

Extreme Spore UV Resistance of *Bacillus pumilus* Isolates Obtained from an Ultraclean Spacecraft Assembly Facility

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Received: 29 April 2003 / Accepted: 23 June 2003 / Online publication: 17 September 2003

Abstract

Recent environmental microbial sampling of the ultraclean Spacecraft Assembly Facility at NASA Jet Propulsion Laboratory (JPL-SAF) identified spores of *Bacillus pumilus* as major culturable bacterial contaminants found on and around spacecraft. As part of an effort to assess the efficacy of various spacecraft sterilants, purified spores of 10 JPL-SAF *B. pumilus* isolates were subjected to 254-nm UV and their UV resistance was compared to spores of standard *B. subtilis* biosimetry strains. Spores of six of the 10 JPL-SAF isolates were significantly more resistant to UV than the *B. subtilis* biosimetry strain, and one of the JPL-SAF isolates, *B. pumilus* SAFR-032, exhibited the highest degree of spore UV resistance observed by any *Bacillus* spp. encountered to date.

Introduction

Because the search for life on other planets will involve ultrasensitive technologies for the detection of cells and biomarkers, contamination of extraterrestrial samples with cells or biomarkers originating from Earth (forward contamination) would seriously compromise the interpretation that life signatures, if found, were indeed of extraterrestrial origin. This concern has been heightened by recent data indicating routine meteorite exchange between Earth and Mars [3]; the probability that living microbes, particularly bacterial spores, could survive interplanetary transfer [6, 10]; and the resulting possibility that martian life could resemble life on Earth [14]. Consequently, current planetary protection protocols require that spacecraft be constructed and assembled under conditions as nearly as possible approaching sterility. To achieve disinfection, robotic spacecraft are as-

sembled in clean rooms where air circulation is controlled and strict hygienic practices are implemented to minimize microbial contamination. In addition to physical containment methods, a number of sterilants including hydrogen peroxide (H₂O₂) vapor and ultraviolet radiation (UV) are considered to be viable options for sterilization of spacecraft hardware because they are low-heat sterilization processes compatible with various modern-day spacecraft electronics and components [1, 2].

As part of the NASA planetary protection program, recent monitoring of microbial diversity in the NASA Jet Propulsion Laboratories Spacecraft Assembly Facility (JPL-SAF) resulted in the isolation of a number of microbial species inhabiting various parts of the facility [16]. Whereas a highly diverse collection of microbes was isolated from entrance areas, microbial diversity decreased as samples were obtained from successively cleaner interior areas of the JPL-SAF. Interestingly, predominant isolates found to have penetrated deepest within the cleanest parts of the JPL-SAF were various strains of spore forming bacteria identified by biochemical testing and 16S rDNA analysis as being most closely related to *Bacillus pumilus* [16]; Kempf, MJ, Quigley MS, Chen F, Satomi M, Kern R, and Venkateswaran K, unpublished data). Not only were *B. pumilus* spores found to survive and persist in the extreme environment of the JPL-SAF, characterized by very low nutrient levels, strictly controlled humidity, and periodic disinfection (extensive details of JPL-SAF clean room disinfection procedures can be viewed at the Web site <http://dnp-lib.jpl.nasa.gov/dnp-lib/dscgi/ds.py/Get/File-1671/Toc495979317>), but *B. pumilus* isolates have also been recently recovered from hardware surfaces and air particles aboard the International Space Station (ISS) [15]. Taken together, the data strongly suggest that spores of *B. pumilus* are capable of escaping current spacecraft disinfection regimens and being inadvertently transported into space.

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Table 1. Bacterial strains used in this study and their resistances to 254-nm UV in the preliminary screen

Strain designation			Resistance to UV	
WN	JPL	Description (ref.)	$[\log(N/N_0) \text{ at } 1 \text{ kJ/m}^2]$	Relative to reference strain
WN624	(-)	<i>B. subtilis</i> [8]	n.d.	n.d.
WN667	ATCC7061	<i>B. pumilus</i> type strain	-2.69	1
WN669	SAFR-032	<i>B. pumilus</i>	-0.16	338.8
WN671	SAFN-029	<i>B. pumilus</i>	-1.40	19.5
WN672	SAFN-037	<i>B. pumilus</i>	-1.49	15.8
WN673	SAFN-036	<i>B. pumilus</i>	-1.09	39.8
WN674	SAFN-001	<i>B. pumilus</i>	-2.40	2.0
WN675	FO-036b	<i>B. pumilus</i>	-1.14	35.5
WN676	FO-033	<i>B. pumilus</i>	-2.30	2.5
WN677	FO-038	<i>B. pumilus</i>	-1.58	12.9
WN678	SAFN-027	<i>B. pumilus</i>	-2.30	2.5
WN679	KL-052	<i>B. pumilus</i>	-2.15	3.5

n.d., Not determined.

In this communication we tested spores of 10 JPL-SAF *B. pumilus* isolates for their resistance to germicidal (254-nm) UV and report that a subset of these strains exhibit elevated levels of UV resistance. The results are discussed in light of their important implications for spacecraft sterilization, planetary protection, and our understanding of spore UV resistance mechanisms.

Materials and Methods

Bacterial Strains and Culture Conditions. The bacterial strains used are listed in Table 1. All JPL-SAF isolates are described in detail elsewhere [16]; Kempf MJ, Quigley MS, Chen F, Satomi M, Kern R, and Venkateswaran K, unpublished data). All bacteria were sporulated by incubation in liquid Schaeffer Sporulation Medium (SSM) [13] at 37°C for 48 h in a rotary shaker with vigorous aeration. Cultures were harvested by centrifugation (10,000 g, 10 min, 25°C); spores were purified using the lysozyme and buffer washing method described by Nicholson and Setlow [11], heat-shocked (80°C, 10 min) and stored in deionized water at 4°C. All spore preparations were determined to be essentially free of vegetative cells and to consist of >99.9% phase-bright spores by phase-contrast microscopy. The viable titer of all spore preparations was determined by serial 10-fold dilution in phosphate-buffered saline (PBS) (10 mM potassium phosphate, 150 mM NaCl, pH 7.4) and plating on SSM solidified with 1.7% agar.

Spore UV Resistance Assay. UV irradiation was performed using a commercial low-pressure mercury vapor lamp (model UVGL-25, UV Products, Upland, CA) with the filter removed, producing predominantly 254-nm UV-C radiation. Throughout the experiments the lamp was placed at a constant height of 42 cm above the target. Lamp UV output was measured using a UVX

radiometer fitted with the UVX-25 filter (UV Products), recently calibrated and traceable to the National Institute of Standards and Technology standard. Purified spores were diluted to a final concentration of 10^6 colony-forming units (cfu)/mL in 10 mL of PBS; the spore suspension was pipetted into the bottom of an uncovered 6-cm diameter Petri dish with a sterile magnetic stir bar added, and the dish was placed on a magnetic stir plate beneath the UV lamp. Spores were exposed to UV with constant mixing and samples removed from the suspension at the desired UV dose, calculated from the fluence rate and the time of exposure. Spores were diluted serially 10-fold in PBS, plated in duplicate on solid SSM medium, and incubated at 37°C for 48 h, after which colonies were counted.

Data Analysis. The surviving fraction of spores was calculated as $\log_{10}(N/N_0)$, in which N is the viable spore titer at any given UV dose and N_0 is the spore titer obtained from the untreated suspension. For establishing dose-response curves, each experiment was repeated at least three times; averages and standard deviations of each set of data points were calculated and compared by analysis of variance (ANOVA) using Minitab version 10.5 software for Macintosh.

Results

In order to screen for extreme UV resistance, 10 JPL-SAF *B. pumilus* isolates were sporulated in SSM medium and their purified spores were exposed to a final UV dose of 1000 J/m^2 . From this preliminary screen it was determined that spores of four isolates (SAFN-001, FO-033, SAFN-027, and KO-052) exhibited approximately the same level of UV resistance as did spores of the *B. pumilus* type strain ATCC 7061 (Table 1). Spores of five JPL-SAF isolates (SAFN-029, SAFN-037, SAFN-036,

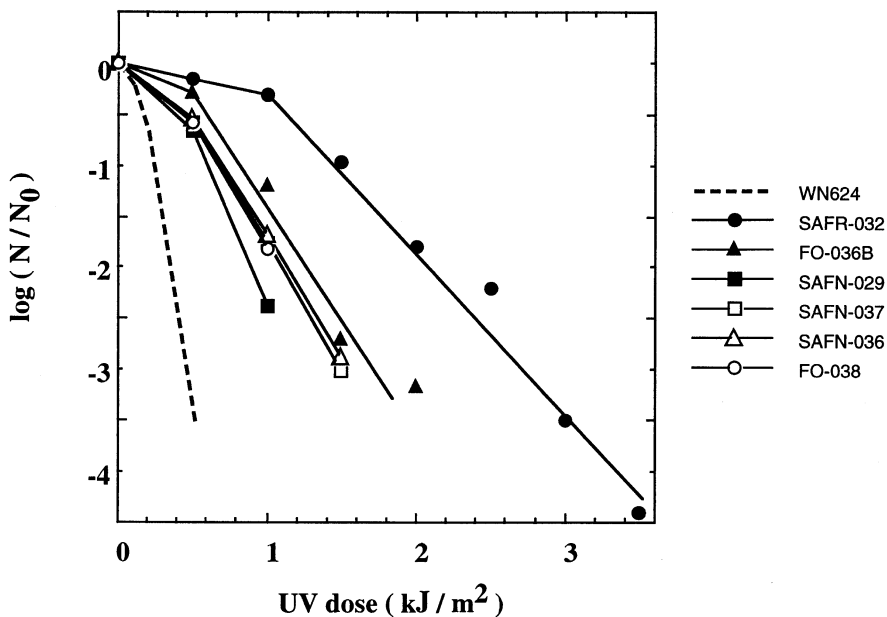


Figure 1. UV inactivation curves of spores of JPL-SAF isolates SAFR-032 (filled circles), SAFN-029 (filled squares), SAFN-037 (open squares), SAFN-036 (open triangles), FO-036B (filled triangles), and FO-038 (open circles). The dashed line represents the spore UV inactivation curve of *B. subtilis* biosimetry strain WN624 [8] for comparison. All curves represent averages of three independent determinations (four independent determinations for WN624). Error bars are omitted for clarity, as the data points differed by less than 10%.

FO-036b, and FO-038) exhibited between 10-fold and 100-fold greater survival at 1000 J/m² than did the *B. pumilus* type strain (Table 1), and spores of one JPL-SAF isolate (SAFR-032) proved to produce the most UV-resistant spores tested, exhibiting ~340-fold greater survival to UV at 1000 J/m² than did spores of the *B. pumilus* type strain ATCC 7061 (Table 1).

In order to confirm and extend the above observations, UV dose-response curves were generated for the six JPL-SAF isolates which exhibited elevated levels of spore UV resistance in the preliminary screen, and the resulting UV inactivation curves were compared with that of the standard laboratory UV biosimetry strain, *B. subtilis* WN624 [8] (Fig. 1). From the data of Fig. 1 it appeared that spores of all JPL-SAF isolates tested were more UV-resistant than spores of the *B. subtilis* biosimetry standard strain WN624. Moreover, it appeared that strains SAFN-029, SAFN-037, SAFN-036, FO-036b, and FO-038 formed a loose cluster exhibiting rather similar UV resistances, but that strain SAFR-032 was distinct in producing by far the most UV-resistant spores of all of the SAF-JPL isolates (Fig. 1). This notion was further tested quantitatively as follows. Spore UV inactivation curves characteristically consist of a “shoulder” at low UV doses followed by an exponential decline in viability at higher UV doses (see Fig. 1). To quantify spore resistance properties two parameters, the LD₉₀ value and the *D* value, are often used [7, 8]. The LD₉₀ value is defined as the lethal dose for 90% of the population and reflects mostly the length of the “shoulder”; the *D* (“decimal reduction”) value, defined as the dose producing 1 log₁₀ of inactivation, is derived from the exponential portion of the inactivation curve and reflects its slope [5]. The LD₉₀ and *D* values were derived from the

inactivation curves of each strain ($n = 3$ for SAF-JPL isolates; $n = 4$ for *B. subtilis* WN624), the averages and standard deviations for each data set were calculated, and the data set from each strain was compared to all other strains in pairwise combination by ANOVA. Differences among the resulting LD₉₀ and *D* values of $P \leq 0.05$ were considered significant, and isolates were grouped accordingly (Fig. 2). When compared by either LD₉₀ (Fig. 2A) or *D*-value (Fig. 2B), spores of the standard *B. subtilis* biosimetry strain WN624 were significantly less UV resistant than spores of all JPL-SAF isolates tested; thus WN624 formed its own group (“a” in Fig. 2). Spores of JPL-SAF isolates SAFN-029, SAFN-036, SAFN-037, and FO-038 were found to form a second cluster (“b”) of similar UV resistance when compared using either LD₉₀ (Fig. 2A) or *D*-value (Fig. 2B) as the criterion. Spores of strain FO-036b also belonged to cluster “b” when *D*-value was used as the comparison criterion (Fig. 2B), but formed its own, slightly more UV-resistant, group (“d”) when evaluated using LD₉₀ as the criterion (Fig. 2A). Spores of strain SAFR-032 were found to be significantly more UV resistant than spores of all other strains tested, thus forming their own group (“c” in Fig. 2). It is noteworthy that spores of strain SAFR-032, exhibiting LD₉₀ and *D* values of 1689 ± 279 and 686 ± 123 J/m², respectively, are the most highly UV-resistant spores yet to be discovered and are at least five-fold more UV-resistant than spores of *B. subtilis* reference strains commonly used for UV biosimetry [8].

Is there a relationship between the high UV resistance of *B. pumilus* SAF isolates and their resistance to H₂O₂? To assess H₂O₂ resistance, both vegetative cells and purified spores of *B. pumilus* SAF isolates were exposed to 5% liquid H₂O₂ for 60 min. As expected, spores

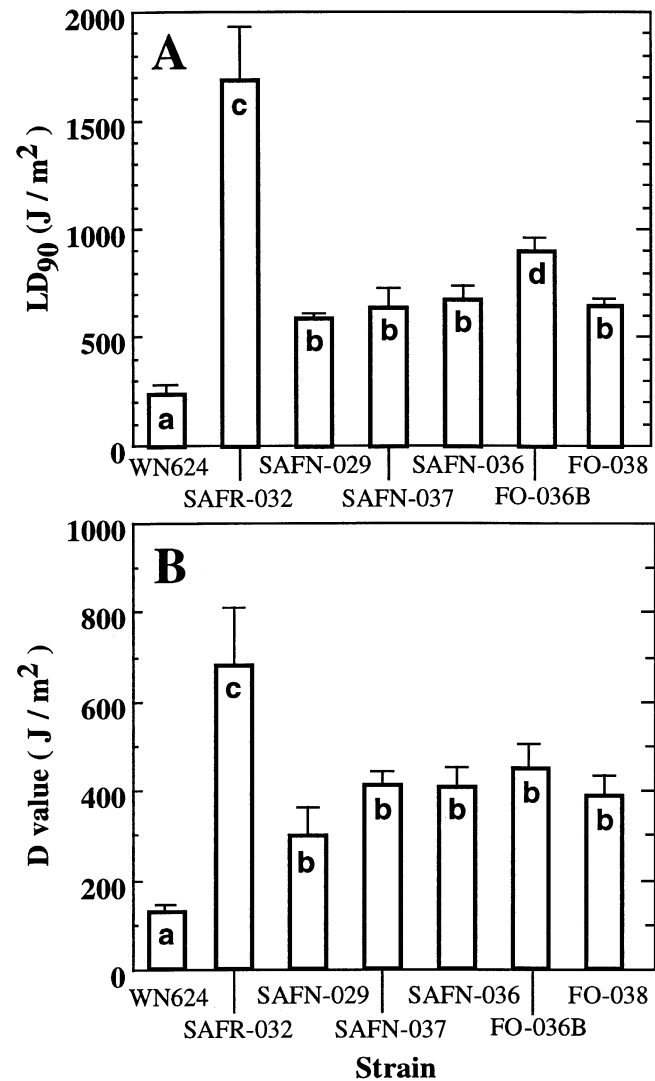


Figure 2. Comparison of LD₉₀ values (A) and D values (B) of JPL-SAF isolates by ANOVA. Bars and error bars represent averages and standard deviations, respectively. Strains with $13 P \leq 0.05$ were judged as belonging to separate groups (lowercase letters).

were more highly resistant than were their vegetative counterparts. Although purified spores of the *B. pumilus* type strain ATCC 7061 did not show any survivors after exposure to liquid H₂O₂, spores of all *B. pumilus* SAF isolates exhibited survivors to the H₂O₂ treatment, as did spores of the standard laboratory strain *B. subtilis* 168. Spores of *B. pumilus* SAF isolates SAFN-036 and FO-038 were inactivated by three to five orders of magnitude, as was the *B. subtilis* 168 laboratory strain. In contrast, spores of *B. pumilus* SAF isolates SAFR-032, SAFN-001, FO-036b, and FO-033 were observed to be more resistant to 5% H₂O₂, exhibiting only zero to two orders of magnitude of reduction in viability. However, although spores of *B. pumilus* strains FO-33 and SAFN-001 exhibited the highest degree of resistance to liquid H₂O₂ (Kempf MJ, Quigley MS, Chen F, Satomi M, Kern R, and

Venkateswaran K, unpublished data), spores of these two strains were essentially no more resistant to UV than the type strain of *B. pumilus*, ATCC 7061 (Table 1). Therefore, there appeared to be no strict correlation between high spore H₂O₂ resistance and high spore UV resistance among the *B. pumilus* SAF isolates.

Discussion

In this article we report that a number of *B. pumilus* strains isolated from the interior of the JPL-SAF produce spores which demonstrated an elevated level of resistance to inactivation by germicidal (254-nm) UV. Spores of one strain in particular, SAFR-032, exhibited the highest degree of UV resistance yet encountered. These observations have profound implications for efforts at prevention of forward bacterial contamination during planetary missions, and for UV disinfection of recycled drinking water and wastewater during long-duration space flight. For example, current standards for UV disinfection of municipal drinking water require a biologically effective dose of 400 J/m² [4], which reduces the viability of *B. subtilis* spore UV biosimetry strains, including ATCC 6633 and WN624, by approximately two orders of magnitude [4, 9]. For spores of JPL-SAF isolate SAFR-032 treated under our laboratory conditions, it would require a UV dose of ~2000–2500 J/m² to achieve the same reduction in viability. In addition, it should be stressed that spore resistance properties can be affected by the sporulation environment. In the current study, SAFR-032 spores were produced in laboratory medium, but in earlier work, we have found that *Bacillus* spp. spores isolated and purified directly from the soil environment demonstrated an additional two- to three-fold higher UV resistance than when the same isolates were sporulated in laboratory medium [9]. Thus it is possible that our measurements underestimate the intrinsic spore UV resistance of SAFR-032 in its natural setting, which raises an interesting ecological issue. It is reasonable to assume that all of the *B. pumilus* isolates originally inhabited the soil environment outside the ultraclean JPL-SAF and were inadvertently brought inside the JPL-SAF along with myriad other bacteria and fungi. It appears that the rigorous maintenance of clean-room conditions within the JPL-SAF has resulted not in an absolute barrier to contamination, but the establishment of a selective “bottleneck” which is sufficient to prevent penetration or survival of all but the hardiest microbes, which not surprisingly are bacterial spores.

In conclusion, recent environmental sampling of the JPL-SAF resulted in the isolation of a number of *B. pumilus* strains whose spore resistance properties are being actively investigated ([16]; Kempf MJ, Quigley MS, Chen F, Satomi M, Kern R, and Venkateswaran K, unpublished data). In this communication we report that a

subset of the JPL-SAF *B. pumilus* isolates exhibit elevated spore resistance to UV. Repeated isolation of highly resistant strains of *B. pumilus* in clean rooms is problematic because their persistence in SAFs and on spacecraft might compromise planetary protection efforts and extraterrestrial life-detection experiments. Elimination of such forward contamination will require novel cleaning and sterilization technologies which are not only compatible with modern spacecraft, but which can successfully remove or inactivate the most resistant of the microbes found in the SAFs. An understanding of the microbial diversity of spacecraft assembly areas, and any extreme characteristics these microbes might possess, is necessary to develop these technologies.

Acknowledgments

The authors thank Michael Kempf for sharing data prior to publication. This work was supported by contract No. 1238194 from NASA-JPL and grant NCC2-1342 from the NASA Exobiology program to W.L.N., and by a University of Arizona-NASA Space Grant to J.S. A portion of this work (K.V.) was carried out at the Jet Propulsion Laboratory, California Institute of Technology, under contract with NASA.

References

1. Anonymous (2000) Prevention of forward contamination of Europa. National Academies Press [online at <http://www.national-academies.org/publications>]
2. Chung, S, Echeverria, C, Kern, R, Venkateswaran, K (2000) Low-temperature H₂O₂ gas plasma—a sterilization process useful in space exploration technology. Abstracts of the 100th General Meeting of the American Society for Microbiology, May 21–25, Los Angeles, CA, USA
3. Gladman, BJ, Burns, JA, Duncan, M, Lee, P, Levinson, HF (1996) The exchange of impact ejecta between the terrestrial planets. *Science* 271: 1387–1392
4. Hoyer, O (2000) The status of UV technology in Europe. *IUVA News* 2: 22–27
5. Joslyn, L (1983) Sterilization by heat. In: Block, SS (Ed.) *Disinfection, Sterilization, and Preservation*. Lea & Febiger, Philadelphia, pp 3–46
6. Mileikowsky, C, Cucinotta, FA, Wilson, JW, Gladman, B, Horneck, G, Lindgren, L, Melosh, HJ, Rickman, H, Valtonen, M, Zheng, JQ (2000) Natural transfer of viable microbes in space. Part 1: From Mars to Earth and Earth to Mars. *Icarus* 145: 391–427
7. Nicholson, WL (2003) Using thermal inactivation kinetics to calculate the probability of extreme spore longevity: implications for paleomicrobiology and lithopanspermia. *Orig Life Evol Biosphere* (in press)
8. Nicholson, WL, Galeano, B (2003) UV resistance of *Bacillus anthracis* spores revisited: validation of *Bacillus subtilis* spores as UV surrogates for spores of *B. anthracis* Sterne. *Appl Environ Microbiol* 69: 1327–1330
9. Nicholson, WL, Law, JF (1999) Method for purification of bacterial endospores from soils: UV 2 resistance of natural Sonoran desert soil populations of *Bacillus* spp. with reference to *B. subtilis* strain 168. *J Microbiol Methods* 35: 13–21
10. Nicholson, WL, Munakata, N, Horneck, G, Melosh, HJ, Setlow, P (2000) Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. *Microbiol Mol Biol Rev* 64: 548–572
11. Nicholson, WL, Setlow, P (1990) Sporulation, germination, and outgrowth. In: Harwood, CR, Cutting, SM (Eds.) *Molecular Biological Methods for Bacillus*. John Wiley & Sons, Sussex, England, pp 391–450
12. Riesenman, PJ, Nicholson, WL (2000) Role of the spore coat layers in *Bacillus subtilis* resistance to hydrogen peroxide, artificial UV-C, UV-B, and solar radiation. *Appl Environ Microbiol* 66: 620–626
13. Schaeffer, P, Millet, J, Aubert, J-P (1965) Catabolic repression of bacterial sporulation. *Proc Natl Acad Sci USA* 54: 704–711
14. Thomas-Keppta, KL, Clemett, SJ, Bazylynski, DA, Kirschvink, JL, McKay, DS, Wentworth, SJ, Vali, H, Gibson, Jr EK, Romanek, CS (2003) Magnetofossils from ancient Mars: a robust biosignature in the martian meteorite ALH84001. *Appl Environ Microbiol* 68: 3663–3672
15. Valadez, VA, Thrasher, AN, Ott, CM, Pierson, DL (2002) Evaluation of bacterial diversity aboard the International Space Station. Abstracts of the 102nd General Meeting of the American Society of Microbiology, May 2002, Salt Lake City, UT, USA
16. Venkateswaran, K, Satomi, M, Chung, S, Kern, R, Koukol, R, Basic, C, White, D (2001) Molecular microbial diversity of a spacecraft assembly facility. *Syst Appl Microbiol* 24: 311–320