Assessing the Cleanliness of Surfaces: Innovative Molecular Approaches vs. Standard Spore Assays

Moogega Cooper[1], Myron T. La Duc[1], Alexander Probst[1], Parag Vaishampayan[1], Christina Stam[1], James N. Benardini[1], Yvette M. Piceno[2], Gary L. Andersen[2], and Kasthuri Venkateswaran[1*]

[1]Jet Propulsion Laboratory, California Institute of Technology, Pasadena, CA 91109, USA 5
[2]MS 70A-3317, One Cyclotron Rd., Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA.

Key words: Spacecraft, spore, DNA microarray, PhyloChip, planetary protection, astrobiology, cleanliness

*Author for correspondence: Kasthuri Venkateswaran
Present address and Mailing address:
Biotechnology and Planetary Protection, NASA Jet Propulsion Laboratory, California Institute of Technology, Mail Stop 89, Oak Grove Dr., Pasadena, CA 91109, USA 15
Tel: (818) 393-1481; Fax: (818) 393-41760
Email: kjvenkat@jpl.nasa.gov

ABSTRACT

A bacterial spore assay and a molecular DNA microarray method were compared for their ability to assess relative cleanliness in the context of bacterial abundance and diversity on spacecraft surfaces. Colony counts derived from the NASA standard spore assay were extremely low on spacecraft surfaces. However, PhyloChip G3 DNA microarray resolved the genetic signatures of a highly diverse suite of microorganisms in the very same sample set. Samples completely devoid of cultivable spores were shown to harbor the DNA of more than 100 distinct microbial phylotypes. Furthermore, samples with higher numbers of cultivable spores did not necessarily give rise to a greater microbial diversity upon analysis with the DNA microarray. The findings of this study clearly demonstrated that there is not a statistically significant correlation between the cultivable spore counts obtained from a sample and the degree of bacterial diversity present. Based on these results it can be stated that a validated state-of-the-art molecular techniques, such as DNA microarrays, can be utilized in parallel with classical culture-based methods to further describe the cleanliness of spacecraft surfaces.

INTRODUCTION

Progress in good manufacturing techniques and precision cleaning practices within sectors including medical, industrial, and domestic settings (2) has emphasized the importance of defining clear metrics for evaluating its products. Along these lines, it is important to verify that microbial burden requirements accounting for space exploration niche concerns are imposed and met for a particular spacecraft prior to launch, as advocated by the international Committee on Space Research (COSPAR). In compliance with COSPAR planetary protection policy, National Aeronautics and Space Administration (NASA) monitors the total microbial burden of spacecraft as a means for minimizing the inadvertent transfer of viable contaminant microorganisms to extraterrestrial environments, especially those of interest relative to understanding the process of chemical evolution and to origins of life in the solar system (forward contamination) (5). To this end, a substantial research effort funded by NASA devised a standard procedure in the 1960s for evaluating the "cleanliness" of spacecraft surfaces, which became the NASA standard spore assay (24). This standard spore assay is a cultivation-dependent, bacterial spore-based method of estimating the microbial burden specifically aerobic mesophilic, heterotrophic spore-forming organisms on spacecraft hardware surfaces (24). Resulting counts are used both as a proxy for relative surface cleanliness and to estimate overall microbial burden as well as to assess whether forward planetary protection risk criteria are met for a given mission, which vary by the planetary body to be explored and whether or not life detection missions are present. Specifically, lander missions to Mars are limited to 300 spores/m^2 and an analysis plan which details variables such as the probability of impact and orbital lifetimes are considered (8).

An extensive study which identified the presence of microorganisms using their rRNA sequences versus the traditional plate count method revealed that over 99% of microorganisms are yet to be cultured (1), and for the remaining 1%, concocting the appropriate combination of carbon and energy

source, and incubation time and temperature in a laboratory is often a significant challenge (37). Non-culture-based approaches can be employed to explore the remaining microbial population. The yet-to-be-cultured members of a microbial consortium may exist in a viable but non-culturable state (VBNC) (28), in an active but non-culturable state (ABNC which is more defined and potentially reversible) (16), or other states of dormancy (15, 16). It is apparent that opportunity exists to update standards pertaining to microbial burden and federal directives using techniques developed by the rapidly evolving biomedical industry. In particular, a molecular-based culture-independent method to comprehensively survey, archive, and quantitate the microbial genetic information associated with spacecraft surfaces is of interest to NASA in the context of future concepts for the study of Mars, Europa, and other icy satellites.

Recently, advanced molecular methods have augmented traditional, cultivation-based procedures for assessing the microbial burden and diversity of cleanrooms and other low-biomass environments (20, 29). An exhaustive 16S rRNA gene clone library-based analysis of typical spacecraft assembly facility was performed, and demonstrated that such facilities harbor highly diverse bacterial populations (20). However, in subsequent studies, PhyloChip generation 2 (G2) DNA microarrays detected the presence of 9 to 70-fold more bacterial taxa than the cloning technique (20, 29). As all this microbial diversity would have gone undetected by the standard spore assay alone, there is clearly a need to incorporate innovative molecular technologies to more fully examine spacecraft surfaces particularly in the context of understanding the cleanliness required for sample return missions. It is not feasible, nor logical, to gauge the cleanliness of spacecraft surfaces, which is almost entirely dependent on associated microbial diversity and abundance, by resulting raw spore-counts alone. It is equally unrealistic to assess forward contamination risk by DNA alone without an indication of who is active (and who is inactive) and hence, both assays are necessary and complimentary. Whereas the standard spore assay is extremely limited in

sensitivity and detection of phylogenetic breadth, PhyloChip DNA microarrays are able to provide detailed information on microbial diversity and composition, and can detect the presence of organisms in amounts below 10^{-4} relative abundance of the total sample (12). In addition, both approaches facilitate the tracking of microbial community dynamics as a function of time and/or sterilization process (3, 14).

The PhyloChip G3 is an improvement over the PhyloChip G2 in a number of ways, to include an increase from 500,000 probes to 1.1 million probes, the inclusion of strain-specific probe sets, the ability to detect over 50,000 operational taxonomic units (OTUs) instead of ~ 9,000 OTUs (14) using the G2 PhyloChip, and the G3 utilized over 320,000 sequences in the reference database, which is over 10-times greater than G2. The analysis software was also refined and the validation approach has been improved (3, 7, 14). To evaluate the validity of the sole use of the standard spore assay in assessing spacecraft surface cleanliness, with regard to microbial diversity and total cell numbers, total spore counts resulting from the standard spore assay were compared directly with PhyloChip generation 3 (G3) DNA microarray data arising from the very same samples. Presented herein are the first ever findings of PhyloChip G3 DNA microarray analyses of microbial communities associated with spacecraft surfaces.

MATERIALS AND METHODS

Sample characteristics. The surface of 107 different components of flight hardware (~0.7 m^2 each) was sampled. Appropriate handling, negative, and environmental controls, addressing the purity of buffers and molecular reagents, sampling materials, and even facility airborne contaminants, were included at all stages of sample collection, processing, and downstream analysis. All 107 samples collected were subdivided into three distinct categories based on the raw spore counts yielded at the time of collection (Category A: 0 spores, Category B: 1 – 50 spores, and Category C: > 50 spores). Samples were further subdivided and pooled based on spore counts and/or sampling location, and were denoted GI-XX, where

XX represents the sample identification number. Most GI-XX sets were comprised of several pooled samples due to the presence of low concentrations of DNA (20). However, limiting spore counts resulted in the representation of some GI-XX sets by a sole sample (e.g. GI-24, containing 151 spores). The number of sampling wipes pooled per sample, total surface area sampled, average spore count, and total nucleic acid concentration yielded/analyzed are given in Table 1.

Sampling. All samples were collected with polyester wipes (9"x9" ITW Alpha polyester wipes, Texwipe TX1009) as per the NASA sampling protocol (25). In preparation for sampling, all wipes were folded and rolled, and placed in glass test tubes containing 15 ml of sterile water, which were then autoclaved. Immediately prior to sampling, wipes were aseptically removed from tubes with sterile forceps and handled with sterile-gloved hands. The wipe is unrolled and successively folded into quarters such that the sampling surface is approximately 1/8 the total surface area of the wipe. Spacecraft surfaces of 0.7 m^2 were then sampled via successive wiping, first in a unidirectional horizontal, then in a unidirectional vertical, and finally in a unidirectional diagonal manner, each time with an unused quarter of the wipe. Sample-laden wipes were then placed in sterile 500 ml glass bottles, which were immediately transported to the laboratory where the standard spore assay was carried out within 2 h of sample collection.

Cultivation of aerobic spores. After sample collection, 200 ml of sterile rinse solution (85 mg/l potassium dihydrogen phosphate, 200 mg/l Tween 80; pH 7.2) were added to each wipe-containing bottle, which was then subjected to vortex mixing at maximum power for 5 s and sonication at 19-27 kHz for 2 min ± 5 s. The samples were subjected to heat-shock (80°C ± 2°C for 15 min) and 2 ml aliquots (24 replicates) from each heat-shocked portion were aseptically placed in sterile petri dishes to which sterile molten (55°C) trypticase soy agar (TSA, BD Co., Franklin Lakes, NJ) was added. The remaining rinse fluid (~152 ml) was frozen at −20°C for subsequent downstream DNA-based analyses. Plates were

incubated aerobically at 32°C in an inverted position, and colony forming units (CFU) were enumerated following 24, 48, and 72 h of growth.

Molecular microbial community analysis. As downstream DNA-based analyses restrict the total volume able to be analyzed to ~ 1 ml, the 152 ml sample portions set aside for such examination were concentrated via filtration with sterile 0.22 µm membrane filters. Filters were aseptically transferred to 50 ml Falcon tubes containing 10 ml of sterile water, which were vigorously agitated via vortex mixing at maximum speed for 5 min. This 10 ml eluent was then further concentrated to ~400 µl with Amicon Ultra-15 Centrifugal Filter Units (50 kDa molecular weight cut-off; Millipore, Billerica, MA) by centrifugation at 4,000 × g for 15 min (20). Half of each sample was subjected to bead-beating mechanical abrasion (MP Bio Lysing Matrix E; using the MP Bio FastPrep-24 Homogenizer, 1 min at the rate of 5.0 motions/s) and the other half not to ensure the sample would contain DNA released from robust and labile cell types (21). A Maxwell® 16 automated DNA extraction system was used per manufacturer's specification (Maxwell 16, Promega, Madison, WI, USA) for the DNA extraction process.

Quantitative PCR downstream analysis. Specific 123 bp regions (*E. coli* positions 1369 to 1492) of the bacterial small subunit 16S rRNA gene were subjected to SYBR-green based quantitative PCR (qPCR) (36) using a BioRad CFX-96 qPCR instrument. This is a method to conservatively estimate the total live and dead cells present, as it is assumed that all gene copies detected originated entirely from a cell. All samples were analyzed in triplicate and were exposed to the following thermal cycling conditions: an initial melting at 95°C for 120 s, followed by 40 cycles of 95.0°C for 15 s, 53°C for 30 s, 72.0°C for 30 s coupled with an ensuing plate read, and a final melt curve analysis ranging from 60°C to 95°C (increments of 0.2°C every 5 s). Each 25 µl reaction volume consisted of 12.5 µl BioRad 2X iQ SYBR Green Supermix (Cat 1708882), 1 µl, each, of 1369F and 1492R primers (to a final concentration of 700 nM), 9.5 µl of nuclease free Sigma water, and 1 µl of purified DNA template. Ribosomal rRNA gene standards,

spanning 10^8 – 10^2 gene copies/µl, were generated by serially diluting *rrn*::pCR4-TOPO plasmids of known concentration.

Phylochip G3 analysis. Due to the low biomass nature of spacecraft surface samples (≤surfaces (≤10^5 16S rRNA copies/m^2), the concentration of 16S rDNA amplicons loaded into, and analyzed via PhyloChips varied greatly from sample to sample (Table 1). Ideally, 350 ng of resulting amplicons from each sample set were added to each corresponding PhyloChip DNA microarray. For samples with less DNA than this, the total amount of amplified product was loaded and analyzed. Ribosomal rRNA genes were amplified from DNA extracts using a gradient PCR (annealing temperatures of 48.0, 48.8, 50.1, 51.9, 54.4, 56.3, 57.5, and 58.0°C) with the bacterial-directed primers 27f (5'-AGA GTT TGA TCC TGG CTC AG) and 1492r (GGT TAC CTT GTT ACG ACT T), as described previously (20). Amplicons were then analyzed using PhyloChip G3 (14), whereas details concerning target fragmentation, biotin labeling, PhyloChip hybridization, scanning, and staining, as well as background subtraction, noise calculation and detection, and quantification criteria were given in the abovementioned reference (11, 20). All arrays included a spike-mix with known amounts of non-16S rRNA genes (total 202 ng). Fluorescence intensities of these positive controls were used to normalize total array intensities among samples.

PhyloChip data processing. In order to determine whether an OTU is present in a sample, Stage 1 and 2 analysis was performed as described explicitly in Hazen et al. (14). Briefly, in stage 2 analysis every OTU must possess a minimum of 18 positively responding, perfect-match probe pairs. For calculation of the relative hybridization intensities of each OUT, the mean of the aforementioned probe pairs was determined but not taking into account the two probe pairs with the highest and the lowest value. Differently than stated in Hazen et al. (14), quartiles of the ranked *r* scores (response score to determine the potential of a probe pair responding to a target and not to the background) were set to: $rQ_1 \geq 0.913$, $rQ_2 \geq 0.972$, $rQ_3 \geq 0.985$. These more stringent parameters helped to decrease the detection of false

positive which is sought after in planetary protection considerations. Moreover, the possibility of cross-hybridization was addressed by calling only those subfamilies (and their OTUs) present that had an r_xQ_2 value (cross-hybridization adjusted response score) of ≥0.66. Only those OTUs that passed all these criteria were considered as present in a microarray analysis and were processed for downstream analysis (difference graph and environmental clustering).

Environmental clustering of PhyloChip data was performed using $\log_2$-transformation*1x10^3 hybridization intensities (14) of all OTUs that were called present in at least one of the samples. NMDS (non-metric multidimensional scaling) based on the Bray-Curtis distance was calculated using the R environment (http://www.R-project.org) in combination with the Vegan package.

Controls and lower detection limits of assays employed. Extensive controls were used at each stage of the sample collection, processing, and analysis regimes described herein to ensure that resulting data was of the highest quality. Wearing sterile gloves, polyester wipes that were exposed to the sampling environment but not used for active sampling were placed directly into sterile rinse solution, and processed in a blind fashion as handling (negative) controls in all molecular assays. In the same manner, ultraclean, molecular-grade sterile water served as a blank to monitor reagent cleanliness. Purified DNA from *Bacillus pumilus* SAFR-032 was included as a positive control in all PCR amplification protocols. In this manner, samples containing inhibitory substances were monitored for false negative results. None of the sample matrices employed in this study inhibited PCR, as was evident by the amplification of internal DNA standards (1 pg extracted *B. pumilus* genomic DNA).

RESULTS AND DISCUSSION

A simple and reliable means of estimating the total microbial population associated with spacecraft hardware is of immense value to those tasked with the bioreduction and decontamination of surfaces in general. Simplicity and reproducibility should not trump accuracy, however, and safeguards must be in

place to ensure that suitable tools are being used for the task at hand. NASA relies on bacterial spore counts as a proxy by which to gauge the microbial burden of spacecraft hardware, whereby for example a total spore threshold of $<5 \times 10^5$ on the entire spacecraft is mandated for all spacecraft destined for Mars, at the time of launch. Whether spore counts derived by NASA standard protocols correlate well with total spacecraft-associated bioburden inclusive of all microbial diversity, however, has never been thoroughly examined in the modern era. Current understanding is limited to the NASA specification which permits surface densities of vegetative organisms to be estimated at ten times the value obtained for spores in the standard spore assay. In this study, microbial diversity profiles resulting from PhyloChip G3 DNA microarray analyses were directly compared with spore counts arising from the same spacecraft surface samples. The results summarized here suggest that the presence of a certain subset of the microbial population will fail to be accounted for if the standard spore assay alone is used to assess spacecraft bioburden. This underestimation of bioburden will in turn increase the probability of transferring terrestrial microorganisms to Mars. The next generation of missions will include life-detection instruments which have the possibility to be compromised if the appropriate tools and standards are not in place prior to their conception and assembly.

Bacterial spore abundance. In the context of complying with planetary protection requirements, it is important to address spore abundance with respect to the total area sampled. A mere 385 spores were collected from the total surface area sampled (65.6 m^2) in this study, resulting in a mean 5.8 spores/m^2. The COSPAR planetary protection specifications limit average spacecraft surface bioburden to a maximum of 300 spores/m^2 for missions to Mars. Samples within Cat A, approximately 60% of the samples analyzed (64 out of 107 samples), and representative of ~40 m^2 of the total spacecraft surface area sampled, failed to yield any measurable spore counts. In addition, less than 2% (~1.4 m^2) of the total 65.6 m^2 sampled gave rise to spore counts in excess of 50 (Cat B; 2 out of 107 samples). A negative

control, consisting of a pooled collection of 14 polyester wipes and appropriate PBS volumes yielded no detectable spores, while three handling controls harbored between 1 and 8 spores per wipe. The source of such contaminants in the handling controls was unlikely the sampling material, since these materials were sterilized prior to sampling. A more plausible explanation may be the random capture of airborne spore-bearing particles from the air within the cleanroom where the spacecraft was assembled and sampled. The low incidence of detectable spores on the surfaces of the spacecraft suggests that good manufacturing practices in place are satisfactory for maintaining hardware-associated bioburden levels below COSPAR policy requirements (6).

Ribosomal RNA gene abundance. The total microbial burden of spacecraft surfaces, as assessed via rDNA-based qPCR, ranged from below detection limits to 4.6 x 10^8 16S rRNA gene copies/m^2. When 16S rDNA-based qPCR-derived bacterial burden was compared directly with spore count data resulting from the exact same sample set (Table 1), the comparative data showed that there was not a statistically significant difference in the distribution of the bioburden values (n=14, t= 0.301; p=0.786 based on the null hypothesis that the populations were not equal). Within Cat A, ribosomal rRNA gene abundances remained at consistent levels ($\leq 10^5$ 16S rRNA gene copies /m^2) with the exception of two samples (GI-15 and GI-17; Table 1) which also gave the highest diversity. Previous studies have demonstrated that whenever spacecraft-associated surface samples yield fewer than ~1 x 10^5 16S rDNA copies/m^2, they can be expected to give rise to very few, if any, cultivable cells or spores (13, 18). The results of this study support this observation.

Bacterial diversity. Although an exhaustive characterization of all 385 strains isolated via NASA standard spore assay was not carried out, random identification of colonies revealed the presence of both spore-forming and heat-shock resistant non-spore-forming bacteria (data not shown). Spore-forming, cultivable bacterial species of the genera *Bacillus, Paenibacillus,* and *Sporosarcina* were observed,

alongside non-spore-forming species of *Staphylococcus* and *Pseudomonas,* as had been shown previously (13, 18). In direct comparison, spore-forming taxa detected via PhyloChip include *Alicyclobacillus, Halobacillus, Bacillus, Clostridium, Lactobacillaceae, Myxococcus, Sulfobacillus,* and *Planococcaceae.* Of particular interest, PhyloChip analysis of Cat A samples revealed the presence of DNA from aerobic spore-forming *Bacillus* and *Alicyclobacillus* spp*.,* which are capable of growing on TSA, while their presence went undetected via standard spore assay. The extent of the diversity of taxa detected via PhyloChip was not at all dependent upon the spore count-based categorization of samples.

It would be predicted that an increasing number of detectable OTUs, especially those representing spore-forming taxa, would be observed with increasing spore counts; however, many of the findings of this study were to the contrary. Often the number of OTUs detected was greatest for samples belonging to Cat A, and yet these samples did not have detectable spores. This contradictory trend observed between the categories may be attributed to the unequal amount of wipes pooled per category. Each wipe is able to collect both intact spores as well as exogenous genetic material. Cat A consisted of 64 wipes which possessed a total of $4.6x10^8$ 16S rRNA gene copies/m^2, whereas Cat C consisted of only 2 samples accounting for a total 16S rRNA gene content of $7.2x10^6$ gene copies/m^2, which is 64-fold lower. Since molecular techniques employed in this study could not differentiate DNA from dead versus viable cells, there is a possibility that the cleaning procedures adapted by NASA could lyse the microbes but leave behind cell debris. Furthermore, the DNA from viable but yet-to-be cultured cells as well as heat labile vegetative cells may also have contributed to an increased signal in the Cat A wipes when assayed by the PhyloChip DNA microarray. In the case of Cat B samples (41 samples), where the 16S rRNA gene copy numbers were low but have more spore counts than Cat A samples, more spore-forming taxa were observed. Such a trait might not only due to the higher spore counts but also most of the samples grouped in Category B (23 out of 41 wipes) were collected from surfaces prior to passing quality control

standards to certify it as flight hardware. Upon certification as flight hardware, maintenance (cleanliness and handling) of the flight hardware is known to be superior compared to the materials still in line for flight certification.

Finally, a comparison between viable spore counts using the standard spore assay and microbial bioburden assessed by PhyloChip DNA microarray hybridization intensities was made. Hybridization intensities are indicative of the OTU presence of the DNA of that particular species within the sample of interest as shown in previous studies (11, 20). Fig. 1a differentiates the OTUs, which are relatively more abundant when bacterial spores are present and at/below 50 spores (Cat B), compared to samples with a zero spore count (Cat A), which was calculated using the following equation:

$$y = \mathrm{HybInten}\left(\mathrm{CatB}(x_i) - \mathrm{CatA}(x_i)\right),$$

where HybInten(CatA(x_i)) designates the hybridization intensity of a particular OTU, x_i, within the category. Fig. 1b differentiates the relative abundance in species when spore counts are greater than 50 (CatC) versus Cat B, which was calculated using the following equation:

$$y = \mathrm{HybInten}\left(\mathrm{CatC}(x_i) - \mathrm{CatB}(x_i)\right).$$

The PhyloChip fluorescence intensity plotted on the Y-axis (Fig. 1) is proportional to the number of OTUs, and thus, the calculated number of live and dead cells based on the amplified DNA molecules that were present. These hybridization intensity differences suggest that *Alicyclobacillus* and *Moraxellaceae* were present in a greater relative abundance in Cat B (Fig. 1a), whereas in Cat C, an increase in alphaproteobacterial OTUs was noticed (Fig. 1b). Proportional to spore counts, a higher number of OTUs belonging to the Alphaproteobacteria class and *Moraxellaceae* family were observed in Cat C compared to Cat B and Cat A samples. However, such logical trend was not observed in majority of the bacterial classes or phyla (both spore-forming and non spore-forming). The increase in *Enterobacteriaceae*

related OTUs found in samples devoid of viable spores (Cat A) is of interest, as these bacteria were commonly associated with humans and have been documented as the major source of contaminants in spacecraft-associated surfaces (23, 30).

Subsequent to the hybridization intensity-based comparison, a deeper understanding of the bacterial community structure was investigated and samples within Categories A, B, and C were analyzed using NMDS analysis (Fig. 2). All 18 sets of GI samples (Table 1) were arranged according to their ordinate data categories (Category A, B, and C based on spore counts) and non-metric similarities were shown to exist between each dataset. Furthermore, the NMDS analysis reveals that the distance between each cluster was low, indicating the categories do not form distinct communities, and the samples collected from the spacecraft surfaces have similar community structures. In other words, there was no dependence of the PhyloChip results on the spore category parameter. The subfamily level richness as detected using the PhyloChip microarray for each GI sample is given in Fig. 3a. Spore counts from spacecraft surfaces were directly compared to corresponding hybridization intensity and the amount of 16S rRNA gene copies present in a particular sample (Fig. 3b). This correlation allows for a direct comparison to be made between spores counted in a sample and a estimation of the cells present using the average 16S rRNA gene copies present in bacterial samples, which can be estimated as 3.6 gene copies per cell (17, 22, 33). The lack of a trend in the increase of spore counts, hybridization intensity, and the cell population reflects that there was not a direct relationship.

The total bacterial subfamilies present in all 107 samples examined are shown in Fig. 4. Gammaproteobacteria comprises of the largest phylum (2,081 OTUs) present on the spacecraft surfaces and include nitrogen fixors and chemoautotrophs, which are able to find suitable nutrition in extreme environments (4). Firmicutes contributed the largest fraction, 20% of the sub-families detected, and have been reported to survive extreme environments including spacecraft-associated surfaces (13, 18,

19, 26), desiccation (10, 32), and other stressors (34) possibly through the production of endospores (9, 27, 35). A specific phylum, Actinobacteria, which was present at reasonably high incidence in this study (11%) was known to be commonly found in soil and associated with human skin (23, 30). The presence of Firmicutes and Actinobacterial OTUs supported earlier findings where it was discussed that the main contaminant sources might be dirt from the local external environment adjacent to clean rooms where spacecraft components were assembled and humans (23, 31).

There is great value in a standard spore assay methodology; however, in an effort to assess the diversity of spacecraft and associated surfaces, a combination of molecular technique with conventional methods should be used. In summary, as expected, the spore counts of the spacecraft-associated surfaces were extremely low in the majority of samples; however, the detected OTUs determined by the PhyloChip DNA microarray method revealed the presence of genetic signatures from a greater number of bacteria. In many cases, the number of OTUs present was at its greatest in samples shown to have a zero spore count (over 100 different microbial OTUs), whereas samples greater than zero spores did not necessarily harbor an increased number of bacterial OTUs. Interpretation of the average hybridization intensity, total live and dead cells present (determined via qPCR), and corresponding spore count associated with that sample in the same frame allows for a direct comparison to be made, showing no relationship between spore counts, estimated concentration of cells based on qPCR, and abundance.

Due to practical limitations, it is not presently feasible to sterilize every component of a spacecraft due to material complexity. For this reason, methods to accurately determine the microbial contaminants, from whole cells to genetic signatures, must be employed. Considering that future life-detection and sample-return missions are already on the drawing boards, it is timely that state-of-the-art molecular techniques should be developed for use to ascertain the presence of microbial signatures in parallel with classical culture-based methods whenever assessing planetary protection risk. The combination of a

classical method traditionally used in federal agencies with a validated state-of-the-art biomolecular technology will have valuable implications in fields (e.g. microbial monitoring, product development, etc.) to include the food, medical, pharmaceutical, aeronautical, and homeland security industries.

ACKNOWLEDGMENTS

Part of the research described in this paper was carried out by the Jet Propulsion Laboratory, California Institute of Technology, under contract with the National Aeronautics and Space Administration. This research was funded by the Mars Program Office at JPL. We are grateful to K. Buxbaum for funding, J.A. Spry for encouragement, R. Koukol for spacecraft sampling, L. Tom and T. DeSantis for PhyloChip analysis. The DNA microarray portion of this study was performed under the auspices of the U.S. Department of Energy by the University of California, Lawrence Berkeley National Laboratory under Contract No. DE-AC02-05CH11231.

REFERENCES

1. **Amann, R., W. Ludwig, and K. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143-169.
2. **ASTM.** 2009. ASTM E2362-09, Standard Practice for Evaluation of Pre-saturated or Impregnated Towelettes for Hard Surface Disinfection. Subcommittee: E35.15, p. 1622, ASTM Book of Standards, Volume 11.05: Water and Environmental Technology: Biological Effects and Environmental Fate, Biotechnology; Pesticides, vol. 11.05. ASTM International.
3. **Brodie, E. L., T. Z. DeSantis, J. P. M. Parker, I. X. Zubietta, Y. M. Piceno, and G. L. Andersen.** 2007. Urban aerosols harbor diverse and dynamic bacterial populations. Proceedings of the National Academy of Sciences **104:**299-304.
4. **Chivian, D., E. L. Brodie, E. J. Alm, D. E. Culley, P. S. Dehal, T. Z. DeSantis, T. M. Gihring, A. Lapidus, L. H. Lin, S. R. Lowry, D. P. Moser, P. M. Richardson, G. Southam, G. Wanger, L. M. Pratt, G. L. Andersen, T. C. Hazen, F. J. Brockman, A. P. Arkin, and T. C. Onstott.** 2008. Environmental genomics reveals a single-species ecosystem deep within Earth. Science **322:**275-8.
5. **Committee on Preventing the Forward Contamination of Mars, N. R. C.** 2006. Preventing the forward contamination of Mars. National Academy of Sciences.
6. **COSPAR.** 2002. Planetary Protection Policy, October 2002, as amended, March 2008. COSPAR, Houston, TX.
7. **DeSantis, T. Z., E. L. Brodie, J. P. Moberg, I. X. Zubieta, Y. M. Piceno, and G. L. Andersen.** 2007. High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. Microb. Ecol. **53:**371-383.
8. **DeVincenzi, D. L., P. Stabekis, and J. Barengoltz.** 1996. Refinement of planetary protection policy for Mars missions. Advances in Space Research **18:**311-316.
9. **Driks, A.** 2003. The dynamic spore. Proc. Natl. Acad. Sci. USA **100:**3007-3009.
10. **Edwards, J. S., and J. R. Battista.** 2003. Using DNA microarray data to understand the ionizing radiation resistance of *Deinococcus radiodurans*. Trends in Biotechnology **21:**381-382.
11. **Flanagan, J. L., E. L. Brodie, L. Weng, S. V. Lynch, O. Garcia, R. Brown, P. Hugenholtz, T. Z. DeSantis, G. L. Andersen, J. P. Wiener-Kronish, and J. Bristow.** 2007. Loss of bacterial diversity during antibiotic treatment of intubated patients colonized with *Pseudomonas aeruginosa*. J. Clin. Microbiol. **45:**1954-1962.
12. **Fodor, S., J. Read, M. Pirrung, L. Stryer, A. Lu, and D. Solas.** 1991. Light-directed, spatially addressable parallel chemical synthesis. Science **251:**767-773.
13. **Ghosh, S., S. Osman, P. Vaishampayan, and K. Venkateswaran.** 2009. Recurrent isolation of extremo-tolerant bacteria from the clean room where Phoenix spacecraft components are assembled. Astrobiology **10:**325-335.
14. **Hazen, T. C., E. A. Dubinsky, T. Z. DeSantis, G. L. Andersen, Y. M. Piceno, N. Singh, J. K. Jansson, A. Probst, S. E. Borglin, J. L. Fortney, W. T. Stringfellow, M. Bill, M. E. Conrad, L. M. Tom, K. L. Chavarria, T. R. Alusi, R. Lamendella, D. C. Joyner, C. Spier, J. Baelum, M. Auer, M. L. Zemla, R. Chakraborty, E. L. Sonnenthal, P. D'Haeseleer, H. Y. Holman, S. Osman, Z. Lu, J. D. Van Nostrand, Y. Deng, J. Zhou, and O. U. Mason.** 2010. Deep-sea oil plume enriches indigenous oil-degrading bacteria. Science **330:**204-8.
15. **Kaprelyants, A. S., J. C. Gottschal, and D. B. Kell.** 1993. Dormancy in non-sporulating bacteria. FEMS Microbiology Letters **104:**271-286.

16. **Kell, D. B., A. S. Kaprelyants, D. H. Weichart, C. R. Harwood, and M. R. Barer.** 1998. Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. Antonie van Leeuwenhoek **73:**169-187.
17. **Klappenbach, J. A., P. R. Saxman, J. R. Cole, and T. M. Schmidt.** 2001. rrndb: the Ribosomal RNA Operon Copy Number Database. Nucleic Acids Research **29:**181-184.
18. **La Duc, M. T., A. E. Dekas, S. Osman, C. Moissl, D. Newcombe, and K. Venkateswaran.** 2007. Isolation and characterization of bacteria capable of tolerating the extreme conditions of clean-room environments. Appl. Environ. Microbiol. **73:**2600-2611.
19. **La Duc, M. T., W. Nicholson, R. Kern, and K. Venkateswaran.** 2003. Microbial characterization of the Mars Odyssey spacecraft and its encapsulation facility. Environ. Microbiol. **5:**977-985.
20. **La Duc, M. T., S. Osman, P. Vaishampayan, Y. Piceno, G. Andersen, J. A. Spry, and K. Venkateswaran.** 2009. Comprehensive census of bacteria in clean rooms by using DNA microarray and cloning methods. Applied and Environmental Microbiology **75:**6559-67.
21. **La Duc, M. T., S. Osman, and K. Venkateswaran.** 2009. Comparative analysis of methods for the purification of DNA from low-biomass samples based on total yield and conserved microbial diversity. J. Rapid Meth. Auto. Microbiol. **17:**350-368.
22. **Lee, Z. M.-P., C. Bussema, and T. M. Schmidt.** 2009. rrnDB: documenting the number of rRNA and tRNA genes in bacteria and archaea. Nucleic Acids Research **37:**D489-D493.
23. **Moissl, C., S. Osman, M. T. La Duc, A. Dekas, E. Brodie, T. DeSantis, and K. Venkateswaran.** 2007. Molecular bacterial community analysis of clean rooms where spacecraft are assembled. FEMS Microbiology Ecology **62:**131-131.
24. **NASA.** 1980. NASA Standard Procedures for the Microbial Examination of Space Hardware. NASA Procedures and Guidelines. NPG: 5340.1D. *In* N. Headquarters (ed.), Washington, DC.
25. **NASA.** 1980. Standard Procedures for the Microbiological Examination of Space Hardware, NHB 5340.1B, Rev. B. National Aeronautics and Space Administration, Washington, D.C.
26. **Newcombe, D. A., A. C. Schuerger, J. N. Benardini, D. Dickinson, R. Tanner, and K. Venkateswaran.** 2005. Survival of spacecraft-associated microorganisms under simulated martian UV irradiation. Appl. Environ. Microbiol. **71:**8147-8156.
27. **Nicholson, W. L., N. Munakata, G. Horneck, H. J. Melosh, and P. Setlow.** 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiology and Molecular Biology Reviews **64:**548-572.
28. **Oliver, J. D.** 2010. Recent findings on the viable but nonculturable state in pathogenic bacteria. FEMS Microbiology Reviews **34:**415-425.
29. **Probst, A., P. Vaishampayan, S. Osman, C. Moissl-Eichinger, G. Andersen, and K. Venkateswaran.** 2010. Microbial Diversity of Anaerobes from Spacecraft Assembly Clean Rooms. Appl. Environ. Microbiol.**:**AEM.02167-09.
30. **Puleo, J. R., G. S. Oxborrow, N. D. Fields, C. M. Herring, and L. S. Smith.** 1973. Microbiological profiles of four Apollo spacecraft. Appl. Microbiol. **26:**838-845.
31. **Puleo, J. R., G. S. Oxborrow, N. D. Fields, C. M. Herring, and L. S. Smith.** 1973. Microbiological Profiles of Four Apollo Spacecraft. Appl. Environ. Microbiol. **26:**838-845.
32. **Rainey, F. A., K. Ray, M. Ferreira, B. Z. Gatz, M. F. Nobre, D. Bagaley, B. A. Rash, M. J. Park, A. M. Earl, N. C. Shank, A. M. Small, M. C. Henk, J. R. Battista, P. Kampfer, and M. S. da Costa.** 2005. Extensive diversity of ionizing-radiation-resistant bacteria recovered from Sonoran Desert soil and description of nine new species of the genus *Deinococcus* obtained from a single soil sample. Appl Environ Microbiol **71:**5225-35.

33. **Schippers, A., L. N. Neretin, J. Kallmeyer, T. G. Ferdelman, B. A. Cragg, R. John Parkes, and B. B. Jorgensen.** 2005. Prokaryotic cells of the deep sub-seafloor biosphere identified as living bacteria. Nature **433:**861-864.
34. **Setlow, B., and P. Setlow.** 1998. Heat killing of *Bacillus subtilis* spores in water is not due to oxidative damage. Appl Environ Microbiol **64:**4109-12.
35. **Setlow, P.** 1995. Mechanisms for the prevention of damage to DNA in spores of *Bacillus* species. Annual Review of Microbiology **49:**29-54.
36. **Suzuki, M. T., O. Beja, L. T. Taylor, and E. F. Delong.** 2001. Phylogenetic analysis of ribosomal RNA operons from uncultivated coastal marine bacterioplankton. Environmental Microbiology **3:**323-331.
37. **Vesley, D., K. M. Keenan, and M. M. Halbert.** 1966. Effect of time and temperature in assessing microbial contamination on flat surfaces. Appl. Environ. Microbiol. **14:**203-205.

Figure legends:

Fig. 1. PhyloChip analysis of hybridization intensity difference of the three "spore abundance" categories. (a) Hybridization intensity difference of Cat B (1-50 spores) compared to Cat A (0 spores). (b) Hybridization intensity difference of Cat C (>50 spores) compared to Cat B. Bars above the zero line represent bacteria that increased in abundance relative to Cat A and Cat B for Fig. 1a and Fig. 1b, respectively; bars below represent those bacterial DNAs that declined in abundance.

Fig. 2. NMDS analysis based on $\log_2$-transformation*1x10^3 hybridization intensity of OTUs reveal the spore categorical groupings are from a similar community structure, as they greatly overlap.

Fig. 3. (a) Comparison of subfamily richness and spore counts grouped by the "spore abundance" categories and based on the NASA standard spore assay. The results show there is no strong correlation between sample category and subfamily count. (b) Calculated cell density based on 16S rRNA gene copies compared to spore counts and total hybridization intensity. Average 16S rRNA gene copies are 3.6 per cell (17, 22, 33). Gene copies correlate similarly to hybridization intensities but show no correlation to spore counts.

Fig. 4. Overall microbial diversity grouped by taxa. Number of subfamilies and OTUs detected per taxon are given in the parenthesis. Percentages lower than 2% are not displayed.

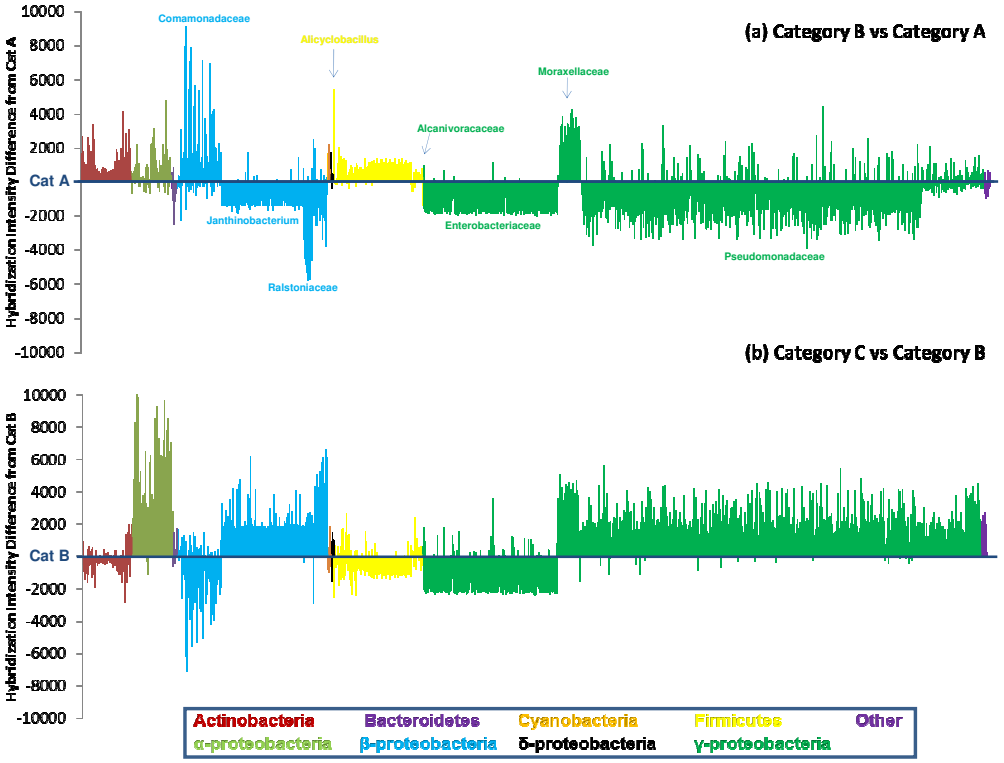
(a) Category B vs Category A
Hybridization Intensity Difference from Cat A
10000
8000
6000
4000
2000
Cat A
-2000
-4000
-6000
-8000
-10000
Comamonadaceae
Alicyclobacillus
Moraxellaceae
Alcanivoracaceae
Janthinobacterium
Enterobacteriaceae
Ralstoniaceae
Pseudomonadaceae
(b) Category C vs Category B
Hybridization Intensity Difference from Cat B
10000
8000
6000
4000
2000
Cat B
-2000
-4000
-6000
-8000
-10000
Actinobacteria
Bacteroidetes
Cyanobacteria
Firmicutes
Other
α-proteobacteria
β-proteobacteria
δ-proteobacteria
γ-proteobacteria

Fig. 1

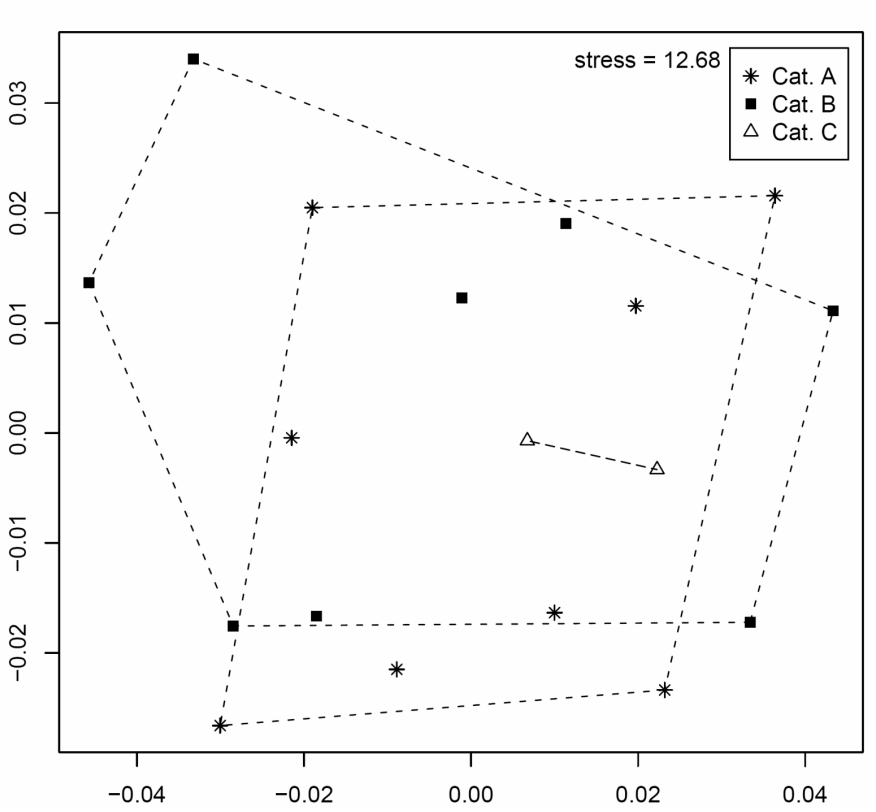
stress = 12.68
Cat. A
Cat. B
Cat. C
0.03
0.02
0.01
0.00
−0.01
−0.02
−0.04
−0.02
0.00
0.02
0.04

Fig. 2

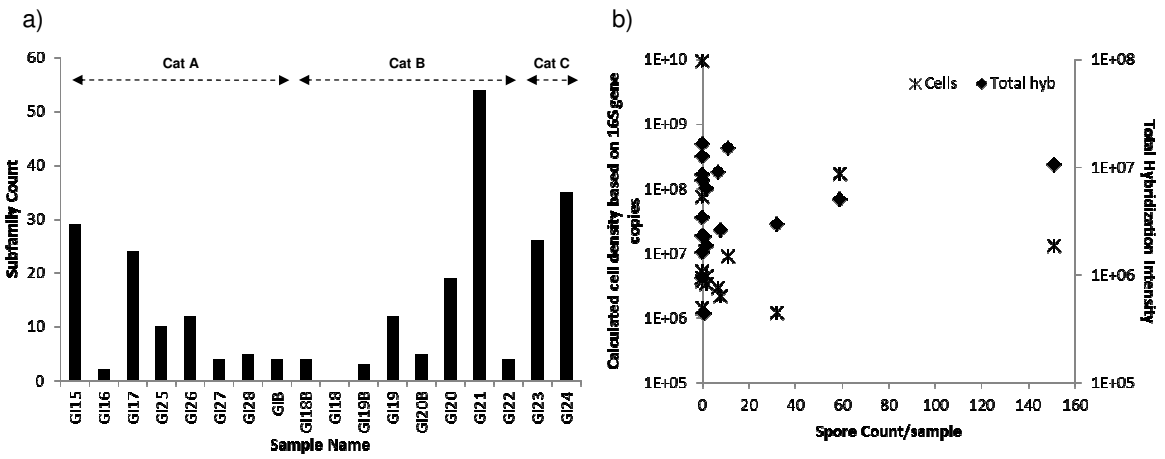
a)
Cat A
Cat B
Cat C
Subfamily Count
60
50
40
30
20
10
0
GI15
GI16
GI17
GI25
GI26
GI27
GI28
GIB
GI18B
GI18
GI19B
GI19
GI20B
GI20
GI21
GI22
GI23
GI24
Sample Name
b)
Cells
Total hyb
Calculated cell density based on 16S gene copies
1E+10
1E+09
1E+08
1E+07
1E+06
1E+05
Total Hybridization Intensity
1E+08
1E+07
1E+06
1E+05
0
20
40
60
80
100
120
140
160
Spore Count/sample

Fig. 3

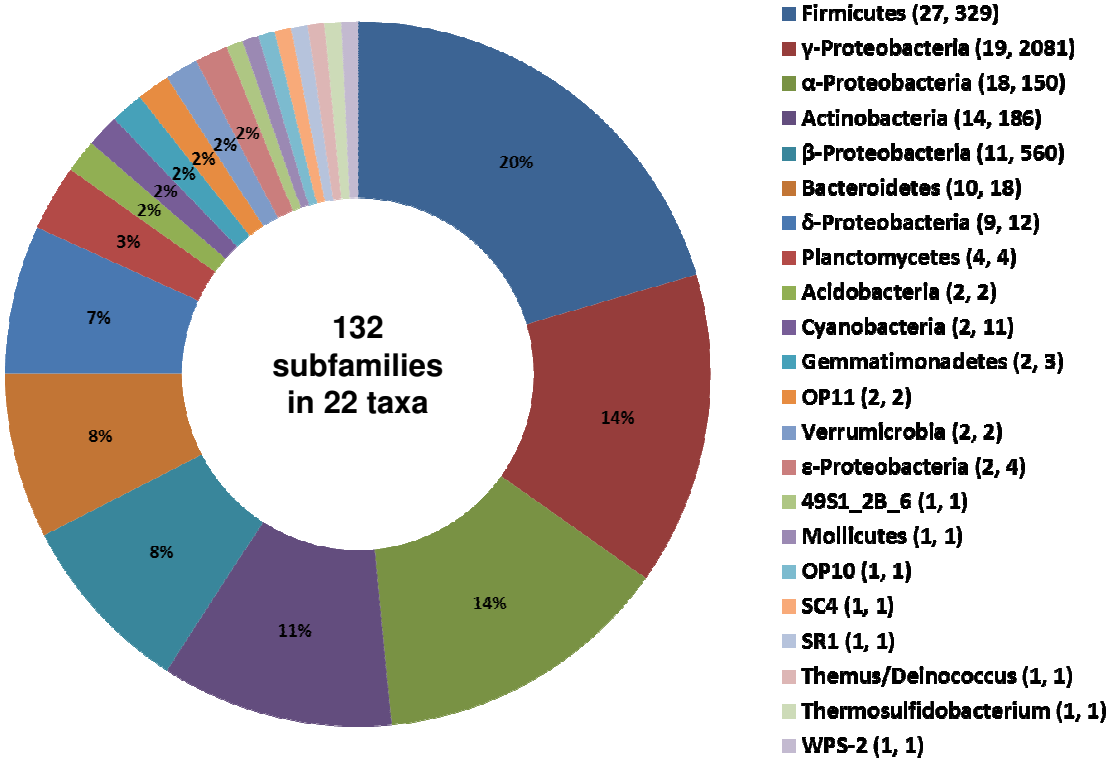
132
subfamilies
in 22 taxa
20%
14%
14%
11%
8%
8%
7%
3%
2%
2%
2%
2%
2%
2%
Firmicutes (27, 329)
γ-Proteobacteria (19, 2081)
α-Proteobacteria (18, 150)
Actinobacteria (14, 186)
β-Proteobacteria (11, 560)
Bacteroidetes (10, 18)
δ-Proteobacteria (9, 12)
Planctomycetes (4, 4)
Acidobacteria (2, 2)
Cyanobacteria (2, 11)
Gemmatimonadetes (2, 3)
OP11 (2, 2)
Verrumicrobia (2, 2)
ε-Proteobacteria (2, 4)
49S1_2B_6 (1, 1)
Mollicutes (1, 1)
OP10 (1, 1)
SC4 (1, 1)
SR1 (1, 1)
Themus/Deinococcus (1, 1)
Thermosulfidobacterium (1, 1)
WPS-2 (1, 1)

Fig. 4

Table 1. Categorical grouping of Mars Science Laboratory Spacecraft samples based on spore population, as determined by the NASA Standard Spore Assay.

	Sample	Sampling category	No. of wipes pooled	Total surface area sampled [m^2]	Total spore count/m^2	Total 16s rRNA gene copies/m^2	16S rRNA amplicons used in the DNA microarray analyses whose:	
							initial concentration [ng/µL]	mass added to the PhyloChip [ng]
Cat A								
	GI-15	S	9	5.7	0	$4.56x10^8$	468	350
	GI-16	S	6	4.2	0	$2.74x10^5$	BDL	NMA
	GI-17	S	10	7	0	$2.94x10^6$	188.2	350
	GI-25	S	8	7	0	$1.64x10^5$	7.4	281
	GI-26	S	7	3.9	0	$3.69x10^5$	24.8	350
	GI-27	S	4	7.8	0	BDL	4.8	183
	GI-28	S	6	3.3	0	BDL	3.7	141
	GI-b	NC	14	0	0	$1.02x10^6$	BDL	NMA
Cat B								
	GI-18b	HC	3	0		BDL	BDL	NMA
	GI-18	S	10	8.5	1.2	BDL	BDL	NMA
	GI-19b	HC	3	0		BDL	BDL	NMA
	GI-19	S	14	9.9	2.8	$1.22x10^5$	17.5	350
	GI-20b	HC	1	0		BDL	3.8	144
	GI-20	S	5	3.4	10.3	BDL	6.7	256
	GI-21	S	4	2.8	15.7	$8.86x10^5$	39.8	350
	GI-22	S	1	0.7	45.7	BDL	BDL	NMA
Cat C								
	GI-23	S	1	0.7	84.3	$6.70x10^7$	548.4	350
	GI-24	S	1	0.7	215.7	$5.13x10^6$	36.1	350

Abbreviations: NC-negative control, S- spacecraft surfaces, and HC-handling control, BDL- below detection limit, NMA-no measured amplicon but the products were processed for PhyloChip analysis