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**Survivability of Psychrobacter cryohalolentis K5 under Simulated Martian Surface Conditions**

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9 3 **Survivability of *Psychrobacter cryohalolentis* K5 under**  
10 4 **Simulated Martian Surface Conditions**  
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**1 ABSTRACT**

2           Spacecraft launched to Mars can retain viable terrestrial microorganisms  
3 onboard that may survive the interplanetary transit. Such biota might compromise  
4 the search for life beyond Earth if capable of propagating on Mars. The current  
5 study explored the survivability of *Psychrobacter cryohalolentis* K5, a  
6 psychrotolerant microorganism obtained from a Siberian permafrost cryopeg,  
7 under simulated martian surface conditions of high ultraviolet (UV) irradiation,  
8 high desiccation, low temperature and low atmospheric pressure. First, a  
9 desiccation experiment compared the survival of *P. cryohalolentis* cells  
10 embedded, or not embedded, within a medium/salt matrix maintained at 25 °C for  
11 24 hrs within a laminar flow hood. Results indicated that the presence of the  
12 medium/salt matrix enhanced survival of the bacterial cells by 1 to 3 orders of  
13 magnitude. Second, tests were conducted in a Mars Simulation Chamber to  
14 determine the UV tolerance of the microorganism. No viable vegetative cells of  
15 *P. cryohalolentis* were detected after 8 hrs of exposure to Mars-normal conditions  
16 of 4.55 W/m<sup>2</sup> UVC irradiation (200-280 nm), -12.5 °C, 7.1 mbars and a Mars gas  
17 mix composed of CO<sub>2</sub> (95.3%), N<sub>2</sub> (2.7%), Ar (1.6%), O<sub>2</sub> (0.2%) and H<sub>2</sub>O  
18 (0.03%). Third, an experiment was conducted within the Mars chamber in which  
19 total atmospheric opacities were simulated at  $\tau = 0.1$  (dust free CO<sub>2</sub> atmosphere at  
20 7.1 mbars), 0.5 (normal clear-sky with 0.4 = dust opacity and 0.1 = CO<sub>2</sub> only  
21 opacity) and 3.5 (global dust storm) to determine the survivability of *P.*  
22 *cryohalolentis* to partially shielded UVC radiation. The survivability of the  
23 bacterium increased with the level of UVC attenuation, although population levels

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1 still declined several orders of magnitude compared to UVC absent controls over  
2 an 8-hr exposure period.  
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## 1 INTRODUCTION

2           Spacecraft that leave Earth inadvertently carry microscopic life forms  
3 onboard embedded within surface defects, wiring, electronic boards, and metal  
4 crevices (Schuerger, 2004). These microorganisms can endure the space  
5 environment, including conditions of high vacuum, extreme temperature  
6 fluctuations, high doses of solar UV irradiation, and other conditions hostile to  
7 living cells (Horneck *et al.* 2002; National Research Council 2005). Microbial  
8 contamination of spacecraft may pose a serious concern for space missions with  
9 life-detection payloads. A primary concern for near-term Mars surface missions  
10 is how to eliminate the risks of false-positives derived from the launched  
11 terrestrial bioloads (Rummel and Meyer, 1996; Schuerger, 2004). Thus,  
12 measurements of the survivability of bacteria under simulated martian surface  
13 conditions are essential to understanding which kinds of terrestrial  
14 microorganisms – if any – can potentially propagate on the surface of Mars  
15 (National Research Council 2005).

16           Recent studies have documented a high diversity of microorganisms in  
17 NASA spacecraft assembly facilities, which could readily attach to spacecraft (La  
18 Duc *et al.*, 2003; 2004; 2007; Link *et al.*, 2004; Moissl *et al.*, 2007;  
19 Venkateswaran *et al.*, 2001; 2003). Crawford (2005), Nicholson *et al.* (2005), and  
20 Tauscher *et al.* (2006) have hypothesized that maintaining a hygienic environment  
21 inadvertently selects for oligotrophic microbes. For example, spores of *Bacillus*

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1 sp. and *Clostridium* sp. isolated from spacecraft clean rooms have indeed shown  
2 unusual resistance to UV irradiation (Link *et al.*, 2004; Newcombe *et al.*, 2005).  
3 These microorganisms and possibly others may be conditioned and pre-adapted to  
4 the harsh conditions of spaceflight (La Duc *et al.*, 2004). Survival of terrestrial  
5 microbes in interplanetary space requires resistance to extreme low pressures,  
6 extreme temperatures, (ranging from -171 °C to +111 °C), severe desiccation,  
7 solar UV irradiation, solar particle/radiation events, and cosmic rays (Schuerger,  
8 2004). Despite these conditions, it has been estimated that a typical Mars  
9 spacecraft might retain  $3 \times 10^4$  –  $2 \times 10^7$  viable bacteria, located deep inside the  
10 vehicle, by the time it reaches the surface of the planet (Schuerger, 2004).

11 Schuerger *et al.* (2003; 2006) showed that after reaching the surface,  
12 99.9% of *Bacillus subtilis* spores exposed to simulated martian conditions were  
13 killed within 30 sec, and greater than six orders of magnitude reductions were  
14 observed after 180 min. Results suggested that direct exposure to UV irradiation  
15 was the most limiting environmental factor for *B. subtilis* survival on the surface,  
16 whereas simulated Mars gas composition, pressure and temperature had no  
17 measurable effects (Schuerger *et al.*, 2003). Cockell *et al.* (2005) had similar UV-  
18 dependent results when studying the survivability of the cyanobacterium  
19 *Chroococcidiopsis* sp. 029 under simulated martian conditions. Together, these  
20 experiments predict a low probability of bacterial surface contamination on Mars,  
21 provided that terrestrial organisms are exposed to an ambient, daytime UV flux

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1 (Schuerger *et al.*, 2003; 2006; Cockell *et al.*, 2005). The absence or attenuation of  
2 UV light, however, might create very different survivability scenarios. Both  
3 Schuerger *et al.* (2003) and Cockell *et al.* (2005) found that the survival of  
4 bacteria increased significantly when shielded from UV irradiation by thin layers  
5 of dust or rocks. In fact, *Chroococcidiopsis* sp. 029 retained viability after 8 hrs  
6 under rock coverage only 1 mm thick (Cockell *et al.*, 2005) and *B. subtilis*  
7 survived 8 hrs of UV irradiation when covered by only a 0.5 mm coating of dust  
8 (Schuerger *et al.*, 2003). In addition, Morozova *et al.* (2006) found that after a 22  
9 d exposure to simulated thermo-physical conditions at martian low- and mid-  
10 latitudes, up to 90% of methanogenic archaea from Siberian permafrost survived  
11 in pure cultures as well as in environmental samples. It is conceivable that  
12 microbes on the external surfaces of Mars spacecraft will be protected from  
13 radiation by atmospheric dust events that deposit dust on the spacecraft surface, or  
14 by mission operations that involve drilling into rocks or driving through regolith.

15 Psychrophilic and psychrotolerant bacteria may possess unique capacities  
16 for dealing with the martian surface environment (Cockell *et al.*, 2005; National  
17 Research Council, 2005; Nicholson *et al.*, 2005). Throughout extremely cold  
18 climates on Earth, these specially-adapted microorganisms not only survive but  
19 thrive in conditions with low temperatures, oligotrophic nutrient regimes, and  
20 limited water resources (Cavicchioli, 2002). Recently cultured bacteria from

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1 Siberian permafrost and Antarctic ice sheets can metabolize at temperatures down  
2 to  $-20^{\circ}\text{C}$  (Rivkina, 2000; Bakermans *et al.*, 2006).

3 The primary objective of the current study was to determine whether the  
4 psychrotolerant bacterium, *Psychrobacter cryohalolentis* K5, could survive under  
5 simulated Mars conditions if provided with adequate levels of nutrients, salts, and  
6 moisture. Survivability was defined by the viability of bacterial cells. The  
7 experiments herein did not measure growth or replication of *P. cryohalolentis*.  
8 The goal was to determine whether the survivability of *P. cryohalolentis* was  
9 greater than the mesophilic species tested previously in simulated martian  
10 conditions (Schuerger *et al.*, 2003; Cockell *et al.*, 2005; Newcombe *et al.*, 2005;  
11 Schuerger *et al.*, 2005; Schuerger and Nicholson, 2006; Tauscher *et al.*, 2006),  
12 particularly when protected from UV irradiation.

13

## 14 MATERIALS and METHODS

### 15 *Strain isolation and physiology*

16 *Psychrobacter cryohalolentis* K5 was isolated from a  $-12^{\circ}\text{C}$  subsurface  
17 hypersaline cryopeg in the Kolyma lowlands of Siberia, Russia. Bakermans *et al.*  
18 (2006) characterized the bacterium as a gram-negative, strictly aerobic,  
19 coccobacillus that was non-motile, non-pigmented, non-spore forming, and  
20 capable of growth in a pH range from 6.0 to 9.5 and a salinity range of 0 to 1.7 M  
21 NaCl. Cell dimensions were 0.9-1.3  $\mu\text{m}$  long and 0.5-0.8  $\mu\text{m}$  wide. The optimal



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1 growth temperature for the species was 22 °C, but the growth range stretched  
2 from -10 to 30 °C (Bakermans *et al.*, 2006). At its optimal growth temperature,  
3 generation time was just under 5 hrs (Bakermans and Neelson, 2004).

#### 4 *Standard microbiological procedures*

5 The *P. cryohalolentis* K5 strain was cultivated at room temperature (25  
6 °C) in a liquid growth medium made from the following chemicals in 1 L of  
7 deionized water: 1 g of Bacto™ yeast extract, 5 g of Bacto™ peptone, and 17 g of  
8 sea salts. For solid media, 16.5 g of Bacto™ agar were added to 1 L of solution.  
9 All media ingredients were obtained from Becton Dickinson and Company  
10 (Sparks, MD, USA), except for the sea salts, which were obtained from Sigma-  
11 Aldrich Chemical Company (St. Louis, MO, USA). Inoculum of *P.*  
12 *cryohalolentis* was prepared 24 hrs prior to use by incubating vegetative cells in a  
13 test tube with 10 mL of liquid media at 25 °C. On solid media at 25 °C, colonies  
14 grew as circular, smooth, opaque and ~ 1 mm diameter after 36-48 hrs.

15 In order to simulate bacterial adhesion to, and survival on, spacecraft  
16 surfaces, vegetative cells of *P. cryohalolentis* were placed on aluminum coupons  
17 (2 cm x 1 cm x 1 mm) previously coated with a chromate conversion film (Iridite  
18 14-2, MacDermid, Inc., Waterbury, CT, USA) (see Schuerger *et al.*, 2005; 2008).  
19 The Iridite surface treatment reduces corrosion on aluminum and is often used on  
20 spacecraft aluminum components (Schuerger *et al.*, 2005). The aluminum  
21 coupons were dry-heat sterilized at 130 °C for 24 hrs and allowed to cool to 25 °C

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1 before bacteria were deposited onto the aluminum surfaces. Under aseptic  
2 conditions, vegetative cells of *P. cryohalolentis* from liquid cultures were applied  
3 to the center of coupons in 100- $\mu$ L spots and allowed to dry at 25 °C in a laminar  
4 flow hood for 2 hrs (NuAire Inc., model NU-201-430, Plymouth, MN, USA).  
5 Once dried the media/salt residue would form a crystalline matrix that both  
6 encased bacteria and provided nutrients to sustain cells (Fig. 1). This coupon  
7 preparation procedure was standardized and used for all experiments. Media/salt  
8 residues on coupons were imaged using a high-resolution video microscope  
9 (model VH-7000, Keyence Corp. of America, Woodcliff Lake, NJ, USA).

#### 10 ***Desiccation experiment for comparing coupon preparation techniques***

11 A 24-hr desiccation experiment was conducted with *P. cryohalolentis* at  
12 room temperature (25 °C) and pressure (1013 mbar). The purpose of the  
13 experiment was twofold: (i) to evaluate the desiccation resistance of the bacterium  
14 on simulated spacecraft surfaces, and (ii) to determine if survivability was  
15 enhanced by encasement within the medium/salt matrix. Two sets of coupons  
16 were prepared for the experiment, hereafter referred to as 'media' and 'non-  
17 media' coupons. The media coupons contained *P. cryohalolentis* vegetative cells  
18 embedded in the medium/salt matrix and dried onto the surface of the coupons, as  
19 described in the previous section. For the non-media coupons, cells were grown  
20 on solid sea-salt media for 24 hrs at 25 °C, mechanically harvested and diluted in  
21 sterile deionized water (SDIW) to densities of  $3.19 \times 10^9$  cells per mL as

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1 quantified by a Spectronic-20 spectrometer (model 4001, Spectron Instruments,  
2 USA) set at 400 nm. Quantified cell suspensions were then spotted onto  
3 spacecraft coupons in 100- $\mu$ L volumes per coupon. The T = 0 samples for both  
4 the media and non-media coupons were sampled, while the remaining coupons  
5 were placed in a Petri dish to desiccate in a dark laminar flow hood for 24 hours.  
6 The surviving bacteria from the media coupons for T = 0 and T = 24 hour samples  
7 were estimated by the Most Probable Number (MPN) method. The minimum  
8 detection limit of the MPN assay was 10 cells per coupon. Cells were re-  
9 suspended in 100  $\mu$ L of liquid media, followed immediately by processing  
10 through a series of 10-fold liquid media dilutions, in which each diluted sample  
11 was plated onto solid media. For the non-media coupons, the method described  
12 by Schuerger *et al.* (2003; 2006) was followed, in which coupons were placed  
13 into plastic tubes with autoclaved deionized water and shaken with 1 g of heat-  
14 sterilized silica sand (24 h at 130 °C). Next, 10-fold serial dilutions with sterile  
15 water were performed and aliquots were dispensed onto solid media and the  
16 number of viable cells estimated by MPN. For both the media and non-media  
17 coupons, the numbers of viable bacteria were estimated after cultures had  
18 incubated at 25 °C for 42 hr.

### 19 *Mars Simulation Chamber experiments*

20 The Mars Simulation Chamber (MSC) is located in the Space Life  
21 Sciences Laboratory at Kennedy Space Center, FL, USA. Details of the MSC

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1 construction and mechanical function have been published elsewhere (Schuerger  
2 *et al.*, 2008). The MSC system was capable of reproducing martian surface  
3 environmental conditions, including: pressure (down to 0.1 mbar), temperature (-  
4 100 to +200 °C), atmospheric gas composition (top five gases; see below), and  
5 atmospheric dust loads (optical depths [ $\tau$ ] from dust-free skies [0.1] to global dust  
6 storms [3.5]). Temperature for all experiments was maintained at -12.5 °C, based  
7 on average daytime highs recorded by the Viking 1 and 2 landers (Owen, 1992)  
8 and just below the minimum growth temperature reported for *P. cryohalolentis*  
9 (Bakermans *et al.*, 2006). Pressure was fixed at 7.1 mbar. A gas mixture (Boggs  
10 Gases, Titusville, FL) representing the martian atmosphere was proportioned into  
11 the chamber using a mass-flow controller and consisted of: CO<sub>2</sub> (95.3%), N<sub>2</sub>  
12 (2.7%), Ar (1.6%), O<sub>2</sub> (0.2%) and H<sub>2</sub>O (0.03%). Ultraviolet irradiation was  
13 generated within the spectral range of 200-400 nm based on a Mars UV model  
14 described previously (Schuerger *et al.*, 2003; 2006). Fluence values for UVC  
15 (200-280 nm), UVB (280-320 nm), and UVA (320-400 nm) were 4.55, 8.82 and  
16 36.95 W/m<sup>2</sup>, respectively. Total UV flux from this calibration was 50.32 W/m<sup>2</sup>.

17 For the first Mars simulation experiment, UV light was passed through  
18 neutral density filters (Maier Phototonics, Inc., Manchester Center, VT) to create  
19 an optical depth of  $\tau = 0.1$ , which simulated the surface of equatorial Mars at its  
20 mean orbital distance from the Sun, under dust-free skies (Schuerger *et al.*, 2003;  
21 2006). Bacteria were prepared using the standard spotting technique on

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1 aluminum coupons in which vegetative cells were embedded within a  
2 medium/salt matrix. The number of bacteria applied in 100- $\mu$ L drops onto  
3 coupons was estimated to be  $1.8 \times 10^{10}$  from 9 replicates. Coupons were loaded  
4 into the MSC in pre-sterilized glass Petri dishes, which sat directly on the upper  
5 surface of the LN<sub>2</sub> cold-plate. The first experiment was designed to measure  
6 bacterial survival over increasing time exposures, where one sol was the  
7 equivalent of 8 hrs UV exposure and 16 hrs of darkness. Time-steps for the  
8 experiment were 0.5, 1, 3 and 9 sols. Each time-step had bacterial coupons  
9 divided into one of three groups (each with triplicate samples) designed to  
10 pinpoint the effect of UV irradiation on survivability: Mars (+UV), Mars (-UV),  
11 and Earth controls (-UV). The Mars (+UV) coupons were inside the MSC and  
12 exposed directly to UV irradiation; the Mars (-UV) coupons were also inside the  
13 MSC but completely protected from UV light; and the Earth control (-UV)  
14 coupons were placed outside the MSC, wrapped with aluminum foil, and left at  
15 room conditions (25 °C, 1013 mbar). Following the martian simulation, all  
16 coupons were assayed for viability using the MPN method described above. The  
17 experiment was repeated 1 week later under identical conditions, yielding a total  
18 of 6 replicates per treatment.

19 The second Mars simulation experiment was designed to determine how  
20 atmospheric dust loading might affect survivability of bacteria cells embedded  
21 within the dried media/salt matrix. Neutral density filters (Fig. 2) composed of

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1 silica glass and a thin Ni-Cr-Fe alloy coating were used to establish optical depths  
2 ( $\tau$ ) of 0.1, 0.5 or 3.5 for UV irradiation, effectively simulating (i) a dust-free CO<sub>2</sub>  
3 atmosphere, (ii) a typical clear sky condition with low levels of dust, or (iii) a  
4 global dust storm condition (sensu Schuerger *et al.*, 2003; 2006). A UV  
5 spectrometer (model IL1400A, International Light, Newburyport, WA, USA),  
6 was used to measure UVC values of 4.55, 3.57 and 0.21 W/m<sup>2</sup> for optical depths  
7 of 0.1, 0.5, and 3.5, respectively. Alongside the three different simulated dust  
8 loading conditions, additional bacterial coupons were shielded from all UV  
9 irradiation within the MSC, placed in a sample holder fitted with an opaque  
10 aluminum plate instead of a neutral density filter (Fig. 2). A set of coupons were  
11 held as Earth controls outside of the MSC and maintained at room temperature  
12 conditions without UV exposures. Coupons were exposed to martian conditions  
13 of 7.1 mbar, -10 °C, and Mars gas composition for 24 hrs with a 1-sol UVC  
14 simulation (i.e., 8 hrs of UV irradiation), and immediately assayed for survival.  
15 The experiment was repeated under identical conditions, for a total of 6 replicates  
16 per treatment.

#### 17 *Statistical treatment of data*

18 Analysis of survivability values (log-transformed means) was performed  
19 using the statistical program R version 2.3.1 (The R Foundation for Statistical  
20 Computing, Vienna, Austria, 2006). Data were subjected to one-way permutation  
21 tests, both rank and pairwise, to compare mean population differences across

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1 groups and controls at a 95% confidence level ( $P < 0.05$ ). For the Mars  
2 simulations, the Wilcox test was used to examine population values across time  
3 between different treatment groups. The Kruskal-Wallis rank sum test was used  
4 to analyze population values across time within individual treatment groups.

## 5 **RESULTS**

### 6 *Survivability from desiccation experiment*

7 The desiccation experiment sought to determine the effects of drying  
8 vegetative cells of *P. cryohalolentis* onto aluminum coupons either alone (i.e., no  
9 buffers or medium/salt matrix) or embedded in a medium/salt matrix. Cells of *P.*  
10 *cryohalolentis* in both the media and non-media treatments were desiccated under  
11 identical environmental conditions for 24 hrs. Survivability assays showed a  
12 decline of several orders of magnitude for both experimental groups over the  
13 desiccation interval (Fig. 3). Although both groups declined, cells of *P.*  
14 *cryohalolentis* embedded within the media/salt matrix survived better than the  
15 non-media cells (permutation test:  $P = 0.032$ ); a 4-order of magnitude reduction  
16 compared to a 6-order of magnitude reduction, respectively, was observed. The  
17 rate of decline for the bacteria in the media group was consistent with the results  
18 reported in the Mars simulations. Although a difference in initial population  
19 values for media and non-media coupons existed due to the disparate preparation  
20 techniques, this did not affect measuring survivability as a proportional value.

### 21 *Survivability from Mars Simulation Chamber experiments*

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1           The survivability of *P. cryohalolentis* was negatively affected by  
2 exposures to UV irradiation under simulated Mars low-dust sky conditions ( $\tau =$   
3 0.1) (Fig. 4). Survivability was dependent upon the treatment type and length of  
4 time exposure (Kruskal-Wallis rank sum test:  $P < 0.05$ ). The number of viable  
5 cells after the Mars (+UV) treatment were significantly lower compared to the  
6 Mars (-UV) and Earth control (-UV) groups (Wilcox Test:  $P < 0.001$ ), and fell  
7 below the detection limits of the MPN assay by the end of sol 1. The difference  
8 between the number of viable cells on the Mars (-UV) chamber coupons and  
9 Earth control (-UV) coupons was not statistically significant (Wilcox Test:  $P =$   
10 0.156). Although surviving populations of *P. cryohalolentis* for both (-UV)  
11 treatments persisted for the 9 sol simulation, the number of viable cells still  
12 declined by 100x over the course of the experiment (Kruskal-Wallis rank sum  
13 test: Mars (-UV) coupons  $P = 0.0149$ ; Earth control (-UV) coupons  $P = 0.0120$ ).

14           In the second MSC experiment the recovered numbers of vegetative cells  
15 in each dust treatment were significantly lower than control treatments (Kruskal-  
16 Wallis rank sum test:  $P < 0.001$ ) (Fig. 5). No viable cells of *P. cryohalolentis*  
17 were recovered from coupons exposed to a dust simulation of  $\tau = 0.1$ .  
18 Furthermore, the  $\tau = 0.5$  dust simulation experiment yielded approximately  $1 \times$   
19  $10^3$  viable cells per coupon, a 4-order of magnitude decline from the (-UV) Mars  
20 controls. The greatest numbers of viable bacteria were recovered from the  $\tau = 3.5$



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1 coupons even though it was several orders of magnitude lower than that of the  
2 initial time-step.

### 3 DISCUSSION

4 The desiccation experiment demonstrated that *P. cryohalolentis* was  
5 especially sensitive to drying, by exhibiting a > 6 order of magnitude population  
6 reduction in recovered cells desiccated for 24 hrs. In order for a terrestrial  
7 microbe to survive and proliferate on Mars, it would need to tolerate, at minimum,  
8 desiccating conditions during the six-month long interplanetary transit, and also  
9 on the dry planet surface for periods long enough to become dispersed into niches  
10 conducive for growth. Hence, the importance of the *P. cryohalolentis* population  
11 decline in the desiccation experiment was threefold: (1) some of the Mars  
12 Simulation Chamber viability reductions could be attributed to desiccation alone,  
13 (2) *P. cryohalolentis* did not appear tolerant to the kinds of desiccating conditions  
14 that would arise in a forward contamination context during a Mars robotic  
15 mission, and (3) even the presence of a media/salt matrix (i.e., biofilm) did not  
16 provide sufficient long-term protection from water stress to dramatically enhance  
17 survival over non-media treatments.

18 The inability of *P. cryohalolentis* to maintain its population in the 24-hr  
19 desiccation period was unexpected, considering that this bacterium was recovered  
20 from salty, oligotrophic permafrost (Bakermans *et al.*, 2006). When bacteria  
21 inoculated into liquid media were applied onto coupons, the dried residues were

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1 composed of a crystalline matrix that (1) encased bacteria with an environmental  
2 barrier, and (2) provided nutrients to cells. The desiccation experiment revealed  
3 the extent of enhanced survivability was minimal, by showing in parallel, the  
4 declining population of the same *P. cryohalolentis* cells without the media/salt  
5 matrix. It is also worth noting that the desiccation experiment was conducted  
6 outside the MSC in a laminar flow hood at room-normal humidity [40-50% at 23  
7 °C)], not the 0.03% H<sub>2</sub>O present in the martian atmosphere. True desiccation  
8 would have required a more sophisticated drying device, such as the instrument  
9 described by Kendrick and Kral (2006), which would likely have further reduced  
10 the survivability of *P. cryohalolentis*.

11 Even assuming that *P. cryohalolentis* could tolerate interplanetary  
12 desiccation and reach the surface of Mars in viable form, the current study  
13 indicates that UV irradiation would kill any sun-exposed cells within 8 hrs or less.  
14 This death rate is slightly slower than previously reported values for the bacterium  
15 *Bacillus subtilis* HA101 (Schuerger *et al.*, 2003; 2006) and *Chroococcidiopsis* sp.  
16 029 (Cockell *et al.*, 2005). The slightly increased length of time (up to 8 hrs)  
17 observed for survival with *P. cryohalolentis* might be related to the protective  
18 medium/salt matrix that surrounded the vegetative cells in the current study. For  
19 example, with *B. subtilis* HA101 (Schuerger *et al.*, 2003), no microbial biofilm  
20 was associated with endospores exposed to martian conditions, but some  
21 extracellular material was observed associated with cells of *Chroococcidiopsis* sp.

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1 029 in other similar Mars simulations (Cockell *et al.*, 2005). Comparing these  
2 two papers with the results from the current study, the numbers of recovered  
3 viable cells may be correlated with an increase of extracellular material (*B.*  
4 *subtilis* < *Chroococciopsis* < *P. cryohalolentis*).

5 The first Mars simulation experiment isolated UV irradiation as the  
6 primary biocidal factor by showing significantly different survivability across  
7 Earth control (-UV), MSC (-UV), and MSC (+UV) treatments. Bacteria exposed  
8 to direct UV irradiation died within 1 sol, whereas cells exposed to identical  
9 conditions but shielded from UV light survived beyond that exposure length; yet  
10 did fall by 2 orders of magnitude by 9 sols. It is worth noting that the dramatic  
11 reductions observed for (+UV) exposed Mars samples occurred while the  
12 vegetative cells were embedded in a medium/salt matrix that would have been  
13 expected to partially or fully attenuate the UV irradiation. Previous studies  
14 (Mancinelli and Klovstad, 2000; Schuerger *et al.*, 2005) have suggested that  
15 biofilms might significantly enhance the survival of microorganisms under  
16 martian conditions by attenuating solar UV irradiation. However, the results of  
17 the current study do not support this conclusion.

18 Since Earth (-UV) and Mars (-UV) populations of *P. cryohalolentis*  
19 changed similarly across time and were sustained through the final time-step (sol  
20 9), it was concluded that martian temperature, pressure, and gas composition had  
21 little effect on *P. cryohalolentis* survival. This result is consistent with previous

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1 reports using a variety of different microorganisms (Schuerger *et al.*, 2003;  
2 Cockell *et al.*, 2005; Nicholson *et al.*, 2005; Schuerger *et al.*, 2006) and may be  
3 attributed primarily to water stress.

4         Increasing simulated dust levels enhanced the survivability of vegetative  
5 cells of *P. cryohalolentis* over 1 sol. The low-dust sky treatment ( $\tau = 0.1$ ) killed  
6 all bacteria by 1 sol; reproducing results from the first MSC experiment Mars  
7 (+UV) coupons. Only one-sixth of the coupons under the normal clear-sky dust  
8 scenario ( $\tau = 0.5$ ) retained viable bacteria after 1 sol simulations. The global dust  
9 storm scenario ( $\tau = 3.5$ ) yielded higher survivability, but with only moderate  
10 significance, suggesting that even trace amounts of UV irradiation on Mars may  
11 dramatically reduce bacterial survival.

## 12

### 13 CONCLUSIONS

14         Psychrophilic and psychrotolerant microorganisms have been regarded as  
15 serious contamination threats to Mars (National Research Council, 2005). *P.*  
16 *cryohalolentis*, however, had virtually no tolerance to the martian environment,  
17 even during scenarios where UV irradiation was partially or fully attenuated.  
18 Since survival of *P. cryohalolentis* was strongly inhibited by martian surface  
19 conditions, subsequent growth, replication, and proliferation seem unlikely.  
20 Desiccation and UV irradiation were identified as the primary lethal factors in  
21 these simulations; consistent with results reported for mesophilic bacteria tested

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1 under similar martian conditions (Cockell *et al.*, 2005; Newcombe *et al.*, 2005;  
2 Schuerger *et al.*, 2003; 2005; 2006; Tauscher *et al.*, 2006).

3 While simulated atmospheric dust conditions in the current study  
4 permitted small amounts of biocidal UV light to reach cells of *P. cryohalolentis*,  
5 natural UV-shields that more thoroughly block light on the martian surface are  
6 imaginable. Future research should continue to explore how the survivability of  
7 psychrophilic and psychrotolerant bacteria on the surface of Mars changes with  
8 UV-shielding. Covering bacteria with a thin layer of dust, for example, should  
9 increase survivability (Mancinelli and Klovstad, 2000; Schuerger *et al.*, 2003), as  
10 would embedding bacteria into rocks or crevices within spacecraft material  
11 (Schuerger *et al.*, 2005). If UV irradiation was totally eliminated as an  
12 environmental factor, desiccation could become the major constraint on  
13 survivability of *P. cryohalolentis*. In the results presented here, viable *P.*  
14 *cryohalolentis* cells at the final MSC experimental time-step (sol 9) should not  
15 necessarily be considered Mars-adapted, since our results, and those of (Beatty *et*  
16 *al.*, 2006) indicate that water stress might continue to decrease bacterial  
17 survivability over time. More research, therefore, is needed to understand the  
18 survivability of psychrophilic and psychrotolerant microbes over time in cold,  
19 extremely desiccating conditions. Identification of purported biogenic trace gases  
20 and microbial cells in ice cores suggest that certain bacteria can survive hundreds  
21 of thousands of years at  $-40\text{ }^{\circ}\text{C}$ , maintaining DNA and protein integrity so that

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1 when conditions improve cells can reactivate and grow (Price and Sowers, 2004;  
 2 Napolitano and Shain, 2004). This might mean that contaminating bacteria cells  
 3 which do not grow or proliferate on Mars during the period of robotic or human  
 4 exploration could eventually be revitalized on a geological timescale (Willerslev  
 5 *et al.*, 2004). Therefore, until the survivability of additional extremophile life  
 6 forms has been evaluated under simulated martian conditions, the effort to  
 7 classify microorganisms found in spacecraft assembly facilities and to develop  
 8 economical methods to sanitize spacecraft seems to be an appropriate  
 9 conservative measure (Schuerger *et al.*, 2003; La Duc *et al.*, 2004; Tauscher *et al.*,  
 10 2006; Moissl *et al.*, 2007).

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5 instrumentation; and Bill Parsons at Kennedy Space Center (NASA).

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**1 FIGURE LEGENDS****2 Fig. 1. Coupon preparation method for *Psychrobacter cryohalolentis* K5. (A)**

3 The standard aluminum coupon preparation procedure used for all experiments,  
4 with the 100- $\mu$ L liquid media/salt matrix spotted on each coupon and containing  
5 *P. cryohalolentis* vegetative cells. (B) Microscopic view of a coupon surface after  
6 the liquid media spot had dried; i.e., media technique. (C) Microscopic view of a  
7 coupon surface prepared with liquid water; i.e., non-media technique.

**8 Fig. 2. Experimental setup for Mars Simulation Chamber (MSC) atmospheric**

9 dust experiment. Treatments are shown resting on the liquid-nitrogen (LN<sub>2</sub>) cold  
10 plate within the MSC system with neutral density filters calibrated to simulate  
11 different atmospheric dust loads. The Mars (-UV) control is located at the far  
12 back, while the three dust scenarios are in the foreground: Mars dust load  
13 simulations are shown for  $\tau = 0.1$  (upper left);  $\tau = 0.5$  (upper right); and  $\tau = 3.5$   
14 (bottom right). The coupons within the glass Petri dish (lower left) can be  
15 disregarded. The experiment delivered 8 hrs of UV irradiation during a total Mars  
16 simulation time of 24 hrs.

17  
18 **Fig. 3. Effect of preparation technique on *P. cryohalolentis* K5 desiccation over a**  
19 24-hr period. The standard liquid media preparation technique (used throughout  
20 this study) was compared to a water-diluted, non-media procedure which left  
21 bacterial cells unprotected on coupon surfaces. Both coupon preparation  
22 treatments were desiccated at 25 °C for 24 hrs and were then assayed for survival

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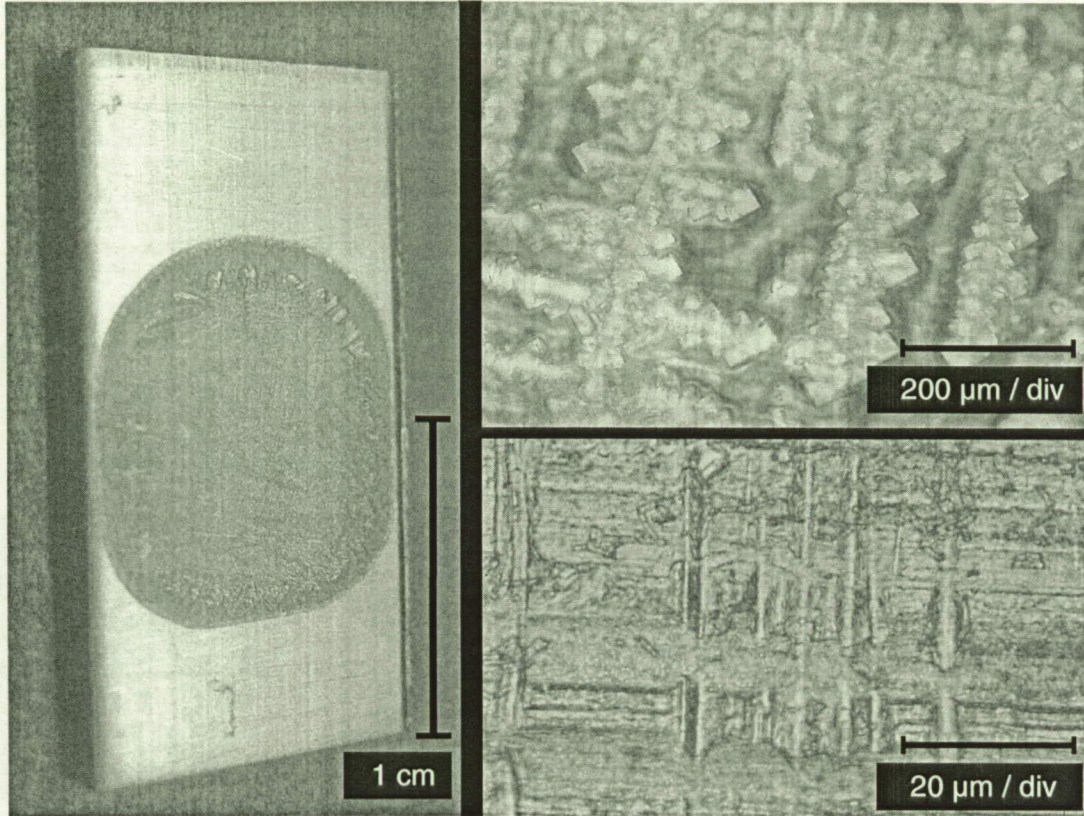
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1 by the MPN assay. Values are means of multiple replicates; *bars* represent  
2 standard errors of the means.

3 **Fig. 4.** Mars Simulation Chamber experiment under simulated low-dust sky ( $\tau =$   
4 0.1) conditions. *Psychrobacter cryohalolentis* K5 doped coupons (i.e., embedded  
5 in the medium/salt matrix) were exposed to simulated martian conditions for five  
6 distinct time intervals. Values are means of six replicates; *bars* represent standard  
7 errors of the means.

8 **Fig. 5.** Atmospheric dust experiment varying irradiation exposure in MSC.  
9 *Psychrobacter cryohalolentis* K5 doped coupons were exposed to martian  
10 conditions under different simulated atmospheric conditions for 1 sol (i.e., 24-hr  
11 in MSC with 8-hr UV irradiation). For  $\tau = 0.1$ , the asterisk indicates that the  
12 number of bacteria recovered were below the MPN detection limit of 10 cells per  
13 coupon (dotted line) for all replicates (i.e., 100% of coupons were negative).  
14 Values are means of six replicates; *bars* represent standard errors of the means.  
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**Fig. 1.**

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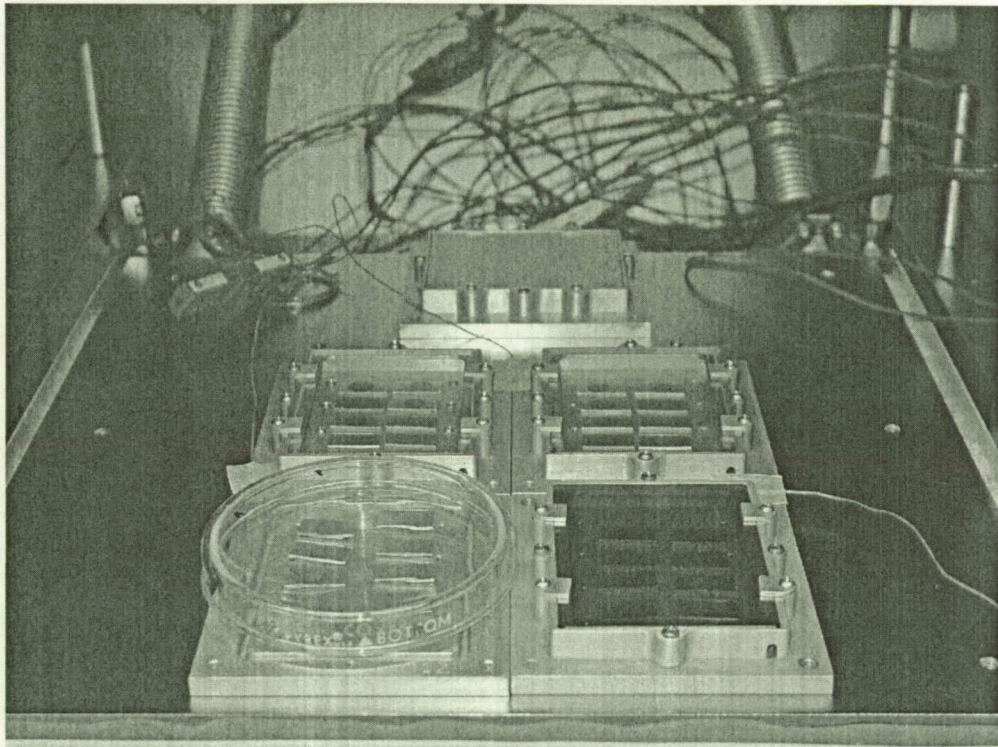


Fig. 2.

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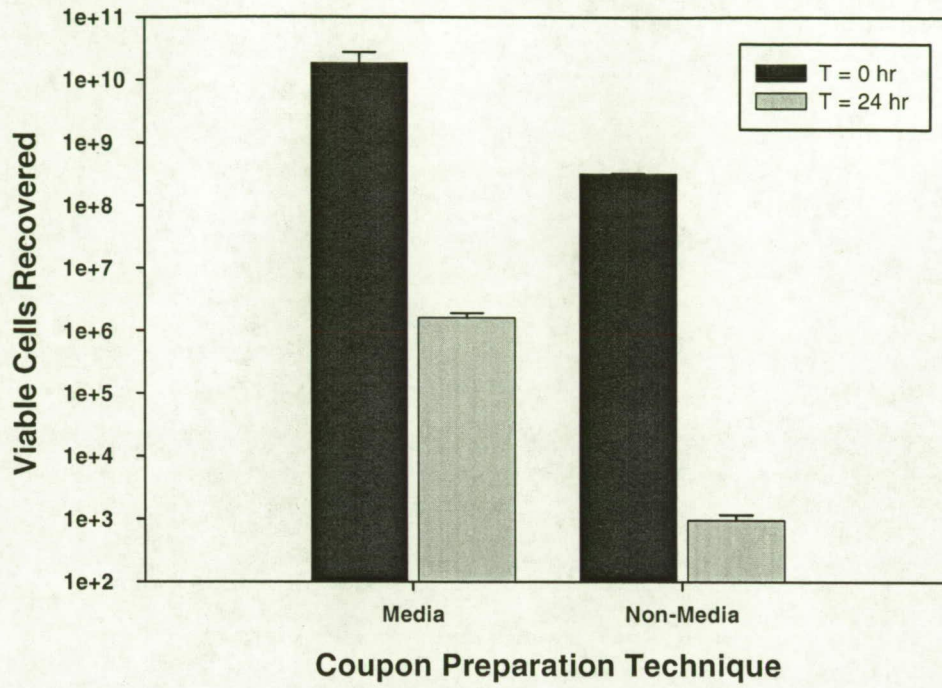


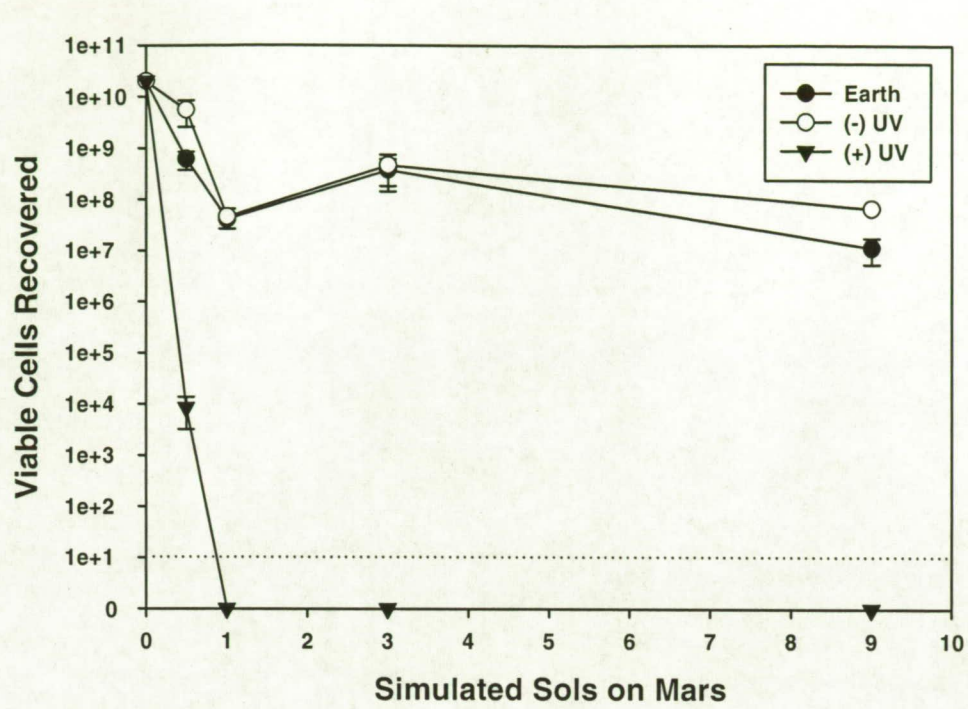
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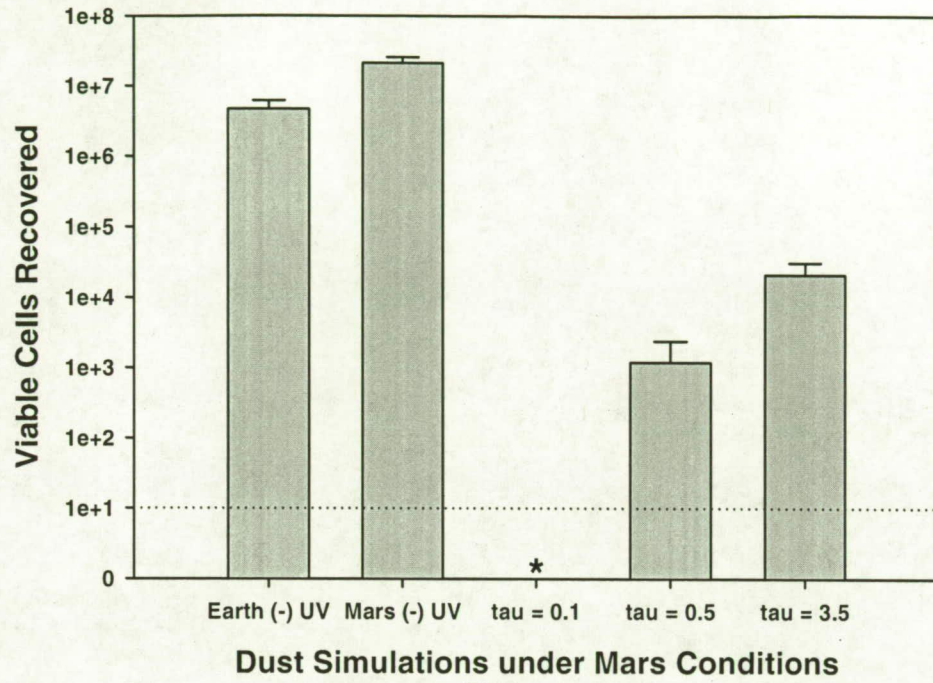
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12 Fig. 5.  
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**FIGURE LEGENDS**

**Fig. 1. Coupon preparation method for *Psychrobacter cryohalolentis* K5.** (A) The standard aluminum coupon preparation procedure used for all experiments, with the 100- $\mu$ L liquid media/salt matrix spotted on each coupon and containing *P. cryohalolentis* vegetative cells. (B) Microscopic view of a coupon surface after the liquid media spot had dried; i.e., media technique. (C) Microscopic view of a coupon surface prepared with liquid water; i.e., non-media technique.

**Fig. 2.** Experimental setup for Mars Simulation Chamber (MSC) atmospheric dust experiment. Treatments are shown resting on the liquid-nitrogen (LN<sub>2</sub>) cold plate within the MSC system with neutral density filters calibrated to simulate different atmospheric dust loads. The Mars (-UV) control is located at the far back, while the three dust scenarios are in the foreground: Mars dust load simulations are shown for  $\tau = 0.1$  (upper left);  $\tau = 0.5$  (upper right); and  $\tau = 3.5$  (bottom right). The coupons within the glass Petri dish (lower left) can be disregarded. The experiment delivered 8 hrs of UV irradiation during a total Mars simulation time of 24 hrs.

**Fig. 3.** Effect of preparation technique on *P. cryohalolentis* K5 desiccation over a 24-hr period. The standard liquid media preparation technique (used throughout this study) was compared to a water-diluted, non-media procedure which left bacterial cells unprotected on coupon surfaces. Both coupon preparation treatments were desiccated at 25 °C for 24 hrs and were then assayed for survival by the MPN assay. Values are means of multiple replicates; *bars* represent standard errors of the means.

**Fig. 4.** Mars Simulation Chamber experiment under simulated low-dust sky ( $\tau = 0.1$ ) conditions. *Psychrobacter cryohalolentis* K5 doped coupons (i.e., embedded in the

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4 medium/salt matrix) were exposed to simulated martian conditions for five distinct time  
5 intervals. Values are means of six replicates; *bars* represent standard errors of the means.  
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7 **Fig. 5.** Atmospheric dust experiment varying irradiation exposure in MSC.  
8 *Psychrobacter cryohalolentis* K5 doped coupons were exposed to martian conditions  
9 under different simulated atmospheric conditions for 1 sol (i.e., 24-hr in MSC with 8-hr  
10 UV irradiation). For  $\tau = 0.1$ , the asterisk indicates that the number of bacteria recovered  
11 were below the MPN detection limit of 10 cells per coupon (dotted line) for all replicates  
12 (i.e., 100% of coupons were negative). Values are means of six replicates; *bars* represent  
13 standard errors of the means.  
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