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Survival of bacterial and fungal spores in the expose-r2 experiment

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Abstract: The primary goal of the Biodiversity experiment, which was part of the Expose-R2 study, was to assess the effects of a prolonged exposure to the space environment on the survival of dormant microorganisms. To do this, units containing samples of bacterial and fungal spores were mounted on a support platform attached to the exterior wall of the Russian Zvezda Module of the International Space Station (ISS). After a 15-mon exposure to outer space the samples were returned to Earth. The experiment demonstrated that microbial spores had the capability to survive and remain viable after the exposure to outer space for the time period comparable to the Earth-Mars-Earth mission duration. This finding is not only of theoretical importance but also of practical significance as related to the development of adequate measures of planetary protection for deep space missions.

Keywords: Dormant forms of various microorganisms, Outer space, Planetary quarantine

Introduction

The Biodiversity study performed by the Institute of Biomedical Problems, Russian Academy of Sciences (IMBP) in cooperation with Roscosmos was part of the Expose-R2 experiment implemented by the European Space Agency (ESA).

The purpose of the Biodiversity experiment was to evaluate the effects of an extended (from 12 to 18 mon) exposure to the space environment on the survival of dormant organisms at different stages of evolutionary development (bacteria, fungi, animals and plants).

The search for extra-terrestrial life forms or their precursors made possible by the use of free flying spacecraft has recently become part and parcel of space exploration programs of many countries. These efforts are associated with certain risks related to the potential transfer of biological matter across interplanetary space because unmanned and manned vehicles may carry on their exterior surfaces millions of microbial cells whose spores may be highly resistant to the deleterious effects of the space environment.

In this context it is highly important to focus on the protection of planets of the solar system to which space probes are sent and the Earth itself as well as on the survival capabilities of various forms of biological matter. In the course of evolution not only microbes but also many species of multi-cellular organisms have developed the ability to transform into dormant stages allowing them to survive hostile environmental effects. Some specimens can remain dormant for long periods of time varying from several months to hundreds or thousands of years [1]. With the purpose of investigating the survival of dormant organisms from different taxonomic groups, Russian scientists carried out Biorisk and Expose-R experiments in which dormant forms of bacteria, fungi, plants and animals were exposed to the space environment for an extended time period. The Biorisk experiment demonstrated [2,3] that not only microbial spores but also dormant forms of other organisms at higher evolutionary stages of development (higher plant seeds, mosquito larvae, lower crustacean eggs) remained viable after a prolonged exposure (up to 33 mon) to the space environment.

The Expose-R experiment revealed the deleterious effects of cosmic ultraviolet (UV) radiation (at a wavelength of >200 nm) on the dormant forms of various biological specimens exposed on the exterior walls of the Russian Segment of the ISS for 22 mon: from March 2009 to January 2011 [4]. Microbial spores, higher plant seeds, dried crustacean embryos survived the exposure when they were shielded from and died when exposed to UV radiation. However, fungal spores (*Aspergillus sydowii, Aspergillus versicolor*) and mosquito larvae (*Polypedilum vanderplanktii*) remained viable after the exposure. This can be attributed either to their higher resistance or to the fact that the survived specimens were shielded from UV radiation by other spores and larvae.

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In contrast to the Expose-R experiment, the Expose-R2 experiment was performed using "windows" covered with filters that significantly reduced UV radiation intensity. This setting allowed the study of survival of dormant forms of various organisms exposed to lower UV radiation doses. Another difference was that in the Expose-R2 experiment some dormant forms were exposed in the gas mixture simulating the atmosphere of Mars.

METHODS

The Expose-R2 hardware was developed and manufactured by Kayser-Threde GmbH (Germany). It is a metal box containing three trays (Fig. 1), two of which carry four compartments and one-three compartments. They are covered by MgF2 "windows" (Fig. 2).

The trays are closed with top windows and sealed by O-rings. The compartments in each tray are connected by a gas exchange line sealed by a valve, which opens when the unit is installed on the exterior surface of the ISS. Each tray contains three microbial sample layers (exposed in the light and in the dark) of different geometry (16 to 64 wells in a layer) and is equipped with covers made of various types of glass, filters and dosimeters.

According to the experimental design, dormant forms of microbial samples were exposed either to open space per se or to the simulated Mars atmosphere (95.55% CO_2, 2.70% N_2, 1.60% Ar, and 0.15% O_2). The sample containing compartments were covered with filters that shielded them from UV radiation of >200 nm. A ground control experiment was run with a 2-mon delay that allowed a precise simulation of spaceflight effects.

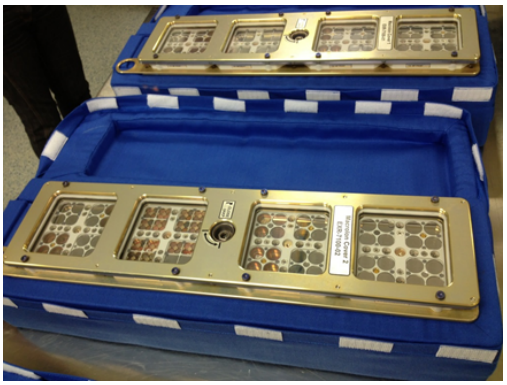

Figure 1: General view of trays (top–tray 1 and bottom–tray 2).

Figure 2: General view of bio-samples in a tray of the Russian experiment.

The samples for the flight Expose-R2 experiment were selected after experiment verification tests (EVT-1, EVT-2) and Sequence Verification Test (SVT) that simulated various environmental effects, including the Martian atmosphere.

Bacterial spores were packed in small (7 mm x 7mm or 10 mm x 10 mm) plastic bags. *Bacillus licheniformis* (strain *B. licheniformis*-24) and *Bacillus pumilus* (strain *B. pumilus*-25) spores were accumulated on filter paper and enclosed in the bags. The bacterial strains selected for the Expose-R2 experiment were previously isolated from the ISS interior modules. The strains typically showed greater resistance to various environmental effects and were therefore of particular interest.

Microbial samples were exposed to the entire spectrum of solar UF radiation and adequate filters blocking portions of the spectrum, which allowed a study of the role the ozone layer plays in protecting our biosphere as well as the survival probability of extremophiles in space. In order to better understand habitability of Mars, some samples were exposed to the effects mimicking the Martian environment (pressure, atmosphere, UV radiation) using simulated Martian soil with and without a protective cover.

Unlike the situation with bacterial spores, dried fungal spores used in verification tests as well as in flight and ground control experiments were loaded directly into plastic bags of 7 mm x 7 mm or 10 mm x 10 mm in size.

Dried fungal spores were placed into the bags by means of a sterile loop, the open side being sealed ultrasonically. To ensure that the spores were of a specific species, their cultural and morphological properties were analyzed. After that each culture was grown in Petri dishes containing Czapek medium at 28°C for 14 days that allowed maximum spore-forming activity. Dried spores of each culture were collected into a sterile plastic container. Spores of different fungal species were collected on different days to exclude the possibility of cross-culture contamination.

Fungal spore containing bags were loaded into pre-assigned compartments of the tray. Every fungal species was investigated 3 to 5 times.

Microbial sensitivity to antibiotics was measured following verification, flight and ground control tests [5]. The experiments were performed using standard disks impregnated with different antibiotics. Second passage cultures were used. Antibiotic sensitivity was measured with respect to the growth inhibition zone around disks.

The Expose-R2 assembly loaded with biological samples was mounted on a support platform attached to the exterior wall of the ISS Russian Segment on August 18th 2014 and the protective cover (blocking UV radiation exposure) was removed on October 22th 2014. The assembly was brought back inside the ISS on February 3th 2016. Several experimental units were returned to Earth onboard Soyuz 44S on March 2th, 2016 and the remaining units onboard Soyuz 45S on June 18th, 2016. Thus, microbial samples were exposed to outer space behind the protective cover for 17.5 mon and without it for 15.5 mon. It should be noted here that in the Expose-R experiment biospecimens were exposed to outer space for over 22 mon.

Results

Bacteria

In outer space solar rays propagate without any obstacles and may produce deleterious effects on microorganisms. This is why in the Expose-R2 experiment preference was given to the microbial strains that allowed a study of their resistance to UV radiation. They were spore-forming microbes isolated from the interior of the ISS, which displayed the highest survival rate after exposure to a laboratory UF emitter.

In our verification tests *Bacillus* bacteria were used to determine their resistance to the expected effects, the major component of which being UV radiation (Fig. 3).

It can be seen from Fig. 3 that in terms of UV-resistance *Bacillus pumilus*-25 was the best (11% spores survived the exposure), being followed by *Bacillus licheniformis*-24. These two strains were therefore selected for the flight and ground control experiments.

In the flight experiment however only individual *Bacillus licheniformis*-24 specimens survived: they were found only in 10 mm x 10 mm bags located in bottom and top layers of the trays (Table 1).

It should be noted here that the number of samples was too small (one for each of the experimental settings) for good statistics. This is why it is not useful to compare the quantities of survived spores in different settings but it is important to notice that there were individual survivals.

In the compartments containing smaller (7 mm x 7 mm) wells, where spores were arranged in four layers, not a single viable cell was detected in the top layer (Table 2).

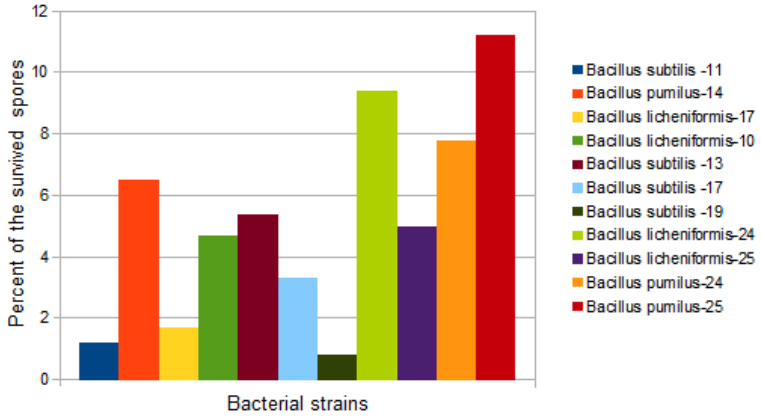

Figure 3: Spore survival rate after UV irradiation.

Table 1: Survival rate of *Bacillus licheniformis*-24 spores after exposure to outer space (survived spores as a percentage of the baseline taken to be 6 x 10^7 per sample).

Code	Description	Ground control %	Space experiment %
Tray #1 comp 3 IBMP\BOSS UV (top layer)	Space-UF (top layer)	0.0045	0.0092
Tray#1 comp 3 IBMP\BOSS dark (bottom layer)	Space-dark (bottom layer)	0.00045	0.0013
Tray#2 comp 3 IBMP\BOSS UV (top layer)	Mars-UF (top layer)	0.0000083	0.0028
Tray#2 comp 3 IBMP\BOSS dark (top layer)	Mars-dark (bottom layer)	0.28	0.38

It can therefore be concluded that UV radiation produces a sterilization effect on specimens exposed to direct solar irradiation. The bacterial strains used in the experiment were isolated from the ISS. They have the capability to form spores or, in other words, to transform into a dormant stage in which they can survive hostile environmental effects and remain viable. However, the bacterial spores used failed to survive prolonged exposure to space: no viable cells were detected in the top layer. However, viable cells of both bacterial strains were identified in the second and third layers. The number of survivals was negligible, *viz.*, less than 1 percent of the baseline. Nonetheless, this shows that bacterial spores when shielded can survive open space exposure though in low quantities.

It is worth mentioning here that exposure on the ground of bacterial strains to the simulated space environment had different effects. In contrast to the real spaceflight experiment, viable spores were seen in the top layer. Quantitatively, the number of viable spores in the middle and bottom layers in both the space-flown and ground control experiments was very close, the difference being less than 1%. It is interesting to note that in space the number of viable spores was greater in the middle layer whereas on the ground it was in the bottom layer. This can be explained either by the fact that the experimental facility inadequately simulated real space effects or by our incomplete understanding of outer space effects.

In addition to the study of bacterial spore viability, *Bacillus licheniformis*-24 was also used to investigate its antibiotic resistance after outer space exposure. The parameter was compared to antibiotic sensitivity shown by the cultures never used in the Expose-R2 flight or simulation experiment.

Bacterial sensitivity to the following nine antibiotics was tested by means of the disk method:

- Ampicillin
- Ampicillin/Sulbactam
- Imipenem
- Gentamicin
- Amikacin
- Ofloxacin
- Trimethoprim-sulfamethoxazole (Co-trimoxazole)
- Cefotaxime
- Ceftriaxone

The data on antibiotic resistance assessed in terms of the growth inhibition zone around disks are illustrated in Fig. 4.

Analysis of sensitivity of experimental and control strains to the above antibiotics showed that after space exposure their resistance tended to increase. The sensitivity to only one (gentamicin) out of the above 9 antibiotics remained almost unchanged or slightly increased. With respect to all other antibiotics bacterial resistance was enhanced.

It can be postulated that after exposure to the space environment defense mechanisms are subject to

Table 2: Survival rate of *Bacillus pumilus*-25 and *Bacillus licheniformis*-24 spores after exposure to outer space.

Experimental design		Pre-flight contamination	Flight experiment					
			Top layer		Middle layer		Bottom layer	
			abs	%	abs	%	abs	%
Space	*B. pumilus*-25	6×10^6	None detected		5.5×10^1	0.0009	1×10^1	0.00017
	B. licheniformis-24	6×10^6	None detected		6.3×10^1	0.001	2×10^1	0.00033
Ground	*B. pumilus*-25	6×10^6	1.3×10^1	0.0002	1.0×10^1	0.00017	5×10^1	0.0007
	B. licheniformis-24	6×10^6	1.3×10^1	0.0002	1.5×10^1	0.0003	1×10^2	0.0017

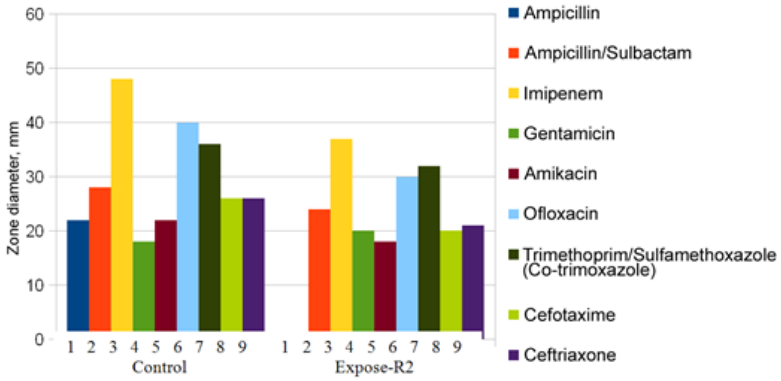

Figure 4: Zones of growth inhibition of *Bacillus licheniformis*-24.

changes even in bacterial spores, which are dormant cells, i.e., cells where growth, physiological reactions and division are arrested. In space, an organism is exposed to a large number of various physical and chemical effects some of which cannot be adequately simulated on the ground. This is why it is extremely difficult to identify specific mechanisms and pathways of physiological and biochemical changes even in the simplest organism, i.e., a bacterial cell. It is however well known that a non-specific stress, such as a prolonged storage of collection cultures, results in enhanced antibiotic resistance seen over several generations. Nonetheless, our observations are important in terms of man-microorganisms co-existence in an enclosed environment, which requires further study as related to long-term exploration missions.

Fungi

Dried spores of the following fungi were used in verification tests:

- *Cladosporium cladosporioides* 2-3
- *Cladosporium cladosporioides* 20-1
- *Ulocladium botrytis* 22-21
- *Ulocladium botrytis* 16-12
- *Ulocladium botrytis* 19-33
- *Ulocladium botrytis* 20-4
- *Aspergillus versicolor* 4-3-4
- *Aspergillus versicolor* 12-2
- *Aspergillus sydowi* 22-11
- *Aspergillus sydowi* 9-6
- *Aspergillus sydowi* 19-11
- *Aspergillus sydowi* 22-1-15
- *Penicillium expansum* 19-30
- *Penicillium expansum* 19-37
- *Penicillium expansum* 4-3-3
- *Alternaria alternata* 20-2

The results of the space-flown and ground simulation experiments are presented in Tables 3 and 4. It can be seen that a large portion of fungal spores, particularly *Aspergillus* spores, remained viable after spaceflight. It appears that in space the effects of UV radiation and other environmental parameters impacted the survival rate of *Aspergillus versicolor* and *Aspergillus sydowi* to a lesser degree and *Ulocladium botrytis* to a greater degree than on the ground. It should be noted that no spores in the top and middle layers survived in the simulation experiment.

Table 3: Survival of fungal spores in the Expose-R2 space experiment (% of the baseline).

Microorganisms	Layer in the compartment		
	Top	Middle	Bottom
Aspergillus versicolor 12-2	19.7	55.6	76.9
Aspergillus versicolor 4-3-4	20.1	62	72.1
Aspergillus sydowi 22-1-5	17	61.3	78.2
Aspergillus sydowi 19-11	16	49.2	76
Aspergillus sydowi 37-12	34.2	69.8	79
Ulocladium botrytis 16-12	0.2	56	68
Ulocladium botrytis 22-21	0.4	63.4	60.1
Penicillium expansum 19-30	9.1	64	69

Table 4: Survival of fungal spores in the Expose-R2 ground simulation experiment (% of the baseline).

Microorganisms	Layer in the compartment		
	Top	Middle	Bottom
Aspergillus versicolor 12-2	0	0	37
Aspergillus versicolor 4-3-4	0	0	65
Aspergillus sydowi 22-1-5	0	0	50.1
Aspergillus sydowi 19-11	0	0	73
Aspergillus sydowi 37-12	0	0	58
Ulocladium botrytis 16-12	0	0	6.8
Ulocladium botrytis 22-21	0	0	2.1
Penicillium expansum 19-30	0	0	0.8

Table 5 summarizes antibiotic sensitivity of fungal strains that survived verification, space and ground simulation experiments. It can be seen that:

- all space-flown strains were resistant to amphotericin B
- space-flown *Aspergillus versicolor* strains showed higher sensitivity to ketoconazole and clotrimazole and lower sensitivity to itraconazole
- space-flown *Aspergillus sydowi* strains showed lowest sensitivity to itraconazole
- space-flown *Penicillium expansum* strains showed higher sensitivity to ketoconazole and *Ulocladium botrytis* to clotrimazole

Table 5

Note: growth inhibition zone of 7 mm in size shows minimal antibiotic sensitivity

It should be emphasized that all fungal strains used in verification, flight and ground control tests were isolated from the ISS interiors, which means that they were previously exposed to spaceflight effects. It can therefore be speculated that their repeated exposure to the space environment may have modified their antibiotic sensitivity resulting from changes in cell membrane permeability induced by cosmic radiation and, probably, microgravity. In all the tests second passage specimens were used. In real spaceflight cell membrane permeability may change; this is why it is important to use space-flown strains of the first or

Table 5: Antibiotic sensitivity of fungal spores in the Expose-R2 experiment (in terms of growth inhibition zone around disks in mm).

Fungal strain	Antibiotic	Verification test		Space experiment		Ground
		Top	Bottom	Top	Bottom	Bottom
Aspergillus versicolor 12-2	ketoconazole	15	21	38	40	27
	clotrimazole	25	30	40	42	32
	itraconazole	10	20	7	7	14
	amphotericin B	0	7	0	0	0
Aspergillus versicolor 4-3-4	ketoconazole	30	30	40	40	28
	clotrimazole	25	30	40	40	32
	itraconazole	15	12	8	0	10
	amphotericin B	7	8	0	0	7
Aspergillus sydowi 22-1-5	ketoconazole	20	26	30	28	20
	clotrimazole	21	20	20	16	25
	itraconazole	12	14	7	7	12
	amphotericin B	0	8	0	0	0
Aspergillus sydowi 19-11	ketoconazole	-	-	36	30	22
	clotrimazole	-	-	25	22	22
	itraconazole	-	-	7	0	14
	amphotericin B	-	-	0	0	0
Penicillium expansum 19-30	ketoconazole	7	7	27	22	12
	clotrimazole	18	10	13	14	14
	itraconazole	8	10	14	14	12
	amphotericin B	11	7	0	0	7
Ulocladium botrytis 16-12	ketoconazole	20	14	30	35	10
	clotrimazole	0	7	18	20	12
	itraconazole	12	8	10	10	10
	amphotericin B	10	7	0	0	0
Ulocladium botrytis 22-21	ketoconazole	30	7	-	-	-
	clotrimazole	0	0	-	-	-
	itraconazole	16	7	-	-	-
	amphotericin B	14	9	-	-	-

second passage when measuring fungal sensitivity to antimycotic and fungicidal agents. In subsequent passages permeability of the cell membrane may undergo modification as it gets adapted to the Earth environment.

In summary, our space and ground experiments demonstrated that fungal spore viability increased as the UF radiation intensity decreased. Strains exposed to open space developed more significant changes in their antibiotic resistance. It can be speculated that exposure to the space environment effects, which were not simulated on the ground (cosmic radiation and microgravity), caused changes in the cell membrane permeability of fungi which resulted in their higher or lower antibiotic sensitivity. This important observation should be taken into consideration when discussing medical risks of future exploration missions.

Conclusions

Study of viability of bacterial and fungal spores and evaluation of their antibiotic resistance after exposure to the hostile effects of outer space allow the following conclusions:

- The Expose-R2 experiment, in which microbial spores were exposed to outer space effects for the time period comparable to the duration of Earth-Mars-Earth missions, demonstrated that some of them could remain viable. This finding is important not only from the theoretical point of view but also in relation to the development of adequate measures of planetary protection.
- The Expose-R2 experiment revealed a deleterious effect of cosmic UV radiation (with a wavelength of >200 nm) on microbial spores. Some bacterial spores survived the exposure to outer space, when shielded from UV radiation, and died when exposed to UV radiation. A small number of fungal spores remained however viable after exposure.
- Most space-flown bacterial strains showed higher antibiotic resistance when compared to the controls.
- After outer space exposure mold fungi displayed more significant changes in antibiotic resistance that were not detected in ground control experiments, probably, due to changes in cell membrane permeability caused by the effects which were not

adequately simulated on the ground (cosmic radiation and microgravity).

Comparison of the results obtained in the Expose-R and Expose-R2 experiments, in which microbial spores were exposed to UV radiation for 22 and 15.5 mon, respectively, allows the following conclusions.

Cosmic UV radiation is a powerful hostile factor of the space environment affecting living matter. In the Expose-R experiment, only isolated *Aspergillus* colony-forming units in the top layer of the compartment survived the exposure. In contrast, in the Expose-R2 experiment, in which spore containing compartments were shielded by filters blocking UV radiation of >200 nm, not only *Aspergillus* but also other fungi survived, though in lesser amounts. In both Expose-R and Expose-R2 experiments, bacterial spores located in the top layer did not survive (some survived but the sample was so small that the data were not statistically significant). In the middle and lower layers of the compartments all microbes, particularly, micromycetes survived, the number of survivors increasing with the distance away from the top. Although the data obtained are limited, it can be agreed that the shielding effect (filtering cover) contributes to microbial survival in outer space.

To sum up, our observations of survivability of biological matter in outer space have both theoretical and applied application.

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