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Research Article

Effects of Long-Term Simulated Martian Conditions on a Freeze-Dried and Homogenized Bacterial Permafrost Community

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Abstract

Indigenous bacteria and biomolecules (DNA and proteins) in a freeze-dried and homogenized Arctic permafrost were exposed to simulated martian conditions that correspond to about 80 days on the surface of Mars with respect to the accumulated UV dose. The simulation conditions included UV radiation, freeze-thaw cycles, the atmospheric gas composition, and pressure. The homogenized permafrost cores were subjected to repeated cycles of UV radiation for 3 h followed by 27 h without irradiation. The effects of the simulation conditions on the concentrations of biomolecules; numbers of viable, dead, and cultured bacteria; as well as the community structure were determined. Simulated martian conditions resulted in a significant reduction of the concentrations of DNA and amino acids in the uppermost 1.5 mm of the soil core. The total number of bacterial cells was reduced in the upper 9 mm of the soil core, while the number of viable cells was reduced in the upper 15 mm. The number of cultured aerobic bacteria was reduced in the upper 6 mm of the soil core, whereas the community structure of cultured anaerobic bacteria was relatively unaffected by the exposure conditions. As explanations for the observed changes, we propose three causes that might have been working on the biological material either individually or synergistically: (i) UV radiation, (ii) UV-generated reactive oxygen species, and (iii) freeze-thaw cycles. Currently, the production and action of reactive gases is only hypothetical and will be a central subject in future investigations. Overall, we conclude that in a stable environment (no wind-/pressure-induced mixing) biological material is efficiently shielded by a 2 cm thick layer of dust, while it is relatively rapidly destroyed in the surface layer, and that biomolecules like proteins and polynucleotides are more resistant to destruction than living biota. Key Words: Astrobiology—UV radiation—Mars—Simulation—Bacteria—Biomolecules. Astrobiology 9, 229–240.

Introduction

Observational data collected by the Mars Odyssey spacecraft have provided substantial evidence for the presence of vast permafrost areas on the surface of Mars (Boynton *et al*., 2002; Mellon *et al*., 2004; Litvak *et al*., 2006). Compared to terrestrial permafrost, martian permafrost is about 1000 times older (3–4 billion years) (Smith and McKay, 2005) and penetrates several kilometers deeper into the subsurface [depending on the internal heat flux (Frolov, 2003)].

The capacity of terrestrial permafrost to preserve living cells and biological material in general (Vorobyova *et al*., 1997; Vishnivetskaya *et al*., 2000; Johnson *et al*., 2007) has led to speculation that martian permafrost could be a potential reservoir of living biota or its remains. Thus, martian permafrost has been proposed as a target for future life-investigating missions (Horneck, 2000; Gilichinsky, 2001; Frolov, 2003; Smith and McKay, 2005). While missions are planned to investigate martian permafrost, terrestrial permafrosts can be studied *per se* or in simulation experiments as an analogue

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to martian permafrost. Data obtained from such experiments will provide new information on the mechanisms evolved by terrestrial organisms to cope with the challenges of this harsh environment. Such information will be useful both in the preparation of future missions and in the selection of landing sites. However, care must be taken when extrapolating results obtained from experiments that use simulated martian conditions, since information on the physical and chemical characteristics of martian permafrost is minimal.

The effects of martian conditions on biological material have been investigated in Mars simulation experiments, where terrestrial bacteria are incubated in either simple or sophisticated incubation chambers in which proxy-Mars conditions can be maintained (*e.g.*, Schuerger *et al*., 2003; Cockell *et al*., 2005; Hansen *et al*., 2005; Jensen *et al*., 2008). Most of these studies have used monocultures of laboratory strains or simple organic molecules as models (Mancinelli and Klovstad, 2000; Schuerger *et al*., 2003; Nicholson and Schuerger, 2005; ten Kate *et al*. 2006; Osman *et al*., 2008), while only a few experiments have addressed the effect of simulated martian conditions on complex bacterial soil communities from soils with special focus on mineralogical similarities to martian soil (Fulton, 1958; Packer *et al*., 1963; Green *et al*., 1971; Hansen *et al*., 2005). It is probable that environmental conditions, such as temperature and water availability, may be more important, when investigating the effect of simulated martian conditions on living organisms, than the mineralogy of the soil in which they live. Therefore, permafrost bacteria, pre-adapted to constant subzero temperatures and low water availability, may be more appropriate model organisms than bacteria from iron-rich, temperate soils.

The UV radiation that reaches the surface of Mars has been identified as the most harmful single factor to terrestrial microorganisms. Studies such as those of Cockell *et al*. (2005), Newcombe *et al*. (2005), and Schuerger *et al*. (2006) on cyanobacteria and *Bacillus* strains have shown that cells do not survive more than 180 minutes of exposure to Mars-equivalent solar radiation. Encouraging for life-seeking missions, however, is the fact that soil layers of only 0.5–1 mm are enough to protect vegetative cells and endospores against short-term exposure to direct UV radiation (Green *et al*., 1971; Mancinelli and Klovstad, 2000; Schuerger *et al*., 2003). Whether the protection is maintained on longer timescales, from months to years, has yet to be established in long-term simulation experiments or direct studies on Mars.

Most studies have evaluated the effect of simulated martian conditions on terrestrial microorganisms by simply determining the survival rate of the exposed bacterial cells as a function of exposure time or radiation dose (*e.g.*, Newcombe *et al*., 2005; Nicholson and Schuerger, 2005; Osman *et al*. 2008). More extended and detailed studies that investigate, for example, the differential persistence of both biomolecules and complex bacterial communities in their indigenous soil matrix have yet to be undertaken. Studies of this kind will provide an important insight into how natural biological systems respond to the imposed conditions and, more importantly, will allow the identification of scenarios that are supportive for the preservation of extant and extinct life signatures as well as organisms that are particularly persistent under these conditions.

The present simulation experiment, which lasted for 31 days and applied an accumulated UV dose that corresponds to about 80 days on the surface of Mars, is one of the longest Mars simulation experiments that has been carried out. We investigated the effects of simulated martian conditions on the indigenous bacteria and biomolecules of a freeze-dried and homogenized Arctic permafrost by quantifying the viable, dead, and cultivable fractions of the bacterial community and by measuring the concentrations of DNA and amino acids in different depth layers of the incubated permafrost and in control samples. In addition, surviving bacteria were isolated and identified by 16S rDNA sequence analysis, and changes in community structure of anaerobic bacteria were assessed by denaturant gradient gel electrophoresis (DGGE) on serially diluted most probable number (MPN) cultures.

Materials and Methods

Soil sample and bacterial community

The experiment was carried out with a permafrost from Spitsbergen (for a detailed description see Hansen *et al*., 2007). Prior to experimentation, the soil was frozen at −20°C and freeze-dried for 14 days, sieved through a 250 μm mesh size sieve, and finally freeze-dried again for a further three days. The soil was stored at -80°C until used in the simulation experiment. The water content of the freeze-dried permafrost was 0.7% (n = 5), as determined by the weight loss of freeze-dried soil dried at 105°C for 24 h.

Incubation under simulated martian conditions

The Mars simulation chamber described by Jensen *et al*. (2008) was used for incubation of the permafrost under simulated martian conditions. Ten soil cores of homogenized freeze-dried permafrost (4 cm diameter and 4 cm depth) were prepared in inert glass tubes and placed inside stainless steel cylinders. Freeze-dried permafrost was used for the experiments, as freeze-drying is an inherited result of the martian environmental conditions (low pressure and low temperature). Further, the soil samples were homogenized prior to experimentation to reduce the variability between the soil cores exposed to martian conditions.

The samples were mounted in the sample carousel of the simulation chamber. The atmosphere of the chamber was evacuated with an external vacuum pump (Edwards RV 5, BOC Edwards, UK), and CO_2 (>99.9% purity, AGA, Denmark) and a gas mixture of CO, O_2, Ar, and N_2 (1:1.5:17.5:30; > 99% purity, Air Liquide, Denmark) were introduced to establish a final pressure of 8 mbar. During the experiment, the gas composition in the simulation chamber was monitored with a rest gas analyzer (Table 1; Microvision plus, MKS Instruments, USA) and regulated when necessary. The temperature in the simulation chamber was automatically regulated by injection of liquid N_2 into the cooling jacket of the chamber. The wall temperature of the chamber varied between −60 and −48°C. The temperature in the soil varied between −41°C in the bottom of the soil core and 22°C in the surface of the soil during UV exposure (Fig. 1). The soil temperature decreased to −41°C throughout the soil when the soil cores were not exposed to UV radiation. The soil samples were irradiated by a 150 W mercury-xenon lamp (Hamamatsu Photonics L2482, D), which was operated at ~135 W and generated light between 200

Table 1. Environmental Conditions in the Mars Simulation Chamber and on the Surface of Present-Day Mars

Parameter	*Simulated environment*	*Present-day Mars*[a]
Temperature range (°C)[b]	−41 to +22	−123 to +25
Pressure range (mbar)	7.6 to 9.7	6.7 to 9.9[c]
UV radiation (nm)	>200	>200
UV intensity at 239 nm (W m^{-2} nm^{-1})	0.207	0.006[d]
Gas composition (%)		
CO_2	91.4	95.3
N_2	4.8	2.7
Ar	2.8	1.6
O_2	0.24	0.13
H_2O	0.14	0.02
CO	0.16	0.07
Other	0.41	0.25

[a]From Horneck (2000).
[b]At the soil surface. See Fig. 1.
[c]From Tillman *et al*. (1993).
[d]Annual average intensity at 11.6°N (Patel, M., personal communication).

and 1000 nm (for details see Jensen *et al*., 2008). After 31 days incubation, including 29 days with the UV lamp on, the UV lamp and N_2 cooling were turned off, respectively, after which temperature, pressure, and atmospheric composition were returned to laboratory conditions. The soil cores were transferred to a clean cabinet where they were sliced into eight depth horizons: H1 (0–1.5 mm); H2 (1.5–3 mm); H3 (3–6 mm); H4 (6–9 mm); H5 (9–15 mm); H6 (15–21 mm); H7 (21–27 mm) and H8 (27–33 mm). Horizons obtained from the 10 soil cores were pooled, which resulted in eight samples (H1–H8). These were analyzed separately in all subsequent experiments, which were carried out to evaluate the effects of simulated martian conditions at the different soil depths.

Each of the 10 permafrost cores was individually exposed for a total of 57 ± 6 h of UV radiation divided into 20 cycles of 3 h UV irradiation followed by 27 h with the UV lamp off during the 29 days of UV exposure. The UV intensity of the lamp applied in the experiment was 35 times greater than the average diurnal flux on Mars (Table 1). Hence, the accumulated dose of UV received by each soil core during the experiment was equivalent to a martian dose of 80 ± 9 Sol (Sol, martian day: 24 h 37 min).

Biomolecules and nitrogen compounds

DNA (n = 3) was extracted directly from the eight soil horizons and the control soil with the FastDNA spin kit for soil (Bio 101, California, USA) with the following modifications: (I) samples were thoroughly ground prior to DNA extraction, (II) the centrifugation time after bead beating was 10 min, (III) samples containing the DNA binding matrix were shaken for 1 h, and (IV) the mixture of DNA binding matrix and the elution water were incubated for 5 min followed by vortex mixing. The DNA yield was quantified fluorometrically (TD-700, Turner BioSystems, California, USA) after staining with PicoGreen (Molecular Probes, Invitrogen) as described in Sandaa *et al*. (1998).

The concentration of total hydrolyzable amino acids (THAA) in the soil samples (n = 1) was determined by high-performance liquid chromatography (Waters Chromatographic System) of *o*-phthaldialdehyde-derivatized products (Lindroth and Mopper, 1979). In short, the THAA samples [0.5 gram dry weitht (gdw)] were hydrolyzed with 10 ml 6 *N* HCl at 105°C for 24 h under a N_2 atmosphere. Hydrolysates (200 μl) were dried in a vacuum desiccator for 24 h. The sample residues were dissolved in Milli-Q water, dried again, and analyzed after resuspension in 0.2 ml Milli-Q water.

Concentrations of total organic carbon (TOC) and total nitrogen (TN) were determined on H_2SO_3 (5–6% w/w) treated soil via a Carlo-Erba NA 1500 CHN analyzer (n = 3).

Bacterial numbers

The total number of bacterial cells (n = 5) in the permafrost was determined by epifluorescence microscopy (Zeiss Axiovert 200M) after staining with either SYBR-Gold (Molecular Probes, Invitrogen) for total cell counts or with the stains of the LIVE/DEAD® *Bac*Light™ kit (Molecular Probes, Invitrogen) (Boulos *et al*., 1999) for differentiation between viable and dead cells. Prior to staining, the samples were suspended in Na pyrophosphate (10 m*M*) and sonicated in an ultrasonic bath for 2 × 20 s at 42 kHz (Branson 5510).

The numbers of aerobic colony-forming units (CFUs) were determined on R2A agar (Difco) (Reasoner and Geldreich, 1985), containing nystatin (50 mg L^{-1}) to suppress fungal growth. Prior to inoculation on agar plates, the permafrost was incubated for 15 min in Na pyrophosphate (2 m*M*), vigorously vortex mixed, and finally sonicated as described previously. Agar plates were inoculated from serial dilutions of the inocula (1:600–1:20000) and incubated at 15°C for 12 weeks.

The number of anaerobic bacteria in H1, H2, H8, and the control soil was determined from 10-fold serial dilutions (1:1, 1:5–1:500000) in anoxic R2A-broth (n = 3). The medium was prepared under anoxic conditions, and the headspace of the incubation vials was a mixture of N_2 and CO_2 (9:1). The tubes were incubated for 15 weeks at 15°C, after which growth was evaluated by microscopy. Most probable numbers (MPN) were calculated with use of index tables (Taras *et al*., 1971).

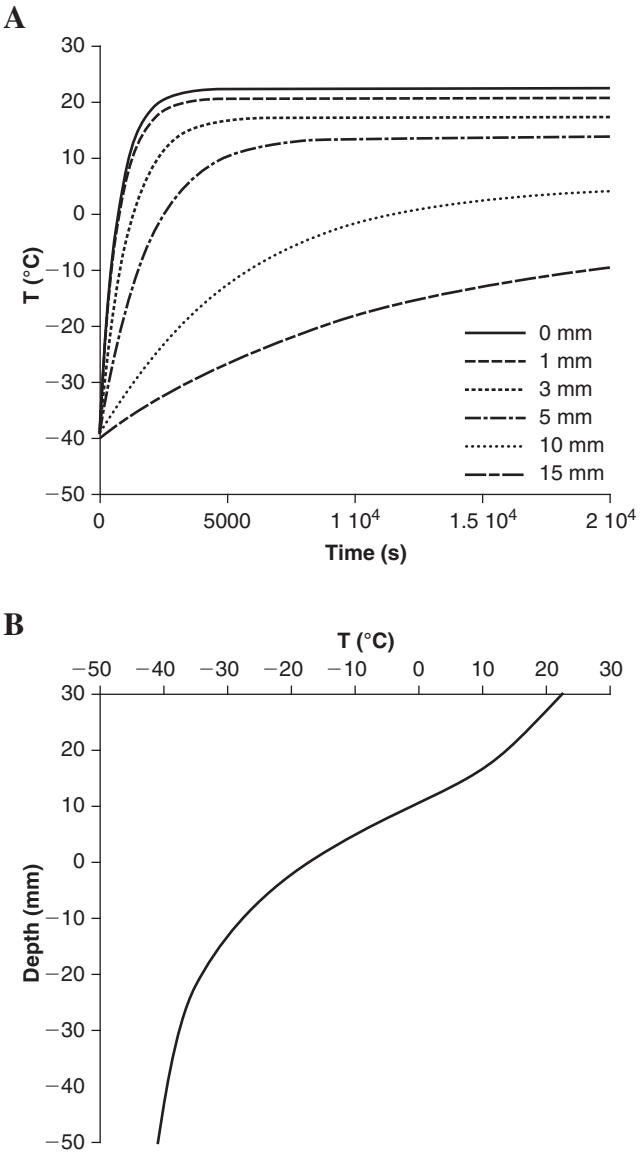

FIG. 1. (**A**) Temperatures at distinct depths of the permafrost core during exposure to the UV light source. *x*-axis gives the time exposed to UV radiation in seconds. The temperatures were calculated from the model described in Jensen *et al*. (2008). (**B**) Calculated temperature profile after 3 hrs (10,800 s) of exposure to the UV light source.

Pure cultures

Pure cultures of aerobic bacteria were obtained from randomly picked colonies from R2A agar plates incubated with 100 μl of a soil suspension (1:60) from the upper 6 mm of the soil. In total, 11 pure cultures were obtained: 2 from H1, 3 from H2, and 6 from H3. 16S rDNA gene fragments from the pure cultures were obtained by direct PCR amplification of the DNA from bacterial cells with the bacterial primer 26F (Hicks *et al*., 1992) and the universal primer 1390R (Zheng *et al*., 1996), following a protocol described previously in Hansen *et al*. (2005). Sequencing of the amplified 16S rDNA fragments from the pure cultures was carried out by Macrogen Inc. (Seoul, Korea, www.macrogen.com) with the 26F and 1390R primers and the BigDye™ terminator cycle sequencing kit on an ABI3730XL sequencer (Applied Biosystems). Consensus sequences of each pure culture were constructed from the forward and reverse reads by use of the ARB software package (Ludwig *et al*., 2004). The sequences were compared to the current database of rRNA gene sequences from GenBank by use of BLAST [Basic Local Alignment Search Tool (Altschul *et al*., 1997)].

Denaturant gradient gel electrophoresis

The community composition of the anoxic MPN enrichments from H1, H2, H8, and the control soil (C) was analyzed by DGGE. DNA was extracted from the cell-pellet from all MPN dilutions (n = 3) by use of the FastDNA spin kit for soil with modifications II–IV described previously (Hansen *et al*., 2005). DGGE was carried out according to Muyzer *et al*. (1998) on 16S rDNA fragments amplified with the primers 341F-GC (Muyzer *et al*., 1993) and 907R (Lane, 1991), following the procedures described previously (Hansen *et al*., 2005). Selected bands were excised from the gel, re-amplified with 341F-GC and 907R, and repeatedly run on DGGE gels until clean. Clean bands were re-amplified with 341F (without GC-clamp) and 907R primers and sequenced as described previously with the 341F primer. Cultures with only one band were amplified directly from the DNA extract and sequenced with the 26F and 1492R primers (Lane, 1991). Sequences were compared to the current database of rRNA gene sequences from GenBank by use of BLAST (Altschul *et al*., 1997).

Data analysis

Statistical analysis was conducted by use of SPSS version 11.0 for Windows (SPSS Inc., Chicago, Illinois, USA). Differences in TOC concentrations were tested by use of one-way analysis of variance (ANOVA) ($P < 0.05$). Differences in DNA concentration were tested by use of one-way ANOVA followed by Fisher's least significant difference test ($P < 0.05$). Total bacterial counts and viable cell counts had unequal variances and were therefore tested by use of one-way ANOVA followed by Games-Howell test ($P < 0.05$). The TN concentrations and the dead cell counts were not normally distributed and were therefore tested by use of the non-parametric Kruskal-Wallis test ($P < 0.05$) and Mann-Whitney U test ($P < 0.05$).

Nucleotide sequence accession numbers

Sequences of the 11 isolates were deposited in GenBank with accession numbers EF187347-187357. Sequences of 89 DGGE bands were cropped to remove primer sequences and deposited in GenBank with accession numbers EF187358-187426.

Results

Biogeochemical characterization of the freeze-dried control soil

Concentrations of TOC, TN, and THAA in the 8 horizons of the freeze-dried permafrost are given in Table 2. The C:N ratio in the bulk freeze-dried permafrost was 19 mol mol^{-1}. THAA contributed ~9% to TOC and 46% to TN on a molar basis. Glycine was the most important of the 18 identified

TABLE 2. CONCENTRATION OF TOTAL ORGANIC CARBON (TOC), TOTAL NITROGEN (TN) AND TOTAL HYDROLYSABLE AMINO ACIDS (THAA) AT DIFFERENT DEPTHS OF THE SOIL CORE INCUBATED UNDER SIMULATED MARTIAN CONDITIONS AND THE CONTROL SOIL

Depth (mm)	*TOC*[a] *(μmol gdw^{-1})*	*TN*[a] *(μmol gdw^{-1})*	*THAA*[b] *(μmol gdw^{-1})*
0–1.5	1777 ± 55	96 ± 2	25.3
1.5–3	1791 ± 34	96 ± 4	37.8
3–6	1803 ± 83	97 ± 4	39.9
6–9	1742 ± 54	95 ± 2	40.4
9–15	1721 ± 67	94 ± 3	37.2
15–21	1719 ± 66	94 ± 3	39.8
21–27	1736 ± 77	95 ± 3	39.2
27–33	1742 ± 107	93 ± 8	43.7
Control	1883 ± 153	98 ± 13	38.6

[a]Mean ± standard deviation, $n = 3$. No significant difference due to incubations was revealed ($P > 0.05$).
[b]THAA measurements were based on single samples, which did not allow statistical analysis, gdw, gram dry weight.

THAA (13.6 mol %), followed by alanine (11.2 mol %), aspartatic acid (9.6 mol %), and glutamic acid (8.6 mol %). The other identified amino acids and the unidentified OPA-reactive compounds (named others) contributed <8 mol % to THAA each.

Effect of simulated martian conditions on biomolecules

There was a significant decrease in the DNA concentration in the upper 3 mm of the soil after exposure to simulated martian conditions compared to the control soil (Fig. 2). In the upper 1.5 mm of the soil, the DNA concentration was reduced to 50% of the initial concentration in the freeze-dried control, whereas 72% of the initial DNA concentration remained in the soil horizon H2. Below H2, the DNA concentration was not significantly affected by the simulation conditions (Fig. 2).

The concentration of THAA decreased to 65% of its initial concentration within H1 during the incubation period (Table 2). There was a reduction in the concentration of all individual THAAs, with the exception of tyrosine (Fig. 3A–C). The individual protein amino acids that were mostly affected by the simulation conditions were the acidic amino acids aspartatic acid and glutamic acid, which were reduced to 45 and 50% of their initial concentrations, respectively. The non-protein amino acids β-alanine, taurine, γ-amino butyric acid, and ornithine were reduced to 60, 51, 56, and 62% of their initial concentrations, respectively. The concentration of all other identified and unidentified OPA-reactive compounds remained at > 68% of their initial concentration (Fig. 3A–C).

The concentrations of TOC and TN were not significantly affected by the simulation conditions (Table 2).

Effect of the simulation martian conditions on bacterial numbers

There was a significant decrease in the total number of bacterial cells in the upper 9 mm of the soil (Fig. 4A). The most pronounced reduction was observed in H1, where the number of cells was reduced to 20% of the number determined in the control soil (1.4×10^9 cells gdw^{-1}). In the 1.5 mm to 9.0 mm depth interval, the bacterial number decreased to <54% of the cell number in the control soil. An enumeration of the total number of bacterial cells via LIVE/DEAD® BacLight™ staining revealed similar reductions in the total number of bacterial cells in the upper 9 mm of the soil core (Fig. 4B). In the upper 15 mm of the soil, the number of viable bacteria was significantly reduced to <44% of the number in the control, whereas the reduction in the number of dead bacteria was only significant within the upper 3 mm of the soil (Fig. 4B). However, the lack of significant reductions in the number of dead bacteria in the depth interval from 3 to 15 mm was due to the large standard deviation (for which we have no explanation) on the number of dead bacteria in the control soil (Fig. 4B). The number of viable bacteria in the freeze-dried control soil accounted for 18% of the total viable + dead cells.

The simulation conditions greatly affected the number of aerobic CFUs. There were no CFUs recovered from H1 (Fig. 5). However, two aerobic colonies (equivalent to 120 CFUs gdw^{-1}) were obtained from a single 60 times dilution of soil from H1, which indicated that the lowest dilution used for aerobic CFUs (600 times) was too dilute. In H2 and H3, 100 CFU gdw^{-1} were recovered, which was equivalent to 7% of the CFUs obtained from the control soil (Fig. 5). At depth >6 mm, the numbers of CFUs ranged from 404–808 CFUs gdw^{-1}, which corresponds to 27–53% of the numbers of CFUs recovered from the control soil (Fig. 5).

In the upper 3 mm of the soil, the number of cultivable anaerobic bacteria was reduced to 226 cells gdw^{-1} (24%) compared to 926 cells gdw^{-1} in the control soil. At the bottom of the soil core, the number of cultivable anaerobic bacteria was only slightly lower (750 cells gdw^{-1}; 81%) than the number of anaerobic bacteria in the control soil.

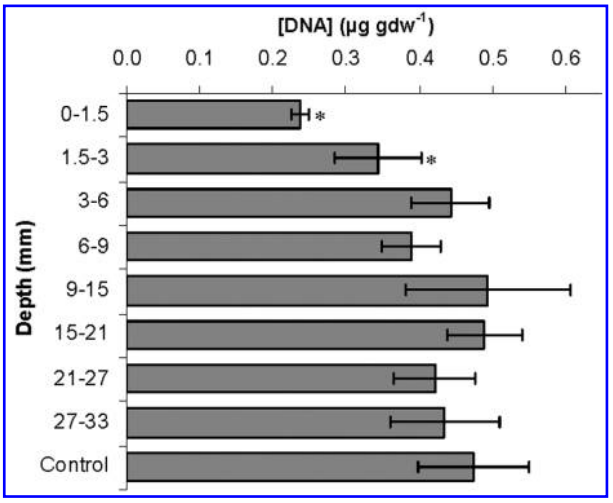

FIG. 2. Concentration of DNA at different depth in the soil core after exposure to simulated martian conditions and in the freeze-dried control soil. Asterisks (*) indicate significant difference from the freeze-dried control soil based on one-way ANOVA followed by Fisher's least significant difference test ($P < 0.05$).

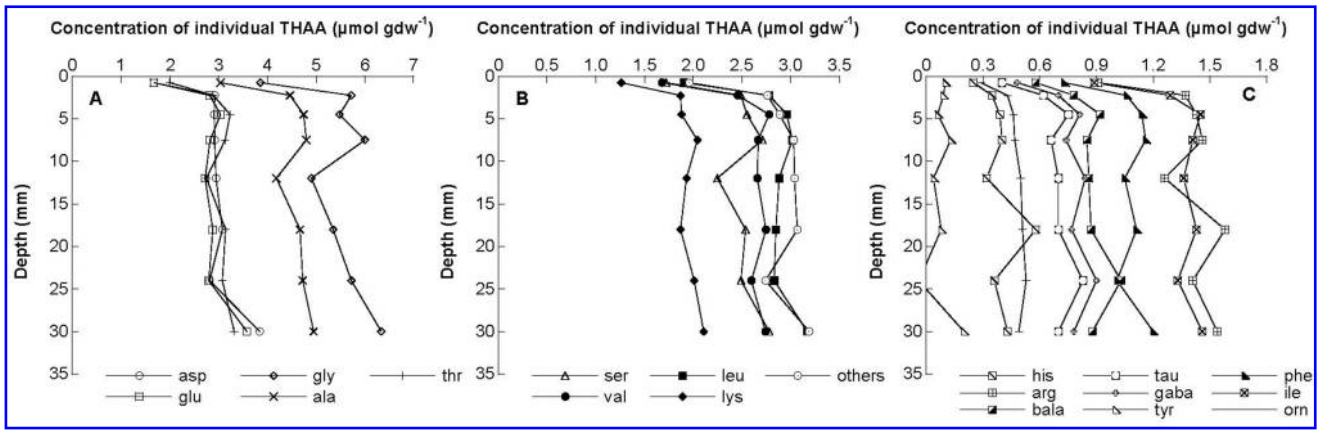

FIG. 3. Concentration of individual THAA at different depths in the soil core after exposure to simulated martian conditions. The concentration of individual THAA (μmol gdw^{-1}) in the freeze-dried control soil was the following: aspartic acid 3.7, glutamic acid 3.3, serine 2.3, histidine 0.3, glycine 5.3, threonine 2.9, arginine 1.3, β-alanine 1.0, taurine 0.8, alanine 4.3, γ-amino butyric acid 0.9, tyrosine 0.1, valine 2.4, phenylalanine 1.0, isoleucine 1.2, leucine 2.7, ornithine 0.5, lysine 1.8, and unidentified OPA-reactive compounds (named others) 2.8.

Identification of cultures

In total, 11 aerobic cultures were isolated from the upper 6 mm of the soil. The two colonies obtained from H1 were purified and identified as Gram-positive *Actinobacteria* with closest affiliation to *Friedmaniella antartica* (92–93%; Table 3). Randomly picked colonies were isolated from H2 (3 colonies) and H3 (6 colonies). One of the three isolates obtained from the 1.5–3.0 mm depth horizon was Gram-positive (G^+), and the other two isolates were Gram-negative (G^-; Table 3). The pure cultures were closely affiliated to *Spirosoma linguale* (94%), a *Sphingobacteria* (G^-); *Micrococcus luteus* (99%), an *Actinobacteria* (G^+); and *Panaciterramonas fulva* (98%), a *Beta-Proteobacteria* (G^-). The six pure cultures isolated from H3 belonged to the *Actinobacteria* (G^+). These cultures affiliated with five different genera (Table 3). The closest relatives of the cultures were: *Cryocola antiquus* (96%), *Cellulomonas cellasea* (98%), *Nocardioides kribbensis* (93%), *Microlunatus phosphovorus* (93%), *Mycobacterium aichiense* (97%), and *Mycobacterium hodleri* (96%).

The community structure of the anoxic MPN enrichments from the upper 3 mm of the soil, the bottom of the soil core (27–33 mm), and the control soil (C) were analyzed by DGGE fingerprinting; and bands were excised and identified by sequencing (Table 4). Growth was detected up to dilution 1:5000 and dilution 1:500 of H8 and C, respectively, and up to dilution 1:50 of H1 and H2. Comparable numbers of operational taxonomic units (OTUs) were detected in the respective samples (8, 7, 5, and 10 OTUs from horizons H1, H2, H8, and C, respectively). In undiluted soil enrichments (1:1), 6 OTUs were observed in the control soil, while 3, 4, and 3 OTUs were identified in H1, H2, and H8, respectively. All but one of the identified OTUs were present in the control soil, whereas 9 of the OTUs could be identified in the soil after exposure to simulated martian conditions (Table 4). The OTUs were affiliated with the Gram-positive bacterial classes *Clostridia*, *Bacilli*, and *Actinobacteria* (Table 4). The dominant bacteria in the highest dilution of H1 soil (1:50) were affiliated with the *Actinobacteria*, *Propioniferax innocula*, *Cellulomonas fermentans*, and *Cellulomonas cellasea* (Table 4). The latter two were also identified in H2 and H8. OTUs in the control soil were affiliated to the *Clostridia*, *Desulfosporosinus orientis*, and the *Actinobacterium, Propioniferax innocua* (Table 4). Bacteria affiliated with the endospore-forming *Clostridia* and *Bacilli* were only detected in the lowest dilutions of the soil samples.

Discussion

The present study combines the results of biogeochemical, microbiological, and molecular approaches to investigate the long-term effect of simulated martian conditions on the indigenous biomolecules and the bacterial community in a freeze-dried and homogenized Arctic permafrost. At the end of the incubation period, the concentrations of biomolecules were reduced down to a depth of 1.5 mm (THAA) and 3 mm (DNA), respectively. The numbers of total bacterial cells, viable cells, and the CFUs were reduced in the upper 9, 15, and at least 6 mm of the soil, respectively. The diversity of the cultured anaerobic bacterial community was not affected by the simulation conditions, except that there was a reduction in cell numbers of the least-abundant members of the bacterial community.

Physico-chemical factors affecting biomolecules and cell viability

Cockell and coworkers showed that a layer of 1 mm of gneiss protects bacteria from direct UV light damage (Cockell *et al*., 2002, 2005). Also, Mancinelli and Klovstad (2000) reported a protective effect with a Mars analog soil. In addition, Schuerger *et al*. (2003) showed that a layer of 0.5 mm of soil protected *Bacillus subtilis* endospores during 8 hours of exposure to a Mars equivalent UV flux. The present investigation differs from previously published studies (*e.g*., Schuerger *et al*., 2003; Cockell *et al*., 2005) by investigating a complex microbial community, by the long incubation period (29 days, equivalent to 80 martian Sol), and by the high UV intensity. However, considering that Cockell *et al*. (2002) showed that 1 mm of gneiss reduced the UV flux by about 2 orders of magnitude, it is unlikely that UV radiation was the primary cause of the effects observed below H1 in our

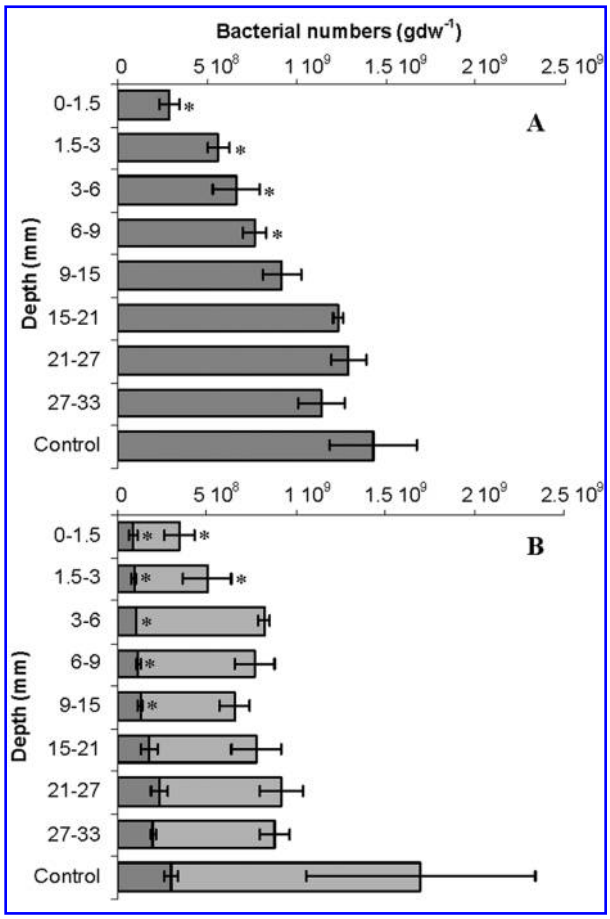

FIG. 4. Number of bacterial cells at different depth of the soil core incubated under simulated martian conditions and the control soil. (**A**) Total number of bacterial cells determined by SybrGold and (**B**) total number of viable (dark gray) and dead (light gray) bacterial cells as determined by the LIVE/DEAD® *Bac*Light™. Asterisks (*) indicate significant difference from the freeze-dried control soil and depth horizon 27–33 mm based on one-way ANOVA followed by Games-Howell test ($P < 0.05$) (total number of bacteria and viable cells) or based on Mann-Whitney U test ($P < 0.05$) (dead bacterial cells).

study. Among other factors that may have been responsible for the reduction in the concentration of biomolecules and bacterial cell numbers at depths >1.5 mm were UV-induced production of reactive oxygen species and freeze-thaw cycles.

In previous Mars simulation experiments, UV-generated reactive oxygen species have been proposed to be the most likely cause of the degradation of amino acids (Oro and Holzer, 1979; ten Kate *et al*., 2005; Garry *et al*., 2006), nucleic acids (Oro and Holzer, 1979), and humic acids (McDonald *et al*., 1998). They have also been proposed to be the likely cause of the inhibition of bacterial activities (Hansen *et al*., 2005). Recently, Yen *et al*. (2000) demonstrated that superoxide radical ions (O_2^-) formed on Mars-analog mineral surfaces exposed to UV radiation at oxygen concentrations equivalent to that in the martian atmosphere. They argued that these reactive ions were able to diffuse into deeper soil strata along the adsorption sites of mineral surfaces when no organic material was present. They proposed that superoxide ions were the source of reactivity observed in the soils studied during the Viking mission. They further proposed that superoxide ions might explain the absence of organics in the martian subsoil, as they have been demonstrated to react with a series of organic compounds, including aromatics and hydrocarbons (Gasymov *et al*., 1984; Lunsfort, 1984). These superoxide radicals may also be candidates to explain the decrease in amino acid and DNA concentrations in the uppermost horizons in our study. Reactive oxygen species may have been formed from the trace of atmospheric oxygen (0.24%) and atmospheric water vapor (0.14%) in the simulation chamber, the thin water film on the soil grains, and indigenous humic substances present in the soil. Humic substances can effectively absorb UV light and, thereby, act as sensitizers for the photolytic production of reactive oxygen species (Frimmel, 1994; Aguer *et al*., 1999). As the concentration of biomolecules was only affected in the upper 3 mm of the soil, we propose that the effect of reactive oxygen species was restricted to this soil depth. Although the conditions that support the formation of these compounds prevailed during our simulation experiments, experimental evidence for their production needs to be provided in future work.

Finally, alternating freeze-thaw cycles may have effected the survival of the bacterial cells down to a depth of 10 mm. Freeze-thaw cycles have previously been shown to have a negative effect on bacterial survival during Mars simulation experiments, especially when no moisture was available (Hawrylewicz *et al*., 1965; Hagen *et al*., 1967). Moreover, freeze-thaw cycles are used as a standard procedure to lyze bacterial cells during DNA extraction (Moré *et al*., 1994). In the present simulation study, the soil cores were exposed to an average of 20 freeze-thaw cycles during the simulation experiment. During each UV exposure cycle, the soil temperature increased to 20, 15, 0, and −15°C at 1, 3, 10, and 15 mm depths, respectively (Fig.1), compared to a soil surface temperature of *ca*. −40°C when the soil cores were not exposed to UV radiation. Although we did not make any salinity measurements in the soil, it is likely that liquid water was present at 0°C, due to the salt content and other impurities

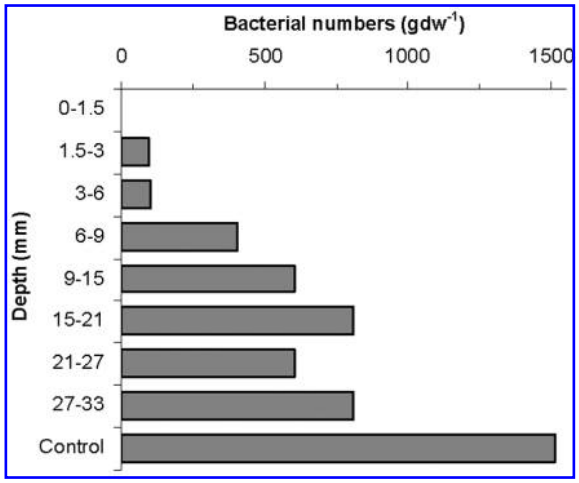

FIG. 5. Number of aerobic CFUs at different depths of the soil core after incubation under simulated martian conditions and from the control soil.

Table 3. Phylogenetic Affiliation of Bacterial Isolates from the Upper 6 mm of the Soil Exposed to Simulated Martian Conditions[a]

Horizon (mm)	Isolate	Taxomonic affiliation	Closest relative[b]	Accession no.[b]	Similarity[b] (%)	Length (bp)
H1 (0–1.5)	MH1-1	*Actinobacteria*	*Friedmanniella antarctica*	Z78206	93	1304
	MH1-2	*Actinobacteria*	*Friedmanniella antarctica*	Z78206	92	1356
H2 (1.5–3)	MH2-1	*Bacteroidetes*	*Spirosoma linguale*	AM000023	94	1315
	MH2-2	*Actinobacteria*	*Micrococcus luteus*	AJ717368	99	1280
	MH2-3	*Proteobacteria*	*Panaciterramonas fulva*	AB245357	98	1355
H3 (3–6)	MH3-1	*Actinobacteria*	*Cryocola antiquus*	AF505513	96	1306
	MH3-2	*Actinobacteria*	*Cellulomonas cellasea*	X83804	98	1305
	MH3-3	*Actinobacteria*	*Nocardioides kribbensis*	AY835926	93	1270
	MH3-4	*Actinobacteria*	*Microlunatus phosphovorus*	Z78207	93	1378
	MH3-5	*Actinobacteria*	*Mycobacterium aichiense*	AF498656	97	1328
	MH3-6	*Actinobacteria*	*Mycobacterium hodleri*	X93184	96	1359

[a]16S rRNA gene sequence similarity by use of BLAST in the GenBank database (Altschul *et al.*, 1997).
[b]The closest culture with the highest bitscore is listed.

in the soil. Hence, there may have been an effect of freeze-thaw cycles in the upper 10 mm of the soil core, which was introduced into the experiment by the ~7-fold higher water content in the experimental atmosphere compared with the average martian environment. In the present study, soil surfaces may, on average, have been covered by a layer of 4–5 water molecules, assuming the diameter of water molecules to be ~2.8 Å and the average surface area of soil particles to be 5.9 $m^2\ gdw^{-1}$ (Jensen, 2006).

Effect of exposure to simulated martian conditions

A summary of estimated half-lives of biomolecules, cell numbers, viable cells, and CFUs in H1 is given in Table 5

Table 4. Phylogenetic Affiliation of Anaerobic Bacteria from the Soil Horizons <1.5 mm; 1.5–3 mm and 27–33 mm and the Control Soil (C)[a]

Taxomonic affiliation	Closest relative[b]	Soil horizon and dilution[c] H1	H2	H8	C	Accession no.	Similarity[d] (%)	Length (bp)
Clostridia	*C. hydroxybenzoicum*[e]		1:1		1:1	L11305	96	482–495
	C. populeti[e]	1:1			1:5	X71853	96–97	495–527
	C. bowmanii[e]	1:1	1:1	1:5	1:5	AJ506120	84–100	492–924
	C. vincentii[e]	1:5	1:5	1:5	1:5	X97432	97–99	491–539
	Desulfosporosinus orientis[e]	1:5	1:1		1:500	AJ493052; Y11570	84–97	558–857
Bacilli	*Paenibacillus antarcticus*[e]		1:5	1:5	1:5	AJ605292	98–99	519–524
Actinobacteria	*Propioniferax innocua*	1:50				AF227165	95–96	504–545
	Friedmaniella spumicola				1:500	AF062535	93	918
	Atopobium parvulum				1:1	X67150	88	503
	Cellulomonas fermentans	1:50	1:50	1:500	1:50	X83805; X79458	93–99	491–922
	Cellulomonas cellasea	1:50	1:50	1:5000	1:50	X83804	96–100	499–923

[a]16S rRNA genes from anaerobic enrichments of diluted soil were separated on DGGE gels. Sequences were obtained from all DGGE bands. 16S rRNA gene sequences were affiliated on the basis of similarity by use of BLAST in the GenBank database (Altschul *et al.*, 1997).
[b]The closest culture with the highest bitscore is listed.
[c]Highest dilution of soil horizons where the 16S rRNA genes from the taxonomic groups were detected.
[d]Range illustrates different similarities of sequences to the closest relative.
[e]Endospore formers.

Table 5. Half-Lives of Biomolecules and Bacterial Cells in the Upper 1.5 mm of the Soil Core Exposed to Simulated Martian Conditions Extrapolated to Mars Equivalent Irradiation Compared to Half-Lives Obtained from the Literature

Parameter	*Temperature (°C)*	*Pressure (mbar)*	*UV radiation (nm)*	*UV intensity dose*	*Lamp*	*Protection of sample*	*Mars half-life (Sol)*[a]	*Reference*
DNA	−41 to 22	8	200–1000	35 times Mars	Hg-xenon, 135 W	Soil matrix 0–1.5 mm	78	Present study
THAA	−41 to 22	8	200–1000	35 times Mars	Hg-xenon, 135 W	Soil matrix 0–1.5 mm	132	Present study
Aspartic acid in THAA	−41 to 22	8	200–1000	35 times Mars	Hg-xenon, 135 W	Soil matrix 0–1.5 mm	69	Present study
Arginine in THAA	−41 to 22	8	200–1000	35 times Mars	Hg-xenon, 135 W	Soil matrix 0–1.5 mm	147	Present study
Glycine in THAA	−41 to 22	8	200–1000	35 times Mars	Hg-xenon, 135 W	Soil matrix 0–1.5 mm	178	Present study
Cell number[b]	−41 to 22	8	200–1000	35 times Mars	Hg-xenon, 135 W	Soil matrix 0–1.5 mm	35	Present study
Viable cells[c]	−41 to 22	8	200–1000	35 times Mars	Hg-xenon, 135 W	Soil matrix 0–1.5 mm	45	Present study
Dead cells (net)[c]	−41 to 22	8	200–1000	35 times Mars	Hg-xenon, 135 W	Soil matrix 0–1.5 mm	33	Present study
CFUs[d]	−41 to 22	8	200–1000	35 times Mars	Hg-xenon, 135 W	Soil matrix 0–1.5 mm	21	Present study
Glycine	21	$\sim 10^{-7}$	190–325	1/12 of Mars	Deuterium	None	1.5	ten Kate *et al.*, 2006
Glycine	21	7	190–325	1/12 of Mars	Deuterium	None	1.5	ten Kate *et al.*, 2006
Glycine	−63	$\sim 10^{-7}$	190–325	1/12 of Mars	Deuterium	None	10.2	ten Kate *et al.*, 2006
Glycine	21	4×10^{-6}	190–325	1/25 of Mars	Deuterium	None	0.9	ten Kate *et al.*, 2005
D-Alanine	21	4×10^{-6}	190–325	1/25 of Mars	Deuterium	None	0.1	ten Kate *et al.*, 2005

[a]Sol, martian day.
[b]SYBR Gold enumerated cells.
[c]Viable cells enumerated with the LIVE/DEAD® *Bac*Light kit™.
[d]After exposure to simulated martian conditions, the CFUs in the upper 1.5 mm of the soil were judged from a single 60 times dilution of the soil.

together with half-lives of biomolecules obtained from the literature. We have assumed that the reduction in the concentration of biomolecules and cell numbers follows first-order rate kinetics. In H1, where a combination of UV radiation, putative reactive oxygen species, and repeated freeze-thaw cycles had the greatest impact on the persistence of the biological material, a clear trend was evident in all data sets: CFUs, which represented the fraction of the surviving bacterial community that grew on standard R2A agar medium after exposure to simulated martian conditions, had the shortest half-life (21 Sol), followed by the dead cells (33 Sol), the total cell number (35 Sol), and viable cells (45 Sol). Total cell numbers included both dead DNA-containing cells and viable DNA-containing cells with an intact cell membrane. The half-lives of cell numbers and biomolecules were up to 8 times longer than the half-life of CFUs, which indicates that, though living cells were killed during exposure to simulated martian conditions, gross cell structures and biomolecules survived for longer time periods. These data are in agreement with observations reported by Cockell *et al*. (2005), which show that phycobilisomes and esterases from *Chroococcidiopsis* sp. remained intact hours after cell viability was lost due to exposure to simulated martian UV flux. The half-lives of biomolecules varied, with glycine having the longest half-life of 178 Sol. Glycine has been used as a model compound in previous studies of the persistence of biomolecules (see Table 5). The half-life of glycine was, in our experiment, 18 times longer than the half-life of unprotected glycine (10 Sol) determined by ten Kate *et al*. (2006). The difference in half-lives is even more significant when taking into account that the UV dose applied by ten Kate *et al*. (2006) corresponded to only 8% of the dose that the sample would have received on Mars within the duration of the experiments, while we applied a flux that corresponded to approximately 35 times the martian UV dose. We postulate that the longer half-life determined in our study is due to the protection of biomolecules by soil particles.

In H2, where we propose that reactive oxygen species and freeze-thaw cycles were the primary detrimental factors, the half-lives of CFUs and viable cells were similar to the half-lives obtained in H1, whereas the half-life of total cell num-

bers and DNA were 1.7 and 2.2 times longer, respectively, than the half-lives estimated in the surface soil layer.

In the 3.0–15.0 mm soil horizon, where we consider that repeated alternating freezing and thawing was the primary detrimental factor, the half-life of CFUs was similar to that in H1. We can only estimate the half-life of CFUs down to a soil depth of 6 mm, as the lack of replication did not allow us to carry out a statistical test of whether the CFUs obtained from greater depths were significantly lower than the CFUs obtained from the freeze-dried control soil. The half-lives of viable cells were 1.2–1.5 times longer at 3–15 mm depth than in H1, whereas the half-life of total cell numbers was 2 times higher in H3 than that in H1.

At soil depth greater than 15 mm, none of the investigated parameters differed from the control soil. This indicates that an extended period of vacuum and low temperature were not detrimental for either the cells or biomolecules. Previous studies have shown that freeze-drying of cells reduces bacterial viability (Stan-Lotter *et al*., 2003; Hansen *et al*., 2005). Freeze-drying also caused a slight reduction in the number of viable cells in the present study (3.2×10^8 cells gdw^{-1} in the control) compared to 4.4×10^8 cells gdw^{-1} in the original permafrost (Hansen *et al*., 2007). However, as the soil was freeze-dried prior to our Mars simulation experiment, our results indicate that additional periods of freeze-drying, which was an effect of the simulated martian environmental conditions, did not further reduce bacterial viability.

Diversity of the bacterial community

As expected, the majority of the bacteria cultured from the soil exposed to simulated martian conditions were Gram-positive bacteria, which are well documented to have a high tolerance to desiccation and radiation (Miyamoto-Shinohara *et al*., 2000; Nicholson et *al*., 2000). *Actinobacteria* was the dominant bacterial class among the cultured survivors from the soil exposed to simulated martian conditions. Indeed, strains belonging to that class have recently been shown to have high UV resistance and desiccation tolerance (Osman *et al*., 2008), which may be a consequence of their ability to generate resting cysts when exposed to unfavorable conditions (Dmitriev *et al*., 2004; Soina *et al*., 2004; Suzina *et al*., 2004). Cyst formation has not been demonstrated in our isolates.

Interestingly, two Gram-negative pure cultures were isolated from the 1.5–3.0 mm soil horizon, which illustrates that some Gram-negative strains have the ability to survive exposure to simulated martian conditions. Only a few Gram-negative bacteria have previously been reported to survive such conditions: *e.g*., *Chroococcidiopsis* sp. (Cockell *et al*., 2005) and *Deinococcus radiodurans* (Pogoda de la Vega *et al*., 2007). However, this is a consequence of the limited number of investigations that have included Gram-negative bacteria in Mars simulation experiments. Thus, the survivability of Gram-negative bacteria under simulated martian conditions needs more attention in future Mars simulation experiments.

Two strains that were present in the anoxic enrichment cultures from the control soil were not identified in soil samples after exposure to simulated martian conditions. They affiliated with *Atopobium parvulum* and *Friedmaniella spumicola*, the latter of which was dominant in the control soil. It is difficult to assess whether the disappearance of these strains from the soil was a result of the simulated martian conditions or a result of the enrichment method.

Most of the pure cultures obtained from the soil samples after exposure to simulated martian conditions were pigmented, which suggests that pigmented bacteria may possess a yet unknown protection mechanism against the simulated martian conditions. This is in agreement with previous studies, which showed that pigmented soil bacteria were more UV resistant than nonpigmented soil bacteria (Arrage *et al*., 1993; Jacobs and Sundin, 2001). However, care should be taken not to overemphasize the value of pigmentation based on the 11 isolates, as we do not know whether the remaining part of the surviving bacterial community was pigmented.

Conclusions

The results of our comprehensive, multi-methodological investigation on the response of indigenous biomolecules and bacteria from an Arctic permafrost to simulated martian conditions indicate that there may be at least three different detrimental factors that affected the persistence of biomolecules and the bacterial cells: (i) UV radiation, (ii) production of reactive oxygen species, and (iii) freeze-thaw cycles. We suggest that UV radiation and reactive oxygen species caused a significant reduction in the concentration of biomolecules in the surface, whereas freeze-thaw cycles may have been responsible for the reduction in the number of CFUs in the deeper soil layers.

The employed UV intensity and oxygen pressure was higher than on present-day Mars, which in combination with the presence of organic matter in the soil may have amplified the photolytic production of reactive oxygen species. This is unlikely to happen on present-day Mars, as organic matter appears to be absent at the surface (Biemann *et al*., 1977). The broader conclusions that can be drawn from the present experiment are as follows:

- The martian UV flux makes it unlikely to find cells or their remains in the upper mobile fraction (dust) of the martian lithosphere
- The search for biomolecules on Mars should take place at soil depths that are not impacted by UV radiation and oxidizing agents, and the search for living organisms should take place at soil depths not impacted by UV radiation and freeze-thaw cycles.
- A persistent forward contamination of Mars is unlikely as long as the cells are not introduced into deeper strata of the lithosphere.

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Abbreviations

ANOVA, analysis of variance; CFUs, colony-forming units; DGGE, denaturant gradient gel electrophoresis; gdw,

gram dry weight; MPN, most probable number; OTUs, operational taxonomic units; THAA, total hydrolyzable amino acids; TN, total nitrogen; TOC, total organic carbon.

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