RESEARCH ARTICLE

Molecular bacterial community analysis of clean rooms where spacecraft are assembled

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Abstract

Molecular bacterial community composition was characterized from three geographically distinct spacecraft-associated clean rooms to determine whether such populations are influenced by the surrounding environment or the maintenance of the clean rooms. Samples were collected from facilities at the Jet Propulsion Laboratory (JPL), Kennedy Space Flight Center (KSC), and Johnson Space Center (JSC). Nine clone libraries representing different surfaces within the spacecraft facilities and three libraries from the surrounding air were created. Despite the highly desiccated, nutrient-bare conditions within these clean rooms, a broad diversity of bacteria was detected, covering all the main bacterial phyla. Furthermore, the bacterial communities were significantly different from each other, revealing only a small subset of microorganisms common to all locations (e.g. *Sphingomonas*, *Staphylococcus*). Samples from JSC assembly room surfaces showed the greatest diversity of bacteria, particularly within the *Alpha-* and *Gammaproteobacteria* and *Actinobacteria*. The bacterial community structure of KSC assembly surfaces revealed a high presence of proteobacterial groups, whereas the surface samples collected from the JPL assembly facility showed a predominance of *Firmicutes*. Our study presents the first extended molecular survey and comparison of NASA spacecraft assembly facilities, and provides new insights into the bacterial diversity of clean room environments .

Introduction

Aside from the practical concern of maintaining spacecraft integrity, the assembly and processing of spacecraft in clean room environments is essential for the prevention of forward contamination, that is, the contamination of extraterrestrial environments with terrestrial microorganisms or biomolecules (NASA, 2005). To prevent the confounding of future life detection experiments on extraterrestrial bodies, it is crucial to minimize biological contamination of spacecraft components. The low nutrient levels (oligotrophic), desiccated, and clean (low particle per square foot air) conditions of the certified clean rooms limit microbial presence and proliferation. Rigorous maintenance procedures such as regular cleaning (NASA-KSC, 1999; Henderson, 2000), the high-efficiency particle air (HEPA) filtering of air, and constant control of humidity and temperature, render these facilities inhospitable to microbial life. Much like similarly maintained facilities in medical centers and industry (Favero *et al*., 1968a), these settings have been dubbed 'extreme,' in the context of microbial survival (Venkateswaran *et al*., 2001; Crawford, 2005).

In these aforementioned artificial environments, microbial contaminants are expected to be closely associated with human activity. However, previous studies have shown these facilities to harbor microbial communities that thrive in desiccated and oligotrophic conditions (La Duc *et al*., 2007). Oligotrophs are microorganisms adapted for growth under low nutrient conditions, and survive by absorbing trace amounts of nutrients from the air or substratum (Wainwright *et al*., 1991). Many oligotrophic microorganisms are capable of colonizing inorganic surfaces like metal (Nagarkar *et al*., 2001) or glass and the presence of such microorganisms may lead to many problems for space missions, including biocontamination, biofouling, and biodeterioration (Wainwright *et al*., 1993). Strains isolated from these

environments have also been shown to tolerate decontamination strategies, such as UV and gamma radiation treatment (Puleo *et al.*, 1978; La Duc *et al.*, 2003, 2007; Newcombe *et al.*, 2005).

Prior to this study, the bulk of published data pertaining to microbial communities present in medical, industrial or spacecraft-associated clean rooms has been derived from culture-based assays (Favero *et al.*, 1966, 1968b). Although cultivation offers a straightforward means of enumerating some portion of the viable microbial population via colony counting, its usefulness is inherently limited as only a minor fraction of all known microorganisms is detectable with any single (or combination of) media (La Duc *et al.*, 2007). A rapid culture-independent method (intracellular-ATP assay) to estimate the number of viable microorganisms, including yet-to-be cultivated microorganisms (Venkateswaran *et al.*, 2003; La Duc *et al.*, 2004), has shown that only ~10% of viable cells in clean room samples were able to grow in a defined culture medium (La Duc *et al.*, 2007). As only a fraction of all free-living microorganisms have been grown in pure culture (Amann *et al.*, 1995), a culture-dependent approach provides very limited information on the physiological and genetic capabilities of the microbial communities present in a particular sample, and fails to reveal the noncultivable diversity of the microbial population. In contrast, molecular rRNA gene sequence analyses provide a far more comprehensive microbial inventory, facilitating life detection exploration by identifying a wide range of potential terrestrial contaminants.

Previous attempts at describing the bacterial diversity housed within spacecraft assembly facility surfaces suggest that the geographic placement of such clean rooms influences the composition and abundance of microbiota (La Duc *et al.*, 2003). To date, however, few data exist to support or reject this speculation. Here, the results of bacterial diversity analyses performed on three distinct spacecraft assembly facilities are compared to provide insight into the effect of geographical variation.

Materials and methods

Sampling locations and facilities

Samples were collected from a total of nine surface areas within spacecraft assembly clean rooms at three distinct NASA facilities: Jet Propulsion Laboratory, High Bay 1 (JPL-SAF), Kennedy Space Center, Payload Hazardous and Servicing Facility (KSC-PHSF) and Johnson Space Center, Genesis Curation Laboratory (JSC-GCL). In addition, a total of three air samples were taken outside of each facility. The JPL-SAF in Pasadena, California, specializes in the assembly of spacecraft components associated with robotic explorations. The PHSF is part of the KSC in Cape Canaveral, Florida, where all mission vehicles are launched and prelaunch verification processes are conducted. The JSC-GCL, located in Houston, Texas, was constructed to accommodate spacecraft components from the NASA Genesis mission, which returned to Earth in 2004 after 2.5 years of spaceflight. Details of sampling locations, area coverage, clean room certification, and other characteristics are presented in Table 1.

Spacecraft assembly facilities sampled during this study are clean rooms of classes 10–100 K [number of particles of size $\geq 0.5\,\mu m\,ft^3$ (Administration, 1992)]. All samples of JPL-SAF and KSC-PHSF were collected from Class 100 K clean rooms, whereas one sample from each of the Class 10, 1 K, 5 K clean rooms was sampled from JSC-GCL. Samples from a $1\,m^2$ area were collected from JPL and KSC locations and samples from a $0.37\,m^2$ area were obtained from JSC. This difference in sampling area was primarily due to the constrained sampling conditions of the JSC facility. All clean rooms were kept at a constant temperature of $20 \pm 5\,^{\circ}C$. However, the relative humidity was maintained at different levels at various facilities. The JPL-SAF relative humidity was constant at $40 \pm 5\%$ and the JSC-GCL at $50 \pm 5\%$, whereas the KSC-PHSF was maintained at $55 \pm 5\%$.

Air entering through HEPA filters mounted in the ceilings of the clean room are tested and guaranteed as class 5000 air (for class 100 K clean rooms at JPL and KSC). Air volume for these facilities is exchanged a minimum of four times per hour, with positive pressure maintained at all times. An Ultra Low Particle Air (ULPA) filtration system was used to maintain the JSC facility at the appropriate clean room certification. The linear flow rate from ULPA is $100\,ft\,min^{-1}$ with a ceiling coverage of 100%. Furthermore, the floor of the class 10 clean room is ventilated to facilitate air processing and minimize the accumulation of particles. The particle count data during the time of sampling at JSC locations complied with, if not exceeded, clean room certification requirements.

Surface sample collection

Samples were taken from each sampling location using wipes (Table 1). Sterile wipes (Texwipe, Mahwah, NJ) were premoistened with 3 mL of phosphate-buffered saline (PBS) and stored in sterile 50 mL tubes until further processing. During the sampling procedure, sterile handling and processing of equipment was enforced. The particulate materials collected through wiping were suspended in 35 mL (200 mL for JPL samples) of sterile PBS and the samples were processed within hours of collection. The wipes containing microcosms were agitated using vortex for at least 1 min and the wipes were removed after sonication followed by

Table 1. Locations and characteristics of sampling points and samples collected from various spacecraft assembly facilities

Facility	Location	Sample #	Description	Area sampled	Clean room classification*	No. of clones analyzed (N)†	No. of RFLP patterns	No. of OTUs identified	$n1$‡	Coverage (C) [1-($n1$/N)]∗100
Jet Propulsion Laboratory - Spacecraft Assembly Facility	Pasadena, CA; West Coast; dry desert-like	JPL-1	Floor; Center	1 m^2	100 K	56	22	22	11	80.4
		JPL-2	Floor; Inside East entrance	1 m^2	100 K	47	16	16	5	89.4
		JPL-Air	Air from outside entrance	750 L	No	80	ND§	10	8	90.0
Kennedy Space Center - Payload Hazardous Servicing Facility	Cape Canaveral, FL; East Coast; swamp-like	KSC-2	Floor; Southwest entrance	1 m^2	100 K	55	19	18	8	85.5
		KSC-3	Floor; Center	1 m^2	100 K	46	11	11	0	100.0
		KSC-4	Floor; North side of bay doors	1 m^2	100 K	71	21	18	7	90.1
		KSC-6	Top of lockers; Southeast	1 m^2	100 K	82	26	24	10	87.8
		KSC-Air	Air from inside facility¶	750 L	No	73	ND	17	8	89.0
Johnson Space Center - Genesis Curation Laboratory	Houston, TX; Gulf Coast; swamp-like	JSC-2	Subfloor	0.37 m^2	10	73	22	22	10	86.3
		JSC-7	Threshold; Inside entrance	0.37 m^2	1 K	98	25	23	6	93.9
		JSC-8	Floor; Garment change room	0.74 m^2	5 K	80	31	30	14	82.5
		JSC-Air	Air from outside entrance	750 L	No	77	ND	34	19	75.3

*Classification is defined by the maximum number of particles of the size $> 0.5\,\mu m$ in 1 ft^3 of air.

†Number of fully sequenced, bacterial clones per sample. Sequences of chloroplasts were not included for calculation. For KSC, clones also obtained from the blank sample were not included.

‡Number of OTU's appearing only once in the library.

§All clones were sequenced without performing RFLP pattern analysis.

¶Air was collected when the facility was not maintained and classified.

additional mixing. The resulting reaction fluid was used for various analyses.

Air sample collection

The BioCapture BT-550 (Mesosystems Technology Inc., Kennewick, WA) sampler employed in this study collects particles in the size range of 0.5–10 μm from ambient air. The flow rate of this portable, lightweight (4.5 kg) sampler is 150 L min^{-1} (5.3 ft^3 min^{-1}). Air parcels of 750 L (roughly equivalent to the volume of air human lungs exchange every 2 h) were impinged in 5 mL of sterile buffered saline by running the sampler for 5 min. Samples were collected just outside the JPL and JSC facilities entrance and indoors at the KSC facility air sample at a time when HEPA filtration was not in operation. The sampling device was centrally positioned for all sampling events to ensure the uniformity of the air parcels collected. Immediately following collection, samples were frozen in dry ice for further analysis. In total, three air samples, one from each facility and a control blank cartridge, were analyzed for bacterial diversity.

Molecular bacterial community analysis

DNA extraction, amplification, and clone library construction

The collected sample (35–200 mL for surface samples and 5 mL for air samples) was concentrated to 200 μL via centrifugation (Amicon 50, Millipore, Billerica, MA) before extracting DNA using standard phenol–chloroform procedures (Ausubel *et al.*, 2001). Bacterial 16S rRNA genes (~1.5 kb) were PCR-amplified with the forward primer 27F (5′-GAGTTTGATCMTGGCTCAG-3′) and the reverse primer 1492R (5′-AAGGAGGTGATCCANCCRCA-3′). The PCR was performed under the following conditions: 95 °C for 4 min; 33 cycles of 95 °C for 50 s, 55 °C for 50 s, and 72 °C for 1 min 30 s; and final incubation at 72 °C for 10 min.

Amplicons were ligated into pCR4-TOPO cloning vectors (Invitrogen, Carlsbad, CA) and transformed into chemically competent *Escherichia coli* Top10 cells (Invitrogen) according to manufacturer's protocols.

RFLP screening and sequencing

For each of the samples, at least 96 randomly picked clones were either sequenced directly (Agencourt, Beverly, MA) or subjected to RFLP analyses. The presence of inserts of the expected size was analyzed by direct PCR screening of 96 transformants. Inserts from each clone were amplified as described above with T7 and M13R primers targeted to vector regions flanking the insert. Amplicons were digested with HhaI restriction endonuclease (Promega, Madison, WI) for 3 h at 37 °C and analyzed on a 2% low melting point agarose gel (Shelton Scientific, Prosta, IA). Clones were grouped according to similarity of banding patterns and representative purified plasmids (Qiaprep kit, Qiagen, Chatsworth, CA) of each group was fully, bi-directionally sequenced.

Controls

Negative controls were included at each step in all of the procedures described herein. Premoistened sterile wipes were exposed for 5 s to the air of each facility without active collection of particulates. These served as a sampling negative control (blanks) and were processed using the same DNA extraction protocols as surface samples. For DNA extraction and subsequent PCR amplification, sampling blanks (wipe), water blanks, and unused air-sample collection cartridges were used. The KSC sampling blank (wipe) revealed slight positive 16S rRNA gene fragment amplification. None of the JPL and JSC controls revealed any amplification or were successfully cloned. The PCR product of the KSC sampling blank (wipe) obtained was cloned and analyzed in the same manner as the experimental samples, and 96 colonies were subjected to restriction fragment length polymorphism (RFLP) and representative clones were sequenced. The identical clone sequences ($>$ 99% sequence similarities) obtained from KSC sampling blank (wipe) were subtracted from the KSC clone libraries and are not included in our calculations. Likewise, BioCapture BT-550 blank cartridges exhibited positive amplifications and resulting clones were excluded in this study. Clones from these blanks belong to the species *Acinetobacter junii*, *Aeromonas* sp., *Brevundimonas vesicularis*, *Caulobacter crescentus*, *Delftia acidovorans*, *Janthinobacterium lividum*, *Peptostreptococcus magnus*, *Providencia heimbachae*, *Pseudomonas poae*, *Pseudomonas trivialis*, *Serratia proteamaculans*, *Staphylococcus epidermidis*, and *Stenotrophomonas maltophilia*.

Phylogenetic analyses

All sequences were submitted to the CHECK_CHIMERA program of the Ribosomal Database Project (Cole *et al.*, 2003) to detect possible chimeric artifacts. The phylogenetic relationships of organisms were determined by comparison of individual 16S rRNA gene sequences with the public database (http://www.ncbi.nlm.nih.gov/). For more extended phylogenetic analyses, an alignment of *c.* 30 000 homologous full and partial sequences available in public databases was used. The 16S rRNA gene sequences obtained in this study were integrated in the above-mentioned 16S rRNA gene alignment using the ARB software package (Ludwig *et al.*, 2004). The resulting alignment was checked manually and corrected if necessary. For tree reconstruction, methods were applied as implemented in the ARB software package. The 16S rRNA gene sequences of the clones were deposited in the NCBI nucleotide sequence database. The accession numbers are given in Table 2.

Statistical analyses

Rarefaction analysis (Heck *et al.*, 1975), and coverage calculations (Good, 1953) were applied to estimate the representation of the phylotypes in bacterial libraries. Operational taxonomic units (OTUs) were defined as clones sharing $>$ 97.5% sequence identity (Stackebrandt & Goebel, 1994; Rossello-Mora & Amann, 2001; Lawley *et al.*, 2004). The rarefaction curve was produced by plotting the number of OTU observed against the number of clones screened using the ANALYTIC RAREFACTION 1.3 software (http://www.uga.edu/~strata/software/index.html). The coverage of clone libraries was calculated according to Good (1953) using the equation: $C = [1-(n1/N)]^{*}100$, where C is the homologous coverage, $n1$ is the number of OTUs appearing only once in the library, and N is the total number of clones examined. Unifrac analyses (Cluster Environments) were implemented as described elsewhere (Lozupone & Knight, 2005; Lozupone *et al.*, 2006). For the calculations, bacterial 16S rRNA gene sequences from all samples were combined into one maximum parsimony tree using the ARB software package. Jackknifing (100 permutations) was carried out as described (Lozupone & Knight, 2005; Lozupone *et al.*, 2006).

Results

The bacterial community structure of each of three geographically distinct spacecraft assembly facilities, encompassing nine clone libraries (Table 2), was elucidated and 16S rRNA gene sequences retrieved from each facility were compared to one another and to sequences publicly available in GenBank. A detailed overview of the sequences obtained

Table 2. Molecular microbial community structure of various spacecraft assembly facilities

		Percent clones retrieved from the spacecraft assembly facilities that are:									
		JPL-SAF		KSC-PHSF				JSC-GCL			
Phylum/Family	Genus	100 K 1	100 K 2	100 K 2	100 K 3	100 K 4	100 K 6	10 2	1 K 7	5K 8	Accession numbers of representative clones (DQ532. . .)*
Alphaproteobacteria											
Acetobacteriaceae	*Craurococcus*, uncultivable						11.0	8.0			333, 166
Aurantimonadaceae	*Fulvimarina*			1.8							290
Bradyrhizobiaceae	*Bosea, Bradyrhizobium, Rhodopseudomonas*			1.8		1.4	2.4				347, 286, 328
Brucellaceae	*Ochrobactrum*					1.4					314
Caulobacteraceae	*Brevundimonas, Caulobacter*			12.7	13.0	18.1		2.7	3.1		288,179, 299, 309, 316, 201, 251
Hyphomicrobiaceae	*Rhodoplanes*								1.0		207
Methylobacteriaceae	*Methylobacterium, Roseomonas*	1.2				1.4		1.3	2.0	7.6	136, 323, 181, 188, 217, 254
Rhizobiaceae	*Agrobacterium, Rhizobium*			1.8	4.3					1.3	296, 301, 238, 253
Rhodobacteraceae	*Loktanella, Paracoccus, Roseobacter*	2.4						1.3		3.8	142, 178, 218, 274
Rhodospirillaceae	*Skermanella*							1.3			177
Sphingomonadaceae	*Kaistobacter, Sphingomonas, Sphingopyxis, Sphingosinicella*		2.7	1.8	23.9	12.5	3.7		10.2	2.6	205, 159, 300, 315, 340, 202, 222, 255, 295, 237
Betaproteobacteria											
Alcaligenaceae	*Achromobacter, Pigmentiphaga*	1.2				2.8					134, 327
Burkholderiaceae	*Burkholderia, Wautersia*	7.1	5.5	10.9							128, 153, 283
Comamonadaceae	*Delftia, Hydrogenophaga, Curvibacter, Comamonas, Acidovorax, Variovorax, Pseudomonas*		5.5	1.8	6.5	33.3		32.0		8.8	160, 169, 216, 151, 294, 306, 329, 268, 219, 325
Oxalobacteraceae	*Herbaspirillum, Janthinobacterium, Massilia*	3.5	5.5		10.9	2.8					247, 305, 319, 163
Uncl. *Betaproteobacteria*	*Imtechium*		1.4								149
Gammaproteobacteria											
Enterobacteriaceae	*Pantoea, Serratia*			7.3				4.0			292, 172
Moraxellaceae	*Acinetobacter*	1.2		23.6	32.6	20.8	2.4			2.5	127, 284,307, 313, 351, 225
Pasteurellaceae	*Terrahaemophilus*	2.4									144
Pseudomonadaceae	*Pseudomonas*			1.8		4.2	1.2	5.4			291, 318, 339, 187
Xanthomonadaceae	*Dyella, Lysobacter, Stenotrophomonas*							2.7		10.0	183, 272, 220
Deltaproteobacteria											
Polyangiaceae	*Polyangium*									7.5	213
Acidobacteria											
Acidobacteriaceae	Uncultivable	1.2					31.7	1.3	3.1	5.1	145, 358, 185, 211, 229, 263
Actinobacteria											
Corynebacteriaceae	*Corynebacterium*							2.6	13.3	1.3	184, 191, 239
Kineosporiaceae	*Kineosporia*						1.2				344
Microbacteriaceae	*Curtobacterium*								1.0		209

Table 2. Continued.

		Percent clones retrieved from the spacecraft assembly facilities that are:									
		JPL-SAF		KSC-PHSF				JSC-GCL			
Phylum/Family	Genus	100 K 1	100 K 2	100 K 2	100 K 3	100 K 4	100 K 6	10 2	1 K 7	5K 8	Accession numbers of representative clones (DQ532. . .)*
Micrococcaceae	*Arthrobacter*									1.3	243
Mycobacteriaceae	*Mycobacterium*							4.0			174
Nocardioidaceae	*Friedmanniella, Nocardioides*							1.3		3.8	175, 242
Rubrobacteraceae	*Rubrobacter*							2.6		1.3	226, 248
Uncl. *Actinobacteria*	*Nostocoida*								2.0		192
Chloroflexi											
Anaerolinaceae	Uncultivable						7.3				243
Uncl. *Chloroflexi*	Uncultivable									3.8	221
Cyanobacteria											
Nostocaceae	*Anabaena, Nostoc*							2.6		2.5	167, 182, 230
Rivulariaceae	*Calothrix*									1.3	224, 280
Deinococcus-Thermus											
Deinococcaceae	*Deinococcus*	2.4	1.4				6.1		3.0		141, 164, 354, 195, 262
Trueperaceae	*Truepera*									1.3	234, 276
Miscellaneous											
Chloroplasts	*Chloroplast (Alnus, Nicotiana, Pinus)*	34.1	35.6			1.4					133, 154, 320, 258
Firmicutes											
Acidaminococcaceae	*Veillonella*								3.1		206
Bacillaceae	*Bacillus, Exiguobacterium*			10.9				5.0		12.5	282, 171, 223, 287
Carnobacteriaceae	*Dolosigranulum*	4.7	8.2								131, 156
Clostridiaceae	*Clostridium, Eubacterium,* uncultivable *Subdoligranulum*	8.3	2.7	1.8						3.8	132, 215, 140, 285, 267, 165
Erysipelotrichaceae	*Bulleidia*		2.7								150
Lachnospiraceae	*Roseburia, Ruminococcus*	11.7	6.9								269, 143, 152
Lactobacillaceae	*Lactobacillus*	9.4	13.7								126, 157
Peptostreptococcaceae	*Anaerococcus, Finegoldia, Helococcus, Peptostreptococcus, Tissierella*		6.8				4.9				162, 260, 161, 264, 342
Staphylococcaceae	*Staphylococcus*	2.4	1.4	21.8	8.7		6.0	21.3	38.8	6.3	139, 155, 281, 303, 350, 170, 200, 231, 266
Streptococcaceae	*Streptococcus*	5.9					8.5		9.2	11.3	146, 346, 198, 233, 270
Uncl. *Bacillales*	*Aerosphaera*	1.2							4.1		135, 196
Gemmatimonadetes											
Uncl. *Gemmatimonadetes*	Uncultivable						13.4		2.0	1.3	334, 210, 241, 275
Planctomycetes											
Planctomycetaceae	*Gemmata*								3.1		212
Candidate division											
OP10	Uncultivable								1.0		193

*Accession numbers of selected clones are shown. All clones submitted are available at GenBank (DQ532126–DQ532358).

Table 3. Major higher taxonomic grouping (phylum or division) of various spacecraft assembly facility surfaces

	Percent clones retrieved from the spacecraft assembly facilities that are:											
	JPL-SAF			KSC-PHSF					JSC Genesis Curation Lab			
Phylum	100K 1	100K 2	Average	100K 2	100K 3	100K 4	100K 6	Average	10 2	1K 7	5K 8	Average
Alphaproteobacteria	3.6	2.7	3.2	19.9	41.2	34.8	17.1	28.3	14.6	16.3	15.3	15.4
Betaproteobacteria	11.7	17.8	14.8	12.7	17.4	38.9		17.3	32.0		8.8	13.6
Gammaproteobacteria	3.6		1.8	32.7	32.6	25.0	3.6	23.5	12.1		12.5	8.2
Deltaproteobacteria											7.5	2.5
Acidobacteria	1.2		0.6				31.7	7.9	1.3	3.1	5.1	3.2
Actinobacteria							1.2	0.3	10.5	16.3	7.7	11.5
Chloroflexi							7.3	1.8			3.8	1.3
Cyanobacteria									2.6		3.8	2.1
Deinococcus-Thermus	2.4	1.4	1.9				6.1	1.5		3.0	1.3	1.4
Firmicutes	43.6	42.5	43.0	34.5	8.7		19.4	15.7	26.3	55.2	33.9	38.5
Gemmatimonadetes							13.4	3.4		2.0	1.3	1.1
Planctomycetes										3.1		1.0
OP10 candidate division										1.0		0.3
Miscellaneous (Chloroplasts)	34.1	35.6	34.9			1.4		0.4				

from these surface samples, their phylogenetic positions, and their percent occurrence are given in Tables 2 and 3. Of all the clones analyzed, 193 were of unique bacterial sequence. However, the sequences of about 8.4% of the clones (56 of 664 clones) were phylogenetically affiliated with the 16S rRNA genes of various chloroplasts (*Alnus*, *Nicotiana*, *Pinus*) and were therefore not included in the tree or in statistical calculations (Tables 1 and 2).

These clone sequences spanned 14 different bacterial phyla, 52 families and 81 genera (Table 2). Blast analysis of these sequences revealed that ~45% of the clones represented new phylotypes, whereas 55% of the clones were previously described microorganisms. Approximately 7% of the clones had $< 90\%$ sequence homology with sequences arising from cultivated nearest neighbors in the GenBank database, suggesting a significant presence of novel bacterial taxa. Following the grouping of RFLP patterns and subsequent sequencing of clones representing each pattern group, a strong correlation was observed between RFLP pattern and OTU. Only one of the nine libraries (KSC-4) exhibited less than a 90% correlation between RFLP patterns and unique OTUs.

Coverage values ranged from 80% to 100% for all of the facility surface samples. Coverage values for clone libraries arising from air samples collected from JPL and KSC were $> 90\%$ but only 75% for those collected at JSC. Figure 1 shows the rarefaction curves created for each of the assembly facility sampling locations. A plateau, indicating more complete coverage in sampled biodiversity, was approached only for the KSC-3 location. While clone library coverage values for most of the sampling locations were similar to the KSC-3 location, rarefaction curves did not reach a similar plateau, indicating an incomplete sampling of bacterial diversity. Similar slopes for JPL-1, KSC-2, and KSC-4 samples from class 100 K were obtained, while the slopes of JSC-2 and JSC-7 exhibited modest variation.

The 16S rRNA genes from *Alpha-*, *Beta-* and *Gammaproteobacteria* and *Firmicutes* were the most prominent sequences detected from the clean rooms (Tables 2 and 3; Fig. 2). In some instances up to 40% (JPL-1 and JPL-2) and 50% (JSC-7) of the retrieved clones belonged to the members of the *Firmicutes* groups. In contrast, 65% (KSC-2) to $> 90\%$ (KSC-3 and KSC-4) of the total clones retrieved from three of the four KSC locations belonged to the proteobacterial groups. A single KSC location (KSC-6) was dominated by members of the acidobacteria (~32%, Table 3). In general, surface samples collected at the JSC facility revealed the greatest bacterial diversity (Fig. 3). This was the only facility to reveal members of the *Deltaproteobacteria*, *Cyanobacteria*, *Gemmatimonadetes*, *Planctomycetes* and OP10 group. JPL facility surface samples were characterized by the predominance of *Firmicutes* and showed the highest clone rate of chloroplast 16S rRNA genes (~35%). The bacterial genera common between facilities are shown in Fig. 2. Sequences belonging to members of the genus *Acinetobacter*, *Deinococcus*, *Methylobacterium*, *Sphingomonas*, *Staphylococcus*, and *Streptococcus* were retrieved from the surfaces of all three facilities (Fig. 3). Overall, overlap of sequences between the facilities was minimal (Fig. 3) and was mainly identified on the genus level. The cleaner (lower particulate level) JSC facility locations (class 10, 1 K, and 5 K) contained a greater variety of bacteria (30 genera) than the JPL (17 genera) and

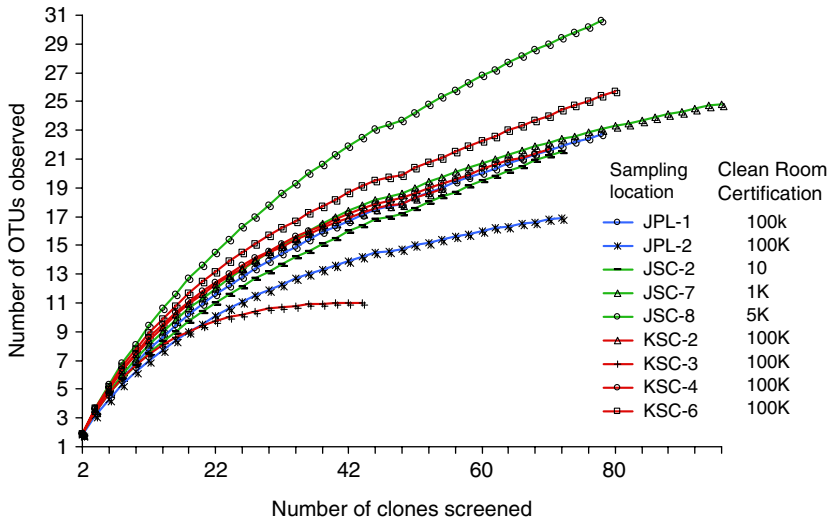

Fig. 1. Rarefaction curves constructed for bacterial clone libraries from nine clean room locations. Clones were grouped into phylotypes at a level of sequence similarity of > 97.5%.

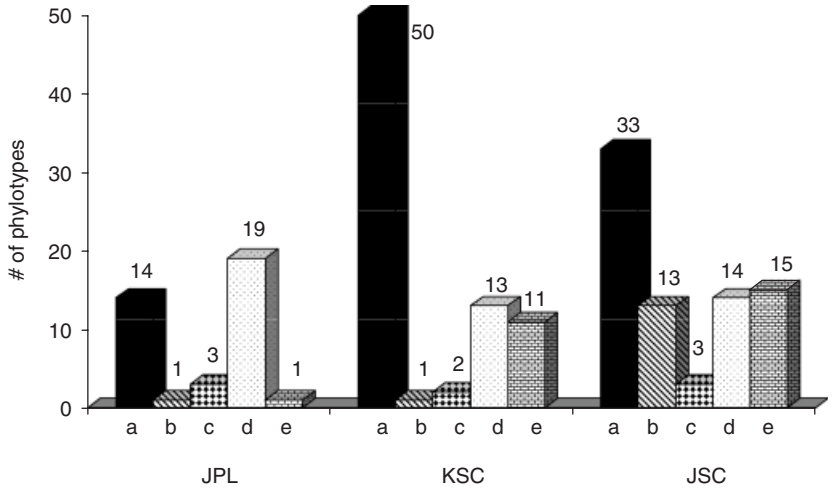

Fig. 2. Number of clones of phylotypes found in various spacecraft assembly facilities. (a) *Proteobacteria*, (b) *Actinobacteria*, (c) *Deinococcus-Thermus* group, (d) *Firmicutes*, and (e) other groups of bacteria are shown. Numbers above the bars indicate the total number of OTUs in each group found at a given facilities.

KSC (15 genera) 100 K clean rooms. Conversely, the class 5 K location (JSC-8) housed more bacterial members (17 genera) compared with nine genera retrieved from the JSC-7 (class 1 K) and JSC-2 (class 10) samples. Environmental cluster analysis (Fig. 4) showed that the clone library composition of different facility surfaces clustered by geographic location and was confirmed by Jackknife analysis. The only exception was the clone library from KSC-6, which clustered with the JSC clone libraries.

The bacterial communities collected from air samples of each of the three facilities are tabulated in Table 4. The majority of sequences retrieved from these three distinct air samples were not represented in the bacterial diversity of the corresponding facility surface samples. These observations were also supported by environment cluster analyses (Fig. 4b). Among 10 bacterial species retrieved from the JPL air sample, only one sequence (*Massilia*) was observed in corresponding surface samples. Sequences retrieved from the KSC surfaces and from the KSC-air clone library were limited (*Agrobacterium*, *Janthinobacterium*, *Wautersia*). Air samples from JSC exhibited the most diverse bacterial incidence (34 OTUs) amongst the three facilities, consequently a larger diversity of sequences were retrieved from both inside (surface) and outside (air) the facility (*Acidovorax*, *Agrobacterium*, *Deinococcus*, *Delftia*, *Gemmata*, *Methylobacterium*, and *Stenotrophomonas*).

Discussion

The clean room facilities tested in this study were located in geographically distinct environments and were 1500–4000 km away from each other. The KSC and JSC facility surrounding environments are characterized by humid, brackish conditions, whereas the JPL facility is

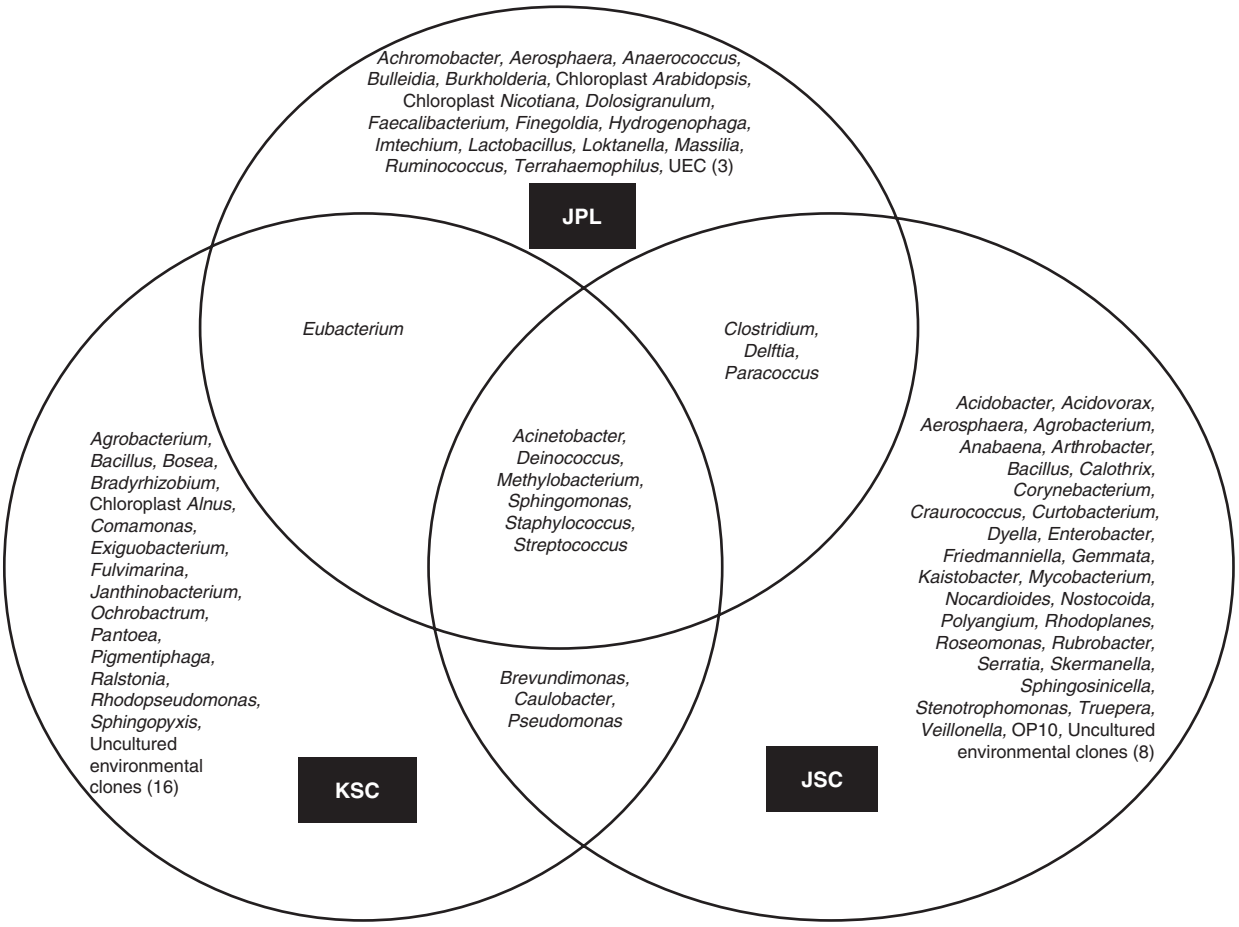

Fig. 3. Schematic drawing showing the detected bacterial genera found in various spacecraft assembly facilities. Overlaps between the facilities are indicated by the arrangement of the circles.

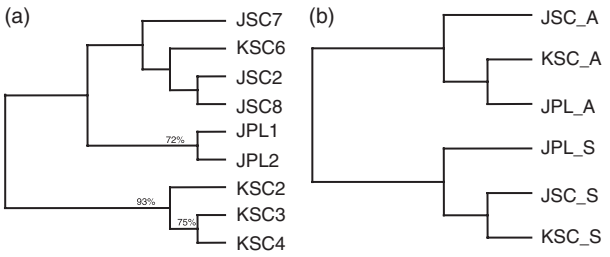

Fig. 4. Environment cluster analyses, showing the relationship of different samples. (a) Clustering of clone libraries from different locations. According to their source, the different libraries cluster together, the only exception being KSC6, which resembles more the JSC sampling libraries. Significant Jackknife values are given. (b) Cluster analyses of clone libraries from the inside and outside the facilities. Surface samples (S) are more similar to each other than to the corresponding air sample (A).

situated in an arid, desert-like setting. It was expected that the influence of environmental characteristics would be reflected in the biodiversity of each facility, but such correlation was not seen in this study. Although proof of endemism is not possible, our results suggest that very little commonality exists between the study sites. Environment cluster analyses supported the comparisons of surface and air samples: clone libraries from each facility resembled each other and clustered together. With one exception (KSC-6, clustering with the JSC libraries), the facility surfaces seem to have characteristic bacterial communities unique to each facility location. These distinct communities, however, seem independent of the bacterial diversity of the surrounding air (Fig. 4). These results suggest that clean room certification procedures such as air filtration removed the majority of the bacterial contaminants from the outside environments. The observed difference in the bacterial diversity amongst the three clean rooms was therefore mainly due to facility maintenance (filter types, relative humidity, temperature, etc.), human activity, and perhaps cleaning agents. As information regarding the cleaning agents used in these facilities was not available, it was not possible to determine the influence of detergents on bacterial species composition.

Table 4. Sequences of bacterial species retrieved from the surrounding air of various spacecraft assembly facilities

Sample	Bacterial 16S rRNA gene sequences retrieved from the air
JPL-Air*	*Acidovorax temperans, Bradyrhizobium betae, Geothrix fermentans, Hyphomicrobium zavarzinii, Massilia timonae, Pantoea stewartii, Pseudomonas lanceolata, Rhodopseudomonas palustris, Roseomonas fauriae, Wautersia insidiosa*
KSC-Air†	*Agrobacterium tumefaciens, Burkholderia cepacia, Burkholderia fungorum, Janthinobacterium lividum, Methylobacterium mesophilicum, Wautersia basilensis, Wautersia metallidurans*
JSC-Air‡	*Acidovorax temperans, Agrobacterium sanguineum, Cellulomonas hominis, Cylindrospermum stagnale, Deinococcus geothermalis, Delftia acidovorans, Flavobacterium ferrugineum, Gemmata obscuriglobus, Janthinobacterium agaricidamnosum, Janthinobacterium lividum, Leptospira parva, Leptothrix mobilis, Loktanella hongkongensis, Methylobacterium fujisawaense, Nitrosomonas oligotropha, Novosphingobium subarcticum, Oceanicola granulosus, Paracoccus yeei, Phyllobacterium myrsinacearum, Pseudomonas carboxydohydrogena, Pseudomonas citronellolis, Pseudonocardia yunnanensis, Rhizobium etli, Rhizobium huautlense, Rhodoferax ferrireducens, Roseomonas fauriae, Shigella flexneri, Sphingomonas aquatilis, Sphingomonas koreensis, Sphingomonas phyllosphaerae, Sphingopyxis witflariensis, Stenotrophomonas maltophilia, Vibrio calviensis, Wautersia paucula*

Microorganisms detected also inside the corresponding spacecraft assembly facilities are underlined.
*Air (~750 L) was collected outside the JPL-SAF facility, Pasadena, CA.
†Air (~750 L) was collected inside the KSC-PHSF facility when the clean room was not maintained.
‡Air (~750 L) was collected outside the JSC-GCL facility, Houston, TX.

Despite its low number of air particulates (class 10–5 K) the JSC facility samples showed the greatest bacterial diversity among the facilities tested. This higher bacterial diversity might be due to the fact that in nutrient-poor environments, slow-growing bacterial species persist and are not out-competed by fast-growing, high biomass, nutrient-dependent species. Previous studies of oligotrophic marine surface waters, for example, showed bacterial abundance to decrease with depth, with no significant change in the richness of community structures, suggesting an independence of biomass and diversity (Hewson & Fuhrman, 2006). In contrast to JSC, JPL samples revealed a relatively low bacterial diversity, possibly due to the low relative humidity (40%) in the JPL-SAF (Lighthart & Frisch, 1976; La Duc *et al.*, 2007). It has been shown that microorganisms at low relative humidity are not likely to persist due to the withdrawal of structural water molecules present in the microbial cells (Theunissen *et al.*, 1993). Half of the OTUs identified in JPL-SAF (19 of 38 phylotypes) were related to *Firmicutes* genera containing either spore-forming or desiccation-resistant bacteria. Cyanobacteria, which are aquatic and photosynthetic bacteria, were exclusively found at JSC. *Chloroflexi*, which are also aquatic and photosynthetic, were detected in samples from both KSC and JSC. The detection of these bacteria may reflect the more humid environments of these facilities (50–55% relative humidity for KSC and JSC), as none of these microorganisms was detected in JPL (~40% relative humidity) samples.

Proteobacteria (*Alpha*, *Beta* and *Gamma*) and *Firmicutes* were the most prominent groups of bacteria detected in our study (Fig. 2), but a small subset of microorganisms (six genera) was common to all facilities (Fig. 3). Members of the genus *Staphylococcus*, commonly associated with human skin, were particularly widespread and were detected in every sample location except for KSC-4. The ubiquity of *Staphylococcus* species was also apparent in independent culture-based studies of the facility samples discussed in this report (La Duc *et al.*, 2007). The high percentage of *Staphylococcus* clones in the class 10 to class 5 K portions of the JSC facility samples (6.3–38.8%; two-thirds of total *Staphylococcus* clones) is particularly noteworthy. Although the ULPA filtration system utilized in JSC clean rooms should have removed most bacterial contaminants, human activity may have reintroduced the *Staphylococcus* species in these ultra clean rooms. *Staphylococci* are among the most prevalent bacteria in clean room settings, including spacecraft assembly facilities (Venkateswaran *et al.*, 2001), surgical operating rooms (Wise *et al.*, 1959), and industrial clean rooms (Favero *et al.*, 1966, 1968b). They have also been frequently found in closed habitat systems such as the MIR space station (Kawamura *et al.*, 2001), ISS (Pierson, 2001; Pierson *et al.*, 2002; La Duc *et al.*, 2007), and the mock-up of ISS modules kept on Earth to test various support equipment (Moissl *et al.*, 2007).

Additionally, members of the family *Sphingomonadaceae* were detected in samples from seven of nine locations including all KSC samples. Sphingomonads, widely distributed in nature, are one of the most abundant contaminants in clean rooms and spacecraft-associated facilities (La Duc *et al.*, 2007) and were even detected as a major cultivable bacteria in ISS potable water (Novikova, 2004; Novikova *et al.*, 2006). Members of the genus *Methylobacterium* were also detected in the samples from all facilities, including all of the JSC clone libraries. This abundance was also confirmed by cultivation assays (La Duc *et al.*, 2007). *Methylobacterium* is frequently found in oligotrophic environments, such as water reservoirs and drinking water vessels as well as clean habitats (La Duc *et al.*, 2007). Their resistance to high levels of chlorine has been described (Hiraishi *et al.*, 1995), and it is possible that cleaning agents used in these assembly

facilities may actually have promoted their growth. The frequent detection of the members of the *Caulobacteriaceae*, *Commamonadaceae*, and *Moraxellaceae* in the clean-rooms of the present study were also reported in the oligotrophic or nutrient-poor environments, such as potable drinking water and hospital surgical rooms (Zinder & Dworkin, 2001; Ireland *et al*., 2002).

Members of *Acinetobacter* were detected in all samples collected from KSC and one of each of the JPL and JSC locations, and were represented in our cultivation study (Venkateswaran *et al*., 2004b; La Duc *et al*., 2007). Members of the genus *Acinetobacter* are frequently found in air, soil and water samples, but are also opportunistic pathogens, causing wound infections, pneumonia and meningitis (Rahal & Urban, 2000). Species of *Deinococcus* were also noticed in each of the three facilities. These bacteria are known for their resistance to desiccation and high doses of ionizing radiation (Saffary *et al*., 2002) and their role in space-related subjects has been discussed extensively (Mileikowsky *et al*., 2000). The role of spore-forming microorganisms has frequently been the topic of discussion in spacecraft and associated environments (La Duc *et al*., 2003). Spores are considered the likeliest candidates to survive in extraterrestrial environments, as they can tolerate a variety of stresses including UV radiation, γ-radiation, hydrogen peroxide exposure, and desiccation (Venkateswaran *et al*., 2004a). *Bacillus* spores in particular have been used for extended resistance studies (Newcombe *et al*., 2005), but similar abilities can be predicted for *Clostridium* and other spore formers. Members of both *Bacillus* and *Clostridium* were detected in this study, and seem to easily persist in harsh clean room conditions, as demonstrated by their successful cultivation from clean rooms. These molecular bacterial community analyses revealed the presence of not only aerobic but also anaerobic spore-formers, as well as unusual and yet to be classified members of the *Bacillales* such as *Aerosphaera*.

Not surprisingly, problematic bacteria reported as endemic to the International Space Station (ISS; *Methylobacterium*, *Sphingomonas*, *Staphylococcus*) are frequently detected as major contaminants of spacecraft assembly facilities on Earth (Novikova *et al*., 2006). Nearly 50% of the bacterial genera cultivated from the ISS environment (Novikova *et al*., 2006) were also detected using this molecular approach. Moreover, all of the predominant groups of microorganisms in the ISS (*Bacillus*, *Corynebacterium* and *Staphylococcus*) were among the clone sequences found in these facility-borne samples. Thus the initial contamination of space flight materials during manufacture and assembly can still be detected later in the space operations phase. Other sources of contamination can be traced to the delivery of supplies, personnel, and biological materials to the station (Novikova *et al*., 2006).

In summary, during this study two different types of bacteria were detected in elevated amounts: those that are human commensals and/or pathogens, and those that thrive in the harsh clean room environment. Collectively, humans might be the sole carrier for the members of the family *Staphylococcaceae* and *Streptococcaceae* in these clean room facility surfaces as these bacteria were not detected in air samples collected outside the facility. In contrast, members of the genera *Methylobacterium*, *Sphingomonas*, and *Acinetobacter* may have originated from the surrounding air particles and that may have escaped the filtration. The presence of deinococci might be due to the controlled humidity of these facilities as members of this group were reported to be prevalent in arid places (Nagy *et al*., 2005) as well as in spacecraft assembly facility (Venkateswaran *et al*., 2001). It should be noted that although the presence of spore forming bacteria is used as a proxy for determining overall clean room cleanliness (NASA, 2005), *Bacillus* clones were often absent in samples obtained during this investigation. Further research on the capabilities of non-*Bacillus* microorganisms, and their potential impact on clean room operations, is warranted.

The presence and persistence of microbial contaminants on spacecraft and in their assembly facilities underscores the need for comprehensive cleaning and maintenance protocols and frequent surveys of bacterial communities. This study yields the first insight into the bacterial diversity present in spacecraft-associated clean rooms, but the results may also be applicable to other (industrial or medical) clean rooms as well. Based on these data, potential sources of contamination can be identified, characterized and confined and problems such as pathogen outbreak and biodeterioration can be addressed prior to the initiation of assembly, testing, and launching operation stages.

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