RETRACTED: Twenty-Three Species of Hypobarophilic Bacteria Recovered from Diverse Ecosystems Exhibit Growth under Simulated Martian Conditions at 0.7 kPa

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Abstract

On 27 April 2016, after acceptance and publication of the above article, the senior author learned that the University of Florida (UF) Institutional Review Board (IRB) review and approval process for using human subjects in a minimal risk scientific research project was not properly followed. Since that date, the senior author has worked closely with the UF/IRB team, the editorial staff at the journal *Astrobiology*, and the publisher Mary Ann Liebert, Inc. to discuss and rectify the situation. At the time the research was performed (Nov. 2012 through March 2013), the three human volunteers mentioned in the article were clearly informed of the intended use of the samples outlined in the study, were informed that the results (if meritorious) would be published in a peer-reviewed scientific journal, and were informed that all samples would be destroyed by autoclaving and then incineration at the conclusion of the research. No identifiable metadata related to the volunteers was recorded. The authors stand by the original results and interpretations of the research, but must now retract the paper because the guidelines for working with human subjects were not followed. We apologize for the error, and look forward to reworking the manuscript into a new paper with the human data removed, and submitting it to an appropriate peer-reviewed astrobiology publication venue.

Astrobiology commends Dr. Schuerger for his honesty and transparency.

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. ex-

hibited growth down to 2.5 kPa in an Earth-normal O₂/N₂ 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 O₂/N₂ 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 in 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 semisolid media incubated under Mars surface conditions of 0.7 kPa, 0°C, and CO₂-enriched anoxic atmosphere. Taken together, a number of studies (Schuerger and Nicholson, 2006; Schuerger *et al.*, 2006, 2013; Nicholson *et al.*, 2010, 2013; Fajardo-Cavazos *et al.*, 2012; 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

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growth for most species at 2.5–3.5 kPa. Only a few species including *Carnobacterium* spp. (0.7 kPa; Nicholson *et al.*, 2013), *Pseudomonas fluorescens* (1.5 kPa; Schuerger, unpublished data), 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 whether 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.*, 0.7–1.0 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°C (most soils) or -70°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, seawater 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 0.5 $\times$ trypticase soy agar (TSA). Hand rinsates were collected by submerging hands in 500 mL of sterile deionized water for 60 s (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. Seawater 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 seawater 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

TABLE 1. NUMBERS OF HYPOBAROPHILES RECOVERED FROM DIVERSE SOIL AND NON-SOIL NICHES

Samples	No. of samples	Units ^a	Hypobarophiles at 0.7 kPa, 0°C, and CO ₂ (½ TSA) ^b	No. culturable bacteria at 101.3 kPa, 25°C, and O ₂ (½ TSA)	No. culturable fungi at 101.3 kPa, 25°C, and O ₂ (PDATC ^c)	Source of soils or samples
Soils						
Atacama Desert, Chile	1	g	0	1.3×10^2	5.0×10^1	C.P. McKay
Battleship Promontory, Antarctica	2	g	0	5.8×10^1	0	C.P. McKay
Mt. Baker, Washington	2	g	1.9×10^2	1.2×10^8	7.2×10^2	D.J. Smith
Colour Lake, Axel Heiberg Island	2	g	5.1×10^4	1.0×10^7	2.2×10^2	C.P. McKay
Devon Island, Canada	2	g	2.5×10^2	3.4×10^4	0	R.J. Ferl
Mojave Desert, California	1	g	0	6.8×10^5	2.0×10^3	C.P. McKay
PHSF, KSC, FL ^d	4	g	0	9.8×10^6	3.7×10^5	A.C. Schuerger
Río Tinto, Spain	1	g	0	3.5×10^3	3.4×10^3	V. Parro
Siberian permafrost, Russia	6	g	2.8×10^4	1.5×10^8	0	K. Krivushin
Non-soils						
Coral bean leaves, KSC, FL	2	10 cm ²	0	5.0×10^1	2.5×10^1	A.C. Schuerger
Forearm hair, human	1		0	NT ^e	NT	2 volunteers
Lab benches, SLSL ^f , KSC, FL	3	100 cm ²	0	4.3×10^1	0	A.C. Schuerger
Saliva, human	2	mL	5.0×10^2	6.4×10^6	0	3 volunteers
Seawater, 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

^aUnits used in the population numbers for hypobarophiles, bacteria, and fungi recovered from various sources. g=grams; cm=centimeters; mL=milliliters.

^bTSA=trypticase soy agar.

^cPDATC=potato dextrose agar supplemented with Tergitol NP-10 at 1 mL/L and chlortetracycline HCl at 50 mg/L.

^dPHSF=Payload Hazardous Servicing Facility, Kennedy Space Center, FL.

^eNT=not tested.

^fSLSL=Space Life Sciences Lab, Kennedy Space Center, FL.

the Space Life Sciences Lab (SLSL; Merritt Island, FL). The leaves were inverted and the adaxial surfaces pressed into agar surfaces for 30 s. 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 semiliquid when stirred and acted to keep soil particles in suspension. The soil/agar suspensions (200 μ L/plate) were plated directly onto double-thick (30 mL per plate) 0.5 $\times$ 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°C), and CO₂-enriched anoxic (low ppO₂) 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 μ m 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 ($\pm$ 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 CO₂ for 5 min. The samples were then held in the low-PTA conditions for 28 days, with the anaerobic sachets changed every 7 days. After 4 weeks, the plates were inspected visually for observable colonies (Round 1 hypobaric assay). Visible colonies were picked, streaked-purified on the same media (0.5 $\times$ TSA or R2A) used in the Round 1 assays, and incubated at lab conditions of 101.3 kPa, 24°C, and Earth-normal O₂/N₂ atmosphere. The pure cultures were then applied to fresh media with heat-sterilized loops and incubated under low-PTA conditions for 14 days (Round 2). Single isolated colonies from Round 2 were picked, restreaked onto fresh plates of the same medium, and incubated a third time under low-PTA conditions for 14 days (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 0.5 $\times$ TSA for 7 days at 15°C under either an anoxic atmosphere (CO₂-enriched anaerobic chambers) or a lab-normal oxygenated atmosphere (O₂/N₂; 21%/78%, respectively). Bacterial colonies were imaged at identical magnifications and lighting conditions.

If either 0.5 $\times$ 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, 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°C 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 h 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 with 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'-AAGGAGGTGATCCANCCRCA-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 $\geq$ 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

Schuerger *et al.* (2013) demonstrated that the type-strain *Serratia liquefaciens* (ATCC 25792) was capable of growth under low-PTA conditions at 0.7 kPa, and Nicholson *et al.* (2013) demonstrated the same capability for nine type-strains in the genus *Carnobacterium*. To determine whether 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 0.5 $\times$ TSA for 35 days. Four additional bacterial strains were grown at 0.7 kPa as either positive controls (*Carnobacterium inhibens* subsp. *gilichinskyi*, strain WN1359; Nicholson *et al.*, 2015) or as negative controls (*Bacillus subtilis*, *Escherichia coli*, and *Sporosarcina aquamarina*; Schuerger *et al.*, 2013). 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°C, and Earth-normal O₂/N₂ atmosphere (21%/78%, respectively). Ratings in Table 3 are given as [+] indicating weak growth to [++++] indicating vigorous growth (see Fig. 1); [–]=no growth. All bacteria with a recorded [–] growth response after 35 days were returned to lab conditions for 48 h. In all cases, the strains

TABLE 2. HYPOBAROPHILE BACTERIA RECOVERED FROM SOIL AND NON-SOIL NICHES

Source ^{a,b,c}	16S rRNA RDP identification	Strain #	GenBank accession #	RDP closest match	Phylum	Family
Soil hypobarophiles						
Colour Lake soil	<i>Bacillus</i> sp.	ASB-86	KR857399	0.844	Firmicutes	Bacillaceae
Colour Lake soil	<i>Bacillus</i> sp.	ASB-88	KR857401	0.880	Firmicutes	Bacillaceae
Colour Lake soil	<i>Clostridium</i> sp.	ASB-85	KR857398	0.953	Firmicutes	Clostridiaceae
Colour Lake soil	<i>Clostridium</i> sp.	ASB-95	KR857406	0.921	Firmicutes	Clostridiaceae
Colour Lake soil	<i>Cryobacterium</i> sp.	ASB-87	KR857400	0.952	Firmicutes	Microbacteriaceae
Colour Lake soil	<i>Cryobacterium</i> sp.	ASB-84	KR857397	0.954	Firmicutes	Microbacteriaceae
Colour Lake soil	<i>Paenibacillus antarcticus</i>	ASB-59	KR857372	0.987	Firmicutes	Paenibacillaceae
Colour Lake soil	<i>Paenibacillus antarcticus</i>	ASB-67	KR857380	0.989	Firmicutes	Paenibacillaceae
Colour Lake soil	<i>Paenibacillus antarcticus</i>	ASB-90	KR857403	0.985	Firmicutes	Paenibacillaceae
Colour Lake soil	<i>Paenibacillus antarcticus</i>	ASB-94	KR857405	0.992	Firmicutes	Paenibacillaceae
Colour Lake soil	<i>Paenibacillus antarcticus</i>	ASB-98	KR857409	0.990	Firmicutes	Paenibacillaceae
Colour Lake soil	<i>Paenibacillus antarcticus</i>	ASB-99	KR857410	0.995	Firmicutes	Paenibacillaceae
Colour Lake soil	<i>Paenibacillus macquariensis</i>	ASB-55	KR857368	0.986	Firmicutes	Paenibacillaceae
Colour Lake soil	<i>Paenibacillus</i> sp.	ASB-56	KR857369	1.000	Firmicutes	Paenibacillaceae
Colour Lake soil	<i>Paenibacillus</i> sp.	ASB-57	KR857370	0.929	Firmicutes	Paenibacillaceae
Colour Lake soil	<i>Paenibacillus</i> sp.	ASB-66	KR857379	0.935	Firmicutes	Paenibacillaceae
Colour Lake soil	<i>Paenibacillus</i> sp.	ASB-68	KR857381	0.996	Firmicutes	Paenibacillaceae
Colour Lake soil	<i>Paenibacillus</i> sp.	ASB-91	KR857404	0.965	Firmicutes	Paenibacillaceae
Colour Lake soil	<i>Rhodococcus qingshengii</i>	ASB-89	KR857402	1.000	Actinobacteria	Norcardiaceae
Colour Lake soil	<i>Streptomyces aureus</i>	ASB-61	KR857374	0.980	Actinobacteria	Streptomycetaceae
Colour Lake soil	<i>Streptomyces aureus</i>	ASB-62	KR857375	0.980	Actinobacteria	Streptomycetaceae
Colour Lake soil	<i>Streptomyces aureus</i>	ASB-63	KR857376	0.978	Actinobacteria	Streptomycetaceae
Colour Lake soil	<i>Streptomyces aureus</i>	ASB-64	KR857377	0.976	Actinobacteria	Streptomycetaceae
Colour Lake soil	<i>Streptomyces vinaceus</i>	ASB-65	KR857378	0.995	Actinobacteria	Streptomycetaceae
Devon Island soil	<i>Paenibacillus</i> sp.	ASB-58	KR857371	0.988	Firmicutes	Paenibacillaceae
Devon Island soil	<i>Paenibacillus</i> sp.	ASB-69	KR857382	0.996	Firmicutes	Paenibacillaceae
Devon Island soil	<i>Paenibacillus</i> sp.	ASB-70	KR857383	0.991	Firmicutes	Paenibacillaceae
Devon Island soil	<i>Paenibacillus</i> sp.	ASB-71	KR857384	0.988	Firmicutes	Paenibacillaceae
Siberian permafrost, #42	<i>Carnobacterium</i> sp.	WN-1483	KR857411	0.988	Firmicutes	Carnobacteriaceae
Siberian permafrost, #42	<i>Carnobacterium</i> sp.	WN-1501	KR857427	0.996	Firmicutes	Carnobacteriaceae
Siberian permafrost, #8	<i>Carnobacterium</i> sp.	WN-1484	KR857412	0.984	Firmicutes	Carnobacteriaceae
Siberian permafrost, #8	<i>Carnobacterium</i> sp.	WN-1490	KR857418	0.998	Firmicutes	Carnobacteriaceae
Siberian permafrost, #9	<i>Carnobacterium</i> sp.	WN-1491	KR857419	0.986	Firmicutes	Carnobacteriaceae
Siberian permafrost, #9	<i>Cryobacterium</i> sp.	ASB-53	KR857366	0.965	Firmicutes	Microbacteriaceae
Siberian permafrost, #9	<i>Cryobacterium</i> sp.	ASB-60	KR857373	0.995	Firmicutes	Microbacteriaceae
Siberian permafrost, #35	<i>Cryobacterium</i> sp.	WN-1504	KR857430	0.996	Firmicutes	Microbacteriaceae
Siberian permafrost, #8	<i>Cryobacterium</i> sp.	WN-1485	KR857413	0.972	Firmicutes	Microbacteriaceae
Siberian permafrost, #9	<i>Cryobacterium</i> sp.	WN-1502	KR857428	0.980	Firmicutes	Microbacteriaceae
Siberian permafrost soil, #42	<i>Exiguobacterium sibiricum</i>	ASB-51	KR857364	1.000	Firmicutes	Bacillales_Incertae Sedis XII
Siberian permafrost, #8	<i>Exiguobacterium sibiricum</i>	WN-1486	KR857414	0.991	Firmicutes	Bacillales_Incertae Sedis XII
Siberian permafrost, #8	<i>Exiguobacterium sibiricum</i>	WN-1488	KR857416	0.991	Firmicutes	Bacillales_Incertae Sedis XII
Siberian permafrost, #9	<i>Exiguobacterium sibiricum</i>	WN-1492	KR857420	1.000	Firmicutes	Bacillales_Incertae Sedis XII
Siberian permafrost, #9	<i>Exiguobacterium sibiricum</i>	WN-1493	KR857421	1.000	Firmicutes	Bacillales_Incertae Sedis XII
Siberian permafrost, #8	<i>Trichococcus pasteurii</i>	WN-1489	KR857417	0.996	Firmicutes	Carnobacteriaceae
Siberian permafrost, #35	<i>Trichococcus collinsii</i>	WN-1503	KR857429	0.996	Firmicutes	Carnobacteriaceae
Siberian permafrost, #35	<i>Trichococcus collinsii</i>	WN-1505	KR857431	0.998	Firmicutes	Carnobacteriaceae
Siberian permafrost, #35	<i>Trichococcus pasteurii</i>	WN-1494	KR857422	0.986	Firmicutes	Carnobacteriaceae
Siberian permafrost, #35	<i>Trichococcus pasteurii</i>	WN-1497	KR857423	0.992	Firmicutes	Carnobacteriaceae
Siberian permafrost, #35	<i>Trichococcus pasteurii</i>	WN-1498	KR857424	0.984	Firmicutes	Carnobacteriaceae
Siberian permafrost, #35	<i>Trichococcus pasteurii</i>	WN-1500	KR857426	0.990	Firmicutes	Carnobacteriaceae
Siberian permafrost, #35	<i>Trichococcus pasteurii</i>	WN-1506	KR857432	0.990	Firmicutes	Carnobacteriaceae
Siberian permafrost, #45	<i>Trichococcus pasteurii</i>	WN-1507	KR857433	0.990	Firmicutes	Carnobacteriaceae
Siberian permafrost, #45	<i>Trichococcus pasteurii</i>	WN-1509	KR857435	0.991	Firmicutes	Carnobacteriaceae
Siberian permafrost, #47	<i>Trichococcus pasteurii</i>	WN-1508	KR857434	0.989	Firmicutes	Carnobacteriaceae
Siberian permafrost, #8	<i>Trichococcus pasteurii</i>	WN-1487	KR857415	0.993	Firmicutes	Carnobacteriaceae
Siberian permafrost, #35	<i>Trichococcus</i> sp.	WN-1499	KR857425	0.985	Firmicutes	Carnobacteriaceae

(continued)

TABLE 2. (CONTINUED)

Source ^{a,b,c}	16S rRNA RDP identification	Strain #	GenBank accession #	RDP closest match	Phylum	Family
Non-soil hypobarophiles						
Hand rinsate, volunteer #1	<i>Leuconostoc gelidum</i>	ASB-96	KR857407	0.992	Firmicutes	Leuconostocaceae
Hand rinsate, volunteer #1	<i>Leuconostoc gelidum</i>	ASB-97	KR857408	0.992	Firmicutes	Leuconostocaceae
Saliva, volunteer #1	<i>Leuconostoc gasicomitatum</i>	ASB-72	KR857385	1.000	Firmicutes	Leuconostocaceae
Saliva, volunteer #1	<i>Leuconostoc gasicomitatum</i>	ASB-74	KR857387	1.000	Firmicutes	Leuconostocaceae
Saliva, volunteer #1	<i>Leuconostoc gasicomitatum</i>	ASB-75	KR857388	0.991	Firmicutes	Leuconostocaceae
Saliva, volunteer #1	<i>Leuconostoc gelidum</i>	ASB-77	KR857390	0.989	Firmicutes	Leuconostocaceae
Saliva, volunteer #1	<i>Leuconostoc gelidum</i>	ASB-78	KR857391	1.000	Firmicutes	Leuconostocaceae
Saliva, volunteer #1	<i>Leuconostoc gelidum</i>	ASB-79	KR857392	0.996	Firmicutes	Leuconostocaceae
Saliva, volunteer #1	<i>Leuconostoc gelidum</i>	ASB-80	KR857393	0.996	Firmicutes	Leuconostocaceae
Saliva, volunteer #1	<i>Leuconostoc gelidum</i>	ASB-82	KR857395	1.000	Firmicutes	Leuconostocaceae
Saliva, volunteer #1	<i>Leuconostoc inhae</i>	ASB-73	KR857386	0.995	Firmicutes	Leuconostocaceae
Saliva, volunteer #1	<i>Leuconostoc inhae</i>	ASB-76	KR857389	0.992	Firmicutes	Leuconostocaceae
Saliva, volunteer #1	<i>Leuconostoc inhae</i>	ASB-81	KR857394	0.991	Firmicutes	Leuconostocaceae
Saliva, volunteer #1	<i>Leuconostoc inhae</i>	ASB-83	KR857396	0.995	Firmicutes	Leuconostocaceae
Hypobarophiles in the genus <i>Serratia</i>						
DSM	<i>Serratia ficaria</i>	4569	AJ233428	na	Proteobacteria	Enterobacteriaceae
DSM	<i>Serratia fonticola</i>	4576	AJ233429	na	Proteobacteria	Enterobacteriaceae
ATCC	<i>Serratia grimesii</i>	14460	AX109622	na	Proteobacteria	Enterobacteriaceae
ATCC	<i>Serratia liquefaciens</i>	27592	AX109623	na	Proteobacteria	Enterobacteriaceae
DSM	<i>Serratia plymuthica</i>	4540	AJ233433	na	Proteobacteria	Enterobacteriaceae
ATCC	<i>Serratia rubidaea</i>	27593	AX109627	na	Proteobacteria	Enterobacteriaceae

^aAbbreviations: DSM=Leibniz Institute DSMZ—German Collection of Microorganisms and Cell Culture, Braunschweig, Germany; na=not applicable; ATCC=American Type Culture Collection, Manassas, VA, USA.

^bSources for all samples are given in Table 1.

^cSiberian permafrost sample locations are given in the work by Nicholson *et al.* (2013).

TABLE 3. GROWTH OF SPECIES TYPE-STRAINS UNDER DIVERSE PRESSURE, TEMPERATURE, AND GAS CONDITIONS

Bacteria ^a	Strains ^b	101.3 kPa 30°C O ₂ /N ₂ ^{c,d}	101.3 kPa 0°C O ₂ /N ₂	101.3 kPa 0°C CO ₂	0.7 kPa 0°C CO ₂
(1) <i>Serratia ficaria</i>	DSM 4569	++++ ^e	+++	- ^f	++
(2) <i>S. fonticola</i>	DSM 4576	++++	++++	++	+++
(3) <i>S. grimesii</i>	ATCC 14460	+++	++	+	++
(4) <i>S. liquefaciens</i>	ATCC 27592	++++	++++	++	+++
(5) <i>S. marcescens</i>	ATCC 13880	+++	-	-	-
(6) <i>S. plymuthica</i>	DSM 4540	+++	+++	+	+
(7) <i>S. quinivorans</i>	DSM 4597	++++	+++	+	++
(8) <i>S. rubidaea</i>	ATCC 27593	+++	-	-	-
(9) <i>Bacillus subtilis</i>	168	++++	-	-	-
(10) <i>Carnobacterium inhibens</i> subsp. <i>gilichinskyi</i>	WN1359	++	++	+++	+++
(11) <i>Escherichia coli</i>	ATCC 35218	++++	-	-	-
(12) <i>Sporosarcina aquamarina</i>	SAFN-008	++++	++++	-	-

^aNumbers are used in Fig. 2 to delineate species grown under diverse pressure, temperature, and gas conditions.

^bBacterial 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).

^cBacteria grown at 101.3 kPa, 30°C, and Earth-normal O₂/N₂ atmosphere were considered lab controls.

^dAssays for 0.7 and 101.3 kPa were conducted for 35 days at the conditions indicated above.

^eGrowth 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 Fig. 2); [-] = no growth.

^fAll bacteria with a recorded [-] growth response after 35 days grew normally (*i.e.*, similar to lab controls) after cultures were transferred and incubated 48 h at 101.3 kPa, 30°C, and lab-normal O₂/N₂ atmosphere. In all cases, the bacteria grew normally under lab conditions.

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°C, and Earth-normal O₂/N₂ 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 Río Tinto, Spain; Atacama Desert, Chile; Colour Lake, Axel Heiberg Island, Canada; and Mt. Baker, Washington, 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 5.8×10^1 colony-forming units (cfu)/g for the Antarctica soils from Battleship Promontory and 1.5×10^8 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 3.7×10^5 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 5.1×10^4 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 0.5× 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 whether 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; 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 ppO₂ at 101.3 kPa, except two strains of the obligate anaerobe *Clostridium* sp. (Table 2).

FIG. 1. Cultures of soil dilution plates incubated under lab (A) or martian (B) conditions. Soil dilution plates of soils were incubated on 0.5× TSA or R2A media under lab (101.3 kPa, 25°C, and 21% ppO₂) or martian (0.7 kPa, 0°C, and CO₂-enriched anoxic atmosphere) conditions. (A) Bacterial (b) and fungal (f) colonies were abundant on agar surfaces when maintained 28 days under lab conditions. (B) In contrast, only bacterial colonies (arrows) were observed at low numbers on soil dilution plates incubated under martian 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). (Color graphics available at www.liebertonline.com/ast)

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 seawater (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 seawater were not recovered during Round 2 subculturing 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 nonquantitative manner, so no estimates of background populations of culturable bacteria or fungi were possible. However, coral bean surfaces (10 cm² basis) and lab benches (100 cm² 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 (6.4×10^6 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 $\geq 97.5\%$ 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; 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).

FIG. 2. Growth of *Serratia* spp. on TSA under diverse conditions of pressure, temperature, and gas composition. Cultures were incubated at (A) Earth-lab conditions of 101.3 kPa, 30°C, and O₂/N₂ atmospheres, (B) low-temperature conditions of 101.3 kPa, 0°C, and O₂/N₂ atmospheres, (C) low-temperature and high-CO₂ conditions of 101.3 kPa, 0°C, and CO₂-enriched anoxic atmospheres, and (D) Mars conditions of 0.7 kPa, 0°C, and CO₂-enriched anoxic atmospheres. 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). (Color graphics available at www.liebertonline.com/ast)

Hypobarophilic bacteria picked from 0.5× 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°C, and Earth-normal O₂/N₂ 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°C in a O₂/N₂ atmosphere, but then returned to a nonpigmented condition when *S. plymuthica* was grown at 101.3 kPa, 0°C, and CO₂-enriched anoxic atmosphere (Fig. 2). Results suggest that both temperature and low pO₂ 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°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°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

FIG. 3. Growth and colony morphology of permafrost isolates after 7 days of growth at 15°C. Cultures were incubated on 0.5× TSA either in the presence [(+)O₂] or absence [(-)O₂] 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). (Color graphics available at www.liebertonline.com/ast)

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 soil 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, Washington, and from seawater obtained from coastal surf near the Kennedy Space Center, Florida, 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 seawater suggests that geochemical or microbial metabolites from the soils or seawater 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 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 hypo-

barophilic 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 0.5× 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 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°C per kilometer. The temperature remains stable near –60°C through the tropopause, increases through the stratosphere approaching 0°C at the stratopause, and then decreases to –80°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°C and maintenance metabolism down to perhaps –33°C (*e.g.*, Rivkina *et al.*, 2000; Panikov and Sizova, 2007; Clarke *et al.*, 2013; see review by Rummel *et al.*, 2014).

Based on these low-temperature limits for microbial activity and growth, the upper altitude threshold for microbial growth at –18°C is likely between 5 and 6 km in the troposphere (54–47 kPa) with maintenance metabolism extending perhaps to 7–8 km (41–36 kPa) (Fig. 4). Recent data (Nicholson *et al.*, 2010; Fajardo-Cavazos *et al.*, 2012; Schuerger *et al.*, 2013; Waters *et al.*, 2014) suggest that pressures down to 5 kPa are not limiting for microbial growth for most species; thus microbial activity will be constrained by other parameters in the atmosphere such as UV irradiation, temperature, desiccation effects, and access to nutrients and liquid water. Based on temperature constraints alone, the only other plausible region in Earth's atmosphere where microbial growth might resume would be

FIG. 4. Temperature (left) and pressure (right) profiles from sea level to 80 km 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°C and extends beyond the graph axis on the right into thermophilic conditions above 100°C . The box depicting conditions conducive for microbial growth on Mars is bounded on the left by the temperature minimum for growth (-18°C) and on the right by the stability of liquid water (0°C at 0.7 kPa). The altitudes in Earth's atmosphere that approximate the surface pressures on Mars are between 30 and 48 km ($0.1\text{--}1.0\text{ kPa}$); 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.)

in the upper stratosphere and stratopause between 40 and 50 km in which temperature once again approaches 0°C . However, the pressure range in the upper stratosphere from 40 to 50 km is ~ 0.28 to 0.076 kPa (2.8 to 0.76 mbar), a pressure range that has not yet been shown capable of supporting microbial growth.

Work here with hypobarophiles at 0.7 kPa and other research with mesobarophiles down to 2.5 kPa (Schuerger and Nicholson, 2006; Schuerger *et al.*, 2006, 2013; Nicholson *et al.*, 2010; Fajardo-Cavazos *et al.*, 2012; Waters *et al.*, 2014) suggests that most airborne bacteria are capable of metabolic activity and growth up to the middle troposphere where temperature, not pressure, becomes limiting at 5–8 km. This should hold for bacteria present as individual cells, aggregates of cells, cell/aerosol assemblages, or embedded cells in ice crystals. Although the atmosphere warms up again approaching 0°C in the upper stratosphere, it has not yet been demonstrated that bacteria are capable of acquiring nutrients and water under the ultralow pressures in the upper stratosphere near 0.28 kPa to permit cellular metabolism and growth. Thus, the lower to middle troposphere may be designated an airborne microbial habitat as suggested by Diehl (2013), but higher altitudes may serve only as conduits for microbial dispersal between terrestrial and marine ecosystems. 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 34 the number of bacterial species capable of growth under martian conditions at 0.7 kPa (current results; Nicholson *et al.*, 2013; Schuerger *et al.*, 2013) 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 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 (SAFs) (Moissl *et al.*, 2007; Probst *et al.*, 2010; Vaishampayan *et al.*, 2010), and human missions to the International Space Station or the Mir space station (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 days 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 carry out metabolism and growth?

Rummel *et al.* (2014) discussed the thermodynamics of liquid water on the martian surface in order to model the occurrence of Special Regions on Mars, which are 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 ($<-18^{\circ}\text{C}$ 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°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). At higher latitudes, a_w may begin to approach conditions conducive for microbial activity (>0.60), but the temperature quickly falls below -18°C . Several possible exceptions were noted by Rummel *et al.* (2014), with recurring slope lineae (RSL; most likely caused by subsurface seepage down steep slopes by liquid brines at temperatures $>-20^{\circ}\text{C}$), shallow subsurface ices, and partially collapsed lava caves being the most likely to provide stable hydrated niches that might support terrestrial microbial activity and growth. Thus, diurnal and seasonal fluctuations in temperature and a_w are likely to inhibit active metabolism and growth of terrestrial microorganisms on most martian terrains, with possible exceptions of hypobarophiles colonizing locations with stable hydrated brines that fall within temperature and pressure ranges conducive for growth.

One such example of a stable liquid environment near the martian surface may be active RSL recently identified with spectral signatures of hydrated perchlorate and chlorate salts consistent with liquid brines, although other salts could not be ruled out (Ojha *et al.*, 2015). Perchlorates can be both toxic to, and utilized as an energy source by, microorganisms (Coates and Achenbach, 2004). Previously, we have demonstrated the growth of *Bacillus subtilis* and *B. pumilus* in hydrated soil solutions extracted from a Mars analog soil modeled after the Phoenix landing site with 1.5 wt % sodium perchlorate salt (Nicholson *et al.*, 2012). And of the 23 hypobarophilic species described here, we have tested the growth of *S. liquefaciens* in nonperchlorate salt solutions relevant to the surface of Mars. Vegetative cells of *S. liquefaciens* were shown capable of growth between 5°C and 30°C in brines up to 5% for MgCl_2 and NaCl , and up to 10% for MgSO_4 (Berry *et al.*, 2010). The presence of hydrated salts in RSL may provide liquid niches conducive for growth of terrestrial hypobarophiles delivered to the surface by spacecraft, and may provide accessible locations in which to search for extant life on Mars. If hydrated brines are present as shallow subsurface features in active RSL sites, salt tolerance becomes an additional parameter that terrestrial hypobarophiles must overcome in order to grow near the martian surface. Future research should explore interactive effects of low pressures near 0.7 kPa, low temperatures required for stable liquid brines at 0.7 kPa, and high salt concentrations that mimic surface regolith conditions on Mars.

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

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Abbreviations Used

cfu = colony-forming units

low-PTA = low-pressure (0.7 kPa), low-temperature (0°C), and CO₂-enriched anoxic (low ppO₂)

RDP = Ribosomal Database Project

RSL = recurring slope lineae

SAFs = spacecraft assembly facilities

TSA = trypticase soy agar