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Carnobacterium Species Capable of Growth at Pressures Ranging Over 5 Orders of Magnitude, from the Surface of Mars (10^3 Pa) to Deep Oceans (10^7 Pa) in the Solar System

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Abstract

Several permanently cold solar system bodies are being investigated with regard to their potential habitability, including Mars and icy moons. In such locations, microbial life would have to cope with low temperatures and both high and low pressures, ranging from $\sim 10^2$ to 10^3 Pa on the surface of Mars to upward of $\sim 10^8$–10^9 Pa in the subsurface oceans of icy moons. The bacterial genus *Carnobacterium* consists of species that were previously shown to be capable of growth in the absence of oxygen at low temperatures and at either low pressure or high pressure, but to date the entire pressure range of the genus has not been explored. In the present study, we subjected 14 *Carnobacterium* strains representing 11 species to cultivation in a complex liquid medium under anaerobic conditions at 2°C and at a range of pressures spanning 5 orders of magnitude, from 10^3 to 10^7 Pa. Eleven of the 14 strains showed measurable growth rates at all pressures tested, representing the first demonstration of terrestrial life forms capable of growth under such a wide range of pressures. These findings expand the physical boundaries of the capabilities of life to occur in extreme extraterrestrial environments. Key Words: *Carnobacterium*—Growth—High pressure—Low pressure. Astrobiology 23, 94–104.

1. Introduction

The search for habitable locations in our solar system concentrates on those environments capable of supporting liquid water, which is a prerequisite for life as we know it (Benner *et al.*, 2004). Habitability has been explored by cataloging our knowledge of the limits of terrestrial microbial life with respect to four basic physicochemical parameters: temperature, pH, salinity, and pressure (Harrison *et al.*, 2013). While our current understanding is incomplete concerning how the limits to habitability are influenced by the actions of multiple extremes applied simultaneously (Harrison *et al.*, 2013), we can explore such limits using a combination of simplified experimental systems to probe the growth limits of known extremophiles. For example, at low atmospheric pressures typical of the martian surface (ranging from $\sim 10^2$ to $\sim 10^3$ Pa; average $\sim 6 \times 10^2$ Pa), thermodynamic considerations essentially preclude pure water from existing in the liquid phase (Nair and Unnikrishnan, 2020). However, the presence of various dissolved salts found in the martian regolith (*e.g.*, salts of chloride, perchlorate, sulfate, sodium, potassium, calcium, magnesium) (Toner *et al.*, 2014) would depress the freezing point of water such that liquid brines could exist under Mars surface pressures at temperatures ranging from approximately −93°C to +25°C (Nair and Unnikrishnan, 2020), a range partially within the temperature limits for growth of known terrestrial microbes. Similarly, increased pressure elevates the boiling point of water, and several hyperthermophilic piezophiles (*i.e.*, microbes capable of growth at high temperatures and

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pressures) have been described that live under the extreme hot and high-pressure conditions prevailing within and around suboceanic hydrothermal vents (Jebbar *et al.*, 2015).

In contrast to isolated hot environments, the vast majority of Earth's global biosphere consists of the ''cold biosphere'' (*i.e.*, environments permanently at temperatures below 5°C), including most of the deep ocean, alpine and polar environments, regions of permafrost, and some subterranean caverns (Siddiqui *et al.*, 2013). In an astrobiological context, most potentially habitable locations in the solar system identified to date are permanently cold, including the surface/near-subsurface of Mars, and the subsurface oceans of icy moons such as Europa, Enceladus, Callisto, or Ganymede (Shematovich, 2018). These permanently cold extraterrestrial environments exist at pressures encompassing a wide range extending from $\sim 10^2$ Pa (Mars) to $\sim 10^9$ Pa (putative subsurface ocean of Titan) (Table 1).

Terrestrial microorganisms have evolved the ability to inhabit diverse environments over a wide array of pressure conditions on Earth's surface, from the highest mountains ($\sim 3.3 \times 10^4$ Pa) to the bottom of deep ocean trenches ($\sim 10^8$ Pa) (Picard and Daniel, 2013). The study of prokaryotes that have optimal growth rates in very high pressures, that is, piezophiles (Greek: *piezo* = to press), has helped to explain how organisms grow in and adapt to high-pressure environments such as the deep ocean (Yayanos, 1995). The adaptations used by piezophiles to grow at high pressure differ from those employed by pressure mesophiles (reviewed in Mota *et al.*, 2013). Indeed, certain obligate piezophiles have been isolated that are incapable of growth at an ambient sea-level pressure of $\sim 10^5$ Pa (Birrien *et al.*, 2011; Kusube *et al.*, 2017). Conversely, most pressure mesophiles do not naturally possess adaptations needed to grow under high pressures (Fang *et al.*, 2010; Mota *et al.*, 2013; Kusube *et al.*, 2017).

Much less is known about microorganisms capable of growth at pressures below Earth's sea-level pressure of $\sim 10^5$ Pa. Examples of natural low-pressure environments on Earth are biomes situated at high altitudes (Zhou *et al.*, 2017) and the upper atmosphere (Smith *et al.*, 2011; DeLeon-Rodriguez *et al.*, 2013), while human-made low-pressure environments include various low-pressure chambers designed for food storage (Burg, 2004), medical use (Dillard *et al.*, 2005), or laboratory investigations (Schuerger *et al.*, 2003; Zhou *et al.*, 2017). Interest in low-pressure microbiology has, in recent years, been stimulated by investigations of potentially habitable extraterrestrial planetary bodies such as Mars, which exhibits surface and near-subsurface conditions of low temperature and pressure (reviewed in Schwendner and Schuerger, 2020; Verseux, 2020).

There is increasing interest in examining the molecular mechanisms that microorganisms utilize to survive and grow in low pressures on planetary surfaces such as Mars. Very few characterized microorganisms are capable of active growth below a certain pressure (reviewed in Schwendner and Schuerger, 2020), and these pressure thresholds are typically higher than the average global pressure on Mars, $\sim 6 \times 10^2$ Pa (Haberle, 2015).

Until recently, studies at low pressures approaching the levels on the martian near subsurface were limited. However, various low-pressure environmental chambers have been designed and utilized to probe the capacity of microorganisms to survive and grow in low-pressure environments (reviewed in Schwendner and Schuerger, 2020). This advance in technology has led to the identification of several terrestrial bacterial genera capable of growth at a cold temperature (0°C) and low pressure (7×10^2 Pa) representative of the Mars surface (Schuerger and Nicholson, 2016; reviewed in Schwendner and Schuerger, 2020).

Taken together, the abovementioned studies have identified a set of microbes capable of growth at low pressures and a different set of microbes capable of high-pressure growth. But do prokaryotes exist that can grow at both low and high pressures? Evidence from the literature and from our respective laboratories at the University of Florida (UF) and the University of California (UC) have led us to hypothesize that species of the psychrotolerant Gram-positive genus *Carnobacterium* might be capable of growth at such a wide range of pressures.

In an astrobiological context, permafrost is considered a terrestrial analog to the present-day martian subsurface environment due to the low temperatures and consequent

Table 1. Pressure Ranges of Potentially Habitable Worlds in the Solar System

World	*Pressure range*	*References*
Earth	Atmosphere at surface: 3.3×10^4 Pa (Mt. Everest) to 1.06×10^5 Pa (Dead Sea) Ocean: 1.03×10^5 Pa (sea level) to 1.1×10^8 Pa (Challenger Deep)	West (1999); Kudish and Evseev (2006); Greenaway *et al.* (2021)
Mars	Atmosphere at surface: 1×10^2 Pa (Olympus Mons) to 1.2×10^3 Pa (Hellas Basin); average 6.1×10^2 Pa Oceans: none verified at present	Taylor *et al.* (2010); Rummel *et al.* (2014)
Europa	Atmosphere at surface: negligible ($\sim 10^{-6}$ Pa) Ocean: up to $\sim 1.2 \times 10^8$ Pa	Hall *et al.* (1995); Marion *et al.* (2003)
Ganymede	Atmosphere at surface: negligible ($\sim 7 \times 10^{-7}$ Pa) Ocean: $\sim 4.0 \times 10^7$ to $\sim 9.3 \times 10^8$ Pa	Prentice (1996); Vance *et al.* (2018)
Callisto	Atmosphere at surface: negligible ($\sim 8 \times 10^{-7}$ Pa) Ocean: poorly constrained at present	Carlson (1999); Journaux *et al.* (2020)
Titan	Atmosphere at surface: $\sim 1.5 \times 10^5$ Pa Ocean: $\sim 1.5 \times 10^5$ to $\sim 1 \times 10^9$ Pa	Sotin *et al.* (2021)
Enceladus	Atmosphere at surface: rarefied, but variable due to south polar plumes Ocean: up to $\sim 7 \times 10^6$ Pa	Dougherty *et al.* (2006); Barge and White (2017)

Note that many of these values are estimates based on remote sensing and/or modeling, and at present are poorly constrained.

frozen state of water. At UF we screened ~10,000 microbial isolates obtained from Siberian permafrost for growth under low pressure (7×10^2 Pa), low temperature (0°C), and anoxic CO_2-enriched conditions simulating the atmosphere of Mars (Nicholson *et al.*, 2013). Six isolates that successfully grew under these conditions were found to belong to the genus *Carnobacterium*. We then demonstrated that two of these *Carnobacterium* spp. permafrost isolates (strains 8 and 14, Table 2), as well as nine *Carnobacterium* type species obtained from culture collections, demonstrated active growth at 0°C and 7×10^2 Pa in a CO_2-enriched anoxic atmosphere (Nicholson *et al.*, 2013). Subsequent review of the literature revealed that three of the *Carnobacterium* type species (strains 3, 5, and 13; Table 2) had originally been isolated from low-temperature, low-pressure environments—as spoilage organisms of refrigerated, vacuum-packed meats (cited in Table 2).

In the context of high pressure, in a separate study at UC, we reported *Carnobacterium* sp. strain AT7, a Gram-positive psychrotolerant facultative anaerobe isolated from the Aleutian Trench at a depth of 2.5 km, at 2.5×10^7 Pa (Lauro *et al.*, 2007). Laboratory cultivation revealed that this strain exhibited growth at high pressures ranging from 10^5 Pa to 6×10^7 Pa (Lauro *et al.*, 2007).

Before this study, the low-pressure limit for *Carnobacterium* sp. AT7 growth was unknown. Furthermore, none of the *Carnobacterium* spp. strains previously demonstrated to grow in low pressure had been tested for growth at high pressure. The ability of individual *Carnobacterium* spp. to grow in either low or high pressure led us to hypothesize that some species within this genus may be capable of growth at pressures both higher and lower than ambient sea-level pressure. To test this hypothesis, we cultivated the 14 *Carnobacterium* strains listed in Table 2 in increments of pressure ranging from 10^3 to 10^7 Pa to determine their growth capabilities. We report in this study that 11 of the 14 *Carnobacterium* spp. strains tested were capable of growth at all pressures tested, ranging over 5 orders of magnitude. To our knowledge, this is the first demonstration of organisms capable of growth at such a wide range of extreme pressures.

2. Materials and Methods

2.1. Carnobacterium *spp. strains, medium, and growth conditions*

A list of the *Carnobacterium* spp. strains examined in this study is presented in Table 2. At present, 13 *Carnobacterium* type species are recognized, 12 of which were used in the present study. The most recently characterized type species, *Carnobacterium antarcticum* CP1^T (Zhu *et al.*, 2018), was not included because it was published after this study was initiated. In addition, two environmental *Carnobacterium* spp. isolates, strain WN1374, isolated from Siberian permafrost (Nicholson *et al.*, 2013), and strain AT7, isolated from the Aleutian trench (Lauro *et al.*, 2007), were studied.

Medium used throughout was Trypticase Soy Yeast Extract (TSY) medium, which consists of (per L): Tryptone, 17 g; Soytone, 3 g; NaCl, 5 g; Yeast Extract, 3 g; K_2HPO_4, 2.5 g; glucose, 2.5 g; final pH 7. For plates, agar was added at 15 g/L. For routine cultivation, strains were grown at

Table 2. *Carnobacterium* spp. Strains Used in This Study

No.	*Strain*	*Source*	*NCBI Accession No.*	*References*
1	*Carnobacterium alterfunditum* pf4^T	Ace Lake, Antarctica	NZ_JQLG00000000.1	Franzmann *et al.* (1991)
2	*Carnobacterium* sp. AT7	Aleutian trench, Pacific Ocean	NZ_ABHH00000000.1	Lauro *et al.* (2007)
3	*Carnobacterium divergens* 66^T	Refrigerated, vacuum-packed meat	NZ_JQLO00000000.1	Holzapfel and Gerber (1983); Collins *et al.* (1987)
4	*Carnobacterium funditum* pf3^T	Ace Lake, Antarctica	JQLL01000001.1	Franzmann *et al.* (1991)
5	*Carnobacterium gallinarum* MT44^T	Refrigerated, vacuum-packed meat	NZ_JQLU00000000.1	Collins *et al.* (1987)
6	*Carnobacterium iners* LMG26642^T	Forlidas Pond, Antarctica	NZ_FXBJ00000000.1	Snauwaert *et al.* (2013)
7	*Carnobacterium inhibens* subsp. *inhibens* K1^T	Intestine of Atlantic salmon	NZ_JQIV01000006.1	Jöborn *et al.* (1999); Nicholson *et al.* (2015)
8	*C. inhibens* subsp. *gilichinskyi* WN1359^T	Permafrost, Siberia	NC_022606.1	Nicholson *et al.* (2015)
9	*Carnobacterium jeotgali* MS3^T	Traditional Korean fermented seafood	NZ_JQNF00000000.1	Kim *et al.* (2009)
10	*Carnobacterium maltaromaticum* MX5^T	Milk with malty flavor	NZ_JQMX01000001.1	Mora *et al.* (2003)
11	*Carnobacterium mobile* MT37L^T	Irradiated chicken meat	NZ_JQMR00000000.1	Collins *et al.* (1987)
12	*Carnobacterium pleistocenium* FTR1^T	Permafrost, Alaska	NZ_JQLQ00000000.1	Pikuta *et al.* (2005)
13	*Carnobacterium viridans* MPL-11^T	Refrigerated, vacuum-packed bologna	NZ_FNJW01000008.1	Holley *et al.* (2002)
14	*Carnobacterium* sp. WN1374	Permafrost, Siberia	NZ_JQNG00000000.1	Nicholson *et al.* (2013)

^{T}Denotes type strain.
NCBI = National Center for Biotechnology Information.

laboratory-ambient temperature ($\sim$21–22°C) and sea-level pressure ($\sim 1.013 \times 10^5$ Pa), with the exception of *Carnobacterium iners* (strain 6, Table 2), which did not grow at ambient temperature and, thus, was routinely cultivated at 2°C.

2.2. Average nucleotide identity analyses

Whole-genome sequences of all the strains listed in Table 2 are publicly available in the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov) and the Integrated Microbial Genome and Microbiome (IMG/M) database (https://img.jgi.doe.gov/cgi-bin/mer/main.cgi). Their NCBI accession numbers are listed in Table 2. All 14 whole-genome sequences were analyzed in pairwise combination using the ''Pairwise ANI'' function on the IMG/M server.

2.3. Growth at ambient pressure

Cultivation of strains at laboratory-ambient sea-level pressure ($\sim 10^5$ Pa) was performed anaerobically in 12 mL liquid TSY medium in Klett tubes sealed with neoprene rubber stoppers leaving no head space. Growth of each strain was determined in triplicate by measuring optical density with a Klett-Summerson colorimeter fitted with the red No. 66 (660 nm) filter. For comparison with spectrophotometric readings, 100 Klett units is equal to 1 optical densities at 660 nm (OD_{660}). Measurements were recorded every 2 h for strains grown at room temperature and once per day for strains grown at 2°C.

2.4. Cultivation at low pressure

Low-pressure cultivation was carried out at UF. Seed cultures grown in liquid TSY were used to inoculate 14 mL of fresh medium in sterile disposable 15 mL conical screw cap tubes in triplicate. Tube caps were fitted loosely to allow for exchanges in gas pressure and composition. Tubes were placed inside a 4 L polycarbonate vacuum desiccator (model 08-642-7; Fisher Scientific, Pittsburg, PA). To generate an anoxic CO_2 atmosphere during incubation, four anaerobic pouches (Mitsubishi AnaeroPouch; Fisher Scientific), and an anaerobic indicator tablet were placed inside the desiccator. For all incubations, the desiccator was first flushed with pure CO_2 for 3–4 min, then sealed and placed into a 2°C incubator. Low pressures of 10^3 or 10^4 Pa were achieved with the use of an external programmable vacuum pump and low-pressure control system (model PU-842; KNF Neuberger, Trenton, NJ) connected to the desiccator. To prevent undesirable boiling of the medium due to rapid decrease in pressure, for the 10^3 Pa incubation, the pressure was sequentially brought down to 10^4 Pa, 5×10^3 Pa, and finally 10^3 Pa. Between each pressure decrease, the samples within the desiccator were allowed to equilibrate for 15 min.

When low-pressure samples at UF showed visible growth, the desiccator was vented, and the samples were removed and placed immediately onto ice. After gentle resuspension, a 0.1 mL aliquot was removed from each sample, and OD_{660} were determined with a spectrophotometer (Shimadzu). Samples that had not yet entered exponential growth phase were returned to the desiccator and allowed to continue incubating in their pressure condition.

2.5. Cultivation at high pressure

At UC, high-pressure cultivation at $\sim 10^5$, 10^6, and 10^7 Pa was performed in TSY medium at 2°C. Sterile 5 mL polyethylene transfer pipettes (Samco™ Narrow Stem Transfer Pipettes) filled with TSY medium were inoculated from frozen stock, heat-sealed, and incubated at atmospheric pressure and 2°C until mid-log phase was reached. Each liquid culture was then diluted 1:200 in quadruplicate into fresh TSY medium in 15 mL sterile transfer pipettes, heat-sealed, and placed into custom-designed stainless steel pressure vessels ($\sim$365 mL internal volume) (Yayanos, 2001) for incubation as described previously (Lauro *et al.*, 2007; Marietou *et al.*, 2014). For each pressure condition, several 5 mL sealed transfer pipettes containing TSY medium were inoculated to monitor growth over time. Periodically, one transfer pipette was removed and opened for growth measurement by optical density at 600 nm (OD_{600}, Genesystem 10S ultraviolet [UV]-Visible spectrophotometer), and the culture discarded. Once the target OD_{600} was reached, a 1 mL aliquot was removed from each 15 mL replicate to determine OD_{600}, perform cell counts through microscopy, and sequence the 16S ribosomal RNA (rRNA) gene for strain verification.

The remainder of the culture was harvested through centrifugation. Briefly, the culture was centrifuged at 7000*g* for 10 min at 2°C, and the supernatant was discarded. A second centrifugation at 7000*g* at 2°C for 2 min was performed and remaining supernatant was removed. The cell pellets were frozen at −80°C and shipped overnight on dry ice to UF.

2.6. Postharvest analyses

At UC, harvested high-pressure samples were analyzed by Sanger Sequencing (Retrogen, Inc.) of the 16S rRNA gene after polymerase chain reaction amplification using bacterial primers 27F and 1492R (Lane, 1991) to confirm the taxonomic identity of each culture and control for contamination. At UF, all samples were further analyzed by RNA-seq (to be described elsewhere). It is important to note here that bioinformatic analyses of the RNA-seq data revealed that some cultures contained mixtures of *Carnobacterium* spp. strains other than those intended. These samples were considered invalid for inclusion in the study and are labeled as ''nd'' (for ''not determined'') in the corresponding data figures.

2.7. Growth rate determinations and statistical analyses

Growth rates were calculated using the exponential model $ln\ (N_2/N_1)/t_2 - t_1$ where N is the OD and t is the time in either hours or days. Data were obtained from $n = 3$ (UF) or $n = 4$ (UC) samples. Means, standard deviations, and statistical analyses (analysis of variance) were computed using Kaleidograph v. 4.5.4 (Synergy Software). Differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Taxonomic classification of environmental Carnobacterium *spp. isolates*

Most of the *Carnobacterium* spp. used in this study belonged to taxonomically well-characterized type strains

(Table 2), with the two exceptions of *Carnobacterium* sp. AT7 isolated from the Aleutian Trench (Lauro *et al.*, 2007) and *Carnobacterium* sp. WN1374 isolated from Siberian permafrost (Nicholson *et al.*, 2013). Due to the uncertain taxonomic positions of these two environmental isolates, we deemed it desirable to determine their relatedness to the collection of known *Carnobacterium* spp. type strains. With the increasing availability of whole-genome prokaryotic sequences, Average Nucleotide Identity (ANI) has risen to prominence as a highly robust and accurate means of placing the genomes of newly isolated strains within the taxonomic context of well-characterized type strains (Ciufo *et al.*, 2018). We, therefore, performed pairwise ANI analyses of the 14 *Carnobacterium* spp. whole genomes, the results of which are presented in Table 3.

As proposed previously (Kim *et al.*, 2014; Ciufo *et al.*, 2018), genomes exhibiting >96% similarity by ANI are considered to belong to the same species. Examination of the data in Table 3 shows that, as expected, most strains are distinct species, exhibiting ANI values ranging from 73% to 88%; these results supported the concordance of ANI with species classifications previously established using 16S rRNA and phenotypic characteristics (cited in Table 2). However, three pairwise comparisons yielded ANI values greater than the 96% species threshold. First, the species *Carnobacterium inhibens* was recently divided into two subspecies, *C. inhibens* subsp. *inhibens* (strain 7) and *C. inhibens* subsp. *gilichinskyi* (strain 8), based on 16S rRNA sequence and phenotypic comparisons (Nicholson *et al.*, 2015). ANI analysis also placed these two strains within the same species with an ANI score of 98% (Table 3), further confirming that these two strains belong to the same species. Second, the two environmental isolates used in this study were also matched closely with known type species by ANI (Table 3).

Carnobacterium sp. AT7 (strain 2), originally isolated from 2.5 km below the surface of the Pacific Ocean (Lauro *et al.*, 2007), demonstrated an ANI score of 99% when compared with the whole-genome sequence of the type strain *Carnobacterium jeotgali* MS3^T (strain 9) (Table 2), a strain originally isolated from jeotgal, a traditional fermented Korean seafood (Kim *et al.*, 2009). *Carnobacterium* sp. WN1374 (strain 14), originally isolated from Siberian permafrost (Nicholson *et al.*, 2013), exhibited an ANI score of 98% when compared with the type strain *Carnobacterium viridans* MPL-11^T (strain 13), originally isolated from refrigerated, vacuum-packed bologna (Holley *et al.*, 2002). Thus, by ANI, the environmental isolates, AT7 and WN1374, likely belong to the species *C. jeotgali* and *C. viridans*, respectively.

As a further test of taxonomic relatedness, it has previously been established that two bacterial isolates are considered the same species if their 16S rRNA gene sequences are >97% similar (Stackebrandt and Goebel, 1994). Pairwise alignment of 16S rRNA sequences showed that environmental isolates AT7 and WN1374 demonstrated 99.37% and 99.86% similarity to type strains of *C. jeotgali* and *C. viridans*, respectively (data not shown). Taken together, the ANI and 16S rRNA data strongly support the notion that *Carnobacterium* spp. Strains, AT7 and WN1374, belong to the species *C. jeotgali* and *C. viridans*, respectively, although their official taxonomic classification would require more extensive phenotypic characterizations.

3.2. Growth of strains in TSY

For purposes of standardization, we deemed it desirable to measure the growth of all 14 *Carnobacterium* spp. strains in the same medium. TSY medium was previously used to successfully cultivate most of the *Carnobacterium* spp. in Table 2 at low temperature (0°C) and low pressure (7×10^2 Pa) (Nicholson *et al.*, 2013), so this was used for growth rate and yield determination of all 14 study strains (Fig. 1). Ten out of the 14 strains were found to be psychrotolerant and could be cultivated at ambient laboratory temperature ($\sim$21°C) and pressure ($\sim10^5$ Pa). Strains 2, 3, 5, 7, and 8 (Fig. 1A) and strains 9, 10, 11, 13, and 14 (Fig. 1B) grew well in TSY: (1) exhibiting exponential growth rates ranging from 0.23 to 0.64 h^{-1}; (2) reaching stationary phase in 5–18 h; and (3) growing to final OD_{660} values of 0.8–2.0 (Fig. 1A, B).

Four *Carnobacterium* spp. strains isolated from permanently cold environments (strains 1, 4, 6, and 12) grew poorly or not at all at 21°C and were therefore cultivated in TSY at 2°C (Fig. 1C). At 2°C, strains 1, 4, 6, and 12

Table 3. Average Nucleotide Identity Values of the 14 *Carnobacterium* Strains Used in This Study

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
1	*													
2	84	*												
3	73	73	*											
4	76	74	73	*										
5	73	74	76	73	*									
6	75	74	74	84	73	*								
7	83	85	73	75	73	75	*							
8	83	85	73	75	73	74	*98*	*						
9	84	*99*	73	74	73	74	85	84	*					
10	73	73	76	73	79	73	73	74	74	*				
11	75	75	73	77	73	77	75	75	74	73	*			
12	88	84	73	74	73	74	82	82	84	73	75	*		
13	83	84	74	74	74	75	85	85	84	73	75	83	*	
14	83	84	73	75	74	74	85	85	84	73	75	83	*98*	*

Numbers in bold refer to the *Carnobacterium* spp. strains denoted in Table 2. ANI values are presented as percentages. Italics values denote ANI values above the threshold for consideration as the same species (>96%) (Kim *et al.*, 2014; Ciufo *et al.*, 2018).

Asterisks denote intersection of strain v. itself.

ANI = average nucleotide identity.

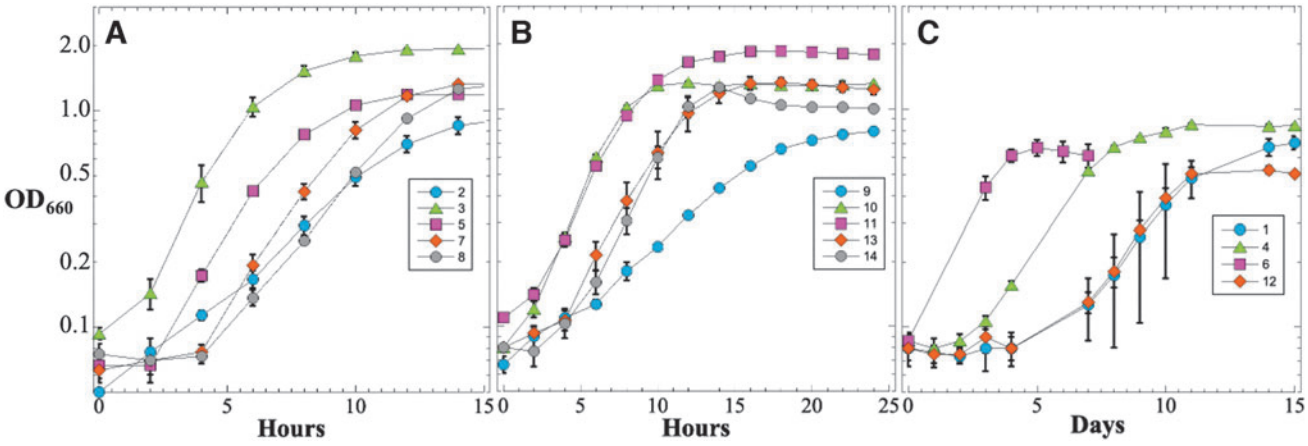

FIG. 1. Growth of 14 *Carnobacterium* sp. strains in liquid TSY medium at atmospheric pressure ($\sim 10^5$ Pa) and 23°C **(A, B)** or 2°C **(C)**. Strain numbers are as denoted in Table 2. Data points are averages ± standard deviations ($n = 3$). Error bars not visible are smaller than the plot symbols. TSY, Trypticase Soy Yeast Extract.

grew in TSY medium, although they exhibited reduced exponential growth rates ranging from 0.021 to 0.028 h^{-1} and lower final growth yields at or slightly above 0.5 OD_{660} units (Fig. 1C). With the exception of strain 6, the strains grown at 2°C also exhibited extended lag phases of up to 4 days before entering the exponential phase (Fig. 1C). From this point on, all 14 *Carnobacterium* spp. strains were cultivated in TSY medium at 2°C.

3.3. Growth of strains at 2°C and ambient pressure ($\sim 10^5$ Pa)

In our comparisons of *Carnobacterium* spp. growth with respect to pressure, our aim was to maintain as many equivalent environmental parameters as possible between cultures grown at UF (low-pressure) and UC (high-pressure). We first established that all strains were capable of anaerobic growth at 2°C, and that all strains could utilize TSY as the growth medium (Fig. 1). However, the methods of strain cultivation were slightly different at UF versus UC; high-pressure cultivations at UC were performed in sealed transfer pipettes with no headspace, whereas low-pressure cultivations at UF were by necessity performed in a vacuum chamber under CO_2 atmosphere (see Section 2 for details). To assess how differences in atmospheric conditions at UF versus UC affected cell growth, we performed a comparative growth experiment in which cells were cultivated in identical conditions of medium (TSY), temperature (2°C), and ambient pressure ($\sim 10^5$ Pa), but under CO_2 (UF) or no head space (UC).

For most strains, growth rate varied dramatically under the two conditions, by as much as nearly eightfold in the case of *Carnobacterium divergens* (strain 3) (Fig. 2). In only 3 strains out of 14 tested (strains 2, 4, and 13) were growth rates similar under both atmospheric conditions (Fig. 2). From these results, it was evident that the presence or absence of CO_2 exerted a differential effect on growth, and quantitative growth rate comparisons could only be made among cultures grown under the same atmospheric condition.

3.4. Growth of strains at low pressures

At UF, all 14 *Carnobacterium* spp. strains were cultivated in liquid TSY medium at 2°C and low pressures of 10^3 and 10^4 Pa, as well as at an ambient pressure of $\sim 10^5$ Pa (Fig. 3). All 14 strains were capable of growth at low pressures; however, their growth rates varied markedly, ranging from ~ 0.15 to ~ 1.6 day^{-1} (Fig. 3). In addition, it was noted that their patterns of growth with respect to pressure differed substantially. For example, the majority of strains (strains 2, 4, 7, 8, 9, 12, 13, and 14) exhibited increased growth rates with decreasing pressure (Fig. 3A). The observation of increased growth of *Carnobacterium* spp. at lowered pressure is consistent with, and expands, prior observations (Nicholson *et al.*, 2013). In contrast, the growth rates of strains 5 and 11 decreased as pressure was

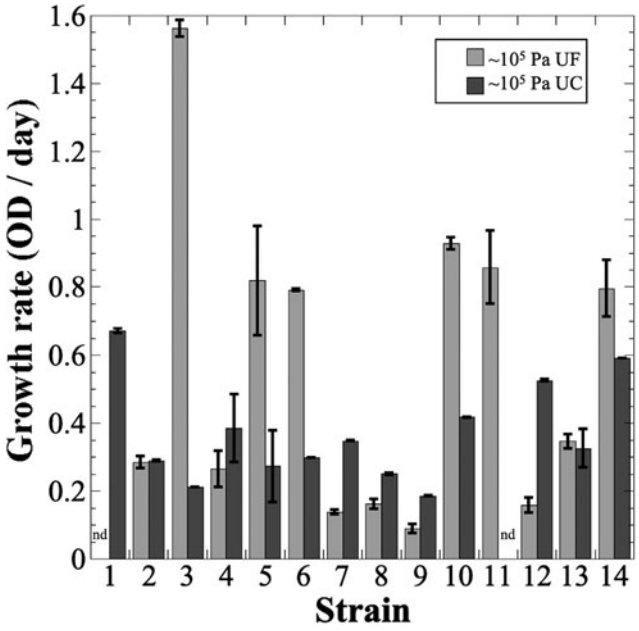

FIG. 2. Growth rates of 14 *Carnobacterium* spp. in TSY medium at 2°C and laboratory-ambient pressure ($\sim 10^5$ Pa) at UF (light gray bars) or UC (dark gray bars). Strain numbers are as denoted in Table 2. Data are averages ± standard deviations of $n = 3$ (UF) or $n = 4$ (UC) replicates. nd, not determined; UC, University of California; UF, University of Florida.

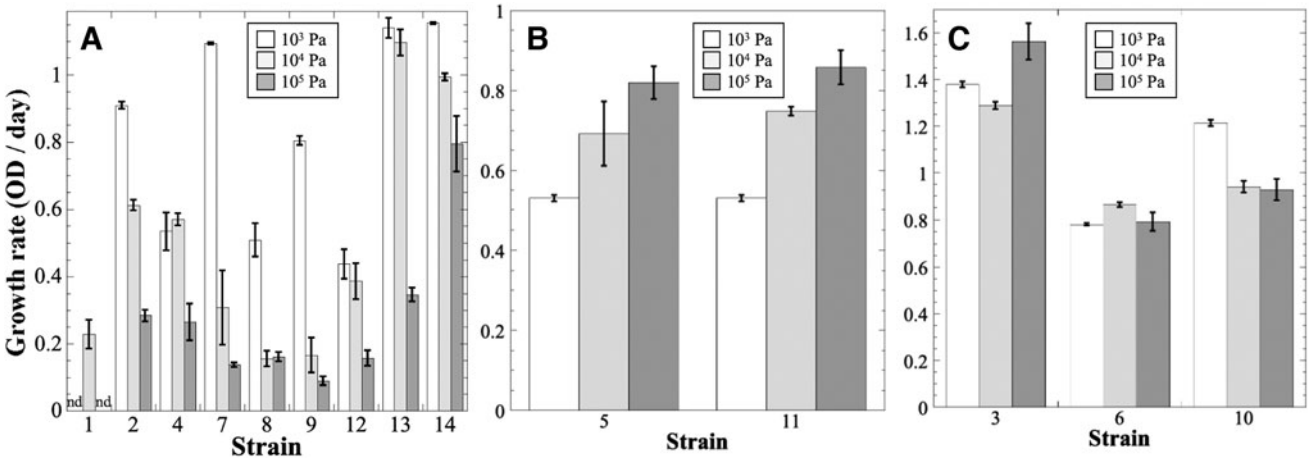

FIG. 3. Growth rates of *Carnobacterium* spp. at low pressures of 10^3 Pa (white bars), 10^4 Pa (light gray bars), and $\sim 10^5$ Pa (gray bars). Strain numbers are as denoted in Table 2. Experiments were performed at UF. Data are averages ± standard deviations of $n = 3$ replicates. Panel letters are described in the text.

lowered (Fig. 3B). Growth of strains 3, 6, and 10 appeared to be essentially indifferent to low pressure, as they grew at relatively high rates under all pressures tested (Fig. 3C). We were unable to determine the response of strain 1 to the full range of low pressure (Fig. 3A), as two of the cultures, grown at 10^3 and $\sim 10^5$ Pa, were subsequently found by later testing not to be pure. However, strain 1 was able to grow at 10^4 Pa (Fig. 1A).

3.5. Growth of strains at high pressures

At UC, all 14 *Carnobacterium* spp. strains were cultivated in liquid TSY medium at 2°C and high pressures of 10^6 and 10^7 Pa, as well as at ambient pressure of $\sim 10^5$ Pa (Fig. 4). In addition, an attempt was made to cultivate all 14 strains in TSY at 3×10^7 Pa; however, cells failed to grow to an appreciable OD_{600} after several months of incubation at this pressure (data not shown). While strains were capable of growth at high pressures, their growth rates varied markedly from ~ 0.12 to ~ 0.68 day^{-1} (Fig. 4). It was noted that, for the majority of strains (strains 1, 3, 5, 6, 7, 8, 10, 13, and 14), growth rate decreased as pressure increased (Fig. 4A), while a handful of strains (strains 2, 4, and 12) appeared relatively indifferent to pressure (Fig. 4B). No strains were observed to increase growth rate with increasing pressure when grown at 2°C. We were unable to determine a growth pattern for strains 9 and 11 (Fig. 4B), as later testing revealed that these samples were not pure cultures.

4. Discussion

The genus *Carnobacterium* was originally established to encompass atypical lactic acid bacteria isolated as contaminants of vacuum-packed refrigerated meats (Collins *et al.*, 1987); in this sense, growth of certain *Carnobacterium* spp. (*C. divergens*, *Carnobacterium gallinarum*, *C. viridans*)

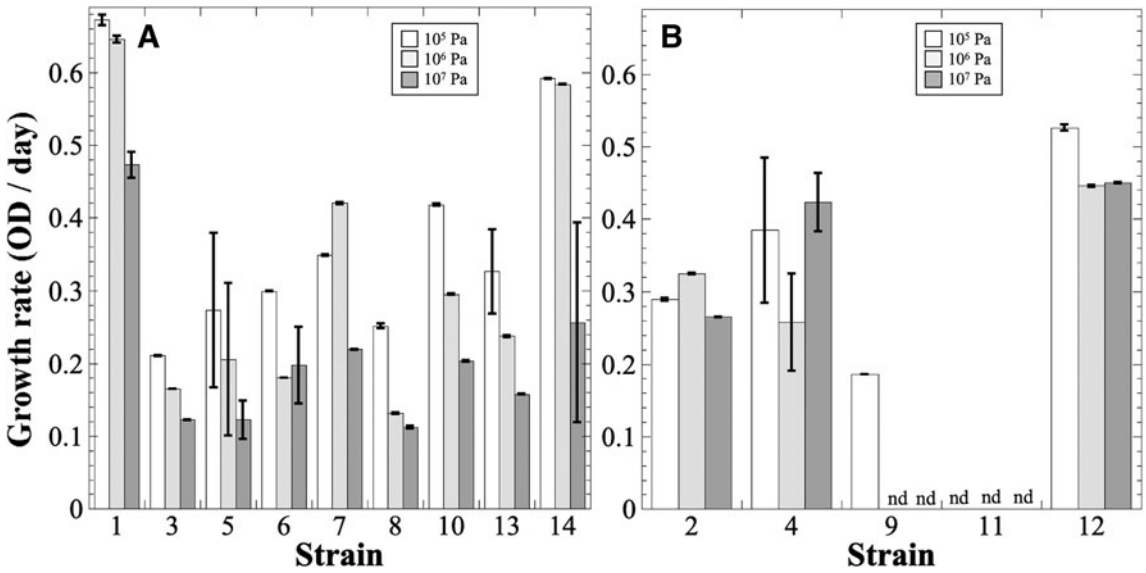

FIG. 4. Growth rates of *Carnobacterium* spp. at high pressures of $\sim 10^5$ Pa (white bars), 10^6 Pa (light gray bars), and 10^7 Pa (dark gray bars). Strain numbers are as denoted in Table 2. Experiments were performed at UC. Data are averages ± standard deviations of $n = 4$ replicates. Panel letters are described in the text.

at low temperature and low pressure had previously been documented in the area of food microbiology (cited in Table 2). Members of the genus have since been isolated from diverse environments ranging from food products and animals to Arctic permafrosts, Antarctic lakes (Table 2), and Antarctic soil (Zhu *et al.*, 2018). Their isolation from such disparate and harsh locations highlights the resilience of these organisms to extreme environmental conditions that prevail in the cold biosphere. In a prior study, most of the 14 *Carnobacterium* spp. isolates used in the study were found to grow under conditions characteristic of the Mars surface (*i.e.*, low-temperature [0°C], low-pressure [7×10^2 Pa], and anoxic CO_2-dominated atmosphere) (Nicholson *et al.*, 2013).

The discovery of *Carnobacterium* sp. AT7 at the bottom of the Aleutian trench—capable of growth over a pressure range of 10^5–10^7 Pa (Lauro *et al.*, 2007)—extended the pressure range of the genus to include high pressures, prompting this study. The present work demonstrates the ability by 11 of the 14 tested isolates from the genus *Carnobacterium* to actively grow in low temperature (2°C) and anoxic conditions at pressures ranging over 5 orders of magnitude, from the surface of Mars (10^3 Pa) to Earth's deep oceans (10^7 Pa). To our knowledge, this represents the largest pressure range for growth demonstrated by any organism.

A surprising finding from this study was the observation that most *Carnobacterium* spp. strains (8 out of 14) grew best at the lowest pressure tested, 10^3 Pa (Fig. 3A). This low pressure does not prevail in natural environments on Earth's surface, but instead corresponds to $\sim$30 km altitude in the stratosphere. Interestingly, this pressure does lie in the pressure range encountered on the surface/near-subsurface of Mars, $\sim10^2$–10^3 Pa (Schuerger and Nicholson, 2016). Growth of *Carnobacterium* spp. strains described in this study, as well as other bacterial species cultivated at Mars-relevant pressures (Schuerger and Nicholson, 2016), lends further credence to the notion that low pressure *per se* is not an impediment for growth of terrestrial microbes inadvertently introduced as forward contaminants into the Mars environment—an ongoing concern for Planetary Protection (reviewed extensively in Rummel *et al.*, 2014).

The observation of robust growth at 10^3 Pa was most strikingly illustrated in the case of *Carnobacterium* sp. AT7 (strain 2), which was originally isolated from 2.5 km ocean depth at a pressure of $\sim2.5\times10^7$ Pa, and which previously exhibited growth at a pressure range of $\sim10^5$ to 6×10^7 Pa (Lauro *et al.*, 2007). Particularly surprising was the observation that this strain's growth rate increased with decreasing pressure below Earth-ambient pressure, showing its highest growth rate of ~0.9 day^{-1} when cultivated at 10^3 Pa (Fig. 3A). In the absence of data to the contrary, we can only presume that the high-pressure environment of the deep ocean is its natural habitat. However, AT7 was shown by ANI to be closely related to the *C. jeotgali* type strain MS3^T (Table 3), which was originally isolated from a traditional Korean fermented food called ''toha jeotgal'' made from salted, fermented freshwater shrimp (Kim *et al.*, 2009), indicating that this species is not limited exclusively to high-pressure environments.

At present, the molecular mechanism(s) for coping with low pressure are much more poorly understood than are those responsible for high-pressure adaptation (reviewed in Michiels *et al.*, 2008; Mota *et al.*, 2013). High pressure exerts effects on multiple cellular functions that include motility, substrate transport, DNA replication, transcription, translation, cell division, and ultimately growth and viability (Bartlett, 1992; Mota *et al.*, 2013). Many, if not most, of these cellular processes are dependent upon, or linked to, lipid membrane fluidity. Increasing pressure and decreasing temperature both tend to compress membranes, making them more rigid and impermeable (reviewed in Michiels *et al.*, 2008; Mota *et al.*, 2013). Conversely, increasing temperature and decreasing pressure both produce the opposite effect, increasing membrane fluidity, permeability, and ultimately disrupting membrane integrity. In addition to lipids, cellular membranes contain integral membrane proteins that perform numerous essential metabolic activities (*e.g.*, nutrient and ion transport, secretion, electrochemical homeostasis, electron transport, ATP generation, and motility, among others).

Both the assembly and optimal function of membrane protein complexes rely intimately on lipid composition and membrane fluidity (Dowhan *et al.*, 2019). These observations introduce the possibility that the combination of low temperature (membrane-rigidizing) and low pressure (membrane-fluidizing) may actually counterbalance each other and result in a physical environment more favorable to growth of psychrotolerant microbes at low pressure.

Homeoviscous adaptation is the process by which cells maintain optimal membrane fluidity in response to environmental changes such as shifts in temperature and pressure. Such optimization is achieved by altering the lipid composition of their membranes (Parsons and Rock, 2013). In the case of high-pressure adaptation, piezophiles often upregulate the production of a variety of modified lipid fatty acids (FAs) such as mono- and polyunsaturated fatty acids (MUFAs and PUFAs, respectively); these increase membrane fluidity to counteract pressure-induced membrane rigidity (reviewed in Fang and Bazylinski, 2008). Although very little data exist regarding membrane lipid composition in bacteria grown at low pressure, in a prior communication we demonstrated in the model organism *Bacillus subtilis* that growth at progressively lower pressures (when temperature was held constant) resulted in a decrease in unsaturated FA and a concomitant increase in saturated FA, consistent with the homeoviscous model (Fajardo-Cavazos *et al.*, 2012).

Future studies using *Carnobacterium* spp. could aid in the effort to uncover fundamental mechanisms by which microbes grow at a wide range of pressures. For example, measurement of membrane FA compositions in the same strain cultivated at a variety of low and high pressures would further probe the homeoviscous adaptation model. In addition, measurements of the global gene expression and physiological responses to a range of pressures would provide a wealth of information from which further hypotheses could be developed. Indeed, in prior studies using both *B. subtilis* (Waters *et al.*, 2014) and *Serratia liquefaciens* (Fajardo-Cavazos *et al.*, 2018), we showed that cultivation at low pressure resulted in the large-scale remodeling of those organisms' transcriptomes. Understanding at a fundamental level how microbes respond and adapt to a wide diversity of pressures in the cold biosphere can give us insights into how life might inhabit permanently cold extraterrestrial environments in our solar system.

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Authors' Contributions

K.M.M.: Methodology, investigation, formal analysis, data curation, writing—original draft, visualization, and writing—review and editing. F.T., S.L., K.K.M., B.R.S., and L.B.: Investigation, formal analysis, and writing—review and editing. D.H.B. and W.L.N.: Visualization, writing—original draft, writing-review and editing, supervision, project administration, and funding acquisition.

Author Disclosure Statement

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Abbreviations Used

ANI = average nucleotide identity
FA = fatty acid
IMG/M = Integrated Microbial Genome and Microbiome
NCBI = National Center for Biotechnology Information
nd = not determined
OD_{600} = optical density at 600 nm
OD_{660} = optical density at 660 nm
rRNA = ribosomal RNA
TSY = Trypticase Soy Yeast Extract
UC = University of California
UF = University of Florida