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Implications of Subzero Metabolic Activity on Long-Term Microbial Survival in Terrestrial and Extraterrestrial Permafrost

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Abstract

The survival of microorganisms over extended time frames in frozen subsurface environments may be limited by chemical (*i.e.,* via hydrolysis and oxidation) and ionizing radiation–induced damage to chromosomal DNA. In an effort to improve estimates for the survival of bacteria in icy terrestrial and extraterrestrial environments, we determined rates of macromolecular synthesis at temperatures down to −15°C in bacteria isolated from Siberian permafrost (*Psychrobacter cryohalolentis* K5 and *P. arcticus* 273-4) and the sensitivity of *P. cryohalolentis* to ionizing radiation. Based on experiments conducted over ≈400 days at −15°C, the rates of protein and DNA synthesis in *P. cryohalolentis* were <1 to 16 proteins cell^{-1} d^{-1} and 83 to 150 base pairs (bp) cell^{-1} d^{-1}, respectively; *P. arcticus* synthesized DNA at rates of 20 to 1625 bp cell^{-1} d^{-1} at −15°C under the conditions tested. The dose of ionizing radiation at which 37% of the cells survive (D_{37}) of frozen suspensions of *P. cryohalolentis* was 136 Gy, which was ~2-fold higher (71 Gy) than identical samples exposed as liquid suspensions. Laboratory measurements of [^{3}H]thymidine incorporation demonstrate the physiological potential for DNA metabolism at −15°C and suggest a sufficient activity is possible to offset chromosomal damage incurred in near-subsurface terrestrial and martian permafrost. Thus, our data imply that the longevity of microorganisms actively metabolizing within permafrost environments is not constrained by chromosomal DNA damage resulting from ionizing radiation or entropic degradation over geological time. Key Words: Extraterrestrial life—Radiation resistance—Cryosphere—DNA—Dormancy. Astrobiology 10, 789–798.

1. Introduction

Microbial permafrost communities provide experimentally tractable analogues to evaluate the likelihood of microbial life surviving in frozen extraterrestrial environments (*e.g.,* Gilichinsky *et al.,* 2007). Relatively high concentrations of organic matter (5–128 g kg^{-1} of soil; Rivkina *et al.,* 2000; Rodionow *et al.,* 2006) and viable cells (up to 10^8 per gram; Rivkina *et al.,* 1998) exist in permafrost. Despite the low temperature (0 to −40°C; *e.g.,* Panikov *et al.,* 2006; Steven *et al.,* 2006) and low water activity conditions (up to 600 g of dissolved salts L^{-1} of unfrozen water; Shouakar-Stash *et al.,* 2007), viable bacteria and fungi are recovered frequently from permafrost (Bakermans *et al.,* 2003, 2006; Gilichinsky *et al.,* 2007; Panikov and Sizova, 2007) and from the highly saline water lenses embedded in permafrost (*i.e.,* cryopegs; Gilichinsky *et al.,* 2003, 2005). In particular, *Psychrobacter arcticus* 273-4 and *P. cryohalolentis* K5 (Gilichinsky *et al.,* 2003; Bakermans *et al.,* 2006) have provided valuable insight into the growth (Bakermans *et al.,* 2003), genomic composition (Bakermans *et al.,* 2006), gene and protein expression (Bakermans *et al.,* 2007; Bergholz *et al.,* 2009), resistance to high salinity (Ponder *et al.,* 2008), and energy metabolism (Amato and Christner, 2009) of cold-adapted bacteria.

For a microorganism to remain viable in a dormant state over an extended time frame, damage to the cell (*e.g.,* chromosomal DNA) must not exceed a level where effective repair is no longer possible. For example, DNA integrity is essential to life, but the molecule is subject to chemical (*i.e.,* via hydrolysis and oxidation) and ionizing radiation–induced damage over geological time frames. McKay (2001) measured natural background gamma radiation [2×10^{-3} Gy yr^{-1} at 25 meters below the surface (mbs)] in Siberian permafrost, presumably a result of the decay of radionuclides such as ^{40}K, ^{232}Th, and ^{238}U (United Nations Scientific Committee on the Effects of Atomic Radiation, 1982) and

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concluded that most dormant bacteria would have received a lethal dosage after 10 million years in permafrost. An investigation of DNA integrity from a chronological sequence of permafrost, ranging in age from 10 thousand to 400 thousand years, revealed that single- and double-stranded breaks in the DNA, extensive interstrand crosslinking, and depurination were all correlated with the age of the sample (Hansen *et al.*, 2006). Using ancient Antarctic ice samples 100 thousand years to 8 million years old, Bidle *et al.* (2007) demonstrated that the mean length of genomic DNA molecules decreased exponentially with age and estimated an average community DNA size half-life of 1.1 million years. It should be noted, however, that the precise age of the samples used in the latter study is a matter of debate (*i.e.*, Ng *et al.*, 2005; Johnson *et al.*, 2007).

The elemental composition of martian permafrost is thought to be similar to that on Earth (Smith and McKay, 2005), so the background gamma irradiation dose rate from this source is likely comparable to terrestrial values. In addition, Mars lacks a substantial atmosphere and magnetic shield, and the surface is continually bombarded with high-energy radiation fields much higher than those on Earth. Dartnell *et al.* (2007) modeled the penetration of cosmic ionizing radiation in the near subsurface of Mars and concluded that at a depth of 2 m a radiation-resistant population of microorganisms (*i.e., Deinococcus radiodurans*) entrapped in permafrost would need to reanimate every 450 thousand years to remain viable; less radiotolerant species (*i.e., E. coli*) would only survive for 30 thousand years.

Implicit to the estimates discussed above is that the microbes entrapped within frozen matrices are metabolically inactive and, therefore, are unable to conduct cellular repair as damage is incurred over time. Hence, as inferred by the results of Johnson *et al.* (2007) on permafrost samples up to 740 thousand years old, a microbial assemblage capable of cellular repair at subzero temperatures would have the potential to remain viable for substantially longer periods under frozen conditions than populations of dormant cells (*i.e.*, metabolically inert cells). There are a number of recent reports documenting microbial metabolism (*i.e.*, respiration and DNA, protein, and lipid synthesis) at temperatures well below the freezing point in permafrost (Rivkina *et al.*, 2000; Jakosky *et al.*, 2003; Panikov *et al.*, 2006), snow (Carpenter *et al.*, 2000), sea ice (Junge *et al.*, 2004), and ices generated in the laboratory (Christner, 2002; Junge *et al.*, 2006; Miteva *et al.*, 2007; Amato *et al.*, 2009). Herein, we report measurements of macromolecular synthesis by *P. arcticus* 273-4 and *P. cryohalolentis* K5 at temperatures of −15°C, together with experiments that examined the sensitivity of *P. cryohalolentis* to controlled dosages of ionizing radiation. Using these data, we estimated the time frame for survival of dormant and active microbial populations of this species in terrestrial and extraterrestrial permafrost. If permafrost environments are active biomes, our calculations imply that DNA damage from cosmic or background ionizing radiation is insufficient to constrain microbial survival in permafrost on Earth or Mars over geological time.

2. Material and Methods

2.1. Bacterial strains and culturing conditions

Pure cultures of *P. cryohalolentis* K5 (DSM 17306 = VKM B-2378) and *P. arcticus* 273-4 (DSM 17307 = VKM B-2377) were grown aerobically (shaking at 200 rpm) at 15°C in M9 mineral medium (Difco) supplemented with 24 m*M* acetate, vitamins (ATCC, ref. MD-VS, 1% v/v), and a trace mineral solution (ATCC, ref. MD-TMS, 1% v/v). Cells in the exponential phase of growth were harvested by centrifugation (3 min, 17,000*g*), washed in deionized H_2O or M9 mineral medium, and resuspended in either deionized water or M9 (80%, 0.8%, 0.08%, and 0.008%) at a final concentration of $\sim 7\times10^6$ colony-forming units (cfu) mL^{-1}. The concentration of cfu mL^{-1} was determined by standard dilution plating of the samples on R2A medium (Difco) and based on the average number of colonies that formed on triplicate plates after 2 days of incubation at 22°C.

2.2. [^{3}H]Leucine and [^{3}H]thymidine incorporation into macromolecules

Measurement of macromolecular synthesis by cells at −15°C was carried out following the procedure described by Christner (2002). A 0.5 mL volume of the cell suspension was amended with 0.1 mL of [^{3}H]leucine (L-leucine [4, 5-^{3}H], 84 Ci $mmol^{-1}$ in ethanol water 2:98; MP Biomedical) or [^{3}H]thymidine (thymidine [Methyl-^{3}H], 61 Ci $mmol^{-1}$ in sterile water; MP Biomedical) to a final concentration of 20 n*M*. Killed controls were amended with ice-cold 50% trichloroacetic acid (TCA) to a final concentration of 7% (w/v). The ice-chilled samples were placed in a −80°C freezer within 30 s after the addition of [^{3}H]leucine or [^{3}H]thymidine. The cooling rate at −80°C was measured with an electronic temperature probe (Hobo) and was 4.5°C min^{-1}, which implies that the samples were frozen <10 min after being placed in the −80°C freezer. After 1 h at −80°C, samples were transferred to a thermally stable freezer with a mean temperature of −15°C (Thermo Isotemp; ± 1.5°C), which is hereafter referred to in the text and figures as the experimental time "zero."

At each experimental time point, frozen samples were removed from the freezer and immediately overlain with 0.1 mL of ice-cold 50% TCA (final concentration of 7%). The frozen suspensions were subsequently allowed to melt directly into the concentrated TCA solution. After melting, the samples were incubated at 4°C for at least 30 min. The acid-insoluble macromolecules were pelleted for 15 min at 17,000*g*, washed with 0.5 mL cold 5% TCA, centrifuged for 10 min (17,000*g*), and rinsed with ice-cold 70% ethanol followed by centrifugation and removal of the supernatant. The pelleted material was resuspended in 1 mL of Cytoscint scintillation cocktail (Fisher, cat. no. BP458-4), and the radioactive material present was quantified with standard liquid scintillation spectrometry (Beckman LS6000IC scintillation counter). Counts per minute were converted to disintegrations per minute (DPM) by determining the counting efficiency with a series of acetone-quenched standards of [^{3}H]toluene (American Radiolabeled Chemicals, cat# ARC182) in the Cytoscint cocktail.

Rates of leucine and thymidine incorporation per cell were calculated over linear portions ($R^2 > 0.80$) of the time-course curves. The rate measurements were converted into gC gC^{-1} d^{-1} (*i.e.*, grams of substrate carbon incorporated, per gram of cell carbon, per day) based on 65 fg C $cell^{-1}$ (Whitman *et al.*, 1998; as in the calculations of Price, 2000, and Junge *et al.*, 2006).

2.3. Biochemical fractionation of macromolecules

The quantity of [^{3}H]leucine incorporated into the lipid, nucleic acid, and protein fraction was determined by sequential biochemical fractionation of the TCA-precipitated material as described by Kelley (1967). The cell material was collected by centrifugation (15 min at 17,000*g*) and was rinsed twice in 0.5 mL deionized H_2O followed by centrifugation for 10 min at 17,000*g*. The lipid fraction was solubilized from the pellet by the addition of 40 μL of a 1:1 ethanol:acetone solution followed by incubation for 30 min at 37°C. The samples were then centrifuged for 10 min at 17,000*g*, and the supernatant was recovered. The nucleic acid fraction was extracted from the remaining pellet by the addition of 40 μL of 5% TCA, incubation for 30 min at 95°C, and centrifugation for 10 min at 17,000*g*. The protein fraction was obtained from the remaining pellet by the addition of 40 μL of 6 *N* HCl, followed by incubation for 15 h at 105°C and centrifugation for 10 min at 17,000*g*. Each fractionation class was quantified by the addition of 1 mL of Cytoscint (Fisher, cat# BP458-4) followed by liquid scintillation spectrometry.

2.4. Determination of ionizing radiation sensitivity

Cells from the exponential phase of growth were harvested by centrifugation (2 min, 17,000*g*), rinsed twice in phosphate-buffered saline (pH 7.2), and resuspended into phosphate-buffered saline to a concentration of $\sim 10^8$ cells mL^{-1}. Triplicate samples containing 0.2 mL of the liquid or frozen cell suspension were exposed to a range (0–1200 Gy) of controlled gamma-radiation exposures with a ^{60}Co irradiator (J.L. Sheppard and Associates Model 484), which permits precise reproducible exposures to the source. For experiments under liquid conditions, irradiations were performed at ambient temperature ($\sim$22°C). For irradiation experiments under frozen conditions, aliquots of the cell suspension were frozen at −80°C as described above and transported in an insulated cooler. The internal cooler temperature was continually monitored (Hobo temperature logger) for the duration of the experiment (50 min) and averaged −6°C (minimum of −14°C, maximum of −3°C). After exposure, the frozen cell suspensions were immediately transferred to the laboratory and melted at room temperature. Twenty-five–microliter aliquots were used for standard dilution plating. The number of cfu mL^{-1} was determined for each dose by counting the colonies that formed on plated dilutions after 2 days of incubation at 22°C. The D_{37} values (*i.e.,* the dose to which 37% of the cells survive) were calculated from linear fits of log (% survival) = *f* (dose) plots.

The precise ionizing radiation dosage was determined by Fricke dosimetry (Fricke and Hart, 1966). Tubes containing 0.2 mL of a filtered solution of 1.4 m*M* $FeSO_4$ and 1 m*M* NaCl in 2.2% H_2SO_4 (v/v) were irradiated simultaneously with the experimental cell suspensions. At designated time points, triplicate samples were removed from the irradiator, and the absorbance at 304 nm was measured. The dose rate was then calculated as follows:

$$\text{Dose rate (Gy min}^{-1}) = 10^{11} \times (A_t - A_0)/(E \times b \times G)$$

where A_t (after exposure) and A_0 (before exposure) are the absorbance at 304 nm measured for the solution exposed for *t* minutes; *E* is the molar extinction coefficient for ferric ion at 304 nm (2174 L mol cm^{-1}); *b* is the path length in centimeters; *G* is the yield term in ions/100 eV (15.6 for ferric ion in an oxygenated solution). A linear fit of the data was calculated from the mean of triplicate measurements from 6 to 9 exposure time points.

3. Results

3.1. Incorporation of macromolecular precursors under frozen conditions

In contrast to the controls, live cells of *P. arcticus* and *P. cryohalolentis* incorporated [^{3}H]leucine and [^{3}H]thymidine in a time-dependent fashion over 404 days at −15°C (Fig. 1). Over the course of the experiment, $\sim 5\times10^8$ molecules of [^{3}H]leucine or [^{3}H]thymidine were incorporated per sample ($1.77 \pm 0.56\times10^6$ cells). The incorporation of [^{3}H]thymidine was continuous and linear ($R^2 > 0.93$) over time for both species. *P. cryohalolentis* incorporated 78% of the total [^{3}H]leucine during the first 15 days of incubation (Fig. 1B), whereas *P. arcticus* [^{3}H]leucine incorporation mirrored the trend observed during incubation with [^{3}H]thymidine (Fig. 1A). Previous studies have documented physiological differences between *P. cryohalolentis* and *P. arcticus* at subzero temperatures (Bakermans *et al.,* 2007; Bergholz *et al.,* 2009). Both species demonstrated very similar patterns and rates of [^{3}H]thymidine and [^{3}H]leucine incorporation at −15°C under the conditions tested. *P. arcticus* did, however, demonstrate an unexplainably high [^{3}H]thymidine incorporation rate (1625 bp cell^{-1} d^{-1}) in deionized water ice compared to nutrient-amended samples (Table 1). Based on these results, *P. cryohalolentis* was chosen for a series of subsequent experimental measurements.

After 123 days at −15°C, the lipid, nucleic acid, and protein fractions of *P. cryohalolentis* cells incubated in the presence of [^{3}H]leucine in deionized water, 80% and 0.8 % M9 medium were separated biochemically, and the radioactivity associated with each fraction was quantified (Fig. 2). For each nutrient treatment, the majority of the radioactive leucine incorporated (74–95%) was associated with the protein fraction, and the lipid and nucleic acid fractions contained each 1–11% of the total incorporated radioactivity, the latter of which was not significantly different from controls. Based on the predicted leucine content and average protein length in the *P. cryohalolentis* genome, this level of activity corresponded to the incorporation of 13–318 [^{3}H]leucine molecules cell^{-1} d^{-1}, which represented the synthesis of <1 to 16 proteins per day.

The rate of [^{3}H]leucine incorporation in *P. cryohalolentis* and *P. arcticus* was determined at temperatures of −15°C, −5°C (frozen), and 4°C ($n = 11$, data not shown) over 3–404 days, depending on the temperature of incubation. Following the Arrhenius rate law, the rate of incorporation decreased exponentially with temperature and ranged from 0.00487 ± 0.00147 to $1.47 \pm 0.481\times10^{-5}$ [$gC_{leucine}$] [gC_{cell}]$^{-1}$ [d]$^{-1}$ at −15°C and 4°C, respectively, for *P. cryohalolentis*. *P. arcticus* incorporated [^{3}H]leucine at rates ranging from 0.131 ± 0.012 to $2.22 \pm 0.180\times10^{-6}$ [$gC_{leucine}$] [gC_{cell}]$^{-1}$ [d]$^{-1}$ at −15°C and −5°C, respectively (no data at 4°C). The rate of [^{3}H]thymidine incorporation at −15°C ranged from 1.84 ± 0.214 to $3.33 \pm 0.745\times10^{-7}$ [$gC_{thymidine}$] [gC_{cell}]$^{-1}$ [d]$^{-1}$ for *P. cryohalolentis* and from 0.0452 ± 0.00273 to $3.63 \pm 0.459\times10^{-6}$ [$gC_{thymidine}$] [gC_{cell}]$^{-1}$ [d]$^{-1}$ for *P. arcticus*,

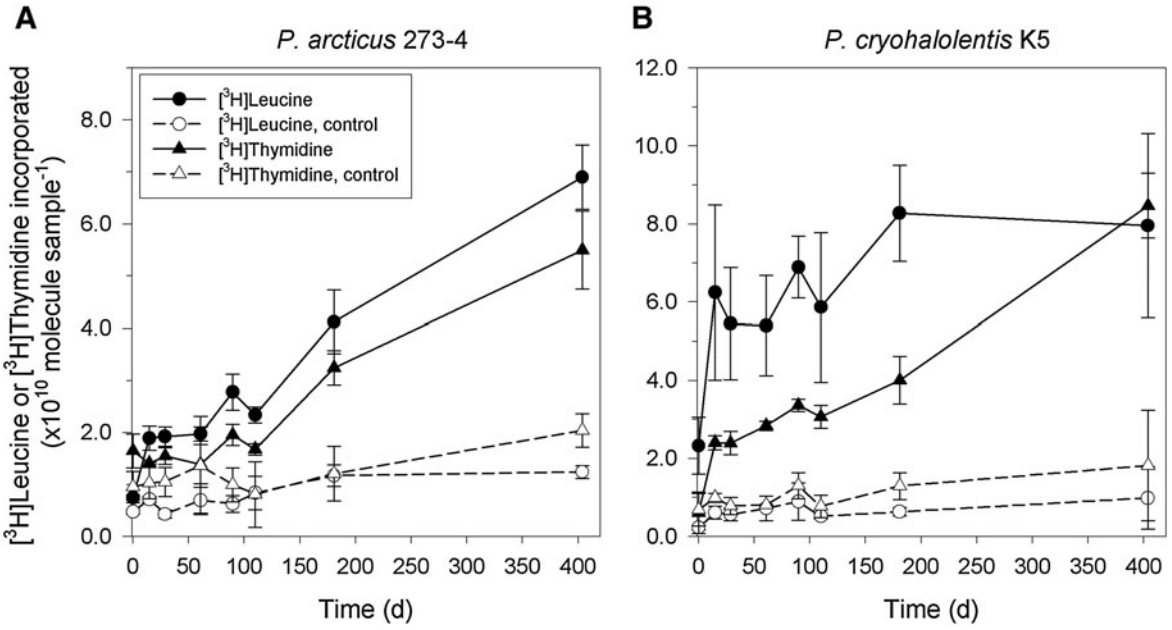

FIG. 1. Incorporation of [^{3}H]leucine (circles) and [^{3}H]thymidine (triangles) under frozen conditions by *P. arcticus* 273-4 **(A)** and *P. cryohalolentis* K5 **(B)** into TCA-precipitable material over 404 days of incubation at −15°C. Cells were suspended ($2.55 \pm 0.76 \times 10^6$ cells mL^{-1}) in 0.7 mL of 0.08% M9 mineral medium supplemented with acetate, trace minerals, vitamins, and one of the radiolabeled compounds. Killed controls (open symbols, dashed lines) consisted of cells pre-treated with 7% TCA. Error bars are the standard deviations from the mean of triplicate samples.

depending on the incubation medium. Table 1 provides a summary of the results at −15°C in comparison with rates reported previously.

Experiments were also conducted to examine the effect of the presence of nutrients on the incorporation rate. Cells suspended in deionized water or diluted M9 mineral medium (0.08% and 0.008%) supplemented with nutrients (acetate and vitamins) were incubated in 20 n*M* of [^{3}H]leucine or [^{3}H]thymidine at −15°C (Fig. 3). The presence of nutrients in the incubation medium significantly increased the rate of [^{3}H]leucine incorporation ($p < 0.05$; $n = 3$; based on data from the first 110 days) and amount of *de novo* protein synthesis (Fig. 2) at −15°C by *P. cryohalolentis.*

3.2. Resistance of P. cryohalolentis to ionizing radiation under liquid and frozen conditions

Liquid (~22°C) and frozen (−6°C) suspensions of *P. cryohalolentis* cells that were recovered from the exponential phase of growth were exposed to control dosages of ionizing radiation ranging from 0 to 450 Gy at a rate of 14.9–17.2 Gy min^{-1}, as determined by Fricke dosimetry. The fraction of surviving cells was determined by standard dilution plating, and the percentage of surviving cells decreased exponentially with increasing exposure dose (Fig. 4). Cells that were irradiated as aqueous suspensions were ~2-fold more sensitive to ionizing radiation than those exposed under frozen conditions ($D_{37} = 71$ Gy and 136 Gy, respectively).

4. Discussion

Permafrost substrates are surrounded with nanometer-thick unfrozen water layers at subzero temperatures, containing a 1–2% unfrozen water content at temperatures as low as −20°C (Rivkina *et al.,* 2000). Similar liquid habitats exist in sea (*e.g.,* Junge *et al.,* 2004) and glacial ice (*e.g.,* Price, 2000), which are comprised of the dissolved and particulate impurities excluded from the ice lattice, forming a network

Table 1. Rates of Incorporation of [^{3}H]Leucine and [^{3}H]Thymidine Reported in the Literature and in This Study for Pure Microbial Strains under Frozen Conditions at −15°C

		Rate of incorporation (gC gC^{-1} d^{-1})	
Reference	*Organism*	*[^{3}H]leucine*	*[^{3}H]thymidine*
Amato *et al.* (2009)	*Cryptococcus* sp. 179-3 (yeast)	5.23×10^{-7}	-
	Cryptococcus sp. 179-4 (yeast)	6.00×10^{-11}	-
Christner (2002)	*Arthrobacter* sp. G200-C1	1.70×10^{-7}	2.25×10^{-6}
	Psychrobacter sp. Trans1	2.67×10^{-7}	2.05×10^{-6}
Junge *et al.* (2006)*	*Colwellia psychrerythraea* 34H	1.20 to 4.80×10^{-6}	-
This study	*Psychrobacter arcticus* 273-4	1.31 to 7.16×10^{-7}	4.52×10^{-8} to 3.63×10^{-6}
	Psychrobacter cryohalolentis K5	0.49 to 6.23×10^{-7}	1.84 to 3.33×10^{-7}

*Visually deduced from graphs in Junge *et al.* (2006).

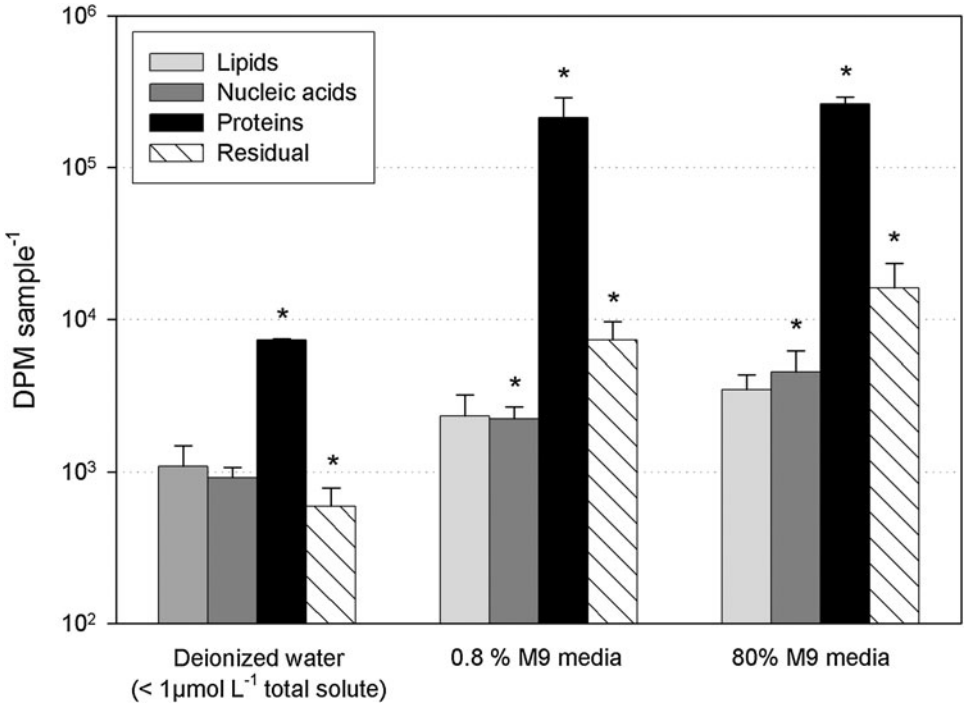

FIG. 2. The amount of radioactivity incorporated into the lipid, nucleic acid, and protein fraction of *P. cryohalolentis* K5. Cells were incubated for 123 days at −15°C in the presence of 20 n*M* of [^{3}H]leucine in either distilled water or M9 mineral medium (0.8% and 80%) supplemented with acetate, trace minerals, and vitamins. Error bars denote standard deviations from the mean of triplicate samples, and an asterisk indicates a significant difference ($p < 0.05$) from control samples.

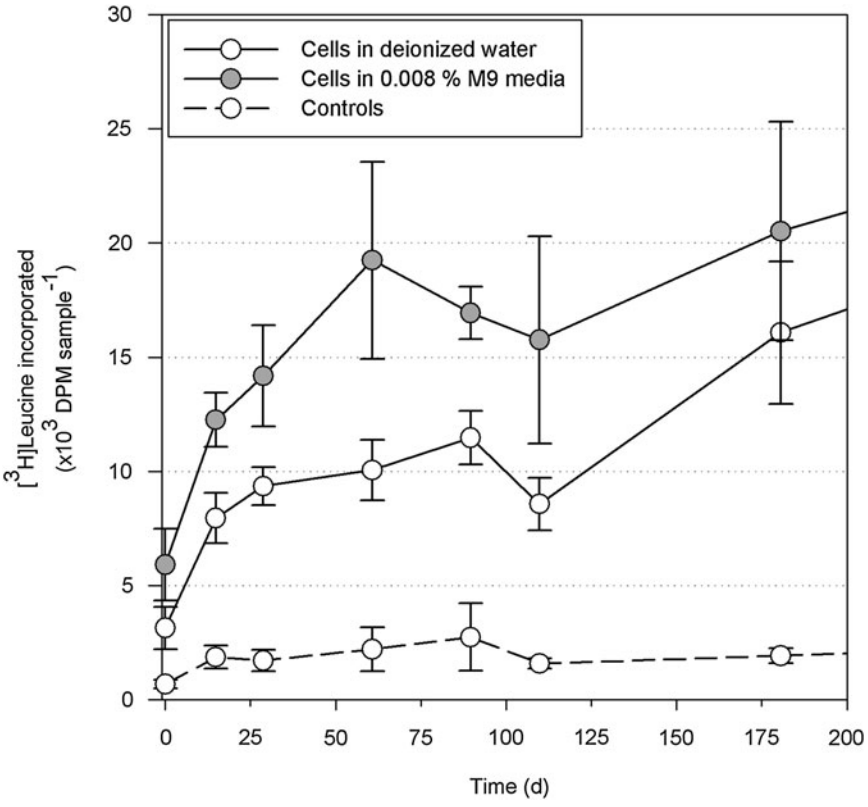

FIG. 3. Incorporation of [^{3}H]leucine by *P. cryohalolentis* K5 into TCA-precipitable material over 180 days at −15°C. Cells were suspended ($2.25 \pm 0.21 \times 10^6$ cells mL^{-1}) in either 0.7 mL of deionized H_2O or diluted M9 mineral medium (0.008%) supplemented with acetate, trace minerals, vitamins, and 20 n*M* of [^{3}H]leucine. Controls (dashed line) contained cell suspensions that were pre-treated with 7% TCA. Error bars are standard deviations from the mean of triplicate samples.

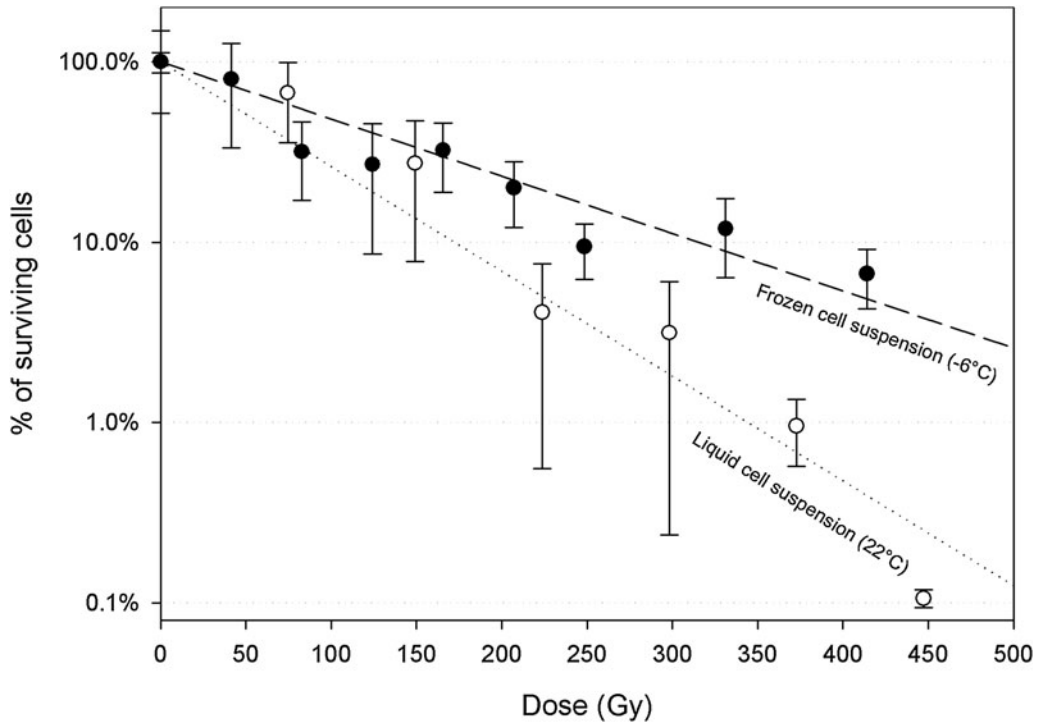

FIG. 4. Survival of *P. cryohalolentis* K5 cell populations after exposure to ionizing radiation under liquid conditions at 22°C (open symbols, dotted fit line) and under frozen conditions at −6°C (closed symbols, dashed fit line). Errors bars are standard deviations from the mean of triplicate samples.

of hypersaline pockets and veins at the ice crystal boundaries (Nye, 1992; Mock and Junge, 2007). The salinity and percentage of liquid water in the ice matrix is a function of the temperature and bulk solute concentration, respectively (Chen, 1987). In freshwater and saline ice, various studies have shown that cells are physically located in the aqueous interstitial veins that exist at grain boundaries (*e.g.,* Junge *et al.,* 2004; Mader *et al.,* 2006; Amato *et al.,* 2009), which supports Price's (2000) hypothesis that ice veins provide a habitat for cells in ice. Physiological studies of permafrost bacteria and fungi indicate that cells are capable of remaining metabolically active to temperatures approaching −40°C (Panikov *et al.,* 2006; Panikov and Sizova, 2007).

DNA and protein precursor incorporation rates at −15°C for *P. arcticus* and *P. cryohalolentis* are compared with published data measured under similar experimental conditions in Table 1. The rates of [^{3}H]leucine incorporation observed for *P. arcticus* and *P. cryohalolentis* ($\sim 10^{-7}$ gC gC^{-1} d^{-1}) were in the range of values reported for bacteria and yeast (Christner, 2002; Amato *et al.,* 2009) and ~10-fold lower than those for *Colwellia psychrerythraea* in saline ice (Junge *et al.,* 2006). [^{3}H]Thymidine incorporation rates were in the same range as those reported previously by Christner (2002) ($\sim 10^{-6}$ gC gC^{-1} d^{-1}). Based on a leucine incorporation rate of 13–318 molecules cell^{-1} d^{-1} under the range of conditions tested (Fig. 2), an average protein length of 267 amino acids (Brocchieri and Karlin, 2005), and assuming a 7.3% leucine content for protein (Kirchman, 1993), each cell synthesized ~80 to 2000 proteins during 123 days of incubation at −15°C. Not surprisingly, the presence of nutrients supporting metabolism correlated with higher rates of leucine incorporation and the total amount of leucine incorporated into the protein fraction (Figs. 2 and 3). Christner (2002) conducted experiments in deionized water supplemented with [^{3}H]leucine and estimated that the bacteria synthesized ~100 proteins during 280 days (~0.4 protein cell^{-1} d^{-1}) of frozen incubation at −15°C. Similarly, Junge *et al.* (2006) calculated that *Colwellia psychrerythraea* 34H incorporated from 79 to 552,000 molecules of leucine cell^{-1} d^{-1} at −196°C and 4°C, respectively. Based on assumptions of leucine content and protein size (discussed above), the activity values reported in the literature correspond to the synthesis of 4–28,400 proteins cell^{-1} d^{-1}, respectively, which are in the range of our estimates for *P. cryohalolentis* at −15°C.

The [^{3}H]thymidine incorporation rate (2×10^{-7} [$gC_{thymidine}$] [gC_{cell}]$^{-1}$ [d]$^{-1}$; Table 1) and thymidine content in the *P. cryohalolentis* genome (29%; http://img.jgi.doe.gov/cgi-bin/pub/main.cgi?), together with the assumption that 80% of the thymidine molecules were incorporated into DNA (Person and Bockrath, 1964; Robarts *et al.,* 1986), allowed us to infer a total DNA synthesis rate of 90 base pairs (bp) cell^{-1} d^{-1}. The low levels of metabolism reported under frozen conditions have led to the conclusion that the activity observed is insufficient for growth and instead may be directed toward repairing damage sustained during freezing or maintenance metabolism, or both (Christner, 2002; Price and Sowers, 2004). It is also possible that the DNA and protein metabolism observed was being directed toward genome and cell replication but that a single generation time exceeded the experimental time frame. Based on the rate of thymidine incorporation in Table 1, at least 94 years would be required to replicate the 3.1 Mb genome of *P. cryohalolentis* at −15°C.

If laboratory measurements of microbial metabolism under frozen conditions accurately reflect their physiological

potential in natural icy environments, a slow metabolic rate may be sufficient to offset cellular and macromolecular damage, which would allow survival over prolonged time frames. To address this experimentally, we examined the sensitivity of *P. cryohalolentis* to ionizing radiation under frozen conditions. The purpose of this experiment was to simulate the damage (*i.e.,* to proteins and DNA) that would accumulate in a cell in the absence of metabolic activity, which would allow a prediction of cellular survival over extended dormancy versus subsistence in a metabolically active state. The D_{37} value for *P. cryohalolentis* at 22°C (71 Gy) is in the range of that reported for lab strains of *E. coli* ($D_{37} = 30$ Gy; Battista, 1997) and approximately 100-fold lower than that for radioresistant species such as *Deinococcus radiodurans* ($D_{37} = \sim 6000$ Gy; Battista, 1997). When cells of *P. cryohalolentis* were irradiated as frozen suspensions, the sensitivity of *P. cryohalolentis* decreased by a factor of 1.9 ($D_{37} = 136$ Gy; Fig. 4). Higher cellular survival in irradiated frozen samples has also been reported by others (*e.g.,* Matsuyama *et al.,* 1964; Gilichinsky, 2001; Sommers *et al.,* 2002) and may be the result of the decreased production of reactive oxygen species (*e.g.,* OH•, H•, and hydrated electrons), which appear to be influenced by the irradiation temperature (*e.g.,* Henriksen, 1966). Importantly, the sensitivity of *P. cryohalolentis* to ionizing radiation indicates that it does not possess extraordinary DNA repair capacity, compared with radioresistant species such as *D. radiodurans,* the latter of which would be predicted to have a greater longevity in the presence of ionizing radiation.

On Earth, the decay of radionuclides, such as ^{40}K, ^{232}Th, and ^{238}U, and cosmic rays results in background ionizing radiation levels averaging 4.4×10^{-4} and 2.8×10^{-4} Gy yr^{-1}, respectively, at ground level (United Nations Scientific Committee on the Effects of Atomic Radiation, 1982). *P. cryohalolentis* was isolated from a 110-thousand-year-old cryopeg in Siberia (Bakermans *et al.,* 2006), and the measured background ionizing radiation rate in permafrost is in the range of ~ 2 mGy yr^{-1} (McKay, 2001). To place this in context, the bacteria recovered by Vishnivetskaya *et al.* (2000) from 3-million-year-old Siberian permafrost are predicted to have received a cumulative ionizing dosage of ~ 6000 Gy; a value equivalent to the D_{37} of highly radioresistant species such as *D. radiodurans* (Battista, 1997). Based on the data in Fig. 4, we estimate that the viability of a metabolically inactive *P. cryohalolentis* population would be reduced by 10-fold after 160 thousand years and by 10^6-fold after 1 billion years. It is important to note that since our calculations are based solely on damage to chromosomal DNA, our predictions for cellular survival in permafrost should be viewed as overestimates.

5. Implications

The ability of cells to conduct DNA and protein synthesis under laboratory conditions at −15°C supports the possibility that, under certain conditions, such processes could also occur in natural terrestrial and extraterrestrial icy environments. Based on the data of Battista (1997), the level of ionizing radiation required to produce a single strand break,

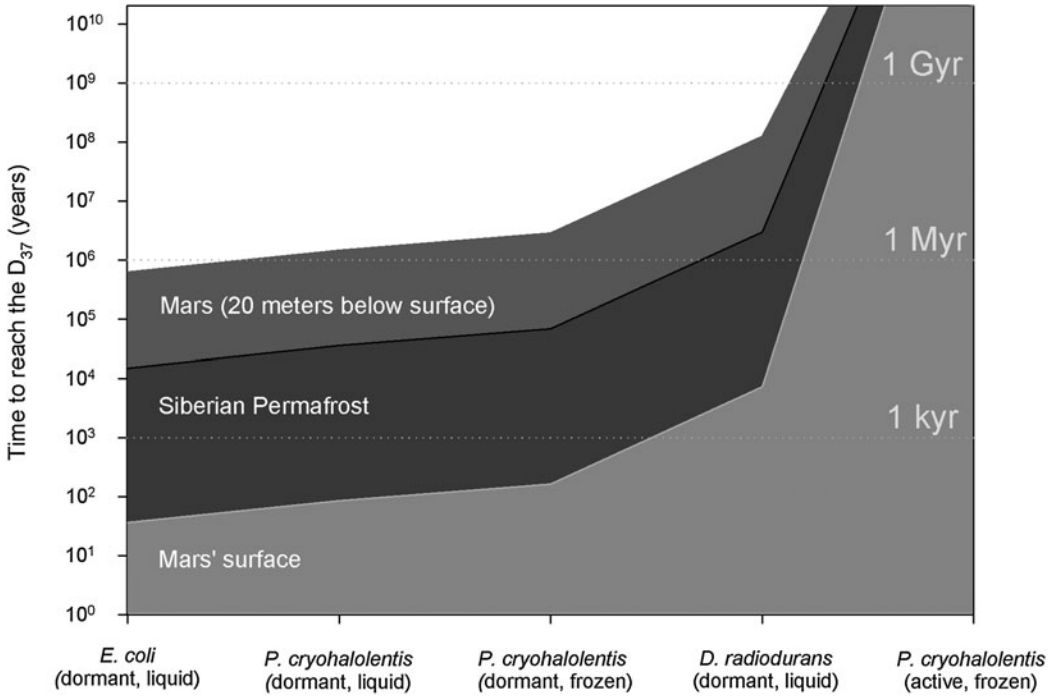

FIG. 5. Inferred time frame to achieve the D_{37} for populations of dormant and active bacteria in terrestrial and extraterrestrial permafrost. Calculations were based on cosmic ionizing radiation exposures of 0.048 and 830 mGy yr^{-1} at 20 m below the surface and at the surface of Mars, respectively (Dartnell *et al.,* 2007), and on the combined rate of cosmic and background gamma radiation measured in Siberian permafrost (2 mGy yr^{-1}; McKay, 2001). The D_{37} value for *E. coli* was 30 Gy (in liquid conditions during room-temperature exposure; Battista, 1997); 71 and 136 Gy for *P. cryohalolentis* under liquid and frozen irradiation conditions, respectively; and 6000 Gy for *D. radiodurans* (in liquid conditions during room temperature exposure; Battista, 1997).

a double strand break, or incidence of nucleotide base damage to the chromosome of *P. cryohalolentis* is estimated to be 5.13×10^{-1} Gy^{-1}, 3.42×10^{-2} Gy^{-1}, and 1.71×10^{-1} Gy^{-1}, respectively. In addition, spontaneous hydrolysis of individual phosphodiester bonds in DNA and DNA depurination occur at rates of 1×10^{-15} bp^{-1} s^{-1} (Wolfenden *et al.,* 1998) and 1×10^{-11} s^{-1} (Lindhal and Nyberg, 1972; Price and Sowers, 2004) at $-15°C$, respectively.

To calculate the D_{37} value for dormant radiosensitive and radioresistant species, as well as those with the capacity to conduct DNA metabolism under frozen conditions, dose rates of ionizing radiation were calculated based on the predicted radiological properties of permafrost on Earth and Mars (McKay, 2001; Dartnell *et al.,* 2007; Fig. 5 in this paper). Dartnell *et al.* (2007) modeled the weighted ionizing radiation dose on the surface to 20 mbs on Mars in two different frozen substrates: pure ice and wet heterogeneous material. Since data from the pure ice model represents the most extreme dosage of ionizing radiation and has the lowest predicted time frame for microbial survival at each of the depths modeled, we chose this substrate for our subsequent calculations. Pure ice has a radionuclide content of zero; therefore, the dosage of background radiation from radionuclide decay is also zero. The level of background radiation in the martian regolith is predicted to be 4×10^{-4} Gy yr^{-1} (Mileikowsky *et al.,* 2000). When the frozen substrate is composed of wet heterogeneous material, the models of Dartnell *et al.* (2007) indicate that the amount of background radiation from radionuclide decay begins to exceed that from cosmic sources at a depth of $\sim$4.5 m. Hence, cells preserved at depths $>$4.5 mbs in wet heterogeneous material (*cf.* with pure ice) are exposed to a constant dosage of background radiation that does not change further with increasing depth.

On Mars, the D_{37} of a dormant *P. cryohalolentis* population in pure ice would be reached in less than 200 years at the surface and in 2.8 million years at 20 mbs (Fig. 5). Dartnell *et al.* (2007) estimated that a population of viable bacteria would be reduced by 6 orders of magnitude in 1200 to 18,100 years on the surface of Mars, depending on their resistance to ionizing radiation. At a depth of 20 mbs, the cosmic ionizing radiation dosage in pure ice decreases to 0.048 mGy yr^{-1} (Dartnell *et al.,* 2007), and the predicted time frame for *P. cryohalolentis* to achieve the D_{37} is 21 million years. Hence, extant microbial life might well be protected and preserved for extended periods at relatively accessible depths within the martian subsurface.

Based on the amount of bulk DNA synthesis needed to repair a known quantity of DNA damage (Slade *et al.,* 2009; D. Slade, personal communication), we estimate that the repair of each double strand break requires the incorporation of 7–11 nucleotides into the DNA. Lacking data on the amount of DNA synthesis necessary to repair a single strand break, we assumed the same amount of nucleotide incorporation required to repair a double strand break. If *P. cryohalolentis* is capable of synthesizing DNA in permafrost environments at the rate observed in Fig. 1 (*i.e.,* 90 bp $cell^{-1}$ d^{-1} at $-15°C$), our calculations imply that ionizing radiation from radionuclide decay or cosmic sources places no constraints on cellular longevity in either terrestrial or extraterrestrial permafrost (Fig. 5). Thus, if permafrost environments are active biomes and biological activity is maintained over geological time, the long-term survival of microbial populations is likely to be limited by other environmental factors (*e.g.,* water activity and availability of suitable redox couples and nutrients). As such, our results imply that the search for extant extraterrestrial life need not be exclusive to environments where water has recently existed in the liquid phase (*e.g.,* 0.5 million years; Dartnell *et al.,* 2007).

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Abbreviations

bp, base pairs; cfu, colony-forming units; DPM, disintegrations per minute; D_{37}, dose of ionizing radiation at which 37% of the cells survive; mbs, meters below the surface; TCA, trichloroacetic acid.

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