PROTECTION OF BACTERIAL SPORES IN SPACE, A CONTRIBUTION TO THE DISCUSSION ON PANSPERMIA

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Abstract. Spores of *Bacillus subtilis* were exposed to space in the BIOPAN facility of the European Space Agency onboard of the Russian Earth-orbiting FOTON satellite. The spores were exposed either in dry layers without any protecting agent, or mixed with clay, red sandstone, Martian analogue soil or meteorite powder, in dry layers as well as in so-called 'artificial meteorites', i.e. cubes filled with clay and spores in naturally occurring concentrations. After about 2 weeks in space, their survival was tested from the number of colony formers. Unprotected spores in layers open to space or behind a quartz window were completely or nearly completely inactivated (survival rates in most cases $\leq 10^{-6}$). The same low survival was obtained behind a thin layer of clay acting as an optical filter. The survival rate was increased by 5 orders of magnitude and more, if the spores in the dry layer were directly mixed with powder of clay, rock or meteorites, and up to 100% survival was reached in soil mixtures with spores comparable to the natural soil to spore ratio. These data confirm the deleterious effects of extraterrestrial solar UV radiation. Thin layers of clay, rock or meteorite are only successful in UV-shielding, if they are in direct contact with the spores. The data suggest that in a scenario of interplanetary transfer of life, small rock ejecta of a few cm in diameter could be sufficiently large to protect bacterial spores against the intense insolation; however, micron-sized grains, as originally requested by Panspermia, may not provide sufficient protection for spores to survive. The data are also pertinent to search for life on Mars and planetary protection considerations for future missions to Mars.

Keywords: bacterial spores, extraterrestrial UV radiation, interplanetary transfer of life, life on Mars, meteorites, Panspermia, space experiments

1. Introduction

About a century ago, Arrhenius (1903) formulated the theory of Panspermia which postulates that microscopic forms of life, e.g. spores, can be dispersed in space by the radiation pressure from the sun, thereby seeding life from one planet to another, or even between solar systems. Since its formulation, this theory has been criticized with reproaches among others that Panspermia cannot be experimentally tested and that spores will not survive long-time exposure to the hostile environment of space, especially vacuum and radiation (Nussinov and Lysenko, 1983, 1992).

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The evidence that SNC meteorites originated from Mars (Becker and Pepin, 1984) has inspired a new scenario of potential transport of living matter from one planet of our solar system to another (Clark *et al*., 1999; Mileikowsky *et al*., 2000; Clark, 2001). Large impacts on Earth – or any other planet – may eject rocks that could eventually fall on Mars – or other planets of our solar system (Melosh, 1988). Since, on Earth, soil and rocks are colonized by microorganisms and spores, it cannot be excluded, that these organisms are swept along into space.

Many spore-forming bacteria are found in terrestrial soils and their spores have been recognized as the hardiest known forms of life on Earth. The developmental pathway from a vegetatively growing bacterial cell to a spore, i.e., the dormant state, is triggered by depletion of nutrients in the bacterial cell's environment (Piggot *et al*., 1994). In the dormant stage, spores undergo no detectable metabolism and exhibit a high degree of resistance to inactivation by various physical insults, such as cycles of extreme heat and cold, extreme desiccation including vacuum, UV and ionizing radiation, as well as oxidizing agents or corrosive chemicals (recently reviewed by Nicholson *et al*., 2000). The high resistance of *Bacillus* endospores is mainly due to two factors: (i) a dehydrated, highly mineralized core enclosed in a thick protective envelop, the cortex and the spore coat layers (Figure 1), and (ii) the saturation of their DNA with small, acid-soluble proteins whose binding greatly alters the chemical and enzymatic reactivity of the DNA (Setlow, 1995). In the presence of appropriate nutrients including liquid water spores respond rapidly by germination and outgrowth, resuming vegetative growth. Hence, spore formation represents a strategy by which a bacterium escapes temporally and/or spatially from unfavorable conditions: spores exhibit extreme longevity and can be relocated e.g., by wind and water, to remote areas. Among the bacterial spores, the endospores of the genus *Bacillus* are the best investigated ones (Nicholson *et al*., 2000). Their responses to the conditions of space has been studied in several space experiments (reviewed by Horneck, 1993; Horneck *et al*., 2001b) as well as in studies using space simulation facilities (Horneck, 1999).

Among the parameters of space, solar UV radiation is the most deleterious one in killing bacterial spores. The incidence of the full spectrum of solar UV radiation (>170 nm) killed 99% of the spores within seconds (Horneck *et al*., 1984). Action spectra of the solar photons (160 nm < λ < 320 nm) for killing *B. subtilis* spores correlate closely with the absorption spectrum of DNA, indicating DNA as the critical target for lethality (Horneck *et al*., 1995). Simultaneous action of solar UV and space vacuum even increased the UV-sensitivity. Photoproducts in the DNA that are not easily amenable to cellular repair processes (Lindberg and Horneck, 1991), such as DNA protein cross-linking and DNA strand breaks were among the most severe injuries produced by solar UV radiation in the spores when in space vacuum. The maximum time microorganisms have been exposed to the space environment was during the LDEF mission which lasted for nearly 6 yr. After recovery, up to 70% of *B. subtilis* spores survived in the vacuum of space, if protected against solar UV by a thin aluminum cover and exposed in a multilayer in the presence of

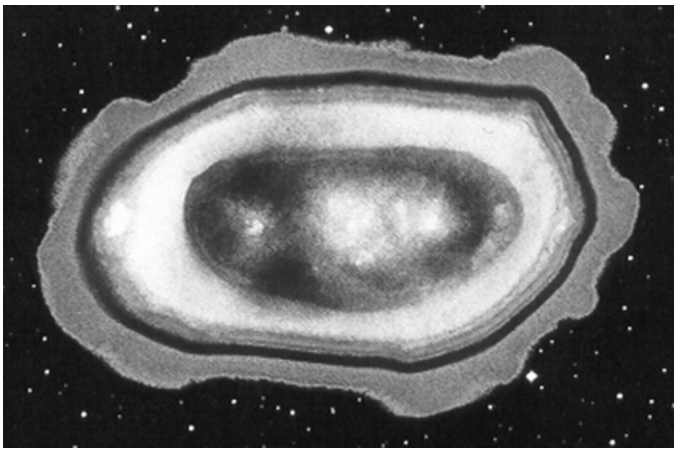

Figure 1. Cross-section of a spore of *B. subtilis*. The DNA is contained in the nucleoid within the spore core. The core is surrounded by the protective cortex and the inner and outer spore coat layers. The long axis of the spore is 1.2 μm; the core area is 0.25 μm^2. (The electron micrograph was kindly provided by S. Pankratz.)

chemical protectants, such as glucose, and up to 10^{-4}, if simultaneously exposed to solar UV of a cumulative dose of roughly 1 GJ m^{-2} (Horneck *et al.*, 1994).

During three space missions, using the BIOPAN facility of the European Space Agency ESA on board of a Russian FOTON satellite we have tackled the question whether and to what extent natural soil or rock material may protect bacterial spores against the harsh environment of space, especially solar UV radiation (Experiment SURVIVAL). For this purpose spores of *B. subtilis* were exposed to space either unprotected, or under a filter of clay, or mixed with different soil, rock or meteorite powders. The data will contribute to the assessment of the probabilities of microorganisms surviving within the surface layers of meteorites.

2. Description of the Space Experiments on BIOPAN

2.1. Biological samples exposed to space

2.1.1. *Bacterial Endospores*

Spores of the following strains of *B. subtilis* were used in the BIOPAN experiments:

(i) *B. subtilis* HA 101 *his B101 met B101 leuA8* as wild type strain with regards to the DNA repair but deficient (auxothroph) in the production of the amino acids histidine, methionine and leucine;
(ii) *B. subtilis* HA F *polA* with the same auxotrophy markers and deficient in DNA repair;

(iii) *B. subtilis* 101 TKJ 6312, *uvrA10 ssp-1*, also with the same auxotrophy markers as strain HA 101 but deficient excision repair and spore photoproduct repair.

The strains and their culture conditions are described by Baltschukat and Horneck (1991). Strains of different DNA repair capacity were used in order to determine the role of DNA repair in survival after exposure to space. The histidine auxotrophy was used to determine the mutagenic efficiency of the space environment by scoring for induced reversions to histidine prototrophy (data not shown). These auxotrophic markers were also used in marker tests to check for potential contamination. Spores in aqueous suspension (about 10^{10} spores mL^{-1}) were stored at 4 °C as stock suspension for preparing the test samples.

2.1.2. *Sample Types*

In order to test the protective effect of different soil or meteorite material against the parameters of space, *B. subtilis* spores were mixed with powder of the following rocks or soil:

(i) clay from Adendorf, Germany (grain diameter 10–200 μm, mean about 80 μm);
(ii) red sandstone from Heidelberg, Germany;
(iii) meteorite Millbillillie (grain diameter 11–226 μm, mean about 93 μm), an eucrite, i.e. an igneous rock of basaltic composition, probably from the asteroid Vesta;
(iv) simulated Martian soil MRTE;
(v) Martian meteorite Zagami (grain diameter 22–230 μm, mean about 113 μm), a shergottite with basaltic composition.

In addition, spores were mixed with glucose which has been found to support survival in space vacuum, probably by helping to prevent damages to the DNA, membranes and proteins by replacing the water molecules during the desiccation process and thereby preserving the three-dimensional structure of the biomolecules (Crowe and Crowe, 1992).

The mixtures were exposed to space in the following arrangements:

(i) so-called 'artificial meteorites': fluffy powder (280 mg dry powder with about 5×10^7 spores) accommodated in a volume of about 1 cm^3 in the sample carrier that was covered by a quartz window (see Section 2.2.1.);
(ii) 'mixed layers': dry layers of mixtures of spores and powder (about 5×10^7 spores, different concentrations of powder) or spores and glucose (5%) mounted on quartz plates of 7 mm in diameter and placed at the bottom of a sample carrier;
(iii) 'shadowed layers': dry layers of spores (about 5×10^7 spores) mounted on quartz plates of 7 mm in diameter and placed at the bottom of the sample

carrier beneath a layer of powdered rock or soil (5.6 mg 0.8 cm^{-2}; yielding approximately 2 layers of grains over the whole area) mounted on the inner side of the quartz window of the sample carrier.

In addition, spores were exposed without any additive as:

(iv) 'inside layers': dry lay of spores (about 5×10^7 spores) mounted on quartz plates of 7 mm in diameter and placed inside the sample carrier beneath a quartz window;
(v) 'unprotected outside layers': dry lay of spores (about 5×10^7 spores) mounted on the outside of the quartz window of the sample carrier facing space.

In these layers, approximately 22% of the spores were non-shadowed by other spores as calculated from

$$f_{NS} = \frac{m \times e^{-m}}{1 - e^{-m}} \tag{1}$$

where f_{NS} is the fraction of non-shadowed spores and m is the ratio of the area covered by spores to the total area of exposure (Lindberg and Horneck, 1991). The area covered by an individual spore was approximated as 1 μm^2.

2.1.3. *Preparation of Samples*
For sample type (i) 'artificial meteorites' 20 μL of an aqueous spore suspension (about 2.5×10^9 mL^{-1}) were mixed with 280 mg of powder, dried at 37 °C over several hours, then homogenized by stirring. For the 'mixed layers' of type (ii) rock or soil powder (different concentrations in the suspension) or glucose (5%) and spores (final concentration in the suspension about 2.5×10^9 mL^{-1}) in distilled water were mixed under vigorous shaking. Twenty μL of these suspensions were transferred onto sterile quartz discs (7 mm diameter) and dried overnight in air at room temperature. A fairly homogeneous distribution of the spores and powder in the layer was achieved by using selectively silanized quartz plates with a hydrophobic outer rim of 1 mm surrounding a hydrophilic inner area of 5 mm in diameter. All other sample types (iii) to (v) were prepared by the same procedure, however without any additive. For each data point, at least 3 parallel samples were used. All materials and equipment, used during sample preparation and analysis, was sterilized according to standard microbiological protocols.

2.1.4. *Sample Analysis*
After retrieval and quick inspection, the powder of sample type (i) 'artificial meteorite' was transferred in 5 mL of distilled water and mixed by vortexing. The dry layers of samples (ii) to (v) were resuspended by the following procedure: the dry spore layer was covered by an aqueous polyvinyl alcohol (PVA) solution (10%),

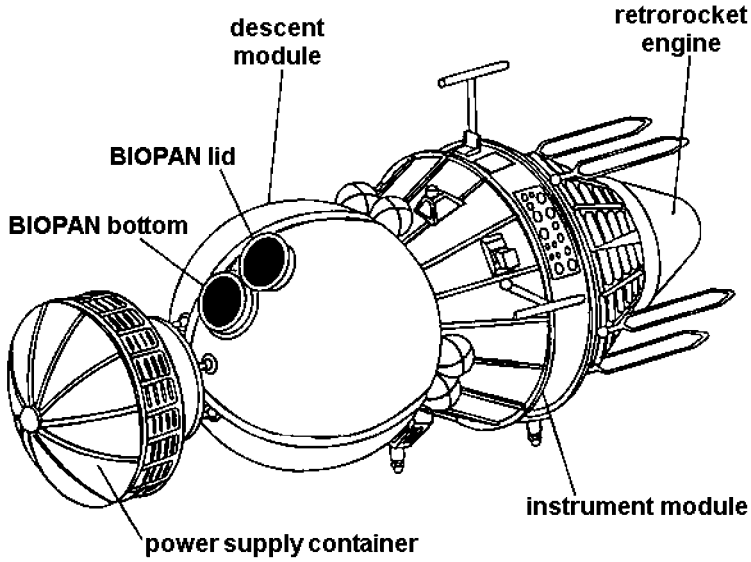

Figure 2. In-flight configuration of the FOTON satellite with the opened facility BIOPAN mounted on the descent module.

which after drying was peeled off the quartz disc together with the spores and resuspended in 1 mL of distilled water.

The colony forming ability was determined by plating diluted spore suspensions on nutrient broth (NB, Difco) agar plates. Colonies were scored after 16 hr of incubation at 37 °C. Surviving fractions were determined as N/N_0 where N was the number of colony formers of the treated samples and N_0 the number of colony formers of the untreated controls.

2.2. EXPOSURE TO SPACE ENVIRONMENT

2.2.1. *Flight Hardware*

The experiment 'SURVIVAL' was accommodated in the lid of the BIOPAN facility of ESA. BIOPAN is a cylindrical, pan-shaped container with a deployable lid, mounted on the outer surface of the sphere-shaped descent module of the Russian FOTON satellite (Figure 2). After attaining the proper orbit, the lid opens through 180°, exposing the experiments accommodated in the bottom and lid to space. To monitor the exposure conditions, BIOPAN is equipped with a built-in solar sensor, a UV-sensor and several temperature sensors. During ascent and reentry the lid is closed and the BIOPAN is hermetically sealed and covered by an ablative heat shield; opening and sealing is initiated by telecommand from the ground (Burger, 1995).

Each sample of the experiment 'SURVIVAL' was accommodated in a hexagonal aluminum sample carrier (height 8 mm, width 17 mm, internal volume approximately 1 cm^3) with a circular quartz window 'Suprasil' (diameter 12.9 mm). The

sample carriers were vented through holes (diameter 1.5 mm) in 4 of their side walls which opened into a channel system. The sample carrier's floor was covered by a teflon disc, the inner side walls were covered by a cylindrical membrane (Du Pont US; pore size 80 nm) to restrain the powder and spores. The teflon disc, the membrane and the quartz window were fixed to the sample carrier by use of Wacker silicon rubber (Delonge, 1993).

Two sets of 61 sample containers were mounted on two aluminum plates, one facing space (sun-exposed tray), and the other one looking inside BIOPAN (dark tray). The sample carriers were positioned by plugging them over small bars and fixed by use of an aluminum cover plate with holes (diameter 12 mm) over each quartz window (Figure 3). To measure to the dose of cosmic radiation, thermoluminscence dosimeters (TLD) were attached to several sample carriers.

2.2.2. *Space Flight Protocol*

Three BIOPAN missions took place on board of a FOTON satellite (Figure 2), in 1994, 1997 and 1999. A typical mission lasted for 2 to 3 weeks with exposure times (lid open) between 10 and 15 days (Table I). During each 90 min orbit the satellite was about 30 min in the Earth's shadow and 60 min in the sun, resulting in temperature fluctuations of about 10 °C per orbit (Figure 4). Because FOTON is a spinning satellite, the samples were arbitrarily insolated for short intervals (minutes) depending on the orientation of the satellite (Figure 4). The total temperature and insolation profile of a typical mission (BIOPAN 3) is shown in Figure 5.

The samples were prepared and integrated in the SURVIVAL experiment hardware about 4 weeks before flight at DLR and then transported to ESA's laboratory MOSLAB in Moscow (BIOPAN 1) or to ESA's technical Center ESTEC in Noordwijk (BIOPAN 2 and 3), where SURVIVAL was integrated in the BIOPAN lid and stored at room temperature. The integrated BIOPAN was transported to the launch site Plesetks in Russia 3–5 days before the launch. Ten min after launch, BIOPAN was evacuated. After about 20 hr in orbit, the lid was opened to expose the samples to space. It was closed about 1 day before landing in the Orsk district, Kazakhstan. BIOPAN was de-integrated at the landing site about 2 hr later and transported back to ESTEC for experiment de-integration. Before the flight, BIOPAN was filled with dry N_2 at atmospheric pressure. After retrieval, BIOPAN maintained the vacuum until it was flushed with dry nitrogen at ESTEC. Temperature was recorded during all ground and space activities.

2.3. GROUND CONTROLS

As ground controls the following identical sets of samples was prepared in parallel: (i) 'Lab control (before)', a laboratory control analyzed one day after sample preparation; (ii) 'Lab control (after)', a laboratory control which was kept DLR in the dark under 1 ATM of air at room temperature for the whole mission period and was

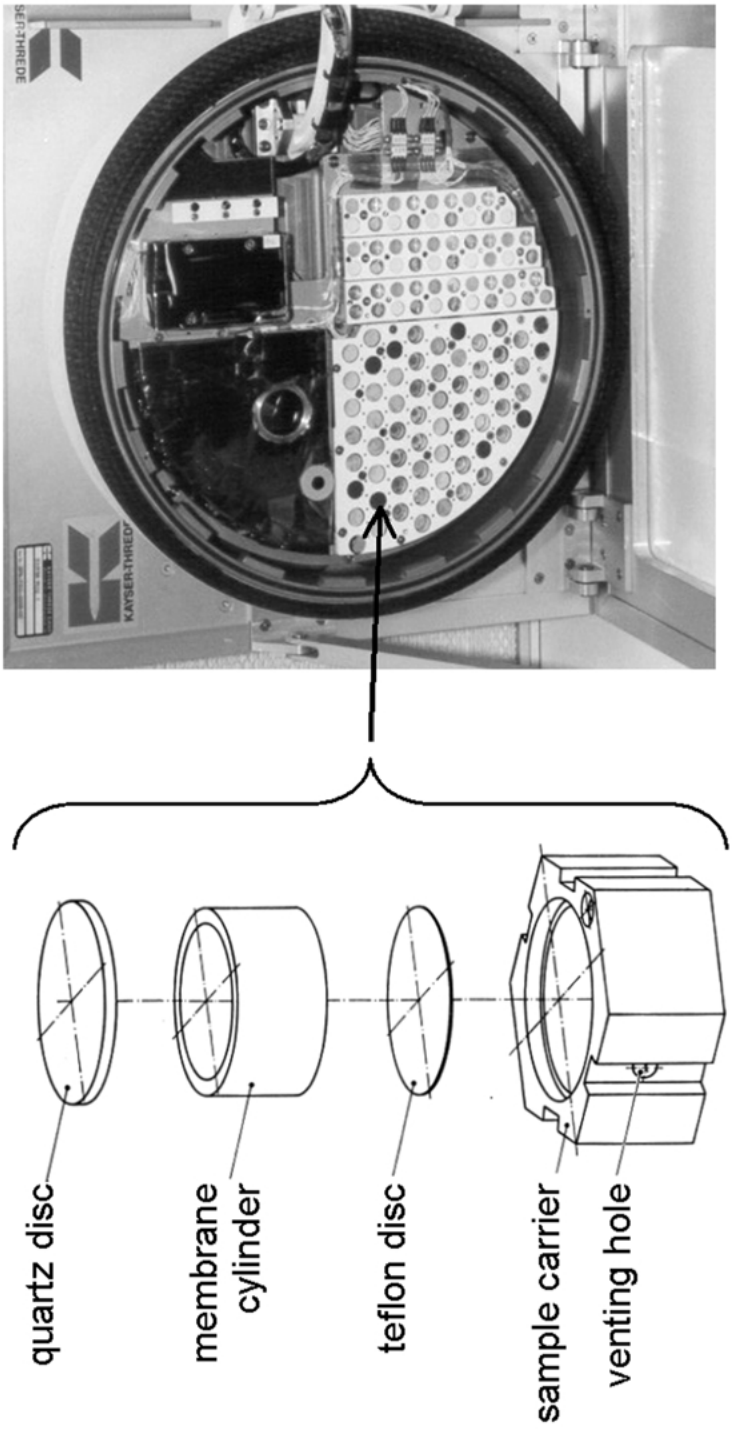

Figure 3. Hardware of experiment 'SURVIVAL' accommodated inside the BIOPAN facility of ESA and exploded view of a sample carrier.

TABLE I

Environmental data of the SURVIVAL experiment during the BIOPAN 1 through 3 space flights

BIOPAN No.	Date Day/month/year	Duration of exposure d	Solar radiation s.c.h.[a]	Solar radiation kJ m^{-2}	Solar UV >170 nm kJ m^{-2}	Cosmic radiation mGy	Temperature range (°C)
1	29/07–17/08/1994	14.81	39.3	192 413	17 317	74.0[b] 5.9[c]	–20–+12
2	09/10–23/10/1997	9.95	27.3	133 661	12 030	29.9[b] 4.0[c]	–38–+10
3	09/09–24/09/1999	12.66	26.1	127 786	11 501	28.2±0.6[b] 4.5±0.1[c]	–17–+15

[a] s.c.h. = Solar constant hours.
[b] Flight unit, sun-exposed (upper layer).
[c] Flight unit, dark (bottom layer).

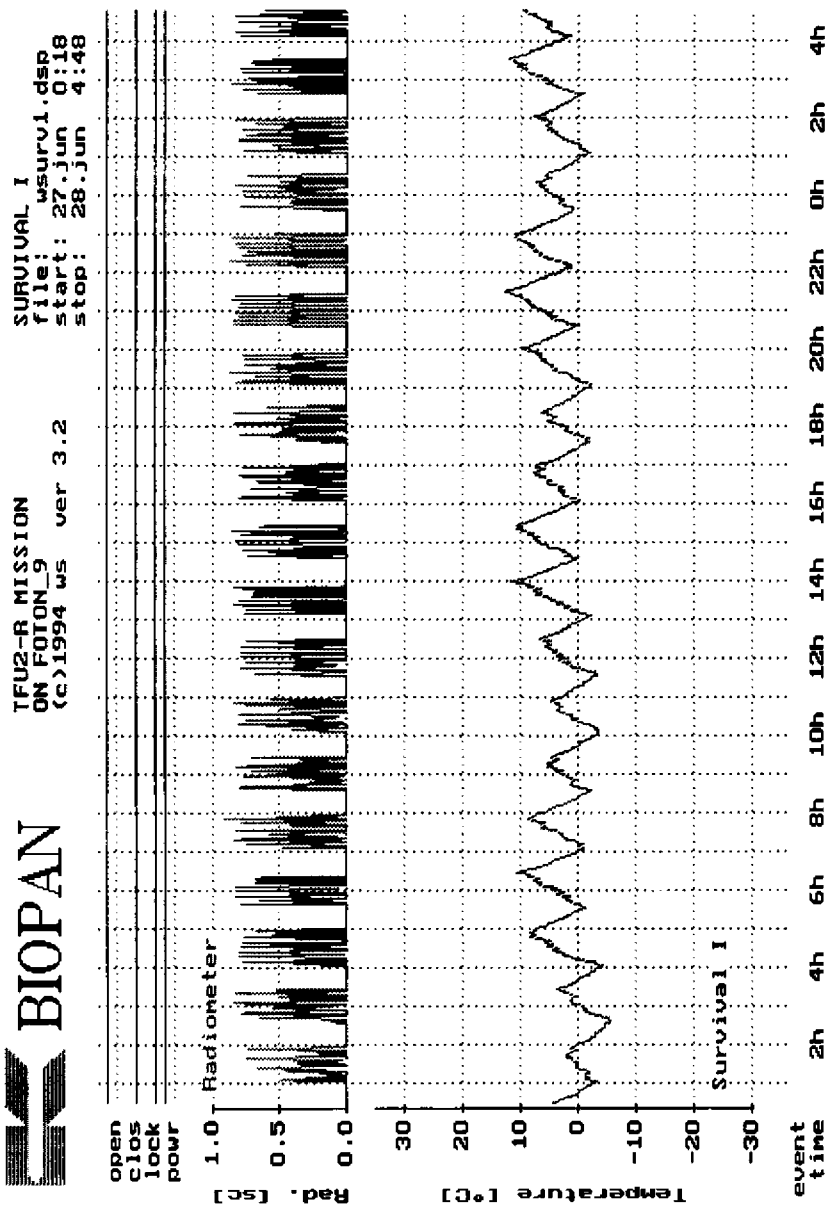

Figure 4. Typical temperature and insolation profile of the SURVIVAL experiment during one day of the BIOPAN 1 mission (s.c. = solar constant).

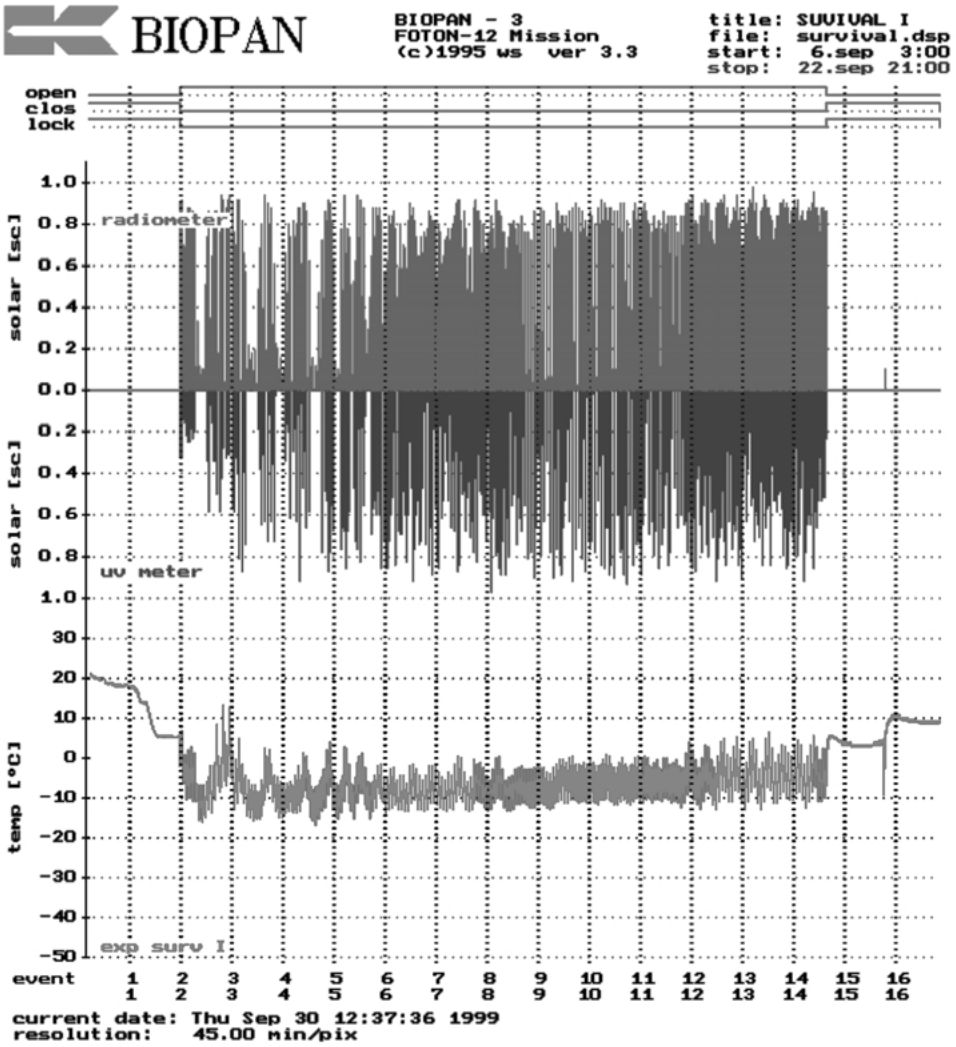

Figure 5. Temperature and insolation profile of the SURVIVAL experiment during the whole BIOPAN 3 mission.

analyzed simultaneously with the flight samples; (ii) 'Ground control (vacuum)', a ground control, kept in the dark under 1 atm of air, which was transported with the flight samples to the launch site and back. Using the space simulation facilities at DLR, these samples were also exposed to vacuum for time periods corresponding to the actual flight time.

3. Results

3.1. Survival of unprotected bacterial spores

During the BIOPAN 1 flight, for the first time bacterial spores were exposed to space without any shielding or protection (Table II). In the 'outside layers' dry layers of spores of *B. subtilis* HA 101 and *B. subtilis* TKJ 6312 were mounted on that side of the quartz windows of the sample carriers that was facing space. Besides space vacuum, they received the full spectrum of extraterrestrial UV-radiation including the highly energetic vacuum-UV at a total fluence of about 17 MJ m^{-2} which is equivalent to 40 hr insolation at the distance of the Earth. They were also exposed without any shielding to cosmic radiation with a dose rate of approximately 5 Gy d^{-1}. This value was determined by Reitz *et al.* (1993) during the BIOPAN test flight using TLDs. Hence, after nearly 15 days in space, the spores had received a total dose of 75 Gy. This dose is not very dramatic, because *B. subtilis* spores are extremely resistant to ionizing radiation with a D_{10} value (dose, causing 10% survival) of about 1500 Gy. From an initial number of about 7×10^7 viable spores per sample of strain HA 101 about 1×10^{-6} spores survived the space journey (actual viable count per sample: 45, 60, 120) tested as colony forming ability. A similar surviving fraction was obtained for spores of strain TKJ 6312 (actual viable count per sample: 65, 78, 110).

A similar low fraction of survivors (10^{-6} or less) was obtained for those spores in dry layers, which were exposed inside the sample carrier beneath a quartz window which is transparent for UV at wavelengths of $\lambda > 170$ nm (Table II). These samples received nearly the same UV dose as the outside samples, because the irradiance of extraterrestrial UV for wavelengths at $\lambda < 170$ nm is about 4 orders of magnitude lower than that e.g., in the range of 260 nm, which is especially genotoxic (Horneck *et al.*, 1995). In 2 samples of strain HA 101 as well as TKJ 6312 not a single spore survived (Table II).

It is interesting to note that the dark flight samples survived very well: between 50 and 97% viable spores were recovered from those samples, which were exposed to all parameters of space except UV radiation (Table II). This survival rate coincides with that of the laboratory controls, stored at room temperature for the whole period, as well as of the ground controls, which in addition were exposed to vacuum of 3×10^{-5} Pa for 15 days (BIOPAN 1) or of 1×10^{-3} Pa for 21 days (BIOPAN 3), respectively.

From the very low – if at all – survival of the sun-exposed spores in both samples, 'outside layer' and 'inside layer', it can be concluded that solar UV radiation at wavelengths of $\lambda > 170$ nm is extremely efficient in killing the spores. Because there was no difference in the responses of spores from the three strains which differ in their repair capacities, the damage seems to be so severe that the functioning DNA repair systems were of no further help. Therefore, during the following BIOPAN missions (2 and 3) only spores of the DNA repair proficient

TABLE II

Survival of *B. subtilis* spores in 'outside layers', 'inside layers' or 'shadowed layers' after exposure to space during the BIOPAN 1 and 3 missions (mean values of 3 parallel samples)

BIOPAN No.	Bacterial strain	Colony formers N					Survival N/N_0	
		Lab control (before)[a]	Lab control (after)	Ground control (vacuum)	Flight unit (dark)	Flight unit (sun exposed)	Flight unit (dark)	Flight unit (sun exposed)
Outside layer								
1	HA 101	$(6.8\pm0.8)\times10^7$	$(4.7\pm0.5)\times10^7$	$(2.7\pm0.7)\times10^7$	No sample	$(7.5\pm4.0)\times10^1$		$(1.1\pm0.6)\times10^{-6}$
1	TKJ6312	$(5.8\pm0.5)\times10^7$	$(4.7\pm1.0)\times10^7$	$(3.7\pm0.3)\times10^7$	No sample	$(8.4\pm2.3)\times10^1$		$(1.4\pm0.4)\times10^{-6}$
Inside layer beneath quartz window								
1	HA 101	$(6.8\pm0.8)\times10^7$	$(4.7\pm0.5)\times10^7$	$(4.3\pm0.4)\times10^7$	$(3.4\pm1.0)\times10^7$	$(5.2\pm3.7)\times10^1$	$(5.0\pm1.5)\times10^{-1}$	$(7.6\pm5.4)\times10^{-7}$
3	HA 101	$(7.8\pm0.2)\times10^7$	$(7.9\pm0.8)\times10^7$	$(5.8\pm0.7)\times10^7$	$(7.6\pm0.5)\times10^7$	$(3.5\pm4.8)\times10^4$	$(9.7\pm0.6)\times10^{-1}$	$(4.5\pm6.2)\times10^{-4}$
						0 (2 samples)		$<10^{-8}$
1	HA F	$(6.8\pm0.5)\times10^7$	$(5.2\pm0.8)\times10^7$	$(3.2\pm0.3)\times10^7$	$(4.1\pm0.4)\times10^7$	$(3.9\pm1.8)\times10^1$	$(6.0\pm0.6)\times10^{-1}$	$(5.7\pm2.6)\times10^{-7}$
1	TKJ6312	$(5.8\pm0.5)\times10^7$	$(4.7\pm1.0)\times10^7$	$(3.7\pm0.7)\times10^7$	$(3.9\pm1.3)\times10^7$	3.1×10^1	$(6.7\pm2.2)\times10^{-1}$	5.3×10^{-7}
						0 (2 samples)		$<10^{-8}$
Shadowed layer beneath a layer of clay plus quartz window								
1	HA 101	$(6.8\pm0.8)\times10^7$	$(4.7\pm0.5)\times10^7$	$(4.6\pm0.4)\times10^7$	$5.5\times10^{7)}$	0	8.0×10^{-1}	$<10^{-8}$
1	HA F	$(6.8\pm0.5)\times10^7$	$(5.2\pm0.8)\times10^7$	$(3.1\pm0.7)\times10^7$	$3.6\times10^{7)}$	0	5.3×10^{-1}	$<10^{-8}$
1	TKJ6312	$(5.8\pm0.5)\times10^7$	$(4.7\pm1.0)\times10^7$	$(3.3\pm1.0)\times10^7$	No sample	$(2.4\pm0.1)\times10^1$		$(4.1\pm0.2)\times10^{-7}$

[a] Taken as untreated control N_0.

[b] The quartz window of this sample carrier which was planned as 'Flight unit sun-exposed' was broken before the flight and fixed again by gluing the cracks with an opaque glue which covered the whole window; therefore, sunlight did not reach this sample.

strain HA 101 were used. The few survivors may be the 'lucky winners' located within clumps of spores where they might be shadowed by the upper layers of dead spores. Marker tests showed that the few colony formers (between 30 and 120 per sample) belonged to the original strains and were free from other bacterial contamination.

3.2. Shielding against extraterrestrial solar UV radiation by dust or soil

3.2.1. *Filtering Sunlight through a Thin Layer of Clay*

If solar UV radiation is the limiting factor in space for spores to survive, then a thin layer of dust or soil might provide sufficient shielding against the harmful extraterrestrial UV radiation. To test this assumption, layers of spores at the bottom of the sample carrier were exposed to space beneath a thin layer of clay that covered the inner side of the quartz windows. This clay layer was opaque for UV and visible light. Table II shows that in these 'shadowed layers' none of the flight spores of strain HA 101 and HA F survived these conditions, and only a few spores of strain TKJ 6312 (actual viable count per sample: 25, 28, 25), whereas the survival rates of the flight dark controls were with 53 to 80% in the same range as those of the lab and ground controls. Hence, the filter of clay did not protect the spores against solar UV radiation, in some cases it made the situation even worse. This unexpected lack of protection by the clay layer may be caused either by microscopic cracks in the clay layer which occurred during the mission and which allowed solar UV to reach the samples, or by toxic volatiles which were photochemically produced in the clay during insolation. To test these two alternatives, in the follow-on mission, the spores were brought in direct contact with clay when exposed to space.

3.2.2. *Mixing Bacterial Spores with Protective Substances*

Dry layers were prepared from a suspension of spores with 100 mg mL^{-1} powdered clay (20 μL per sample) and exposed to space during the BIOPAN 2 mission. A much better survival (by more than 5 orders of magnitude) was achieved, if the spores were exposed in these 'mixed layers', i.e. directly in contact with clay (Table III), than if exposed in 'shadowed layers' under a filter of clay (Table II). These data suggest that clay protects the spores against the harmful solar UV to a certain extent, and that cracks in the clay filter were probably the reason for the complete killing of the spores in the 'shadowed layers'. A likewise good protection (survival rates within the same order of magnitude) was obtained in 'mixed layers' prepared from a suspension of spores with 100 mg mL^{-1} powdered red sandstone, or with 10 mg mL^{-1} powdered meteorites (Millbillillie, Zagami) or simulated Martian soil (MRTE) (Table III). These concentrations of powdered rock, soil or meteorite were selected before the flight, because they allowed stabile 'mixed layers'; higher concentrations caused the layer to crumble away from the quartz support. Probably the protection by these substances is caused by a certain

degree of shielding against solar UV for spores directly attached to the grains (on the average, each grain was about 100 times larger than a single spore). None of the substances was toxic to the spores when kept in the dark or in vacuum as demonstrated by the nearly 100% survival of the flight dark samples as well as the lab and ground controls (Table III). For comparison, the survival of spores in layers mixed with 5% glucose is shown in Table III. The protective potential of glucose for spores in space has been found in previous long-term space missions (Horneck *et al*., 1994, 1995), when up to 10^{-4} spores survived after an insolation with about 1 GJ m^{-2} during the six years lasting LDEF flight. In the latter case the spores were exposed in thick layers (>10^8 spores/sample) with 5% glucose. In the BIOPAN experiments, in all but one case (Millbillillie), spores mixed with soil or meteorite powder survived better than those mixed with glucose.

3.2.3. *Bacterial Spores in 'Artificial Meteorites'*
Terrestrial soil contains roughly up to 10^8 microorganisms per gram. In order to have a comparable ratio of soil to microorganisms, 0.28 g of powdered clay or red sandstone was thoroughly mixed with about 5×10^7 spores (BIOPAN 1) or 3×10^7 spores (BIOPAN 2) and filled in a sample carrier covered by a quartz window. In these 'artificial meteorites', the spores were so well protected against solar UV, that similar survival rates were obtained for all flight samples, whether they were kept in the dark or sun-exposed. After the BIOPAN 2 flight, 100% viable spores were recovered from both, mixtures with clay or with red sandstone. The lower survival rate (12–15%) after the BIOPAN 1 mission was also observed for all dark samples, in-flight as well as lab and ground controls. One possible explanation is that during resuspension several spores may stick to each grain thereby giving rise to only one colony.

4. Discussion

During a hypothetical interplanetary transfer, organisms have to cope with the following three major steps: (1) the escape process, (2) the long-duration exposure to space, and (3) the entering process. In shock recovery experiments using spores of *B. subtilis* and shock pressures of 32 GPa which are in the range experienced by the Martian meteorites, it has been shown that bacterial spores may survive an impact-induced escape process in a scenario of interplanetary transfer of life (Horneck *et al*., 2001a).

Once rocks are ejected from the planet by an impact scenario, they face the hostile environment of space which is characterized by a high vacuum, an intense radiation climate and extreme temperatures (Horneck and Brack, 1992). This extreme environment of space obviously does not support active metabolism and growth of the microorganisms enclosed; however, a variety of organisms exist that are adapted to survive in extreme conditions when in the dormant state, such as

TABLE III

Survival of *B. subtilis* HA 101 spores in 'mixed layers' or 'artificial meteorites' after exposure to space during the BIOPAN 1, 2, and 3 missions (mean value of 3 parallel samples with standard deviation)

BIOPAN No.	Protective substance	Bacterial strain	Colony formers N					Survival N/N_0	
			Lab control (before)[a]	Lab control (after)	Ground control (vacuum)	Flight unit (dark)	Flight unit (sun exposed)	Flight unit (dark)	Flight unit (sun exposed)
Mixed layer									
2	Clay	HA 101	$(2.9\pm0.1)\times10^7$	$(1.6\pm0.4)\times10^7$	$(1.6\pm0.4)\times10^7$	$(2.2\pm0.6)\times10^7$	$(2.2\pm1.5)\times10^5$	$(7.6\pm2.1)\times10^{-1}$	$(7.5\pm5.1)\times10^{-3}$
2	Red sandstone	HA 101	$(3.0\pm0.3)\times10^7$	$(2.1\pm0.3)\times10^7$	$(1.3\pm0.4)\times10^7$	$(2.3\pm0.6)\times10^7$	$(5.1\pm2.3)\times10^4$	$(7.7\pm2.0)\times10^{-1}$	$(1.7\pm0.3)\times10^{-3}$
3	Millbillillie	HA 101	$(6.9\pm0.4)\times10^7$	$(7.1\pm0.2)\times10^7$	$(5.6\pm0.7)\times10^7$	$(6.9\pm0.2)\times10^7$	$(3.6\pm4.1)\times10^4$	1.0 ± 0.1	$(5.2\pm5.9)\times10^{-4}$
3	MRTE	HA 101	$(7.1\pm0.3)\times10^7$	$(7.7\pm0.7)\times10^7$	$(6.2\pm0.4)\times10^7$	$(6.6\pm0.5)\times10^7$	$(7.1\pm4.5)\times10^4$	$(9.3\pm0.1)\times10^{-1}$	$(1.0\pm0.6)\times10^{-3}$
3	Zagami	HA 101	$(7.5\pm0.7)\times10^7$	$(7.7\pm0.7)\times10^7$	$(5.4\pm1.2)\times10^7$	$(7.0\pm0.3)\times10^7$	$(7.9\pm5.5)\times10^4$	$(9.3\pm0.4)\times10^{-1}$	$(1.1\pm0.7)\times10^{-3}$
2	Glucose	HA 101	$(3.8\pm0.2)\times10^7$	$(3.9\pm0.2)\times10^7$	$(2.8\pm0.7)\times10^7$	$(3.9\pm0.1)\times10^7$	3.4×10^3	1.0 ± 0.1	8.9×10^{-5}
							0 (2 samples)		$<10^{-8}$
3	Glucose	HA 101	$(7.3\pm0.6)\times10^7$	$(7.6\pm1.6)\times10^7$	$(6.6\pm1.0)\times10^7$	$(8.6\pm0.7)\times10^7$	$(5.0\pm2.9)\times10^4$	1.2 ± 0.1	$(6.8\pm1.6)\times10^{-4}$
Artificial meteorite									
1	Clay	HA 101	$(4.5\pm0.1)\times10^7$	$(6.4\pm0.8)\times10^6$	$(8.5\pm2.7)\times10^6$	$(8.8\pm3.1)\times10^6$	$(5.6\pm0.2)\times10^6$	$(2.0\pm0.7)\times10^{-1}$	$(1.2\pm0.1)\times10^{-1}$
1	Clay	HA F	$(4.6\pm0.3)\times10^7$	$(7.7\pm1.6)\times10^6$	$(6.6\pm1.3)\times10^6$	$(6.2\pm0.6)\times10^6$	$(5.6\pm0.6)\times10^6$	$(1.3\pm0.1)\times10^{-1}$	$(1.2\pm0.1)\times10^{-1}$
1	Clay	TKJ6312	$(5.5\pm1.8)\times10^7$	$(7.1\pm0.8)\times10^6$	$(7.9\pm1.7)\times10^6$	$(1.1\pm0.2)\times10^7$	$(8.2\pm1.6)\times10^6$	$(2.0\pm0.4)\times10^{-1}$	$(1.5\pm0.3)\times10^{-1}$
2	Clay	HA 101	$(3.0\pm0.2)\times10^7$	$(3.4\pm0.2)\times10^7$	$(3.8\pm0.4)\times10^7$	$(3.6\pm0.4)\times10^7$	$(3.9\pm0.2)\times10^7$	1.2 ± 0.1	1.3 ± 0.1
2	Red sandstone	HA 101	$(3.0\pm0.3)\times10^7$	$(3.2\pm0.4)\times10^7$	$(2.7\pm0.4)\times10^7$	$(2.9\pm0.2)\times10^7$	$(2.9\pm0.5)\times10^7$	$(9.7\pm0.9)\times10^{-1}$	$(9.7\pm1.7)\times10^{-1}$

[a] Taken as untreated control N_0.

bacterial endospores. A crucial question is how the meteorite itself could protect spores against the most harmful parameters of space, above all cosmic and solar radiation.

Concerning shielding against galactic cosmic radiation effects, Mileikowsky *et al*. (2000) have calculated that behind 1 m of meteorite material a substantial fraction of a spore population (10^{-6}) would survive the exposure to cosmic radiation for about 1 million years. The same surviving fraction would be reached after about 600 000 yr without any shielding, after about 300 000 yr behind 10 cm of shielding (maximum dose rate because of secondary radiation) and after 25 million years behind 2 to 3 m of shielding. These calculations are based on a series of experiments on the survival of *B. subtilis* spores exposed to accelerated heavy ions of different mass and energy (Baltschukat and Horneck, 1991). Based on the assumption that a dose of 10–50 kGy is sufficient for complete sterilization – which is given as a typical value for food sterilization (Clark *et al*., 1999), Clark (2001) comes to less optimistic conclusions: he has calculated that after 1 million years in space the outer shell of a meteorite of a thickness of 1 m will be completely sterilized. The same effect will be reached for the outer 3 cm layer within 100 000 yr in space and for 1–3 mm within 10 000 yr. Both calculations are hampered by the fact that they are based on laboratory experiments using irradiations at high dose rates (several Gy or kGy min^{-1}) applied within minutes or hours, whereas the interplanetary travelers have to be confronted to dose rates in the order of 5–250 mGy/a. The discrepancy in these two calculations demonstrates the difficulty to extrapolate from short-term experimental results to a long-term scenario of thousands or millions of years.

Mileikowsky *et al*. (2000) also assume that a few μm of meteorite material would be sufficient to give enough protection against UV if the material is without cracks, a few mm would be required to shield against diffuse X-rays, and about 10 cm against solar particles. Our space studies on the BIOPAN facility provide experimental data on the efficiency of protection against solar UV radiation by the uppermost layers of a meteorite in the μm to mm range. In previous experiments on Spacelab 1 we had shown that a UV dose of 200 kJ m^{-2} at $\lambda > 170$ nm reduced the survival rate of unprotected *B. subtilis* spores by three orders of magnitude (Horneck, 1993). After about 2 weeks in space, the unprotected spores in the BIOPAN facility had received a hundred-fold higher UV dose than during the Spacelab 1 mission. Extrapolation of the survival curves to these high dose values would expect a reduction of the survival rate by more than 15 orders of magnitude. This means, all spores should be dead. Although there was a large scattering between the colony formers of parallel samples, in some cases up to 10 000 spores survived full exposure to the sun (survival rates ranged between 10^{-4} and $<10^{-8}$). Probably, these survivors were located within clumps of spores where they might have been shadowed by the upper layers of dead spores. On the other hand, a thin layer of clay did not protect the spores at all, if it was placed at some distance (about 5 mm) from the spore layer. Probably, tiny cracks in the

clay layer allowed solar UV to reach the spore layer. A certain degree of protection could be reached by mixing the spores in the layer directly with clay or other rock or meteorite material (survival rates ranged between 10^{-3} and 10^{-4}). These results are consistent with observations during the PERSEUS mission on the MIR space station, where spores mixed with similar meteorite material were exposed to space for more than 3 months (Rettberg *et al*., 2001). Probably, soil or rock grains served as a shield against UV for those spores that were located beneath them. Maximum protection was achieved, if the spores were exposed to solar UV within a mixture of clay, rock or meteorite powder in a similar ratio as occurring in terrestrial soil. Within a column of 5 mm, sunlight was attenuated so efficiently that the same high survival rates were observed for both, dark and sun-exposed flight samples.

The BIOPAN results show that bacterial spores can be protected against solar UV radiation, if embedded in clay or rock material in a ratio soil/spores comparable to that normally observed in terrestrial soils. It has further been shown by Franchi *et al*. (1999) that the formation of clay-nucleic acid complexes leads to the preservation of the genetic material in critical conditions, above all UV radiation (Vettori *et al*., 2000). In such naturally occurring mixtures or complexes, even small meteorites, e.g. of a few cm in diameter, could be an appropriate vehicle for interplanetary transfer of life. Because such small rock ejecta with radii between 2 and 80 cm are by 3 orders of magnitude more frequent than very large boulders of 2 m or more in radius (Mileikowsky *et al*., 2000) these results increase the chances for viable transfer of life within our solar system. However, single spores or spores attached to micron-sized grains, as requested by the theory of Panspermia, will not survive in space.

In most space experiments, the exposure time of *B. subtilis* spores to this hostile environment was relatively short: in most cases 1–2 weeks, with the exception of 3 months for PERSEUS on MIR (Rettberg *et al*., 2001), 9 months for EURECA (Horneck *et al*., 1995), and 6 yr for LDEF (Horneck *et al*., 1994). The LDEF results are the first experimental proof that a very high percentage of spores survive at least 6 yr in space, if efficiently shielded against solar UV radiation. As shown in this article, the shielding could be achieved by the outer layers of rocks as used by those microbial communities that inhabit rocks. However, calculations expect thousands or millions of years to be needed for most ejecta before reaching another planet (Mileikowsky *et al*., 2000; Clark, 2001). However, Gladman (1997) has estimated that with very low probability (10^{-7}) fragments from Mars may reach the Earth within one year or even less. For these rare cases, the 6 years may already be a realistic time frame for an interplanetary transfer of life.

The results are also applicable for the discussion of the toxicity of the Martian regolith, because one of the minerals used (MRTE) served as simulated Martian soil and one was a Martian meteorite (Zagami). In layers mixed with MRTE or Zagami the spores survived sun exposure with a similar rate that in those layers mixed with clay or red sandstone. There was no indication of a photochemical production of toxic volatiles in the Martian analogue of meteorite, when exposed

to extraterrestrial UV radiation at $\lambda > 170$ nm, at least not within the short period of the BIOPAN missions. On the contrary, the Martian regolith may shield potential microbial inhabitants from solar UV. Mancinelli and Klovstad (2000) have shown that 1 mm of Martian analogue soil completely protects *B. subtilis* spores from UV radiation at doses comparable to those experienced during the BIOPAN flights, whereas unprotected spores were killed by 100%. These findings are also important for planetary protection considerations, because potential terrestrial contaminants on the surface of spacecrafts could survive on the surface of Mars under a thin layer of Martian dust.

For future research on bacterial spores and other microorganisms in space, the European Space Agency ESA is developing the EXPOSE facility that is to be attached to the External Pallet of the truss structure of the International Space Station for 1.5 yr during the early utilization period of the Space Station. EXPOSE will support long-term *in situ* studies of microbes in artificial meteorites, as well as of microbial communities from special ecological niches, such as endolithic and endoevaporitic ecosystems (Horneck *et al*., 1999). These experiments on the Responses of Organisms to the Space Environment (ROSE) include the study of photobiological processes in simulated radiation climates of planets (e.g. early Earth, early and present Mars, and the role of the ozone layer in protecting the biosphere from harmful UV-B radiation), as well as studies of the probabilities and limitations for life to be distributed beyond its planet of origin. Here-to-fore, the results from the space experiments on BIOPAN or EXPOSE will eventually provide clues to a better understanding of the processes regulating the interactions of life with its environment.

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