Are we There Yet? Understanding Interplanetary Microbial Hitchhikers using Molecular Methods

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Abstract
Since the early time of space travel, planetary bodies undergoing chemical or biological evolution have been of particular interest for life detection missions. NASA's and ESA's Planetary Protection offices ensure responsible exploration of the solar system and aim at avoiding inadvertent contamination of celestial bodies with biomolecules or even living organisms. Life forms that have the potential to colonize foreign planetary bodies could be a threat to the integrity of science objectives of life detection missions. While standard requirements for assessing the cleanliness of spacecraft are still based on cultivation approaches, several molecular methods have been applied in the past to elucidate the full breadth of (micro)organisms that can be found on spacecraft and in cleanrooms, where the hardware is assembled. Here, we review molecular assays that have been applied in Planetary Protection research and list their significant advantages and disadvantages. By providing a comprehensive summary of the latest molecular methods yet to be applied in this research area, this article will not only aid in designing technological roadmaps for future Planetary Protection endeavors but also help other disciplines in environmental microbiology that deal with low biomass samples.

From required standards to cutting-edge microbiome profiling

Since the early days of space travel, space-faring nations had been interested in the detection of extraterrestrial life, may it exist. Missions to foreign celestial bodies that are of interest in the context of biological evolution can be equipped with highly sensitive instruments. The integrity of these instruments must be ensured by keeping them clean from terrestrial contaminants during assembly, testing and launching operations. This is one reason why spacecraft are assembled in cleanrooms and undergo rigorous cleaning procedures as a measure of Planetary Protection guidelines (COSPAR, 2002).

In the 1970s, The Viking spacecraft destined to Mars underwent rigorous heat sterilization at the system level for days to ensure sterility of the lander (Puleo et al., 1977). Alternative sterilization methods are necessary to ensure the cleanliness of modern heat-sensitive technical equipment. These alternative methods can only perform surface sterilization and miss the 'embedded bioburden', increasing the risk of contamination of alien celestial bodies with terrestrial life. Consequently, the cleanliness of the spacecraft is continuously monitored throughout its time being on Earth with standardized methodologies. These have been established in the early stages of Planetary Protection and are based on the enumeration of cultivable bacterial endospores per square meter surface of spacecraft or cleanrooms (also termed "standard spore assay"). The main advantage of this cost-effective method is that they are standardized across time and thus comparable between missions. The main disadvantage, however, is that they only target cultivable, heat resistant microorganisms, most of which are in dormant states like endospores. Currently, it is estimated that 0.1%-1% of all microorganisms detected via molecular methods can be cultivated under defined laboratory conditions (Tyson and Banfield, 2005). Moreover, the cultivable spore load of spacecraft surfaces does not correlate with the absolute quantity of microorganisms detectable via molecular methods (Cooper et al., 2011). Consequently, the spore load is only an estimation of spacecraft and cleanroom cleanliness but molecular methods are necessary to determine the actual microbial load and particularly the microbial diversity. Since these modern methods have undergone drastic development over the past years and will continue to be improved in the future, they enable researchers better insight into the microbiome structure and its functional profile associated with spacecraft and cleanrooms, alongside the standard spore assay. A summary of the molecular methods that have been applied in Planetary Protection research and two key methods that will hopefully find application in the near future are depicted in Figure 1.

Detection starts with collection

In order to study microorganisms from spacecraft or cleanrooms, the cells need to be recovered from the respective matrix. Air sampling is fairly straight forward, since there are commercial air samplers available that

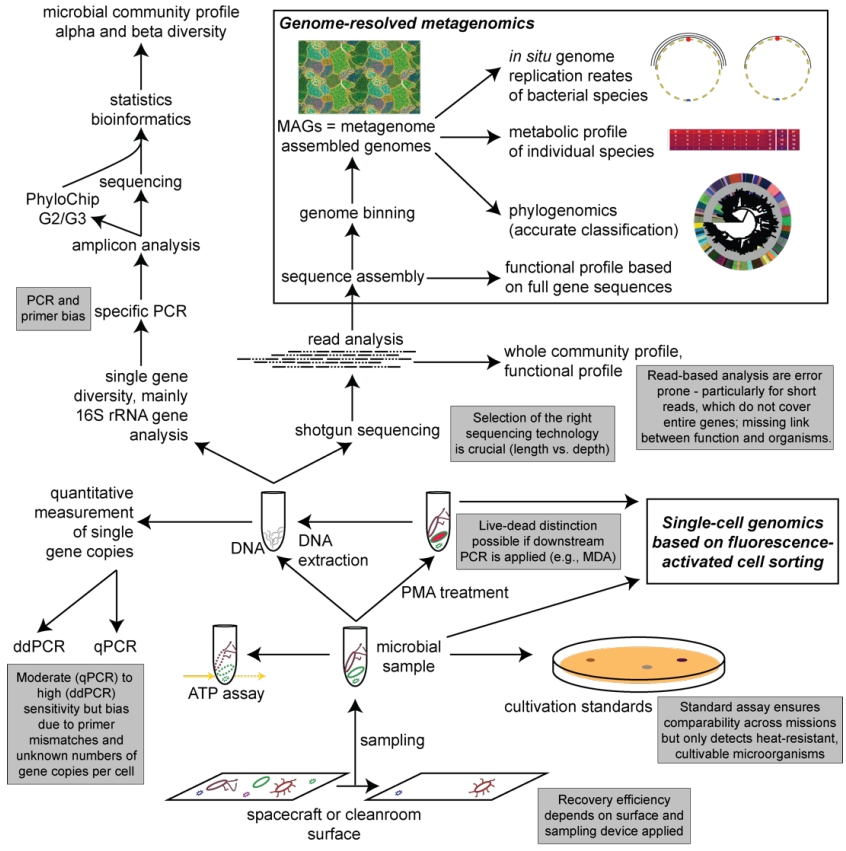

Figure 1. Overview of the molecular methods that have been applied for cleanroom environments and in some cases also for samples from spacecraft hardware. Methods in boxes have not yet been applied but their potential outcomes and limits are discussed in this review. Grey boxes contain comments on the different techniques. Please note that single cell genomics and metagenomics are the only techniques that can result in the detection of viruses because these biological entities do not possess a commonly shared gene that could be used for gene-directed molecular assays.

filter air onto a membrane to catch particles including microorganisms. Retrieving microorganisms from surfaces can be achieved via multiple different methods, whereas wipes and swabs have been the standard for NASA's Planetary Protection over decades (Cooper et al., 2011; La Duc et al., 2012; Ghosh et al., 2010; Probst et al., 2010a, 2010b; Vaishampayan et al., 2010, 2013; Venkateswaran et al., 2001). Other sampling devices (Bargoma et al., 2013; La Duc et al., 2014; Kwan et al., 2011) and their efficiency regarding the retrieval of microorganisms from various surfaces

(Probst et al., 2011) have been investigated and are regularly applied in scientific Planetary Protection studies (La Duc et al., 2004; Mahnert et al., 2015; Moissl-Eichinger et al., 2013; Moissl et al., 2007; Weinmaier et al., 2015). The detection of encapsulated bioburden has proven to be fairly difficult and depending on the matrix, the microbes sometimes cannot be sampled at all (Bauermeister et al., 2014). The evaluation of these techniques has mainly been performed based on endospores or viable bacteria, however, little is known about the recovery efficiency of archaea, eukaryotic cells and viruses from these matrices. In the following paragraphs, we will discuss the analysis of recovered cellular material from any matrix and its downstream analysis and interpretation. An overview of these analyses and the respective methods is provided in Figure 1.

Quantifying contamination

Adenosine triphosphate (ATP) is the main energy currency of all known forms of life. It is fairly stable, for instance, boiling water and alkaline pH over several minutes are necessary to hydrolyze the molecule (Stanley, 1989). Consequently, it is also used as a marker for quantifying microbial activity (Janaszek et al., 1987; Thore et al., 1975; Venkateswaran et al., 2003). Using standardized assays, it is possible to selectively measure the extracellular and intracellular ATP concentration of a sample. This selective ATP quantification technique is used to measure bioburden of spacecraft hardware and associated surfaces in the cleanroom (Figure 1) (Venkateswaran et al., 2003). Total ATP quantification technique was used to rapidly assess cleanliness of spacecraft surfaces in order to assess PP risk (Benardini and Venkateswaran, 2016). However, this quantification is heavily error-prone as i) the sample preparation is dependent on lysozyme-based cell lysis (which does not work for certain microorganisms, e.g. Archaea) and ii) the intracellular ATP concentration depends on the type of microorganisms analyzed and its metabolic state. For example, eukaryotic microorganisms are much larger in cell size and thus usually contain on average many more ATP molecules than prokaryotic cells.

A more accurate way of determining the microbial load of organisms in a cleanroom sample is via quantitative PCR (qPCR; Figure 1). This method has been applied in cleanroom studies to estimate the number of microbes in samples. Usually, this method is based on the amplification of bacterial or archaeal 16S ribosomal RNA genes (rRNA genes or 18S rRNA genes in the case of Eukaryotes) in comparison with standard concentrations. 16S/18S rRNA genes are ubiquitous in all known forms of life on our planet and are a structural component of ribosomes. Targeting individual rRNA genes with qPCR can in theory accurately estimate the number of these genes in a DNA sample. Nevertheless, the method has some disadvantages, which are related to the biology of microorganisms. First of all, the major idea behind bioburden estimation in Planetary Protection is determining the number of microbial cells in a collected sample. However, individual cells can be polyploidic, i.e. have multiple copies of their genomes and thus of

their 16S rRNA gene, leading to an overestimation of microbial abundance. Indeed, some organisms have been reported to have hundreds of chromosomes per cell (Ionescu et al., 2017). Although polyploidy usually occurs during high growth rates, which is unlikely to occur in oligotrophic cleanrooms, halophilic archaea have been reported to become polyploidic to use DNA as a phosphate storage (Zerulla et al., 2014). Scientists can currently only speculate about the activity of microbes and their polyploidity in cleanroom environments. Second, some microbial species are known to have multiple copies of 16S rRNA genes encoded on a single genome, adding another uncertainty to the estimation of the microbial load via 16S rRNA gene quantification for bioburden estimation. For instance, one species of *Paenibacillus* isolated from a cleanroom of the European Space Agency is known to have multiple and different copies of 16S rRNA genes (Behrendt et al., 2010). Scientists tried to correct for the bias of targeting multiple copies of 16S rRNA genes of the same chromosome in a metagenomic sample (Moissl-Eichinger, 2011) by considering the average number of bacterial or archaeal 16S rRNA genes per genome (Klappenbach, 2001). However, the average number used was based on the database assembled from sequenced prokaryotes, which was consequently not designed for the diversity of microbes found in spacecraft assembly cleanrooms and much less for the specific diversity of individual samples. As recently revealed, such a process can introduce severe biases and cause a misinterpretation of data (Louca et al., 2018). Third, qPCR, a quantitative PCR approach, is biased toward the primer pair used. In fact, recent investigations regarding the overall diversity that can be reconstructed using metagenomics indicated that conventional 16S rRNA gene primers miss entire clades of organisms (Brown et al., 2015; Eloe-Fadrosh et al., 2016). To what extent these clades, e.g., the Candidate Phyla Radiation of bacteria (Brown et al., 2015), which is estimated to make up nearly one-third of the entire diversity on our planet (Hug et al., 2016), exist in cleanrooms remains unknown due to the lack of genomic information from these ecosystems. Moreover, as any DNA-based method, qPCR is also biased toward the type of DNA extraction method applied. Nevertheless, researchers of the Jet Propulsion Laboratory, Pasadena, have deeply investigated the DNA extraction biases for molecular analyses and have suggested combinations of methods to capture the greatest diversity of organisms in a sample (Cooper et al., 2011). However, to which extent DNA extractions are quantitative for organisms of uncultivated phyla remains to be shown.

Small subunit ribosomal RNA for classifying microbes

Assessing the diversity of microorganisms on spacecraft and in cleanrooms was one of the major foci of research in Planetary Protection over the last two decades. Initiated by Venkateswaran *et al.*, (Venkateswaran et al., 2001) researchers have been probing the diversity of bacteria and archaea using 16S rRNA gene analyses (Figure 1) and found that this diversity is orders of magnitude greater than the cultivable diversity described until that

day (Ghosh et al., 2010; Vaishampayan et al., 2010). With the emergence of next-generation sequencing platforms, this diversity was further explored with some researchers also focusing on the rare biosphere of these ecosystems using deep sequencing of individual samples (Mahnert et al., 2015). Generally, 16S rRNA gene amplicon analyses suffer from the same biases as qPCR techniques, which include DNA extraction, mismatches of primer pairs, and a biased estimation of diversity due to the possibility of multiple 16S rRNA gene copies on a single genome. Moreover, a general PCR bias – the preferred amplification of genes of high abundance and of preferred primer binding – adds another layer of complexity to these analyses. Generally, 16S rRNA gene diversity analyses can be used to infer alpha diversities (e.g., Shannon-Wiener index (Spellerberg and Fedor, 2003) and beta diversities (e.g., changes of microbial communities over time) but existing PCR biases do not allow the estimation of relative abundance of organisms within one sample (changes of relative abundances across samples is indeed possible). Nevertheless, researchers have extensively used 16S rRNA gene amplicon analyses to determine the most abundant taxonomic groups in individual samples from cleanrooms or spacecraft (La Duc et al., 2012; Mahnert et al., 2015; Vaishampayan et al., 2010).

Beyond 16S rRNA gene sequencing, microarray analyses have been used to study the biodiversity changes between samples and over time (Moissl-Eichinger et al., 2015; Vaishampayan et al., 2010, 2013). While PhyloChip G2 (Brodie et al., 2006) and G3 (Hazen et al., 2010) suffer from the ability to accurately call an organism present or absent in a sample, their sensitivity to changes is several orders of magnitude greater than amplicon sequencing due to the amount of PCR product that is hybridized onto the sample (Probst et al., 2014). Although some researchers have looked into using PhyloChip G3 for the detection of microbes that have not been considered in the initial microarray design by compiling new sets of existing probes (Probst et al., 2014), the vastly expanding diversity would require frequent updates of microarray probes. However, due to the drop in sequencing costs and the necessity to accurately call an organism present or absent in a sample have substantially replaced the use of 16S rRNA gene microarray with amplicon sequencing for Planetary Protection research.

Metaomics

Biomass quantities that can be retrieved from cleanrooms and spacecraft surfaces are generally fairly low rendering RNA, protein, or metabolite analyses difficult. However, sequencing of metagenomic DNA extracted from these environments has been successfully applied. Nevertheless, scientists still have to rely on random amplification of the metagenomic DNA (MDA, multi displacement amplification) to create enough biomass for sequencing (Yilmaz et al., 2010) (Figure 1). Randomized amplification of DNA is problematic as it introduced biases to the community, which are

mainly based on the GC content of the template DNA (Probst et al., 2015). Using this approach, scientists have identified a great diversity of microorganisms, including an entire category of biological entities that were previously not reported in cleanrooms: viruses. Weinmaier and co-workers detected signatures of two phage, a Phi29-like virus and an unclassified Siphoviridae, and several viruses associated with humans and other eukaryotes, such as human herpesvirus 4, Cyclovirus TN12, Dragonfly cyclovirus 2, Hypericum japonicum-associated DNA virus, various Fecal-associated gemycircularviruses, and a *Meles meles* fecal virus (Weinmaier et al., 2015)

To overcome the ultralow biomass limitation for generating regular metagenome libraries for sequencing, a larger surface area was sampled. However, if the microbiome is too heterogeneous, this would likely not result in good sequence assemblies and mask the heterogeneous composition of the ecosystem. Hence, a recent study reported the usage of KatharoSeq for generating low-biomass metagenome libraries from cleanroom samples (Minich et al., 2018). However, the metagenomic sample preparation and sequencing technique was not mentioned in detail. Also it remains unclear if amplification steps were involved in this study. Apart from these issues that make KatharoSeq currently not yet attractive for a detailed metagenome study of the functional cleanroom diversity, it seems a very promising technology. Another interesting approach is the generation of regular metagenome libraries from few nanograms using emulsion PCR (Blow et al., 2008) or from biomass as small as a few femtograms (Rinke et al., 2016). Rinke *et al.* applied this protocol to several samples from different environments and produced reliable metagenome data after removing duplicate reads from the samples. This technology should find application in future Planetary Protection endeavors and might enable researchers to go beyond metagenomics of cleanroom samples and produce the first metagenome libraries from spacecraft hardware.

Live-dead distinction

In nature, microorganisms can have several different states of existence: They can either be alive and metabolically active, in a dormant state (e.g., as an endospore), viable but non-culturable (VBNC) or they can simply be dead (Barer and Harwood, 1999; Oliver, 2005, 2010; Xu et al., 1982). Even though distinguishing viable microbial cells from dead cells will have a paramount effect on ecological inferences in cleanrooms and on spacecraft, very few microbial diversity studies take this into account. A lack of understanding about the viability of a microbial population could have serious and sometimes grave consequences, since this is the portion of the community that contributes or affects the ecosystem. This is of particular importance for microbial diversity analyses in food and medical device manufacturing. Estimates of the viable microbial population in the spacecraft assembly cleanroom would help Planetary Protection engineers to calculate the viable microbial bioburden and in turn the Planetary

Protection risk for forward contamination. In-depth understanding of the viable microbes present on the cleanroom environment will also guide the development of more effective bioburden reduction techniques.

Propidium iodide (PI) represents one of the most commonly used fluorescent dyes to determine cell viability by probing the membrane integrity of microorganisms. PI can penetrate only compromised cell membranes and intercalate with DNA resulting in a red fluorescence (excitation 493 nm and emission 636 nm). It is a component of the LIVE/DEAD BacLight Bacterial Viability Kits (ThermoFisher, USA) along with SYTO 9 (Boulos et al., 1999). The latter stains all cells with a different color resulting in a green (viable) versus red (dead, overshadowing the green) differentiation of dead from viable cells for epifluorescence microscopy, flow cytometry, and fluorometry techniques (Williams et al., 1998). Andreas Nocker and colleagues further developed this technique and replaced PI with propidium monoazide (PMA), which also intercalates into DNA of membrane-compromised cells (Fittipaldi et al., 2012; Nocker et al., 2006, 2007). However, the azide group can be photoactivated resulting in a covalent bond between DNA and the fluorescence dye. Followed by DNA-extraction and PCR reaction (e.g., 16S rRNA gene PCR), this method is selective for viable microorganisms, since the DNA of the PMA-tagged cells can no longer be amplified due to a steric hindrance of the DNA polymerase in binding.

Vaishampayan *et al.* reported pre-PCR propidium monoazide (PMA) treatment of samples followed by downstream 16S rRNA gene analyses (via qPCR, pyrosequencing and PhyloChip DNA microarray) to understand the diversity and distribution of the viable bacterial population in spacecraft assembly cleanrooms (Vaishampayan et al., 2010) (Figure 1). Their results demonstrate a substantially lower bioburden of viable cells compared to total cells and a very limited diversity of living microorganisms. One step further, Weinmaier *et al.* published the first viability-linked metagenomic analysis of cleanroom environments resulting in many novel findings including viruses (see above). Subsequently, Mahnert *et al*. published another PMA-based viability study on spacecraft assembly cleanrooms reporting the effect of cleanroom maintenance on microbial diversity and abundance.

Another advantage of PMA is its application in reduction of contaminants during sample processing to ensure the cleanliness of the reagents applied. Here, PMA is used to remove contaminating extracellular DNA present in almost all commercial PCR reagents (Salter et al., 2014), particularly while using low biomass samples such as cleanrooms. PCR reagents can be treated with PMA to exclude contaminating DNA from amplification during the PCR reaction. Thus treatment of both environmental samples and PCR reagents could improve the detection of viable cells from low biomass samples (Schnetzinger et al., 2013). Several

other cultivation-independent techniques such as stable isotope labeling (Dumont and Murrell, 2005; Fischer and Pusch, 1999), respiration detection (Winding et al., 1994), BONCAT (Hatzenpichler et al., 2014) , isothermal microcalorimetary (IMC) (Rong et al., 2007) were recently reviewed (Emerson et al., 2017). These techniques would, however, necessitate the establishment of mesocosms of cleanroom populations and thus result in skewing the community structure. Nevertheless, these techniques could be useful for hypothesis testing regarding the metabolic activity of organisms in response to a certain substrate.

Open questions

Planetary Protection research, in general, has been very descriptive by cataloging the microbial diversity in cleanroom environments and associated spacecraft hardware. Little effort has been performed in understanding the ecology of these built environments. First and foremost, a general understanding of the entire breadth of organisms and viruses in these ecosystem needs to be established. Most of the assays are geared towards the detection of Bacteria, although Archaea (Moissl-Eichinger, 2011; Moissl-Eichinger et al., 2015) and even Eukarya (La Duc et al., 2012) and viruses (Weinmaier et al., 2015) have also been detected in these ecosystems. It is obvious, that current sampling techniques of surfaces can recover these organisms but the actual efficiency has only been established for a few bacterial strains (Bargoma et al., 2013; Probst et al., 2010b).

More importantly, the general nature of the assembly of the ecosystem of the cleanrooms needs to be deciphered. Based on the current understanding, the ecosystem of cleanroom facilities has substantial selective pressures on microbes. These pressures arise from the harsh cleaning procedures and environmental conditions that are maintained within these facilities limiting the survival of organisms. They also result in little nutrient availability (oligotrophy) posing a challenge for most microbes to thrive in these ecosystems, although a recent study reported the growth of bacterial species from a cleanroom on cleaning reagents (Mogul et al., 2018). In theory, only a small portion of the detectable microbiome thrives in these environments and other microbes are random contaminants in cleanrooms. This enables the assumption that cleanroom microbiomes do neither follow a deterministic model nor a pure stochastic ecosystem assembly (Dumbrell et al., 2010; Hubbell, 2001; Langenheder and Székely, 2011; Ofiteru et al., 2010). Indeed, multiple factors like biogeography of skin microbiome, soil composition of the surrounding ecosystem, weather influences on soil and hardware entering the cleanroom serve as sources for microbial dispersal and suggest a stochastic model for the inactive community. In contrast, the active community, microbes that might grow and increase in cell numbers in this oligotrophic environment, should in theory follow a deterministic model for ecosystem assembly.

A few studies have looked into beta diversity changes of the microbiomes in spacecraft assembly cleanrooms (Moissl et al., 2007; Vaishampayan et al., 2010) to understand the temporal or spatial differences of the microbial communities. Moissl and co-workers concluded that the surrounding ecosystem of the cleanroom buildings substantially impacts the detected biodiversity (Moissl et al., 2007). Particularly 16S rRNA genes of microbes putatively originating from soil were detected. This conclusion is based on studying geographically distinct cleanroom facilities. However, the conclusion is questionable considering that a) the cleanrooms had different maintenance procedures (e.g. particulate filtering) and b) different people were working in these cleanrooms. The human microbiome shows highly significant variations between human beings (Kolde et al., 2018; Morgan et al., 2013); ergo, different workers would ultimately mean the transport of different microbiomes into the cleanrooms (e.g. by shedding skin particles) but linking signatures of microbes from cleanrooms to those of the workers has not been performed yet. A first attempt has been done for Archaea, for which it has been shown that certain 16S rRNA genes of Thaumarchaeota are also present on human skin (Probst et al., 2013). Other studies have tried to identify contamination routes and followed microbial signatures from outside the cleanroom (e.g., changing room) into the actual cleanroom (Mahnert et al., 2015) as a first attempt to identify how these ecosystems assemble over time.

In the future, rigorous source tracking of microbes involving sampling the workers' microbiome would need to be performed to understand how human beings impact the cleanroom microbiome. For instance, it is unclear if human skin particles can serve as nutrients for microorganisms that survive under the harsh cleanroom conditions. At the same time, contamination routes of microbes on hardware entering the cleanroom and from the surrounding ecosystem and would need to be explored in detail, which would involve sampling the outside of the assembly facilities. At the same time, the active microbiome in these facilities needs to be identified by going beyond simple live/dead distinction. Activity measurements linked to phylogeny are necessary to understand the active portion of microbes that might assemble via deterministic processes. Measuring activity of microbes in an ecosystem is generally hard to achieve (see below for calculating *in situ* replication rates) without bringing the microorganisms into an enrichment culture. However, enrichment can be used to test important hypotheses like the growth of microbes on cleaning agents as performed recently. Here, Mogul et al 2018 showed that spacecraft cleaning reagents may serve as nutrient sources under oligotrophic conditions (Mogul et al., 2018). The researchers demonstrated that spacecraft associated *Acinetobacter* strains, one of the dominating and recurring microbial species, can grow on ethanol (ethyl alcohol), 2-propanol (isopropyl alcohol), or Kleenol 30 (floor detergent) under minimal conditions in the laboratory. Results of this study enable speculation about the survival

and dynamics of the active microorganisms in spacecraft-associated environments suggesting a partially deterministic ecosystem assembly.

Next steps in microbiome profiling

Planetary Protection research has mostly been lacking behind several years compared to the state-of-the-art in environmental microbiology. For instance, cloning and sequencing of 16S rRNA genes from environmental samples was published in 1990 (Giovannoni et al., 1990), yet it took more than ten years until the technique was applied to cleanroom environments by Venkateswaran et al. (2001). However, NASA's Jet Propulsion Laboratory Planetary Protection research group has generally been employing cutting edge technology ever since to study cleanroom diversity, e.g., 16S rRNA gene microarrays called PhyloChip, next generation sequencing and shotgun metagenome sequencing (La Duc et al., 2009, 2012; Vaishampayan et al., 2010; Weinmaier et al., 2015).

There have been several technical advances in microbiome research in the recent years that will prove useful in the near future for Planetary Protection research and that have not yet been applied. Some of these advances regard 16S rRNA gene analyses, including the usage of long-read sequencing for a fairly accurate micro-diversity measure. For instance, PacBio sequencing of circularized 16S rRNA gene amplicons can detect microdiversity of bacteria in environmental samples (Singer et al., 2017). Another 16S rRNA gene-based technology is its accurate and very sensitive quantifications using digital droplet PCR (ddPCR) (Hu et al., 2014; Lin et al., 2017; White et al., 2009); Multiple 16S rRNA genes can occur per genome and the restricted diversity cannot be represented by an overall correction for this phenomenon as performed earlier (see above). We suggest that a comprehensive database of genomes from cleanrooms and their relative distribution based on amplicon sequencing could be used to qualitatively correct for the occurrence of multiple 16S rRNA genes in genomes. Such a genome database could be generated either from public reference genomes matching cleanroom 16S rRNA gene data or directly by resolving population genomes via environmental genomics. Particularly the latter is a major advance in the field of environmental microbiology but was already introduced in 2004 (Tyson et al., 2004) and has made substantial advantages since (Sieber et al., 2018).

The low biomass of cleanroom environments has so far been very challenging in producing high-quality metagenomes and has so far not enabled researchers to perform genome-resolved metagenomics (Figure 1). However, there are multiple promising techniques available that will help to achieve this goal as outline above. Having population genomes from cleanrooms and/or spacecraft surfaces at hand would not only enable the above-mentioned correction of 16S rRNA gene surveys but also bolster the understanding of the metabolic diversity of microorganisms in cleanrooms. Moreover, the known diversity of mobile genetic elements in these

environments would be greatly enhanced leading to important insights for pharmaceutical sciences. A recent bioinformatics technology that enables researchers to calculate genome replication rates of microorganisms from sequencing reads (Korem et al., 2015) would provide further insight into the ecology of cleanrooms by deciphering which organism actively replicates under these harsh conditions. During this procedure non-MDA biased metagenomic sequence reads are mapped to microbial genomes and the relative abundance of sequence information of the origin of replication is compared to the terminus of replication. The difference in the relative abundance can be interpreted as the presence of replication forks running from the origin to the terminus of replication. The result is an average of the entire population of a representative genome and can also be applied to genomes from metagenomes (Brown et al., 2016).

Last but not least, a really elegant and probably the most feasible approach for generating genomes from cleanroom samples would be the use of single-cell genomics, which has in the past lead to the discovery of several hundred novel lineages from environmental samples (Rinke et al., 2013). This approach is based on the sorting of particles that have been stained with a DNA-intercalating dye, and might even be combined with PMA-treatment of samples to selectively sort living microorganisms (Figure 1). After sorting, the cell is lysed and the DNA is amplified using an MDA approach, followed by sequencing (Rinke et al., 2014). Interestingly, this approach can, in theory, also result in the detection of viruses if these are integrated as prophages, attached to their host or have infected the host. Compared to other techniques, this method is particularly useful for low-biomass samples as retrieved from cleanroom environments or even spacecraft hardware and could provide substantial information on the metabolism of microorganisms that reside there and even resolve their strain distribution (Blainey, 2013).

Molecular methods have provided substantial insight into the cultivable and not-yet-cultivable microbiome of cleanrooms and spacecraft surfaces over the past two decades. These methods enabled researchers multiple break throughs including the detection of viruses, identifying contamination routes and potential sources of contaminants, deciphering the viable microbiome and investigating the dynamics of the microbiome in these ecosystems. Although the ecosystem assembly of cleanroom facilities has not yet been deciphered, there are already novel technologies on the horizon that are just waiting to be applied by Planetary Protection researchers to fully understand these ecosystems and minimize the risk of microbial hitchhikers on spacecraft destined to foreign planetary bodies.

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