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RESEARCH ARTICLE

Surviving Mars: new insights into the persistence of facultative anaerobic microbes from analogue sites

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Abstract

Mars analogue environments are some of the most extreme locations on Earth. Their unique combination of multiples extremes (e.g. high salinity, anoxia and low nutrient availability) make them valuable sources for finding new polyextremophilic microbes, and for exploring the limits of life. Mars, especially at its surface, is still considered to be very hostile to life but it probably possesses geological subsurface niches where the occurrence of (polyextremophilic) life is conceivable. Despite their well-recognized relevance, current knowledge on the capability of (facultative) anaerobic microbes to withstand extraterrestrial/Martian conditions, either as single strains or in communities, is still very sparse. Therefore, space experiments simulating the Martian environmental conditions by using space as a tool for astrobiological research are needed to substantiate the hypotheses of habitability of Mars. Addressing this knowledge gap is one of the main goals of the project MEXEM (Mars EXposed Extremophiles Mixture), where selected model organisms will be subjected to space for a period of 3 months. These experiments will take place on the Exobiology facility (currently under development and implementation), located outside the International Space Station. Such space experiments require a series of preliminary tests and ground data collection for the selected microbial strains. Here, we report on the survivability of *Salinisphaera shabanensis* and *Buttiauxella* sp. MASE-IM-9 after exposure to Mars-relevant stress factors (such as desiccation and ultraviolet (UV) radiation under anoxia). Both organisms showed survival after anoxic desiccation for up to 3 months but this could be further extended (nearly doubled) by adding artificial Mars regolith (MGS-1S; 0.5% wt/v) and sucrose (0.1 M). Survival after desiccation was also observed when both organisms were mixed before treatment. Mixing also positively influenced survival after exposure to polychromatic Mars-like UV radiation (200–400 nm) up to 12 kJ m^{-2}, both in suspension and in a desiccated form.

Contents

Introduction

Earth hosts many natural extreme environments, characterized by e.g. extremes in temperature, pH, salinity and hydrostatic pressure, as well as in the availability of water, nutrients and oxygen. Nevertheless, these environments are colonized by specifically adapted and very successful life forms, also called extremophilic organisms or extremophiles (Rothschild and Mancinelli, 2001; Rampelotto, 2013).

Taking these extreme conditions into consideration and based on their similarities with conditions prevailing on other parts of our Solar System, some of these extreme sites were defined as analogue environments. A significant focus has been specifically placed on Mars analogue environments, with many locations highlighted across the world, namely in Tibet, USA, Argentina, Antarctica and India, just to name a few (e.g. Szponar *et al*., 2013; Forte *et al*., 2016; Xiao *et al*., 2017; Pandey *et al*., 2019; Favaro *et al*., 2020). European Mars analogue environments were selected and investigated in detail in the project MASE (Mars Analogues for Space Exploration) with their characterization from a microbial, physical, mineralogical and chemical point of view (Cockell *et al*., 2018; Bashir *et al*., 2021).

One of these sites is the Islinger Mühlbach in the Sippenauer Moor, located in southern Germany and hosting several cold sulphidic springs. These are part of a network of springs that emanate from the subsurface, and are characterized by low organic content, low temperatures, anoxia and having sulphur compounds as possible energy sources. The springs have been extensively investigated from a microbiological point of view for the past 20 years, illustrating their relevance (e.g. Moissl *et al*., 2002; Rudolph *et al*., 2004; Probst *et al*., 2014; Cockell *et al*., 2018). More recently, several new organisms have been isolated from this site during the MASE project (Cockell *et al*., 2018), including *Buttiauxella* sp. MASE-IM-9 (abbreviated as *Buttiauxella* sp. hereinafter). *Buttiauxella* sp. is a Gram-negative, facultative anaerobic and motile bacterial species within the family *Enterobacteriaceae*, and consists of rod-shaped cells. This strain has been tested under diverse Martian-relevant stress factors, such as desiccation, ionizing radiation and oxidizing compounds such as perchlorates (Beblo-Vranesevic *et al*., 2018, 2020).

An additional interesting analogue site is provided by deep-sea brine pools. Given their unique combination of high salinity, anoxia and varying levels of hydrothermal input (Antunes *et al*., 2011*a*), they have recently been identified as new analogue environments of relevance for astrobiological studies (Antunes *et al*., 2020). The deep-sea brines are further characterized by very sharp brine–seawater interfaces, with drastic transition in environmental conditions and significant particle accumulation, fuelling unique ecosystems and harbouring several new organisms (e.g. Antunes *et al*., 2011*a*; Oliver *et al*., 2015; Kaartvedt *et al*., 2016). One of the most striking new microbes from these locations is *Salinisphaera shabanensis*, isolated from the brine–seawater interface of the Shaban Deep, in the northern Red Sea (Antunes *et al*., 2003). *S. shabanensis* belongs to a deeply branching lineage within

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the γ-Proteobacteria, and is described as a Gram-negative, facultative anaerobic and monotrichous cocci. This species was shown *via* lab-based testing and genome-based analysis to be particularly versatile and resilient, likely an adaptation and advantage for living in the brine–seawater interface (Antunes *et al*., 2003, 2011*b*; Vetriani *et al*., 2014).

The harsh conditions on the surface of Mars are characterized by low water and nutrient availability, high salinity (including oxidizing compounds), high ultraviolet (UV) radiation influx, low ionizing radiation influx and the presence of Mars regolith itself. Although the Mars solar constant is, on average, only 43% of that observed on Earth, the biologically effective fluence rate of UV irradiation on the Martian surface is up to three orders of magnitude higher than on the surface of Earth due to its atmosphere, which is a thin, carbon dioxide (CO_2)-dominated atmosphere, with only trace amounts of oxygen and the lack of a protecting ozone layer. Thus, and in contrast to Earth, biologically damaging UV (200–400 nm) radiation reaches the surface of Mars at relatively high levels. The total polychromatic UV fluence rate for low dust scenarios on the surface of equatorial Mars ranges between 42 and 55 W m^{-2} (Schuerger *et al*., 2006). Considering the theoretical surface flux model as described in Patel *et al*. (2003) the UV flux in summer at the destined latitude of 60 lies around 38 W m^{-2}. In general, the UVC fluence rates were estimated to be approximately 8–10% of the total UV flux. Based on these models, between 3.2 and 5.5 W m^{-2} of UVC irradiation is likely to reach the surface of equatorial Mars under clear-sky conditions (Schuerger *et al*., 2006). However, the Martian regolith and dust particles can contribute to a UV-protection scenario on the surface of Mars today (Vicente-Retortillo *et al*., 2020). Due to the lack of a magnetic field, on Mars there is also an increased dose of ionizing radiation. Indeed, the radiation dose rate of ionizing radiation on the surface of Mars was measured and calculated to be up to 0.21 mGy day^{-1} (Matthiä *et al*., 2016) approximately ten times higher than on Earth.

From previous missions to Mars, the composition of Martian regolith is quite well known for different locations, permitting the description of different Mars soil simulants which are commercially available for purchase (Cannon *et al*., 2019). One of them is the Mars Global Simulant (MGS-1) standard mixture, resembling basaltic soils on Mars, and based on quantitative mineralogical data obtained from the Curiosity rover/Mars Science Lab mission (Grotzinger *et al*., 2012). MGS-1 is designed to replicate the Rocknest windblown soil in Gale crater on Mars, which is chemically similar to other basaltic soils at disparate landing sites and thus constitutes a 'global' basaltic soil composition. MGS-1S is a modified version of the MGS-1 simulant and is enriched in the polyhydrated sulphate gypsum.

Current knowledge on the capability of (facultative) anaerobic microbes to withstand extraterrestrial conditions is still very sparse and is one of the main goals of the project MEXEM (standing for Mars EXposed Extremophiles Mixture). The proposal for the space experiment MEXEM was selected by ESA in 2014, and at the moment the implementation is foreseen for 2025 at the new Exobiology facility that will be installed on the upcoming Bartolomeo platform outside the European Columbus module on the International Space Station (ISS). It is worth noting that space experiments often have long lead times, so it is even more important to prepare the experiments accurately. With MEXEM the long and successful tradition of passive exposure experiments on satellites and on the outside of the ISS will be continued. Thereby, biological samples will be exposed to a simulated anoxic Martian environment, i.e. a combination of Mars-like UV radiation from the Sun, simulated Martian atmosphere and Martian atmospheric pressure. Bacterial, archaeal and eukaryotic single species as well as natural communities, and mixtures will be evaluated. Besides other bacterial strains, *Buttiauxella* sp. and S. shabanensis were selected a part of the 'passenger list'. The general aim of the space exposure experiments is to determine and analyse in detail the limits of terrestrial life. The results of MEXEM will contribute to our understanding of the capability of life to persist in extreme environments on Earth, on other planets (e.g. Mars), and moons of our Solar System.

According to the literature, past attempts to investigate the response of microorganisms to Martian extremes have generally been focused on aerobic organisms adapted to life under oxic conditions. Only a few studies have been conducted on (facultative) anaerobic microorganisms obtained from, and are thereby adapted to, Mars analogue environments (e.g. Serrano *et al*., 2019). This will be the first time

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that anaerobic polyextremophilic microbial isolates, i.e. bacteria, archaea, ciliates and viruses, will be investigated utilizing space as a tool for astrobiology.

The direct comparison of the survival strategies of phylogenetically different microbial species will also give new insights into the adequacy of actual planetary protection measures and may support the development of new life detection technologies for space application. When we know which terrestrial microorganisms can survive Martian conditions or can even live under these conditions, we can further improve our efforts and refine control protocols to prevent contamination of Mars and other planets during future missions.

The overall aim is the assessment of the survivability of microorganisms from extreme environments on Earth using the space environment as a tool. The purpose of the present work is a general data collection and detailed analysis of the desiccation and radiation tolerance of two new model organisms in the presence of added protective substances. The generated data serve on the one hand as an accurate preparation for the implementation of the space experiment itself and on the other hand the data are also essential information for future comparison with the results of the space experiments.

Materials and methods

Cultivation of the strains

In this study, *Buttiauxella* sp. MASE-IM-9 (DSM 105071) and *S. shabanensis* E1L3A^T (DSM 14853) were investigated. Both strains were cultivated under microoxic conditions in liquid medium (less than 1% v/v O_2). Therefore, the protocol by Miller and Wolin (1974) was used with the adaptation, that the medium was portioned (20 ml) in 120 ml serum bottles without prior addition of a reducing agent within the anaerobic chamber (Coy Laboratory Products Inc.; $[O_2] < 5$ ppm, relative humidity 13 ± 0.5%). The headspace was filled with N_2/CO_2 (80 : 20 v/v) with a pressure of 1 bar. *Buttiauxella* sp. was grown in Sigma Aldrich tryptic soy broth No. 2 (TSB), *S. shabanensis* was grown in BD Difco™ marine broth 2216 (MB) adjusted by the addition of sodium chloride (NaCl) to a final concentration of 10% wt/v. Alternatively, the cells were cultivated on tryptic soy agar plates for *Buttiauxella* sp. and marine agar with 10% NaCl wt/v for *S. shabanensis*. The cultivation temperature, either in liquid or on plates was 30°C, the optimal growth temperature for both strains. The strains were grown in broth until reaching the stationary growth phase (24 h for *Buttiauxella* sp. and 24–48 h for *S. shabanensis*); the plates remained in the incubator for up to 1 week.

Added substances

In this study, three different substance classes were added to the liquid medium/cultures: sugars (sucrose and trehalose), low-molecular substances (ectoine and betaine) and different Mars regolith simulants. The sucrose, trehalose, ectoine and betaine were prepared as aqueous stock solutions, gassed with N_2/CO_2 (80 : 20 v/v) and sterile filtered under anoxic conditions. The appropriate amount of added substances (end concentrations 0.1, 0.5 and 1 M) was added to the liquid medium/culture within the serum bottle *via* injection with a syringe.

Three Martian regolith simulants (MGS-1, MGS-1S and MGS-1C) were used in this study; detailed description of their components can be found at the homepage of CLASS Exolith Lab (Center for Lunar and Asteroid Surface Science at the University of Central Florida; https://sciences.ucf.edu/class/exolithlab/). MGS-1S, a modified version of MGS-1 (with a grain size of >0.04–600 µm), is described in detail by Cannon *et al.* (2019). According to the suppliers, MGS-1S has the following mineralogy in % (wt) as mixed: gypsum 40.0, plagioclase 16.4, basaltic glass 13.7, pyroxene 12.2, olivine 8.2, epsomite 2.4, ferrihydrite 2.1, hydrated silica 1.8, magnetite 1.1, anhydrite 1.0, siderite 0.8 and haematite 0.3. Additionally, MGS-1S has the following bulk chemistry by % (wt), as measured by X-ray fluorescence: SiO_2 29.3, TiO_2 0.3, Al_2O_3 6.4, Cr_2O_3 0.1, FeO_T 11.3, MnO 0.1, MgO 16.2, CaO 14.8, Na_2O 2.1, K_2O 0.5, P_2O_5 0.3 and SO_3 18.7.

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Prior to being used in the experiments, Martian regolith was portioned and autoclaved under oxic conditions. The addition of regolith to the liquid medium/cultures in the appropriate quantities (end concentration of 0.1, 0.5 and 1% wt/v) was done under anoxic conditions within the anaerobic chamber. Therefore, the sterile serum bottles containing the medium/culture were opened under forming gas in the anaerobic chamber, regolith (MGS-1/MGS-1S/MGS-1C) was added, the serum bottles were closed again and the gas headspace was changed again to N_2/CO_2 (80 : 20 v/v).

Microscopic observations

Light and fluorescence microscopy was performed with 4′,6-diamidino-2-phenylindole (DAPI)-stained cells on a Zeiss Axio Imager.M2 including a DAPI filter set. For the fluorescent dye, we used the protocol previously described by Küper *et al*. (2010) (adapted from Huber *et al*., 1985). Briefly, prior to microscopy observations, 8 µl of the grown cultures were mixed with 1 µl of DAPI-staining solution, and incubated for 1 h at room temperature (RT).

Scanning electron microscopy was used to analyse the granular structures of the simulated Martian regolith. Samples were examined using a Hitachi TM3000 tabletop microscope with an accelerating voltage of 15 kV. Energy-dispersive spectroscopy (EDS) was performed using the Quantax70 program (Bruker).

Desiccation of the cells

For all experiments described below the strains were cultivated until early stationary phase (24 h for *Buttiauxella* sp., 48 h for *S. shabanensis*). Growth and cell concentrations before the exposure experiments were determined microscopically by cell counting using a Thoma counting chamber (depth: 0.02 mm). The desiccation experiment was carried out as described before (Beblo *et al*., 2009). Briefly, the cell suspension (1 ml) was used directly without any washing or concentration steps, applied evenly on five glass discs (Ø 1.2 mm) and allowed to dry overnight at RT inside the anaerobic chamber (% $O_2 < 5$ ppm, relative humidity $13 \pm 0.5\%$, 10^5 Pa). The desiccation experiment was set up for 5 months (day 140), after 24 h of desiccation the first dataset (day 1) was evaluated. For the evaluation of the surviving fraction, the five glass discs with the attached dried cells were placed into 5 ml sterile anoxic phosphate-buffered saline (PBS). The PBS solution for *Buttiauxella* sp. had the following composition: NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.44 g, KH_2PO_4 0.24 g per litre, while the modified PBS solution for *S. shabanensis* consisted of: NaCl 100 g, KCl 0.2 g, Na_2HPO_4 1.44 g, KH_2PO_4 0.24 g per litre. The cells were resuspended from the glass discs by shaking at RT. Afterwards, the cells were diluted in the corresponding PBS and plated on strain-specific agar plates (N). Control cells from liquid cultures were treated the same way: 1 ml of the grown culture was diluted in 5 ml of the corresponding sterile PBS, diluted further and plated on strain-specific plates (N_0). After incubation of all plates the number of grown colonies was noted.

Mixture and desiccation experiments

Both strains were cultivated under optimal cultivation conditions in their corresponding liquid medium. The cell concentration of both strains was determined before aliquots of the two liquid cultures were mixed and stored at RT for 24 h. Additionally, 1 ml of the mixture was desiccated on glass discs under anoxic conditions as described above. After 24 h, the cell numbers of both strains present in the liquid mixture and in the desiccated mixture was investigated. Thereby, the desiccated cells on glass discs were resuspended in PBS (adjusted to a final NaCl concentration of 5% wt/v), further diluted and plated on the corresponding agar plates of the strains. Preliminary tests confirmed that each strain was only able to grow on their strain-specific agar plates.

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Irradiation treatment

Exposure to polychromatic UV (200–400 nm) in liquid

To avoid any absorbance due to the medium, or any of the individual medium components (e.g. organic material), cells were diluted 1 : 10 within the anaerobic chamber, and in the corresponding anoxic PBS, before the radiation treatment (Table 1). The absorption was measured with a Hitachi spectrophotometer (U-3310, Hitachi High Technologies America Inc.).

The polychromatic UV exposure was performed with the SOL2 lamp and conducted in UV transparent hermetically closed quartz cuvettes (type 1/GL14/S; Starna GmbH), including a magnetic stir bar. The SOL2 lamp was equipped with a UV 500S irradiation source (Dr Hönle AG, UV-Technologie). A detailed description of the irradiation facility is given in Rabbow *et al*. (2005). The biologically relevant spectrum for cellular damage (200–400 nm) was used to calculate the fluence rate and the exposure time to reach predetermined fluence values up to 12 000 J m^{-2}. For the polychromatic UV experiments described here, a measured fluence rate of 10.35 W m^{-2} was used with a distance of 128 cm between the UV source and the sample. To ensure a homogenous irradiation, a magnetic stirrer was installed and the cultures were mixed during irradiation. All steps were done under anoxic conditions and at RT. After the irradiation with an appropriate fluence an aliquot of the irradiated cells was taken, further diluted in the corresponding PBS, plated on strain-specific plates, incubated and the grown colonies were counted (N). A non-irradiated dark control was processed in the same way as a reference (N_0).

Exposure to monochromatic UVC and ionizing radiation in liquid

Cells were treated for the exposure with monochromatic UVC (254 nm) in the same way as described above. For the anoxic UVC irradiation a low-pressure mercury lamp (model NN 8/15 Heraeus) with a major emission line at 254 nm (fluence rate 86.7 W m^{-2}) was used at RT as described by Beblo *et al*. (2011).

The exposure to ionizing radiation was performed with the X-ray source Gulmay RS 225A (Gulmay Medical Ltd.) at 200 kV and 15 mA following Beblo *et al*. (2011). Microbial cell cultures were transferred anoxically into high-performance liquid chromatography vessels and irradiated at a distance of 19.5 cm below the X-ray source with approximately 20 Gy min^{-1} up to 500 Gy. The dose rate was measured with a UNIDOS dosimeter (PTW Freiburg GmbH) and all irradiation experiments were performed under anoxic conditions at RT.

Exposure of dried cells to polychromatic UV (200–400 nm)

For the combination of desiccation and polychromatic irradiation, cells were treated as described above for the desiccation experiments under anoxic conditions. After 24 h of desiccation, the glass discs were transferred within the anaerobic chamber into the transport and exposure box (Trex-box; Beblo-Vranesevic *et al*., 2017*a*). The Trex-box was covered with a UV transparent quartz window and hermetically sealed. The exposure to polychromatic UV radiation was done under anoxic conditions at RT within the Trex-box.

Evaluation of the survival fraction

All experiments were repeated independently at least three times to represent biological replicas for each time point, fluence, dose and strain, respectively. N_0 (non-desiccated/non-irradiated liquid control) and N (desiccated and/or irradiated sample) were determined from the number of counted colonies on the plates. The mean of the data with standard error was calculated. Within the graphs the survival fraction ($S = N/N_0$) is given. The graphs and survival curves were created with Sigmaplot 13.0. Additionally, the F_{10} values/D_{10} values were calculated from regression lines of the exponential slopes of the survival curves as previously described (Harm, 1980). They provide the fluence/dose where the survival after treatment is reduced by one order of magnitude.

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Table 1. *Absorption of undiluted and diluted media at the wavelengths of 200 and 254 nm and at 400 nm*

	Absorption at		
	200 nm	254 nm	400 nm
TSB	4.000	4.000	0.864
TSB 1 : 10	1.314	1.135	0.018
TSB with MGS-1S 1 : 10 in PBS (0.8% NaCl)	1.407	1.135	0.027
MB	4.000	4.000	0.242
MB 1 : 10	0.972	0.663	0.025
MB with MGS-1S 1 : 10 in PBS (10% NaCl)	0.885	0.711	0.015

The measurement limit of the used spectrophotometer is at 4.

Table 2. *Calculated* F_{10} *values (254; 200–400 nm) and* D_{10} *values in liquid*

Organism	F_{10} values (254 nm) in J m^{-2} in liquid	F_{10} values (200–400 nm) in kJ m^{-2} in liquid	D_{10} values in kGy in liquid
Buttiauxella sp. MASE-IM-9	42	4.4	0.4 (a)
Salinisphaera shabanensis	56	4.3	1.7
Aquifex pyrophilus	63 (m)	n.d.	2.8 (m)
Bacillus subtilis (vegetative)	27 (b)	1.3 (c)	0.1 (c)
B. subtilis (spore)	273 (d)	12.4 (e)	0.8 (d)
Deinococcus radiodurans	660 (f)	20.0 (g)	5.6 (f)
Escherichia coli	40 (h)	n.d.	0.3 (i)
Halobacterium salinarum	280 (j)	10.8 (k)	5.0 (l)
Halococcus hamelinensis	80 (m)	1.6 (k)	2.0 (n)
Hydrogenothermus marinus	67 (o)	n.d.	0.8 (o)

n.d., not determined/no data available.
(a) Beblo-Vranesevic *et al.* (2018); (b) Wassmann *et al.* (2011); (c) Wassmann *et al.* (2010); (d) Moeller *et al.* (2007); (e) Wassmann *et al.* (2012); (f) Bauermeister *et al.* (2011); (g) Pogoda De La Vega *et al.* (2005); (h) Arrage *et al.* (1993); (i) Clavero *et al.* (1994); (j) Shahmohammadi *et al.* (1998); (k) Leuko *et al.* (2015); (l) Kottemann *et al.* (2005); (m) Leuko *et al.* (2011); (n) Leuko and Rettberg (2017); (o) Beblo *et al.* (2011).

Results

Effects of added substances on desiccation tolerance

In a pretest series, we examined three different Mars regolith simulants (MGS-1, MGS-1S and MGS-1C), two sugars (sucrose and trehalose), as well as two low-molecular substances (ectoine and betaine) which are known to possibly influence the desiccation tolerance as intracellular accumulated compatible solutes. These substances were added to the cultures in different concentrations (0.1, 0.5, 1% wt/v for regolith, and 0.1, 0.5, 1 M for the other substances) both before and after inoculation (data not shown). The combination of MGS-1S (0.5% wt/v) and sucrose (0.1 M) revealed the greatest influence on desiccation tolerance after 24 h in *Buttiauxella* sp. and in *S. shabanensis*. Therefore, this combination was used for the evaluation of the long-term desiccation tolerance of both strains under anoxic conditions.

The desiccation tolerance under anoxic conditions of standard cultivated *Buttiauxella* sp. cells could be reproduced (Beblo-Vranesevic *et al.*, 2018). Additionally, a general tolerance to desiccation was determined for standard cultivated cells of *S. shabanensis* under anoxic conditions up to 88 days (Fig. 1). After 88 days the survival rate of *S. shabanensis* was reduced by six orders of magnitude

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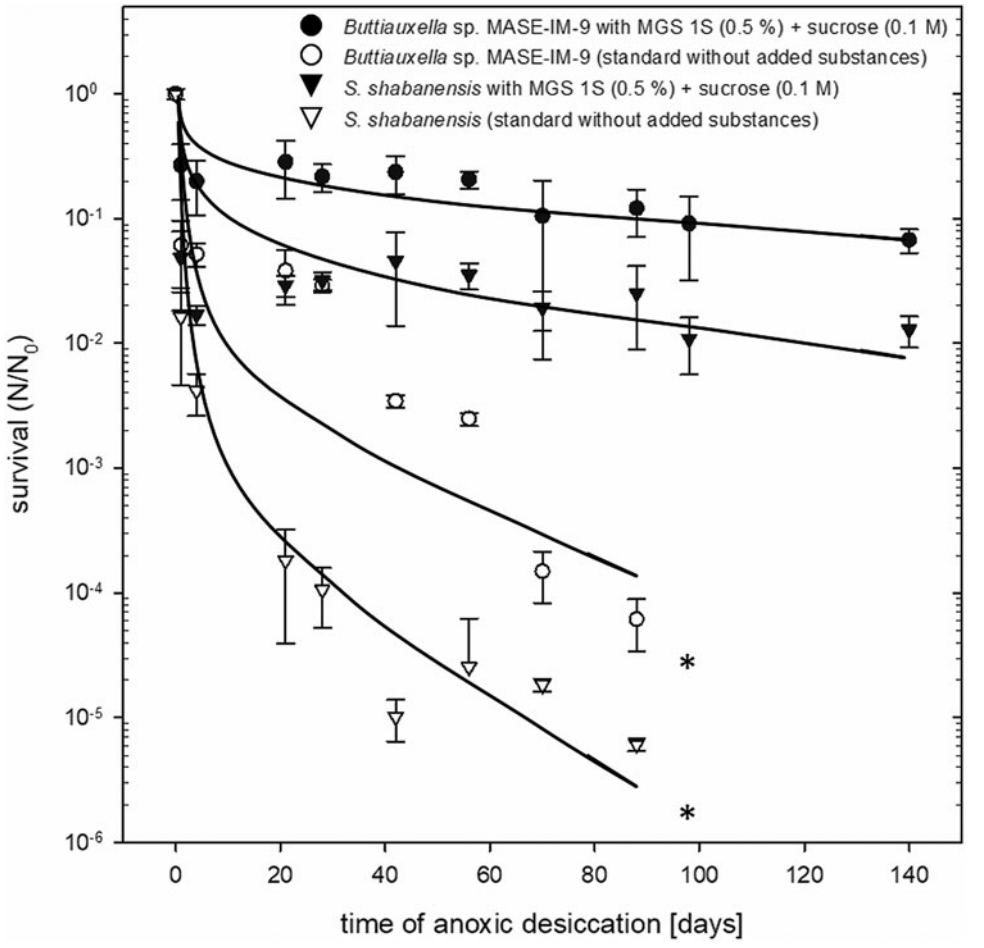

Fig. 1. *Survival of* Buttiauxella *sp. (circles) and* Salinisphaera shabanensis *(triangles) after anoxic desiccation up to 5 months. Strains were cultivated either under standard conditions (open symbols) or in the presence of Martian regolith (MGS-1S; 0.5% wt/v) and sugar (sucrose; 0.1 M) (filled symbols) before desiccation. *No survival was detectable. Trendlines were fitted by hand.*

($S = 6.1 \times 10^{-6}$). However, under standard conditions no growth after reactivation could be shown for both strains after 98 days of desiccation. For both strains, the desiccation tolerance under anoxic conditions was influenced by the addition of 0.5% (wt/v) MGS-1 and 0.1 M sucrose. In the presence of Martian regolith and sugar both strains survived at least 140 days (longer time periods were not tested in this experiment). The survival rate for *Buttiauxella* sp. decreased by only one order of magnitude over the time of 5 month ($S = 0.6 \times 10^{-1}$), while for *S. shabanensis* this decrease was about two orders of magnitudes ($S = 1.3 \times 10^{-2}$) for the same time span.

Desiccation tolerance of the mixed cultures

The storage of the mixed strains together, as an artificial community, slightly influences their absolute cell numbers. The survival of *Buttiauxella* sp. and *S. shabanensis* after mixture and after desiccation (24 h) was in the range of the survival of the strains alone or even slightly better (*Buttiauxella* sp.: $S = 6.1 \times 10^{-2}$ (alone) versus $S = 1.5 \times 10^{-1}$ (mixed); *S. shabanensis*: $S = 1.6 \times 10^{-2}$ (alone) versus $S = 7.9 \times 10^{-3}$ (mixed)) (Fig. 2).

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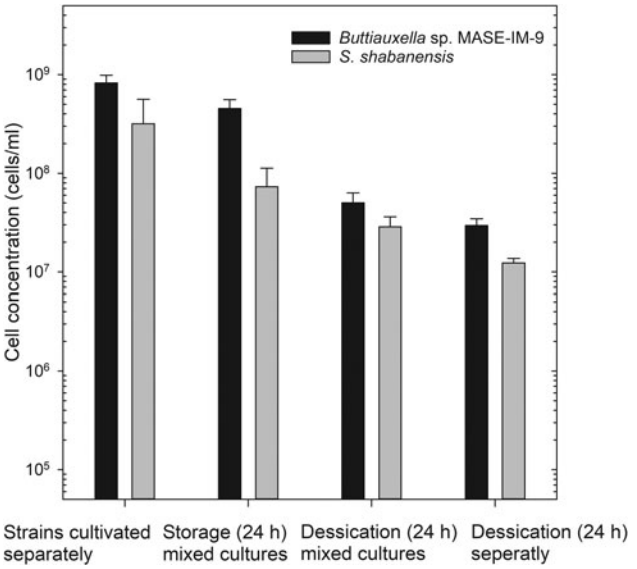

Fig. 2. *Cell numbers of* Buttiauxella *sp. (black) and* S. shabanensis *(grey) after cultivation under optimal conditions separately for 24 h (first columns). Cells numbers of both strains after being mixed for 24 h at RT (second columns). Cells numbers after desiccation of the mixed cultures for 24 h at RT under anoxic conditions (third columns). Cell numbers after desiccation of the strains separately (fourth columns). Cell concentration was evaluated by plating on strain specific solidified medium.*

Survival after polychromatic UV in liquid and after desiccation

Survival of cells in a liquid suspension (Fig. 3(a)) and in a desiccated state (Fig. 3(b)) was determined after irradiation with Mars-relevant polychromatic UV radiation (200–400 nm). The cells were treated under standard conditions, as well as in the presence of Mars regolith (MGS-1S 0.5% wt/v) and sugar (sucrose). In general, the same concentrations were used as in the long-term desiccation experiments.

Survival of *Buttiauxella* sp. in liquid after irradiation with polychromatic UV was visible in all cases up to 12 kJ m^{-2}, the highest tested fluence (Fig. 3(a)) and displaying typical shouldered survival curves. For *S. shabanensis* survival up to the highest tested fluence of 10 kJ m^{-2} was recognizable and the curves were less shouldered than those of *Buttiauxella* sp. (Fig. 3(c)). Nevertheless, the survival curves of both organisms bundle together. There is a marginal difference in the survival curves if MGS-1S was present. In the presence of Martian regolith, as well as in the presence of MGS-1S and sugar the survival of *Buttiauxella* sp. and *S. shabanensis* in liquid was about one order of magnitude higher than in the standard samples or in the samples with sugar only (Figs 3(a) and (c)).

In general, the elevated survival after desiccation in the presence of Martian regolith and sugar is visible here (Fig. 3(b)). The addition of sucrose or MGS-1S alone seems to have a minor effect on drought tolerance. More obvious is the difference in the presence of the added substances after irradiation at higher fluences and desiccation. In the samples of *Buttiauxella* sp. with Martian regolith either alone or in combination with sugar the survival rate is two orders of magnitude higher than in the standard samples (Figs 3(b) and (d)).

Survival after exposure to UVC (254 nm) and ionizing radiation in liquid

The survival after exposure to UVC and to ionizing radiation is shown here *via* the calculated F_{10} values (254 nm; 200–400 nm) and D_{10} values of *Buttiauxella* sp. MASE-IM-9 and *S. shabanensis* (Table 2). These values were compared with other spaceflight relevant strains, such as *Bacillus subtilis*,

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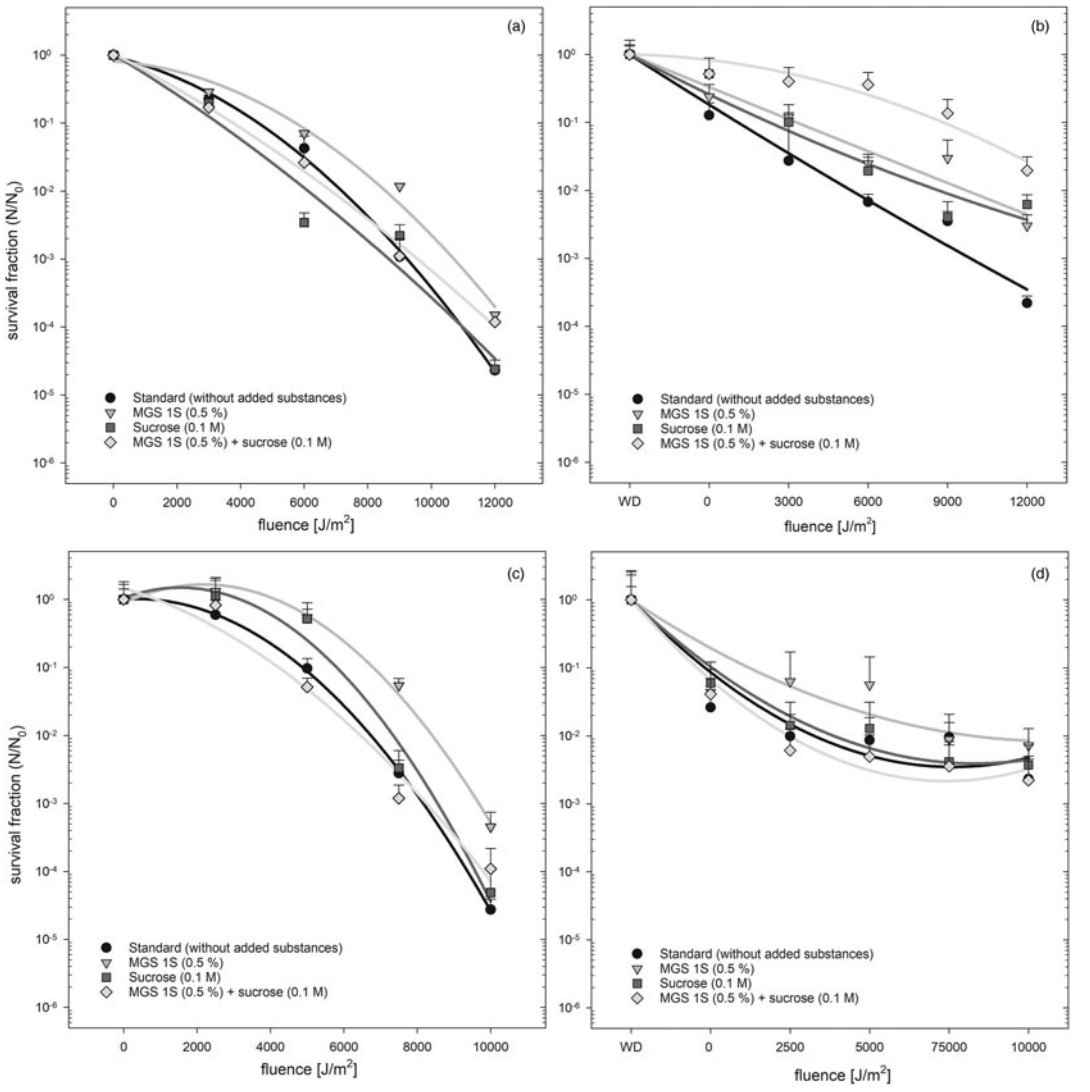

Fig. 3. *Survival after anoxic irradiation with polychromatic UV (200–400 nm) in liquid (a:* Buttiauxella *sp.; c:* S. shabanensis*) and after desiccation and polychromatic irradiation in combination (b:* Buttiauxella *sp.; d:* S. shabanensis*). In both irradiation treatments the standard conditions are marked with black bullets. The addition of Martian regolith (MGS-1S; 0.5% wt/v) is indicated with a grey triangle, the addition of sucrose (0.1 M) is indicated with a dark grey square. The addition of both substances is marked with a light grey diamond. In (b) and (d): WD (without desiccation) is the survival without desiccation and irradiation. The non-irradiated samples (0 J m^{-2}) is showing the survival after anoxic desiccation (24 h) only.*

as spores and as vegetative cells, *Deinococcus radiodurans*, and other slightly halophilic/aquatic microorganisms (*Aquifex pyrophilus* and *Hydrogenothermus marinus*).

The F_{10} values and therefore the tolerance to monochromatic UVC and polychromatic UV of *Buttiauxella* sp. and *S. shabanensis* are comparable and were in the range of other vegetative cells such as *B. subtilis* and *Escherichia coli*. The D_{10} value of *Buttiauxella* sp. is in the range of *E. coli*, which is also affiliated to the *Gammaproteobacteria*. The D_{10} values of *S. shabanensis* (also a gammaproteobacterial) is even higher than the D_{10} value of *B. subtilis* spores, but clearly lower than

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those of the halophilic archaeon *Halobacterium salinarum* and the radiation tolerant model organism *D. radiodurans*.

Microscopic observations

Survival after UV irradiation in the presence of Martian regolith was drastically increased in both organisms. In the following, the distribution of microbes in samples with Martian regolith was analysed microscopically to assess the potential shielding by mineral grains, especially in case the microbes would attach to the grains, and by potential aggregate formation where outer cells would shield inner cells from UV radiation.

The cells were stained with DAPI, a fluorescence dye which binds to adenine- and thymine-rich areas in the DNA and is indicating thereby the location of the DNA-containing cells on particles. To avoid technical errors a DAPI-staining protocol without centrifugation steps was used for our experiments.

The Martian regolith simulant MGS-1S has a granular structure with particles of different sizes (Figs 4(a)–(c)). EDS measurements of the regolith samples identified silicon in a significant amount, present in the form of diatomaceous earth, also known as diatoms or kieselgur particles with characteristic shapes (arrow in Fig. 4(c)) visible even with phase contrast microscopy (arrow in Fig. 4(d)). Cells of *Buttiauxella* and *Salinisphaera* cultivated in the presence of MGS-1S were observed as either swimming separately in broth or clustering together on particles including the kieselgur particles (arrow in Fig. 4(e)). This extensive clustering or even colony/biofilm-like growth is especially visible after DAPI-staining of the cells of both strains (Figs 4(e), (h), (f) and (i)). However, the particles themselves do not stain with DAPI and a fluorescence of the particles could be excluded (arrow in Fig. 4(d) and arrow in Fig. 4(g)).

Discussion

The conditions on the surface of Mars seem to be very harmful or even deadly for terrestrial life. To recap a few, the anoxia, the aridity, the Martian regolith itself, the unfiltered short wavelength radiation and the cosmic radiation influx. In the following, the results of the stress tests with the two polyextremophilic organisms *Buttiauxella* sp. and *S. shabanensis* will be discussed with respect to desiccation, Martian-relevant radiation in combination with Martian regolith.

The detailed analysis of the survivability under the different conditions mentioned above is the basis for the preparation of the MEXEM space experiment.

Both strains showed desiccation tolerance under anoxic conditions

First of all, it should be noted that both studied organisms are facultative anaerobes and they can cope with the reduced oxygen levels in the experiments conducted under anoxic conditions. The known tolerance of *Buttiauxella* sp. under standard conditions to desiccation was confirmed (Beblo-Vranesevic *et al.*, 2018). Additionally, the newly investigated non-spore forming bacterium *S. shabanensis* showed desiccation tolerance, although there are no periods of drought in its natural habitat. It is surprising that organisms originating from aqueous environments possess the ability to survive periods of water loss, as it was already shown for other organisms such as *Yersinia intermedia* MASE-LG-1 (Beblo-Vranesevic *et al.*, 2017*a*). One possible explanation for this phenomenon in *S. shabanensis* can be the very high salinity (between 21.2 and 23%), found in the brine immediately below the brine–seawater interface of Shaban Deep, in the Red Sea, where *S. shabanensis* has been isolated (Antunes *et al.*, 2003). Previous studies have already shown that osmotic stress has a positive influence on the desiccation tolerance of bacteria and archaea (Beblo-Vranesevic *et al.*, 2017*b*). Here, the role of compatible solutes is discussed in particular (Galinski *et al.*, 2014; Zeidler and Müller, 2019). *S. shabanensis* was reported as capable of accumulation of remarkably high concentrations of the compatible

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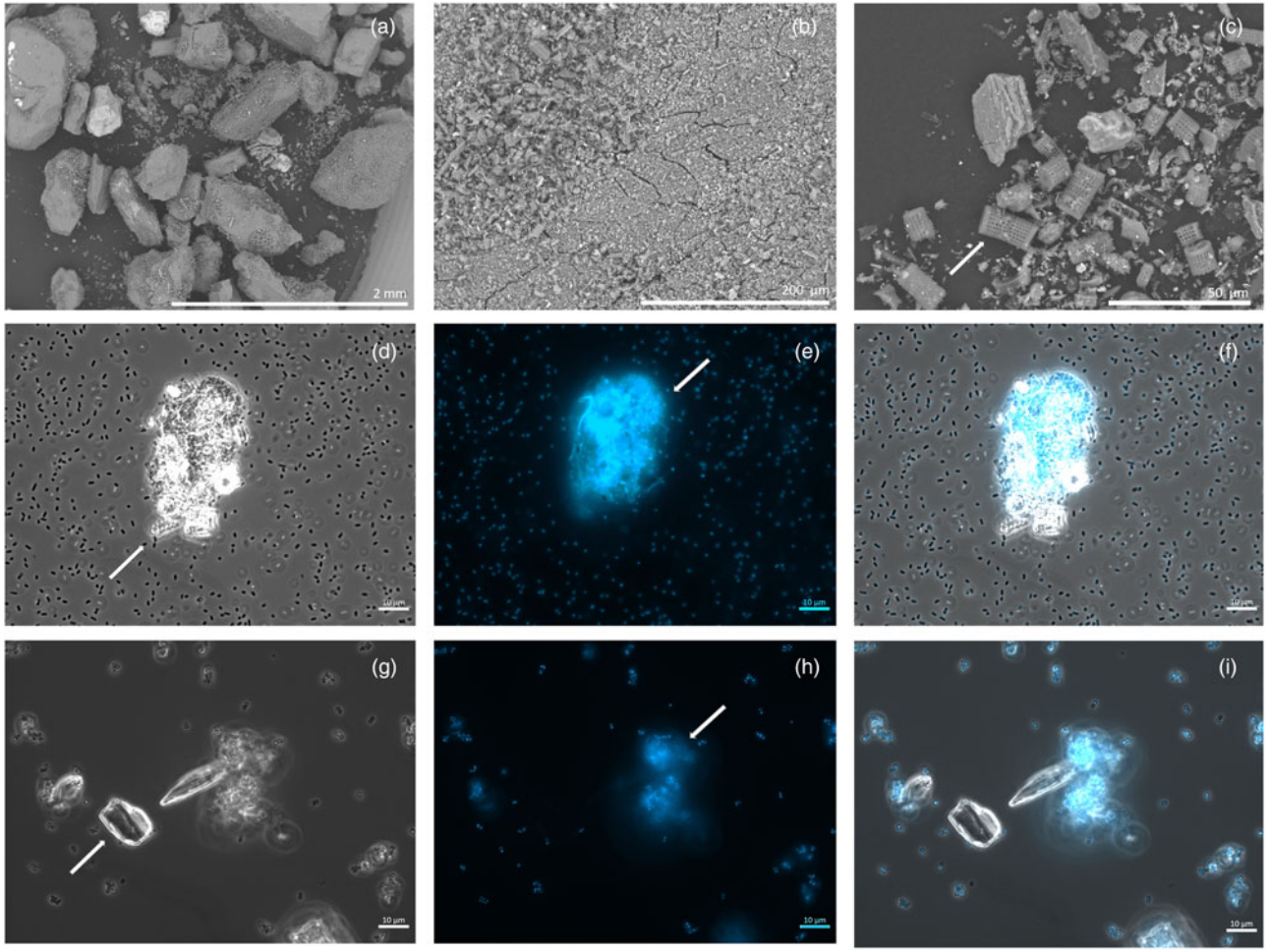

Fig. 4. *Scanning electron microscopic pictures of the used Martian regolith simulant MGS-1S (a–c). Phase contrast light microscopic pictures of grown cultures (d, g), DAPI-stained cultures (e, h) and merged multi-channels picture (f, i) of* Buttiauxella *sp. (d–f) and* S. shabanensis *(g–i) grown in the presence of Martian regolith simulant MGS-1S.*

solutes ectoine and betaine (above 4 M); additionally, it represented the first report of a prokaryote capable of accumulating glycerol (Antunes *et al*., 2003).

The drought tolerance of *Buttiauxella* sp. and *S. shabanensis* was also detectable and even slightly elevated when the two strains were previously mixed and dried together. This is of particular interest because communities are foreseen to be exposed in the space experiment MEXEM. This experiment should show whether the individual strains of the community can support or even protect each other during exposure under Martian conditions and thus survive better than the individual strains alone. For example, for the halophilic archaeon *Halococcus morrhuae* and the biofilm-forming bacterium *Halomonas muralis*, co-existing in natural environments, it could be shown that the biofilm of *H. muralis* can protect *H. morrhue* in space exposure experiments. A mixture of both strains was advantageous only for the survival of *H. morrhuae*, as shown when growing this microbe exposed independently and in combination with *H. muralis* to space conditions outside of the ISS for 534 days during the EXPOSE-R2 mission (Leuko *et al*., 2020). For other halophilic Archaea co-entombment in salt crystals showed improved viability of mixed-cultures versus isolated ones (Gramain *et al*., 2011).

If this protective effect of co-exposure of two strains is also true after irradiation treatment, as it was shown for *Bacillus* strains, it should be further investigated in the future. Thereby, spores of different *Bacillus* species were shown to have different survival pattern after polychromatic UV irradiation (200–400 nm), with *Bacillus pumilus* being more resistant than *B. subtilis*. The tolerance of *B. subtilis* was higher if the strains were irradiated together in equal amounts (Newcombe *et al*., 2005).

Martian regolith and sugar are influencing the survivability of both strains after desiccation positively

If we are thinking of the habitability of Mars, there is of course the Martian regolith itself which could be influencing possible life there. In the past, different (negative, neutral and positive) effects of Martian regolith on various life forms could be shown. For example, plants grow worse in MGS-1 than in other artificial Martian soils (Eichler *et al*., 2021). In other experiments, it has been shown that basalt as a Mars analogue regolith and also silicates in combination with a movement that mimics wind-driven saltation can have a negative or even deadly influence on microorganisms (Bak *et al*., 2017, 2019). A slight negative influence of Martian regolith for the fungal species *Cryomyces antarcticus* was recently demonstrated by Aureli *et al*. (2020). There, cells exhibited a reduced growth capability after exposure to higher doses of heavy ion particles (iron) up to 1 kGy when mixed with different Martian regolith analogues. This effect would be explainable with the production of secondary particles which are produced during irradiation of the regolith material (Dartnell *et al*., 2007).

On the other hand, there are studies where the Martian regolith did not influence microbial life negatively. For example, an aqueous extract of a Mars analogue regolith (including significant amounts of perchlorates) did not inhibit the germination rate of spores of *B. subtilis* and *B. pumilus* (Nicholson *et al*., 2012). Another relevant case is the survival after desiccation (400 days) of methanogenic Archaea in the presence of regolith analogues which was investigated by Schirmack *et al*. (2015).

However, there can also be a positive effect of Martian regolith analogues on different organisms. A positive influence on the survivability of fungi has been shown in the EXPOSE-R2 mission with different Martian and Lunar rock analogues (Onofri *et al*., 2019; Pacelli *et al*., 2020).

The tolerance to anoxic desiccation in our experiments could be increased in both organisms by adding Martian regolith and sugar. In both strains, this results in only slightly reduced survival rate over months, therefore making the strains suitable for the 3 month space exposure experiments as planned and specified by us in the MEXEM mission. An increased survival in the presence of Martian regolith was also simultaneously shown for some human-associated microbial species (*Pseudomonas*, *Klebsiella*, *Serratia* and *Burkholderia*). Here, the desiccation-sensitive organisms survived periods of dehydration better in the presence of MGS or even only survived desiccation in the presence of Martian regolith (Zaccharia, 2021). One reason for the positive effect could be the water retention potential of some of the regolith particles (Jänchen *et al*., 2014).

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This is of particular interest when thinking about various future Mars missions. Humans are surrounded by microorganisms and if these, including drought-sensitive organisms, are allowed to reach Martian soil and are eventually covered with Martian regolith by the ever-present Martian wind, they could survive the prevailing arid conditions.

It is known for some time that the addition of sugar increases the drought tolerance for example in *E. coli* and *Bradyrhizobium japonicum* (Louis *et al*., 1994; Streeter, 2003). This effect could be confirmed in our experiments, even if only in combination with MGS as a long-term experiment. The reason behind this could be that the sugars protect the membranes and proteins in bacteria during drying as described by Leslie *et al*. (1995). Additionally, it has recently been shown that lipid bilayers are also stabilized by sucrose during desiccation (Stachura *et al*., 2019), so this could also be a relevant aspect.

Both strains are able to withstand desiccation combined with polychromatic UV radiation

Buttiauxella sp. and *S. shabanensis* were able to survive polychromatic irradiation in a dried form and in liquid. For *D. radiodurans*, it has been shown that their cell withstand radiation better when in the dried state and in a monolayer under standard conditions rather than when in liquid and irradiation with polychromatic wavelength (Bauermeister *et al*., 2011). This phenomenon was not confirmed in the model organisms studied here. This is more an additive effect of drying and radiation than a synergistic/interactive one.

The addition of sucrose had only a marginal influence on the survival of *Buttiauxella* sp. and *S. shabanensis* after polychromatic irradiation in liquid. However, for *Halococcus hamelinensis* it has been shown that the addition of 100 mM trehalose to the growth medium elevated the tolerance to polychromatic UV and the F_{10} value tripled (Leuko *et al*., 2015). The addition of the powdery, fine-grained Martian regolith increases the tolerance to radiation in liquid medium in both strains. Comparable results have been shown in experiments with other materials such as ores or desert soil (Osman *et al*., 2008; Beblo *et al*., 2011).

The addition of the two additives also increases the survival rate after the combination of desiccation and radiation, probably due to physical protection properties of the regolith material. The added Martian regolith protects against polychromatic radiation, as it would also occur on the surface of Mars. UV radiation can only penetrate a few hundred micrometres deep into the soil (Cockell *et al*., 2000; Mancinelli and Klovstad, 2000). This means that organisms that would present a little deeper in the soil are not only protected from the radiation, but are also better protected from desiccation due to the Mars regolith. Similar effects could also be shown with *E. coli*. There too, components of the Martian soil were added to the cultures and it was concluded that its cells may be able to survive, but not grow, in surficial soils on Mars (Berry *et al*., 2010).

Previous space experiments have shown the protective properties of powdered material (rock, sandstone, meteorite and simulated Martian regolith) on spores exposed to solar UV radiation (Rettberg *et al*., 2004). Additionally, it was shown that none of these 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 (Rettberg *et al*., 2004). In contrast, for *D. radiodurans* the UV irradiation was very efficient in reducing the microbial populations under the investigated surface conditions. Neither the strain, whether mixed with nano-crystalline haematite or not, survived the exposure to Mars-like solar radiation. Viability rates were only measured when Goldenrod B-6090, a commercial mineral pigment produced by blending red haematite and yellow goethite pigments, was added to the bacteria (Morris and Golden, 1998; Pogoda De la Vega *et al*., 2007).

Both tested model organisms, *Buttiauxella* sp. and *S. shabanensis*, have an average to high tolerance to ionizing radiation. With a dose of 0.21 mGy day^{-1} (Matthiä *et al*., 2016) the majority of microbial life will be affected only in longer timescales such as decades. However, a potential influence of the Martian regolith in combination with ionizing radiation was not tested until now. It was shown that *B. subtilis* 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 (Moeller *et al*., 2010).

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The expected radiation environment of the MEXEM experiment

In space exposure experiments, mounted on the outside of the ISS, high fluences of UV radiation and rather small doses of ionizing radiation occur. For comparison, in the EXPOSE-R2 experiment with a mission duration of 469 days (approximately 17 months) 536 ± 116 MJ m^{-2} (5.36×10^8 J m^{-2}) in the wavelength range of 200–400 nm were modelled. Additionally, during this mission values up to 1 Gy were detected (Rabbow *et al.*, 2017). Taking the UV fluences measured in the EXPOSE-R2 mission into account, and even though the MEXEM mission will only be exposed for 3 months, the estimated UV (200–400 nm) fluence will be about 9.5×10^7 J m^{-2}. Therefore, the need of attenuation of the incoming UV with neutral filters is unavoidable as it is proposed in the mission description. This is still necessary even if the cells are mixed with protecting Martian regolith. The ionizing radiation that will occur in a 3 month exposure experiment in low Earth orbit plays a negligible role for microorganisms due to the low dose rate and total dose.

Conclusion

In general, the strategy of searching for new polyextremophilic and resistant organisms in extreme environments on Earth has proven successful for finding microbes capable of surviving Martian environmental conditions. *Buttiauxella* sp. had been previously shown to be tolerant of Mars relevant conditions such as perchlorate exposure and Martian gas composition and pressure (Beblo-Vranesevic *et al.*, 2020). *S. shabanensis* was also tolerant to Martian atmospheric conditions and pressure over 5 months even when combined with exposure to UV at an altitude of 36 km (Cortesão *et al.*, 2021). Our experiments further support that *Buttiauxella* sp. and *S. shabanensis* are two promising candidates that are suitable for the MEXEM space experiment.

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Conflict of interest. None.

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