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Characterization of Hydrogen Peroxide–Resistant *Acinetobacter* Species Isolated during the Mars Phoenix Spacecraft Assembly

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Abstract

The microbiological inventory of spacecraft and the associated assembly facility surfaces represent the primary pool of forward contaminants that may impact the integrity of life-detection missions. Herein, we report on the characterization of several strains of hydrogen peroxide–resistant *Acinetobacter*, which were isolated during the Mars Phoenix lander assembly. All Phoenix-associated *Acinetobacter* strains possessed very high catalase specific activities, and the specific strain, *A. gyllenbergii* 2P01AA, displayed a survival against hydrogen peroxide (no loss in 100 m*M* H_2O_2 for 1 h) that is perhaps the highest known among Gram-negative and non-spore-forming bacteria. Proteomic characterizations reveal a survival mechanism inclusive of proteins coupled to peroxide degradation (catalase and alkyl hydroperoxide reductase), energy/redox management (dihydrolipoamide dehydrogenase), protein synthesis/folding (EF-G, EF-Ts, peptidyl-tRNA hydrolase, DnaK), membrane functions (OmpA-like protein and ABC transporter–related protein), and nucleotide metabolism (HIT family hydrolase). Together, these survivability and biochemical parameters support the hypothesis that oxidative tolerance and the related biochemical features are the measurable phenotypes or outcomes for microbial survival in the spacecraft assembly facilities, where the low-humidity (desiccation) and clean (low-nutrient) conditions may serve as selective pressures. Hence, the spacecraft-associated *Acinetobacter*, due to the conferred oxidative tolerances, may ultimately hinder efforts to reduce spacecraft bioburden when using chemical sterilants, thus suggesting that non-spore-forming bacteria may need to be included in the bioburden accounting for future life-detection missions. Key Words: Extremophiles—Planetary protection—Spacecraft assembly facility—Stress proteins—Microbe. Astrobiology 14, 837–847.

1. Introduction

In an effort to minimize the biological contamination of Mars and bolster the integrity of future science exploration missions, Mars-destined landers are required to adhere to strict assembly procedures, cleaning regimes, and biological burden limits (National Research Council, 2006; NASA, 2011). Together, these constraints reduce the potential for terrestrial microorganisms to be carried along with the spacecraft and interfere with life-detection analyses as false-positive signals of life (National Research Council, 2006). The general procedures utilized in maintaining spacecraft cleanliness include assembly in clean-room environments, surface wiping with alcohol solvents, and the use of alkaline floor detergents (NASA-KSC, 1999), with sterilization of spacecraft and spacecraft components used for Mars lander spacecraft (and for spacecraft that do not meet impact avoidance constraints) (National Research Council, 2006; NASA, 2011). Consequently, the microbial inventories of spacecraft and associated environments are typically quite low (Venkateswaran *et al.*, 2012) yet surprisingly diverse and inclusive of microorganisms possessing extreme tolerances toward radiation, oxidation, and desiccation (La Duc *et al.*, 2007; Ghosh *et al.*, 2010). Cultivation and DNA-based studies on these facilities delineate microbial ecologies inclusive of non-spore-forming bacteria, spore-forming bacteria, archaea, anaerobes, and fungi (Vasin and Trofimov, 1995; La Duc *et al.*, 2003, 2004a, 2007, 2012; Kempf *et al.*, 2005; Rettberg *et al.*, 2006; Moissl *et al.*, 2008; Ghosh *et al.*, 2010; Probst *et al.*, 2010). Moreover, studies show that the spore-forming bacteria, such as specific strains of *Bacillus pumilus* and *Bacillus odysseyi*, possess the most extreme tolerances toward simulated and actual space conditions among the spacecraft-associated microorganisms and, hence,

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carry a high potential for survival in extraterrestrial environments (La Duc *et al.*, 2004b; Newcombe *et al.*, 2005; Vaishampayan *et al.*, 2012b). Accordingly, assessments of spacecraft bioburden are typically obtained by using the NASA standard assay, which includes a heat-shock step followed by enumeration of the surviving microorganisms, typically dominated by spore formers (NASA, 2011; Frick *et al.*, 2014).

Many reports suggest that the recurrent isolation of extremotolerant microorganisms from spacecraft and associated environments is the result of the selective pressures manifested by the oligotrophic and low-humidity conditions of the assembly facilities (La Duc *et al.*, 2004a; Kempf *et al.*, 2005; Probst *et al.*, 2010). Further, quantitative studies show that the relative microbial abundances within the clean-room facilities are influenced by the different phases of the assembly process (Ghosh *et al.*, 2010; Vaishampayan *et al.*, 2010). For instance, molecular microbial community analyses of the Mars Phoenix lander facilities have shown that, just prior to assembly, the genera of *Ralstonia*, *Pseudomonas*, and *Sphingomonas* were the most abundant in the facilities, whereas, upon conclusion of the assembly, the *Acinetobacter*, *Ralstonia*, *Brevundimonas*, and *Mycoplana* were the most abundant (Vaishampayan *et al.*, 2010). Further, throughout the assembly process, the total operational taxonomic units (OTUs) represented by Gram-negative bacteria reduced from $\sim$82% (representing at least 32 genera) to $\sim$45% (representing 8 genera) during the assembly, and upon conclusion of assembly increased to an abundance of $\sim$98%, (representing 16 different genera). As for the Gram-positive bacteria, the abundances increased from $\sim$6% (representing at least 13 genera) to $\sim$55% during the assembly, where the only detectable OTUs were those represented by *Streptococcus*, and significantly decreased to $\sim$1% (representing 5 genera) upon conclusion. For the spore-forming and Gram-positive *Bacillus*, the relative abundances decreased throughout the assembly process from 0.6% to 0.3% (Vaishampayan *et al.*, 2010), which is meaningful, as the enumeration of spore formers such as *Bacillus* is typically employed as a proxy for spacecraft bioburden (NASA, 2011; Frick *et al.*, 2014). Furthermore, these studies demonstrate that $\geq$90% of the microbial community members after assembly were Gram-negative and non-spore-forming bacteria, which may be an important consideration for future lander and life-detection missions to Mars, as non spore formers, which are presumably heat-intolerant, are not adequately measured by the NASA standard assay (La Duc *et al.*, 2007; Frick *et al.*, 2014). Lastly, the presence of a core microbial community within the Mars Phoenix assembly facility was indicated by the observation of *Acinetobacter*, *Ralstonia*, *Brevundimonas*, and *Mycoplana* (the most abundant members in the facility upon assembly conclusion) in all phases of the assembly, thus suggesting that the core community possessed a tolerance toward the assembly conditions (Ghosh *et al.*, 2010; Vaishampayan *et al.*, 2010), with the *Acinetobacter* being the most abundant among these microorganisms (and perhaps the most favored) as indicated by an $\sim$11-fold increase in abundance throughout the assembly process.

The *Acinetobacter* are broadly associated with soil, water, and clinical environments (Gerischer, 2008) and are now commonly isolated and genetically detected from spacecraft and associated environments (La Duc *et al.*, 2003, 2004a, 2012; Castro *et al.*, 2004; Vaishampayan *et al.*, 2010; Moissl-Eichinger *et al.*, 2013). We recently reported that *Acinetobacter radioresistens* 50v1, which was isolated from the surface of the prelaunch Mars Odyssey orbiter, is extremotolerant toward Mars-like conditions, including combined exposures to desiccation, ultraviolet radiation, and oxidizing conditions (McCoy *et al.*, 2012). Biochemically, the survival of *A. radioresistens* 50v1 against hydrogen peroxide (H_2O_2) correlates with a decreased abundance in ATP synthase (F1 beta subunit) and an increase in activity and abundance of catalase and alkyl hydroperoxide reductase, respectively (McCoy *et al.*, 2012). Further, Schuerger *et al.* reported that *A. radioresistens* 50v1 not only survives exposure to martian atmospheric conditions (*e.g.*, pressure, gas composition, and temperature) but resumes proliferation after reintroduction to oxic conditions (Schuerger *et al.*, 2013). Therefore, despite being obligate aerobes, the survival and metabolic properties of the *Acinetobacter* indicate a potential for persistence under martian conditions, especially when considering the potential for shielding (or shadowing) from the martian surface radiation by spacecraft materials, the regolith, or both.

In this study, therefore, we present the characterization of several *Acinetobacter* strains isolated during the Mars Phoenix assembly and examine the significances between oxidative extremotolerance, survival in the clean-room facilities, and spacecraft bioburden. All Phoenix-associated *Acinetobacter* species exhibited very high catalase specific activities, with the specific strain, *A. gyllenbergii* 2P01AA, displaying a survival against H_2O_2 that is perhaps the highest known among Gram-negative and non-spore-forming bacteria (Miller, 1969; Ichise *et al.*, 1999; Buckova *et al.*, 2010; Tondo *et al.*, 2010; McCoy *et al.*, 2012). Proteomic analyses revealed the role of several redox homeostasis proteins in the survival against H_2O_2, including alkyl hydroperoxide reductase, dihydrolipoamide dehydrogenase, peptidyl-tRNA hydrolase, and DnaK. In fact, the molecular and survival properties of the Phoenix-associated *Acinetobacter* strains are comparable to those exhibited by radiation- and oxidation-resistant bacteria when grown under nonlimiting nutrient conditions (Arrage *et al.*, 1993; Wang and Schellhorn, 1995; Ichise *et al.*, 1999; Davies and Walker, 2007; Liedert *et al.*, 2010; Soares *et al.*, 2010). Hence, this comparison supports the hypotheses that (A) the spacecraft assembly facilities are extreme environments (La Duc *et al.*, 2007; Vaishampayan *et al.*, 2010; Moissl-Eichinger, 2012) and (B) the conditions of the facilities serve as selective pressures toward oxidative tolerance (La Duc *et al.*, 2004a, 2012; Benardini *et al.*, 2014). Within the context of spacecraft cleanliness, these results additionally support the notion that the *Acinetobacter* are potential forward contaminants and suggest that non-spore-forming bacteria may be important components of spacecraft bioburden, as the conferred oxidative tolerances of bacteria such as the *Acinetobacter* may hamper bioburden reduction efforts when using vapor-phase H_2O_2 as a sterilant.

2. Materials and Methods

2.1. Materials

Control bacterial isolates (Table 1) included *Deinococcus radiodurans* R1 [American Type Culture Collection (ATCC),

Table 1. Taxonomic Descriptions of the *Acinetobacter* and Control Microbial Strains

Species ID	*Strain*	*Catalase (Units/mg)*	*Physiology*[1]	*16S rRNA*	*Location isolated*
A. guillouiae	2P07PB[2]	890	Psychrophile	99.93%	Floor of PHSF (KSC) during assembly of the Mars Phoenix lander (Ghosh *et al.*, 2010)
A. guillouiae	2P07PC[2]	650	Psychrophile	99.65%	
A. gyllenbergii	2P01AA	1800	Alkaliphile	98.88%	
A. oryzae	2P01MB	1000	Mesophile	98.83%	
A. johnsonii	2P02AB	1100	Alkaliphile	99.49%	
A. oryzae	2P05ME	850	Mesophile	98.82%	
A. johnsonii	2P07AA	910	Alkaliphile	99.50%	
A. johnsonii	2P08AA	860	Alkaliphile	99.42%	
A. oryzae	2P08MC	910	Mesophile	99.23%	
A. radioresistens	50v1	120	—	—	Surface of prelaunch Mars Odyssey (SAEF-II, KSC) (La Duc *et al.*, 2003)
A. radioresistens	43998^T	41	Type strain	—	Cotton and soil (Nishimura *et al.*, 1988)
D. radiodurans	R1	1100	—	—	Canned meat (Cox and Battista, 2005)
V. rumoiensis	$S\text{-}1^T$	3900	—	—	Drain pool, fishery plant (Ichise *et al.*, 1999)
E. coli	DH5α	7.6	—	—	Invitrogen

[1]Definitions for the physiological conditions are reported elsewhere (Ghosh *et al.*, 2010).
[2]16S rRNA similarity is >97.5% with *A. gyllenbergii* type strain; however, polytaxonomic characterization indicates that this strain is a novel *Acinetobacter* species (Vaishampayan *et al.*, unpublished data).

Manassas, VA], *Vibrio rumoiensis* $S\text{-}1^T$ (received from Dr. I. Yumoto), *A. radioresistens* ATCC 43998^T (ATCC) (henceforth referred to as the type strain), and *Escherichia coli* DH5α (Invitrogen, Carlsbad, CA). Non-stabilized 30% w/w H_2O_2 (Sigma-Aldrich, St. Louis, MO) was used to eliminate the impacts of the stabilizers (*e.g.*, phenol, acetanilide, and sodium stanate); the H_2O_2 solutions were immediately stored as aliquots at −20°C and assayed for consistent concentration prior to use by absorbance spectroscopy ($\varepsilon = 43.6\ M^{-1}\ cm^{-1}$ at 234 nm). Reagents included Bugbuster Master Mix (EMD4Biosciences, USA), Halt Protease Inhibitor Cocktail (Thermo Scientific, USA), bovine liver catalase (Sigma-Aldrich), HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer (VWR, Radnor, PA), NaCl (VWR), and phosphate-buffered saline (PBS; 10× PBS: 100 m*M* potassium phosphate, 100 m*M* NaCl, pH 7.4) (VWR). Lysogeny broth (LB) was prepared (per liter) by using 5.0 g of yeast extract (Becton Dickinson, Franklin Lakes, NJ), 10.0 g of tryptone (Becton Dickinson), and 10.0 g of NaCl. Tryptone glucose yeast (TGY) broth was prepared (per liter) by using 5.0 g of yeast extract, 5.0 g of tryptone, 1.0 g of glucose (VWR), and 1.0 g of K_2HPO_4 (VWR). All media were autoclaved at 121°C for 30 min, buffers and solutions were sterile filtered (0.22 μm), and pure water (18 MΩ cm^{-1}) was used throughout.

2.2. Sampling and isolation

Microbiological sample sets were collected from the Payload Hazardous Servicing Facility (PHSF), a Class 100 clean-room facility at the Kennedy Space Center, during three phases of the Mars Phoenix assembly. These assembly phases occurred (1) *prior* to the arrival of the spacecraft components (Phase 1; April 25, 2007), (2) *during* the assembly and testing operations of the spacecraft but prior to launch (Phase 2; June 27, 2007), and (3) *after* removal of the spacecraft to the launch pad (Phase 3; August 1, 2007). Details of the sample collection and molecular taxonomic identifications are described elsewhere (Ghosh *et al.*, 2010). From this microbiological sample set, nine *Acinetobacter* strains, isolated during Phase 2 from the floor of the PHSF, were selected for further characterization.

2.3. Catalase specific activities

Catalase specific activities were measured from nine Phoenix-associated *Acinetobacter* strains and five controls including *A. radioresistens* 50v1, the *A. radioresistens* type strain, *D. radiodurans* R1, *V. rumoiensis* $S\text{-}1^T$, and *E. coli* DH5α. Cultures of the *Acinetobacter* and *E. coli* DH5α were grown in LB at 32°C, *V. rumoiensis* $S\text{-}1^T$ in LB at 18°C, and *D. radiodurans* in TGY at 32°C with constant agitation (200 rpm). Mid-log phase cells were harvested (5445 × *g* for 10 min at 4°C, Beckman Coulter Allegra 21R centrifuge) and washed twice in 50 m*M* HEPES buffer (pH 7.5) containing 100 m*M* NaCl. Extracts of each cell line were prepared by ultrasonication with a Virsonic 600 ultrasonic cell disrupter. While immersed in an ice bath, the fully resuspended cells [~0.5 g wet cells in 10 mL of 50 m*M* HEPES buffer (pH 7.5) containing 100 m*M* NaCl] were lysed by using two 30 s cycles at a power setting of 5, with an incubation of 30 s on ice in between the cycles. Supernatants of the samples were then collected (5445 × *g*, 10 min, 4°C), stored on ice, and immediately analyzed.

Kinetic assays for H_2O_2 degradation were performed as described by using three independent samples with at least six measurements of rates each (McCoy *et al.*, 2012). In summary, reactions were performed by using 20 m*M* H_2O_2 in 50 m*M* HEPES (pH 7.5) with 100 m*M* NaCl at 22°C, and were thoroughly mixed. All reactions were initiated by the addition of 100 μL of the extract, and the changes in absorbance at 240 nm measured every 2 s for a minimum of

30 s/sample (Perkin-Elmer DU640 UV-vis spectrometer). Catalase specific activities were calculated with the initial linear rates, a molar absorptivity (ε) of 43.6 M^{-1} cm^{-1}, and total protein contents as measured with a Bio-Rad Protein Assay (Bio-Rad, Richmond, CA). Specific activities were expressed in Units per milligram protein, where a Unit of activity represents the micromoles of substrate converted per minute.

2.4. Survival in H_2O_2

Survival of *A. gyllenbergii* 2P01AA, which contained the highest catalase specific activity, was measured before and after exposure to aqueous H_2O_2. Growth conditions were in LB at 32°C for various time periods with constant agitation at 200 rpm (La Duc *et al.*, 2003, 2007; Ghosh *et al.*, 2010). Exposures (1 h) were performed on mid-log phase cultures ($\sim 1.6 \times 10^9$ cfu/mL) by using 100 and 320 m*M* H_2O_2. Upon conclusion, 100 μL aliquots were removed, diluted to 1.0 mL in 100 μg/mL bovine liver catalase in PBS (10 m*M* potassium phosphate, 10 m*M* NaCl, pH 7.4), and incubated for 5 min at 22°C. Samples were then decimally diluted with LB (10–10,000 fold) and spread onto LB media agar plates. Plates bearing 25–300 colonies (FDA, 1998) after incubation (overnight at 32°C) were enumerated by using triplicate measurements on three independent samples.

2.5. Proteomics of H_2O_2 exposure

The effects of H_2O_2 exposure on the proteome of *A. gyllenbergii* 2P01AA were measured with two-dimensional polyacrylamide gel electrophoresis and mass spectroscopy. Aliquots (10 mL) of mid-log phase cultures (LB, 32°C) were exposed to 1 m*M* H_2O_2 for 15 min (22°C), or treated as the unexposed sample, and the cell pellets collected (5445 × *g*, 10 min, 4°C) and thoroughly washed three times in PBS. Cell pellets ($\sim$1 g wet cells) were prepared for proteomics by resuspension in 5.0 mL Bugbuster Master Mix (containing $\sim$50 μL of Halt Protease Inhibitor Cocktail), followed by gentle mixing and incubation for 20 min at 22°C, with collection (5445 × *g*, 20 min, 4°C) and concentration of the supernatants to $\sim$5.0 mg/mL. Exposures were carried out by using three independent samples of $\sim 2 \times 10^9$ cfu/mL. Plating of the unexposed and exposed samples indicated no loss in survival after the exposure for all strains (LB agar plates, overnight at 32°C).

Proteomics were performed as described with 300 μg protein/gel, pH 3–10 gradients, separation by using 12% polyacrylamide gels, staining with Coomassie blue, and imaging and quantification with the Progenesis SameSpots analysis software (Nonlinear Dynamics Limited, USA) (McCoy *et al.*, 2012). Target proteins were digested by trypsin and masses obtained on an Applied Biosystems 4800 MALDI/TOF spectrometer, and sequences were matched by using confidence intervals of >95%, with the corresponding accession number for the matches listed in Table 2. Protein volumes from the exposed and unexposed samples were compared with a paired Student *t* test. The proteomic profiles of *A. gyllenbergii* 2P01AA were also compared to those of *A. radioresistens* 50v1 and the *A. radioresistens* type strain (McCoy *et al.*, 2012) by using pairwise two-way ANOVA and Holm-Sidak tests. For both sets of analyses, expression differences of $\geq$1.2-fold were considered significant when $p \leq 0.05$, and inferred as a trend, for the Student *t* test, when $p \leq 0.1$ (Ethen *et al.*, 2006; Trost *et al.*, 2009; Jovanovic *et al.*, 2010).

3. Results

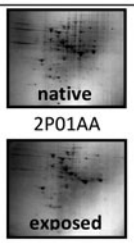
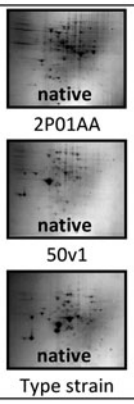
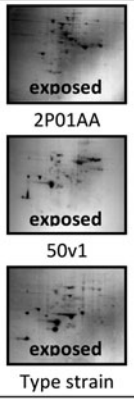
3.1. Cultivation and characterization of PHSF samples

Several *Acinetobacter* strains were isolated during the assembly process of the Mars Phoenix lander. Interestingly, no *Acinetobacter* strains were isolated during Phase 1 (before the assembly) due to a possible overwhelming presence of competing and more highly abundant microbial strains that were present in the PHSF just prior to assembly and testing. The cultivation studies also suggest that the cleaning conditions enforced during the Phoenix assembly were sufficient to remove a majority of the predominant bacterial species. These statements are supported by the decrease in OTUs from 132 to 42 between Phase 1 and 2 during the Mars Phoenix assembly (Vaishampayan *et al.*, 2010), which subsequently increased to 100 OTUs in Phase 3. The reduction in microbial abundance between Phase 1 and 2 is also supported by the 1-log difference in viable microbial burden values, measured by culture-independent ATP analyses (Phase 1, $\sim 10^5$ cells/m^2; Phase 2, $\sim 10^4$ cells/m^2) (Ghosh *et al.*, 2010). Hence, during Phase 1, *Acinetobacter* isolates were not retrieved during colony picking or in agar plates (due to the dilution series in plate count assays). In sharp contrast, a total of 36 *Acinetobacter* strains were isolated from the PHSF when more stringent cleaning conditions were enforced, including 12 strains isolated during Phase 2, after arrival of the Phoenix spacecraft components, and 15 strains from Phase 3, after the assembly process had concluded. Molecular microbial community analyses (16s rRNA gene cloning) (Vaishampayan *et al.*, 2010) showed that the *Acinetobacter* significantly increased in relative abundance from 4.3% in Phase 1 to 38.2% and 48.3% in Phase 2 and Phase 3, respectively. In corroboration, DNA microarray analyses showed substantial increases in the relative abundance of the Moraxellaceae family members (including *Acinetobacter*) in Phases 2 and 3 (Vaishampayan *et al.*, 2010). Together, these results support the potential proliferation of the robust microorganisms that remain during the assembly. Among the collected *Acinetobacter* isolates, nine strains from Phase 2 were selected for further study. In all cases, taxonomic profiles, which are provided in Table 1, were obtained by using sequence matches of >98% for the 16s rRNA. The isolation conditions of the *Acinetobacter* strains included low-temperature (4°C; two strains), high-alkaline (pH 11; four strains), and mesophilic (25°C; three strains) conditions. The *A. gyllenbergii* 2P01AA strain was isolated by inoculating the clean-room samples directly on R2A agar plates, which were adjusted to pH 11 (Ghosh *et al.*, 2010).

3.2. Catalase specific activities

The specific activities of H_2O_2 degradation, inferred as catalase specific activity due to gaseous evolution, were measured from nine different Phoenix-associated *Acinetobacter* strains and from *D. radiodurans* R1, *V. rumoiensis* S-1^T, *A. radioresistens* 50v1, the *A. radioresistens* type strain, and *E. coli* DH5α. As shown in Fig. 1, all Phoenix-associated

TABLE 2. COMPARATIVE PROTEOMICS OF *A. GYLLENBERGII* 2P01AA

Protein (2P01AA)	ID[1]	Function	Analysis	Gels[2]
Comparison of exposed versus native proteomes: 2P01AA				
Dihydrolipoamide dehydrogenase	Q6F8L3	TCA cycle, pyruvate dehydrogenase	Student's *t* test	
HIT family hydrolase	Q6MP02	Nucleotide hydrolase		
ABC transporter–related, hypothetical protein	YP_916717	Macrolide-specific ABC-type efflux carrier		
Comparison of native proteomes: 2P01AA, 50v1, and 43998^T				
Alkyl hydroperoxide reductase	Q6FAK2	Peroxide degradation	2-way ANOVA, Holm-Sidak	
EF-G	Q6FDS6	Translocation, translation		
OmpA-like protein	Q8VPR9	Bioemulsifier, porin		
DnaK	Q6F6N3	Chaperone, folding catalyst		
Comparison of exposed proteomes: 2P01AA, 50v1, and 43998^T				
Peptidyl-RNA hydrolase	YP_001762048.1	RNA release, translation	2-way ANOVA, Holm-Sidak	
EF-Ts	BAC89770	Protein elongation, translation		

[1]UniProt or Accession Number.
[2]Representative gels for the native and exposed proteomes of each tested strain.

Acinetobacter exhibited very high catalase specific activities in the range of 650–1800 Units/mg. These values were similar to those obtained from *D. radiodurans* R1 (1100 ± 39 Units/mg) and *V. rumoiensis* S-1^T (3900 ± 500 Units/mg), which is significant since *D. radiodurans* R1 (Gram-positive) is known for its extreme radiation resistance (Daly, 2009) whereas *V. rumoiensis* S-1^T (Gram-negative) was isolated from a fish processing plant where H_2O_2 is utilized as the bleaching agent (Yumoto *et al.*, 1999). Among the Phoenix-associated strains, *A. gyllenbergii* 2P01AA possessed the highest catalase specific activity (1800 ± 350 Units/mg), which was ~1.6-fold higher than that of *D. radiodurans* R1 and ~50% of *V. rumoiensis* S-1^T.

Comparisons to *A. radioresistens* 50v1 (120 ± 4 Units/mg), which was isolated from the preflight Mars Odyssey spacecraft, revealed that the Phoenix-associated strains contained 5- to 15-fold higher catalase specific activities. This is noteworthy, as the Mars Phoenix lander and Odyssey orbiter spacecraft (and associated facilities) were subjected to differing assembly, cleaning, and bioburden constraints. Lastly, and as expected, the catalase specific activities from the Phoenix-associated strains were a minimum of 1-log and ~2-log greater than that of the *A. radioresistens* type strain (41 ± 4 Units/mg) and *E. coli* (7.6 ± 3.3 Units/mg), respectively. In summary, the trend in catalase specific activities was as follows: *V. rumoiensis* S-1^T > all Phoenix-associated

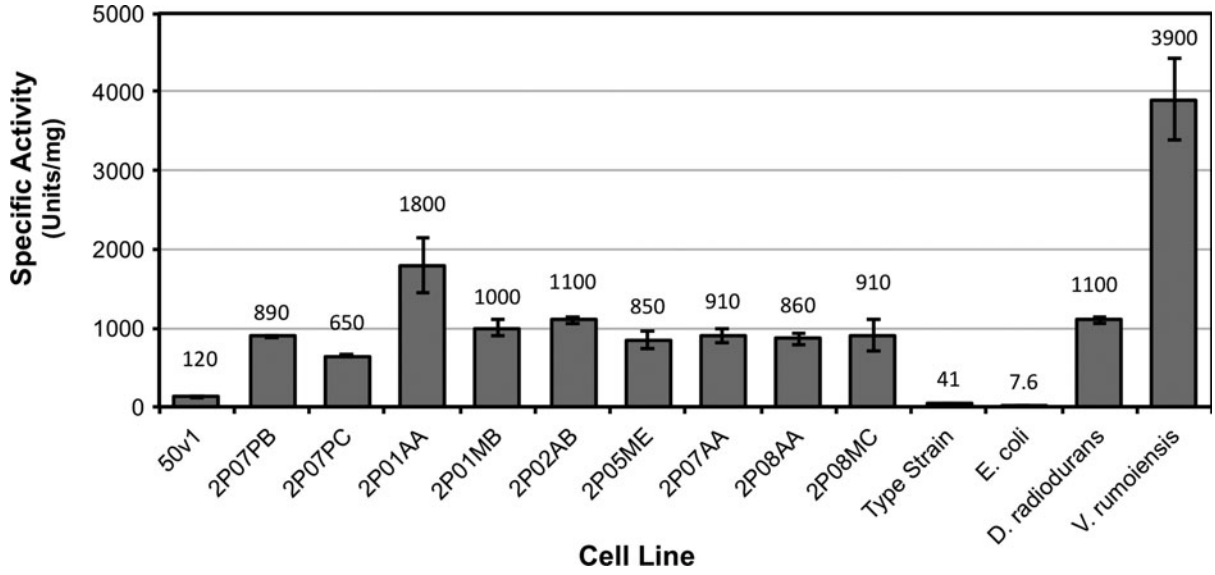


FIG. 1. Catalase specific activities from nine Phoenix-associated *Acinetobacter* strains (strains: 2P07PB, 2P07PC, 2P01AA, 2P01MB, 2P02AB, 2P05ME, 2P07AA, 2P08AA, and 2P08MC) and the controls of *A. radioresistens* 50v1, *D. radiodurans* R1, *V. rumoiensis* S-1^T, *A. radioresistens* ATCC 43998^T (type strain), and *E. coli* DH5α; all samples were grown under nonlimiting nutrient conditions and analyzed by using parallel procedures, and assay conditions included 20 m*M* H_2O_2 in 50 m*M* HEPES (pH 7.5) with 100 m*M* NaCl ($n \geq 3$; error bars display the standard deviation; specific activity measurement of Units/mg represents the μmol of substrate converted per min per mg of protein).

Acinetobacter ≥ *D. radiodurans* R1 >> *A. radioresistens* 50v1 > *A. radioresistens* type strain > *E. coli*.

3.3. H_2O_2 survivability

The survivability of *A. gyllenbergii* 2P01AA (~10^9 cfu/mL) was measured after exposures to 100 and 320 m*M* H_2O_2 for 1 h in conditions typically used when characterizing spacecraft-associated microorganisms (La Duc *et al.*, 2003, 2007; Ghosh *et al.*, 2010). As displayed in Fig. 2, the 100 m*M* H_2O_2 (~0.3% w/v) exposures yielded no loss in survival of *A. gyllenbergii* 2P01AA, whereas the 320 m*M* H_2O_2 (~1% w/v) exposures reduced the surviving cells by ~2-log. In comparison, 100 m*M* H_2O_2 exposures (1 h) yielded ~2-log reductions for *A. radioresistens* 50v1 and eradicated the *A. radioresistens* type strain, with 320 m*M* H_2O_2 ultimately eradicating the 50v1 strain. In contrast, studies on *D. radiodurans* R1 (Arrage *et al.*, 1993) and *V. rumoiensis* S-1^T (Ichise *et al.*, 1999) show ≥1-log reductions in survival after exposures to only 33 and 0.4 m*M* H_2O_2 when isothermally cultivated in nonlimiting nutrient conditions (*e.g.*, LB, tryptic soy broth, and peptone-yeast

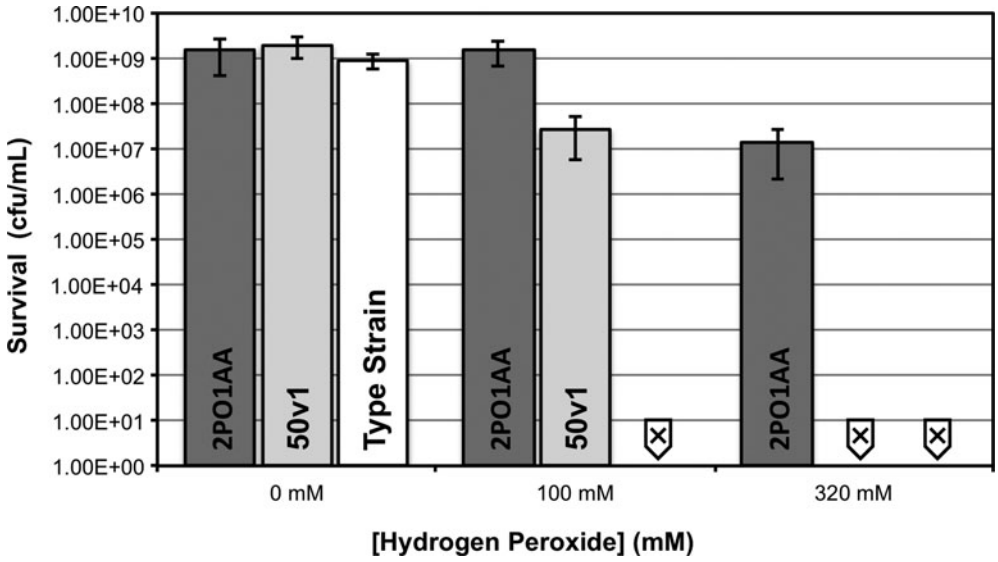


FIG. 2. The survivals of *A. gyllenbergii* 2P01AA, *A. radioresistens* 50v1, and *A. radioresistens* type strain after exposure to 100 and 320 m*M* H_2O_2 (~0.3 and ~1% w/w H_2O_2) for 1 h during mid-log phase in LB medium (cfu/mL represents the colony-forming units per mL of sample; $n = 3$; error bars display the standard deviation; and X represents < 25 colonies/plate).

extract-starch) in culture and agar plates, respectively. For *V. rumoiensis* S-1^T, optical density measurements suggest a slight loss in survival at 100 m*M* H_2O_2 (Ichise *et al.*, 2008). Lastly, the 2P01AA strain was reduced to undetectable levels (by plate count assay) by exposure to 490 m*M* H_2O_2 for 1 h (1.5% w/w H_2O_2 or ~5% v/v from a 30% w/w solution).

3.4. Proteomics

The effects of H_2O_2 exposure on the proteome of *A. gyllenbergii* 2P01AA were analyzed by two-dimensional gel electrophoresis and mass spectrometry (Table 2), and the combined results were statistically compared to those from *A. radioresistens* 50v1 and the *A. radioresistens* type strain (McCoy *et al.*, 2012). Experimental conditions included sublethal exposures to H_2O_2 (1 m*M*, 15 min), which yielded no loss in survival for *A. gyllenbergii* 2P01AA, *A. radioresistens* 50v1, or the *A. radioresistens* type strain. Hence, these conditions allowed for a maximum number of viable cells to be included in the analysis and minimized any deleterious oxidation. For *A. gyllenbergii* 2P01AA, comparison across the unexposed and exposed proteomes yielded changes to three identifiable proteins. A ~1.2-fold increase in expression was observed for dihydrolipoamide dehydrogenase, which is a component of the pyruvate and α-ketoglutarate dehydrogenase complexes and involved in NADH production. Upward trends in expression (~1.2-fold) were also observed for the HIT family hydrolase protein, which is a member of the histidine triad superfamily of nucleotide hydrolases. Trends were also observed in the downward expression (~0.75-fold) for the ATP-binding cassette (ABC) transporter–related protein, which belongs to a superfamily of proteins involved in the energy-dependent export and import of solutes in bacteria.

Comparative analyses of the native (or unexposed) proteomes of *A. gyllenbergii* 2P01AA and *A. radioresistens* 50v1 (hereafter referred to as the 2P01AA and 50v1 strains, respectively) indicated that the 2P01AA strain possessed a ~1.9-fold abundance in alkyl hydroperoxide reductase, which reductively degrades alkyl- and hydro-peroxides, and a ~1.6-fold abundance in EF-G, which is involved in translocation at the ribosome. The 2P01AA strain also possessed a ~0.4-fold lowered abundance in the OmpA-like protein, which is a membrane protein involved in solute transport in Gram-negative bacteria; for reference purposes, the 50v1 strain displayed a ~2-fold higher abundance in OmpA over the *A. radioresistens* type strain. In addition, the 2P01AA strain displayed a ~0.6-fold lowered abundance in DnaK, which is a molecular chaperone and folding catalyst.

Further, comparison across the H_2O_2-exposed proteomes revealed that the 2P01AA strain, as compared to the 50v1 strain, displayed a ~1.7-fold abundance in peptidyl-tRNA hydrolase, which is involved in protein synthesis and serves to release the RNA from the elongated peptide during translation, and a ~0.6-fold lowered abundance for EF-Ts, which is involved in guanine nucleotide exchange during protein elongation (or translation). In addition, the abundances of alkyl hydroperoxide reductase and EF-G were statistically equivalent across the exposed proteomes of the 2P01AA and 50v1 strains, indicating that both proteins were up-regulated in the 50v1 strain due to the H_2O_2 exposure (which supports the results of McCoy *et al.*).

4. Discussion

Our studies indicate that the Gram-negative and non-spore-forming *Acinetobacter* were constituents of the persistent microbial burden in the Mars Phoenix clean-room facility, as several strains were isolated during Phases 2 and 3 of the assembly process. The robust survival of these *Acinetobacter* strains is presumably due to metabolic tolerances toward the environmental, oligotrophic, and cleaning conditions. To measure the impact of oxidative stress and to reveal insights into the mechanisms of survival, a suite of survival, enzymatic, and proteomic interrogations were performed on the Phoenix-associated *Acinetobacter* strains. For the specific strain *A. gyllenbergii* 2P01AA, our survival studies revealed an extremotolerance toward H_2O_2 (no loss in 100 m*M* H_2O_2) that is perhaps the highest known among Gram-negative and non-spore-forming bacteria (Miller, 1969; Ichise *et al.*, 1999; Buckova *et al.*, 2010; Tondo *et al.*, 2010; McCoy *et al.*, 2012). In fact, the tolerance toward H_2O_2 for *A. gyllenbergii* 2P01AA surpasses that of *D. radiodurans* R1 (Arrage *et al.*, 1993) and *V. rumoiensis* S-1^T (Ichise *et al.*, 1999) under nonlimiting nutrient conditions, which is noteworthy, as these microorganisms are radiation- and oxidation-resistant bacteria, respectively. Enzymatic studies reveal that catalase is in high abundance in these strains, with the Phoenix-associated *Acinetobacter* possessing specific activities that were 40–160% of *D. radiodurans* R1 and 17–50% of *V. rumoiensis* S-1^T (where all strains, including controls, were cultivated, prepared, and analyzed using parallel procedures).

Comparison of the catalase and survival parameters (Fig. 3) also revealed that the extremotolerance is dependent

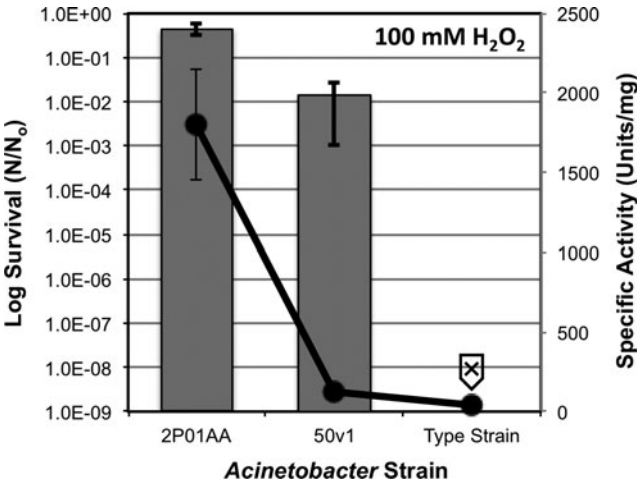


FIG. 3. Comparison of the trends in survival and catalase specific activities between *A. gyllenbergii* 2P01AA, *A. radioresistens* 50v1, and the *A. radioresistens* type strain: (1) the column graph represents the ratio of survivals (N/N_0, where $N_0 \sim 10^9$ cfu/mL) after exposure to 100 m*M* H_2O_2 for 1 h during mid-log phase in LB medium, where N/N_0 was derived from the averaged survivals and initial titers for each strain; (2) the line graph represents the trend in catalase specific activities across the strains (Units/mg represents the μmol of substrate converted per min per mg of protein; $n \geq 3$; error bars display the standard deviation; and X represents <25 colonies/plate).

on, but not directly proportional to, catalase specific activity. In these analyses, comparisons were conducted across *A. gyllenbergii* 2P01AA (Phoenix) and *A. radioresistens* 50v1 (Odyssey), which respectively possessed the highest and lowest catalase specific activities among the spacecraft-associated *Acinetobacter*. Evaluations showed that a 15-fold difference in catalase specific activities correlated to a ~2-log difference in survival in 100 m*M* H_2O_2. However, comparison of *A. radioresistens* 50v1 and the *A. radioresistens* type strain revealed that a >7-log difference in survival (in 100 m*M* H_2O_2) related to only a 3-fold difference in catalase specific activity. This is significant, as the trends illustrate that, while catalase is important, additional biochemical factors are compulsory for extremotolerance. This is further supported by the differing apparent survivals for *V. rumoiensis* S-1^T, when exposed to H_2O_2 in plate and culture conditions (≥1-log reduction in 0.4 m*M* H_2O_2 in plate assays and minimal loss in culture as inferred from optical density) (Ichise *et al.*, 1999, 2008), which additionally suggests that factors other than catalase play significant roles in H_2O_2 exposure.

Accordingly, proteomic interrogations confirmed that several additional proteins were involved in the oxidative homeostasis of *A. gyllenbergii* 2P01AA. Exposures to H_2O_2 resulted in increases in expression for proteins related to energy/redox management (dihydrolipoamide dehydrogenase) and nucleotide metabolism (HIT family hydrolase) and a decrease in expression for a protein involved in solute transport (ABC transporter–related protein). Further insights were gained by statistical comparison of the proteomic profiles from *A. gyllenbergii* 2P01AA to those from *A. radioresistens* 50v1 (and the *A. radioresistens* type strain). Comparative analysis across the native states of *A. gyllenbergii* 2P01AA and *A. radioresistens* 50v1 indicated that the 2P01AA strain possessed higher abundances of proteins related to peroxide degradation (alkyl hydroperoxide reductase) and protein synthesis (EF-G), and lower abundances of proteins related to solute transport (OmpA-like protein) and protein folding (DnaK). In contrast, comparison across the exposed proteomic profiles indicated that the 2P01AA strain had higher abundances in peptidyl-tRNA hydrolase and lower abundances in EF-Ts (with both proteins playing roles in protein biosynthesis).

Together, the proteomics suggest that the native physiology of the 2P01AA strain is well suited to manage sublethal oxidative exposure, while the 50v1 strain must biochemically adjust to accommodate an equivalent exposure. For instance, both the 2P01AA and 50v1 strains manage peroxide degradation by using high catalase specific activities, relative to the type strain, as well as alkyl hydroperoxide reductase, which is in high abundance in the native 2P01AA strain and up-regulated to equivalent levels in the exposed 50v1 strain. Similarly, protein synthesis is presumably impacted by EF-G, which is found in high abundance in the native 2P01AA strain and up-regulated to statistically equivalent levels in the exposed 50v1 strain. The utilization of protein folding catalysts was also similar, though unique in each case, with the native state of the 2P01AA strain possessing lower abundances of DnaK and the 50v1 strain down-regulating peptidyl-prolyl isomerase upon exposure (McCoy *et al.*, 2012). Lastly, proteins involved with energy production were managed in opposing manners, with H_2O_2 exposures yielding an increase in abundance of a NADH-producing enzyme (dihydrolipoamide dehydrogenase) for the 2P01AA strain and a decrease in abundance in the F1 beta subunit of ATP synthase for the 50v1 strain. However, dihydrolipoamide dehydrogenase also plays a role in redox homeostasis through regulation of NADH and lipoic acid (De Graef *et al.*, 1999).

Thus, the Phoenix-associated *Acinetobacter* strains display survival and molecular properties consistent with the physiology or adaptations to cope with oxidative stress. Namely, this includes (1) high activities and abundances of enzymes related to oxidative stress and redox homeostasis as found in *D. radiodurans*, *V. rumoiensis* S-1^T, *D. geothermalis* E50051, and *A. baumannii* (Wang and Schellhorn, 1995; Yumoto *et al.*, 1999; Liedert *et al.*, 2010; Soares *et al.*, 2010); (2) the up-regulation of factors related to protein synthesis as shown for *S. meliloti*, *E. sakazakii*, and *D. radiodurans* R1 (Davies and Walker, 2007; Riedel and Lehner, 2007; Lu *et al.*, 2009); and (3) the extremotolerant survival under oxidizing conditions as observed for *D. radiodurans* ATCC 13939 and *V. rumoiensis* S-1^T (Arrage *et al.*, 1993; Ichise *et al.*, 1999). Further, these studies demonstrate that spacecraft-associated microorganisms, particularly those from the same genus, display similarities in biochemistry. When considered together, therefore, these overall comparisons among non-spore-forming clean-room, environmental, and control isolates, which display radiation, oxidation, and desiccation tolerances under nonlimiting nutrient conditions, support the hypothesis that oxidative tolerance is the measurable phenotype (or outcome) of survival in the clean-room facilities, where selective pressures influence the microorganisms at the biochemical level.

Potential sources of selective pressure toward oxidative tolerance include the low-humidity conditions of the facilities and the cleaning reagents used in the assembly process, including solvent wipes (isopropanol and ethanol) and alkaline floor detergents (*e.g.*, Kleenol 30) (NASA-KSC, 1999; Vaishampayan *et al.*, 2010, 2012a). In support of this premise are the known relationships between oxidative stress and exposures to desiccating (or low-humidity) conditions (Fredrickson *et al.*, 2008; Slade and Radman, 2011), alkyl peroxides [a known product of isopropanol degradation (Kelly, 1996; Clark, 2001)], and the chemical components of Kleenol 30 (Park *et al.*, 2002; Urakawa *et al.*, 2008). Further, our measurements of the catalase specific activities suggest that the large disparity between the Phoenix- and Odyssey-associated *Acinetobacter* strains is possibly due to the differing assembly constraints for lander and orbiter spacecraft (NASA, 2011), which include differing cleanliness levels and cleaning schedules; however, additional studies are needed to confirm this correlation.

Nevertheless, the isolation of *Acinetobacter* strains from floor and spacecraft surfaces suggests that the total surfaces within spacecraft assembly facilities share similar microbial inventories and, hence, may be used to estimate the composition of spacecraft bioburden. Additionally, these results demonstrate that the spacecraft-associated *Acinetobacter*, which are the most abundant bacteria in the facility (after conclusion of assembly), are extremely tolerant toward oxidative stress, which thus supports the potential for these microorganisms to be forward contaminants. Within the

context of spacecraft contamination, therefore, the conferred oxidative extremotolerances of non-spore-forming bacteria, which are typically not measured by the NASA standard assay, may hamper future life-detection missions that use vapor-phase H_2O_2 as a chemical sterilant, which has been recently approved for surface sterilization of spacecraft and spacecraft components (Chung *et al.*, 2008; Chen *et al.*, 2013).

5. Conclusion

In conclusion, this study provides key insights into the biochemistry of extremotolerance for the Phoenix-associated *Acinetobacter* spp., where survival against H_2O_2 includes enzymatic degradation of peroxide (catalase and alkyl hydroperoxide reductase), regulation of redox reactive fuels (dihydrolipoamide dehydrogenase), modulation of protein synthesis and folding (EF-G, EF-Ts, peptidyl-tRNA hydrolase, DnaK), regulation of solute transport across the membranes (OmpA-like protein and ABC transporter–related protein), and nucleotide metabolism (HIT family hydrolase). Given the commonality of oxidative and radiation tolerance among spacecraft-associated microorganisms (La Duc *et al.*, 2004b, 2007; Link *et al.*, 2004; Newcombe *et al.*, 2005; McCoy *et al.*, 2012), our findings support the hypothesis that selective pressures impact the microbial ecology and dynamics of the clean-room facilities. Thus, this study indicates that non-spore-forming bacteria, such as the *Acinetobacter* spp., may need to be included in the bioburden accounting for future life-detection missions, particularly those subject to experimentally driven bioburden constraints (NASA, 2011).

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Abbreviations

ABC, ATP-binding cassette; ATCC, American Type Culture Collection; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LB, lysogeny broth; OTUs, operational taxonomic units; PBS, phosphate-buffered saline; PHSF, Payload Hazardous Servicing Facility; TGY, tryptone glucose yeast.

References

Arrage, A., Phelps, T., Benoit, R., and White, D. (1993) Survival of subsurface microorganisms exposed to UV radiation and hydrogen peroxide. *Appl Environ Microbiol* 59:3545–3550.

Benardini, J.N., III, La Duc, M.T., Beaudet, R.A., and Koukol, R. (2014) Implementing planetary protection measures on the Mars Science Laboratory. *Astrobiology* 14:27–32.

Buckova, M., Godocikova, J., Zamocky, M., and Polek, B. (2010) Screening of bacterial isolates from polluted soils exhibiting catalase and peroxidase activity and diversity of their responses to oxidative stress. *Curr Microbiol* 61:241–247.

Castro, V.A., Thrasher, A.N., Healy, M., Ott, C.M., and Pierson, D.L. (2004) Microbial characterization during the early habitation of the International Space Station. *Microb Ecol* 47:119–126.

Chen, F., McKay, T., Spry, J.A., Colozza, A.J., and DiStefano, S. (2013) Planetary protection concerns during pre-launch radioisotope power system final integration activities. In *Proceedings of Nuclear and Emerging Technologies for Space 2013,* Document ID 20130010386, NASA Glenn Research Center, Cleveland, OH.

Chung, S., Kern, R., Koukol, R., Barengoltz, J., and Cash, H. (2008) Vapor hydrogen peroxide as alternative to dry heat microbial reduction. *Adv Space Res* 42:1150–1160.

Clark, D.E. (2001) Peroxides and peroxide-forming compounds. *Chem Health Saf* 8:12–22.

Cox, M.M. and Battista, J.R. (2005) *Deinococcus radiodurans*—the consummate survivor. *Nat Rev Microbiol* 3:882–892.

Daly, M.J. (2009) A new perspective on radiation resistance based on *Deinococcus radiodurans*. *Nat Rev Microbiol* 7:237–245.

Davies, B.W. and Walker, G.C. (2007) Identification of novel *Sinorhizobium meliloti* mutants compromised for oxidative stress protection and symbiosis. *J Bacteriol* 189:2110–2113.

De Graef, M.R., Alexeeva, S., Snoep, J.L., and de Mattos, M.J.T. (1999) The steady-state internal redox state (NADH/NAD) reflects the external redox state and is correlated with catabolic adaptation in *Escherichia coli*. *J Bacteriol* 181:2351–2357.

Ethen, C.M., Reilly, C., Feng, X., Olsen, T.W., and Ferrington, D.A. (2006) The proteome of central and peripheral retina with progression of age-related macular degeneration. *Invest Ophthalmol Vis Sci* 47:2280–2290.

FDA. (1998) *Bacteriological Analytical Manual,* U.S. Food and Drug Administration, Silver Spring, MD. Available online at http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/default.htm.

Fredrickson, J.K., Li, S.M., Gaidamakova, E.K., Matrosova, V.Y., Zhai, M., Sulloway, H.M., Scholten, J.C., Brown, M.G., Balkwill, D.L., and Daly, M.J. (2008) Protein oxidation: key to bacterial desiccation resistance? *ISME J* 2:393–403.

Frick, A., Mogul, R., Stabekis, P., Conley, C.A., and Ehrenfreund, P. (2014) Overview of current capabilities and research and technology developments for planetary protection. *Adv Space Res* 54:221–240.

Gerischer, U. (2008) Acinetobacter *Molecular Microbiology,* Caister Academic Press, Norfolk, UK.

Ghosh, S., Osman, S., Vaishampayan, P., and Venkateswaran, K. (2010) Recurrent isolation of extremotolerant bacteria from the clean room where Phoenix spacecraft components were assembled. *Astrobiology* 10:325–335.

Ichise, N., Morita N., Hoshino T., Kawasaki K., Yumoto I., and Okuyama, H. (1999) A mechanism of resistance to hydrogen peroxide in *Vibrio rumoiensis* S-1. *Appl Environ Microbiol* 65:73–79.

Ichise, N., Hirota, K., Ichihashi, D., Nodasaka, Y., Morita, N., Okuyama, H., and Yumoto, I. (2008) H_2O_2 tolerance of *Vibrio rumoiensis* S-1^T is attributable to the cellular catalase activity. *J Biosci Bioeng* 106:39–45.

Jovanovic, M., Reiter, L., Picotti, P., Lange, V., Bogan, E., Hurschler, B.A., Blenkiron, C., Lehrbach, N.J., Ding, X.C., Weiss, M., Schrimpf, S.P., Miska, E.A., Grosshans, H., Aebersold, R., and Hengartner, M.O. (2010) A quantitative targeted proteomics approach to validate predicted microRNA targets in *C. elegans*. *Nat Methods* 7:837–842.

Kelly, R.J. (1996) Review of safety guidelines for peroxidizable organic chemicals. *Chem Health Saf* 3:27–36.

Kempf, M.J., Chen, F., Kern, R., and Venkateswaran, K. (2005) Recurrent isolation of hydrogen peroxide-resistant spores of *Bacillus pumilus* from a spacecraft assembly facility. *Astrobiology* 5:391–405.

La Duc, M.T., Nicholson, W., Kern, R., and Venkateswaran, K. (2003) Microbial characterization of the Mars Odyssey spacecraft and its encapsulation facility. *Environ Microbiol* 5:977–985.

La Duc, M.T., Kern, R., and Venkateswaran, K. (2004a) Microbial monitoring of spacecraft and associated environments. *Microb Ecol* 47:150–158.

La Duc, M.T., Satomi, M., and Venkateswaran, K. (2004b) *Bacillus odysseyi* sp. nov., a round-spore-forming bacillus isolated from the Mars Odyssey spacecraft. *Int J Syst Evol Microbiol* 54:195–201.

La Duc, M.T., Dekas, A., Osman, S., Moissl, C., Newcombe, D., and Venkateswaran, K. (2007) Isolation and characterization of bacteria capable of tolerating the extreme conditions of clean room environments. *Appl Environ Microbiol* 73:2600–2611.

La Duc, M.T., Vaishampayan, P., Nilsson, H.R., Torok, T., and Venkateswaran, K. (2012a) Pyrosequencing-derived bacterial, archaeal, and fungal diversity of spacecraft hardware destined for Mars. *Appl Environ Microbiol* 78:5912–5922.

Liedert, C., Bernhardt, J., Albrecht, D., Voigt, B., Hecker, M., Salkinoja-Salonen, M., and Neubauer, P. (2010) Two-dimensional proteome reference map for the radiation-resistant bacterium *Deinococcus geothermalis*. *Proteomics* 10:555–563.

Link, L., Sawyer, J., Venkateswaran, K., and Nicholson, W. (2004) Extreme spore UV resistance of *Bacillus pumilus* isolates obtained from an ultraclean spacecraft assembly facility. *Microb Ecol* 47:159–163.

Lu, H., Gao, G., Xu, G., Fan, L., Yin, L., Shen, B., and Hua, Y. (2009) *Deinococcus radiodurans* PprI switches on DNA damage response and cellular survival networks after radiation damage. *Mol Cell Proteomics* 8:481–494.

McCoy, K., Derecho, I., Wong, T., Tran, H., Huynh, T., La Duc, M., Venkateswaran, K., and Mogul, R. (2012) Insights into the extremotolerance of *Acinetobacter radioresistens* 50v1, a Gram-negative bacterium isolated from the Mars Odyssey spacecraft. *Astrobiology* 12:854–862.

Miller, T.E. (1969) Killing and lysis of Gram-negative bacteria through the synergistic effect of hydrogen peroxide, ascorbic acid, and lysozyme. *J Bacteriol* 98:949–955.

Moissl, C., Bruckner, J.C., and Venkateswaran, K. (2008) Archaeal diversity analysis of spacecraft assembly clean rooms. *ISME J* 2:115–119.

Moissl-Eichinger, C. (2012) Extremophiles in spacecraft assembly clean rooms. In *Adaption of Microbial Life to Environmental Extremes: Novel Research Results and Application*, edited by H. Stan-Lotter and S. Fendrihan, Springer, New York, pp 231–261.

Moissl-Eichinger, C., Pukall, R., Probst, A.J., Stieglmeier, M., Schwendner, P., Mora, M., Barczyk, S., Bohmeier, M., and Rettberg, P. (2013) Lessons learned from the microbial analysis of the Herschel spacecraft during assembly, integration, and test operations. *Astrobiology* 13:1125–1139.

NASA. (2011) *Planetary Protection Provisions for Robotic Extraterrestrial Missions,* NPR 8020.12D, National Aeronautics and Space Administration, Washington, DC. Available online at http://nodis3.gsfc.nasa.gov/displayDir.cfm?Internal_ID=N_PR_8020_012D_&page_name=main&search_term=8020%2E12.

NASA-KSC. (1999) Launch Site Requirement Planning Group. In *Facilities Handbook of Payload Hazardous Servicing Facility (PHSF),* Vol. KSTSM-14.1.15 rev D, NASA Kennedy Space Center, Cape Canaveral, FL.

National Research Council. (2006) *Preventing the Forward Contamination of Mars,* The National Academies Press, Washington, DC. Available online at http://www.nap.edu/catalog.php?record_id=11381.

Newcombe, D.A., Schuerger, A.C., Benardini, J.N., Dickinson, D., Tanner, R., and Venkateswaran, K. (2005) Survival of spacecraft-associated microorganisms under simulated martian UV irradiation. *Appl Environ Microbiol* 71:8147–8156.

Nishimura, Y., Ino, T., and Iizuka, H. (1988) *Acinetobacter radioresistens* sp. nov. isolated from cotton and soil. *Int J Syst Bacteriol* 38:209–211.

Park, J., Kamendulis, L.M., and Klaunig, J.E. (2002) Effects of 2-butoxyethanol on hepatic oxidative damage. *Toxicol Lett* 126:19–29.

Probst, A., Vaishampayan, P., Osman, S., Moissl-Eichinger, C., Andersen, G.L., and Venkateswaran, K. (2010) Diversity of anaerobic microbes in spacecraft assembly clean rooms. *Appl Environ Microbiol* 76:2837–2845.

Rettberg, P., Fritze, D., Verbarg, S., Nellen, J., Horneck, G., Stackebrandt, E., and Kminek, G. (2006) Determination of the microbial diversity of spacecraft assembly, testing and launch facilities: first results of the ESA project MiDiv. *Adv Space Res* 38:1260–1265.

Riedel, K. and Lehner, A. (2007) Identification of proteins involved in osmotic stress response in *Enterobacter sakazakii* by proteomics. *Proteomics* 7:1217–1231.

Schuerger, A.C., Ulrich, R., Berry, B.J., and Nicholson, W.L. (2013) Growth of *Serratia liquefaciens* under 7 mbar, 0°C, and CO_2-enriched anoxic atmospheres. *Astrobiology* 13:115–131.

Slade, D. and Radman, M. (2011) Oxidative stress resistance in *Deinococcus radiodurans*. *Microbiol Mol Biol Rev* 75:133–191.

Soares, N.C., Cabral, M.P., Gayoso, C., Mallo, S., Rodriguez-Velo, P., Fernández-Moreira, E., and Bou, G. (2010) Associating growth-phase-related changes in the proteome of *Acinetobacter baumannii* with increased resistance to oxidative stress. *J Proteome Res* 9:1951–1964.

Tondo, M.L., Petrocelli, S., Ottado, J., and Orellano, E.G. (2010) The monofunctional catalase KatE of *Xanthomonas axonopodis* pv. *citri* is required for full virulence in citrus plants. *PloS One* 5:e10803.

Trost, M., English, L., Lemieux, S., Courcelles, M., Desjardins, M., and Thibault, P. (2009) The phagosomal proteome in interferon-gamma-activated macrophages. *Immunity* 30:143–154.

Urakawa, H., Matsumoto, J., Inaba, K., and Tsuneda, S. (2008) DNA microarray mediated transcriptional profiling of *Nitrosomonas europaea* in response to linear alkylbenzene sulfonates. *FEMS Microbiol Lett* 282:166–173.

Vaishampayan, P., Osman, S., Andersen, G., and Venkateswaran, K. (2010) High-density 16S microarray and clone library-based microbial community composition of the Phoenix spacecraft assembly clean room. *Astrobiology* 10:499–508.

Vaishampayan, P., Probst, A.J., La Duc, M.T., Bargoma, E., Benardini, J.N., Andersen, G.L., and Venkateswaran, K. (2012a) New perspectives on viable microbial communities in low-biomass cleanroom environments. *ISME J* 7:312–324.

Vaishampayan, P.A., Rabbow, E., Horneck, G., and Venkateswaran, K.J. (2012b) Survival of *Bacillus pumilus* spores for a prolonged period of time in real space conditions. *Astrobiology* 12:487–497.

Vasin, V.B. and Trofimov, V.I. (1995) The experimental study of microbial contamination of the space hardware. *Adv Space Res* 15:273–276.

Venkateswaran, K., La Duc, M.T., and Vaishampayan, P. (2012) *Genetic Inventory Task Final Report,* Vol. 1, Jet Propulsion Laboratory, NASA, Pasadena, CA, pp 1–117. Available online at http://hdl.handle.net/2014/42319.

Wang, P. and Schellhorn, H.E. (1995) Induction of resistance to hydrogen peroxide and radiation in *Deinococcus radiodurans*. *Can J Microbiol* 41:170–176.

Yumoto, I.I., Iwata, H., Sawabe, T., Ueno, K., Ichise, N., Matsuyama, H., Okuyama, H., and Kawasaki, K. (1999) Characterization of a facultatively psychrophilic bacterium, *Vibrio rumoiensis* sp. nov., that exhibits high catalase activity. *Appl Environ Microbiol* 65:67–72.

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