Exploring Microbial Activity in Low-pressure Environments

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Abstract
The importance of hypopiezophilic and hypopiezotolerant microorganisms (i.e., life that grows at low atmospheric pressures; see section 2) in the field of astrobiology cannot be overstated. The ability to reproduce and thrive at Martian atmospheric pressure (0.2 to 1.2 kPa) is of high importance to both modeling the forward contamination of its planetary surface and predicting the habitability of Mars. On Earth, microbial growth at low pressure also has implications for the dissemination of microorganisms within clouds or the bulk atmosphere. Yet our ability to understand the effect of low pressure on microbial metabolism, growth, cellular structure and integrity, and adaptation is still limited. We present current knowledge on hypopiezophilic and hypopiezotolerant microorganisms, methods for isolation and cultivation, justify why there should be more focus for future research, and discuss their importance for astrobiology.

1. Introduction

Earth's global average atmospheric pressure at sea level is 101.3 kPa (0.1 MPa) and can reach as high as 120 MPa at the bottom of the Mariana Trench 11 km below sea level (Picard and Daniel, 2013). On Earth, low-pressure environments below 101.3 kPa, are only present at high alpine sites in mountainous regions; the highest peak, Mt. Everest in Nepal, has a peak-height pressure of 33.0 kPa. With increasing altitude, the pressure drops to 1 kPa in the middle stratosphere and <1 Pa above 80 km. Therefore, life on Earth typically encounters pressures that range from 0.1 to 120 MPa (Oger and Jebbar, 2010; Yayanos, 1995). The pressure in interplanetary space is 1.32×10^{-8} kPa.

On Mars, the atmospheric composition and pressure differ dramatically compared to Earth. The average atmospheric pressure on the surface is approx. 0.7 kPa, which is equivalent to the atmospheric pressure at approx. 27 km altitude on Earth. The pressure on Mars varies between 0.1 kPa at the summit of Olympus Mons and 1.2 kPa in Hellas Basin (e.g., Barth et al., 1992; Rummel et al., 2014; Taylor et al., 2010). The gas-phase pressure (e.g., within interstitial spaces) increases very slowly in the lithosphere on Mars reaching 2.5 kPa at 13.8 km. In contrast, the lithographic overburden pressure can reach 2.5 kPa at only 19.5 cm below the surface in a confined niche (e.g., ice or salt inclusions) covered by rock or regolith (Schuerger et al., 2013).

Furthermore, the atmosphere on Mars consists mainly of CO_2 (approx. 96%) with low partial pressures of nitrogen (2%), argon (1.7%), and O_2 (0.13%; Mahaffy et al., 2013). In contrast, Earth's atmosphere is composed of nitrogen (78%), oxygen (21%), argon (1%), and trace amounts of CO_2 and other gases.

Mars, as a candidate for finding life elsewhere in the Solar System, has been of interest for space fairing nations for several decades, and remains a central goal in astrobiology. With data about the planet's geology, atmosphere, etc. returned from different Mars missions since the 1960s, and the in-depth knowledge about life in extreme terrestrial environments, Martian habitability has become a key focus (Cockell et al., 2016). To consider any environment or extraterrestrial body as habitable, a plethora of different requirements need to be met in order to allow life to survive and eventually thrive. Potential energy sources, ambient geochemical composition, the availability of a life-sustaining solvent, protection from biocidal factors, and the availability of carbon sources all need to be taken into account. In fact, at least 17 biocidal factors have been identified that potential life on Mars would encounter (Beaty et al., 2006; Rummel et al., 2014; Schuerger et al., 2012). Examples of global environmental hazards on Mars include a CO_2-dominated anoxic atmosphere, UV solar irradiation, hypobaria (0.1 – 1.2 kPa), low global temperatures (-61 °C), and extremely low water activity (a_w) of the surface regoliths. Other more episodic or randomly distributed factors include high salinity, low pH in certain soils, unknown or poorly described redox potentials (Eh) in hydrated brines, oxidizing compounds such as perchlorates prevalent in the regolith, the presence of heavy metals, UV-induced volatile oxidants (O^{2-}, O^{-}, H_2O_2, NO_x, O_3), solar particle events, and galactic cosmic rays.

Consequently, it has to be evaluated whether the combination of these factors provide environmental conditions suitable to build microbial biomass. Various environmental stressors have been extensively studied on different forms of life since the 1930s. These include simulation experiments (reviewed by Olsson-Francis and Cockell, 2010; Rothschild and Mancinelli, 2001), flight missions in Earth orbit using rockets and

balloons (DasSarma et al., 2017; Pulschen et al., 2018), outside Earth's orbit during NASA's Apollo program (reviewed by Horneck et al., 2010) and exposure experiments on the EXPOSE platform mounted outside the International Space Station (Rabbow et al., 2009; 2012; 2017). The stressors tested include UV radiation, gamma-rays, galactic cosmic rays, vacuum, high and low temperatures, and freeze-thaw cycling and combinations thereof. In addition, exposure to oxidizing chemicals, vacuum and subsequent survivability has been explored in a myriad of experiments (e.g., Horneck 1981; Horneck et al., 1994; Paulino-Lima et al., 2010; 2011; Sancho et al., 2007). The model organisms were mostly bacteria (e.g., *Bacillus subtilis*, *Deinoccoccus radiodurans*), but also archaea, viruses, fungi, lichens, and tardigrades have been tested (see reviews by Mileikowsky et al., 2000; Horneck et al., 2010; Nicholson et al., 2000). While numerous publications exist on the effects of high pressures on microbial biology (see section 8), surprisingly, little information is published on the effects of hypobaria on microbial metabolism, growth, cell integrity, and adaptation.

Herein, we review the current status of knowledge on microorganisms capable of metabolism and cellular replication at pressures below the average atmospheric pressure on Earth of 101.3 kPa down to 0.7 kPa. The literature cited serves as a brief introduction to concepts of hypobaric microbiology. Furthermore, we propose a redefinition of the word hypobarophiles (coined by Schuerger et al., 2013). Next we give a short overview about microbial survival and growth at low pressures, and describe the results of the molecular studies done on the topic. We also briefly present the effects of high pressure on microorganisms focusing on the major adaptations of piezophiles to cope at extreme oceanic depths. Lastly, we discuss the implications of low-pressure microbiology for astrobiology (e.g., Des Marais et al., 2008). This review hopes to convince readers that studies on microbial activity in low-pressure environments are of high importance to gain insights into basic biological mechanisms, the factors involved in adaption to low-pressure environments, the likelihood of microbial growth in the upper atmospheric boundaries of Earth's biosphere (0.7 kPa is equivalent to 34 km in the middle stratosphere of Earth), and the potential to assess habitability of extraterrestrial planetary bodies like Mars. In addition, the information may allow us to improve the development of planetary protection guidelines for robotic and manned space missions to Mars.

2. Definitions: Piezophilic, hypopiezotolerant, and hypopiezophilic microorganisms

Low-pressure microbiology is a nascent field in extreme environments because there are no low pressure ecological niches on Earth, except for the possibility of mid-troposphere cloud microbiology. But even here, it is debatable whether the mid-troposphere (or stratosphere) can function as a unique ecological niche, with long-term microbial survival, growth, and

adaptations, or merely as a conduit between ecosystems (DasSarma and DasSarma, 2018; Diehl, 2013; Griffin et al., 2018; Smith, 2013). Perhaps the best chance of finding a microbial community truly adapted to low-pressure niches will be the discovery of extraterrestrial life in the shallow subsurface of Mars (Schuerger et al., 2013) at a pressure range of 0.1 to 1.2 kPa (Rummel et al., 2014; Taylor et al., 2010). Here we would like to reevaluate the term *hypobarophile* for bacteria growing under low-pressure conditions between 0.7 and 1.2 kPa, and propose new terms that are consistent with microbial species able to reproduce under a wide spectrum of pressures on Earth from sea level (0.1 MPa) to the deep hadal regions in oceanic trenches (<120 MPa; see Yayanos, 1995).

The term *barophile* was coined by ZoBell and Johnson (1949), and has been used historically to refer to microorganisms growing above 10 MPa (Eisenmenger and Reyes-De-Corcuera, 2009; Picard and Daniel, 2013). However, Yayanos (1995) proposed the term *piezophile* (Greek: *piezo* = to press, and *philo* = love) instead to be consistent with the use of the prefix *piezo*- in physics and chemistry. Thus, piezophiles refer to high-pressure microbial species that optimally grow between 10 and < 60 MPa found in the deep lithosphere or oceanic benthic regions which are defined as the piezosphere (Jannasch and Taylor, 1984; Fang et al., 2010; Oger and Jebbar, 2010). The *piezosphere* which starts 1 km below sea level, excludes the upper 1 km because it is considered too well mixed (Fang et al., 2010; Oger and Jebbar, 2010). Yayanos (1995) further proposed adding the term *hyper*- to piezophile to refer to microbial species that optimally grow at pressures between 60 and 120 MPa.

To be consistent with the use of the term piezophile for a microorganism adapted to high-pressure environments, we will use the term *hypopiezophile* to refer to a microorganism that grows optimally under hypobaric conditions < 2.5 kPa (0.025 MPa). To date, no true hypopiezophile has been described. However, currently 62 bacterial isolates have been identified that can tolerate low-pressure conditions and grow at pressures down to 0.7 kPa (section 5, Nicholson et al., 2013a; Schuerger et al., 2013; Schuerger and Nicholson, 2016) and are therefore considered hypopiezotolerants. We propose to withdraw the term hypobarophile as being inaccurate because the bacteria described do not optimally grow at low pressures, but instead tolerate pressures down to 0.7 kPa in which growth rates are significantly retarded compared to normal growth at 101.3 kPa. Thus, the term *hypopiezotolerant* is more appropriate and consistent with the terms proposed by Yayanos (1995).

3. Experimental methods - how to isolate hypopiezotolerant microorganisms

3.1. Desiccators

To grow microorganisms at low pressures the construction of hypobaric systems holding pressure as low as 0.7 kPa at 0 °C are required (Figure 1). A simple system (Schuerger et al., 2013) was developed to simulate three conditions (pressure, P; temperature, T; and atmosphere, A) found on the surface of Mars, henceforth called *low-PTA* conditions, that are defined as: 0.7 kPa (close to the average surface pressure on Mars; Rummel et al., 2014), 0 °C (to maintain stable liquid water near its triple point; Haberle et al., 2001), and a CO_2-enriched anoxic atmosphere (96% CO_2 on Mars; Mahaffy et al., 2013). The systems can also accommodate Earth-normal pO_2/pN_2 atmospheres of 21% and 78%, respectively, by allowing room air to diffuse into the desiccators instead of flushing the chambers with CO_2 or including anaerobic pouches in the system.

Three approaches have been used to isolate and identify hypopiezotolerant bacteria from culture collections and environmental samples (see Schuerger et al., 2013; Schuerger and Nicholson; 2016, respectively). First,

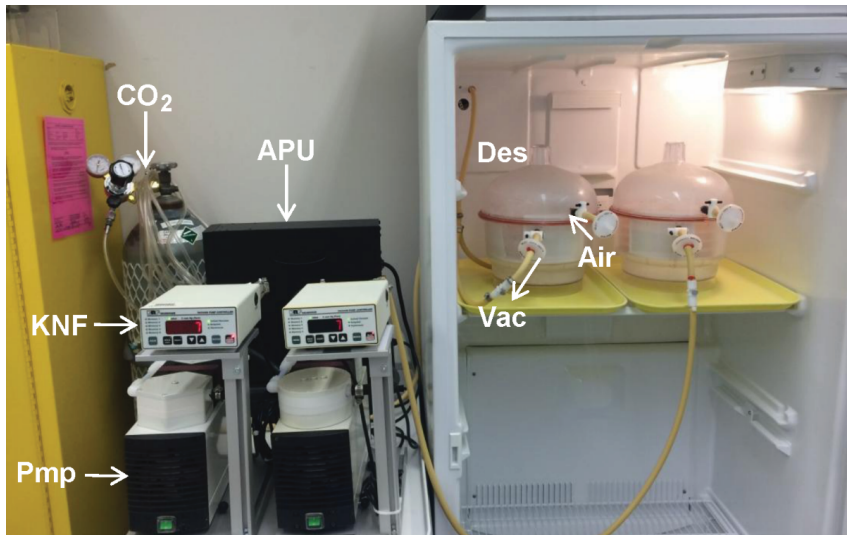

Figure 1. Two vacuum control systems (KNF) and pumps (Pmp, model PU-842, KNF Neuberger, Trenton, NJ, USA) were connected to individual 4-L desiccators (Des, model 08-642-7, Fisher Scientific, Pittsburgh, PA, USA) via vacuum (Vac) lines (described by Schuerger et al., 2013). The KNF controllers should be connected to a battery back-up (APU) unit to prevent losing the KNF controller program if any power glitches occur. The vent lines (Air) are used to repressurize the desiccators. Ultra-high purity carbon dioxide (CO_2) gas is used to flush room air out of the desiccators (2-3 minutes) prior to sealing the desiccators and connecting the vacuum lines.

the purified isolates are either streaked (Figure 2) or spotted on the media of choice (Nicholson et al., 2013a), placed in the hypobaric desiccators and either flushed with CO_2 or air, prior to closing the vent lines. When anoxic conditions are required, four anaerobic pouches are placed in each desiccator to continuously maintain low pO_2 < 0.1% (Van Horn et al., 1997). Pumping directly down to 0.7 kPa, would lead to cracks, bubbles, or distortions in the agar, and boiling of liquid media. Thus, a slow pump-down protocol was developed that sequentially reduces the internal atmospheric pressures down to 10, 5, 2.5, and finally 0.7 kPa in 15 minute increments. This procedure allows for adequate time to outgas internally trapped gases in agars or liquids, and permits a slow cool down to 0 °C of the media. When stabilized at 0 °C and 0.7 kPa, agar surfaces and liquid media can be stable for 28-35 days. When using semi-solid media, it is important to use double-thick layers of agar (≈ 30 mL) in deep petri dishes.

Up to 100 strains (Nicholson et al., 2013a; Schuerger et al., 2013) could be evaluated per petri dish at low-PTA conditions using the streak or spot technique. Each 4-L desiccator can hold up to 12-15 double-deep petri dishes which are stored at 4 °C until all plates are ready for insertion into the low-pressure desiccators. This process prevents strains from initiating growth at room temperature while other plates are prepared. It is important to include both positive hypopiezotolerant controls (e.g., *Carnobacterium* sp. in position #10 in Figure 2A and 2B; Nicholson et al., 2013a) and

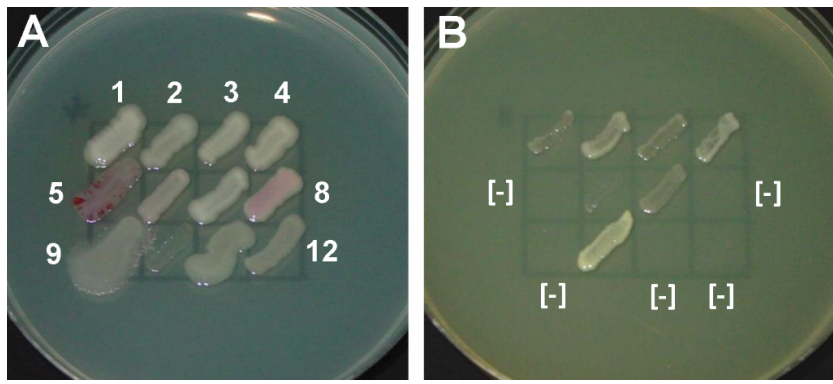

Figure 2. Example of the streak technique to check for growth of hypopiezotolerants at low-PTA conditions. Displayed is the growth of eight *Serratia* spp. and appropriate controls under Earth-normal conditions at 101.3 kPa and 30 °C (**A**) and low-PTA conditions used to simulate the Martian surface at 0.7 kPa (**B**). Positions 1 to 8 include (from left to right, and top to bottom): *S. ficaria* DSM4569, *S. fonticola* DSM4576, *S. grimesii* ATCC14460, *S. liquefaciens* ATCC27592, *S. marcescens* ATCC13880, *S. plymuthica* DSM4540, *S. quinivorans* DSM4597, and *S. rubidaea* ATCC27593. The bottom row was composed of negative [-] and positive controls that included (from #9 to #12): *Bacillus subtilis* 168 (negative), *Carnobacterium* sp. WN1359 (positive), *Escherichia coli* ATCC35218 (negative), and *Sporosarcina aquamarina* SAFN-008 (negative) (adapted from Schuerger and Nicholson, 2016).

negative controls (e.g., *Bacillus subtilis* 168 in position #9; Schuerger et al., 2013) in the assays.

In the second method, a soil-dilution protocol was developed to screen environmental samples such as arctic and alpine soils (Figure 3; Schuerger and Nicholson, 2016). The soil-dilution protocol combines 1.0 g of thawed soil and 25 mL of a pre-autoclaved 0.1% agar solution in a 125 mL Erlenmeyer flask. The soil suspension is vigorously agitated using a magnetic stirrer for 10-20 minutes to form a very dilute but semisolid agar matrix that will keep all soil particles in suspension for several minutes after stopping the agitation. Two hundred µL of the agar/soil suspension is then pipetted and spread onto the agar surface using sterile pre-cut 1000 µL pipette tips with the bottom 2-3 mm of the tips cut off prior to sterilization to avoid clogging of the tips by soil particles. The plates are then transferred into the desiccators and incubated as described above.

In the third method, the hypobaric desiccator set-up is used for growing hypopiezotolerant microbes in liquid medium down to 0.7 kPa. This protocol requires that the depth of the liquid medium be kept to a minimum

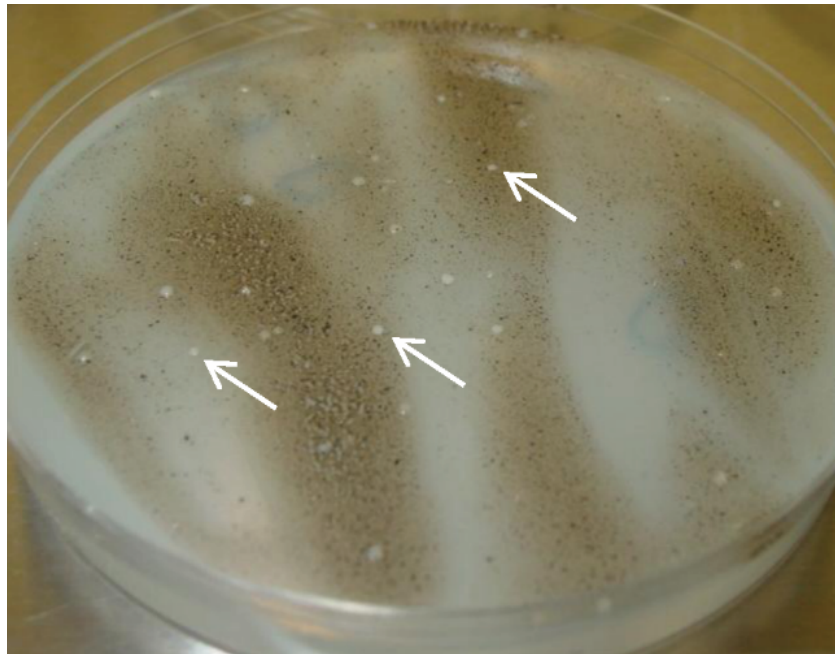

Figure 3. Isolation of hypopiezotolerant bacteria from environmental soil samples incubated under low-PTA conditions for 4 weeks. Hypopiezotolerant bacterial colonies (arrows) were recovered from an arctic soil from Colour Lake, Axel Heiberg, Canada (Schuerger and Nicholson, 2016).

(≤ 7 cm) to maintain pressure at the bottom of the culture tube consistent with the atmospheric pressure in the desiccator (e.g., Fajardo-Cavazos et al., 2018), and that the tubes are not sealed in order to permit the equalization of internal and external pressures in the desiccator. Another liquid medium protocol using 96-well plates was recently developed to examine the metabolic fingerprint of *Serratia liquefaciens* utilizing 95 carbon sources in Biolog GN2 microarray plates (Schwendner and Schuerger, 2018).

3.2. Hydrologic and thermodynamic issues related to growth of microbes at low pressures

The classic paper by Haberle et al. (2001) was the first to describe the triple point of water in the context of the Mars surface environment. It identified that the pressure (0.61 to 1.24 kPa) and temperature (0.1 to 10°C) ranges for stable liquid water on Mars severely constrain the habitability of the terrain to thin films of water plausibly formed during short-term melting of ice. Based on these findings we like to emphasize the limits of agar or liquid medium habitability in the hypobaric protocols described above.

As the pressure is lowered from 2.5 kPa to 0.7 kPa, temperature must also be concomitantly lowered from 30 to 0 °C (compare Schuerger and Nicholson, 2006 to Schuerger et al., 2013, respectively). Attempts to incubate double-thick agar plates at 30 °C and pressures below 2.5 kPa resulted in the agar surfaces splitting, pitting, and desiccating over only 48-72 hours (Schuerger and Nicholson, 2006). Within the stable liquid water "zone" of pressure and temperature on Mars (see above), the agar was stable for at least 35 days. In essence, incubations at low pressures near the surface "range" on Mars (0.1 to 1.2 kPa) are controlled by the thermodynamics of stable liquid water close to the triple point. At pressures lower than 0.5 kPa, liquid water cannot be maintained unless liquid brines are used with concurrent depression of the freezing point of water (Heinz et al., 2018), which in turn changes osmotic pressure and water activity that may also inhibit microbial activity for some microbes. Although extreme halophiles can tolerate extremes of osmotic pressure and low water activity (DasSarma and DasSarma, 2015; Fox-Powell et al., 2016).

Haberle et al. (2001) also cautioned that even though the pressure and temperature ranges in an ecological setting on Mars might fall within the range for stable liquid water, evaporation of liquid water will still occur. The best way to envision this process is to watch how the low-pressure control systems work near 0.7 kPa. After the samples are placed in the desiccators (or in Mars simulations chamber, see below), it generally takes 60-90 minutes to stabilize the pressure and temperature to 0.7 kPa and 0 °C, respectively. But over time, water from the samples evaporates and increases the pressure in the chamber/desiccator systems, which causes the hypobaric control systems to kick-in to lower the pressure. During this

process, the water vapor is removed and consequently, the medium slowly desiccates. Thus, these hypobaric assays are a thermodynamic struggle in which adequate microbial growth must occur before the water reserves of the agar or liquid media are exhausted.

3.3. Other Mars simulation chambers

Many designs have been published for hypobaric Mars simulation chambers, but few have been used to attempt to grow bacteria, archaea, or fungi under low-PTA conditions. Most of the more complex and instrumented Mars chambers have been used to study the survival of microorganisms, biosignature molecules, or geochemical processes under diverse conditions found on Mars (e.g., dos Santos et al., 2016; Gomez et al., 2010; Schuerger et al., 2008; Stan-Lotter et al., 2003).

Table 1 lists 14 Mars simulation chambers, constructed of stainless steel tanks placed in either a vertical or horizontal orientation with numerous ports, electrical connector feeds, cooling systems, and UV illumination sources, that have been evaluated for the control of low pressure, gas composition, UV irradiation, and atmospheric composition. Most chambers can be adapted for microbial metabolism, growth, and adaptation experiments, but in general, the more complex Mars chambers are primarily relevant when UV exposures are required in the simulations. However, the same thermodynamic issues described above for agar and liquid media in the 4-L desiccators will also hold for maintaining hydrated growth media in the more complex chambers.

For studying active metabolism and growth with hydrated media, the systems need to be compatible with water vapor, otherwise the listed chambers only work effectively for microbial survival, desiccation, and UV irradiation experiments. Only one chamber was specifically designed to handle liquid medium under simulated Martian conditions (i.e., the Planetary Environmental Liquid Simulator (PELS) chamber; Martin and Cockell, 2015). The PELS chamber can accommodate six independently controlled liquid samples in isolated vessels at low pressures down to 0.1 kPa between –50 and +70 °C. To collect aliquots from *in situ* experiments without venting the PELS chamber, separate sampling lock-out chambers for each reaction vessel were installed. Most of the chambers described in Table 1 appear to have extra ports that could accommodate internal sampling lock-out systems.

The Mars chambers in Table 1 were selected in part because they are likely to be available for new research into the survival, metabolism, growth, and adaptation of hypopiezotolerant microorganisms relevant to near-term robotic and human missions. Thus, the details provided here are offered as a brief primer for new Mars astrobiology research.

Table 1. List of Mars simulation chambers described in the literature since 2000. These chambers were selected here due to their plausible adaptability to conduct microbial survival and growth experiments at pressures < 10 kPa, and the inclusion of drawings, photographs, and specifications of the chambers in the cited papers.

Chamber name	Lowest pressure reported (kPa)	Temperature range (°C)	UV bands	Location	References
Andromeda Chamber	0.07	–25 to 24	none	Univ. of Arkansas, Fayetteville, AR, USA	Sears et al., 2002; 2005
LISA and mini-LISA Mars Chambers	5 x 10^{-3}	–123 to 57	UVC, UVB, UVA	Astronomical Observatory of Padua, Italy	Galletta et al., 2011
Mars Chamber Simulator	0.6	–80 to 20	UVC, UVB, UVA	Open University, Milton Keynes, UK	dos Santos et al., 2016
Mars Environmental Simulation Chamber	0.01	–140 to 25	UVB, UVA	Univ. of Aarhus, Aarhus, Denmark	Jensen et al., 2008
Mars Simulation Chamber (MSC)	0.01	–85 to 70	UVC, UVB, UVA	Univ. of Florida, Kennedy Space Center, FL	Schuerger et al., 2008
Mars Simulation Chamber	1.5	–123 to 25	UVC, UVB, UVA	Univ. of Maryland, MD, USA	Ertem et al., 2017
Mars Simulation Facility	0.7	–0 to 20	UVC, UVB, UVA	German Aerospace Center (DLR), Berlin, Germany	De la Torre Noetzel et al., 2018
Mars Simulation Vacuum Chamber	5 x 10^{-4}	–196 to 24	UVC, UVB, UVA	Leiden Univ., Leiden, The Netherlands	ten Kate et al., 2003
MaSimKa Chamber	1 x 10^{-6}	–20 to 85	UVC, UVB, UVA	German Aerospace Center (DLR), Cologne, Germany	Rabbow et al., 2016
Pegasus Planetary Simulation Chamber	5	24 (ambient only)	none	Univ. of Arkansas, Fayetteville, AR, USA	Kral et al., 2011
PELS Chamber	0.1	–50 to 70	UVB, UVA	Univ. of Edinburgh, Edinburgh, UK	Martin and Cockell, 2015
Planetary Atmosphere Simulation Chamber	0.7	–120 to 24	UVC	Centro de Astrobiologia Centre, Madrid, Spain	Gomez et al., 2010
Shot Mars Chamber	5	–80 to 26	UVB, UVA	Techshot, Inc., Greenville, IN, USA	Thomas et al., 2008
SRI Mars Chamber	1 x 10^{-3}	–60 to 24	none	Austrian Academy of Science, Graz, Austria	Stan-Lotter et al., 2003

4. Microbial survival experiments in low pressure or space vacuum

The majority of experiments under low-pressure conditions have reported on the survivability of microorganisms rather than actual growth. Test organisms for survivability studies initially included mainly spores from bacteria and fungi, but later microbial vegetative cells were also investigated. Portner et al. (1961) was among the first to demonstrate the survival of microorganisms after exposure to ultra-high vacuum (down to 2.6 x 10^{-11} kPa). Additional Earth-based studies on bacterial spores of *Bacillus stearothermophilus*, *B. megaterium*, *B. subtilis*, *Clostridium sporogenes*, and *Aspergillus niger* as well as vegetative cells of *Staphylococcus epidermidis* and *Micrococcus sp*. exposed to ultra-high vacuum (10^{-3}-10^{-10} kPa) showed evidence of survival, but also revealed a temperature-effect (Brueschke et al., 1961; Hagen et al., 1971; Morelli et al., 1962; Silverman et al., 1964).

After Earth-based tests showed that microorganisms can survive ultra-high vacuum, space studies were conducted (see reviews by Horneck et al.,

2010; Nicholson et al., 2000). For example, following six years in space and shielded from solar UV irradiation, vacuum-only effects on monolayers of *Bacillus subtilis* showed that only 1-2% of spores survived; but multilayered aggregates of spores coated in either glucose or buffering salts revealed increased survival to 80% (Horneck et al., 1994). Similar effects on microbial survival for monolayers versus multilayers have been reported for other bacterial species exposed to low pressures (Mancinelli and Klovstad, 2000; Osman et al., 2008; Schuerger et al., 2005). In a series of experiments investigating the effects of several simulated Martian conditions on bacterial survival, Schuerger et al. (2003) observed ~30% inactivation of *B. subtilis* spores due to low pressure alone (0.7 kPa). Moeller et al. (2012) exposed *B. subtilis* and eight mutants to a simulated Martian atmosphere at 0.7 kPa and reported up to a 1.5 log reduction for certain mutants while the majority of tested *Bacillus* strains exhibited only minor reductions in spore survival. In all of these examples, spore survival rates under low pressures were enhanced if the cells were present as multi-layered aggregates.

A few studies have tested the survival of archaeal species to low-pressure environments, in combination with other simulated Martian conditions (e.g., Kral et al., 2011; Mickol and Kral, 2017; Mancinelli, 2015; Morozova et al., 2007). For example, *Methanothermobacter wolfeii*, *Methanosarcina barkeri,* and *Methanobacterium formicicum* survived up to 120 days of desiccation and *Methanococcus maripaludis* survived 60 days at 0.6 kPa.

Survival studies are of high importance and great value with regard to planetary protection and the prediction of habitability (see section 9). However, they do not give an indication whether the microorganisms have the ability to grow in low-pressure environments.

5. Current status of hypopiezotolerants

5.1. Microorganisms able to grow in low-pressure environments (101.3 < 2.5 kPa)

Low-pressure environments are defined as environments with pressures below the Earth-normal sea level pressure of 101.3 kPa. Initially, a low-pressure threshold of 2.5 kPa for microbial growth was proposed by Schuerger and Nicholson (2006) based on early results with the growth of seven *Bacillus* spp. Later experiments established the growth of hypopiezotolerant bacteria down to 0.7 kPa in which *Bacillus* spp. were in general unable to grow below 2.5 kPa (section 5.2; Nicholson et al., 2013a; Schuerger et al., 2013; Schuerger and Nicholson, 2016).

The current observed low-pressure threshold for microbial growth is 0.7 kPa, though this may not be the actual limit. Table 2 summarizes microbial species that have been observed to grow under hypobaric conditions (i.e., < 10 kPa) in various growth media and hypobaric chambers. The data were selected based on our interpretation that the presented data showed

unequivocal evidence of microbial growth at pressures < 10 kPa. However, there were a few notable exceptions to the data in Table 2 that are worth mentioning.

First, Hawrylewicz et al. (1968) reported growth of *Staphylococcus aureus* in both O_2 and CO_2 atmospheres between 1 and 4 kPa, but failed to give details on the temperatures used during incubation. Furthermore, sealed tubes were used that were initially pressurized to 1, 2.5, or 4 kPa, but no mechanism was mentioned to either verify or adjust the pressure during the course of the experiments. This shortfall makes it difficult to assess whether growth occurred at the pressures indicated or at higher pressures resulting from evaporated water from the growth media.

Second, Pokorny et al. (2005) demonstrated growth of *Escherichia coli* and *Bacillus subtilis* at 33 kPa, much higher than the upper limit of hypopiezotolerant bacteria being considered here. These results were eventually superseded by several other studies in Table 2 growing both species down to 2.5 kPa.

Third, Pavlov et al. (2010) reported growth of a *Vibrio* sp. at 0.001 to 0.01 kPa. However, this study is problematic for three reasons: (1) starting cell densities (10^5 to 10^6 cells per assay) were always higher than the measured cell densities at the end of the Mars simulations (≤ 2.63×10^4 cells per assay). (2) There was no data that could be used to verify that the pressures being measured were in fact present in sample. Often pressures measured by a gauge external to a chamber can be several kPa lower than the actual pressures present inside the hypobaric chambers due to local effects of water evaporation or ice sublimation. And (3), the purported pressure range used was significantly below the triple point of water on Mars (section 3.2), and thus, water could only persist as either ice or vapor in the assays. Based on these reasons we conclude that Pavlov et al. (2010) actually observed cell survival and not growth at the low pressures reported.

Fourth, several papers describe problems with maintaining low pressures due to evaporation of water from hydrated media at temperatures greater than the Mars "water stability window" described in section 3.2 (Haberle et al., 2001). For example, Bauermeister et al. (2014) used the MaSimKa chamber for experiments on the growth of *Acidithiobacillus ferrooxidans* at low pressures. The microbial incubations were planned for 20 °C and 0.7 kPa, but only a pressure of 1.5 kPa could be maintained at that temperature due to the evaporation of water from the growth matrix. Similar problems with desiccation of media at low pressures and elevated temperatures have been described by Thomas et al. (2008) and Schuerger and Nicholson (2006).

And lastly, we want to point out that in the study reported by Kral et al. (2011), growth was indirectly measured for *Methanothermobacter wolfeii*, *Methanosarcina barkeri* and *Methanobacterium formicicum* by monitoring methane evolution. Methane production rates which were linked to growth without cell counts being performed, were reported down to 5.0 kPa, but not tested at lower pressures. Methane production in archaea does not always correlate to actual growth and cell proliferation.

Additionally, a number of studies have explored the effects of both Low-Earth Orbit space and Martian conditions on the survival of a diversity of lichens (e.g., Brandt et al., 2015; de la Torre Noetzel et al., 2018; Meeßen et al., 2015; and citations within). However, no studies to date have measured growth of lichens directly under hypobaric conditions similar to the Martian surface. A few studies have tried to correlate photosynthetic activity to growth through monitoring the chlorophyll fluorescence of PS I and PS II systems, and have revealed varying results (increased fluorescence in de Vera et al., 2010; stable fluorescence in Sanchez et al., 2014; or decreased fluorescence over time in Sanchez et al., 2014) when exposed to 0.7 to 1.0 kPa and UVC fluence rates found on Mars. In general, lichen viability, photosynthesis, and cellular structures all appear to decrease over time when exposed to simulated conditions found on the surface of Mars (see Sanchez et al., 2014; Meeßen et al., 2014, de Vera et al., 2014). It remains to be shown if lichens can actually acquire hydrated nutrients, carry out metabolism, and increase cell numbers under simulated Martian conditions at 0.7 kPa.

As indicated above, other important factors to consider are the impacts of temperature on growth rates at low pressures, atmospheric composition in the assays, and whether spores or vegetative cells are investigated. Schuerger and Nicholson (2006) demonstrated that although vegetative cells of *B. pumilus* (SAFR-03, FO-36B), *B. subtilis* (HA-101, 42HS-1), *B. nealsonii*, and *B. licheniformis* were able to grow slowly at 2.5 kPa at 30 °C in O_2/N_2 or CO_2 atmosphere, at 20 °C growth was inhibited indicating a temperature effect. *B. megaterium* was not able to grow at 2.5 kPa. In addition, their endospores were, in general, only able to germinate and subsequently grow at atmospheric pressures higher ≥ 5.0 kPa in Earth-normal O_2/N_2 atmosphere at 30 °C. Interestingly, endospores of one strain, *B. subtilis* HA 101, were able to germinate and grow at 3.5 kPa, 30 °C, and Earth-normal pO_2/pN_2 conditions, but not in CO_2-enriched anoxic atmospheres at the same pressures and temperatures. In contrast, endospores of *B. nealsonii* and *B licheniformis* were able to germinate and grow at 3.5 kPa, 30 °C, and CO_2 atmospheres, but not in pO_2/pN_2 atmospheres (Schuerger and Nicholson, 2006). Thus, atmospheric composition during the low-pressure growth assays had a markedly different effect on the germination of endospores for seven *Bacillus* spp.

Table 2. Examples of low-pressure microbial growth at which clear signs of growth were reported. Examples given here were selected based on the testing of microbial growth at or below 10 kPa. Lowest pressures and temperatures are indicated.

Species	Pressure (kPa)	Temperature (°C)	Gas Mix
Chorella ellipsoidea[a]	10	–80 night +29 day	CO_2
Chroococcidiopsis sp[a]	10	–80 night +29 day	CO_2
Plectonema boryanum[a,b]	10	–80 night +29 day	CO_2
Anabaena sp.[a,b]	10-5	–80 night +29 day	CO_2
Synechocystis sp.[b,c,d,e]	10-5	32	5% CO_2
Bacillus subtilis [f,g,h]	5	27-37	O_2
Methanothermobacter wolfeii [i]	5	35	50:50 H_2:CO_2
Methanosarcina barkeri [i]	5	35	50:50 H_2:CO_2
Methanobacterium formicicum [i]	5	35	50:50 H_2:CO_2
Pseudomonas aeruginosa [j]	5	30	O_2 or CO_2
Synechococcus sp.[b]	5		5% CO_2
Bacillus (6 spp.)[k]	2.5	30	O_2 or CO_2
Enterococcus faecalis [j]	2.5	30	O_2 or CO_2
Escherichia coli [l]	2.5	20	CO_2
Staphylococcus aureus [j]	2.5	30	O_2 or CO_2
Bacillus sp.[m]	0.7	0	CO_2
Carnobacterium (10 spp. [m,n]	0.7	0	CO_2
Clostridium sp.[m]	0.7	0	CO_2
Cryobacterium sp.[m]	0.7	0	CO_2
Exiguobacterium sibiricum.[m]	0.7	0	CO_2
Paenibacillus (3 spp.)[m]	0.7	0	CO_2
Rhodococcus qingshengii.[m]	0.7	0	CO_2
Serratia (6 spp.)[m]	0.7	0	CO_2
Serratia liquefaciens [j,m]	0.7	0	CO_2
Streptomyces (2 spp.)[m]	0.7	0	CO_2
Trichococcus (3 spp.)[m]	0.7	0	CO_2

[a]Thomas et al., 2008; [b]Thomas et al., 2005; [c]Kanervo et al., 2005; [d]Pokorny et al., 2005; [e]Sakon and Burnap, 2006; [f]Waters et al., 2014; [g]Nicholson et al., 2010; [h]Fajardo-Cavazos et al., 2012; [i]Kral et al., 2011; [j]Schuerger et al., 2013; [k]Schuerger and Nicholson, 2006; [l]Berry et al., 2010; [m]Schuerger and Nicholson, 2016; [n]Nicholson et al., 2013a

5.2. Bacteria able to grow at low-PTA conditions

A set of experiments investigated microbial growth under low-PTA conditions, i.e., 0.7 kPa, 0 °C, and a CO_2 atmosphere (Figure 4, Table 2; Schuerger et al., 2013; Nicholson et al., 2013a; Schuerger and Nicholson, 2016). In order to reduce the pressure to 0.7 kPa and maintain a stable hydrated growth medium, the temperature had to be concomitantly lowered to 0 °C (triple point of water; see section 3) and therefore the cells were exposed to two or more stresses simultaneously when determining the low-pressure limit for growth. Another factor that has a negative effect on growth in combination with low pressure and temperature is the atmospheric environment (e.g. oxygenated versus CO_2-enriched environments) in which the experiments are conducted. Results indicated

that the majority of the tested microbial species remained in an inactive or dormant state at 0.7 kPa, but were not killed by the low-PTA conditions. When returned to optimal growth conditions at 30 °C, 101.3 kPa and Earth-normal atmosphere, microbial growth was resumed and colony development observed. The underlying mechanism for the inhibition at 0.7 kPa is currently not known. Thus, low-PTA conditions should be considered as non-lethal for the majority of microorganisms tested.

The first hypopiezotolerant microorganisms being described to grow at 0.7 kPa were isolates of the genera *Carnobacterium* (Nicholson et al., 2013a) and *Serratia* (Figure 2; Schuerger et al., 2013). When exposing nine type-strains of *Carnobacterium* (*C. alterfunditium*, *C. divergens*, *C. funditum*, *C. gallinarum*, *C. inhibens*, *C. maltaromaticum*, *C. mobile*, *C. pleistocenium*,

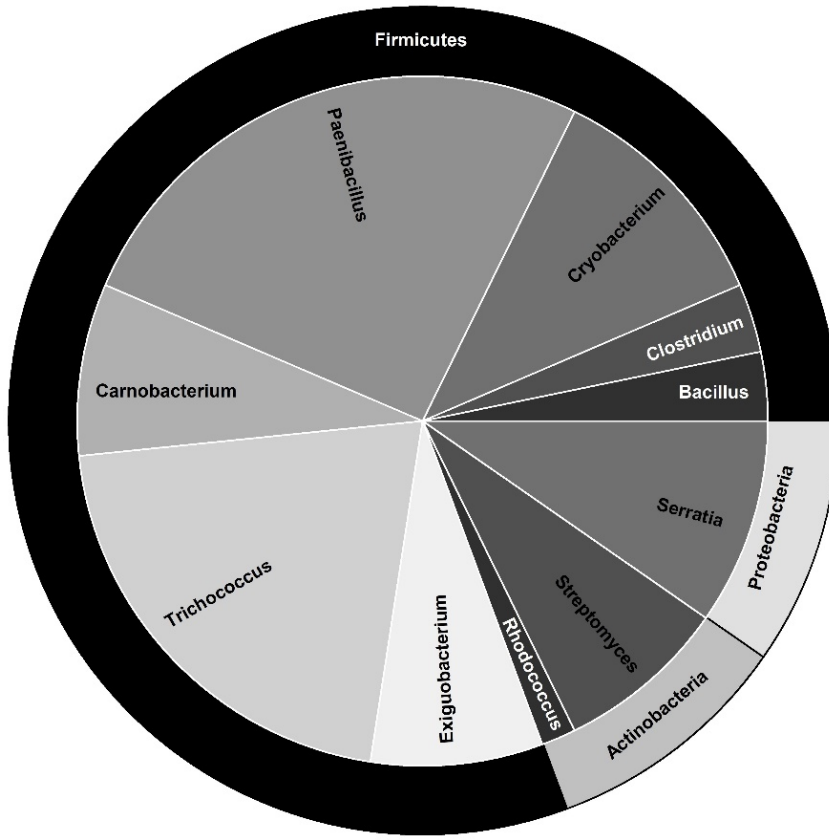

Figure 4. Microbial diversity of hypopiezotolerant bacteria on phylum and genus level (adapted from Schuerger and Nicholson, 2016).

and *C. viridans*) and eight type-strains of *Serratia* (*S. ficaria*, *S. fonticola*, *S. grimesii*, *S. liquefaciens*, *S. marcescens*, *S. plymuthica*, *S. quinivorans*, and *S. rubidaea*) to low-PTA conditions, all tested type strains of *Carnobacterium*, but only six of eight *Serratia* species were able to grow at 0.7 kPa (e.g., Figure 2 for *Serratia* spp.; Schuerger and Nicholson, 2016). In contrast, seven *Bacillus* spp. were not able to grow at pressures < 3.5 kPa in one study (Schuerger and Nicholson, 2006), while two undescribed *Bacillus* isolates were able to grow at 0.7 kPa in a second study (Schuerger and Nicholson, 2016). These results suggest that the capability of growing at 0.7 kPa may be species-specific and not ubiquitously manifested within a genus.

Schuerger and Nicholson (2016) described 62 bacterial isolates that grow at 0.7 kPa (Figure 4). The bacteria belonged to three different phyla and grouped within 10 bacterial genera. Fifty-eight percent of these isolates were identified on species level including *Paenibacillus antarcticus, P. macquariensis, Rhodococcus qingshengii, Streptomyces aureus, S. vinaceus, Exiguobacterium sibiricum, Trichococcus pasteurii, T. collinsii, Serratia liquefaciens, S. ficaria, S. fonticola, S. grimesii, S. plymuthica, S. rubidaea*. To date, no Archaea, fungi nor other eukaryotic organisms have been reported capable of growth in low-PTA conditions at 0.7 kPa. *Serratia liquefaciens* has become the most studied model organism for growth at low-PTA conditions, and had its whole genome sequenced (Nicholson et al., 2013b), paving the way for more complex molecular studies under low-PTA conditions (e.g., Fajardo-Cavazos et al., 2018).

Additional cultivation approaches to detect the total number of culturable hypopiezotolerants compared to the total viable microorganisms led to the enrichment of hypopiezotolerant bacteria, but not archaea or fungi, from a range of soils including permafrost and a nonglacial high arctic lake (Table 3). Samples from mesophilic environments were negative for indigenous hypopiezotolerant bacteria (Schuerger and Nicholson, 2016). The results indicated a general low percentage of culturable hypopiezotolerant microorganisms in these samples compared to the total culturable fraction incubated at 101.3 kPa, 25 °C and Earth-normal atmosphere.

6. Exploring the microbial "dark matter" of hypopiezophiles and -tolerants

The portion of microorganisms that cannot be cultivated in the laboratory are described as the microbial "dark matter". The problems associated with soil assay protocols (section 3.2) illustrate the severe limitations to predicting the amount/number of hypopiezophiles/-tolerants in samples using only cultivation basal methods. The primary limitation to the agar media assays to date is that it only works effectively for culturable microorganisms with visually observable colonies that grow under low-PTA conditions (e.g., Figure 3). It is plausible that many other culturable strains did, in fact, grow under low-PTA conditions but were lost in the soil particles present on the agar because their colony sizes were below the limits of

Table 3. Portion of hypopiezotolerant bacteria in various soil samples including permafrost and a non-glacial high arctic lake incubated at low-PTA conditions (0 °C, CO_2, 0.7 kPa) in the hypobaric chamber compared to the total viable microorganism count at 25 °C, 21% pO_2, 101.3 kPa. Cultivation was done on agar plates [adapted from Nicholson et al., 2013a; Schuerger and Nicholson, 2016]. Only bacterial colonies were observed.

Sample	Hypopiezotolerants	Total viable cells at 25°C, O_2, 101.3 kPa
Siberian permafrost	6[a]	9.3×10^3
Mt. Baker, Washington	1.9×10^2	1.2×10^8
Devon Island, Canada	2.5×10^2	3.4×10^4
Siberian Permafrost, Russia	2.8×10^4	1.5×10^8
Colour Lake, Axel Heiberg Island	5.1×10^4	1.0×10^7

[a]Given as number of colony forming units (cfu) per gram of soil

visual detection using 5x jeweler glasses (Schuerger and Nicholson, 2016). In addition, estimates revealed that 85-99% of bacteria and archaea, respectively, cannot yet be grown in the laboratory and the numbers can vary highly depending on the environment sampled and media used (Lok, 2015). Furthermore, the hypobaric protocols are not yet being fine-tuned to finding non-culturable hypopiezotolerant microorganisms. Thus, there is a crucial need for new assay protocols that can explore the so-called microbial dark matter of non-culturable microorganisms in environmental samples, and to identify slow-growing fastidious hypopiezotolerant microorganisms.

7. Cultivation and molecular approaches to unravel influences of low-pressure environments on and within the microbial cell

Microorganisms have evolved abilities to sense, respond, and adapt to a variety of physical parameters in the environment. However, little is known about how bacteria sense low pressure, how they acclimatize to pressure alterations and whether they possess pressure-specific adaptations, marker genes, or metabolic pathways. The following studies investigated the effects of hypobaria on the genome, gene expression, protein synthesis, lipid composition and metabolism of bacteria grown under low-pressure conditions. Furthermore, currently it is unknown whether these potential adaptations are species-specific or ubiquitous.

7.1. Adaptation experiments and genomic changes

The findings that microorganisms, which did not grow at low pressure but resumed growth when returned to Earth-normal pressure (Schuerger et al., 2013; Schuerger and Nicholson, 2006) led to the hypothesis that target molecules might exist within cells that are reversibly inactivated at low pressure. To understand the response and adaptation of microorganisms to low pressure, adaptation experiments with *Bacillus subtilis* were conducted

for over 1,000 generations at the near-inhibitory low pressure of 5.0 kPa. Populations of the evolving strains were sampled every 50 generations, and led to the isolation of a low-pressure evolved strain that had developed higher growth rates at 5.0 kPa compared to wild-type strains maintained at 101.3 kPa (Nicholson et al., 2010). Compared to its ancestral strain, the evolved strain rapidly acquired increased fitness for higher growth rates at 5.0 kPa starting at 200 generations. The adaptations were akin to steps in punctuated equilibrium (defined by Gould, 2002) for the evolution of a low-pressure adapted *B. subtilis* strain over time.

To identify genomic alterations, like changes or mutations that were induced by the low-pressure treatments, whole-genome sequencing of the adapted strain and respective mutants was performed (Waters et al., 2015). The genomic adaptations to low pressure were found to be a dynamic process and revealed complex kinetics, i.e., different patterns of mutations that appeared in either early or late stages of the experiment with some of the earlier mutations not being detected in the end. During the 1,000 generations, final amino acid-altering mutations of seven genes and a single 9-bp in-frame deletion in a RNA degradosome encoding gene were detected (Waters et al., 2015). However, data on genomic changes are still scarce and only available from one strain at 5.0 kPa. There is still a lack of data from other hypopiezotolerant microorganisms grown at low-PTA conditions between 0.7 and 1.0 kPa. Apart from changes in the whole genome, adaptation to low pressure can be expressed on multiple levels, as for example gene expression.

7.2. Gene expression studies

Another step towards the identification of molecular mechanisms in hypopiezotolerant microorganisms adapted to novel environmental stresses at low pressure is to investigate the gene expression under hypobaric conditions. Currently, there are two separate studies available on two different bacterial strains.

Fajardo-Cavazos et al. (2012) investigated the gene expression of a low-pressure-adapted *B. subtilis* strain (section 7.1) to explore the mechanisms that enabled the previously low-pressure inhibited strain to grow at 5.0 kPa. A cluster of three candidate genes (*des*, *desK*, and *desR*) was up-regulated. The *des* gene encoding a Des membrane fatty acid (FA) desaturase, the *desK* encoding a DesK sensor kinase and *desR* genes encoding a DesR response regulator are involved in the maintenance of membrane fluidity. Inactivation of the *des* gene achieved by a knockout mutation, resulted in decreased fitness of the evolved strain to 5.0 kPa.

The first transcriptomics study under low-PTA conditions (0.7 kPa, 0 °C, CO_2 atmosphere) was performed using the hypopiezotolerant bacterium, *Serratia liquefaciens* ATCC 27592 (Fajardo-Cavazos et al., 2018) to search for new insights into the molecular mechanisms responsible for microbial

adaptation to alterations in their pressure environment. RNA-seq and subsequent transcriptome analyses revealed significant differentially expressed transcripts. Up-regulation in genes that encode transporters (ABC and PTS transporters), genes that are involved in translation (ribosomes and their biogenesis, biosynthesis of tRNAs and aminoacyl-tRNAs), DNA repair and recombination, and non-coding RNAs were observed. More specifically, several amino acid, purine, and sugar carbohydrate pathways were up-regulated at 0.7 kPa. Genes down-regulated included transporters (mostly ABC transporters), flagellar and motility proteins, transcription factors, and two-component systems. Despite the observed changes, the alterations did not reflect a major rearrangement of the transcriptome nor were pressure-specific genes identified. However, the data rather indicated that the transcriptome profile from *S. liquefaciens* at 0.7 kPa was a complex process altering gene expression and resulting in a stress response triggered by several environmental stressors acting simultaneously.

7.3. Membrane structure

The gene expression studies (section 7.2) suggest an effect of low pressure on the cell membrane. At high hydrostatic pressures (> 10 MPa), membranes become more rigid due to increased packing of membrane fatty acids (FAs) and proteins, leading to cellular adaption in which unsaturated FAs increase and saturated FAs decrease in an attempt to maintain membrane fluidity and functionality (Oger and Jebbar, 2010). In contrast, at low pressures a less ordered packing of membrane components is assumed when compared to Earth-normal atmospheric pressure, which likely increases the fluidity of the membrane. Consequently, it might affect the FA composition in an opposite manner to the effects reported under high pressure. In turn, the membrane processes including proton pumping, nutrient and ion transport as well as protein translocation are altered due to the increased disordering.

For example, analysis of membrane FA composition of *Bacillus subtilis* vegetative cells grown at the Earth-normal pressure of 101.3 kPa compared to 5.0 kPa revealed a decrease in the ratio of unsaturated to saturated FAs but an increase in the ratio of anteiso- to iso-FAs (Fajardo-Cavazos et al., 2012). Currently, it is not known whether these effects are widespread in the domains Bacteria, Archaea, and Eukarya, and valid for all hypopiezotolerants/philes, or whether they are species-specific. In addition, there are no data available on the membrane composition of other bacteria grown at low-PTA-conditions near 0.7 kPa. More research is required to unravel the effects of hypobaria on membrane structure.

7.4. Carbon source utilization

The changes of the metabolic fingerprint of *Serratia liquefaciens* ATCC 27592, a hypopiezotolerant model organism, was investigated under decreasing atmospheric pressures to 2.5 kPa and low-PTA conditions

(Schwendner and Schuerger, 2018). Currently, this is the only study that has investigated microbial metabolism under low pressures. To outline the metabolic changes, Biolog GN2 microarray plates were used to test the utilization of 95 different single carbon sources. Apart from temperature and atmospheric composition, decreasing the atmospheric pressure revealed a distinct effect on the metabolic fingerprint. More specifically, above 10 kPa *S. liquefaciens* utilized the various carbon sources in a similar manner as observed at an Earth-normal pressure of 101.3 kPa; whereas below 10 kPa, significant changes were observed indicating that the cells may have undergone physiological alterations. In particular, *S. liquefaciens* preferred to utilize a range of carbohydrates while the cells lost the ability to metabolize the majority of the provided carbon sources with a significant decrease in the oxidation of amino acids. These alterations were suggested to be the result of several potential stress-induced reactions such as potential physiological changes, the alteration of gene expression, or changes in the membrane which in turn affected the uptake of nutrients. Data on the metabolic responses to different carbon sources are of great value to identify nutritional constraints that support cellular replication in low-pressure habitats.

8. Looking at the other end of the spectrum - what can we learn from piezophiles?

We believe that we have not yet determined the limits to life in high- and low-pressure environments. An upper limit of growth at pressures between 130-150 MPa was described for *Pyrococcus yayanosii* (Zeng et al., 2009), but most other hyperpiezophiles are only able to grow up to 60-110 MPa (Oger and Jebbar, 2010; Picard and Daniel, 2013). Piezophiles and hyperpiezophiles have been found in both bacterial and archaeal domains. Strains have been isolated from the subseafloor oceanic crusts, deep subterranean environments such as the Mariana Trench with a maximum depth of 11 km (~ 110 MPa), and many other high-pressure marine locations on Earth (Fang et al., 2010; 2017; Inagaki et al., 2015; Margosch et al., 2006; Nogi et al., 1998; Russell et al., 2016). Studies on how microorganisms adapt to high pressures reveal alterations in gene expressions, protein synthesis, and membrane lipid composition (reviewed in Bartlett et al., 1995; Kato and Bartlett, 1997; Oger and Jebbar 2010).

With increasing pressure, double stranded DNA becomes more rigid which negatively affects the transition into single strands; a prerequisite for replication, transcription, and translation. Consequently, protein and nucleic acid synthesis is hindered (Oger and Jebbar, 2010; Simonato et al., 2006). In addition, increased packing occurs in lipid membranes leading to decreased fluidity; which in turn affects the permeability of cells for water and nutrient uptake, and protein-lipid interactions (Oger and Jebbar, 2010; Winter and Jeworrek, 2009). Similarly, proteins adapt their conformations according to volume restrictions caused by high pressures, which negatively affect multimer associations, stability, and catalytic sites, leading

to a loss of enzymatic function and metabolic activity (Balny et al., 2002; Northtrop, 2002). Without adaptation mechanisms to cope with high-pressures and its induced alterations of cellular architectures, these effects would eventually cause cell death.

Three mechanisms have been proposed to counteract the damages experienced at high pressures: (1) upregulation of gene expression to compensate for the loss of biological activity leading to an increase in specific components to manage energy and osmotic stability as well as chaperons aiding protein folding (Campanero et al., 2005; Fernandes et al., 2004); (2) expression of pressure-inducible genes such as those involved in the ToxR/S two–component system (Bartlett, 1991; Kato and Qureshi, 1999); and (3), structural changes of biomolecules by increasing the proportion of unsaturated fatty acids in cell membranes at high pressures (Chilukuri and Bartlett, 1997; Fang and Bazylinksi, 2008). Microorganisms not adapted to but able to withstand high pressures can synthesize pressure inducible proteins (PIPs) which are "stress" proteins previously identified as heat shock, cold shock, or ribosomal proteins or proteins with unknown function (Bartlett et al., 1995).

We predict that the genomic, metabolic, and physiological adaptive trends for piezophiles growing at lower pressures (down to 0.1 MPa) would be similar to microorganisms which are optimally adapted to Earth-normal pressure growing at hypobaric conditions close to 0.7 kPa. For example, saturated fatty acid (FA) levels in general decrease and unsaturated FA levels increase in piezophiles as the hydrostatic pressure is increased (see reviews by Oger and Jebbar, 2010; Picard and Daniel, 2013). Conversely, we would expect to see saturated FAs to increase and unsaturated FAs to decrease at low pressures < 10 kPa in bacteria ecologically adapted to sea-level pressures of 0.1 MPa. Such a situation was reported for *B. subtilis* strains grown at low pressures down to 5.0 kPa (Fajardo-Cavazos et al., 2012). Thus, the study of piezophiles and (hyper-)piezophiles growing at pressures lower than their normal ecological niches may provide insights on the genomic, metabolic, and cellular adaptations of mesophilic bacteria, normally adapted to sea-level pressures of 0.1 MPa growing in hypobaric environments down to 0.7 kPa.

9. Implications of hypopiezotolerants to Mars astrobiology

Despite the current planetary protection regulations and the vigorous cleaning efforts taken place before launch, spacecraft launched into space carry finite amounts of viable microorganisms. In fact, on a Category IVa mission to Mars (i.e., soft-landed spacecraft without life-detection payloads) the bioburden on the vehicle is required to be < 3 x 10^5 spores per spacecraft; and on Category IVb and IVc missions (i.e., life-detection payloads present), the bioburden reductions are even more stringent requiring the total surface bioburden of the spacecraft to be < 30 spores (Frick et al., 2014). Both, vegetative cells and spores are present on

spacecraft with spores beingthe most likely to survive interplanetary transfer to Mars, and therefore might pose the greatest potential forward contamination risks (Kempf et al., 2005; Moissl-Eichinger et al., 2013; Nicholson et al., 2009). However, results of investigating the effects of low pressure on spores indicates that spores fail to germinate at low pressure (Schuerger and Nicholson, 2006). Once launched, spacecraft microorganisms face harsh environmental conditions including high UV irradiation, extreme desiccation, ionizing radiation, vacuum, and extreme thermal cycling; which leads to reductions of survivability between 50-70% for spores and up to two orders of magnitude for vegetative cells during a typical 6 to 8-month cruise-phase to Mars (Dose and Klein, 1996; Hagen et al., 1971; Horneck et al., 1994; Koike and Oshima, 1993).

Thus, a small number of viable spacecraft microorganisms are likely to survive the cruise phase to Mars (Horneck et al., 2010; Nicholson et al., 2000) and may be present on spacecraft surfaces after landing. The initial evidence indicated that a few bacteria (e.g., Figure 4), but so far no fungi or archaea, have been shown to grow at pressures down to 0.7 kPa (i.e., the average surface pressure on Mars). However, the astrobiology community has barely scratched the surface characterizing the effects of hypobaria on microbial survival, metabolism, growth, and adaptation under relevant Martian conditions. Nevertheless, low pressure is an emerging extreme environmental factor which needs to be addressed when discussing habitability of Mars and planetary protection issues related to new missions with life-detection payloads to Mars. It is plausible that microorganisms originating from Earth with the capability of growing at 0.7 kPa may have an unwanted or previously unpredicted impact on the search for life on Mars by creating a risk of false positives in the assays.

Some of the studies cited above (e.g., Nicholson et al., 2013a; Schuerger et al., 2013) suggest that some Earth microorganisms possess the physiological range to initiate microbial activity and grow on Mars if they are dispersed to hydrated niches that might support microbial activity (e.g., recurring slope lineae; Ojha et al., 2015). For example, growth of *S. liquefaciens* on Biolog GN2 organics at 0.7 kPa (Schwendner and Schuerger, 2018) suggests that if similar organics are found on Mars from accreted interplanetary dust particles and carbonaceous chondrites (e.g. Sephton and Botta, 2005) or *in situ* organics (Eigenbrode et al., 2018), then heterotrophic metabolic activity using *in situ* organics might occur on Mars if the microorganisms are dispersed to stable, UV-protected, and hydrated niches. Data on the ecological settings and nutrient requirements of microbial hypopiezotolerant strains in a simulated Martian environment are key factors to both ascertain the potential risk of forward contamination and to determine whether Mars is or might have been habitable.

Another aspect to consider is that in the past the atmospheric pressure was much higher than it is now (Brain et al., 2010). The observation that *B.*

subtilis was able to evolve and gain fitness over 1,000 generations when grown at 5.0 kPa (Nicholson et al., 2010) suggests that other microorganisms may have the capability to adapt to low-pressure environments on a short timescale; perhaps even to adapt to low-PTA conditions at 0.7 kPa. Thus, the study of hypopiezotolerant microorganisms growing at pressures similar to the surface of Mars (0.1 to 1.2 kPa) not only allows predictions for forward contamination in general and of Special Regions in particular (i.e., locations that might support the growth of Earth microorganisms, or harbor an extant Mars microbiota; Rummel et al., 2014), but also may help us locate terrains in which to search for an extant microbiota and provide insights into the habitability of Mars. In addition, hypopiezotolerant microorganisms can serve as positive controls for the development of life-detection experiments and payloads.

Consequently, experiments searching for hypopiezophilic and hypopiezotolerant microorganisms on Earth, and investigating their growth and adaptation to Martian conditions, may provide data that strengthens the hypothesis of an extant microbiota on Mars. However, more research is required to examine the ability of Earth microbes to grow and proliferate on Mars under low-PTA conditions that are relevant to future exploration missions.

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