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# Twenty-three Species of Hypobarophilic Bacteria Recovered from Diverse Ecosystems Exhibit Growth under Simulated Martian Conditions at 0.7 kPa

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**Summary**

Bacterial growth at low pressure is a new research area with implications for predicting microbial activity in clouds, the bulk atmosphere on Earth, and for modeling the forward contamination of planetary surfaces like Mars. Here we describe experiments on the recovery and identification of 23 species of bacterial hypobarophiles (def., growth under hypobaric conditions of approximately 1-2 kPa) in 11 genera capable of growth at 0.7 kPa. Hypobarophilic bacteria, but not archaea or fungi, were recovered from soil and non-soil ecosystems. The highest numbers of hypobarophiles were recovered from Arctic soil, Siberian permafrost, and human saliva. Isolates were identified through 16S rRNA sequencing to belong to the genera *Carnobacterium*, *Exiguobacterium*, *Leuconostoc*, *Paenibacillus*, and *Trichococcus*. The highest population of culturable hypobarophilic bacteria ($5.1 \times 10^4$ cfu/g) was recovered from Colour Lake soils from Axel Heiberg Island in the Canadian arctic. In addition, we extend the number of hypobarophilic species in the genus *Serratia* to 6 type-strains that include *S. ficaria*, *S. fonticola*, *S. grimesii*, *S. liquefaciens*, *S. plymuthica*, and *S. quinivorans*. Microbial growth at 0.7 kPa suggests that pressure alone will not be growth-limiting on the martian surface, or in Earth's atmosphere up to an altitude of 34 km.

## 1. Introduction

Earth's global average atmospheric pressure at sea level is 101.33 kPa (i.e., 1013.3 mbar) and decreases with altitude to below 1 Pa above 80 km. In contrast, high pressure environments up to 1.1 GPa (i.e., 1086 bars) are present in deep sea sites like the bottom of the Mariana Trench in the Pacific Ocean. Numerous studies (see reviews in Michiels *et al.*, 2008) have explored how microorganisms respond to increasing pressure, and such piezophiles have been reported capable of metabolic activity up to 16 GPa (e.g., *Shewanella oneidensis* and *Escherichia coli*; Sharma *et al.*, 2002). However, what about low pressure? Are microorganisms metabolically active and capable of growth at significantly reduced atmospheric pressures? The question is relevant for studies into metabolic activity and growth of microorganisms in our terrestrial atmosphere and for extraterrestrial environments with low-pressure atmospheres such as Mars.

From a terrestrial perspective, a few studies have begun to explore metabolic activity of bacteria under partially simulated cloud and tropospheric conditions (e.g., Amato *et al.*, 2007; Santl-Temkiv *et al.*, 2013; Vaitilingom *et al.*, 2013). However, simulations of microbial activity under cloud or tropospheric conditions often have ignored low pressure as an experimental factor. Several exceptions can be noted. First, vegetative cells, but not endospores, of six *Bacillus* spp. exhibited growth down to 2.5 kPa in an Earth-normal $O_2/N_2$ atmosphere (Schuerger and Nicholson, 2006). Recent papers have demonstrated microbial growth and up-regulation of the *des-desKR* system encoding membrane fatty acid desaturase (Fajardo-Cavazos *et al.*, 2012), evolution (Nicholson *et al.*, 2010), and altered global gene expression (Waters *et al.*, 2014) of *Bacillus subtilis* strains cultivated under Earth-normal $O_2/N_2$ atmospheres at 5 kPa. A pressure range of 2.5 to 5 kPa is equivalent to altitudes of 20 to 25 km, respectively, in Earth's atmosphere.

From an extraterrestrial perspective, two recent studies have demonstrated growth of a few bacterial species to even low pressure environments < 2.5 kPa, including seven permafrost isolates and nine type-strains of *Carnobacterium* spp. (Nicholson *et al.*, 2013) and a single type-strain of *Serratia liquefaciens* (Schuerger *et al.*, 2013) that exhibited vigorous to moderate growth, respectively, on semi-solid media incubated under Mars surface conditions of 0.7 kPa, 0 $^{o}$C, and $CO_2$-enriched anoxic atmosphere. Taken together, a number of studies (Fajardo-Cavazos *et al.*, 2012; Nicholson *et al.*, 2010; 2013; Schuerger *et al.*, 2006; 2013; Schuerger and Nicholson, 2006; Waters *et al.*, 2014) have demonstrated that of over 150 bacterial strains tested under a range of decreasing pressures, most bacteria grew nominally between 10.0 and 101.3 kPa, with decreased growth rates noted for most bacteria below 10.0 kPa, and the cessation of growth for most species at 2.5 to 3.5 kPa. Only a few species including *Carnobacterium* spp. (0.7 kPa; Nicholson *et al.*, 2013), *Pseudomonas fluorescens* (1.5 kPa; Schuerger, unpublished), and *Serratia liquefaciens* (0.7 kPa; Schuerger *et al.*, 2013) exhibited growth below 2.5 kPa.

The primary objective of the current research was to determine if other archaea, bacteria, or fungi, from soil and non-soil ecological niches were capable of growth at pressures that approach those found in the middle stratosphere on Earth and the surface of Mars (i.e., 0.7 to 1.0 kPa). In a recent review, Smith (2013) argued successfully that studies into microbial survival, growth, and evolution in the terrestrial middle to upper atmosphere are likely to inform and guide planetary protection and astrobiology studies into potential microbial activity on Mars. The work described here was designed to screen for microbial activity under conditions relevant to the surface of Mars, but is applicable to microbial high-altitude studies in Earth's atmosphere.

We use the terms *hypobarophile* to denote microorganisms capable of metabolic activity and growth at pressures ≤ 1-2% sea level pressure (≤ 1-2 kPa), and *mesobarophile* to denote microorganisms that can only grow at pressures between 2.5 and 101.3 kPa.

## 2. **Materials and Methods**

### *2.1. Sample sources*

Soils were collected by several individuals from nine locations including Arctic, Antarctic, desert, alpine, and Kennedy Space Center sites (Table 1). Soils were shipped and stored at -15 $^{o}$C (most soils) or -70 $^{o}$C (permafrost soils only) until processed. All soils were collected from surface profiles to a depth of 1-2 cm, except the Siberian permafrost soils which were collected from permafrost boreholes (Nicholson *et al.* 2013).

Non-soil samples were collected from diverse ecological settings. Several local volunteers provided saliva, hand-rinsate, forearm hair, or scalp hair for assays. In addition, we collected plant leaf imprints on selective media, sea water samples from the surf along the Florida coast at the Merritt Island National Refuge, and swabs of bench tops in one of our labs (ACS). Saliva samples were collected as 1 mL aliquots in 15 mL sterile polystyrene tubes. Saliva samples (100 µL/plate) were then plated directly onto 0.5x trypticase soy agar (TSA). Hand rinsates were collected by submerging hands in 500 mL of sterile deionized water (SDIW) for 60 sec (with agitation), the water filtered through sterile 0.45 µm nitrocellulose filters (Whatman, 7190-004, GE Healthcare Life Sciences, Kent, England), filters inverted on TSA, and incubated as described below. Hair was collected aseptically in the lab from volunteers and placed directly on selective media. Sea water was collected, and then three 500-mL aliquots were filtered through separate sterile 0.45 µm nitrocellulose filters, the filters inverted on selective media, and incubated at 0.7 kPa, as described below. After 7 days, the filters from both

hand rinsate and sea water samples were removed and discarded. The filter cultures were then returned to the low-pressure conditions. Leaf imprints were created by randomly selecting fully expanded true leaves from a Coral Bean plant (*Erythrina herbacea* L.) adjacent to the Space Life Sciences Lab (SLSL; Merritt Island, FL). The leaves were inverted, and the adaxial surfaces pressed into agar surfaces for 30 sec. The leaf imprints were then incubated at 0. 7 kPa, as described below.

### *2.2. Soil dilution plate assays*

All soils were removed from cold storage and allowed to partially thaw for sample removal. One gram of each soil was added to separate 125 mL flasks containing 25 mL of autoclaved 0.1% water agar, and vigorously mixed with magnetic stir bars for 10 min. The 0.1% water-agar mixtures were semi-liquid when stirred and acted to keep soil particles in suspension. The soil/agar suspensions (200 µL/plate) were plated directly onto double-thick (30 mL per plate) 0.5x TSA (BD Difco, Becton, Dickinson, and Company, Sparks, MD, USA) or R2A agar (BD Difco) plates.

### *2.3. Hypobaric conditions*

After plating, all samples were immediately placed under low-pressure (0.7 kPa), low-temperature (0 $^{o}$C), and $CO_2$-enriched anoxic (low pp$O_2$) conditions (henceforth called low-PTA conditions), as described previously (Schuerger *et al.*, 2013). The hypobaric assays were conducted in 4-L polycarbonate desiccators (model 08-642-7, Fisher Scientific, Pittsburg, PA, USA) fitted with 0.45 µm HEPA filters (Whatman, model 6723-5000) on the top and bottom sections for sterile operation of the vacuum and repressurization processes (Schuerger *et al.*, 2013). The desiccators were connected to an external pump and controller (model PU-842, KNF Neuberger, Trenton, NJ, USA) capable of holding pressure at 0.7 kPa (+/- 0.1 kPa).

Anoxic conditions within the desiccators were achieved by first placing four anaerobic sachets (Remel AnerobicPack, Fisher Scientific) into the vacuum chambers, closing the desiccators, and flushing them with research-grade $CO_2$ for 5 min. The samples were then held in the low-PTA conditions for 28 d, with the anaerobic sachets changed every 7 d. After 4 wks, the plates were inspected visually for observable colonies (Round 1 hypobaric assay). Visible colonies were picked, streaked-purified on the same media (0.5x TSA or R2A) used in the Round 1 assays, and incubated at lab conditions of 101.3 kPa, 24 $^oC$, and Earth-normal $O_2/N_2$ atmosphere. The pure cultures were then applied to fresh media with heat-sterilized loops and incubated under low-PTA conditions for 14 d (Round 2). Single isolated colonies from Round 2 were picked, re-streaked onto fresh plates of the same medium, and incubated a third time under low-PTA conditions for 14 d (Round 3). Isolates that grew through Rounds 1-3 were given strain designations (Table 2), stored at -70˚C as frozen glycerol stocks, and characterized further.

The Siberian permafrost isolates were then processed for a separate study on the effects of low temperature on colony morphology and pigmentation. Permafrost hypobarophiles were grown on 0.5x TSA for 7 d at 15 $^oC$ under either an anoxic atmosphere ($CO_2$-enriched anaerobic chambers) or a lab-normal oxygenated atmosphere ($O_2/N_2$; 21%/78%, respectively). Bacterial colonies were imaged at identical magnifications and lighting conditions.

If either 0.5x TSA or R2A media were placed in the desiccators and pumped directly from 101.3 to 0.7 kPa, the media would often bubble, crack, and deform. Thus, in order to permit the outgassing of dissolved lab air from the agar, a three step pump-down procedure was developed. Media in petri dishes were placed in the 4-L desiccators as described above, set in the 0 $^oC$ incubator, pumped to a range of 7.5 - 10.0 kPa, and allowed to equilibrate for 30 min.

Then the vacuum chamber was pumped to 2.5 kPa for an additional 30 min. After a total elapsed time of 1 hr for outgassing, the vacuum chambers were then allowed to equilibrate at 0.7 kPa.

### *2.4. 16S rRNA sequencing*

Bacteria that demonstrated growth at 0.7 kPa through Rounds 1-3 were labeled as hypobarophiles, and the 16S rRNA genes were sequenced from purified strains. DNA was extracted from each strain with the UltraClean® Microbial DNA Isolation Kit (12224-50, MoBio Laboratories, Inc., Carlsbad, CA, USA) and PCR amplified according to the protocols of Benardini *et al.* (2003). All PCR amplicons were then cleaned using the QIAquick® PCR Purification Kit (model 28104, QIAGEN Sciences, Valencia, CA, USA). Universal bacterial primers for the 16S rRNA region were B27F (5'-GAGTTTGATCMTGGCTCAG-3') and B1512R (5'- AAGGAGGTGATCCA NCCRCA-3') (Lueders *et al.*, 2004). Purified PCR amplicons were Sanger sequenced at the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida (Gainesville, FL, USA).

Strains were assigned taxonomic affiliations based on ≥97.5% similarities (Stackebrandt *et al.*, 2002) to published entries in the Ribosomal Database Project (RDP) library (Release 10, Update 31) (Cole *et al.* 2009) [http://rdp.cme.msu.edu/]. Nucleotide sequences have been deposited in GenBank [http://ncbi.nlm.nih.gov/genbank; National Center for Biotechnology Information, USA] under the accession numbers given in Table 2.

### *2.5. Growth of type-strains for the genus Serratia under low-PTA conditions*

Recently, Schuerger *et al.* (2013) demonstrated that the type-strain *Serratia liquefaciens* (ATCC 25792) and Nicholson *et al.* (2013) demonstrated that nine type-strains in the genus *Carnobacterium* were capable of growth under low-PTA conditions at 0.7 kPa. In order to

determine if a genus-wide capability for low-pressure growth similar to *Carnobacterium* existed for *Serratia*, eight type-strains in the genus *Serratia* were incubated under low-PTA conditions.

Cultures of *Serratia* type-strains (Table 3) were grown under low-PTA conditions on 0.5x TSA for 35 d. Four additional bacterial strains were grown as either positive controls (*Carnobacterium inhibens* subsp. *gilichinskyi*, strain WN1359; Nicholson *et al.*, 2015) or as negative controls (Ba*cillus subtilis*, *Escherichia coli*, and *Sporosarcina aquamarina*; Schuerger *et al.*, 2013) for growth at 0.7 kPa. Growth for each strain was recorded using a simple rating system that ranked growth relative to the size and morphology of the control strains at Earth-normal lab conditions of 101.3 kPa, 30 $^{o}$C, and Earth-normal $O_2/N_2$ atmosphere (21%/78%, respectively). Ratings in Table 3 are given as [+] indicating weak growth to [++++] indicating vigorous growth (see Figure 1); [-] = no growth. All bacteria with a recorded [-] growth response after 35 d, were returned to lab conditions for 48 h. In all cases, the strains exhibiting negative growth at 0.7 kPa responded to the lab conditions and grew normally (data not shown).

## 3. **Results**

### *3.1 Isolation of hypobarophilic bacteria from soils*

Most soil samples grown under lab conditions of 101.3 kPa, 20 $^{o}$C, and Earth-normal $O_2/N_2$ conditions exhibited high diversities of bacteria and fungi on the agar surfaces of the soil-dilution plates. Figure 1A is an example of the high microbial diversity observed in four soils from Rio Tinto, Spain; Atacama Desert, Chile; Colour Lake, Axel Heiberg Island, Canada; and Mt Baker, CA, USA. Both bacterial and fungal colonies were observed with multiple colony morphologies and pigmentations. In contrast, when hypobarophilic bacteria were recovered

from soil-dilution plates maintained at 0.7 kPa only white, translucent, and smooth-margined colonies were observed (Fig. 1B).

Total culturable bacteria in soils were estimated to range between $5.8 \times 10^1$ colony-forming units (cfu)/g for the Antarctica soils from Battleship Promontory and $1.5 \times 10^8$ cfu/g of soil for the permafrost soils (Table 1). The total culturable fungi were estimated to range between zero for three cold-temperature soils from the Arctic or Antarctica and $3.7 \times 10^5$ cfu/g for the Florida soils collected around the Payload Hazardous Servicing Facility at the Kennedy Space Center.

Hypobarophilic bacteria were recovered from soils in a range between zero cfu/g for one-half of the soils tested and $5.1 \times 10^4$ cfu/g of soils from Colour Lake (Table 1). Although fungal colonies were abundant in most soils grown under lab conditions (Fig. 1A), no fungi were observed to grow on either 0.5x TSA or R2A when plates were incubated at low-PTA conditions. In addition, all bacteria-like isolates recovered from TSA or R2A agar surfaces incubated under low-PTA conditions (Fig. 1B) were initially sequenced with 16S primers. In all cases, 16S amplicons were recovered and identified to species in the domain bacteria. Thus, no archaea were recovered from TSA or R2A cultures incubated at low-PTA conditions.

Two hypobarophilic bacteria, *Streptomyces aureus* and *S. vinaceus* (Table 2), were isolated on three separate iterations of Round 1 assays with the Colour Lake soils but failed to grow under low-PTA conditions when streak-purified and tested in Round 2 assays. Although it is unknown if the failure to grow in Round 2 assays for *S. aureus* and *S. vinaceus* was due to missing geochemical or biological components in the soil matrix, the *Streptomyces* species were consistently isolated in three separate Round 1 assays in the presence of the soil particles and undescribed biological communities. Thus, we list them here as presumptive hypobarophiles.

All other bacteria that maintained growth at low-PTA conditions for Rounds 2 and 3 are listed as confirmed hypobarophiles (Table 2).

The focus for the current study was to recover and identify only culturable hypobarophilic species, and thus, no further work was attempted to isolate or identify culturable or nonculturable bacteria, archaea, and fungi that may have been concomitantly present in the soils. Furthermore, all hypobarophiles that grew under anoxic conditions at 0.7 kPa were also able to grow under Earth-normal $ppO_2$ at 101.3 kPa, except two strains of the obligate anaerobe *Clostridium* sp. (Table 2).

### *3.2. Isolation of hypobarophiles from non-soil samples*

After Round 1 assays, hypobarophilic bacterial colonies were observed on agar surfaces used to process human saliva and hand rinsate (volunteer #1 only) and sea water (coastal surf along Florida on the Merritt Island National Refuge). The hypobarophiles from saliva and hand rinsate were identified as *Leuconostoc gasicomitatum*, *L. gelidum*, and *L. inhae* (Table 2). In contrast, the two colonies observed at low-PTA conditions from sea water were not recovered during Round 2 sub-culturing assays (Table 1). Other samples from coral bean plants, forearm hair, lab benches, and scalp hair were negative for the presence of hypobarophilic bacteria. Fungi were not observed on any of the non-soil samples incubated at low-PTA conditions.

Several of the non-soil sample assays were conducted in a non-quantitative manner so no estimates of background populations of culturable bacteria or fungi were possible. However, coral bean surfaces (10 $cm^2$ basis) and lab benches (100 $cm^2$ basis) yielded low numbers of bacteria and fungi per unit areas of the assays (Table 1). In contrast, human saliva yielded no culturable fungi and high numbers of culturable bacteria ($6.4 \times 10^6$ cfu/mL) from all three volunteers.

### *3.3. 16S rRNA sequencing for confirmed hypobarophiles*

Table 2 lists 76 strains of hypobarophilic bacteria from all sources and is arranged in the following order of source materials: soils, non-soil samples, and type-strains of *Serratia* with the soils and then hypobarophiles listed in alphabetical order within each category. Although Table 2 represents primarily confirmed hypobarophiles that successfully completed three rounds of incubation under low-PTA conditions, five strains of the presumptive hypobarophiles *Streptomyces aureus* and *S. vinaceus* are included for completeness. Assigning taxonomic affiliation at the species level was based on the closest matches being at ≥ 97.5% 16S sequence identity (Stackebrandt *et al.*, 2002). If the closest matches were either below 97.5% in the RDP database, or the taxonomic affiliations listed in the RDP database lacked species level identities, the taxonomic affiliations were kept at the genus level.

Hypobarophilic bacteria were identified representing 3 phyla, 10 families, and 11 genera (Table 2), demonstrating the presence of hypobarophiles in diverse and globally distributed ecosystems. The highest numbers of hypobarophiles, with the greatest diversity, were recovered from Colour Lake and Siberian permafrost soils, and human saliva (volunteer #1) representing the genera *Carnobacterium*, *Exiguobacterium*, *Leuconostoc*, *Paenibacillus*, and *Trichococcus*. However, many of the hypobarophiles remain to be characterized at the species level. Hypobarophiles are now identified in the families Bacillaceae, Bacillales_Incertae Sedis XII, Carnobacteriaceae, Clostridiaceae, Enterobacteriaceae, Leuconostocaceae, Microbacteriaceae, Norcardiaceae, Paenibacillaceae, and Streptomycetaceae.

### *3.4 Growth of type-strains in the genus Serratia under low-PTA conditions*

Six of eight type-strains in the genus *Serratia* exhibited growth under low-PTA conditions including *S. ficaria, S. fonticola, S. grimesii, S. liquefaciens, S. plymuthica*, and *S.*

*quinivorans* (Fig. 2; Table 3). In contrast, *S. marcescens* and *S. rubidaea* failed to grow under all low temperature and low pressure conditions, and thus, the effect of low pressure alone could not be separated from the effect of low temperature. The growth of *Carnobacterium inhibens* subsp. *gilichinskyi*, strain WN1359 at low-PTA conditions confirms earlier work on growth of nine type-strains at 0.7 kPa for the genus *Carnobacterium*, including the observation that WN1359 grew slightly better at the low-PTA conditions at 0.7 kPa when compared to growth under aerobic lab conditions at 101.3 kPa (Nicholson *et al.*, 2013). The growth or lack of growth for the positive and negative controls, respectively, (Table 3) matched the growth under low-PTA conditions previously reported for these strains (Nicholson *et al.*, 2013; Schuerger *et al.*, 2013).

Hypobarophilic bacteria picked from 0.5x TSA or R2A media maintained under low-PTA conditions were, in general, white and translucent (Fig. 1B). Similarly, six of eight *Serratia* type-strains that grew under low-PTA conditions lost the pigmentation observed under lab conditions of 101.3 kPa, 30 $^{o}$C, and Earth-normal $O_2/N_2$ atmosphere (Fig. 2). In addition, the pigmentation of two strains, *Serratia plymuthica* and *Sporosarcina aquamarina*, exhibited increased red pigmentation under a lab pressure of 101.3 kPa when incubated at 0 $^{o}$C in a $O_2/N_2$ atmosphere, but then returned to a non-pigmented condition when *S. plymuthica* was grown at 101.3 kPa, 0 $^{o}$C, and $CO_2$-enriched anoxic atmosphere (Fig. 3). Results suggest that both temperature and low $ppO_2$ are responsible for the loss of pigmentation in bacterial cells of *Serratia* spp. tested under low-PTA conditions. Because maintaining stable hydrated media at 0.7 kPa requires low temperatures at or below 0 $^{o}$C (i.e., near the triple-point of water; Haberle *et al.*, 2001), the effects of low-pressure alone on cell pigmentation could not be discerned.

### *3.5. Growth and colony morphology of permafrost strains at 15 ˚C in the presence or absence of oxygen*

Similar to the depigmentation observed at reduced temperatures for the hypobarophiles *S. plymuthica* and *S. aquamarina*, multiple strains of *Cryobacterium* sp. (WN1502 and WN1485) and *Exiguobacterium sibiricum* (WN1486, WN1488, WN1492, and WN1493) were observed to lose their yellow/orange pigmentation when grown under anoxic conditions at 15 $^{o}$C (Fig. 3). The rest of the permafrost hypobarophile isolates produced rather small (<1-mm diameter), cream or light tan colonies; and grew either slightly better without oxygen, or at approximately the same rate regardless of the presence or absence of oxygen (Fig. 3). Thus, all isolates appeared to be either facultative aerobes or facultative anaerobes. And lastly, all permafrost hypobarophiles picked from soil-dilution plates incubated at low-PTA conditions were not pigmented (similar to Fig. 1B).

## 4. **Discussion**

The recovery of 23 species in 11 genera of hypobarophilic bacteria from diverse ecosystems supports the conclusion that such low-pressure adapted bacteria are common on Earth. Most hypobarophiles were detected in arctic, permafrost, and alpine soils, and thus, may indicate that many psychrophiles possess stress regulons that confer dual tolerance to hypobaria. However, hypobarophiles were also recovered from human saliva and hands, and type-strains of the hypobarophilic genera *Carnobacterium* spp. (current study; Nicholson *et al.*, 2013) and *Serratia* (current study; Schuerger *et al.*, 2013) were originally recovered from diverse sources including fig, fish, frozen meat, insect, milk, soil, and water niches (based on cited literature from ATCC and DSMZ culture collections). Thus, hypobarophiles from temperate ecosystems

cannot be ruled out. Intriguingly, no archaea (unknown presence) and no fungi (i.e., confirmed to be present in most soils samples under lab, but not low-PTA conditions; Table 1) were recovered in the Round 1 assays, suggesting that the capability to grow at low pressures might be constrained to the domain bacteria.

All hypobarophilic bacteria except *Clostridium* sp. exhibited growth under both anoxic (Round 1, 2, and 3 assays) and aerobic (during streak-purification under Earth-normal gases) conditions, and thus, can be labeled as facultative anaerobic hypobarophiles. This interpretation is consistent with other studies that found hypo- and mesobarophilic bacteria capable of growth at pressures between 0.7 and 2.5 kPa, respectively, in both anoxic and aerobic low-pressure conditions (Schuerger and Nicholson, 2006; Schuerger *et al.*, 2013). In contrast, two *Streptomyces* spp. were recovered from arctic soils during three separate iterations of Round 1 assays under low-PTA conditions, but would not grow again in Round 2 assays when streak-purified under lab conditions and retested under low-PTA parameters (Table 2). Also, colonies of presumptive hypobarophiles were observed on several Round 1 assays with alpine soils from Mt. Baker, CA and from sea water obtained from coastal surf near the Kennedy Space Center, FL, but failed to grow during attempts to streak-purify the isolates under lab conditions. Taken together, the failure of subsequent growth at 0.7 kPa for the *Streptomyces* spp. and presumptive hypobarophiles from Mt. Baker and sea water suggests that geochemical or microbial metabolites from the soils or sea water were required to support growth of these bacteria under low-PTA conditions. To date, no analytical procedures have been reported in the literature that can screen for nonculturable hypobarophiles.

Demonstration that six of eight hypobarophilic species type-strains in the genus *Serratia* could be grown under low-PTA conditions suggests that tolerance to low pressure is a near-

genus wide trait in *Serratia*. Similar results were reported for the genus *Carnobacterium* in which nine of nine species type-strains tested were capable of growth under low-PTA conditions (Nicholson *et al.*, 2013). Furthermore, multiple hypobarophilic species were found in the genera *Leuconostoc*, *Paenibacillus*, and *Trichococcus* (Table 2). Taken together, these results suggest that tolerance to low-pressure may be widely dispersed in some genera and may not be constrained to unique species.

One of the most intriguing observations in the current work was the recovery of three hypobarophilic *Leuconostoc* spp. from human saliva and hand rinsate from a single volunteer (three volunteers tested). After the detection of the *Leuconostoc* spp. from saliva and hands, Volunteer #1 was retested on two separate occasions with negative results. The recovery on only one of three Round-1 assays from a single volunteer suggests that the detection of *Leuconostoc* hypobarophiles may have been associated with the consumption of food by Volunteer #1 and not indicative of an extant human-associated hypobarophile microbial community.

Future research should be conducted to expand the list of selective media beyond TSA and R2A for culturing hypobarophiles from environmental samples in order to rule out the role media chemistry might play on recovering hypobarophilic bacteria from the samples. Although we present evidence that both temperature and gas composition can alter cell pigmentation (Figs. 2 and 3), the loss of cell pigmentation observed for bacterial cells under diverse conditions may have been partly due to the media-types used. In previous work (Schuerger and Lee, 2015; Schuerger unpublished), we have used 0.5x TSA and R2A successfully to recover bacteria and fungi from oligotrophic environments. At this time, we cannot rule out the recovery of hypobarophilic bacteria, archaea, or fungi under low-PTA conditions if other media and incubation protocols are used.

### *4.1. Implications for growth of bacteria in Earth's atmosphere*

Numerous papers have reported the recovery of terrestrial bacteria and fungi in Earth's atmosphere, with the accepted maximum altitude of detection at 41 km (see review by Smith, 2013). But none of the atmospheric sampling studies cited by Smith (2013) examined metabolic activity at the sampled altitudes, or pressure simulations of those altitudes. It remains a goal of aeromicrobiology to probe the upper limits of the active terrestrial biosphere, but such studies have relied upon sampling of probably dormant airborne cells by aircraft or balloons to predict activity. We report here on a series of experiments to recover and identify hypobarophilic bacteria capable of growth at 0.7 kPa; the pressure equivalent of 34 km in the middle stratosphere. Although the experimental conditions were originally designed to simulate the surface of Mars, the results reported here should be applicable to predicting metabolic activity and growth in the terrestrial atmosphere because all bacterial strains, except *Clostridium* sp., were found to be facultative anaerobes.

Figure 4 depicts the temperature profile of the Earth's atmosphere from the surface to 80 km (based on NASA/NOAA, 1976). Initially the temperature decreases linearly in the troposphere from the surface to the tropopause (12-15 km) with a lapse rate of 6.5 $^{o}$C per km. The temperature remains stable near – 60 $^{o}$C through the tropopause, increases through the stratosphere approaching 0 $^{o}$C at the stratopause, and then decreases to – 80 $^{o}$C at the top of the mesosphere. Recent work on temperature minima for metabolic activity and growth in bacteria, has demonstrated that some species are capable of cell division and growth down to – 18 $^{o}$C; and maintenance metabolism down to perhaps – 33 $^{o}$C (e.g., Clarke *et al.*, 2013; Panikov *et al.*, 2007; Rivikina *et al.*, 2000; see review by Rummel *et al.*, 2014),.

382 Based on these low-temperature limits for microbial activity and growth, the upper
383 altitude threshold for microbial growth at – 18 $^{o}$C is likely between 5 and 6 km in the troposphere
384 (54 - 47 kPa) with maintenance metabolism extending perhaps to 7 - 8 km (41 – 36 kPa) (Fig. 4).
385 Recent data (Farjardo-Cavazos *et al.*, 2012; Nicholson *et al.*, 2010; Schuerger *et al.*, 2013;
386 Waters *et al.*, 2014) suggest that pressures down to 36 kPa are not limiting for microbial growth
387 for most species, and thus, microbial activity will be constrained by other parameters in the
388 atmosphere such as UV irradiation, temperature, desiccation effects, and access to nutrients and
389 liquid water. Based on temperature constraints alone, the only other plausible region in Earth's
390 atmosphere where microbial growth might resume would be in the upper stratosphere and
391 stratopause between 40 and 50 km in which temperature once again approaches 0 $^{o}$C. However,
392 the pressure range in the upper stratosphere from 40 to 50 km is ~0.28 to 0.076 kPa (2.8 to 0.76
393 mbar); a pressure range that has not yet been shown capable of supporting microbial growth.
394 Work here with hypobarophiles at 0.7 kPa and other research with mesobarophiles down
395 to 2.5 kPa (Farjardo-Cavazos *et al.*, 2012; Nicholson *et al.*, 2010; Schuerger *et al.*, 2006; 2013;
396 Schuerger and Nicholson, 2006; Waters *et al.*, 2014) suggests that most airborne bacteria are
397 capable of metabolic activity and growth up to the middle troposphere where temperature, not
398 pressure, becomes limiting at 5 to 8 km. This should hold for bacteria present as individual cells,
399 aggregates of cells, cell/aerosol assemblages, or embedded cells in ice crystals. Although the
400 atmosphere warms up again approaching 0 $^{o}$C in the upper stratosphere, it has not yet been
401 demonstrated that bacteria are capable of acquiring nutrients and water under the ultra low
402 pressures in the upper stratosphere near 0.28 kPa to permit cellular metabolism and growth.
403 Thus, the lower to middle troposphere may be designated an airborne microbial habitat as
404 suggested by Diehl (2013), but that higher altitudes may serve only as conduits for microbial

dispersal between terrestrial and marine ecosystems. In order to fully characterize the upper limits of the active aerial biosphere on Earth, work must be expanded to probe the absolute minima for microbial metabolism and growth for pressures below 0.7 kPa.

### *4.2. Implications for growth of bacteria on the surface of Mars*

Robotic missions to potentially habitable destinations on Mars (i.e., called Special Regions; see Rummel *et al.*, 2014) are required to comply with international guidelines of planetary protection as established by the Committee on Space Research (COSPAR) (Bruckner *et al.*, 2009). In addition, guidelines are established to align future human missions to Mars with existing planetary protection protocols for mitigating forward contamination of the surface (Criswell *et al.*, 2005; Race *et al.*, 2008). Critical to planetary protection guidelines for both robotic and crewed missions to Mars is an understanding of how and to what extent terrestrial microorganisms might survive, grow, and evolve on the surface.

Here we extend to 23 the number of bacterial species capable of growth under martian condition at 0.7 kPa, and widen the source regions for recovery of hypobarophiles to a diversity of arctic, temperate, and even human ecosystems. However, two key questions must be addressed in order to properly model the risk of contaminating the surface of Mars. First, are hypobarophiles present on spacecraft hardware? And second, if present, will they be able to grow on the actual surface of Mars?

Comparing the species listed in Table 2 with microbial surveys of planetary spacecraft and human missions, several of the hypobarophiles identified here may have been present on space hardware prior to launch. For example, species in the genera *Bacillus*, *Clostridium*, *Exiguobacterium*, *Paenibacillus*, *Rhodococcus*, *Serratia* (including *S. liquefaciens*), and *Streptococcus* are reported here as hypobarophiles; and have been recovered from robotic

spacecraft (La Duc *et al.*, 2003), spacecraft assembly facilities (SAF's) (Moissl *et al.*, 2007; Probst *et al.*, 2010; Vaishampayan *et al.*, 2010), and human missions to the ISS or MIR space stations (La Duc *et al.*, 2004; Novikova, 2004). Furthermore, at least three species of *Leuconostoc* were recovered from human saliva and hand rinsate that exhibited growth at 0.7 kPa. Thus, it is reasonable to suggest that hypobarophiles may have been present on some robotic and human spacecraft prior to launch.

Next, can launched hypobarophiles plausibly grow on the surface of Mars? First, our results here and elsewhere (Nicholson *et al.*, 2013; Schuerger *et al.*, 2013) support the conclusion that hypobarophiles are capable of growth under low-PTA conditions near 0.7 kPa that approximate some of the environmental conditions on the surface of Mars. However, the growth rates of the hypobarophiles were extremely slow, and assays often required between 10 and 14 d of growth on continuously stable, hydrated, and nutrient-rich media for small pin-prick sized colonies to appear on the agar surfaces. Thus, can terrestrial hypobarophiles acquire adequate liquid water and nutrients on the thermodynamically unstable surface of Mars over the course of several sols to carryout metabolism and growth?

Rummel *et al.* (2014) have discussed the thermodynamics of liquid water on the martian surface in order to model the occurrence of Special Regions on Mars; defined as "a region within which terrestrial organisms are likely to replicate, or a region which is interpreted to have a high potential for the existence of extant martian life forms." With few exceptions, the martian surface appears to be either too cold (< -18 $^{o}$C minimum), too dry (< 0.60 water activity [$a_w$] minimum), or both for growth of terrestrial microorganisms. At low latitudes, surface temperatures can rise above the -18 $^{o}$C minimum threshold for terrestrial microbial growth, but then $a_w$ is significantly below the 0.60 minimum for microbial activity (often < 0.05 $a_w$ on Mars).

451 At higher latitudes, $a_w$ may begin to approach conditions conducive for microbial activity (>
452 0.60), but the temperature quickly falls below -18 $^oC$. Several possible exceptions were noted by
453 Rummel *et al.* (2014), with recurring slope lineae (most likely caused by subsurface seepage
454 down steep slopes by liquid brines at temperatures > -20 $^oC$), shallow subsurface ices, and
455 partially collapsed lava caves being the most likely to provide stable hydrated niches that might
456 support terrestrial microbial activity and growth. Thus, diurnal and seasonal fluctuations in
457 temperature and $a_w$ are likely to inhibit active metabolism and growth of terrestrial
458 microorganisms on most martian terrains, with possible exceptions of hypobarophiles colonizing
459 locations with stable hydrated brines that fall within temperature and pressure ranges conducive
460 for growth.

461 One such example of a stable liquid environment near the martian surface may be active
462 recurring slope lineae (RSL) recently identified with spectral signatures of hydrated perchlorate
463 and chlorate salts consistent with liquid brines; although other salts could not be ruled out (Ojha
464 et al., 2015). Perchlorates can be both toxic to, and utilized as an energy source by,
465 microorganisms (Coates and Achenbach, 2004). Previously, we have demonstrated the growth
466 of *Bacillus subtilis* and *B. pumilus* in hydrated soil solutions extracted from a Mars analog soil
467 modeled after the Phoenix landing site with 1.5 wt% sodium perchlorate salt (Nicholson et al.,
468 2012). And of the 23 hypobarophilic species described here, we have tested the growth of *S.*
469 *liquefaciens* in non-perchlorate salt solutions relevant to the surface of Mars. Vegetative cells of
470 *S. liquefaciens* were shown capable of growth between 5 and 30 $^oC$ in brines up to 5% for $MgCl_2$
471 and NaCl, and up to 10% for $MgSO_4$ (Berry et al., 2010). The presence of hydrated salts in RSL,
472 may provide liquid niches conducive for growth of terrestrial hypobarophiles delivered to the
473 surface by spacecraft, and may provide accessible locations in which to search for extant life on

474 Mars. If hydrated brines are present as shallow subsurface features in active RSL sites, salt
475 tolerance becomes an additional parameter that terrestrial hypobarophiles must overcome in
476 order to grow near the martian surface. Future research should explore interactive effects of low
477 pressures near 0.7 kPa, low temperatures required for stable liquid brines at 0.7 kPa, and high
478 salt concentrations that mimic surface regolith conditions on Mars.

479 And lastly, Fig.4 depicts a zone in the Earth's upper stratosphere in which temperature is
480 above the microbial growth minimum of – 18 $^{o}$C and lies between the pressure range on Mars of
481 0.1 kPa (top of Olympus Mons) and 1.0 kPa (Hellas Basin). The Mars-like microbial growth
482 zone in the Earth's stratosphere is also bounded by 0 $^{o}$C at the high end of temperature due to the
483 stability of liquid water at low pressures (Haberle et al., 2001; Rummel et al., 2014). Above 0 $^{o}$C
484 at 0.7 kPa, liquid water is not stable and sublimates directly from ice to the gaseous phase.
485 Research into microbial activity and growth of hypobarophiles *in situ* within the terrestrial
486 stratospheric 'Mars zone' by long-duration experiments on high altitude balloons, or in ground
487 simulations at pressure and temperature conditions present in the stratosphere (current study) will
488 inform planetary protection and astrobiology efforts in locating Special Regions on Mars. We
489 demonstrate here that hypobarophiles are present in diverse terrestrial ecosystems and that there
490 may be zones in both the terrestrial atmosphere and the surface of Mars that may support
491 metabolism and growth of terrestrial microorganisms.

492

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**Table 1.** Numbers of hypobarophiles recovered from diverse soil and non-soil niches.

| Samples | No. of samples | Units[1] | Hypobarophiles at 0.7 kPa, 0 °C, & $CO_2$ (½ TSA[2]) | No. culturable Bacteria at 101.3 kPa, 25 °C & $O_2$ (½ TSA) | No. culturable Fungi at 101.3 kPa, 25 °C, & $O_2$ (PDATC[3]) | Source of soils or samples |
|---|---|---|---|---|---|---|
| **Soils** | | | | | | |
| Atacama Desert, Chile | 1 | g | 0 | $1.3 \times 10^2$ | $5.0 \times 10^1$ | C.P. McKay |
| Battleship Promontory, Antarctica | 2 | g | 0 | $5.8 \times 10^1$ | 0 | C.P. McKay |
| Mt. Baker, Washington | 2 | g | $1.9 \times 10^2$ | $1.2 \times 10^8$ | $7.2 \times 10^2$ | D. J. Smith |
| Colour Lake, Axel Heiberg Island | 2 | g | $5.1 \times 10^4$ | $1.0 \times 10^7$ | $2.2 \times 10^2$ | C.P. McKay |
| Devon Island, Canada | 2 | g | $2.5 \times 10^2$ | $3.4 \times 10^4$ | 0 | R. J. Ferl |
| Mojave Desert, California | 1 | g | 0 | $6.8 \times 10^5$ | $2.0 \times 10^3$ | C.P. McKay |
| PHSF, KSC, FL[4] | 4 | g | 0 | $9.8 \times 10^6$ | $3.7 \times 10^5$ | A.C. Schuerger |
| Rio Tinto, Spain | 1 | g | 0 | $3.5 \times 10^3$ | $3.4 \times 10^3$ | V. Parro |
| Siberian permafrost, Russia | 6 | g | $2.8 \times 10^4$ | $1.5 \times 10^8$ | 0 | K. Krivushin |
| | | | | | | |
| **Non-soils** | | | | | | |
| Coral bean leaves, KSC, FL | 2 | 10 $cm^2$ | 0 | $5.0 \times 10^1$ | $2.5 \times 10^1$ | A.C. Schuerger |
| Forearm hair, human | 1 | | 0 | NT[5] | NT | 2 volunteers |
| Lab benches, SLSL[6], KSC, FL | 3 | 100 $cm^2$ | 0 | $4.3 \times 10^1$ | 0 | A.C. Schuerger |
| Saliva, human | 2 | mL | $5.0 \times 10^2$ | $6.4 \times 10^6$ | 0 | 3 volunteers |
| Sea Water, KSC, FL | 1 | 250 mL | 2 | NT | NT | A.C. Schuerger |
| Scalp hair, human | 3 | | 0 | NT | NT | 2 volunteers |
| Washed hands, human | 1 | 250 mL | 2 | NT | NT | 3 volunteers |

[1]Units used in the population numbers for hypobarophiles, bacteria, and fungi recovered from various sources. [g = grams; cm = centimeters; mL = milliliters.]
[2]TSA = trypticase soy agar.
[3]PDATC = potato dextrose agar supplemented with Tergitol NP-10 at 1 mL/L and chlortetracycline HCl at 50 mg/L.
[4]PHSF = Payload Hazardous Servicing Facility, Kennedy Space Center, FL.
[5]NT = not tested.
[6]SLSL = Space Life Sciences Lab, Kennedy Space Center, FL.

**Table 2.** Hypobarophile bacteria recovered from soil and non-soil niches.

| Source[1, 2, 3] | 16S rRNA RDP Identification | Strain # | GenBank accession # | RDP Closest match | Phylum | Family |
|---|---|---|---|---|---|---|
| **Soil hypobarophiles** | | | | | | |
| | | | | | | |
| Colour Lake, soil | *Bacillus sp.* | ASB-86 | KR857399 | 0.844 | Firmicutes | Bacillaceae |
| Colour Lake, soil | *Bacillus sp.* | ASB-88 | KR857401 | 0.880 | Firmicutes | Bacillaceae |
| Colour Lake, soil | *Clostridium sp.* | ASB-85 | KR857398 | 0.953 | Firmicutes | Clostridiaceae |
| Colour Lake, soil | *Clostridium sp.* | ASB-95 | KR857406 | 0.921 | Firmicutes | Clostridiaceae |
| Colour Lake, soil | *Cryobacterium sp.* | ASB-87 | KR857400 | 0.952 | Firmicutes | Microbacteriaceae |
| Colour Lake, soil | *Cryobacterium sp.* | ASB-84 | KR857397 | 0.954 | Actinobacteria | Microbacteriaceae |
| Colour Lake soil | *Paenibacillus antarcticus* | ASB-59 | KR857372 | 0.987 | Firmicutes | Paenibacillaceae |
| Colour Lake, soil | *Paenibacillus antarcticus* | ASB-67 | KR857380 | 0.989 | Firmicutes | Paenibacillaceae |
| Colour Lake, soil | *Paenibacillus antarcticus* | ASB-90 | KR857403 | 0.985 | Firmicutes | Paenibacillaceae |
| Colour Lake, soil | *Paenibacillus antarcticus* | ASB-94 | KR857405 | 0.992 | Firmicutes | Paenibacillaceae |
| Colour Lake, soil | *Paenibacillus antarcticus* | ASB-98 | KR857409 | 0.990 | Firmicutes | Paenibacillaceae |
| Colour Lake, soil | *Paenibacillus antarcticus* | ASB-99 | KR857410 | 0.995 | Firmicutes | Paenibacillaceae |
| Colour Lake soil | *Paenibacillus macquariensis* | ASB-55 | KR857368 | 0.986 | Firmicutes | Paenibacillaceae |
| Colour Lake soil | *Paenibacillus* sp. | ASB-56 | KR857369 | 1.000 | Firmicutes | Paenibacillaceae |
| Colour Lake soil | *Paenibacillus* sp. | ASB-57 | KR857370 | 0.929 | Firmicutes | Paenibacillaceae |
| Colour Lake, soil | *Paenibacillus* sp. | ASB-66 | KR857379 | 0.935 | Firmicutes | Paenibacillaceae |
| Colour Lake, soil | *Paenibacillus* sp. | ASB-68 | KR857381 | 0.996 | Firmicutes | Paenibacillaceae |
| Colour Lake, soil | *Paenibacillus* sp. | ASB-91 | KR857404 | 0.965 | Firmicutes | Paenibacillaceae |
| Colour Lake, soil | *Rhodococcus qingshengii* | ASB-89 | KR857402 | 1.000 | Actinobacteria | Norcardiaceae |
| Colour Lake soil | *Streptomyces aureus* | ASB-61 | KR857374 | 0.980 | Actinobacteria | Streptomycetaceae |
| Colour Lake, soil | *Streptomyces aureus* | ASB-62 | KR857375 | 0.980 | Actinobacteria | Streptomycetaceae |
| Colour Lake, soil | *Streptomyces aureus* | ASB-63 | KR857376 | 0.978 | Actinobacteria | Streptomycetaceae |
| Colour Lake, soil | *Streptomyces aureus* | ASB-64 | KR857377 | 0.976 | Actinobacteria | Streptomycetaceae |

| | | | | | | |
|---|---|---|---|---|---|---|
| Colour Lake, soil | *Streptomyces vinaceus* | ASB-65 | KR857378 | 0.995 | Actinobacteria | Streptomycetaceae |
| Devon Island soil | *Paenibacillus* sp. | ASB-58 | KR857371 | 0.988 | Firmicutes | Paenibacillaceae |
| Devon Island soil | *Paenibacillus* sp. | ASB-69 | KR857382 | 0.996 | Firmicutes | Paenibacillaceae |
| Devon Island soil | *Paenibacillus* sp. | ASB-70 | KR857383 | 0.991 | Firmicutes | Paenibacillaceae |
| Devon Island soil | *Paenibacillus* sp. | ASB-71 | KR857384 | 0.988 | Firmicutes | Paenibacillaceae |
| Siberian permafrost, #42 | *Carnobacterium* sp. | WN-1483 | KR857411 | 0.988 | Firmicutes | Carnobacteriaceae |
| Siberian permafrost, #42 | *Carnobacterium sp.* | WN-1501 | KR857427 | 0.996 | Firmicutes | Carnobacteriaceae |
| Siberian permafrost, #8 | *Carnobacterium* sp. | WN-1484 | KR857412 | 0.984 | Firmicutes | Carnobacteriaceae |
| Siberian permafrost, #8 | *Carnobacterium* sp. | WN-1490 | KR857418 | 0.998 | Firmicutes | Carnobacteriaceae |
| Siberian permafrost, #9 | *Carnobacterium* sp. | WN-1491 | KR857419 | 0.986 | Firmicutes | Carnobacteriaceae |
| Siberian permafrost, #9 | *Cryobacterium* sp. | ASB-53 | KR857366 | 0.965 | Firmicutes | Microbacteriaceae |
| Siberian permafrost, #9 | *Cryobacterium* sp. | ASB-60 | KR857373 | 0.995 | Firmicutes | Microbacteriaceae |
| Siberian permafrost, #35 | *Cryobacterium* sp. | WN-1504 | KR857430 | 0.996 | Firmicutes | Microbacteriaceae |
| Siberian permafrost, #8 | *Cryobacterium* sp. | WN-1485 | KR857413 | 0.972 | Firmicutes | Microbacteriaceae |
| Siberian permafrost, #9 | *Cryobacterium* sp. | WN-1502 | KR857428 | 0.980 | Firmicutes | Microbacteriaceae |
| Siberian permafrost soil, #42 | *Exiguobacterium sibiricum* | ASB-51 | KR857364 | 1.000 | Firmicutes | Bacillales_Incertae Sedis XII |
| Siberian permafrost, #8 | *Exiguobacterium sibiricum* | WN-1486 | KR857414 | 0.991 | Firmicutes | Bacillales_Incertae Sedis XII |
| Siberian permafrost, #8 | *Exiguobacterium sibiricum* | WN-1488 | KR857416 | 0.991 | Firmicutes | Bacillales_Incertae Sedis XII |
| Siberian permafrost, #9 | *Exiguobacterium sibiricum* | WN-1492 | KR857420 | 1.000 | Firmicutes | Bacillales_Incertae Sedis XII |
| Siberian permafrost, #9 | *Exiguobacterium sibiricum* | WN-1493 | KR857421 | 1.000 | Firmicutes | Bacillales_Incertae Sedis XII |
| Siberian permafrost, #8 | *Thrichococcus pasteurii* | WN-1489 | KR857417 | 0.996 | Firmicutes | Carnobacteriaceae |
| Siberian permafrost, #35 | *Trichococcus collinsii* | WN-1503 | KR857429 | 0.996 | Firmicutes | Carnobacteriaceae |
| Siberian permafrost, #35 | *Trichococcus collinsii* | WN-1505 | KR857431 | 0.998 | Firmicutes | Carnobacteriaceae |
| Siberian permafrost, #35 | *Trichococcus pasteurii* | WN-1494 | KR857422 | 0.986 | Firmicutes | Carnobacteriaceae |
| Siberian permafrost, #35 | *Trichococcus pasteurii* | WN-1497 | KR857423 | 0.992 | Firmicutes | Carnobacteriaceae |
| Siberian permafrost, #35 | *Trichococcus pasteurii* | WN-1498 | KR857424 | 0.984 | Firmicutes | Carnobacteriaceae |

| | | | | | | |
|---|---|---|---|---|---|---|
| Siberian permafrost, #35 | *Trichococcus pasteurii* | WN-1500 | KR857426 | 0.990 | Firmicutes | Carnobacteriaceae |
| Siberian permafrost, #35 | *Trichococcus pasteurii* | WN-1506 | KR857432 | 0.990 | Firmicutes | Carnobacteriaceae |
| Siberian permafrost, #45 | *Trichococcus pasteurii* | WN-1507 | KR857433 | 0.990 | Firmicutes | Carnobacteriaceae |
| Siberian permafrost, #45 | *Trichococcus pasteurii* | WN-1509 | KR857435 | 0.991 | Firmicutes | Carnobacteriaceae |
| Siberian permafrost, #47 | *Trichococcus pasteurii* | WN-1508 | KR857434 | 0.989 | Firmicutes | Carnobacteriaceae |
| Siberian permafrost, #8 | *Trichococcus pasteurii* | WN-1487 | KR857415 | 0.993 | Firmicutes | Carnobacteriaceae |
| Siberian permafrost, #35 | *Trichococcus* sp. | WN-1499 | KR857425 | 0.985 | Firmicutes | Carnobacteriaceae |
| | | | | | | |
| **Non-soil hypobarophiles** | | | | | | |
| | | | | | | |
| Hand rinsate, volunteer #1 | *Leuconostoc gelidum* | ASB-96 | KR857407 | 0.992 | Firmicutes | Leuconostocaceae |
| Hand rinsate, volunteer #1 | *Leuconostoc gelidum* s | ASB-97 | KR857408 | 0.992 | Firmicutes | Leuconostocaceae |
| Saliva, volunteer #1 | *Leuconostoc gasicomitatum* | ASB-72 | KR857385 | 1.000 | Firmicutes | Leuconostocaceae |
| Saliva, volunteer #1 | *Leuconostoc gasicomitatum* | ASB-74 | KR857387 | 1.000 | Firmicutes | Leuconostocaceae |
| Saliva, volunteer #1 | *Leuconostoc gasicomitatum* | ASB-75 | KR857388 | 0.991 | Firmicutes | Leuconostocaceae |
| Saliva, volunteer #1 | *Leuconostoc gelidum* | ASB-77 | KR857390 | 0.989 | Firmicutes | Leuconostocaceae |
| Saliva, volunteer #1 | *Leuconostoc gelidum* | ASB-78 | KR857391 | 1.000 | Firmicutes | Leuconostocaceae |
| Saliva, volunteer #1 | *Leuconostoc gelidum* | ASB-79 | KR857392 | 0.996 | Firmicutes | Leuconostocaceae |
| Saliva, volunteer #1 | *Leuconostoc gelidum* | ASB-80 | KR857393 | 0.996 | Firmicutes | Leuconostocaceae |
| Saliva, volunteer #1 | *Leuconostoc gelidum* | ASB-82 | KR857395 | 1.000 | Firmicutes | Leuconostocaceae |
| Saliva, volunteer #1 | *Leuconostoc inhae* | ASB-73 | KR857386 | 0.995 | Firmicutes | Leuconostocaceae |
| Saliva, volunteer #1 | *Leuconostoc inhae* | ASB-76 | KR857389 | 0.992 | Firmicutes | Leuconostocaceae |
| Saliva, volunteer #1 | *Leuconostoc inhae* | ASB-81 | KR857394 | 0.991 | Firmicutes | Leuconostocaceae |
| Saliva, volunteer #1 | *Leuconostoc inhae* | ASB-83 | KR857396 | 0.995 | Firmicutes | Leuconostocaceae |
| | | | | | | |
| **Hypobarophiles in the genus *Serratia*** | | | | | | |
| | | | | | | |
| DSM | *Serratia ficaria* | 4569 | AJ233428 | na[1] | Proteobacteria | Enterobacteriaceae |
| DSM | *Serratia fonticola* | 4576 | AJ233429 | na | Proteobacteria | Enterobacteriaceae |

| | | | | | | |
|---|---|---|---|---|---|---|
| ATCC | *Serratia grimesii* | 14460 | AX109622 | na | Proteobacteria | Enterobacteriaceae |
| ATCC | *Serratia liquefaciens* | 27592 | AX109623 | na | Proteobacteria | Enterobacteriaceae |
| DSM | *Serratia plymuthica* | 4540 | AJ233433 | na | Proteobacteria | Enterobacteriaceae |
| ATCC | *Serratia rubidaea* | 27593 | AX109627 | na | Proteobacteria | Enterobacteriaceae |

[1]Abbreviations: DSM = Leibniz-Institute, DSMZ-German Collection of Microorganisms and Cell Culture, Braunschweig, Germany; ATCC = American Type Culture Collection, Manassas, VA, USA; na = not applicable.

[2]Sources for all samples are given in Table 1.

[3]Siberian permafrost sample locations are given in Nicholson *et al.* (2013).

**Table 3.** Growth of species type-strains under diverse pressure, temperature, and gas conditions.

| Bacteria[1] | Strains[2] | 101.3 kPa<br>30 ºC<br>$O_2/N_2$[3,4] | 101.3 kPa<br>0 ºC<br>$O_2/N_2$ | 101.3 kPa<br>0 ºC<br>$CO_2$ | 0.7 kPa<br>0 ºC<br>$CO_2$ |
|---|---|---|---|---|---|
| 1) *Serratia ficaria* | DSM 4569 | ++++[5] | +++ | –[6] | ++ |
| 2) *S. fonticola* | DSM 4576 | ++++ | ++++ | ++ | +++ |
| 3) *S. grimesii* | ATCC 14460 | +++ | ++ | + | ++ |
| 4) *S. liquefaciens* | ATCC 27592 | ++++ | ++++ | ++ | +++ |
| 5) *S. marcescens* | ATCC 13880 | +++ | – | – | – |
| 6) *S. plymuthica* | DSM 4540 | +++ | +++ | + | + |
| 7) *S. quinivorans* | DSM 4597 | ++++ | +++ | + | ++ |
| 8) *S. rubidaea* | ATCC 27593 | +++ | – | – | – |
| 9) *Bacillus subtilis* | 168 | ++++ | – | – | – |
| 10) *Carnobacterium inhibens* subsp. *gilichinskyi* | WN1359 | ++ | ++ | +++ | +++ |
| 11) *Escherichia coli* | ATCC 35218 | ++++ | – | – | – |
| 12) *Sporosarcina aquamarina* | SAFN-008 | ++++ | ++++ | – | – |

[1]Numbers are used in Figure 1 to delineate species grown under diverse pressure, temperature, and gas conditions.

[2]Bacteial strains were obtained from: (i) American Type Culture Collection (ATCC), (ii) Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSM), (iii) W.L. Nicholson (168 and WN1359), and (iv) K. Venkateswaran (SAFN-008).

[3]Bacteria grown at 101.3 kPa, 30 ºC, and Earth-normal $O_2/N_2$ atmosphere were considered lab controls.

[4]Assays for 0.7 and 101.3 kPa were conducted for 35 days at the conditions indicated above.

[5]Growth for each strain is recorded by plus-signs indicating relative growth compared to the size of the control strain of *Serratia liquefaciens* ATCC 27592 at Earth-normal conditions. Ratings are from [+] indicating weak growth, to [++++] indicating vigorous growth (see Figure 1); [-] = no growth.

[6]All bacteria with a recorded [-] growth response after 35 d grew normally (i.e., similar to lab controls) after cultures were transferred and incuabated48 hrs at 101.3 kPa, 30 ºC, and lab-normal $O_2/N_2$ atmosphere. In all cases, the bacteria grew normally under lab conditions.

**Figures Legends:**

**Figure 1.** Cultures of soil dilution plates incubated under lab (**A**) or Mars (**B**) conditions. Soil dilution plates of soils were incubated on 0.5x TSA or R2A media under lab (101.3 kPa, 25 $^{o}$C, and 21% pp$O_2$) or Mars (0.7 kPa, 0 $^{o}$C, and $CO_2$-enriched anoxic atmosphere) conditions. (**A**) Bacterial (b) and fungal (f) colonies were abundant on agar surfaces when maintained 28 d under lab conditions. (**B**) In contrast, only bacterial colonies (arrows) were observed at low numbers on soil dilution plates incubated under Mars conditions. Compare the microbial growth on lab-incubated soil dilutions with Colour Lake soils (Fig. 1A; lower left) to the growth of only hypobarophiles (arrows) from the Colour Lake soils incubated at 0.7 kPa (Fig. 1B).

**Figure 2.** Growth of *Serratia* spp. on TSA under diverse conditions of pressure, temperature, and gas composition. Control bacterial strains included the following (left to right; bottom row): *Bacillus subtilis* 168, *Carnobacterium inhibens* subsp. *gilichinskyi* WN1359, *Escherichia coli* ATCC 35218, and *Sporosarcina aquamarina* SAFN-008. The numbering system goes left to right and from top to bottom (1 through 12) and coincides with the order in Table 3 (from top to bottom).

**Figure 3.** Growth and colony morphology of permafrost isolates after 7 d of growth at 15 ˚C. Cultures were incubated on 0.5x TSA either in the presence [(+)$O_2$] or absence [(-)$O_2$] of oxygen. Numbers above each paired set of images refer to the WN strain collection numbers in Table 2. Distinctive yellow (Y) and orange (O) pigmented colonies are denoted. Photographs were all taken at the same magnification (see scale bar in lower right corner).

**Figure 4.** Temperature (left) and pressure (right) profiles from sea level to 80 kilometers for a standard Earth atmosphere. The box depicting microbial growth in Earth's troposphere is bounded on the left by a low-temperature threshold of -18 $^{o}$C, and extends beyond the graph axis on the right into thermophilic conditions above 100 $^{o}$C. The box depicting conditions conducive for microbial growth on Mars is bounded on the left by the temperature minimum for growth (-18 $^{o}$C) and on the right by the stability of liquid water (0 $^{o}$C at 0.7 kPa). The altitudes in Earth's atmosphere that approximate the surface pressures on Mars are between 30-48 km (0.1-1.0 kPa), and thus, there is a zone in the upper stratosphere that might fall within temperature and pressure ranges that mimic a portion of the martian surface. [Graph based on the US Standard Atmosphere, 1976; NASA TM-X-74335.]

Figure 1.

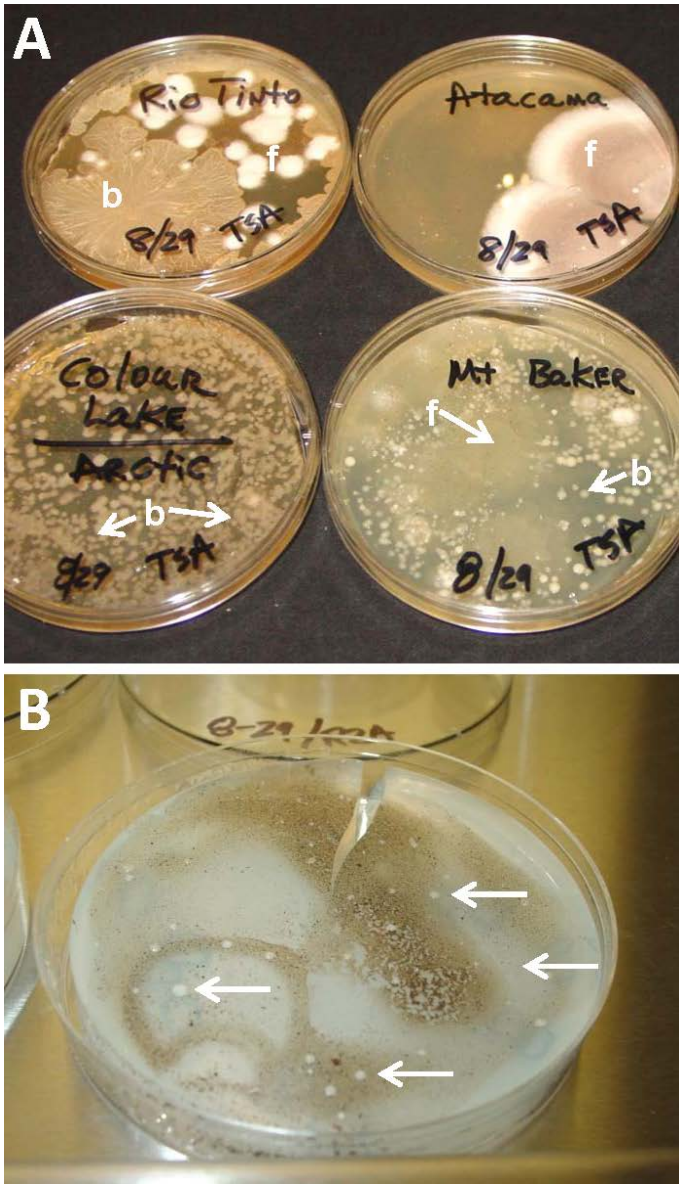

A
Rio Tinto
f
b
8/29 TSA
Atacama
f
8/29 TSA
Colour Lake
Arctic
b
8/29 TSA
Mt Baker
f
b
8/29 TSA
B

Figure 2.

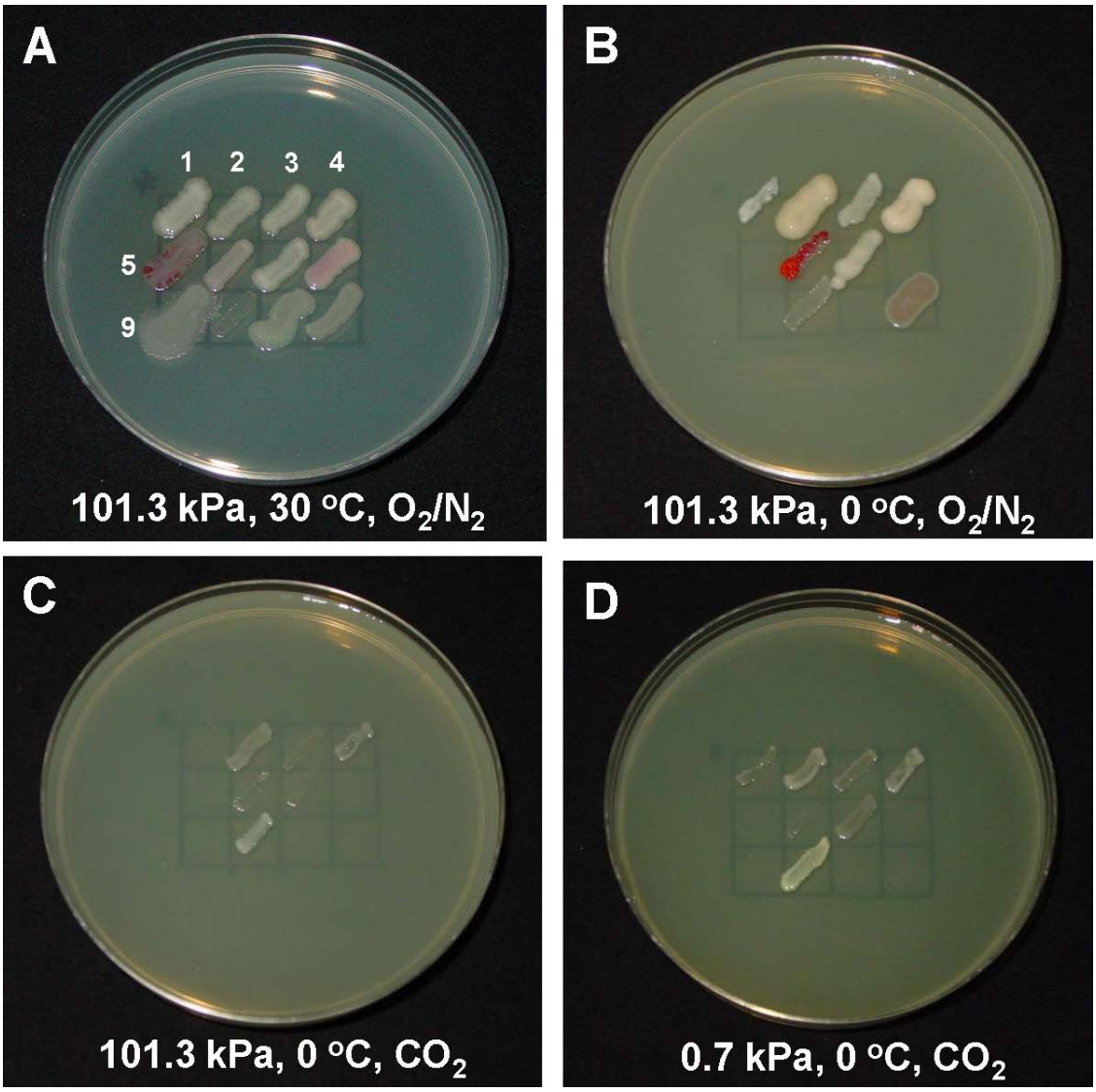

A
1 2 3 4
5
9
101.3 kPa, 30 °C, O2/N2
B
101.3 kPa, 0 °C, O2/N2
C
101.3 kPa, 0 °C, CO2
D
0.7 kPa, 0 °C, CO2

Figure 3.

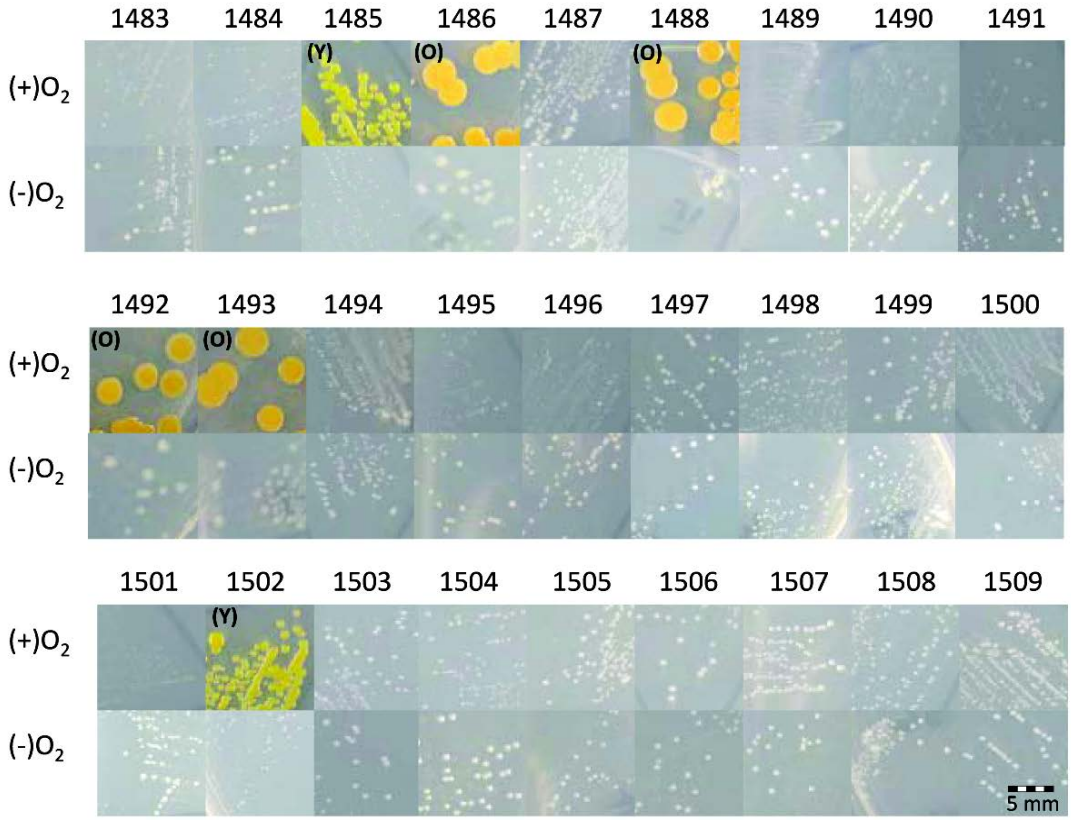
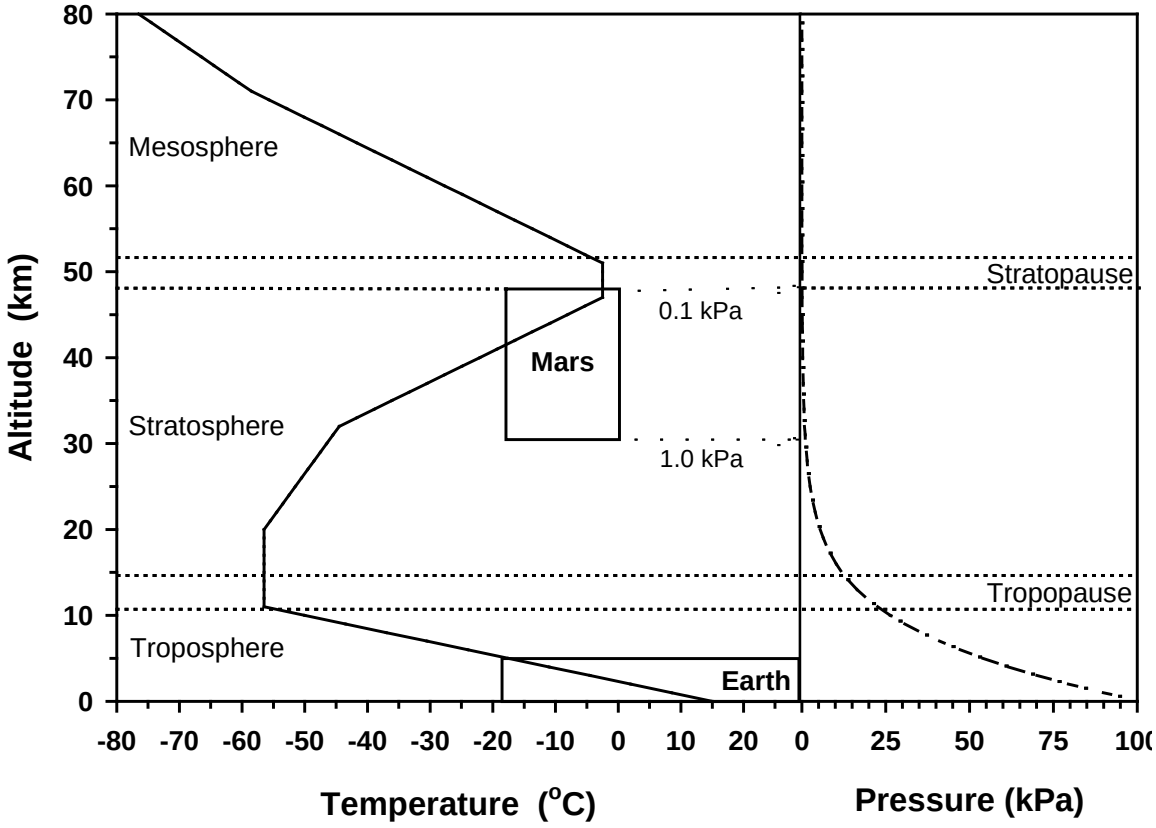

1483
1484
1485
1486
1487
1488
1489
1490
1491
(+)O2
(-)O2
(Y)
(O)
(O)
1492
1493
1494
1495
1496
1497
1498
1499
1500
(+)O2
(-)O2
(O)
(O)
1501
1502
1503
1504
1505
1506
1507
1508
1509
(+)O2
(-)O2
(Y)
5 mm

Figure 4.


80
70
60
50
40
30
20
10
0
Altitude (km)
Mesosphere
Stratosphere
Troposphere
Stratopause
Tropopause
0.1 kPa
1.0 kPa
Mars
Earth
-80 -70 -60 -50 -40 -30 -20 -10 0 10 20
0 25 50 75 100
Temperature (°C)
Pressure (kPa)