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Effects of ionizing radiation on the survival of bacterial spores in artificial Martian regolith

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Running title: *Bacillus* spore survival in Martian regolith

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ABSTRACT

Bacterial spores of *Bacillus subtilis* were used as a model system to study the effects of ionizing radiation on the survivability of spores uncovered and covered with artificial Martian regolith. Spore survival after X-ray exposure was mainly depending on the role of non-homologous end-joining (NHEJ) as the major DNA double-strand break repair pathway during germination, the involvement of major small, acid-soluble spore proteins (SASP) as DNA radioprotectants and the coverage by Martian regolith, whereas spores covered with Martian regolith were significantly more sensitive to X-rays than uncovered spores, which is mainly due to the interaction of X-rays with artificial Martian regolith resulting in the formation of secondary electrons and reactive oxygen species.

Keywords: *Bacillus* spores, Mars, regolith, DNA repair, ionizing radiation.

Bacterial endospores have since been recognized as one of the hardiest known form of life on Earth. Considerable effort has been invested in understanding the molecular mechanisms responsible for the almost unbelievable resistance of spores to environments which exist at (and beyond) the physical extremes which can support terrestrial life (Nicholson et al., 2000, 2002, 2005; Nicholson, 2009). Spores in their metabolic inactive state survive long-term dormancy for thousands of years or even over millennial time spans and environmental damage to spore cellular components accumulates unrepaired until germination and outgrowth (Nicholson et al., 2000 and references therein). Based on their extraordinary high resistance spores have been used as biosimetric system for environmental monitoring and astrobiological studies both in space flight and ground-based simulations in order to obtain information on the biological damage produced by space conditions (Horneck et al., 1994, 2001). Onboard several spacecraft (Apollo 16, Spacelab 1, LDEF, D2, FOTON) spores of *Bacillus subtilis* were exposed to selected parameters of space, such as space vacuum and different spectral ranges of solar UV-radiation and cosmic rays, applied separately or in combination (Horneck et al., 1994; Nicholson et al., 2000; Rettberg et al., 2004). These spores survived extended periods of time in space, up to several years, if protected against the high flux of solar UV-radiation (Horneck et al., 1994, 2001; Rettberg et al., 2004). Recent studies have repeatedly shown that extremely resilient, spore-forming members of the genus *Bacillus* are the most strongly represented microorganisms in samples collected from spacecraft and facility surfaces (Mancinelli, 2003; Crawford, 2005; Newcombe et al., 2005). Spacecraft and associated clean-room assembly-facility surfaces harbor an extremely low biomass, due to stringent maintenance and environmental controls (i.e., humidity and temperature). Persistence of bacterial spores on spacecrafts and spacecraft materials is one of major concern to that commissioning modern-day space-related experimentation. It is the aim of planetary protection to provide international standards on procedures to avoid organic constituent and biological contamination in space exploration (COSPAR Planetary Protection Policy, 2002, amended 2005 and 2008). The search for extraterrestrial life will rely heavily on validated cleaning and bioreduction strategies to ensure that terrestrial microbial contamination does not compromise the scientific integrity of such missions (Mancinelli, 2003; Crawford, 2005; Nicholson et al., 2005). Several environmental factors must be considered when the ability of microbes to survive and proliferate in extraterrestrial environments is assessed (Horneck et al., 1994; Mancinelli, 2003; Schuerger et al., 2006; Osman et al., 2008). In the case of Mars, "special regions" have been designated in the COSPAR planetary

91 protection policy as areas that may support Earth microbes inadvertently introduced to Mars (Rummel,
 92 2009 and references therein). It has been postulated that survival and propagation of terrestrial
 93 microbes on Mars are only possible in case the organisms can withstand the extreme environmental
 94 conditions present on Mars (Schuerger et al., 2003). Previous studies dealt with the effects of simulated
 95 Martian conditions, i.e. atmosphere, temperature diurnal cycles, UV radiation, surface pressure,
 96 humidity, on survival of *B. subtilis* and other *Bacillus* spp. spores (Newcombe et al., 2005; Schuerger
 97 et al., 2006; Osman et al., 2008). Those studies show that, the inactivation kinetics of the tested
 98 *Bacillus* spp. spores were strongly depending on the impinging solar UV radiation and much less of
 99 temperature shifts, relative water activity, atmosphere composition and surface pressure presented on
 100 Mars (Schuerger et al., 2003, 2006; Osman et al., 2008). There has been growing concern that the
 101 hardy nature of spores might allow them to escape their earthly confines, survive in the vacuum of
 102 space, and intercept extraterrestrial bodies (Crawford, 2005; Schuerger et al., 2006; Osman et al.,
 103 2008). In addition to these concerns, the resistance of spores could enable the organisms to escape
 104 sterilization processes and possibly survive aboard spacecraft to contaminate planets such as Mars. The
 105 surface of Mars, shielded only by a thin atmosphere, is exposed to high levels of cosmic radiation
 106 (Kminek et al., 2003; Saganti et al., 2004). While a lot of experiments have been performed under
 107 simulated Martian conditions, mainly to study the microbial response to Martian UV, diurnal
 108 temperature shifts and surface pressure (Rettberg et al., 2004; Newcombe et al., 2005; Osman et al.,
 109 2008), there has been relatively little work on the impact on ionizing radiation as further environmental
 110 stressor for (potential) microbial life on Mars. In this study, we have examined the survival of *B.*
 111 *subtilis* spores uncovered and covered with artificial Martian regolith in response to X-rays and
 112 secondary radiation produce by interaction. To investigate the protective role of α/β -type small, acid-
 113 soluble spore proteins (SASP), as well as the role of non-homologous end-joining (NHEJ) as DNA
 114 double-strand breaks repair mechanism in spore resistance to ionizing radiation exposure, we used
 115 wild-type spores of *B. subtilis* and repair-deficient mutants with mutations in the NHEJ ligase-like gene
 116 *ykoU*, and the NHEJ Ku-like gene *ykoV* (Wang et al., 2006; Moeller et al., 2008a), as well as spores
 117 lacking in α/β -type SASP (encoded by *sspA* and *sspB*) (Moeller et al., 2008a). In our research,
 118 performed under identical experimental conditions, we addressed the question: do increased X-ray and
 119 secondary radiation exposure affect the survival of bacterial spores, when covered with artificial
 120 Martian regolith? To answer this question, endospores of four different isogenic *Bacillus subtilis* 168-

121 derived strains were used for this work: PS832, a Trp⁺ revertant of strain 168 (Wang et al., 2006)
 122 (wild-type strain); PS355, lacking SASP- α and - β (encoded by *sspA* and *sspB*) (Wang et al., 2006),
 123 PS3722, lacking YkoU, a ATP-dependent DNA ligase and YkoV, a Ku-like DNA end-binding protein
 124 (both genes are member of the operon *ykoWVU* encoding functional NHEJ complex in *B. subtilis*;
 125 (Wang et al., 2006)); and PS3751, lacking α/β -type SASP, YkoU and YkoV (Wang et al., 2006). Strain
 126 PS355 is resistant to chloramphenicol (5 $\mu\text{g/ml}$), strain PS3722 is resistant to lincomycin (25 $\mu\text{g/ml}$)
 127 and erythromycin (1 $\mu\text{g/ml}$); and PS3751 is resistant to chloramphenicol (5 $\mu\text{g/ml}$), lincomycin (25
 128 $\mu\text{g/ml}$) and erythromycin (1 $\mu\text{g/ml}$). The used *B. subtilis* strains were kindly provided by P. Setlow
 129 from the University of Connecticut Health Center, Farmington, Connecticut, USA. Spores were
 130 obtained by cultivation at 37°C with vigorous aeration in double-strength liquid Schaeffer Sporulation
 131 Medium (Schaeffer et al., 1965), under identical conditions for each strain, and the spores were purified
 132 and stored as described (Nicholson and Setlow, 1990). Spore preparations were free (> 99%) of
 133 growing cells, germinated spores and cell debris, as determined by phase-contrast microscopy. Test
 134 samples (50 μl) totaling 5×10^8 spores each, were prepared in two ways: (i) spores in phosphate buffer
 135 saline (PBS) were immobilized on 25 $\times$ 25 mm glass slides (as layers approx. 4-5 spores thick), as
 136 described previously (Moeller et al., 2007, 2008a) and air-dried under laboratory conditions (at 20°C
 137 and 33% relative humidity (RH)); (ii) a spore suspension was added to a powder composed of artificial
 138 Martian regolith (MRS07) to final concentration of 10 mg/ml, mixed, homogenous spotted on 25 $\times$ 25
 139 mm glass slides and air-dried, as described earlier. The mineralogical composition of the used artificial
 140 Martian regolith (MRS07) is shown in **Table 1**. Spores were irradiated either uncovered (as layers
 141 approx. 4-5 spores thick; **Fig. 1 A-C**) or covered with artificial Martian regolith (MRS07; **Fig. 1 D-F**)
 142 with X-rays (150 keV/19 mA) generated by a X-ray tube (Mueller Type MG 150, Germany); as
 143 described previously by Moeller et al. (2007)) up to a final dose of 2.5 kGy. For scanning electron
 144 microscopy (SEM), spores in PBS and mixed with artificial Martian regolith (MRS07), were then
 145 sputtered with a gold film of approx. 10 nm and examined with a Zeiss field emission scanning
 146 electron microscope DSM 982 Gemini with an acceleration voltage of 5 kV using the Everhart-
 147 Thornley SE-detector and the inlens SE-detector. As shown clearly in **Fig. 1 D-F**, only few spores were
 148 uncovered (less than 20% of the spores of the upper layer were free-lying), whereas the majority of the
 149 spores were entrapped in aggregates of the artificial Martian regolith (**Fig. 1**). To recover spores from
 150 the quartz discs after X-ray exposure, spore layers were covered by 10% aqueous polyvinyl alcohol

(PVA) and after drying, the spores-PVA layer was removed as described (Horneck et al., 2001). The spore-bearing PVA film was dissolved in 1 ml of sterile distilled water, resulting in > 95% recovery of the spores. The PVA-recovery method was repeated three times. This recovery procedure has no genotoxic or cytotoxic effect on the spore viability (Horneck et al., 2001). Spore survival was determined from appropriate dilutions in distilled water as colony-forming ability after incubation overnight at 37°C on nutrient broth (NB) agar plates (Difco, Detroit, USA) as described (Horneck et al., 2001). When appropriate, chloramphenicol (Cm; 5 µg/ml), lincomycin (Lin; 25 µg/ml) and/or erythromycin (Erm; 1 µg/ml) were added to the NB recovery plates. Spore survival was determined by a standard colony formation assay and the formation of macroscopically visible colonies was used as a parameter for viability (Horneck et al., 2001). Survival of uncovered air-dried layers of spores exposed to X-rays was determined in three separate experiments (**Fig. 2A, Table 1**). The surviving fraction was determined from the quotient N/N_0 , with N being the number of CFU of the irradiated sample and N_0 being that of the non-irradiated controls (Moeller et al., 2007). Spore survival was plotted as a function of the applied dose of X-rays (**Fig. 2**). Data are reported as D_{10} values, the dose lethal for 90% of the initial spore population (Moeller et al., 2007). Typical inactivation curves were obtained for spores of all strains tested in response to X-ray irradiation (**Fig. 2A**), and D_{10} -values were calculated from the survival curves (**Table 1**). Spores of all mutant strains tested were significantly more sensitive to X-rays than wild-type spores, with the *sspA sspB ykoU ykoV* mutant spores being the most sensitive. In comparison to wild-type spores, the order of X-ray sensitivity of spores of the various strains from most- to least-sensitive was: *sspA sspB ykoU ykoV* > *ykoU ykoV* > *sspA sspB* > wild-type (**Table 1**). These observations are in good agreement with the results from previous experiments in which a subset of these mutant spores were exposed to high doses of ionizing radiation of X-ray and high-energy charged-particle bombardment (Moeller et al., 2007, 2008a). Interestingly, all spores, both wild-type, NHEJ- and α/β -type SASP-deficient mutant spores, when covered with artificial Martian regolith exhibited lower survival rates than uncovered spores to X-ray exposure. Again, D_{10} values were calculated and compared by using Student's *t*-test (**Fig. 2B, Table 1**). When comparing the D_{10} values, with artificial Martian regolith covering and relative to wild-type spores, *sspA sspB ykoU ykoV* mutant spores showed the highest rate of inactivation (8.5-fold), followed by the *ykoU ykoV* mutant spores (2.6-fold) and the *sspA sspB* mutant spores (2.1-fold). A comparison of the inactivation constant of wild-type and NHEJ- and α/β -type SASP-deficient mutant spores show their significant difference

181 from wild-type spores in their sensitivity to X-rays in comparison with their D_{10} values (**Table 1**) under
 182 both conditions - covered with artificial Martian regolith and exposed as uncovered bare spores.
 183 However, the D_{10} values with artificial Martian regolith covered spores were significantly higher than
 184 those of uncovered spores (**Table 1**). Bacterial spores persist in a metabolically inactive state and
 185 environmental damage to spore cellular components accumulates unrepaired until germination and
 186 outgrowth. During germination of spores accumulated DNA damage is repaired by interplay of a set of
 187 highly efficient DNA repair mechanisms (Nicholson et al., 2000; Setlow, 2003, 2006). One further key
 188 reason for the high resistance of bacterial spores to environmental extremes lies in the structure of the
 189 spore. Spores possess thick layers of highly-crosslinked coat proteins, a modified peptidoglycan spore
 190 cortex, abundant intracellular constituents such as the calcium chelate of dipicolinic acid (Ca-DPA) and
 191 α/β -type SASP as protectants of spore DNA (Nicholson et al., 2000; Setlow, 2003, 2006). Major
 192 SASP-deficient spores are much more sensitive to extended desiccation, wet and dry heat, oxidizing
 193 agents (Nicholson et al., 2000, 2002, 2005; Moeller et al., 2007, 2008a; Setlow, 2006). Ionizing
 194 radiation can damage cellular components through direct deposition of radiation energy into
 195 biomolecules and also indirectly by generating reactive oxygen species (ROS) (Hutchinson, 1985;
 196 Kiefer, 1990). Hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO\bullet$) are major oxidizing species
 197 produced by the radiolysis of water, and superoxide ions ($O_2^{\bullet-}$) are formed in the presence of dissolved
 198 oxygen (Dianov et al., 2001; Cooper et al., 2008). Generally, the cytotoxic and mutagenic effects
 199 induced by ionizing radiation are thought to be the result of DNA damage caused during the course of
 200 irradiation, which include single-strand breaks (SSB), double-strand breaks (DSB), base modification,
 201 abasic sites, and sugar modification (Hutchinson, 1985; Kiefer, 1990). The data presented in this
 202 communication augment previous data suggesting a number of conclusions about mechanisms of spore
 203 resistance to X-ray. First, it appears that the major and α/β -type SASP are necessary not only for spore
 204 resistance to X-rays as part of complex composition of the galactic cosmic radiation (Saganti et al.,
 205 2004; Reitz, 2008). Second, it could be shown that NHEJ provides an efficient DNA double-strand
 206 break repair pathway during spore germination. Spores of *B. subtilis* contain a single chromosome in
 207 A-DNA conformation arranged in a toroidal shape (Wang et al., 2006; Moeller et al., 2007): therefore
 208 only non-homologous end-joining (NHEJ) can act as repair pathway for DSB induced in spore DNA
 209 (Brissett and Doherty, 2009), whereas the homologous recombination pathway, which requires at least
 210 two homologous chromosomes, cannot operate on DSB repair during spore germination (Wang et al.,

2006; Moeller et al., 2007). And third, spores deficient in NHEJ and lacking in α/β -type SASP formation were significantly more sensitive to ionizing radiation than the respective single mutant spores (**Table 1, Fig. 2**). A further result from this work was the observation that spores covered with artificial Martian regolith (all different tested genotypes) were significantly more sensitive than uncovered spores to X-ray exposure. A body of evidence has accumulated which suggests that spores covered with artificial Martian regolith, a complex mixture of various water-absorbing minerals e.g. montmorillonite, kaolinite, hematite, anhydrite (Squyres et al., 2004; Bibring et al., 2005; Cloutis et al., 2008), were additionally affected by the X-ray caused generation of ROS such as peroxide, superoxide, or hydroxyl radicals in the artificial Martian regolith (Dianov et al., 2001; Saganti et al., 2004; Bibring et al., 2005). This leads us to the assumption that spores were mainly inactivated by direct impact of X-rays leading to DNA SSB and DSB, while ROS formation in the Martian regolith acts as an additional synergistic sporicidal stressor. A potential damage of the X-ray induced ROS could be the destruction, damage, or inhibition of spore coats, core membranes and/or essential germination receptors, leading to a blockage in spore germination and outgrowth (Setlow, 2003, 2006). Further on, as shown by various authors, cellular damage produced by low (linear energy transfer) LET radiation, e.g. X-rays can be attributed to ionization and excitation processes or interactions with radicals (Hutchinson, 1985; Kiefer, 1990; Dianov et al., 2001; Nicastro et al., 2002). The higher inactivation is most likely to be produced by secondary electrons, produced by interaction of the primary X-rays (Fricke and Glasser, 1924) with the artificial Martian regolith and latter with the spores as biological target. Also, it could be shown that the irradiation of spores with electrons lead to the destruction of bacterial spores (Helfinstine et al., 2005). Therefore, it is reasonable to assume that the interaction of the occurring secondary electrons with the spores covered with Martian regolith led to higher inactivation rates compared to those of uncovered spores. Also it should be noted that long-term storage (up to 3 months) under laboratory conditions of both uncovered and with Martian regolith covered spores did not affect the spore vitality in all tested spores (data not shown). Nevertheless, one should consider that uncovered spores will be rapidly inactivated by solar UV radiation as shown previously by various researchers (Horneck et al., 2001; Schuerger et al., 2003, 2006; Rettberg et al., 2004; Newcombe et al., 2005; Osman et al., 2008), therefore only spores, shielded with Martian regolith, would be capable to survive certain exposures to ionizing radiation reaching Martian surface. On present Mars very low dose rates for ionizing radiation are to be expected (Kim et al., 1998; Dartnell et al., 2007), Saganti et al. (2004) recently reported on the

average dose of approx. 0.2 Gy per year for the Martian surface, which would yield in approx. 15550 years (for uncovered) and approx. 14660 years (for with MRS07 covered spores) to reach an inactivation by 6 orders of magnitude (**Fig. 2**, values obtained by extrapolation). These inactivation data are in good agreement with model calculations by Dartnell et al. (2007). In their model calculations, Dartnell and co-workers (2007) could show that homogenous dried bacterial spores survive on surface 10100 years (a yearly dose of 0.83 Gy) and in 0.5 m depth 31700 years (a dose of 0.27 Gy/year) until reaching a 10^{-6} spore inactivation. Based on these data, it can be outlined that the interactive effects of Martian environmental parameters actually acted to decrease the survival of bacterial spores, one of the major candidates involved in a (potential) contamination of Mars. However, since the Martian environment is a complex interaction of the Martian radiation climate (solar UV and galactic cosmic rays), diurnal and seasonal temperature cycles, pressure, atmospheric composition, which are technically very difficult to simulate at the same time, therefore the direct (synergistic or antagonistic) impact of ionizing radiation in the complete simulated Martian environment remain to be investigated. As to our knowledge, up to now no complete Mars simulation was performed in one single experiment including diurnal temperature cycles, UV radiation, different humidity levels, atmospheric compositions, surface pressure, adequate Martian soil analogues, dust particle interactions and ionizing radiation, and creating these challenges to a fascinating and fruitful area for further studies.

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TABLES

Table 1. Survival characteristics of the X-ray exposed *B. subtilis* spores.

Genotype (cover ^a)	D ₁₀ value ^b	Ratio D ₁₀ value ^c
wild-type (unc.)	735.8 ± 43.2	
wild-type (MRS07)	629.9 ± 25.4*	0.85 ± 0.05*
<i>sspA sspB</i> (unc.)	377.1 ± 28.1	
<i>sspA sspB</i> (MRS07)	298.4 ± 22.7*	0.79 ± 0.06*
<i>ykoV ykoU</i> (unc.)	237.6 ± 20.5	
<i>ykoV ykoU</i> (MRS07)	181.4 ± 19.8*	0.76 ± 0.05*
<i>sspA sspB ykoV ykoU</i> (unc.)	105.8 ± 15.4	
<i>sspA sspB ykoV ykoU</i> (MRS07)	73.9 ± 14.3*	0.69 ± 0.07*

^a Spores were either uncovered (unc.) or covered with artificial Martian regolith (MRS07). Powder composition of the artificial Martian regolith (MRS07): 47.7% Na-montmorillonite, 9.9% kaolinite, 21.3% hematite (19.2% Fe₂O₃ with 1.3% SiO₂), 13.0% anhydrite, 7.1% MgSO₄, 1.0% NaCl, 2.5% Na₂O, 3.4% MgO, 14.1% Al₂O₃, 34.6% SiO₂, 5.1% SO₃, 0.2% Cl⁻, 0.2% K₂O, 6.1% CaO, 0.1% TiO, 18.5% FeO; artificially composed according the spectral data and information of the NASA Mars Exploration Rover (MER), Spirit and Opportunity (Squyres et al., 2004) and OMEGA/Mars Express observations (Bibring et al., 2005).

^b D₁₀ value = dose (Gy) of X-ray treatment leading to a 90 % inactivation of the initial CFU (Moeller et al., 2007, 2008a).

^c Ratio of D₁₀ value (uncovered) to the respective D₁₀ value of with artificial Martian regolith covered spores.

Data reported as averages ± standard deviations (*n* = 3). Asterisks indicate D₁₀ values that were significantly different (*P* values of ≤ 0.05) with regard to the respective values of the uncovered spores.

FIGURE LEGENDS

Fig. 1 Scanning electron micrograph images of wild-type *B. subtilis* spores, uncovered (Panel **A-C**) and covered with artificial Martian regolith (MRS07) (Panel **D-F**). Scale bar: 5 μm (A and D), 2 μm (B and E), and 1 μm (C and F).

Fig. 2 Survival curves of *B. subtilis* spores in response to sparsely ionizing radiation, emitted by X-rays. Spores of *B. subtilis* were either uncovered (Panel **A**; open symbols) or covered with artificial Martian regolith (MRS07) (Panel **B**; filled symbols). Strains are wild-type (circles), *sspA sspB* mutant (squares), *ykoV ykoU* mutant (triangles up), and *sspA sspB ykoV ykoU* mutant (triangles down). Data are averages $\pm$ standard deviation ($n = 3$). Otherwise error bars for survival data not shown were smaller than the symbol.

FIGURES

Fig. 1 Moeller et al.

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