal subunit gene (16S rDNA) of *D. arcticum.* The primers used were 27F and 16S R1 which render an amplicon of approximately 1465 bp. The samples were run in a regular agarose gel (0.9%) submerged in TAE (1X) at 100 volts. Furthermore, the lanes represent samples, **1**: Ladder 1Kb; **2**: A5, medium negative control; **3**: A6, growth control with Na_2SO_4; **4**: C2, $CaSO_4$ 0.1 wt %; **5**: C4, $MgSO_4$ 10 wt %; **6**: C6, $MgSO_4$ 18 wt %; **7**: C8, $FeSO_4$ 10 wt %; **8**: C10, $FeSO_4$ 14 wt %; **9**: C12, $Fe_2(SO_4)_3$ 10 wt %; **10**: C14, $Fe_2(SO_4)_3$ 20 wt %; **11**: C16, $Fe_2(SO_4)_3$ 30 wt %; **12**: C18, $Fe_2(SO_4)_3$ 40 wt %; **13**: C20, $Fe_2(SO_4)_3$ 48 wt %; **14**: PCR positive control, **15**: PCR negative control, **16**: 25 bp step ladder.

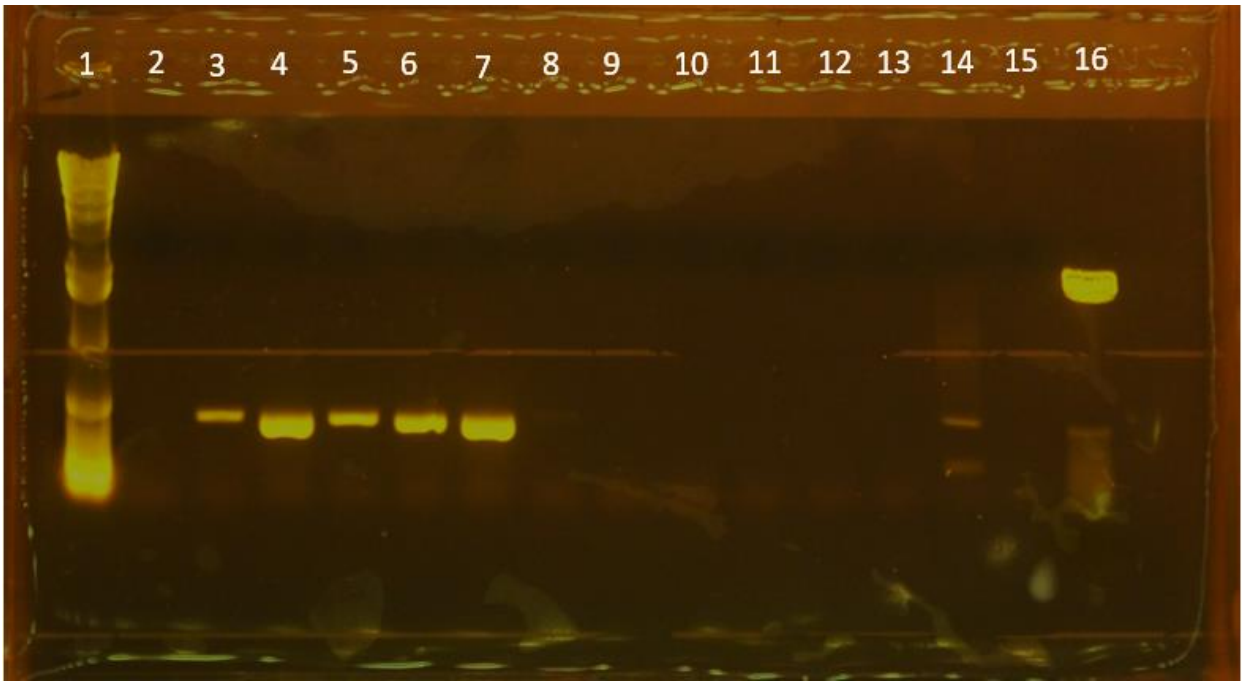

Figure 7. Gel electrophoresis of dsrA PCR products from *D. arcticum* subjected to different sulfate compounds. The primers used were dsrA_FWD and dsrA_REV which render an amplicon of 285 bp. The samples were run in low melting agarose gel (2.5%) submerged in TAE (1X). Furthermore, the lanes represent samples, **1**: Ladder 1Kb; **2**: A5, medium negative control; **3**: A6, growth control with Na_2SO_4; **4**: C2, $CaSO_4$ 0.1 wt %; **5**: C4, $MgSO_4$ 10 wt %; **6**: C6, $MgSO_4$ 18 wt %; **7**: C8, $FeSO_4$ 10 wt %; **8**: C10, $FeSO_4$ 14 wt %; **9**: C12, $Fe_2(SO_4)_3$ 10 wt %; **10**: C14, $Fe_2(SO_4)_3$ 20 wt %; **11**: C16, $Fe_2(SO_4)_3$ 30 wt %; **12**: C18, $Fe_2(SO_4)_3$ 40 wt %; **13**: C20, $Fe_2(SO_4)_3$ 48 wt %; **14**: PCR positive control, **15**: PCR negative control, **16**: 25 bp step ladder.

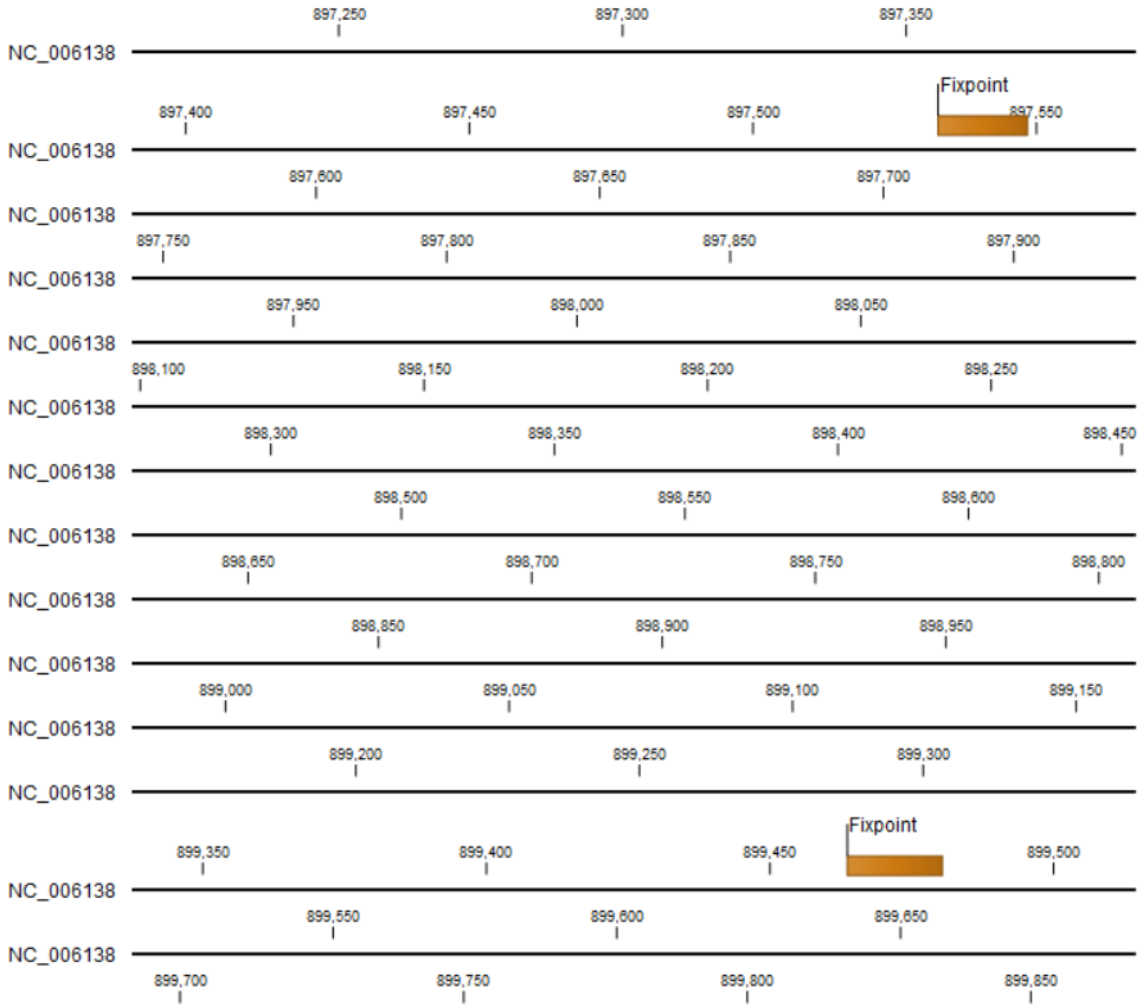

Figure 8. dsrAB operon of *D. psychrophila* that indicates the position of primers DSR1FD, and DSR4RE. Amplicon size of approximately 1900 bp. *D. psychrophila* genome portion obtained from GenBank (accession number NC_006138, Primers' matching performed with CLC Genomics Workbench 10.1.1).

Figure 9. dsrA portion of *D. arcticum* dsrAB operon that indicates the position of primers dsrA_FWD and dsrA_REV. Amplicon size of approximately 285 bp. *D. arcticum* genome portion obtained from GenBank (accession number FOOX01000011.1, Primers' matching performed with CLC Genomics Workbench 10.1.1).

Figure 10. Samples of sulfate reducers producing sulfide (black precipitate) as part of their metabolic activity (sulfate anaerobic respiration).