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Activity and stability of a complex bacterial soil community under simulated Martian conditions

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Abstract: A simulation experiment with a complex bacterial soil community in a Mars simulation chamber was performed to determine the effect of Martian conditions on community activity, stability and survival. At three different depths in the soil core short-term effects of Martian conditions with and without ultraviolet (UV) exposure corresponding to 8 Martian Sol were compared. Community metabolic activities and functional diversity, measured as glucose respiration and versatility in substrate utilization, respectively, decreased after UV exposure, whereas they remained unaffected by Martian conditions without UV exposure. In contrast, the numbers of culturable bacteria and the genetic diversity were unaffected by the simulated Martian conditions both with and without UV exposure. The genetic diversity of the soil community and of the colonies grown on agar plates were evaluated by denaturant gradient gel electrophoresis (DGGE) on DNA extracts. Desiccation of the soil prior to experimentation affected the functional diversity by decreasing the versatility in substrate utilization. The natural dominance of endospores and Gram-positive bacteria in the investigated Mars-analogue soil may explain the limited effect of the Mars incubations on the survival and community structure. Our results suggest that UV radiation and desiccation are major selecting factors on bacterial functional diversity in terrestrial bacterial communities incubated under simulated Martian conditions. Furthermore, these results suggest that forward contamination of Mars is a matter of great concern in future space missions.

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Key words: bacterial community, desiccation, exobiology, Mars, soil, ultraviolet radiation.

Introduction

Since the time of Galileo, scientists have been interested in the nature of the planet Mars and over time there have been numerous speculations on the prospect of life on Mars. Today the surface of Mars is recognized as being hostile to all known life forms (Table 1). However, in the past, 3.5–3.8 Ga ago, when life had evolved on Earth, the climate of Mars was probably much wetter and warmer (McKay & Stoker 1989) and thereby offered conditions suitable for life. If life ever evolved on Mars, extant life may possibly be found in subsurface habitats where liquid water may be present, for example, in permafrost soils and in connection with the polar icecaps. These areas are the largest known water reservoirs on Mars. Moreover, the possible large areas with ground-ice, identified by gamma-spectroscopy (Boynton *et al.* 2002), would be possible environments supporting extant life. On Earth there are several examples of life found in cold and dry habitats similar to those on present-day Mars: endolithic cyanobacteria have been found at the Antarctic under the crust of sandstone and granite (Friedmann 1982); viable heterotrophic, methanogenic, nitrate- and sulphate-reducing bacteria have been found under an Arctic glacier (Skidmore *et al.* 2000); Gram-positive and α- and β-proteobacteria have been isolated from ice cores, possibly originating from accreted water from Lake Vostok, 3600 m below the ground surface (Christner *et al.* 2001), and metabolic activity has been observed down to −20 °C in permafrost soil (Rivkina *et al.* 2000). These findings indicate that life potentially may exist on Mars in similar environments. In the search for life or the remains of life on other planets it is essential to develop methods to avoid contamination with terrestrial bacteria. Better knowledge concerning the probability of contamination, the limits of life and appropriate methods to detect exobiological life can be gained from investigations of the response of terrestrial bacteria to simulated extraterrestrial conditions.

Only a few exobiological simulation experiments have investigated the effect of Martian conditions on terrestrial bacterial soil communities (e.g. Kooistra *et al.* 1958; Packer

Table 1. *Physical conditions in the Mars simulation chamber and on the surface of present-day Mars*

Parameter	Simulated conditions	Martian conditions[a]
Temperature (°C)	−95.4 to +12[b]	−123 to +25
Mean pressure (mbar)	9 and 13[c]	5.6
UV radiation (nm)	>200	>200
UV intensity at 239 nm ($W\ m^{-2}\ nm^{-1}$)	0.207	0.006[d]
Gas composition (%)	CO_2: 77.5; N_2: 8.7; O_2: 1.3[e]	CO_2: 95.3; N_2: 2.7; O_2: 0.13

[a] From Horneck (2000).
[b] See Fig. 1.
[c] Without and with UV radiation, respectively.
[d] Annual average intensity at 11.6° N (M. Patel, personal communication).
[e] Composition when flushed with CO_2 during the experimentation.

et al. 1963; Green *et al.* 1971; Foster *et al.* 1978) and pure cultures (e.g. Hawrylewicz *et al.* 1962; Hagen *et al.* 1964, 1967; Green *et al.* 1971; Koike *et al.* 1995, 1996; Mancinelli & Klovstad 2000; Schuerger *et al.* 2003). The evaluation of these simulation experiments has exclusively been based on traditional cultivation techniques such as colony-forming units (CFUs) or most probable number methods.

The early simulation studies with soil communities (Kooistra *et al.* 1958; Packer *et al.* 1963) failed to create the conditions that are now known to exist on Mars. They included an N_2 atmosphere instead of a CO_2 atmosphere, if ultraviolet (UV) radiation was included it was monochromatic and the pressure was too high compared to the actual Martian pressure. We have only been able to find one study where the effects of the presently know Martian conditions have been evaluated on a bacterial soil community (Green *et al.* 1971). In this study, severe effects were found on bacterial community survival at the soil surface, when the soil was exposed to UV radiation (Green *et al.* 1971). However, no detectable decline in survival was observed in the subsurface soil community, indicating that UV radiation is a primary damaging factor for soil bacteria under simulated Martian conditions.

In recent years, simulation studies have exclusively been carried out with pure cultures. These studies have focused on the survival of endospore-forming bacteria, which are known to be very tolerant to desiccation and UV radiation. In agreement with the community studies, simulation studies with pure cultures have shown that UV radiation is the primary selecting agent on the viability of bacterial cells incubated under Martian conditions. Furthermore, the recovery of viable spores has generally been much higher, when they were protected from direct UV exposure with either cell layers, soil or dust (Green *et al.* 1971; Mancinelli & Klovstad 2000; Schuerger *et al.* 2003). In addition, studies with different pure cultures have shown that Gram-positive cells and spores have a much higher survival rate than cells of Gram-negative bacteria (Koike *et al.* 1995, 1996). Hence, in recent years, bacterial laboratory strains have been the preferred biological model system for space experiments, meaning that insights into the effects of Martian conditions on natural communities remain unclear. However, simulation experiments with natural environmental bacterial communities will by natural selection have the ability to identify the bacterial groups particularly well suited to surviving Martian conditions. Thus, community studies provide an additional tool expanding our knowledge about the response of terrestrial bacteria to extraterrestrial conditions.

In this communication, results are reported from a Mars simulation experiment, where the native bacterial community of the Mars-analogue soil from Salten Skov, Denmark, was studied. To the best of our knowledge this is the first investigation where the short-term effects of Martian conditions on the activity and stability of a complex bacterial soil community have been investigated. Our examination was based on a combined methodological approach including traditional cultivation techniques, metabolic activity studies and physiological and molecular studies. The soil from Salten Skov, Denmark, was used as a Mars-analogue because of its high content of the iron oxides haematite, maghaemite and goethite (Nørnberg *et al.* 2004). This specific soil has previously been used as a Mars-analogue in several simulation experiments studying the properties of Martian dust (Merrison *et al.* 2002, 2004a,b).

Materials and methods

Soil samples and bacterial community

The Mars-analogue iron-rich beech forest soil from Salten Skov, Denmark (Nørnberg *et al.* 2004) was sampled at a depth of 5–20 cm and air-dried at room temperature (air-dried control soil) or stored at 4 °C to prevent water loss (fresh control soil). After sieving (500 μm) the air-dried soil was homogenized by fractionation into equal samples with respect to grain size with a Laborette sample feeder and divider (Fritsch, D), frozen at −20 °C and freeze-dried for 24–48 h (freeze-dried control soil). The freeze-dried soil was stored in 50 ml centrifuge tubes at −80 °C until required.

Incubation under Martian conditions

The Mars simulation chamber used for incubations was a stainless steel cylinder (30 cm diameter and 30 cm height). One soil core of the homogenized freeze-dried Salten Skov soil (4 cm diameter and 6 cm height) was mounted in the middle of the chamber in a tube of inert glass. Thermocouples for monitoring temperature and humidity during incubation were mounted in the soil core at 2, 46 and 60 mm depth in the sample not exposed to UV radiation (chamber (Cha) samples) and at 8.5, 17, 36, 45 and 60 mm depth in the incubation subjected to UV radiation (UV samples). The chamber was evacuated using an external vacuum pump (Edwards rotary pump, model 1SC150B) and the atmosphere replaced with carbon dioxide (CO_2 >99.999% purity, AGA, DK) through a sterile filter (0.22 μm, Osmonics). The mean pressure in the chamber was 13 and 9 mbar in the incubations with and without UV radiation, respectively. However, due to air

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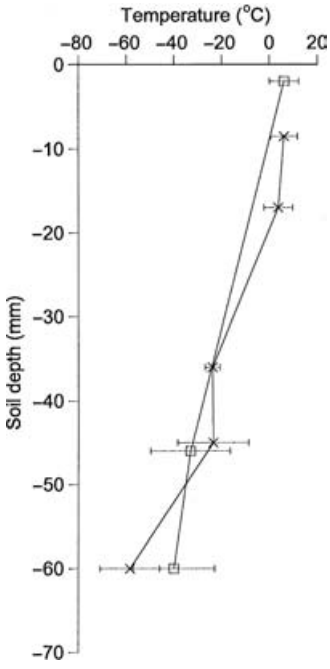

Fig. 1. Temperature profiles in soil cores during incubation in the Mars simulation chamber, with (×) and without (□) UV radiation, respectively. The temperature was measured with thermocouples. Data are shown as means with standard deviations.

leaking into the chamber two short-term increases in pressure were monitored (18 and 21 mbar) during incubation of the soil exposed to UV radiation. These pressure increases lasted for less than 5 min each. The experimental conditions in the simulation chamber are given in Table 1. The UV, visible and near-infrared light (200–1000 nm) was generated with a 200 W mercury–xenon lamp (Hamamatsu Photonics, D) at 99.3 W and was directed to the sample surface through a quartz window by a mirror. The dose of UV radiation applied during the experiment (5.5 h) was equivalent to 8 Martian Sol (Sol = Martian day; 24 h 37 min). The temperature was maintained between −60 and −40 °C at the bottom of the soil core regulated by liquid nitrogen cooling of the steel sample holder. Soil temperature profiles were similar in the two simulation experiments with and without UV radiation (Fig. 1). The gas composition in the simulation chamber was monitored using a rest gas analyser (Microvision plus; Spectra Sensor Tech Ltd, UK) during the experiment without UV radiation (after 27, 105 and 304 min). These measurements showed that temporary changes in the atmospheric composition, due to air leaking into the chamber, increased N_2 from 8.74 to 44.2 % and O_2 from 1.28 to 9.54 % while CO_2 decreased from 77.5 to 35.4 %. Upon leakage, the chamber was immediately flushed with CO_2 resulting in a chamber gas composition as given in Table 1. After 5.5 h of incubation, corresponding to 8 Martian Sol with respect to UV radiation, the chamber temperature, pressure and atmospheric composition were returned to Earth conditions. Air was introduced through a sterile filter (0.22 μm, Osmonics). The soil core was taken out of the chamber and divided into three depth horizons: D1, 0–1 cm; D2, 2–3 cm and D3, 4–5 cm. These horizons were analysed as separate samples in all subsequent experiments, evaluating the effects of Martian conditions at the different soil depths.

Culture media and viable counts

Two growth media were used to estimate the total number of CFUs of aerobic bacteria, Gram-negative bacteria and endospore-forming bacteria. Nutrient broth (NB, Difco) was used as indicated by the manufacturer and solidified with agar (1.5 % w/v). Sodium N-lauroylsarcosinate (SLS, Fluka) was added (1.2 g l^{-1}) to some of the NB media to prevent growth of Gram-positive bacteria (Gould *et al.* 1985). Filter-sterilized nystatin in methanol (50 mg l^{-1}) was added to all media after autoclaving to prevent fungal growth.

The inoculum was prepared from 1.2 g of soil sample mixed with 10 ml of NaCl solution (0.9 % w/v). A dilution series was prepared (10^1–10^7) and triplicate samples (50 μl) were spread on NB plates with and without SLS. The inoculum for enumeration of endospore-forming bacteria was pasteurized at 80 °C for 10 min and spread on NB plates. All plates were incubated at 15 °C and colonies counted (CFUs) until no new colonies developed, the colony density was too high or fungal growth appeared on the plates.

Community analysis with DGGE fingerprints

DNA was extracted directly from the soil samples using the FastDNA spin kit for soil (Bio 101, Vista, CA, USA) with the modification that samples were bead beaten at level 6.0 for 30 s (FastPrep DNA Extractor, Bio101). The DNA yield was evaluated on a 1 % (w/v) SeaKem GTG agarose gel (BioWhittaker Molecular Applications, ME, USA). Region V3–V5 of 16S rDNA was amplified by PCR using the Eubacterial specific primer 341f (Muyzer *et al.* 1993) with a GC-clamp attached (5′-CGCCCGCCGCGCCCCGCGC-CCGTCCCGCCGCC CCCGCCCG-CCTACGGGAGG-CAGCAG-3′) and the universal primer 907r (Lane 1991) (5′-CCGTCAATTCCTTTGAGTTT-3′). The total volume of the PCR mixture was 50 μl (33.5 μl dH_2O, 5 μl buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl; Sigma, D), 5 μl dNTP (1.25 mM of each dNTP; Invitrogen, NL), 3 μl 25 mM $MgCl_2$, 1 μl of each primer (50 pmol μl^{-1}; MWG Biotech, D), 2 U Taq DNA polymerase (Sigma) and 1 μl template). DNA amplification was achieved by the following cycles: 93 °C for 1 min, 30 cycles at 92 °C for 30 s, 57 °C for 1 min and 72 °C for 45 s; the extension time was increased by 1 s per cycle; the last cycle was kept at 72 °C for 5 min. To concentrate the PCR product, two products from each sample were pooled and purified with GenElute PCR Clean-up Kit (Sigma). The optimal denaturing gradient gel electrophoresis (DGGE) gel loading volume of the purified PCR product was evaluated from a 2 % (w/v) Nusieve 3:1 agarose gel (Bio Whittaker Molecular Applications).

DGGE was performed with the D GENE SYSTEM (BioRad, Hercules, CA, USA) (Muyzer *et al.* 1998). Polyacrylamide gels (8 % w/v acrylamide) with gradients of 40–60 % denaturant (100 % denaturant = 7 M urea and 40 %

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v/v formamide) were made using a BioRad gradient maker. Gels were run for 15 h at 100 V in 1×TAE buffer at 60 °C. After electrophoresis, gels were stained for 20 min with Sybr Gold (Molecular Probes, Leiden, NL). Patterns were visualized through UV transillumination and digital images of the gel were made (Gel Doc 2000, BioRad).

DGGE fingerprint analysis was carried out using Quantity One software version 4 (BioRad). The fingerprints were scored by inspection of the lane intensity curves to detect presence or absence of bands independent of intensity. Similarity matrices were generated from binary data for each lane with the Dice coefficient (C_D):

$$C_D = 2j/(a+b)$$

where j is the number of bands shared between samples A and B, and a and b are the total number of bands in sample A and B, respectively (Dice 1945). The percentage similarity is obtained by multiplying this number by 100.

Cultivable community analysis was carried out by molecular analysis of colonies grown on the agar plates described above. DNA was extracted from the total aerobic and endospore-forming colonies harvested from agar plates with the lowest and highest dilution of the inoculated soil suspension. DNA extraction was achieved using the FastDNA spin kit for soil (Bio 101), where all colonies from each agar plate were scraped down in a kit-supplied lysing matrix tube with needles. PCR amplification and DGGE fingerprints were carried out as described above but without pooling and purifying the PCR products and with a 30–70% denaturant gradient instead. The cultivable community composition was quantified as the number of operational taxonomic units (OTUs) in each sample defined as the number of unique bands in each sample on the DGGE gel.

Soil respiration

^{14}C glucose oxidation was measured in four different time course incubations: (1) a short-term (0–24 h) and (2) a long-term (0–118 h) control incubation of freeze-dried control soil, (3) a long-term incubation (0–116 h) of freeze-dried soil after UV exposure under Martian conditions and (4) a long-term incubation (0–120 h) of freeze-dried soil after incubation under Martian conditions, but without UV exposure. Incubations were performed as follows: 0.5 ml ^{14}C-[U]-glucose tracer was injected into ~0.5 g freeze-dried soil in an exetainer; the specific activity of the tracer was 3.0 μCi μmol^{-1} and the activities of tracer per mole of glucose were 2.31, 1.59, 2.31 and 2.35 μCi 0.5 ml^{-1} in incubation 1, 2, 3 and 4, respectively. Glucose oxidation activity was stopped by the addition of 0.5 ml 2.5% (w/v) NaOH. The soil–NaOH suspension was thoroughly mixed and frozen for later analysis.

Processing of samples prior to liquid scintillation counting was in principle as described in Lund & Blackburn (1989). Radioactivity was counted in a Packard Tri-Carb 2900 TR Liquid Scintillation analyser. The mean glucose oxidation rate constant was calculated for the three time intervals (0–21, 21–46 and 46–120 h) according to the method of Lund & Blackburn (1989).

Substrate utilization profiles

Biolog Ecolog™ plates (Biolog Inc., Hayward, CA, USA), containing three replicates of 31 different carbon sources and one negative control, were incubated with a ten times dilution of the soil suspension also used for the plate count. The soil suspension was added to each well (150 μl). Plates were incubated at 15 °C and colour development in each well was recorded as absorbance at 515 nm with a EL309 microplate autoreader (BIO-TEK Instruments Inc., CT, USA). The absorbance in each well was corrected for colour development in the control well. Bacterial activity in each soil sample was expressed as average well colour development (AWCD; $n=3$) for the full plate as described by Garland and Mills (1991).

Data analysis

Principal components analysis (PCA) was performed using PC-ORD software (MjM Software Design, Oregon, USA) to analyse patterns of substrate utilization in Ecolog™ plates. Prior to principal component analysis the corrected absorbance for every Ecoplate™ well was normalized by division by the AWCD (Garland & Mills 1991).

SPSS version 11.0 (SPSS Inc., Chicago, IL, USA) for Windows was used to conduct the statistical analysis. Differences in community activity (AWCD) and diversity (number of substrates utilized) were tested using one-way ANOVA followed by Fisher's least significant difference test (LSD), when a significant treatment effect was observed ($P<0.05$). Differences in bacterial numbers (CFUs) were tested by the non-parametric Mann–Whitney U test, where the raw data were converted into ranks before the test was carried out. This test was used because the CFU data was not normally distributed.

Results

Viable counts

The numbers of culturable aerobic heterotrophic bacteria, Gram-negative bacteria and endospores in the soil samples were determined as CFUs on agar plates at 15 °C (Fig. 2). There was a high recovery of bacteria from all samples incubated in the simulation chamber and compared to the control soils no decrease in survival was observed. The total number of culturable aerobic bacteria recovered from the samples varied from 1.2×10^5 to 1.5×10^6 CFU g soil^{-1} (Fig. 2a). The lowest number of bacteria was obtained with the air-dried control soil and the highest number of bacteria with the fresh control soil, although these were not significantly different. Endospore-forming bacteria, determined after pasteurization of the soil samples, dominated the aerobic bacterial community and were in the range of 1.3–7.2 × 10^5 CFU g soil^{-1} (Fig. 2b). The number of culturable endospore-forming bacteria was not significantly different from the total number of culturable bacteria. They accounted for 82–143% of all culturable bacteria in samples that were incubated in the simulation chamber and for 15, 435 and 44% in the fresh, air-dried and freeze-dried control soils,

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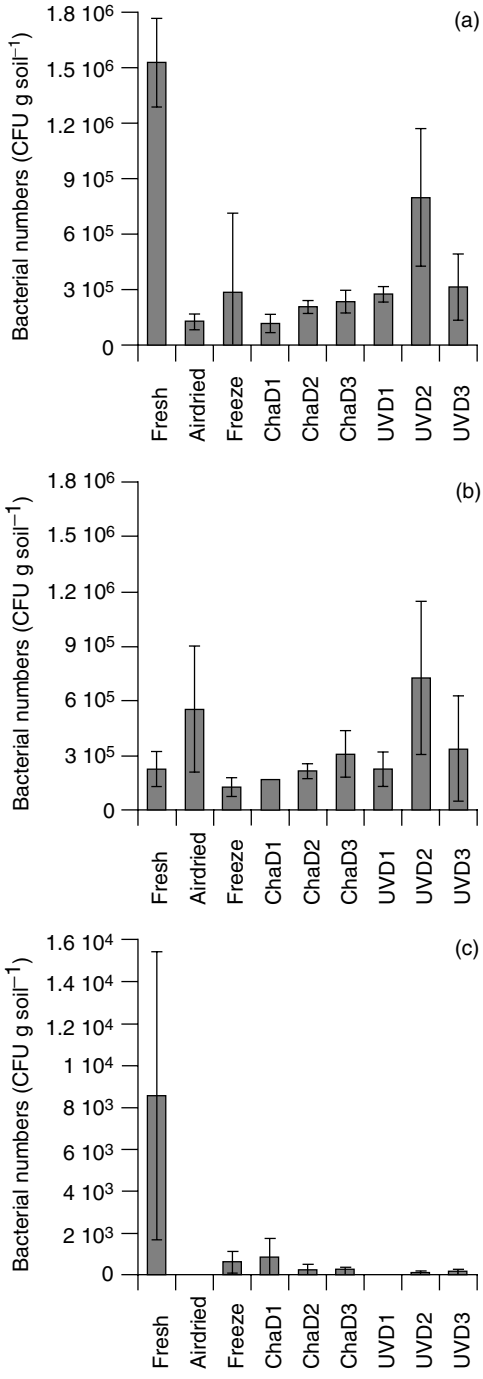

Fig. 2. Bacterial numbers as CFUs at 15 °C ($n=3$). (a) Total number of aerobic bacteria, (b) number of endospores and (c) number of Gram-negative bacteria. Fresh, air-dried and freeze-dried control soils and samples incubated under Martian conditions without (Cha) and with UV radiation (UV) from three horizons of the soil core: D1, 0–1 cm; D2, 2–3 cm; and D3, 4–5 cm. Data are shown as means with standard deviations. No significant difference due to incubations was revealed by the Mann–Whitney U-test ($P>0.05$). Note that the y-axis on plot (c) differs from those of plots (a) and (b).

respectively. The recovery of Gram-negative bacteria was low in all samples and ranged from non-detectable in the air-dried control soil and the upper horizon of the soil incubated under

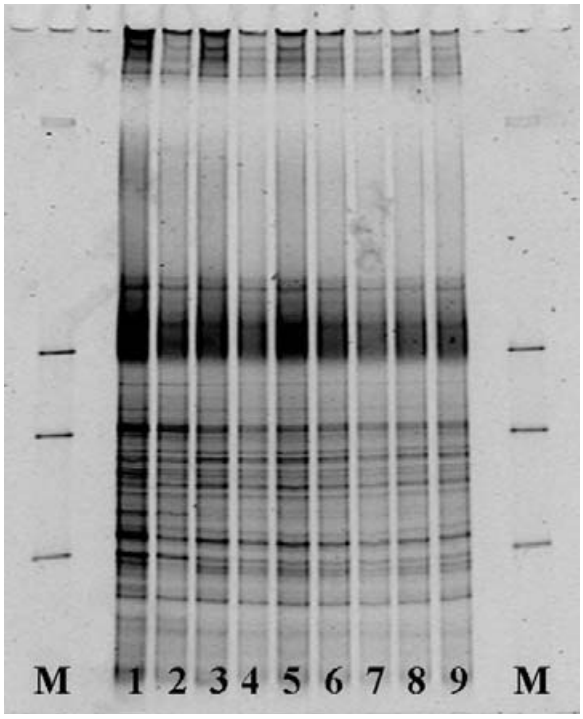

Fig. 3. DGGE community fingerprints of 16S rDNA amplified from DNA extracted from the soil samples. Lanes 1–3: non-incubated fresh, air- and freeze-dried control soils, respectively. Lanes 4–6: soil incubated under Martian conditions without UV radiation from D1 (0–1 cm), D2 (2–3 cm) and D3 (4–5 cm), respectively. Lanes 5–7: soil incubated under Martian conditions with UV radiation corresponding to 8 Martian Sol from D1 (0–1 cm), D2 (2–3 cm) and D3 (4–5 cm). M: marker.

Martian conditions with UV radiation (UVD1) to 8.6×10^3 CFU g soil^{-1} in the fresh control soil (Fig. 2c). The contribution of Gram-negative bacteria to the total number of aerobic bacteria was $<1\%$ in all samples.

Community analysis

Molecular community analysis was achieved with DGGE on DNA extracted from the control soils and incubated soils (Fig. 3). Analysis of the DGGE banding patterns revealed a great similarity between the community structures in all samples (85–100%; Table 2). Only the intensity of the weak bands differed between the fingerprints. Thus, on the molecular level, exposure to Martian conditions did not cause any detectable community change.

Analysis of the culturable bacterial community

Visualized as distinct bands on a polyacrylamide gel by DGGE, the numbers of OTUs of culturable bacteria were similar in the incubated samples and the freeze-dried control soil (Table 3). The 16S rDNA amplicons, loaded on the DGGE gel, were obtained from DNA extracts from bacterial colonies harvested from agar plates inoculated with the lowest and highest dilution of the soil suspensions. Consequently, the OTU number reflects both the total number of taxonomic groups of the culturable community as

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Table 2. *Similarities* (*expressed in %*), *calculated from the Dice coefficient*, *between the DGGE fingerprints* (*Fig. 3*) *from the three control soils* (*fresh*, *air-dried and freeze-dried*), *and the samples incubated under Martian conditions without* (*Cha*) *and with UV radiation* (*UV*). *The soil core was divided into three horizons*: *D1*, *0–1 cm*; *D2*, *2–3 cm and D3*, *4–5 cm*

	Fresh	Air-dried	Freeze-dried	ChaD1	ChaD2	ChaD3	UVD1	UVD2
Fresh								
Air-dried	94.1							
Freeze-dried	92.3	89.8						
ChaD1	94.3	92.0	94.1					
ChaD2	94.3	92.0	94.1	99.8				
ChaD3	89.8	87.0	93.6	91.7	91.7			
UVD1	89.8	87.0	93.6	91.7	91.7	100.0		
UVD2	88.0	85.1	91.7	93.9	93.6	97.8	97.8	
UVD3	89.8	95.7	89.4	91.7	91.7	90.9	90.9	88.9

Table 3. *Genetic composition of the culturable aerobic- and endospore-forming bacteria determined as number of distinct bands on a DGGE-gel* (*OTUs*). *Colonies were harvested from agar plates inoculated with the lowest* (*total number of taxonomic groups*) *and the highest* (*dominant taxonomic groups*) *dilution of the soil suspension. Numbers of substrates utilized and AWCD from Ecolog™ plates* ($n=3$; *mean* $\pm$ *std. dev.*). *Samples*: *fresh*, *air-dried and freeze-dried control soils and samples incubated under Martian conditions without* (*Cha*) *and with UV radiation* (*UV*) *from three horizons of the soil core*: *D1*, *0–1 cm*; *D2*, *2–3 cm*; *and D3*, *4–5 cm*

	Aerobic OTUs (number of bands)		Endospore-forming OTUs (number of bands)			
	Low dilution	High dilution	Low dilution	High dilution	Number of substrates utilized	AWCD
Fresh	na	na	na	na	27.3 ± 0.6^{a}	0.90 ± 0.12^{a}
Air-dried	na	na	na	na	12.9 ± 1.7^{bd}	0.62 ± 0.17^{bd}
Freeze-dried	14	13	10	8	17.6 ± 3.5^{c}	0.6 ± 0.21^{bd}
ChaD1	15	7	13	15	18.0 ± 0.0^{c}	1.23 ± 0.15^{c}
ChaD2	24	18	14	20	15.7 ± 2.1^{bc}	0.84 ± 0.1^{ab}
ChaD3	15	12	10	13	17.3 ± 3.8^{c}	0.82 ± 0.06^{ab}
UVD1	12	7	11	6	9.7 ± 2.1^{d}	0.41 ± 0.23^{d}
UVD2	16	16	13	19	9.7 ± 1.5^{d}	0.36 ± 0.21^{d}
UVD3	11	20	13	11	14.7 ± 1.5^{bc}	0.86 ± 0.14^{ab}

na. Not analysed.
Letters (a–d) indicate differences revealed by Fisher's LSD test ($P<0.05$). Samples with the same letter are not significantly different. The Fisher's LSD was performed if one-way ANOVA revealed significant differences ($P<0.05$).

well as the number of dominant taxonomic groups. Regardless of dilution, similar numbers of OTUs were found with both cultured endospore-forming bacteria and cultured aerobic bacteria (Table 3). Hence, the genetic community composition of the cultured colonies from the lowest and highest dilution was in the same range for all samples. The only exception was the soil sample ChaD2, where the number of OTUs was inexplicably higher than all other soil samples. Thus, UV exposure under Martian conditions was not found to reduce the genetic composition of the culturable bacterial community.

Soil respiration

A temporary decrease in the glucose oxidation rate constants was observed in samples exposed to simulated Martian conditions including UV radiation (Fig. 4). The lowest oxidation rate constant was observed in the upper soil horizon during the three time intervals of the glucose incubation. The oxidation rate constants obtained in soil samples from the two deeper soil horizons, UVD2 and UVD3, were higher than the rate constant obtained in surface soil, UVD1, but they did not reach the rate constant of the freeze-dried control soil during the 120 h incubation. The rate constant remained unchanged with depth in the soil incubated under Martian conditions without UV radiation (Cha D1–3). Furthermore, the glucose oxidation rate constants obtained in the samples Cha D1–3 did not differ from the freeze-dried control soil except from the second time interval, where the activities in Cha D1–3 were incomprehensibly high.

Substrate utilization and functional diversity

Examination of the substrate utilization potential with Ecolog™ plates showed that the bacterial community of the fresh control soil respired 27.3 of the 31 possible substrates. The bacterial communities in UVD1 and UVD2 samples utilized only 9.7 of the 31 possible substrates (Table 3), which were significantly fewer than the substrate utilization abilities of the freeze-dried control soil and the other incubated

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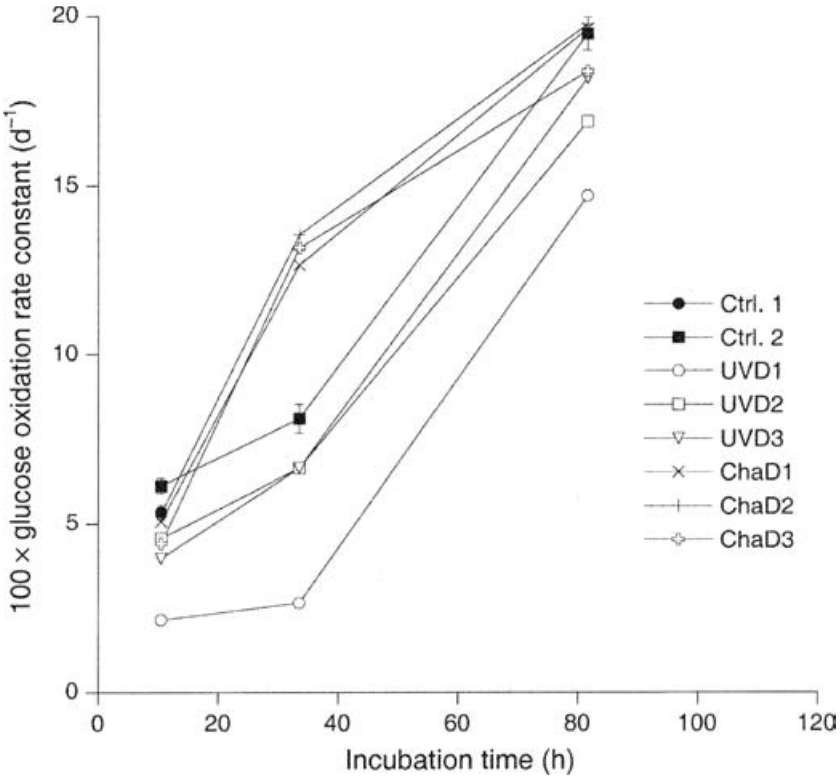

Fig. 4. Soil ^{14}C-glucose oxidation rate constants determined from time courses obtained within the following time intervals after exposure to Martian conditions: 0–21, 21–46 and 46–120 h. The mean oxidation rate constants are plotted in the middle of each time interval as the mean of triplicate measurements. Ctrl. 1, 2: freeze-dried control soil measured in a short- and long-term series. UV: samples incubated under Martian conditions with UV radiation corresponding to 8 Martian Sol from 0–1 cm (D1), 2–3 cm (D2) and 4–5 cm (D3). Cha: samples incubated under Martian conditions without UV radiation from 0–1 cm (D1), 2–3 cm (D2) and 4–5 cm (D3).

samples. Compared to the freeze-dried control soil UVD1 and UVD2 were not able to respire four amino acids (L-asparagine, L-phenylalanine, L-serine and L-threonine), four carboxylic acids (γ-hydroxy butyric acid, α-keto butyric acid, 4-hydroxy benzoic acid and itaconic acid), two carbohydrates (β-methyl-D-glucoside and *i*-erythritol), an amine (phenylethylamine) and an ester (pyruvic acid methyl ester). The highest functional diversity was observed in the fresh control soil compared to the dried and incubated soil samples (Table 3). Thus, both the exposure to Martian conditions including UV radiation and desiccation prior to experimentation decreased the functional diversity of the bacterial community.

Corresponding to the substrate utilization profiles, the lowest AWCD values were observed in Ecolog™ plates inoculated with suspensions from the UVD1 and UVD2 samples (Table 3). Furthermore, the AWCD values from UVD1 and UVD2 were significantly lower than the AWCD from samples incubated under Martian conditions without UV radiation (Cha D1–3). However, the AWCD from UVD1 and UVD2 were not significantly different from the air-dried and freeze-dried control soils. The AWCD of the Ecolog™ plate inoculated with soil suspension of the fresh control soil was significantly higher than the AWCD in the dried control samples (air-dried and freeze-dried). Thus, the bacterial activity was also negatively affected by desiccation of the soil prior to incubation in the simulation chamber. The AWCD of the Ecolog™ plate incubated with the suspension of ChaD1 sample was incomprehensible higher than the AWCD of all other samples.

The first and second axis of the principal component analysis of normalized substrate utilization data explained 78 % of the total variation in the data set (Fig. 5). UVD1 and UVD2 were separated out from the other samples on both PCs, while UVD3 and samples incubated without UV radiation (ChaD1–3) grouped together with the control soils. Hence, the PCA diagram illustrates that the incubation under simulated Martian conditions with UV radiation had a negative effect on the functional diversity and activity in the UVD1 and the UVD2 communities compared to the other samples.

Discussion

In this study we found that the metabolic activities of the bacterial community decreased after incubation under simulated Martian conditions. Glucose oxidation rate constants and substrate utilization determined with Ecolog™ plates were negatively affected when samples were incubated with UV radiation. This effect was detected down to a depth of at least 3 cm of the soil core, equivalent to the samples UVD1 and UVD2. The activity decrease can most likely be

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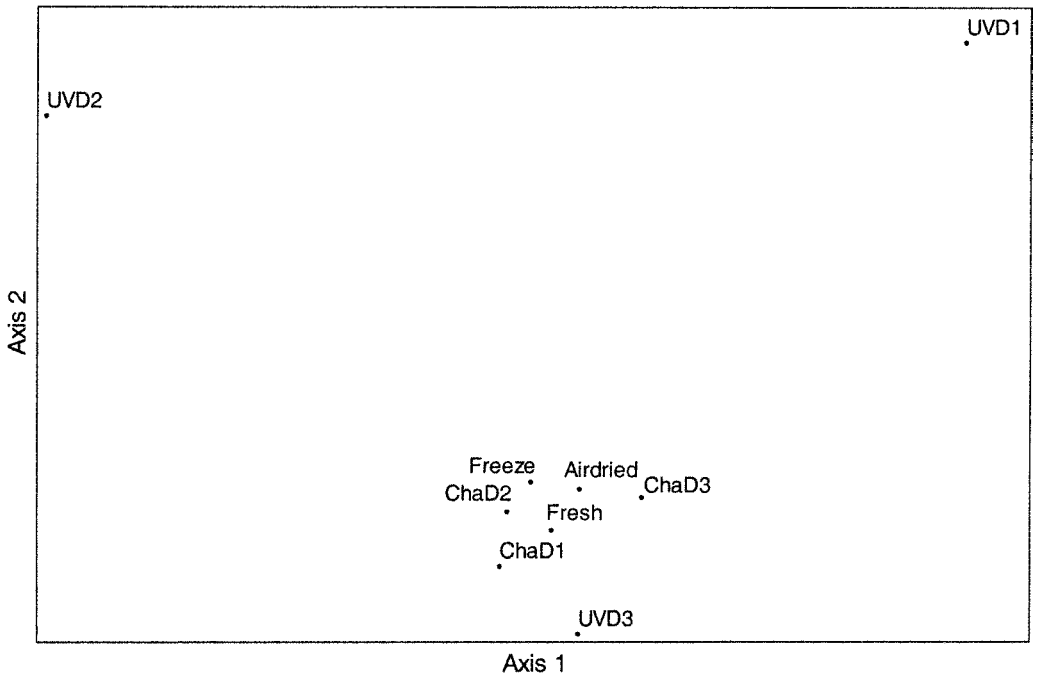

Fig. 5. Principal component analysis based on substrate utilization data from Ecolog™ plates. PC1 and PC2 (Axis 1 and Axis 2) explained 44.1 and 34.3 % of the variance, respectively. UV: soil incubated under Martian conditions with UV radiation corresponding to 8 Martian Sol. Cha: soil incubated in simulation chamber without UV radiation. Soil cores were cut into three horizons 0–1 cm (D1), 2–3 cm (D2) and 4–5 cm (D3). Control soils: unincubated fresh, air-dried and freeze-dried soil.

attributed directly or indirectly to UV radiation since no negative effect was observed on the community incubated under Martian conditions not exposed to UV radiation. Previous studies have shown that the penetration depth of UV radiation is dependent on the properties of the soil or sediment. Garcia-Pichel and Bebout (1996) found that irradiance of UVB was reduced to 1 % at depths <1.25 mm in different sediment types. Moreover, the UV penetration depth is found to be further reduced by stronger scattering in dry sediments (Garcia-Pichel & Bebout 1996) and by absorption of iron compounds (Pierson *et al.* 1993; Kumar *et al.* 1996). Thus, the observed decrease in the community activity down to a soil depth of at least 3 cm in our experiment cannot simply be explained by direct UV radiation effects alone. The subsurface impact must be due to indirect effects such as photochemically produced free oxygen species, a mechanism that Yen *et al.* (2000) recently proposed to occur on the surface of Mars. The relatively high pressure of oxygen in our experiment (1.3 %) compared to present-day Mars (0.13 %) most likely caused formation of harmful free oxygen radicals. However, this potential UV-mediated formation of radicals under our experimental conditions remains to be verified.

Recently, long-term elevated UV radiation exposure of bacterial communities was reported to affect the bacterial community structure and to reduce the bacterial activity (Johnson *et al.* 2002). However, in our short-term experiment UV exposure did not cause detectable changes in the community structure; i.e. the DGGE fingerprint analysis did not reflect the observed decrease in functional diversity demonstrated with EcoLog™ plates. There may be several reasons for this lack of UV effects on the molecular level: (1) DNA in the dead cells was not degraded, (2) the surface soil section analysed was too coarse to reveal surface UV effects and (3) most of the bacterial community was protected by the soil matrix. The subzero temperatures and dry conditions in the simulation chamber probably prevented degradation of DNA from dead cells. Hence, such DNA was extracted and amplified together with DNA from bacteria surviving the UV exposure, thereby frustrating the molecular fingerprint analysis. Persistence of DNA at low temperatures is well known and has recently been demonstrated in a study by Willerslev *et al.* (2003), who PCR-amplified DNA of extinct animals and plants from Siberian permafrost soil samples that were up to 400 000 years old. An effect of UV radiation on the bacterial community structure would possibly have been detected at the molecular level if we had analysed the uppermost millimetre of the surface layer separately. Analysing the upper centimetre as one sample may have obscured a significant extinction or change in the bacterial community in the very surface of the soil.

The relatively coarse sampling procedure used in our investigation may likewise explain why the number of culturable bacteria, determined as CFUs, did not differ between samples incubated in the Mars simulation chamber and the control soils. We suggest that the bacterial cells covered by soil particles were protected against the direct detrimental effects of UV radiation. This is in agreement with earlier Mars simulation studies with soil communities. In these studies it was found that <0.3 % of the community survived on the soil surface when exposed to UV radiation (Packer *et al.* 1963;

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Green *et al*. 1971), while there was no reduction in survival 1 cm below the surface (Green *et al*. 1971). Similarly, Mars simulation studies with *Bacillus subtilis* endospores found that spores protected by soil particles or spore layers had a higher survivability than unprotected spores, which were inactivated after only 15 min of UV exposure (Mars equivalent intensity; Mancinelli & Klovstad 2000; Schuerger *et al*. 2003). Moreover, a 1 mm dust layer gave the endospores full protection against the damaging effects of UV radiation (Mancinelli & Klovstad 2000). Survival experiments with endospores of *Bacillus subtilis* in space have shown similar results, where almost none of the unprotected spores survived (<0.001%), while 2–70% survived when protecting substances were present (e.g. glucose or meteorites; Horneck *et al*. 1994; Rettberg *et al*. 2002).

The majority of the original bacterial community in the Salten Skov soil formed endospores. Hence, the present study adds to the increasing number of studies underlining the high resistance of endospore-forming bacteria to the harsh conditions on present-day Mars. In 1963, Packer *et al*. demonstrated that endospore-forming bacteria survived Martian conditions preferentially compared to other bacterial members of the investigated soil community. This has been confirmed by the studies of Koike *et al*. (1995, 1996), who demonstrated that Gram-positive cells and spores had a profoundly higher survivability (25–72%) than Gram-negative bacteria (<0.8%) when pure cultures were exposed to UV and proton particle doses equivalent to 200 Martian years. We believe that the observed loss of culturability of Gram-negative bacteria in our experiment can be explained by the initial desiccation of the fresh control soil. The preferential survival of the spore-forming part of the soil community was expected, as desiccation tolerance among spores is well known and well documented (e.g. Potts 1994; Nicholson *et al*. 2000; Dose *et al*. 2001). Moreover, with the Ecolog™ plates desiccation of the fresh control soil was shown to significantly affect the functional diversity of the soil community. From these results it is evident that the arid conditions on Mars are important in terms of bacterial survival. However, previous experiments under simulated Martian conditions have not evaluated the effect of desiccation on bacterial communities and comparisons are therefore not possible. Nevertheless, aridity is found to be very selective for bacterial soil communities and recently an investigation of soil samples from the Atacama Desert, Chile (the driest desert on Earth), showed that bacterial numbers, diversity and activity were dependent on moisture (Navarro-Gonzalez *et al*. 2003). According to this investigation, the most arid location was too dry to support bacterial life as neither bacterial colonies, activity nor DNA were recovered from the soil samples. In addition, the cultivated microorganisms from the nearby region were exclusively Gram-positive bacteria (Navarro-Gonzalez *et al*. 2003).

In conclusion, methods operating on the molecular, metabolic and community level were applied for evaluation of the effects of Martian conditions on the activity and stability of a complex soil community. Based on the results, it is apparent that a combined methodological approach provides a more comprehensive analysis of the effects of Martian conditions on terrestrial bacteria. The short duration of our simulation experiment combined with the coarse soil sectioning meant that the methods evaluating metabolic responses had the best suited resolution for evaluation of the effects. The results indicate that UV radiation and desiccation are the major selecting factors on bacterial functional diversity under simulated Martian conditions. The observed effects of UV radiation on bacterial metabolic activity and functional diversity down to 3 cm depth in the soil may be explained by production of free oxygen radicals. Such radical production would be facilitated by the somewhat higher oxygen pressure and UV intensity in this experiment compared to present-day Martian conditions.

Long-term investigations in the range of several weeks to months are needed to gain further insight into the survival potential of bacterial communities under Martian conditions. Future studies should operate with finer depth resolutions than those undertaken in the present study. Also, attempts should be made to identify and quantify reactive gasses in the soil pore volume in order to test whether the suggested indirect UV effect is of any importance.

By including a complex bacterial community our study adds a new dimension to our understanding of the impact of Martian conditions on the survival of terrestrial bacteria. In addition, studies with complex communities will elucidate which microorganisms are particularly well pre-adapted to simulated Martian conditions.

Insight into the mechanisms behind the ability of terrestrial bacteria to survive Martian conditions has a great importance for the development of decontamination procedures for spacecrafts. Consequently, such insights will help to prevent target site contamination with terrestrial bacteria.

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References

Boynton, W.V. *et al*. (2002). Distribution of hydrogen in the near surface of Mars: evidence for subsurface ice deposits. *Science* **297**, 81–85.

Christner, B.C., Mosley-Thompson, E., Thompson, L.G. & Reeve, J.N. (2001). Isolation of bacteria and 16S rDNAs from Lake Vostok accretion ice. *Environ. Microbiol.* **3**, 570–577.

Dice, L. R. (1945). Measures of the amount of ecologic association between species. *Ecology* **26**, 297–302.

Dose, K., Bieger-Dose, A., Ernst, B., Feister, U., Gomez-Silva, B., Klein, A., Risi, S. & Stridde, C. (2001). Survival of microorganisms under the extreme conditions of the Atacama Desert. *Origins Life Evol. Biosphere* **31**, 287–303.

Foster, T.L., Winans, L. Jr., Casey, R.C. & Kirschner, L.E. (1978). Response of terrestrial microorganisms to a simulated Martian environment. *Appl. Environ. Microbiol.* **35**, 730–737.

Friedmann, E.I. (1982). Endolithic microorganisms in the Antarctic cold desert. *Science* **215**, 1045–1053.

Garcia-Pichel, F. & Bebout, B.M. (1996). Penetration of ultraviolet radiation into shallow water sediments: high exposure for photosynthetic communities. *Marine Ecol. Progr. Ser.* **131**, 257–262.

Garland, J.L. & Mills, A.L. (1991). Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. *Appl. Environ. Microbiol.* **57**, 2351–2359.

Gould, W.D., Hagedorn, C., Bardinelli, T.R. & Zablotowicz, R.M. (1985). New selective media for enumeration and recovery of fluorescent pseudomonads from various habitats. *Appl. Environ. Microbiol.* **49**, 28–32.

Green, R.H., Taylor, D.M., Gustan, E.A., Fraser, S.J. & Olson, R.L. (1971). Survival of microorganisms in a simulated Martian environment. *Space Life Sci.* **3**, 12–24.

Hagen, C.A., Ehrlich, R. & Hawrylewicz, E. (1964). Survival of microorganisms in simulated Martian environment. I. *Bacillus subtilis* var. *globigii*. *Appl. Microbiol.* **12**, 215–218.

Hagen, C.A., Hawrylew, E. & Ehrlich, R. (1967). Survival of microorganisms in a simulated Martian environment. 2. Moisture and oxygen requirements for germination of *Bacillus cereus* and *Bacillus subtilis* var. *niger* Spores. *Appl. Microbiol.* **15**, 285–291.

Hawrylewicz, E., Gowdy, B. & Ehrlich, R. (1962). Micro-organisms under a simulated Martian environment. *Nature* **193**, 497.

Horneck, G. (2000). The microbial world and the case for Mars. *Planet. Space Sci.* **48**, 1053–1063.

Horneck, G., Bucker, H. & Reitz, G. (1994). Long-term survival of bacterial spores in space. *Adv. Space Res.* **14**, 41–45.

Johnson, D., Campbell, C.D., Lee, J.A., Callaghan, T.V. & Gwynn-Jones, D. (2002). Arctic microorganisms respond more to elevated UV-B radiation than CO_2. *Nature* **416**, 82–83.

Koike, J., Oshima, T., Kobayashi, K. & Kawasaki, Y. (1995). Studies in the search for life on Mars. *Adv. Space Res.* **15**, 211–214.

Koike, J., Hori, T., Katahira, Y., Koike, K.A., Tanaka, K., Kobayashi, K. & Kawasaki, Y. (1996). Fundamental studies concerning planetary quarantine in space. *Adv. Space Res.* **18**, 339–344.

Kooistra, J.A., Mitchell, R.B. & Strughold, H. (1958). The behavior of microorganisms under simulated Martian environmental conditions. *Astron. Soc. Pacific* **70**, 64–69.

Kumar, A., Tyagi, M.B., Srinivas, G., Singh, N., Kumar, H.D., Sinha, R.P. & Hader, D.P. (1996). UVB shielding role of $FeCl_3$ and certain cyanobacterial pigments. *Photochem. Photobiol.* **64**, 321–325.

Lane, D.J. (1991). 16/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, ed. Stackebrandt, E. & Goodfellow, M., pp. 113–175. Wiley, Chichester.

Lund, B.A. & Blackburn, H.B. (1989). Urea turnover in a coastal marine sediment measured by a ^{14}C-urea short-term incubation. *J. Microbiol. Methods* **9**, 297–308.

McKay, C.P. & Stoker, C.R. (1989). The early environment and its evolution on Mars – implications for life. *Rev. Geophys.* **27**, 189–214.

Mancinelli, R.L. & Klovstad, M. (2000). Martian soil and UV radiation: microbial viability assessment on spacecraft surfaces. *Planet. Space Sci.* **48**, 1093–1097.

Merrison, J.P. *et al.* (2002). Simulation of the Martian dust aerosol at low wind speeds. *J. Geophys. Res. Planets* **107**, art no 5133.

Merrison, J. P., Jensen, J., Kinch, K., Mugford, R. & Nornberg, P. (2004a). The electrical properties of Mars-analogue dust. *Planet. Space Sci.* **52**, 279–290.

Merrison, J.P., Gunnlaugsson, H.P., Jensen, J., Kinch, K., Nørnberg, P. & Rasmussen, K.R. (2004b). A miniature laser anemometer for measurement of wind speed and dust suspension on Mars. *Planet. Space Sci.* **52**, 1177–1186.

Muyzer, G., Dewaal, E.C. & Uitterlinden, A.G. (1993). Profiling of complex microbial-populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16S ribosomal-RNA. *Appl. Environ. Microbiol.* **59**, 695–700.

Muyzer, G., Brinkhoff, T., Nübel, U., Santegoeds, C., Schäfer, H. & Wawer, C. (1998). Denaturing gradient gel electrophoresis (DGGE) in microbial ecology. In *Molecular Microbial Ecology Manual*, ed. Akkermans, A.D.L., Van Elsas, J.D. & De Bruijn, F.J., pp. 3.4.4: 1–27. Kluwer, Dordrecht.

Navarro-Gonzalez, R. *et al.* (2003). Mars-like soils in the Atacama Desert, Chile, and the dry limit of microbial life. *Science* **302**, 1018–1021.

Nicholson, W.L., Munakata, N., Horneck, G., Melosh, H.J. & Setlow, P. (2000). Resistance of Bacillus endospores to extreme terrestrial and extraterrestrial environments. *Microbiol. Molec. Biol. Rev.* **64**, 548–572.

Nørnberg, P., Schwertmann, U., Stanjek, H., Andersen, T. & Gunnlaugsson, H.P. (2004). Mineralogy of a burned soil compared with four anomalously red quaternary deposits in Denmark. *Clay Minerals* **39**, 85–98.

Packer, E., Scher, S. & Sagan, C. (1963). Biological contamination of Mars 2. Cold and aridity as constraints on the survival of terrestrial microorganisms in simulated Martian environments. *Icarus* **2**, 293–316.

Pierson, B.K., Mitchell, H.K. & Ruffroberts, A.L. (1993). *Chloroflexus-Aurantiacus* and ultraviolet-radiation – implications for archean shallow-water stromatolites. *Origins Life Evol. Biosphere* **23**, 243–260.

Potts, M. (1994). Desiccation tolerance of prokaryotes. *Microbiol. Rev.* **58**, 755–805.

Rettberg, P., Eschweiler, U., Strauch, K., Reitz, G., Horneck, G., Wanke, H., Brack, A. & Barbier, B. (2002). Survival of microorganisms in space protected by meteorite material: results of the experiment 'EXOBIOLOGIE' of the PERSEUS mission. *Adv. Space Res.* **30**, 1539–1545.

Rivkina, E.M., Friedmann, E.I., McKay, C.P. & Gilichinsky, D.A. (2000). Metabolic activity of permafrost bacteria below the freezing point. *Appl. Environ. Microbiol.* **66**, 3230–3233.

Schuerger, A.C., Mancinelli, R.L., Kern, R.G., Rothschild, L.J. & McKay, C.P. (2003). Survival of endospores of *Bacillus subtilis* on spacecraft surfaces under simulated Martian environments: implications for the forward contamination of Mars. *Icarus* **165**, 253–276.

Skidmore, M.L., Foght, J.M. & Sharp, M.J. (2000). Microbial life beneath a high Arctic glacier. *Appl. Environ. Microbiol.* **66**, 3214–3220.

Willerslev, E., Hansen, A.J., Binladen, J., Brand, T.B., Gilbert, M.T.P., Shapiro, B., Bunce, M., Wiuf, C., Gilichinsky, D.A. & Cooper, A. (2003). Diverse plant and animal genetic records from Holocene and Pleistocene sediments. *Science* **300**, 791–795.

Yen, A.S., Kim, S.S., Hecht, M.H., Frant, M.S. & Murray, B. (2000). Evidence that the reactivity of the Martian soil is due to superoxide ions. *Science* **289**, 1909–1912.