Diversity of Anaerobic Microbes in Spacecraft Assembly Clean Rooms

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Although the cultivable and noncultivable microbial diversity of spacecraft assembly clean rooms has been previously documented using conventional and state-of-the-art molecular techniques, the occurrence of obligate anaerobes within these clean rooms is still uncertain. Therefore, anaerobic bacterial communities of three clean-room facilities were analyzed during assembly of the Mars Science Laboratory rover. Anaerobic bacteria were cultured on several media, and DNA was extracted from suitable anaerobic enrichments and examined with conventional 16S rRNA gene clone library, as well as high-density phylogenetic 16S rRNA gene microarray (PhyloChip) technologies. The culture-dependent analyses predominantly showed the presence of clostridial and propionibacterial strains. The 16S rRNA gene sequences retrieved from clone libraries revealed distinct microbial populations associated with each clean-room facility, clustered exclusively within gram-positive organisms. PhyloChip analysis detected a greater microbial diversity, spanning many phyla of bacteria, and provided a deeper insight into the microbial community structure of the clean-room facilities. This study presents an integrated approach for assessing the anaerobic microbial population within clean-room facilities, using both molecular and cultivation-based analyses. The results reveal that highly diverse anaerobic bacterial populations persist in the clean rooms even after the imposition of rigorous maintenance programs and will pose a challenge to planetary protection implementation activities.

As mandated by United Nations treaty, space-faring nations enumerate aerobic spore-forming bacteria on spacecraft surfaces as a proxy for measuring the cleanliness of spacecraft intended to land in particular extraterrestrial environments (5, 27). However, recent use of molecular microbial community analyses on clean-room samples has revealed a much higher biodiversity—including the presence of genetic signatures from anaerobic spore-formers—that can be assessed by the standard procedure alone (14–16). During space travel and after inadvertent contamination of Mars, microbes are exposed to low or nonexistent concentrations of oxygen, challenging the survival of aerobic microorganisms. The study of anaerobes is therefore particularly important in the context of space research, since the proliferation of microbes adapted to Mars-like environments would increase the risk of contaminating target planets and would compromise sensitive life detection activities. The Mars Science Laboratory (MSL) mission aims to explore new areas of Mars and will search for probable life on the red planet using highly sensitive biosensors, requiring high cleanliness control to prevent false positives. To overcome present limitations in characterizing the potential threat from anaerobic bacterial diversity, the objective of this study was to utilize both culture-dependent and culture-independent molecular analyses to characterize the obligate anaerobic bacterial communities of the three clean-room facilities used for the MSL rover assembly.

Recent investigations of spacecraft facilities have retrieved 16S rRNA gene sequences from facultative and obligate anaerobic microorganisms from environmental samples (17, 25). Some facultative anaerobes of the genus Paenibacillus and Staphylococcus have been isolated in the course of describing the cultivable diversity of extremotolerant microbes in clean-room facilities (14). A microbial survey of European clean-room facilities has reported the isolation of facultative and strict anaerobes from spacecraft-associated surfaces (36). Typically, 20 to 50% of all isolates from different sampling events and different locations exhibited growth under anaerobic conditions, with a small subset of isolates being strict anaerobes (36). Nevertheless, these analyses have been based only on cultivation, and it has been reported that only ~1% of microorganisms in environmental samples are cultivable in defined media under laboratory conditions (1).

Current molecular cloning techniques targeting the 16S rRNA genes can capture a wide spectrum of bacterial diversity and facilitate the construction of a comprehensive microbial inventory (17, 29). Investigations utilizing these methods have revealed a much higher biodiversity in clean rooms than is detected by the NASA standard assay procedures (15, 16), with approximately 0 to 8% of retrieved sequences belonging to obligate anaerobes (24, 25). This low percentage might be due to the fact that clone library construction is generally limited to hundreds or thousands of sequences, which has proven to be
Sampling locations. The Jet Propulsion Laboratory (JPL) clean-room facilities used for assembly of the MSL spacecraft components were sampled. In four sampling events (JPL Spacecraft Assembly Facility (SAF)-A, building 233-B, JPL-SAF-C, Biomolecule Detection Laboratory (BDL)-D), 53 surface samples from three different clean-room facilities were examined. For each sampling event, 10 surface samples were taken using biological sampling kits (BisKits, see below). In addition, ground support equipment (GSE) of the spacecraft was sampled when present (JPL-SAF-A and JPL-SAF-C) using wipes and BisKits, respectively. Samples were taken from the JPL-SAF clean room on two occasions (at exactly the same locations), since it hosts the majority of the MSL assembly and activities. During sampling, several MSL spacecraft assemblies and GSE structures were present in the JPL-SAF (cruise stage, spacecraft assembly and rotation fixture tower, and cradle), and a high level of human activity was observed (20 to 30 people). Clean-room facility building 233 (Bldg-233) harbored the pressure tanks and heater assemblies of MSL prior to their transfer to the JPL-SAF but also had a high human activity (~10 people). However, this clean room is smaller than JPL-SAF. The BDL, where the last sampling event took place, has low human activity (two people) because it hosted no ongoing assembly. The BDL served as a storage and test location to keep mission components at the same cleanliness level and was therefore studied for comparison. An overview of the specific sampling locations from each facility, physical characteristics, and their corresponding clean-room class designations is given in Table S1 in the supplemental material.

Prior to entering clean rooms, staff take appropriate actions to minimize the influx of particulate matter. The air of all facilities is filtered through HEPA filters. Environmental monitoring system (EMS) sensor sets are located in appropriate locations, and each set contains a temperature sensor, a relative humidity sensor (Vaisala model 260EX), and a laser particle counter that measures particle concentration (Met One model 237A). Air is volumetrically sampled at appropriate locations, and each set contains a temperature sensor, a relative humidity sensor (Vaisala model 260EX), and a laser particle counter that measures particle concentration (Met One model 237A). Air is volumetrically sampled at appropriate locations, and each set contains a temperature sensor, a relative humidity sensor (Vaisala model 260EX), and a laser particle counter that measures particle concentration (Met One model 237A). 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Isolates were retrieved from direct culturing (spread plating). Others were isolated from enrichment cultures. Sequences were assigned to OTUs using DOTUR analysis, and a representative sequence from each OTU was compared against RDP-type strain database using BLAST function.

**TABLE 1. Obligate anaerobes isolated from various spacecraft-associated surfaces**

<table>
<thead>
<tr>
<th>Facility and Media used to isolate Samples</th>
<th>Strain, accession no.</th>
<th>No. of OTUs</th>
<th>DNA fingerprint sequence similarity %</th>
<th>Phylogeny</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Actinobacteria/Propionibacteriaceae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propionibacterium acnes, DSM 1897, X53218</td>
<td>99.0</td>
<td>96.6</td>
<td>0.90</td>
<td>Bacilli</td>
</tr>
<tr>
<td>Propionibacterium acnes, ATCC 3584, X68189</td>
<td>100.0</td>
<td>98.3</td>
<td>1.40</td>
<td>Bacilli</td>
</tr>
<tr>
<td>Clostridium sporogenes, ATCC 585, X68189</td>
<td>100.0</td>
<td>96.3</td>
<td>0.90</td>
<td>Bacilli</td>
</tr>
<tr>
<td>Clostridium saccharolyticum, ATCC 585, X68189</td>
<td>100.0</td>
<td>100.0</td>
<td>0.90</td>
<td>Bacilli</td>
</tr>
<tr>
<td>Clostridium perfringens, ATCC 13124, 59103</td>
<td>99.6</td>
<td>99.6</td>
<td>0.90</td>
<td>Bacilli</td>
</tr>
</tbody>
</table>

**RESULTS**

**Sensitivity of the methods used.** A comparison of wet and dry sampling with the BiSKit indicated that dry sampling was more efficient (efficiency, 18.4%) than wet sampling (efficiency, 11.3%) (4). However, it has been reported that, although the sampling efficiency was lower for the wet sampling method, the wet samples were more concentrated due to the low sample volume and therefore had greater detection sensitivity when they were used directly for biological detection assessment (4). With reference to sample recovery, BiSKit samplers are superior (10 to 20%) to the wipe samples (<10%) because the BiSKit samplers are compact and smaller volumes of sample buffer are required to recover the impacted bacteria. Since cultivation is based on the enrichment methods; theoretically,
even if only one anaerobic cell is present in the sample, it should be enriched. However, it is not possible that all known anaerobes will grow under the conditions used here. Furthermore, there is a consensus that PCR-dependent techniques require ~10^3 initial copies of the target to reproducibly assess microbial diversity for standard PCR and ~10^2 initial copies for gradient PCR (17). Therefore, although theoretical calculations posit the need for as few as one copy of a given target molecule, signal-to-noise ratios demand that target molecules exist in excess of 10^2 to 10^3 copy numbers (due to indigenous DNA in reagents and sampling materials). In the present study, since initial enrichment was employed before extracting DNA and the sample was analyzed via conventional cloning or PhyloChip analyses, sensitivity is not a problem for these assays, but collection of the target organism from the environment needs to be resolved.

**Isolation of strict anaerobes.** A total of 113 anaerobic strains were isolated during this study, including 21 strains isolated exclusively from GSE present within the JPL-SAF. The TS/TG enrichments followed by plating on anaerobic TS agar medium (88 strains) promoted greater isolation of anaerobes compared to the direct agar plating on TS agar (20 strains) without enrichment. However, enrichment in the modified ATCC 591 medium under CO_2_ gas phase yielded only six anaerobic enrichment. The majority of isolates (93 of total 113 strains) exhibited growth in the presence of oxygen and were not further characterized. Obligate anaerobes were successfully isolated from each clean-room facility (Table 1). All 20 strict anaerobic strains (17.7% of all isolates in this study) were Gram-positive bacteria belonging to the genus Clostridium (four species) or the genus Propionibacterium (one species). Three different species (C. colicanis, C. sporogenes, and P. acnes) were retrieved from clean-room floors, while four species were obtained from JPL-SAF GSE (C. perfringens, C. saccharolyticum, C. sporogenes, and P. acnes). Since analysis of the 16S rRNA gene cannot distinguish C. sporogenes from C. botulinum, the presence of the C. botulinum type A toxin gene was also tested using established procedure (21). The strains in question did not yield type A toxin gene amplicons, confirming that they did not belong to C. botulinum (data not shown). Sporulation was observed in all strains of the genus Clostridium (except C. colicanis) after anaerobic incubation for an appropriate duration in TS medium. Some of these clostridial strains were able to grow on modified ATCC 591 medium with CO_2_ gas phase as well as on TS medium with N_2_ gas phase (Table 1).

**Molecular microbial community analyses.** (i) **Conventional clone library analysis.** All four samples tested via gradient PCR showed 16S rRNA gene amplification products. By comparison, only three were successful (the JPL-SAF-A sample did not yield appreciable 16S amplicons) when standard PCR amplification conditions were used. Retrieval of high-quality 16S rRNA gene sequences was similar when different PCR conditions were compared (~66% of 288 clones for standard PCR and 62% of 384 clones for gradient PCR). Although clones were robotically picked and sequenced from each library, after quality control (chimera check, elimination of short sequence, etc.) only 63.5% (JPL-SAF-A), 67.2% (Bdg-233-B), 57.3% (JPL-SAF-C), and 66.1% (BDL-D) of clones yielded high-quality sequences, which were used for subsequent phylogenetic and comparative analysis. The enrichment followed by cloning and sequencing approach revealed sequences related to strict anaerobes (27%; 114 clones) and facultative anaerobes (73%). The sequences retrieved spanned 10 families, 11 genera, and 70 distinct OTUs when analyzed by DOTUR analysis (Table 2). All 70 OTUs detected in this study clustered with 20 known bacterial species. Only members of the phyla Actinobacteria (13 OTUs; 4 species) and Firmicutes (57 OTUs; 16 species) were observed. Sequences of two strictly anaerobic microorganisms (P. propioniciue and Sarcina ventriculi) retrieved via molecular analysis were not isolated using the cultivation methods employed. Likewise, only two species of strict anaerobes (C. colicanis and P. acnes) isolated from enrichment cultures during this study were present in the corresponding clone library, while C. sporogenes could not be detected in the clone library even though it was successfully isolated. All other strict anaerobes isolated from GSE samples were not observed in the clone libraries. About 33% of the sequences (142 of 427 clones) shared lower than 97.5% 16S rRNA gene sequence similarity with closest type strains and may belong to novel species. In addition to the obligate anaerobes, 70 (16%) sequences were related to species of Actinobacteria, 176 (41%) sequences to spore-formers (Paenibacillus, Bacillus, Clostridium, and Sarcina), and 178 (42%) sequences to Staphylococcus species. About 70% of sequences were affiliated to the microbes that are human commensals and/or human pathogens (e.g., Staphylococcus, Propionibacterium, and Dermabacter spp.).

The coverage values of the clone libraries ranged from ~73 to ~97%, and the rarefaction curves for seven clone libraries are shown in Fig. 1. Coverage values for JPL-SAF-A, JPL-SAF-C, and BDL-D were >88% and reached a plateau in rarefaction analysis, indicating that the methods used to pick clones were quite sufficient. Samples from Bdg-233-B exhibited the highest number of OTUs (Table 2) and lowest coverage value (73%) plus a rarefaction curve with a steep slope (Fig. 1), indicating incomplete sampling of clones for sequencing. When standard PCR conditions were used to construct clone libraries, only 36 different OTUs were observed, while the use of gradient PCR revealed 47 OTUs based on DOTUR analysis (Table 2). Furthermore, the standard PCR conditions exhibited lower coverage values (1 to 5% less than gradient PCR) and slightly steeper rarefaction curve slopes compared to libraries constructed from gradient PCR products (Fig. 1). Although a slight increase in the OTUs incidence (one to five OTUs more) was observed in the libraries constructed after gradient PCR, UniFrac analysis indicated marginal to no significant statistical differences in bacterial diversity between standard PCR and gradient PCR clone libraries from all samples. Regardless of PCR protocol, however, UniFrac significant analysis (P, <0.001) and principal coordinate analysis (Fig. 2) revealed significant differences in bacterial populations among the four sampling events, indicating distinct bacterial populations within each clean-room location (Fig. 2).

Among the clean rooms tested, only the JPL-SAF-C locations revealed no 16S rRNA gene sequences of strictly anaerobic microorganisms, but sequences of the facultative anaerobe S. epidermidis were retrieved from all facility floors. S. caprae and S. capitis subsp. capitis sequences were found to be
present in three of the sampling locations. Interestingly, despite the fact that all clean rooms are in the same geographical/climate location, they did not share similar bacterial populations. While members of the *Bacillus* and *Staphylococcus* genera were common at the genus level, JPL-SAF-A and JPL-SAF-C shared no common species except *S. epidermidis*.

**Table 2. Anaerobic bacterial diversity-based clone libraries of enrichments of various spacecraft facilities**

<table>
<thead>
<tr>
<th>Phylum/family (no. of OTUs)</th>
<th>Genus and species (of closest relative type strain), strain, accession no.</th>
<th>OTU no.</th>
<th>JPL-SAF-A</th>
<th>JPL-SAF-C</th>
<th>BDL-D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinobacteria (13)</td>
<td><em>Oerskovia paurometabola</em>, DSM 14281, AJ314851</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>Dermabacter hominis</em>, DSM 7083, X91034</td>
<td>2</td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td><em>Propionibacterium acnes</em>, DSM 1897, X53218</td>
<td>7</td>
<td>24</td>
<td>18</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td><em>Propionibacterium propionicum</em>, DSM 43307, AJ003058</td>
<td>3</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Firmicutes (57)</td>
<td><em>Bacillus licheniformis</em>, DSM 13, X68416</td>
<td>8</td>
<td>3</td>
<td>41</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td><em>Bacillus aerius</em>, MTCC 7303, AJ831843</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Bacillus thuringiensis</em>, ATCC 10792, AF290545</td>
<td>2</td>
<td>24</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Bacillus weihenstephanensis</em>, DSM 11821, AB021199</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Granulicatella adiacens</em>, ATCC 49175, D50540</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clostridiales (19)</td>
<td><em>Clostridium colicanis</em>, DSM 13634, AJ420008</td>
<td>8</td>
<td>11</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Sarcina ventriculi</em>, DSM 286, X76649</td>
<td>11</td>
<td>19</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Leuconostoc lactis</em>, DSM 8581, AF175403</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Paenibacillus odorifer</em>, ATCC BAA-93, AJ223990</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Paenibacillus borealis</em>, DSM 13188, AJ011322</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Staphylococcus capitis subsp. capitis</em>, ATCC 49326T, AB009927</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Staphylococcus capreus</em>, ATCC 35538, AB009935</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td><em>Staphylococcus epidermidis</em>, ATCC 14990, D83363</td>
<td>14</td>
<td>30</td>
<td>18</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td><em>Staphylococcus saccharolyticus</em>, ATCC 14953, L37602</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Staphylococcus warneri</em>, ATCC 27836, L37603</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptococcaceae (1)</td>
<td><em>Streptococcus parasanguinis</em>, ATCC 15912, AJ003093</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* Even though some of the clone sequences retrieved show similarities with the nearest neighbor, the DOTUR analysis considered these sequence as different OTUs.

*b* Numbers of OTUs are given in parentheses. Clones were assigned to OTUs using DOTUR analysis, and a representative clone from each OTU was compared against RDP-type strain database using the BLAST function.

(ii) DNA microarray-based microbial community analysis. Fig. 3 demonstrates the distribution of bacterial diversity detected by DNA microarray technology. Additional raw data based on family-level incidence are given (see Table S2 in the supplemental material). Comparisons of the microbial diversity in each sampling event indicate that JPL-SAF-A possessed the highest diversity (719 OTUs), but when the same facility was sampled again within a span of a month (JPL-SAF-C), the lowest diversity was observed (422 OTUs). Compared to the clone libraries, where only gram-positive bacterial sequences were retrieved, DNA microarray analysis showed that only two-thirds of the total diversity were gram-positives in these facility locations. All families presented in clone libraries were also detected via microarray, with the exception of *Carnobacteriaceae* (*Granulicatella*), which was sequenced from the JPL-SAF-A sample and exhibited 98% similarity with the described
type strain. Unlike the results from the cloning-sequencing methods, DNA microarray technology detected strict aerobes such as members of the family Micrococcaceae.

At the family level, Bacillaceae dominated each sampling, which is consistent with previous cultivation-based studies (32). Interestingly, microarray analysis showed higher diversity of Clostridiaceae in Bldg-233-B compared to the other samples. Furthermore, the diversity of Lachnospiraceae and Peptostreptococcaceae, both clustering within the Clostridiales and capable of growing only under strict anaerobic conditions, were present in all facilities. Several families detected—such as Helicobacteriaceae, Enterococcaceae, Mycobacteriaceae, or Spirochaetaceae—include opportunistic pathogens. Although sequences of the members of Staphylococcaceae and Propionibacteriaceae dominated in clone libraries, only a few OTUs were observed as hybridization signals in the DNA microarray. In addition, the diversity of Propionibacteriaceae was low using either molecular method but still dominant in clone library analysis if present (JPL-SAF-A).

DISCUSSION

Previous microbial surveys of clean-room environments have been analyzed either via a culture-based approach or molecular methods (16, 17, 25, 36, 37). In contrast, this study combined a primary enrichment suitable for anaerobes in oxygen-free media and subsequently analyzed them to elucidate a more complete view on anaerobic microbial diversity using the state-of-the-art molecular methods (13, 38). Although used for anaerobic cultivation, samples were aerobically collected and processed, since on-site handling of anaerobic equipment was not possible due to the ongoing spacecraft assembly. When anaerobic and aerobic sampling procedures were used in parallel for sample collection, there were no significant differences in detection between the two sampling methods (36). In addition, the isolation and retrieval of 16S rRNA gene sequences of non-spore-forming obligate anaerobes in the samples collected under aerobic conditions suggested that when samples are analyzed within an hour of sampling, elaborate use of anaerobic sample handling instruments can be avoided.

The enrichment approach was selected to suppress the growth of aerobes not able to thrive under the anaerobic conditions provided. Clone libraries constructed from enrichment cultures revealed only anaerobes and suggested that all se-

FIG. 1. Rarefaction analysis of anaerobic bacterial diversity from several spacecraft assembly facilities. The coverage values (C) for each clone library are given in parentheses. Rarefaction curves of standard and gradient PCR samples are depicted with asterisks and in italics, respectively.

FIG. 2. Principal coordinate analyses showing the relationships between samples.
quences retrieved were of microbes able to grow under these defined enriched culturing conditions. Moreover, the broad spectrum of strict anaerobes detected in clone libraries compared to cultivation studies indicated the advantage of this technique. Certainly, the enrichment conditions used (media, temperature, gas phase, etc.) might preferentially support growth for only a subset of all known anaerobes; hence, the approach taken during this study does not cover all anaerobes that might possibly be present in a sample. Because a universal primer set(s) to selectively amplify anaerobes does not currently exist, and since the previous molecular studies suggested that only 0 to 8% of the total rRNA gene sequences retrieved belong to anaerobes, the approach used in this study is a step forward in identifying the extent of anaerobic bacterial diversity compared with approaches reported in other studies (25, 36, 37).

Compared to the cultivation approach (two microbial genera), the amplification and cloning of 16S rRNA genes from enrichments revealed a broader diversity of microorganisms (11 microbial genera). Interestingly, the two different amplification methods performed (standard PCR and gradient PCR) resulted in no significant diversity differences. Despite their similarities in geographic location and environmental controls, UniFrac analysis found distinct bacterial populations associated with each clean-room facility. More research is necessary to confirm whether the microbial diversity in the clean rooms is influenced by the environment as has been suggested by previous studies (14, 25). In addition, the analysis presented here indicates that the presence of spacecraft hardware in the clean room and high human activity has a strong influence on the transport of microbes into the clean room. The change in microbial diversity between JPL-SAF-A (before spacecraft components arrival) and JPL-SAF-C (only 4 weeks between the samplings; during spacecraft assembly) might be attributed to the fact that selective pressure can cause the bacterial population to change very fast; but more sampling points are needed to validate this assumption.

The findings of the microarray analysis were consistent with those of the clone libraries concerning the distribution of the detected diversities. In general, the microbial population of the sampled clean rooms seemed to be very similar at the phylum level (Fig. 3), but analysis of bacterial families revealed major differences. The advantages of PhyloChip over clone libraries has already been reported (6, 17); and similar to previous investigations (9- to 70-fold increase), this study found an ~10-fold increase in number of different OTUs when PhyloChips were used. The high number of detected Gram-positive bacterial families indicated that their presence is abundant in all clean rooms analyzed. The results also suggest the presence of a wide range of low-abundance organisms, since one-third of the bacterial types found by the PhyloChip belonged to phyla not observed by clone libraries. Likewise, the diversity of strict anaerobes, especially within the order Clostridiales was found to be more diverse when PhyloChip was used. These results are in contrast to those of a published study (36) that may have underestimated the obligate anaerobes in clean-room facilities due to the use of a cultivation-based analysis only.

In summary, the two-step approach (initial enrichment followed by the molecular analysis) implemented during this study to survey anaerobic bacterial diversity, yielded two advantages. Initially, the dilution of the inocula causes attenuation of aerobic bacterial DNA that might have been available.
for clone library analysis. Second, anaerobic bacteria were preferentially enriched under the cultural conditions used, making anaerobic bacterial DNA more readily available for PCR amplification. These predictions were reflected in the results of this study: aerobic microbial diversity was absent in clone libraries due to cloning biases (for abundant DNAs in ligation and transformation), whereas the PhyloChip detected low-abundance aerobic DNA (e.g., Micrococcaceae) due to its high sensitivity, as reported elsewhere (3, 17). However, the low abundance of Propionibacteriaceae signatures in PhyloChip compared to their dominance in both clone libraries (Table 2) and isolation procedures (Table 1) suggest that more scrutiny may be required in the microarray probe design for this bacterial group.

Results of this study indicate that anaerobic spore-formers such as clostridia may have access to the MSL components. Since many of these anaerobes were isolated from the GSE used to contain and support MSL subsystems during assembly, it is recommended that the quantitative measurement of anaerobic population (and appropriate cleaning protocols) be considered in strategic planning to preserve the scientific integrity of high-sensitivity missions. Although clostridia may not be able to germinate or thrive on Mars due to their fermentation-based metabolism, the high level of resistance of their spores to heat, chemicals, and UV irradiation (particularly noted in C. perfringens) may allow survival and proliferation during space flight; this could potentially cause forward contamination significant enough to confound the results of a life detection mission (28, 30, 34).

The composition of the Martian atmosphere is dominated by CO₂ with traces of N₂, O₂, CO, and H₂O, and the aerobic microbes are unlikely to grow under these conditions. Furthermore, no organic compounds have yet been detected on the Martian surface; the absence of organic compounds limits the potential number of microbes that could thrive on the planet’s surface (39). The isolation of several strict anaerobes that use nitrogen (such as P. borealis) and CO₂ to synthesize organic compounds in European Space Agency clean rooms (7, 36) is a concern since their transport to the exploring planets via human made satellites is possible. However, it is important to note that chemolithotrophs, which could act as pioneers in colonizing Mars, were not detected by either clone libraries or PhyloChips in the anaerobic enrichment cultures prepared over the course of this study. Apparently, an appropriate culture condition enriching chemolithotrophs could be used to confirm this assumption (36). Future studies should consider characterizing resistance of these anaerobes to space conditions and isolating anaerobic chemolithotrophs from spacecraft and associated surfaces. Although the results of this study are of particular interest to space-faring nations, the study will also benefit a wide range of scientific, electronic, homeland security, medical, and pharmaceutical ventures that produce sensitive products or assemble products in clean rooms.

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