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Molecular Microbial Diversity of a Spacecraft Assembly Facility

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Summary

In ongoing investigations to map and archive the microbial footprints in various components of the spacecraft and its accessories, we have examined the microbial populations of the Jet Propulsion Laboratory's Spacecraft Assembly Facility (JPL-SAF). Witness plates made up of spacecraft materials, some painted with spacecraft qualified paints, were exposed for ~7 to 9 months at JPL-SAF and examined the particulate materials collected for the incidence of total cultivable aerobic heterotrophs and heat-tolerant (80ºC for 15-min.) spore-formers. The results showed that the witness plates coated with spacecraft qualified paints attracted more dust particles than the non-coated stainless steel witness plates. Among the four paints tested, witness plates coated with NS43G accumulated the highest number of particles, and hence attracted more cultivable microbes. The conventional microbiological examination revealed that the JPL-SAF harbors mainly Gram-positive microbes and mostly spore-forming *Bacillus* species. Most of the isolated microbes were heat resistant to 80ºC and proliferate at 60ºC. The phylogenetic relationships among 23 cultivable heat-tolerant microbes were examined using a battery of morphological, physiological, molecular and chemotaxonomic characterizations. By 16S rDNA sequence analysis, the isolates fell into seven clades: *Bacillus licheniformis, B. pumilus, B. cereus, B. circulans, Staphylococcus capitis, Planococcus* sp. and *Micrococcus lylae*. In contrast to the cultivable approach, direct DNA isolation, cloning and 16S rDNA sequencing analysis revealed equal representation of both Gram-positive and Gram-negative microorganisms.

Key words: Spacecraft Assembly Facility – Thermo-tolerance – *Bacillus* spores – 16S rDNA analysis – RFLP analysis – Phylogeny

Introduction

As NASA's search for evidence of past or present life beyond Earth continues, an increasing number of in situ life detection and sample return missions to extraterrestrial bodies of interest to life's origins are envisioned. As enabling technologies for these robotic missions emerge, awareness of the need to control spacecraft carried microbial contamination is also growing. One of the NASA planetary protection policy guidelines specifically directed to control the terrestrial microbial contamination associated with robotic space vehicles intended to land, orbit, flyby, or otherwise be in the vicinity of extraterrestrial solar system bodies. Implementation of these planetary protection requirements will ensure that biological safeguards to maintain extraterrestrial bodies as biological preserves for scientific investigations are being followed in NASA's space programs.

The main focus of planetary protection research efforts at the Jet Propulsion Laboratory (JPL) is the development of enabling cleaning and sterilization technologies for spacecraft preparation prior to launch. Knowledge of the microbial diversity of spacecraft assembly facilities (SAF), as well as any extreme characteristics these microbes might possess, are critical to the development of these enabling technologies. We consider the spacecraft assembly facilities to be extreme environments created by the controlled air circulation, desiccation, moderately high temperature, and low-nutrient conditions found in these clean rooms.

The state of knowledge of microbial populations in SAF is currently limited to the results of culture-based studies conducted in the 1970's (PULEO *et al.*, 1973; 1975; 1977). During the 20 year hiatus between the launch of

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the Viking Landers and the current Mars Surveyor Program, microbiology has undergone a fundamental change. Modern methods of microbe detection are based upon molecules not metabolism and growth. By using the methods of modern molecular microbial ecology, supplemented with traditional culture-based techniques, we will provide a more complete understanding of the microbial contaminants associated with the assembly of spacecraft.

The objective of this study is to systematically detect and classify the cultivable and non-cultivable microbes present in SAF at JPL using classical microbial phylogeny and advanced molecular microbial ecology methods. This will provide a detailed understanding of the microbial species most likely to contaminate the surfaces of spacecraft hardware. Furthermore this will also lead to enabling planetary protection cleaning and sterilization technologies for spacecraft preparation. In addition, this approach will help in identifying those terrestrial microbes most likely to contaminate extraterrestrial environments, in-situ life detection studies, or, possibly, returned samples.

Materials and Methods

Spacecraft assembly facility

The dimensions of the JPL-SAF are 80' wide, 120' long, and 44' 4" high. Relative humidity was controlled at 40 ± 5% with a cap at 45% and the average temperature was maintained at 20 ± 5º C. Entry into this facility was minimal and carefully monitored by setting a series of rigorous procedures. For example, people must clean their shoes in a provided mechanized shoe-cleaner, followed by taking an air shower before entering the ante-room. The ante-room provides all clean room garments including shoes, booties, gowns, caps, etc. This JPL-SAF room was controlled by qualified contamination control people with a periodic check to maintain a Class 100,000 clean room level.

Collection of dust particles on witness plates

Witness plates of a specified size (1"×2") were used. Stainless steel (type 304, No.4 finish, 0.08-0.05 cm thick) plates were ultrasonically cleaned in acetone (5 to 10-min) followed by isopropanol (5 to 10-min). After air drying, the plates were then sterilized either by heat at 175º C for 2 hours (ANONYMOUS, 1980) or by hydrogen peroxide gas plasma sterilization (JOHNSON and JOHNSON Co., Calif.). Four commonly used spacecraft paints, coated on aluminum 6061 witness plates were also prepared (ANONYMOUS, 1998) with subsequent sterilization in hydrogen peroxide gas plasma. The pre-sterilized witness plates made of stainless steel and four different paint-coated aluminum were exposed in a JPL spacecraft assembly facility. Stands that carry these witness plates were about 6 feet high to minimize contamination from human exhalation or sweat, thus allowing collection of dust particles that were naturally falling out onto the witness plates in the assembly area. Exposure time, specifications of paints and positions of the witness plates are given in Table 1. After the given exposure period, all witness plates were individually placed into 50-mL polypropylene disposable sterile centrifuge tubes.

Collection of particles and fibers

To determine the extent of particulate contamination on the surfaces, a tape lift technique was performed (ANONYMOUS, 1989). Particulate materials were counted and distinguished individually according to size using an optical microscope. This provides an approximate diameter measurement for particles and a length measurement for fibers. All particulate materials larger than 500 µm in size were identified individually.

Microbial examination

Each witness plate retrieved from JPL-SAF was placed into 30 mL of sterile phosphate buffered rinse solution (ANONYMOUS, 1980). The plate and rinse solution were sonicated for 2 min. The rinse solution was aseptically pipetted out and split into 2 parts (15-mL each). One part of the rinse solution along with the witness plate were subjected to heat-shock (80º C for 15 min) and the other part was not heated. Appropriate aliquots of samples were placed in petridishes and total aerobic counts were enumerated by pour plate technique using TSA as growth medium (32º C for 3 to 7 days). All microorganisms, including fungi, yeasts and bacterial isolates were stored in glycerol until further characterized. Type species of various genera were procured from American Type Culture Collection (ATCC). The ability to grow at various temperatures and sodium chloride concentrations was used as a discriminator to select strains. All the isolates that showed growth at 60º C were further characterized. In addition, bacteria that were morphologically different but not growing at 60º C were included. Although these strains grew well at higher temperature, 32º C was found to be optimum temperature and hence further characterization was carried out at 32º C.

Heat tolerance

Overnight grown cultures were inoculated into 10-mL of TSB and incubated at various temperatures (25, 30, 35, 40, 45, 50, 55, 60, 65 and 80º C) using either microbiological incubator or water bath. The growth of the microorganisms was checked by turbidity at various intervals of incubation period (2 to 7 days). The turbid cultures were then streaked onto TSA agar plate and checked for purity.

Phenotypic characterization and fatty acid analysis

Routine biochemical tests were carried out according to established procedures (CLAUS and BERKELEY, 1986; PRIEST, 1993). The ability to grow at a NaCl concentration of 1 to 10% was determined in T_1N_1 liquid medium (VENKATESWARAN et al., 1989), and the ability to grow without NaCl was determined in 1% sterile tryptone water. The API CHB 50 kit and API 20E were used for the Gram-positive spore-forming rods (75 biochemical tests) and the API STAPH kit was used for Gram-positive coccoid isolates (20 biochemical tests). Identification of the test isolate was carried out by computing and comparing test results from the bioMerieux database (bioMerieux, Inc., St. Louis, Misso.). Fatty acid methyl ester (FAME) profiles were examined as described previously (RINGELBERG et al., 1994).

16S rDNA sequencing

Purified genomic DNA (JOHNSON, 1981) from liquid-grown cultures were quantified and ~10 ng of DNA was used as the template for PCR amplification. Universal primers (Bact 11 and 1,492) were used to amplify the 1.4-kb PCR fragment per protocols established by RUIMY et al. (1994). Amplicons thus generated were sequenced directly following purification on Qiagen columns (Qiagen, Valencia, Calif.). The identity of a given PCR product was verified by sequencing using the dideoxy chain termination method with Sequenase DNA sequencing kit (United States Biochemical Corporation, Cleveland, Ohio) and with an

ABI 373A automatic sequencer as recommended by the manufacturer (Perkin-Elmer Corp., Foster City, Calif.). The phylogenetic relationships of organisms covered in this study were determined by comparison of individual 16S rDNA sequences to other already existing sequences in the public database (GenBank; *http://www.ncbi.nlm.nih.gov/*). Evolutionary trees were constructed with the PAUP (SWOFFORD, 1990).

DNA-DNA hybridization

Cells were suspended in 0.1M EDTA solution (pH 8.0) and digestion of cell wall was carried out by treating the cells with lysozyme (final concentration, 2mg/ml) for bacilli or labiase (final concentration, 2mg/ml) for cocci. The DNA was isolated by standard procedures (JOHNSON, 1981). DNA-DNA hybridization was carried out as described previously (SATOMI et al., 1997).

Direct DNA isolation and cloning

The witness plates were immersed in a Tris-EDTA buffer (50 mM Tris pH 7.5; 20 mM EDTA) and particulate materials were released into the solution by sonication. The contents were then centrifuged (9,000 g; 10-min.; 4° C) and the pellets were resuspended in a small volume of Tris-EDTA buffer before chromosomal DNA were extracted (JOHNSON, 1981). A 493-bp PCR product was amplified using a primer set (27f and 519r). The PCR products were then cloned using TA cloning kit (Invitrogen, Carlsbad, Calif.). Plasmid was isolated from several clones (SAMBROOK et al., 1989) from each witness plate, and the insert was amplified with 27f and 519r primer set to check for correct insert size. Each product of the correct size (493 bp) was digested with *HaeIII* or *MspI* enzyme and electrophoresed through high-sieve quality agarose gels. The restriction fragment length polymorphism (RFLP) patterns from both digests were compared among clones. Clones with the same pattern for both enzymes were grouped, and several clones from each pattern were sequenced (unless only one or two clones comprised a pattern group).

Suitable negative controls were included in all the experiments to avoid any false-positive results.

Results

Collection of witness plates and distribution of particulate materials

Table 1 details the retrieval history of various witness plates deployed in JPL-SAF. About 20 to 25 replicates of 5 different types of witness plates were removed and individually placed into 50-mL screw-capped sterile centrifuge tubes. Although we deployed these witness plates on various dates, we have retrieved the samples on the same day. A clear pattern of particle distribution was noticed in the spacecraft quality paint coated witness plates. In general, particles of the size 5 to 100 µm were collected on all types of witness plates. The smaller size particles were more abundant in the paint coated witness plates and the profusion decreased with increased particle size. In contrast, pure stainless steel witness plates accumulated more mid-range size (26-100 µm) particles and the abundance of particles decreased when the particle sizes decreased. In terms of fibers, the NS43G paint coated surface attracted more fibers followed by stainless steel, S-13GP/LO-1, Z307 and 463-3-8 paint coated surfaces.

Microbial examination

The paint-coated witness plates attracted more microbial population than the pure stainless steel plates (Table 2). Such microbial abundance is well correlated to an abundance of particulate materials trapped on the witness plates. In other words, NS43G-coated witness plates had trapped more particles and the number of microorganisms isolated was also higher when compared to other witness plates. However, the number of fibers on various witness plates showed no relationship with the microbial counts. In general, both heat-resistant (3 to 1.5×10^1 cfu/cm^2) and vegetative (5 to 6.9×10^1 cfu/cm^2) microbial contamination transferred through particulate materials is not high in terms of microbial load. In addition to the bacterial population, spatial distribution of yeast and fungal population were noticed in these witness plates and were insignificant.

Isolation and identification of microbial population

Bacterial colonies of various morphotypes were carefully selected with an emphasis to represent all witness plates (Table 2). Among the total of 89 isolates selected, 39 isolates were picked from the samples that were treated at 80°C for 15-min, while 50 isolates were isolated from unheated samples. About 44% of the 39 heat-tolerant

Table 1. Nature, placement position, and exposure time of various witness plates deployed in a Spacecraft Assembly Facility during this study.

Nature of the witness plate	Nature of the paint	Location	Date & Time Installed	Date & Time Retrieved	# of witness plates Retrieved	Exposure, days (months)
Stainless steel	–	North	6/11/98 3 p.m.	3/16/99 2:00 p.m.	20	278 (9.3)
NS43G-painted	off-white conductive paint (silicate binder)	West	7/27/98 11 a.m.	3/16/99 2:00 p.m.	25	232 (7.7)
S-13GP/LO-1-painted	non-conductive white silicone elastomer based	West	7/9/98 9:30 a.m.	3/16/99 2:00 p.m.	25	250 (8.3)
463-3-8-painted	non-conductive black epoxy	West	6/26/98 3:30 p.m.	3/16/99 2:00 p.m.	20	263 (8.8)
Z307-painted	electrically conductive flat black epoxy	West	7/27/98 11 a.m.	3/16/99 2:00 p.m.	25	232 (7.7)

Table 2. Microbial population of various witness plates exposed in the Spacecraft Assembly Facility.

Materials	Number of microbial population (CFU/ cm^2) that are:		Number of microbes isolated from the assays that are:		Number of microbes[1] identified from the assays that are:	
	Vegetative[2]	Spore formers[3]	Vegetative	Spore formers	Vegetative	Spore formers
Stainless steel	5 ± 0	6 ± 1	3	6	1[4]	1
Z307	16 ± 3	10 ± 1	9	11	3 (2)[5]	2
S13GLO-1	37 ± 9	3 ± 1	13	7	3 (1)	2
NS43G	69 ± 9	15 ± 9	16	9	6[6]	6
463-3-8	31 ± 3	3 ± 1	9	6	2 (1)	3

[1,4]All coccoid and one rod-shaped isolates (FO-092) were picked for their morphological novelty and other isolates were selected for their profound growth at higher temperatures (60°C).
[2]Colonies formed from the samples that are not heat-shocked at 80°C for 15-min. are called vegetative cells.
[3]Colonies formed from the samples that are heat-shocked at 80°C for 15-min. are called spore-formers.
[5]Number in the parenthesis are the coccoid forms.
[6]Not included in the identification because of contamination problem.

Table 3. Microbial identification of various strains isolated from Spacecraft Assembly Facility.

Strain #[1]	Bacterial identification as seen by their:					GenBank accession #
	Phenotype	FAME profiles	16S rDNA sequence	% similarity[2]	DNA-DNA hybridization	
FO-003	*B. licheniformis*	*B. subtilis*	*B. licheniformis*	98.5	*B. licheniformis*	AF234841
FO-011	*B. cereus*	*B. cereus*	*B. cereus*	99.2	*Bacillus* sp.	AF234842
FO-17a	*Micrococcus* sp.	*Micrococcus lylae*	*Micrococcus luteus*	98.0	*Micrococcus luteus*	AF234843
FO-017b	*Bacillus* sp.	*B. licheniformis*	*B. licheniformis*	98.6	*B. licheniformis*	AF234844
FO-022a	*Bacillus* sp.	*B. licheniformis*	*B. licheniformis*	97.9	*B. licheniformis*	AF234845
FO-024	*Bacillus* sp.	*B. licheniformis*	*B. licheniformis*	98.5	*B. licheniformis*	AF234846
FO-026	*Bacillus* sp.	ND*[3]	*B. licheniformis*	98.8	*B. licheniformis*	AF234847
FO-028	*Bacillus* sp.	*B. licheniformis*	*B. licheniformis*	97.0	*B. licheniformis*	AF234848
FO-029a	*B. subtilis*	*B. lentimorbus*	*B. subtilis*	98.0	*B. subtilis*	AF234849
FO-032	*Bacillus* sp.	*B. licheniformis*	*B. licheniformis*	98.0	*B. subtilis*	AF234850
FO-033	*B. pumilus*	*B. pumilus*	*B. pumilus*	98.0	*B. pumilus*	AF234851
FO-035b	*Bacillus* sp.	ND	*B. licheniformis*	97.0	*Bacillus* sp.	AF234852
FO-36a	*S. epidermidis*	*S. epidermidis*	*S. epidermidis*	99.0	*S. capitis*	AF234853
FO-036b	*Bacillus* sp.	*B. pumilus*	*B. pumilus*	99.8	*B. pumilus*	AF234854
FO-037	*Bacillus* sp.	ND	*B. licheniformis*	98.5	*B. licheniformis*	AF234855
FO-038	*B. pumilus*	*B. pumilus*	*B. pumilus*	99.0	*B. pumilus*	AF234856
FO-054	*Bacillus* sp.	*B. licheniformis*	*B. licheniformis*	99.0	*B. licheniformis*	AF234857
FO-74a	*Kocuria varians*	Unidentified	*Planococcus citreus*	96.9	Planococcus citreus	AF234858
FO-74b	*Bacillus* sp.	*B. licheniformis*	*B. licheniformis*	98.0	ND	AF234859
FO-080	*Bacillus* sp.	*B. mycoides*	*B. mycoides*	98.7	*B. mycoides*	AF234860
FO-84a	*Micrococcus* sp.	*Micrococcus lylae*	*Micrococcus luteus*	98.0	Micrococcus luteus	AF234861
FO-085	*B. licheniformis*	*B. licheniformis*	*B. licheniformis*	99.0	*B. licheniformis*	AF234862
FO-092	*B. circulans*	*B. circulans*	*B. circulans*	99.4	*Bacillus* sp.	AF234863
JCM 1252	*B. cereus*	*B. cereus*	ND		*B. cereus*	
ATCC 4513	*B. circulans*	*B. circulans*	ND		*B. circulans*	
ATCC 14580	*B. licheniformis*	*B. licheniformis*	ND		*B. licheniformis*	
ATCC 6462	*B. mycoides*	*B. mycoides*	ND		*B. mycoides*	
ATCC 7061	*B. pumilus*	*B. pumilus*	ND		*B. pumilus*	
IAM 1026	*B. subtilis*	*B. subtilis*	ND		*B. subtilis*	
ATCC 4698	*Micrococcus luteus*	*Micrococcus luteus*	ND		*Micrococcus luteus*	
ATCC 27566	*Micrococcus lylae*	*Micrococcus lylae*	ND		*Micrococcus lylae*	
IFO 15849	Unidentified	Unidentified	ND		*P. citreus*	
ATCC 14990	*S. epidermidis*	*S. epidermidis*	ND		*S. epidermidis*	
ATCC 2784O	*S. epidermidis*	*S. capitis*	ND		*S. capitis*	

[1]Abbreviations: JCM, Japanese Collections of Microorganisms – ATCC, American Type Culture Collection – IAM, Institute for Applied Microbiology – IFO, Institute of Fermentation-Osaka.
[2]As per Wayne et al. (1987) recommendations, the 16S rDNA sequence similarities of >97.5% was considered as the same species.
[3]Not determined.

(80° C for 15-min) isolates and 5 of the 50 strains isolated from samples that were not heat-shocked showed growth at 60° C. Similarly, about 50% of the total 89 isolates showed very good growth at 10% NaCl concentration. All isolates were able to ferment glucose and Gram-reaction positive. Only 5% of these isolates were coccoid; all others were rod shaped. The microbial species identification based on various biochemical reactions, FAME profiles and 16S rDNA sequence accession numbers are depicted (Table 3).

Phenotypic characterization

Representatives of rod-shaped (19 strains; API CHB50/API 20E test strips) and coccoid-shaped (4 strains; API STAPH test strip) bacterial cultures were tested for their physiological characteristics. All coccoid and one rod-shaped isolates (FO-092) were picked for their morphological novelty and other isolates were selected for their profound growth at higher temperatures (60°C). Based on the Biomerieux database, only 7 rods and 1 coccoid-shaped bacteria were identified to its species level. All rod-shaped bacteria were presumptively identified as *Bacillus* sp. and fell into two groups: *B. licheniformis* and *B. pumilus.* The coccoid-shaped isolates were identified by phenotypes as members of the genera *Micrococcus* and *Staphylococcus.*

Fatty acid analysis

Among coccoid-shaped groups, members of *Micrococcus* genus (FO-017a, FO-084a) showed unique FAME patterns (80% as 15:0 anteiso). Likewise, strain *P. citreus* FO-074a (19% monounsaturated) and *S. capitis* FO-036a (41% straight chain saturated) also showed different patterns in their FAME composition. C15:0 anteiso was predominant in *Bacillus* sp. Among various groups of *Bacillus*, *B. licheniformis*-group yielded significant amount of 15:0 anteiso (33 to 41%), 15:0 iso (20 to 34%) and 17:0 anteiso (11 to 16%). However, all *B. licheniformis* strains did not show similar FAME profiles. One unidentified *Bacillus* sp. FO-092 also fell into this FAME category. *B. pumilus*-group (FO-033, FO-036b, FO-038) generated significantly more 15:0 iso (45 to 49%) than 15:0 anteiso (25%). The strains FO-003 and FO-029a were higher in C15:0 anteiso (40 to 41%) than C15:0 iso (13 to 20%). Unlike those seen in any other group, *B. cereus*-group (FO-011, FO-080) produced significant amounts of monosaturates (20 to 30%) among their total FAME composition. However, terminally branched saturates were more abundant (60 to 70%) than straight chain saturates (8 to 9%).

16S rDNA sequence analysis

There were 3 different clades observed among coccoid-shaped bacteria. They were the members of the genera *Micrococcus, Staphylococcus,* and *Planococcus.* Two isolates that have high similarities to *Micrococcus* did exhibit >99% and 98% similarities to *M. luteus* and *M. lylae*, respectively. The phylogenetic tree based on the 16S rDNA analysis indicates that the *Micrococcus* species forms a group independent of the others. *Staphylococcus* and *Planococcus* species form distinct independent groups and these 2 groups were clustered in the main stalk of *Bacillus* species (data not shown). The strain FO-036a showed 99.8% similarities in its 16S rDNA sequence to both *S. epidermidis* and *S. capitis.* The genus *Planococcus* is the closest relative to the strain FO-074a and shows 96.9% similarities to *P. citreus*.

Variation in 16S rDNA sequences among rod-shaped strains revealed 5 groups (Fig. 1). These are related to *B. cereus*-group (2 strains), *B. licheniformis* (12 strains), *B. pumilus* (3 strains), *B. subtilis* (1 strain) and *B. circulans* (1 strain) group. This grouping was drawn on the basis of their proximity to respective type strains. A group of 12 strains that are related to *B. licheniformis*, while somewhat heterogeneous in 16S rDNA sequence (97.2 to 99.8% similarities) appeared to form a cluster. Although the strain FO-003 was identified as *B. subtilis* by FAME analysis, the phylogeny tree grouped this strain among *B. licheniformis*-group. The strain FO-011 that was identified as *B. cereus* exhibited 99.1% similarities to both *B. cereus* and *B. thuringiensis.* Likewise, the other member of this cluster, strain FO-080, exhibiting mucoid morphology, did cluster with *B. mycoides* (99.2%) however

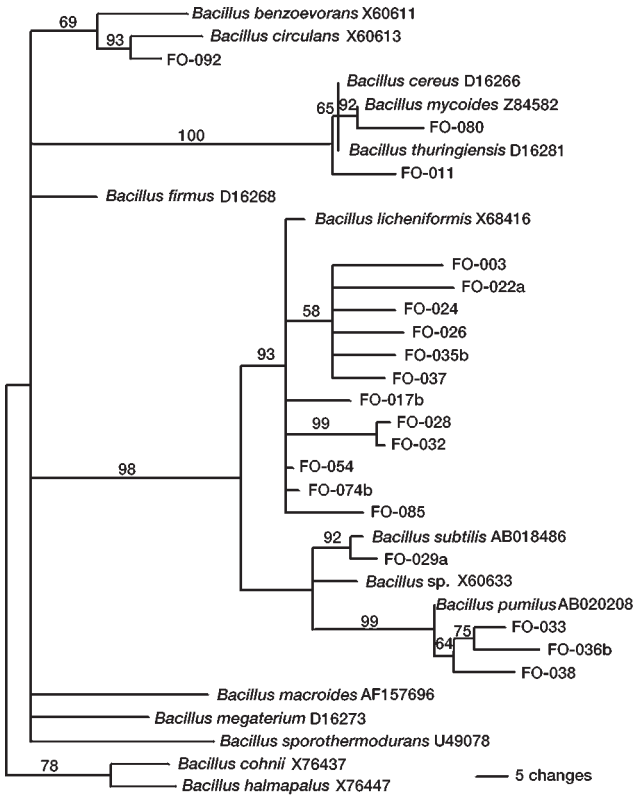

Fig. 1. Phylogenetic tree of the heat-tolerant *Bacillus* strains isolated from JPL-SAF and type strains of various *Bacillus* species based on 16S rDNA sequence comparison by maximum likelihood analysis.

this strain showed 98.9% similarities to both *B. cereus* and *B. thuringiensis.* The lone member of the fifth group, the strain FO-092, with high similarities to *B. circulans,* formed a cluster with *B. benzeovorans* (98.1%) and *B. circulans* (98.7%).

DNA-DNA hybridization

The identity of the bacterial species was determined to be the same species when the DNA-DNA reassociation values between the type species and test strain were above 70% (WAYNE *et al.*, 1987; STACKEBRANDT and GOEBEL, 1994). DNA hybridization studies revealed existence of 4 established *Bacillus* species among 19 strains tested and further supported to describe 3 new *Bacillus* species.

Among the 19 *Bacillus* strains tested, the DNA reassociation percentage range was 77 to 100% for 10 *B. licheniformis* strains (Table 3). The strain FO-035b that show higher 16S rDNA sequence similarities (99%) to *B. licheniformis* did not exhibit higher DNA reassociation values (38%) between them. One of the two strains identified as *B. cereus*-group (FO-080) showed 77% similarities to *B. mycoides* whereas the non-mucoid strain FO-011 exhibited 53 to 61% similarities to the members of *B. cereus*-group. Three strains showed closest match to *B. pumilus* (65 to 85%) and one strain FO-029a showed high 16S rDNA sequence (98%) and DNA hybridization similarities (77%) with *B. subtilis* type strain. However, the other member of the *B. subtilis* group (FO-032) clustered with *B. licheniformis* based on 16S rDNA phyloge-

Table 4. DNA-DNA hybridization of various rod-shaped bacterial species isolated from Spacecraft Assembly Facility.

Bacteria	Strain no.	Percentage similarities to labelled DNA from:													
		B. cereus JCM1252	*B. circurans* ATCC 4513	*B. licheniformis* ATCC 14580	*B. mycoides* ATCC 6462	*B. pumilus* ATCC 7061	*B. subtilis* IAM 1026	*B.thuringens* IAM 12077	*B. licheniformis* FO-085	*B. mycoides* FO-080	*B. pumilus* FO-036b	*B. subtilis* FO-029a	*Bacillus sp.* FO-011	*Bacillus sp.* FO-035b	*Bacillus sp.* FO-092
B. benzoevorans	ATCC 49005	11	5		7	2					2	15	7	20	15
B. cereus	JCM 1252	100	5		51	1		57		41	1	6	51	6	11
B. circurans	ATCC 4513	17	100		15	4					2	12	15	8	19
B. cohnii	ATCC 51227	18	15		18	14					10	23	14	8	10
B. firmus	ATCC 14575	21	9		16	15					11	23	15	10	24
B. licheniformis	ATCC 14580			100		7			89		10	12		28	19
B. megaterium	IAM 13418													12	19
B. mycoides	ATCC 6462	54	5		100	1		52		88	6	9	52	30	17
B. pumilus	ATCC 7061	19	7		22	100					74	21	30	9	22
B. subtilis	IAM 1026	12	2		9	4	100				5	80	5	20	12
B. thuringiensis	IAM 12077	51	6		39	1		100		48	1	6	54	6	13
B. licheniformis	FO-003			100					71			20			
B. licheniformis	FO-017b			86					74			12			
B. licheniformis	FO-022a			95					80			12			
B. licheniformis	FO-024			86					86			8			
B. licheniformis	FO-026			87					83			12			
B. licheniformis	FO-028			85					76			13			
B. licheniformis	FO-037			92					76			16			
B. licheniformis	FO-054			83					77			9			
B. licheniformis	FO-084b			85					71			13			
B. licheniformis	FO-085			77					100			11			
B. mycoides	FO-080	43	1	3	84	1		52		100	2	9	41		11
B. pumilus	FO-033	27	8	22	24	76					90	21	20		19
B. pumilus	FO-036b	15	5	12	12	69					100	16	10		13
B. pumilus	FO-038			5		66					89				
B. subtilis	FO-029a	17	7	11	12	10	97		16		10	100	13	21	14
B. subtilis	FO-032	15	4	19	12	8	88		16		8	84	13		12
Bacillus sp.	FO-011	56	9	14	56	6		61		53	3	9	100		17
Bacillus sp.	FO-035b	14	2	38	18	6	19		28		6	14	12		12
Bacillus sp.	FO-092	22	5	9	20	1					4	13	10		100

ny (99%). One morphologically novel strain (FO-092) that produces extremely big spores (data not shown) showed no relationship with any of the *Bacillus* species tested. It is interesting to note that the strain FO-092 had a clear match with *B. circulans* by phenotypic data, FAME profiles, and 16S rDNA sequence similarities (98.7%). However, DNA hybridization study (21% with *B. circulans*) undoubtedly indicates that the strain FO-092 should be regarded as distinct.

Among the five coccoid strains examined, two strains (FO-017a and FO-084a) showed higher DNA reassociation values among themselves (>80%) and with *Micrococcus luteus* (~81%). The strain FO-074a exhibited 81% value with *Planococcus citreus* IFO 15849. The strain FO-036a revealed 89% and 70% hybridization values with *S. capitis* subspecies *capitis* ATCC 27840 and *S. capitis* subspecies *ureolyticus* ATCC 49326, respectively.

Direct DNA extraction and RFLP analysis

There was insufficient DNA amplification using the bacterial PCR primers 341f and 519r from the witness plates (data not shown). All witness plates yielded at least some PCR product with the 27f and 519r primers. These primers have often yielded amplification when the other primers have not (personal observation) produced PCR products. The negative control lane had a faint band. Only PCR products from witness plates showing greater band intensity than the negative control lane were used for cloning. The PCR products amplified from the DNA of various witness plates and the enzyme digests of the PCR products are shown in Fig. 2. The RFLP patterns from both digests were compared among clones.

Gram-positive and Gram-negative bacterial sequences were retrieved with almost equal frequency (Fig. 3). There were, however, many more Gram-negative than Gram-positive clone sequences because of the overwhelming number of *Alcaligenes* sequences retrieved from one witness plate set (S-13GP/LO-1). The individual witness plate coatings differed in the ratio of Gram-negative to Gram-positive bacterial sequences retrieved [75% of 20 clones for "stainless steel," 60% of 23 clones for "NS43G," 82% of 38 clones for "S-13GP/LO-1," and 50% of 18 clones for "Z307"], however, these ratios could change if more clones were sequenced. Also, it should be kept in mind that cloning may be selective, and even very low abundance sequences can be cloned from a sample (i.e. the sequences reported are not necessarily the most abundant sequences).

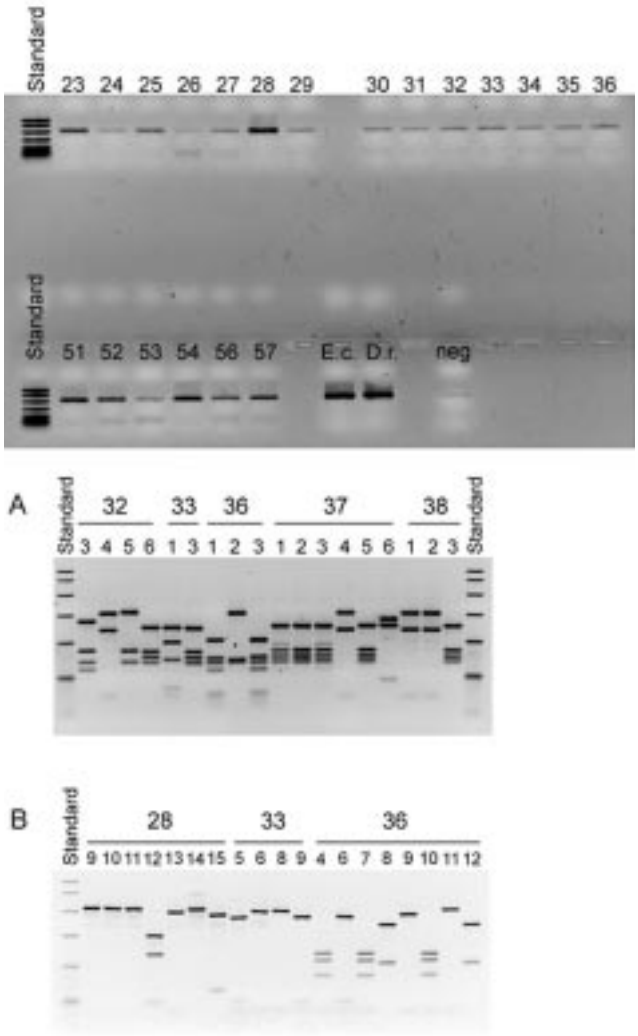

Fig. 2. Agarose gel image of ethidium bromide stained PCR product from SAF room witness plates used for cloning (left panel). Numbers above each lane correspond to the witness plate numbers from which DNA was extracted. PCR products extracted from stainless steel (25 to 29); NS43G (30 to 36) and Z307 (51 to 57) witness plates are shown. S13GLO-painted witness plates are not included in this figure. "E.c." is *Escherichia coli*, "D.r." is *Deinococcus radiodurans*, and "neg" is the PCR negative control. RFLP analysis of several clones from several witness plates (right panel). The PCR products were digested with *HaeIII* (A) or *MspI* (B). Numbers grouped by horizontal lines were taken from the same witness plate (plate number above the line, clone number below the line). Standards are molecular weight markers (50 – 1000 bp).

Discussion

Prokaryotes are believed to have inhabited Earth for more than 3.5 billion years and yet have remained small and simple throughout their evolutionary history. Their diversity is expressed in terms of physiology and metabolism. Prokaryotes have optimized their biochemistry for the uptake and utilization of a wide variety of nutrients thereby creating unique pathways and genetic regulation to meet a variety of conditions within nature. Deep in the subsurface of the earth, new species are being identified that can survive for long periods of time without growing or being metabolically active (HUNTER-CEVERA, 1998). A spacecraft building facility for a life detection mission is often guarded by stringent quality control measures. Because of controlled air circulation,

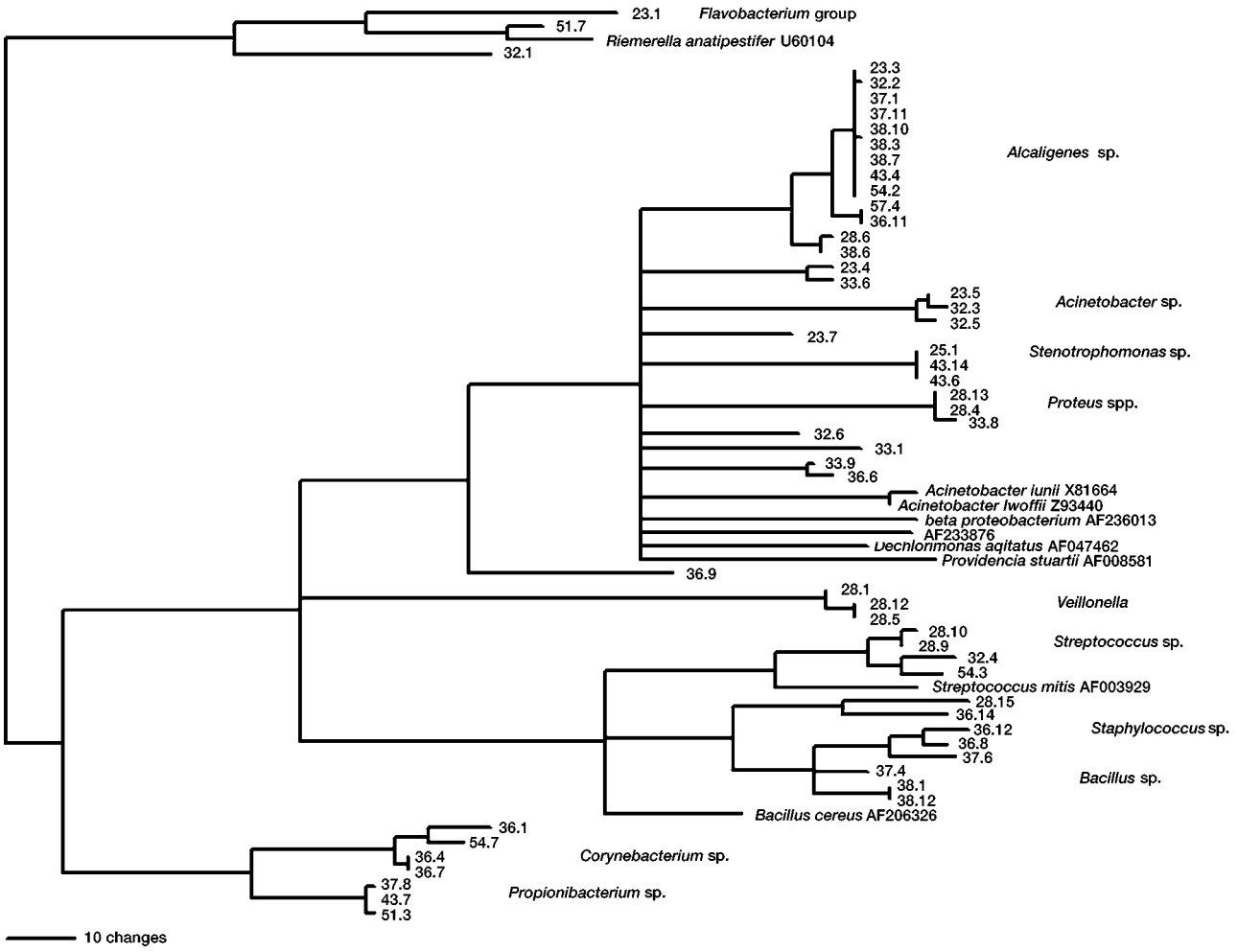

Fig. 3. Phylogenetic tree of the 16S rDNA sequences retrieved directly from various witness plates. Clone numbers retrieved from stainless steel (23, 25, 28), NS43G (32, 33, 36), S-13GP/LO-1 (37, 38, 43), and Z307 (51, 54, 57) painted aluminum are given after the witness plate numbers.

desiccation, moderately high temperature, and low-nutrient conditions, the environment of JPL-SAF can be considered as an extreme environment, and microbes might find it difficult to thrive under such conditions. Evidently, many bacterial strains isolated from the JPL-SAF exhibited intense growth at 60° C (44%), and 10% NaCl (50%). This substantiates the fact that JPL-SAF is an extreme environment. A majority of these thermotolerant and halotolerant isolates were identified as the *Bacillus* species.

Historically, the ability to determine the thermal resistance of naturally occurring airborne bacterial spores associated with the spacecraft and their assembly areas has been hindered by a lack of an effective collecting system. Efforts to collect and concentrate spores with air samplers or from air filters have not been successful (PULEO *et al.* 1975). A fallout method was developed where sterile Teflon ribbons were exposed in pertinent spacecraft assembly areas and subsequently analyzed for microbial population that survived the dry heat environment (125 and 113° C; PULEO *et al.* 1975). Likewise, naturally occurring airborne bacterial spores were collected on Teflon ribbons in selected spacecraft assembly areas where Viking spacecraft hardware was built and subjected to thermal inactivation experiments (105 to 135° C). The majority of heat survivors recovered at these temperatures were phenotypically identified as *Bacillus* species.

Microbiological assessment of two Viking spacecraft during assembly and testing at Cape Canaveral and the Kennedy Space Center was reported. Levels of bacterial spores per square meter on the Viking Lander Capsules were 9.7×10^1 to 1.6×10^2 prior to dry-heat sterilization. The ranges of aerobic mesophilic microorganisms detected on the Orbiters at various sampling events were 2.3×10^2 to $8.9 \times 10^3/m^2$. About 75% of 1,300 isolates were microorganisms considered indigenous to humans (*Staphylococcus* or *Micrococcus*-groups); the remaining isolates were associated with soil and dust (*Bacillus*-group) in the environment (PULEO *et al.* 1977). Similar

results were obtained with previous automated spacecraft but slightly lower than those observed for manned (Apollo) spacecraft (PULEO *et al.* 1973). Our results confirmed that the JPL-SAF High Bay-1 exhibited lower microbial burden and the taxonomical characterization revealed a similar microbial profile as that of the Viking mission. In other words, the Precursor spacecraft that used to verify spacecraft level flight article assembly and test operating procedures at the launching site had high incidence of the members of the *Bacillaceae* family. The witness plates employed in this study mimics the Precursor spacecraft in their microbial profile rather than the Viking 1 and 2 spacecraft.

The conventional biochemical tests, FAME analysis and 16S rDNA sequence results did not firmly define the species status of the given strain during this study. Only 3 out of 19 *Bacillus* strains and one of the 4 coccoid-shaped strains were identified as the same species by all four methodologies. It is clear that the biochemical test profile did not differentiate the Gram-positive bacteria at their species level. This might be due to the fact that the microbes isolated from SAF would have to shed some of their genetic characters to adapt themselves to the extreme environmental conditions thereby changing their phenotypes. The results of FAME profiles and 16S rDNA similarities (75%), FAME profiles and DNA-DNA hybridization similarities (52.6%) and 16S rDNA and DNA-DNA hybridization similarities (73%) were in accordance in identifying them as the same species.

The direct DNA extraction and cloning data clearly suggest there are many Gram-negative bacteria in the gravity collected indoor air material. Often, only Gram-positive bacteria are retrieved by culture analysis of indoor air because they are thought to withstand desiccation better and some produce spores. The abundance of Gram-negative bacterial DNA sequences retrieved through this molecular analysis highlights the advantage of using non-culture-based methods for assessing bacterial community composition from indoor air environments. Several sequences retrieved with this analysis aligned with organisms often cultured from humans (e.g. *Propionibacterium acnes, Streptococcus mitis, Staphylococcus epidermidis*).

The work at hand represents a comprehensive effort to impose a logical phylogenetic framework on the growing collection of SAF isolates. From this study it is clear that the molecular analysis yielded useful information about several kinds of bacteria present in the SAF rooms, which were not known previously, and provides information for future monitoring efforts.

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