

## Microbial Monitoring of Spacecraft and Associated Environments

M.T. La Duc, R. Kern and K. Venkateswaran

Biotechnology and Planetary Protection Group, Jet Propulsion Laboratory, California Institute of Technology, Pasadena, CA 91109, USA

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### Abstract

Rapid microbial monitoring technologies are invaluable in assessing contamination of spacecraft and associated environments. Universal and widespread elements of microbial structure and chemistry are logical targets for assessing microbial burden. Several biomarkers such as ATP, LPS, and DNA (ribosomal or spore-specific), were targeted to quantify either total bioburden or specific types of microbial contamination. The findings of these assays were compared with conventional, culture-dependent methods. This review evaluates the applicability and efficacy of some of these methods in monitoring the microbial burden of spacecraft and associated environments. Samples were collected from the surfaces of spacecraft, from surfaces of assembly facilities, and from drinking water reservoirs aboard the International Space Station (ISS). Culture-dependent techniques found species of *Bacillus* to be dominant on these surfaces. In contrast, rapid, culture-independent techniques revealed the presence of many Gram-positive and Gram-negative microorganisms, as well as actinomycetes and fungi. These included both cultivable and noncultivable microbes, findings further confirmed by DNA-based microbial detection techniques. Although the ISS drinking water was devoid of cultivable microbes, molecular-based techniques retrieved DNA sequences of numerous opportunistic pathogens. Each of the methods tested in this study has its advantages, and by coupling two or more of these techniques even more reliable information as to microbial burden is rapidly obtained.

### Introduction

The microbial colonization of spacecraft and cleanroom assembly facility surfaces is of major concern to those commissioning modern-day space-related experimenta-

tion. The search for life elsewhere in the solar system will rely heavily on validated cleaning and sterility methods. It would be devastating for the integrity of a mission, or far worse for a pristine environment, such as the possible European subsurface ocean to be compromised as a result of terrestrial microbial contamination. To this end, new molecular-based methods are being used to ensure the cleanliness and sterility of mission-critical spacecraft component surfaces.

It is important to emphasize both cleanliness and sterility, and to validate the effectiveness and sensitivity of selected methods. Microorganisms have been found to inhabit nearly every niche imaginable on Earth, including the manmade environments of spacecraft surfaces and cleanrooms. Our previous studies suggest that spacecraft surfaces, during assembly in cleanrooms, contain 0 to 4 spore-forming cells  $\text{cm}^{-2}$  [13, 30]. So, not only is it crucial to work to minimize and, if possible, eradicate such microbial contaminants, but also it is important to characterize and identify the recurring, prevalent microbes associated with such cleanroom and spacecraft assembly environments. Studies have repeatedly shown species of the extremely hardy, spore-forming members of the *Bacillus* genus to be the most highly represented in samples collected from spacecraft and facility surfaces [13, 24]. The extremely oligotrophic, low-humidity, temperature-controlled conditions of a spacecraft assembly facility exert a selective pressure: only microbes able to withstand such unfavorable conditions persist. It is here that we see the overwhelming advantage that spore-forming bacteria possess. They enter a dormant state in the form of spores until favorable conditions permit them to germinate. Indeed, time and time again it has been shown that spore-forming bacteria, particularly species of *Bacillus*, are the most commonly isolated when assaying such environments [13, 22, 24, 30, 32].

The bias in *Bacillus* incidence might be due to the use of current culture-dependent microbial detection technologies. In our studies, we proved that when newly developed molecular microbial detection techniques were

Correspondence to: K. Venkateswaran; E-mail: kjenkat@jpl.nasa.gov

implemented, incidence and prevalence of non-spore-forming microbial species were also observed in both spacecraft and associated environments. Culture-dependent microbial detection methods underestimate the viable microbial population [8, 13, 16, 19, 30] because of the noncultivability of certain microbes and/or the presence of viable, but noncultivable, microbes [7, 25].

Identification and characterization of microbial bio-burden must be restricted to a certain number of detection methodologies because practical limitations exist in detecting every microbe in a given sample. Targeting various “biomolecules” and “biomarkers” through use of the newer molecule-based methods allows for the rapid and thorough monitoring of microbial presence within such spacecraft and cleanroom surface samples [5, 18, 31]. Microbial detection based on enzymatic activity and several gene marker-based techniques were evaluated and are presented here. Recently, these techniques have found application in the assessment of drinking water quality of the International Space Station (ISS).

## Methods

### *Microbial Detection Technologies*

*Samples.* Although many opportunities exist to enhance the collection of samples, a discussion of sampling techniques is beyond the scope of this communication. Samples listed in Table 1 were collected using either spacecraft qualified swabs or wipes. Drinking water samples processed for the STS-113 mission and aboard the ISS were also collected and analyzed. Whenever necessary, suitable negative and positive controls were included. Based on the method, positive controls were either live microbial cultures or pure chemicals.

### *Culture-Dependent Methods*

*NASA Standard Assay.* This assay is intended for the enumeration of spores and heterotrophic microbial populations. A swab sample (25 cm<sup>2</sup> area) was placed into 10 mL of sterile phosphate buffered rinse solution [1] and sonicated for 2 min. The rinse solution was aseptically pipetted out and split into 2 parts. One part of the rinse solution was subjected to heat-shock (80°C for 15 min) and the other part was not. Appropriate aliquots of samples were placed in petri dishes and total aerobic counts were enumerated by the pour plate technique using TSA as growth medium (32°C for 3 to 7 days). All microorganisms isolated, including fungi, yeasts, and bacteria, were stored at -80°C in glycerol until they were further characterized.

*Plate Count Assay.* This assay is routinely used in environmental microbiology for the cultivation of mesophilic and heterotrophic microbial populations [2]. Samples were prepared as described above, spread plated

onto pre-poured R2A agar plates, and incubated at 25°C for 7 days. Samples were not heat-shocked. Although this minimal medium facilitates the growth of a diverse range of microbes, there was not a significant difference in microbial counts between TSA and R2A when “clean” spacecraft surface samples were analyzed.

### *Culture-Independent Methods*

*ATP-Based Microbial Detection.* ATP-based microbial detection was made popular in the early 1960s to evaluate industrial hygiene quality. However, recent innovations have promoted this technique to the very forefront of state-of-the-art microbial monitoring [6]. Today, it is possible to measure strictly intracellular ATP (viable microbes) within a given sample, as well as total ATP (total microbial burden), inclusive of extracellular and intracellular ATP [29]. To determine total ATP, 0.1 mL of sample was combined with 0.1 mL of a detergent for lysing cells (benzalkonium chloride) and then incubated at room temperature for 1 min prior to the addition of 0.1 mL of luciferin-luciferase reagent, in which the luciferase is an enzyme selected for resistance to inactivation by the detergent. The sample was mixed and the amount of bioluminescence measured with a luminometer. To determine intracellular ATP, 0.1 mL of an ATP-eliminating reagent (apyrase, adenosine deaminase) was added to a 1-mL portion of the sample, mixed, and allowed to incubate for 30 min to remove any extracellular ATP, after which the assay for ATP was carried out as described above. This rapid and simple assay (60-min) is commercially available (Kikkoman Corp., San Francisco, CA).

*LPS-Based Microbial Detection.* When microorganisms invade an animal, the immune system responds by initiating a highly specific enzyme cascade in its blood cells (amebocytes). It is known that this cascade is initiated by the presence of lipopolysaccharide found in the outer membrane of Gram-negative bacteria and beta glucan in yeasts, and results in the formation of a gel clot that destroys the invading microbes. The LPS-based microbial detection assay exploits this principle as it occurs in amoebocytes of horseshoe crab (*Limulus polyphemus*) coupled with a chromogenic substrate. Hereafter, we refer to this as the limulus amebocyte assay (LAL). This method is most commonly used to quantify endotoxin and is the basis for the American Society for Testing and Materials method E2144-01 [26]. Commercially available kits (Charles River Laboratories, Wilmington, MA) were used to perform this rapid and simple assay (2-h) where appropriate aliquots of samples were mixed with reaction reagents and clotting was measured colorimetrically.

*DNA-Based Microbial Detection.* DNA was extracted directly from samples and was used to analyze molecular microbial diversity without cultivation. Suitable aliquots

**Table 1. Microbial populations of spacecraft and facility surfaces and ISS drinking water**

Source	Locations or materials	# of samples tested	Number of microbial population (CFU/ cm <sup>2</sup> )			Microbial burden (per cm <sup>2</sup> ) based on:			
			Vegetative	Spore formers	ATP (RLU)	LPS (pg endotoxin)	ATP (RLU)	LPS (pg endotoxin)	
Air particulates trapped on witness plates of spacecraft assembly facility:	Stainless steel	20	5	0.0	15.0	Not done	Not done	Not done	
	Z307	25	8	2.5	5.0	Not done	Not done	Not done	
	S13GLO-1	25	10	2.5	35.0	Not done	Not done	Not done	
	NS43G	20	20	2.5	25.0	Not done	Not done	Not done	
	463-3-8	25	25	0.0	27.5	Not done	Not done	Not done	
Various locations of spacecraft assembly facility:	Jet Propulsion Lab; SAF	50	0	0.0	0.4	0	25	2	50
	Kennedy Space Center; SAEF-II	4	1	51	Not done	37	1.3 × 10 <sup>3</sup>	1	Not done
Cleanroom floors		50	0	0.0	0.3	0	9	1	6
		25	1	5	0.1	5	12	1	16
Cleanroom tables		10	0	130	0.0	0	5.6 × 10 <sup>2</sup>	Not done	Not done
		1	0	1	Not done		Not done	Not done	Not done
Spacecraft or accessories surfaces: Mars Odyssey Orbiter		2	1.3 × 10 <sup>4</sup>	2.0 × 10 <sup>4</sup>	Not done	1.0 × 10 <sup>5</sup>	1.3 × 10 <sup>5</sup>	2.6 × 10 <sup>2</sup>	2.9 × 10 <sup>2</sup>
		3	No CFU	Not done	Not done	No ATP	No ATP	No ATP	No exotoxin
		1	5.1 × 10 <sup>1</sup>	Not done	4.8 × 10 <sup>5</sup>	2.1 × 10 <sup>2</sup>			
Flight-ready circuit boards of future Europa mission		1	No CFU	Not done	Not done	No ATP	No ATP	1.1 × 10 <sup>1</sup>	
		1	No CFU	Not done	Not done	No ATP	No ATP	1.1 × 10 <sup>1</sup>	
International Space Station (ISS): Ground processed; not biocide treated		2	1.3 × 10 <sup>4</sup>	2.0 × 10 <sup>4</sup>	Not done	1.0 × 10 <sup>5</sup>	1.3 × 10 <sup>5</sup>	2.6 × 10 <sup>2</sup>	2.9 × 10 <sup>2</sup>
		3	No CFU	Not done	Not done	No ATP	No ATP	No ATP	No exotoxin
Regenerated water for rehydrating foods aboard ISS (SRV-K hot)		1	5.1 × 10 <sup>1</sup>	Not done	4.8 × 10 <sup>5</sup>	2.1 × 10 <sup>2</sup>			
		1	No CFU	Not done	Not done	No ATP	No ATP	1.1 × 10 <sup>1</sup>	
Potable water aboard ISS (SVOZV)		1	No CFU	Not done	Not done	No ATP	No ATP	1.1 × 10 <sup>1</sup>	

\*Units expressed are for 100 mL.

of each sample were subjected to DNA extraction using either a manual [9] procedure or an automated IG Autolyser DNA extractor (Instant Genetics, Menlo Park, CA) per the manufacturers' protocol ([www.instantgenetics.com](http://www.instantgenetics.com)). Bacterial small-subunit rRNA genes (*rrn*) were PCR-amplified with eubacterially biased primers B27F and B1512R, and eukaryotic 18S rRNA genes were amplified using NS1F and NS8R primers. PCR conditions and cloning procedures were followed as described elsewhere [13]. Ribosomal DNA inserts were PCR-amplified from plasmid clones and amplification products were digested with restriction endonuclease for 3 h at 37°C. Banding patterns were grouped according to similarity, and representative members of each distinct pattern group were fully, bidirectionally sequenced. The phylogenetic relationships of organisms covered in this study were determined by comparison of individual 16S or 18S rDNA sequences to sequences in the public database (<http://www.ncbi.nlm.nih.gov/>). Evolutionary trees were constructed using PAUP software [25].

*Specific Detection of Problematic Microbial Species.* Research has identified spore-forming microorganisms as problematic because of their resistance to varying chemicals and conditions in spacecraft and associated environment. In order to quantify prevalent microbial species, a strategy to detect spore-forming microorganisms was developed. The gene *spkB* is unique to spore-forming bacteria and encodes spore-photoproduct-lyase, an enzyme involved in direct reversal of UV-induced pyrimidine dimerization. Twenty-eight *Bacillus* species were procured from various sources, and their DNA was extracted. The *rrn* and *spkB* genes were then PCR amplified, and species showing positive *spkB* gene amplification were sequenced. Alignment of the *spkB* sequences enabled us to identify highly conserved domains for the design of semidegenerate "universal" *Bacillus spkB* primers for PCR amplification of unknown samples. Spacecraft-qualified titanium plates artificially inoculated with *B. subtilis* ATCC 6051 were swabbed and PCR assayed for *spkB* presence. Number of *B. subtilis* cells using *spkB* and the sensitivity of the TaqMan quantitative-PCR (Q-PCR) method were determined.

## Results

*Microbial Burden.* The aerobic heterotrophic microbial population (total heterotrophs) was enumerated using both R2A and TSA media, whereas TSA was chosen to count endospore-forming microbes since spore-formers tend to grow faster in this medium. The microbial populations aboard naturally falling air particulates both trapped on witness plates and swabbed from floors, tables, and cabinet tops of class 100K (number of particles >0.5  $\mu\text{m}$  in size per cubic foot) cleanroom

spacecraft assembly facility surfaces are depicted in Table 1. This includes culture-dependent microbial counts enumerated for both spore-formers and heterotrophs. In addition, microbial density values derived from assaying two biomolecules, ATP and LPS, are given in Table 1. Based on *Escherichia coli* standard culture counts, microbial densities were calculated on the assumption that one RLU (a measure of ATP) and 1 pg endotoxin (a measure of LPS) is equivalent to one CFU. Number of samples tested in each category is also tabulated.

*Fallout Air Particulates.* The total heterotrophs of the cleanroom air ranged between 5 and  $2.4 \times 10^5$  CFU, and the spore-forming microbes were between 0 and  $2.8 \times 10^1$  CFU per  $\text{cm}^2$  on the surface area of witness plates. Paint-coated witness plates captured more microbes than did stainless steel plates. Witness plates coated with NS43G, an off-white conductive paint, trapped more particulates [28], and the number of microorganisms isolated was also higher with NS43G when compared to other witness plates. In most cases, the number of heterotrophs present ranged from less than the detectable level to  $5.0 \times 10^1$  CFU per  $\text{cm}^2$ .

*Spacecraft and Assembly Facilities.* Twenty-five surface samples of the Mars Odyssey spacecraft were pooled and showed, on average, total heterotrophs and spore-formers at 1 and 0.1 CFU per  $\text{cm}^2$ , respectively (Table 1). The pooled swab samples collected from flight-ready circuit boards (<http://aarchive-lib.jpl.nasa.gov>), prepared for a possible future mission to Europa, contained microbial densities somewhat similar to those observed for the Mars Odyssey [13]. The microbial burden determined by the ATP assay was within an order of magnitude of that dictated by the plate count assay for the Mars Odyssey surfaces.

Floor, table, and cabinet top surface samples from two class 100K cleanroom assembly facilities were examined. In general, the number of heterotrophs present in these locations was in the range of 0 to  $10^2$  CFU, and abundance of spore-formers was less than 1 CFU per  $\text{cm}^2$ . Many of the tested samples had no cultivable microbial counts. The microbial burden associated with the JPL-SAF cleanroom floors, tables, and cabinet tops, as determined by biomolecule enumeration, showed similar microbial densities.

However, the microbial burden of the KSC SAEF-II floors, as measured by the ATP assay, exhibited at least 1 to 2 logs higher viable microbial population than cultivable counts.

*ISS Drinking Water.* Untreated municipal water samples (City of Cocoa, FL) processed for the ISS contained  $1.4 \times 10^4$  CFU/100 mL, whereas cultivable counts were not observed in the biocide-treated samples. The

water used to rehydrate foods on board the ISS revealed  $5.1 \times 10^3$  CFU/100 mL cultivable counts, whereas the drinking water did not show any CFU. The ATP assay exhibited  $1.3 \times 10^5$  microorganisms per 100 mL present in the untreated municipal water samples, an order of magnitude higher. Based on culture-independent analysis (ATP and LPS measurements), the absence of any viable microbial populations in these water samples suggests that biocide treatment is effective in removing all cultivable microbes from the water system. Intracellular ATP determinations were unable to detect any measurable microbial burden in the ISS drinking water samples, unlike LAL analysis that measured  $1.1 \times 10^1$  pg endotoxin. Whether this contaminating endotoxin arose from living or dead cells could not be determined, as this assay detects the presence of all endotoxin within a sample, regardless of its source. Results of the ATP assay in combination with LAL analysis stress the need to develop an approach to analyze the presence of noncultivable microbes [15].

**Cultivable Microbial Diversity.** Excluding the ISS drinking water samples, the most common microbes isolated from the environments sampled in this study were Gram-positive bacteria, particularly species of *Bacillus* (Table 2). Among the members of the *Bacillus*, about 12 known and 10 novel species were encountered. One of these novel species was characterized and named *B. nealsonii* [30], and another round-spore-forming bacillus was described as “*B. odysseyi*” [14]. Without exception, *B. pumilus* was repeatedly isolated from all of the various surface locations analyzed. Although *B. pumilus* was not isolated from the ISS drinking water, this bacterial species was isolated from ISS hardware surfaces (data not shown). With the exception of *B. odysseyi*, all of the *Bacillus* species isolated in this study fall into the *Bacillus* rRNA group-1, phylogenetically.

In addition to *Bacillus*, species of other spore-forming genera such as *Sporosarcina* and *Paenibacillus*, actinomycetes, and fungi (*Aureobasidium*) were isolated. *A. pullulans* was isolated from the Mars Odyssey spacecraft but was not cultivated from any of the other surrounding surfaces. Unlike the surrounding facility surfaces, several genera were cultivated from the surfaces of the Mars Odyssey spacecraft, whose components were manufactured across various geographical locations. Asporogenous rods, such as *Corynebacterium*, *Curto-bacterium*, *Filibacter*, *Microbacterium*, and cocci, *Kyto-coccus*, *Micrococcus*, *Planococcus*, *Staphylococcus*, and *Terracoccus*, were cultivated from the various surfaces assayed in this study.

Comparatively, fewer Gram-negative microbes were isolated from the many surface samples analyzed. Species of the  $\beta$ - and  $\gamma$ -proteobacterial lineages were cultivated

from the spacecraft surfaces only. Of the water collected from the ISS, only the water used to rehydrate foods yielded any cultivable microbes, those being *Acinetobacter radioresistens* and *Acidovorax temperans*. Interestingly, *A. radioresistens* was isolated from both the surface of the Mars Odyssey spacecraft and the ISS drinking water.

**Molecular Microbial Diversity.** Because this method is not dependent on culturing of microbes, a much larger diversity was observed (Table 2). Phylogenetic analyses of the 16S rDNA sequences showed equal representation of Gram-positive and Gram-negative bacteria in the spacecraft and facility surface samples. Among the sequences retrieved directly from the samples analyzed in this study were representatives of  $\alpha$ -,  $\beta$ -,  $\gamma$ -proteobacteria and Gram-positive bacteria. Sequences of *Bacillus* and *Taxeobacter* species are repeatedly retrieved from JPL-SAF surfaces, whereas *Aquaspirillum* and *Variovorax* sequences are the most commonly retrieved from KSC facility surfaces. For the most part, sequences retrieved from JPL-SAF surfaces derive from low-humidity, oligotrophic tolerant microbes, similar to those found in desert-like environments. Conversely, most of the sequences retrieved from KSC facility surfaces arise from microbes more tolerant of humid, brackish environments.

The ISS drinking water samples were quite diverse, as sequences arising from aerobic heterotrophs, halogen-reducing bacteria, and commensals were observed. Of greatest importance was the retrieval of several 16S rDNA sequences of opportunistic pathogens. Sequences of *Afipia*, *Ochrobactrum*, *Stenotrophomonas*, and *Propionibacterium* were all retrieved from the drinking and/or regenerative waters of the ISS. The untreated municipal water harbored sequences of *Pseudomonas* only, while after treatment with iodine sequences of  $\alpha$ - and  $\beta$ -proteobacteria were retrieved. This is suggestive of a post-treatment contamination event, either linked with the storage practices or transport, or otherwise.

**Specific Detection of Problematic Microbial Species.** About 800 base pairs of the *spkB* sequences from 17 *Bacillus* species were sequenced. The *spkB* gene nucleotide sequence is highly heterogeneous, and  $\sim 70\%$  nucleotide sequence similarity was observed among various species of *Bacillus*, as well as between inter-genus spore-forming bacteria (Fig. 1). Such heterogeneity of gene sequences has been exploited to design effective probe-primer sets specific for a given problematic species. For example, a specific TaqMan *spkB* probe-primer set was synthesized that allowed us to perform quantitative real-time PCR to detect *B. subtilis* from environmental surface samples. Surfaces contaminated with as few as  $10^3$  CFU per mL were effectively detected and this TaqMan system was specific to the detection of the microbial species targeted (Fig. 1).

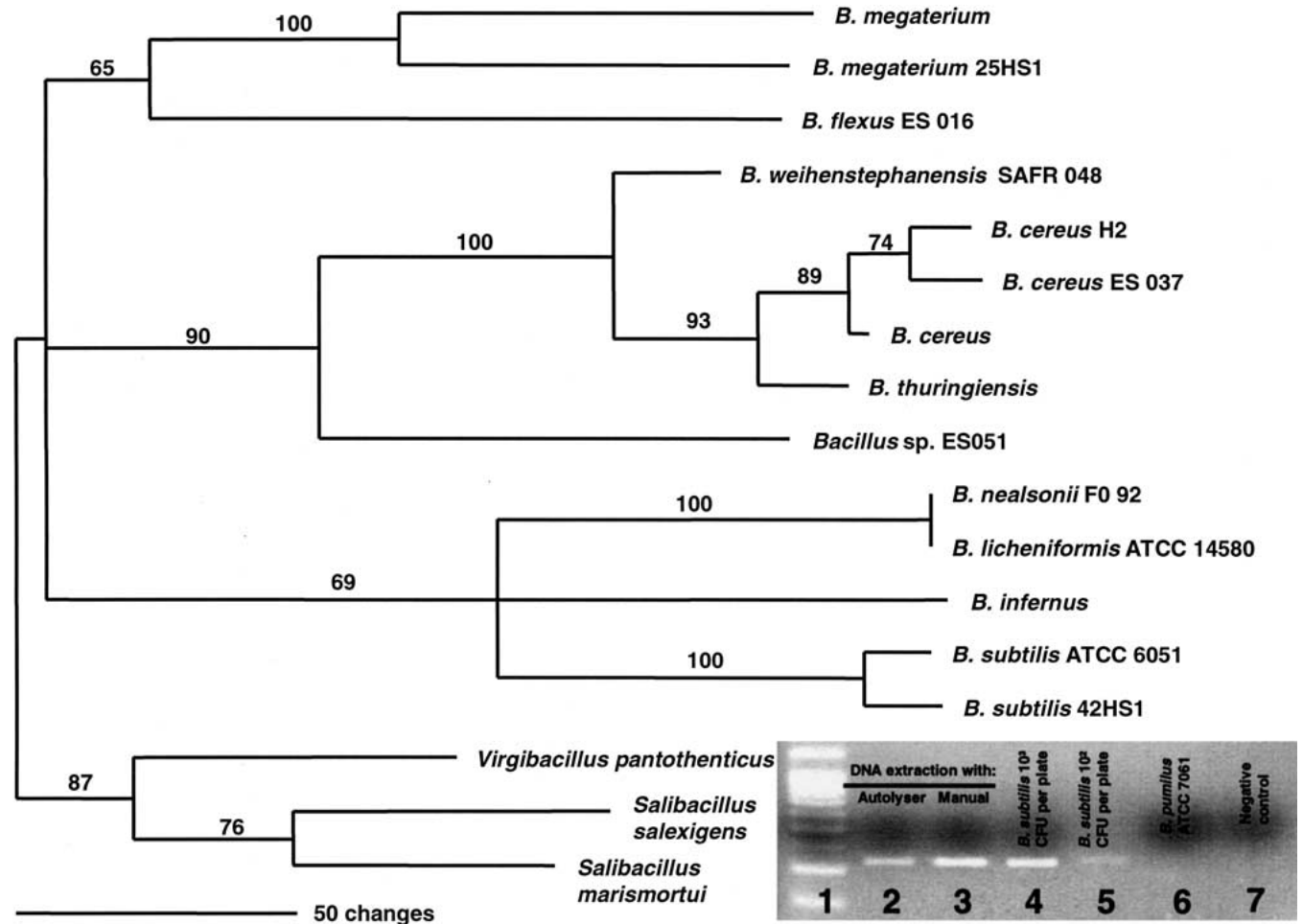
**Table 2. Microbial species either isolated or their sequences retrieved from spacecraft and facility surfaces and ISS drinking water**

Sample description	Microbial species present			
	Culture-dependent	Number of species	Culture-independent	Number of species
Air particles trapped on witness plates of spacecraft assembly facility	<i>Bacillus</i> species	6	$\beta$ -Proteobacteria	3
	<i>Micrococcus luteus</i>	1	$\gamma$ -Proteobacteria	5
	<i>Staphylococcus capitis</i>	1	Gram-positive	6
	<i>Planococcus citreus</i>	1	CFB group	2
Jet Propulsion Lab; SAF	<i>Bacillus</i> species	11	$\alpha$ -proteobacteria	1
	<i>Staphylococcus</i> species	2	$\beta$ -proteobacteria	3
	<i>Streptomyces platensis</i>	1	$\gamma$ -proteobacteria	2
	<i>Filibacter limicola</i>	1	Gram-positive	6
	<i>Sporosarcina aquamarinus</i>	1	CFB group	4
	<i>Paenibacillus</i> species	4		
Kennedy Space Center; SAEF-II	<i>Bacillus</i> species	4	$\alpha$ -Proteobacteria	3
	<i>Staphylococcus warneri</i>	1	$\beta$ -Proteobacteria	6
	<i>Terracoccus luteus</i>	1	Gram-positive	3
Spacecraft or accessories surfaces Mars Odyssey Orbiter	<i>Bacillus</i> species	6	$\alpha$ -Proteobacteria	2
	<i>Curtobacterium</i> species	1	$\beta$ -Proteobacteria	5
	<i>Delftia acidovorans</i>	1	$\gamma$ -Proteobacteria	1
	<i>Microbacterium</i> species	2	Gram-positive	4
	<i>Ralstonia pickettii</i>	1		
	<i>Acinetobacter radioresistens</i>	1		
	<i>Aureobasidium pullulans</i>	1		
Flight-ready circuit boards of future Europa mission	<i>Bacillus</i> species	10	$\alpha$ -Proteobacteria	1
	<i>Staphylococcus</i> species	3	$\beta$ -Proteobacteria	5
	<i>Micrococcus luteus</i>	1	$\gamma$ -Proteobacteria	3
	<i>Paracraurococcus</i> species	1	Gram-positive	9
	<i>Kytococcus sedentarius</i>	1	CFB group	1
	<i>Streptomyces</i> species	1		
	<i>Corynebacterium</i> species	2		
	<i>Pseudomonas</i> species	1		
	<i>Microbacterium</i> species	2		
	<i>Curtobacterium</i> species	1		
	<i>Cellulomonas</i> species	1		
	<i>Brachybacterium</i> species	1		
	<i>Variovorax</i> species	1		
	<i>Dietzia</i> species	1		
	International Space Station (ISS)			
Ground processed; biocide not treated	Not done		$\gamma$ -Proteobacteria	2
Ground processed; biocide treated	None		$\alpha$ -Proteobacteria	1
Regenerated water for rehydrating foods aboard ISS (SRV-K hot)			$\beta$ -Proteobacteria	2
	<i>Acinetobacter radioresistens</i>	1	$\alpha$ -Proteobacteria	2
	<i>Acidovorax temperans</i>	1	$\beta$ -Proteobacteria	3
Potable water aboard ISS (SVOZV)			$\gamma$ -Proteobacteria	1
			$\alpha$ -Proteobacteria	4
			$\beta$ -Proteobacteria	2
			Gram-positive	1

## Discussion

For decades microbiologists and ecologists alike have battled with “the great plate count anomaly,” that is, how to go about culturing microbes from the environment while minimizing the inherent bias associated with using any one particular medium. It is well known that only

~1% of the microorganisms reported are cultivable, so if humanity is to gain a better understanding of the role that microbes play in any given environment, superior methods of investigation will need to be developed and put into practice. In this study we explored several state-of-the-art techniques, ranging from enzyme-based bio-molecule enumeration schemes to universal gene-based



**Figure 1.** Phylogenetic tree of the *splB* sequences (~800 bp each) of 17 *Bacillus* sp. The numbers above the lines are the percent bootstrap values of that branch of the tree after 500 replications. Inset gel image shows *splB* amplification (~150 bp) of varying DNA extractions from artificial inoculations of either *B. subtilis* on Ti plates. Lane 1: DNA ladder; lane 2: automated DNA extraction; lane 3: manual DNA extraction; lane 4:  $10^3$  *B. subtilis* per Ti plate; lane 5:  $10^2$  *B. subtilis* per Ti plate; lane 6: *B. pumilus* ATCC 7061 DNA; lane 7: negative control, no DNA.

cloning strategies. Whereas cultivation-dependent methods are reliant upon prior knowledge of the microbes in question, modern day cultivation-independent techniques require no such foreknowledge, and elicit no such bias for or against previously reported microorganisms.

From the Viking mission (1976) to the present day, each and every attempt at microbial enumeration and isolation from spacecraft and associated environments has been carried out using traditional culture-based techniques [3, 4, 12, 16, 19–21, 23, 24, 30]. In this communication we compared the results generated using both traditional and advanced molecular approaches while examining the abundance of microorganisms from spacecraft and associated environments. As expected, populations were far less diverse when examined by culture-dependent methods, most likely because of the limitations in the methods employed.

One of the culture-independent methods, microscopy, is a powerful tool and is invaluable for qualitatively assessing microbial populations. However, because of the drastically low number of microbes present in spacecraft and associated environments (0 to  $10^2$  CFU per  $\text{cm}^2$ ), and the limitations associated with this technique (autofluorescence, false-positives, nonspecific binding of stain, insufficient penetration resulting in false negatives [21]) it is impractical. Despite an appreciable number of visible cells (2 log higher than spacecraft surfaces) in the assembly floor samples, the nonspecific binding of stains to particulates was problematic. We did not perform *in situ* nucleic acid hybridizations since previous attempts have shown that such techniques necessitate  $>10^7$  cells per reaction (data not shown).

The molecular microbial detection methods revealed the presence of noncultivable microbes in the ISS drinking water, confirming the fact that the measures

taken to cultivate microbes from the drinking water samples were estimating only 10% of the microbial contamination. Although the drinking water collected from the ISS did not yield any measurable microbes, DNA-based procedures retrieved ribosomal sequences of opportunistic pathogens. Likewise, molecular cloning-based methods showed more diverse populations from both the spacecraft and facility samples than culture-dependent methods employed. This is reasonable since recovery of DNA fragments yields information from the total microbial population present, dead or alive, unlike culturing microorganisms that require specific conditions for growth. In this communication, we have shown that Q-PCR approaches can be used to detect and quantify problematic microbial species (*spkB* for spore-forming microbes) once the problem is made known by use of molecular microbial diversity approaches.

The ATP assay has long been recognized as being "by far the most convenient and reliable method for estimating total microbial biomass in most environmental samples" [10]. In many samples, notably cleanroom floors, the levels of free extracellular ATP were extremely high, sometimes 2 or 3 orders of magnitude greater than the intracellular values (data not shown). This ATP is probably attributable to lysed, dead cells. The present study demonstrates the value of knowing culturable as well as total microbial burden. Although it can be presumed that the level of ATP is as valid as are viable cell counts for estimating the cleanliness of a facility, it is evidently not possible for the amount of ATP measured to be used to estimate a cell number or a quantitative bioburden, because of large differences in volumes and metabolic states of different cells. However, the use of culturable cell counts to determine total bioburden is also subject to scrutiny, since with any given medium only a small and indeterminable fraction of the organisms present will grow [11, 26]. Similarly, the rapid inferences derived from the LAL assay allowed quick decisions to be made in regard to recleaning necessary surfaces.

Finally, these molecular microbial detection assays report clumped-cell numbers accurately, whereas plate counts do not. Moreover, the molecule(s)-based assays show no preference to cultivable species; they tell of the presence of any and all contaminating microbes, regardless of growth requirement, nutrient availability, syntrophy, or cellular health. These assays are particularly applicable for monitoring samples from environments with extremely low microbial burden. Single-handedly, each of the above-described technologies has its limitations and can yield only so much relevant information. The real power of such rapid techniques comes in their use in concert, and when combined, they yield additional insight into the microbial population.

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