MOLECULAR IDENTIFICATION OF MICROBIAL CONTAMINANTS

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ABSTRACT

Microorganisms can have significant impacts on the success of NASA's missions, including the integrity of materials, the reliability of scientific results, and maintenance of crew health. Robust cleaning and sterilization protocols are currently in place in NASA facilities, but agency experts agree that microbial contamination is unavoidable and its impact on NASA's missions and science must be minimized. Therefore, it is critical to understand: 1) what specific microorganisms are present, 2) how they may impact scientific objectives, and 3) how to select appropriate mitigation strategies. The Marshall Space Flight Center (MSFC) Planetary Protection (PP) microbiology lab historically relied solely upon enumeration of culturable microbial contamination associated with spacecraft materials or cleanrooms. However, this process is time consuming, many microbes cannot be cultured, and very few can be identified with any fidelity using NASA standard microbiological methods. The work described in this white paper includes the establishment of molecular identification capabilities at MSFC, including DNA isolation, amplification, purification, and Sanger sequencing. This capability will not only improve planetary protection efforts at MSFC (i.e. by identifying contaminating microorganisms in cleanrooms or on spacecraft) but also offers a service center-wide for the identification of contaminants that arise in other projects, processing locations, or during set up and roll out of spacecraft. This work also lays the foundation for higher throughput efforts to identify large populations of microbes across the lifetime of a project and serves as the starting point for future work into whole genome sequencing, non-culture based methods, or additional characterization studies. Ultimately, accurate identification informs appropriate mitigation strategies, increasing the chances of success for NASA's missions and objectives.

INTRODUCTION

Microbiology within NASA typically falls into a handful of categories that include but are not limited to space biology, Environmental Control and Life Support Systems (ECLSS), astrobiology, and Planetary Protection (PP). In each of these fields, it is critical to gain a thorough understanding of the microbes of interest. Historically, microbiologists studied microbes by growing them on solid nutrient media and then performing biochemical characterization to provide the most specific information about the isolate. However, in the past few decades, more advanced techniques of identification have become available, including molecular identification.

In terms of NASA's PP, great care is taken to ensure the cleanliness of spacecraft landing on sensitive solar system bodies. However, the methods used to achieve acceptable levels of cleanliness are largely based on culturing microbes [1]. While this is an established method, it is time consuming and limited in detection. New efforts are underway within the Office of Planetary Protection at NASA Headquarters to advance NASA's PP into the realm of molecular identification. Specifically, this would help scientists and project engineers gain a truer understanding of contamination on spacecraft, not just the contaminants that can be cultured (estimated at ~1% of all microbes) [2]. Furthermore, the identification of microbial contaminants could help inform risk (i.e. are extremophiles present?) and direct mitigation strategies (e.g. is heat required or will an ethanol wipe suffice?). Therefore, as NASA pushes to use molecular strategies in PP, individual centers must be able to meet that need.

The dawn of the molecular age in microbiology occurred with the development of polymerase chain reaction (PCR) and Sanger sequencing. PCR allowed the rapid and now automated production of numerous copies of a particular gene relatively inexpensively [3]. The 16S rRNA gene from bacteria/archaea and the Internal Transcribed Spacer (ITS) region between the 18S and 28S ribosomal

subunit genes within fungi, respectively are commonly used templates because they contain both conserved and variable sequences [4, 5]. The conserved regions act as the site for primer adherence, allowing amplification of the variable regions which help to identify and differentiate the unknown microbe. Sanger sequencing was the first method to allow rapid determination of DNA sequences, and while next generation sequencing methods have become more popular, Sanger sequencing is still commonly employed for smaller scale projects [6].

The process of molecular identification followed in this study is shown in Figure 1. While much of the work described in the diagram can be completed in a basic microbiology laboratory, three primary capabilities must be available: 1) DNA quantification, 2) DNA amplification, and 3) DNA visualization. Prior to the onset of the work described in this white paper, we were awarded additional funding from Jacobs Space Exploration Group for the procurement of three pieces of equipment that enable these three capabilities—capabilities that were not achievable in the MSFC PP Lab before this grant was awarded. These instruments were critical in the completion and success of the work described in the later sections of this white paper. Finally, this white paper also describes the collection and curation of a large library of cleanroom and material-associated microbes which can be used in future studies both at MSFC and beyond.

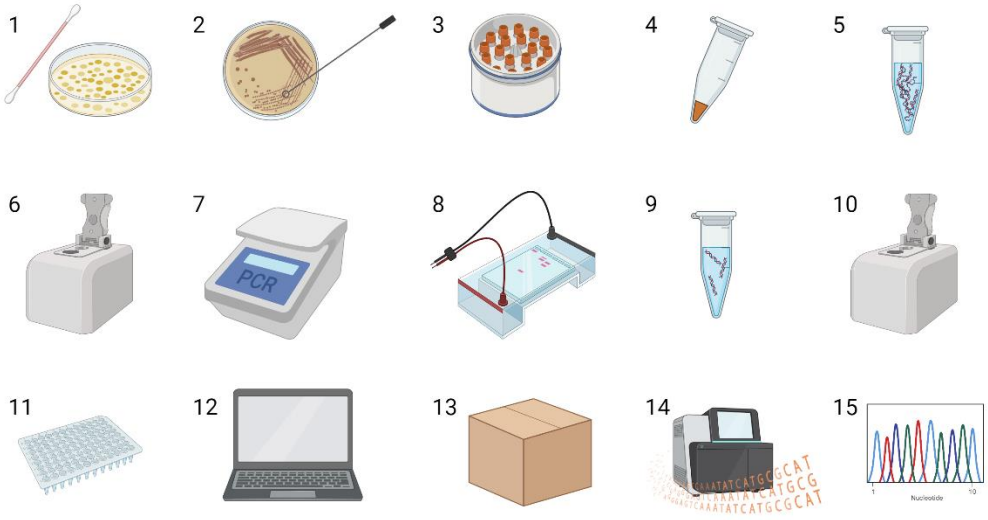

Figure 1: The Process of Molecular Identification
1) Collect samples and plate; 2) Isolate pure cultures; 3) Freeze strains and curate an organism library; 4) Accumulate biomass for all strains; 5) Perform DNA extraction; 6) Quantify DNA on Nanodrop; 7) Amplify targeted gene sequence using PCR; 8) Confirm PCR using gel electrophoresis; 9) Purify DNA; 10) Quantify DNA on Nanodrop 11) Array samples in 96 well plate; 12) Set up account and create order with commercial sequencing service; 13) Ship samples to commercial sequencing service; 14) Sanger sequencing; 15) Perform DNA analysis to identify organisms. Figure created with BioRender.com.

METHODS

MICROBIAL COLLECTION AND ISOLATION

Four cleanrooms at MSFC were selected for sampling, in addition to the uncontrolled lab spaces (Table 1 Locations A and B). One cleanroom, (Table 1 Location F) is not maintained as a cleanroom but still has controlled access. In all cases, samples were taken from the air into 10 ml sterile water using a Bertin Coriolis µ air sampler or by waving a swab through the air. Surface samples were collected using either dry

or sterile water-wetted swabs (Puritan), or sterile water-wetted wipes (TexWipe). The room descriptions and locations are listed in Table 1 with their various classifications and collection locations.

Table 1: Location Characteristics for Microbial Isolate Collection

Location	Cleanliness Rating	Collection Date	Collection Method	CFU
A	Uncontrolled	5/9/2022	air sample	6
A	Uncontrolled	5/12/2022	dry swab (table)	27
A	Uncontrolled	5/12/2022	wet swab (table)	12
A	Uncontrolled	5/12/2022	wet wipe (table)	TMTC
B	Uncontrolled	5/12/2022	wet swab (air)	0
B	Uncontrolled	5/12/2022	dry swab (air)	0
B	Uncontrolled	5/12/2022	wet swab (BSL2 hood)	0
B	Uncontrolled	5/12/2022	dry swab (BSL2 hood)	0
C	ISO 8	5/12/2022	air sample (air)	0
C	ISO 8	5/12/2022	wet swab (air)	0
C	ISO 8	5/12/2022	wet swab (floor)	7
C	ISO 8	5/12/2022	wet wipe (table)	463
D	ISO 8	5/12/2022	wet swab (floor)	26
D	ISO 8	5/12/2022	wet swab (table)	11
D	ISO 8	5/12/2022	wet wipe (table)	154
E	ISO 8	5/12/2022	air sample	0
E	ISO 8	5/12/2022	wet swab (air)	0
E	ISO 8	5/12/2022	wet swab (floor)	2
E	ISO 8	5/12/2022	wet wipe (table)	229
F	Uncontrolled	9/22/2022	air sample	0
F	Uncontrolled	9/22/2022	wet swab (floor)	0
F	Uncontrolled	9/22/2022	wet swab (floor)	4
F	Uncontrolled	9/22/2022	wet wipe (floor)	2

Air samples (300 liters/min) were collected for 5 minutes into 10–15 ml sterile water. After completion, collection cones were capped and stored at 4 °C until processed. Swabs were collected and then stored in sterile tubes at 4 °C until processed. Wipes were dampened in 10 ml sterile water within a sterile petri dish and following collection, stored in sterile glass jars at 4 °C until processed.

Media used for culturing strains was limited to Tryptic Soy Agar (TSA) (BD Biosciences) and Tryptic Soy Broth (TSB) (BD Biosciences). All swabs were applied directly to the surface of a TSA plate and then incubated at 32 °C for at least two days. Wipes were submerged in 35 ml of sterile water and then sonicated. 250 µl of liquid was then pipetted onto TSA, spread using a plastic hockey-stick, and incubated at 32 °C for at least two days. Last, 100 µl of air sample solution was spread on TSA using plastic hockey sticks and incubated at 32 °C for at least two days. If overgrowth was observed, or too many colonies to count (TMTC), dilutions were made of the liquid into sterile water and replated until countable colonies were observed (with the exception of the wetted wipe from Table 1 Location A).

Furthermore, thirteen isolates originated from a paint-coated aluminum plate exposed to the beach near Kennedy Space Center (KSC) for six months. These were collected using a dry swab which was then contained in non-sterile sample bags and transported back to MSFC. Swabs were stored at 4 °C until they were applied directly to TSA plates and incubated at 25 °C for approximately seven days.

Finally, we included strains of *Bacillus atrophaeus* and *B. subtilis* from American Type Culture Collection (ATCC) as positive controls. We also included three water and three DNA purification kit blanks as negative controls.

MICROBIAL LIBRARY CURATION

For all plates, images were taken and colony forming units (CFU) were counted (Table 1). Representative colonies were selected and entered into the PP Lab organism library with a unique identifier as well as a description of the colony and its origin. Selected colonies were all streaked for isolation on TSA and incubated at either 25 °C or 32 °C until sufficient growth and individual colonies were observed. In the event that multiple colony morphologies were detected, colonies were both streaked for isolation and re-named. This process was repeated until pure cultures were obtained. In the event that molds were observed, single colonies could not be obtained and therefore attempts were made to subculture until no other growth was detected. Images of all pure colonies were taken and saved within the PP Lab organism library. When possible, strains were subcultured from one colony using a sterile loop into 1.5–3 ml TSB and grown stationary incubated at either 25 °C or 32 °C. When turbid growth was observed, the strains were frozen with a 1:1 volume of 25% sterile glycerol in cryotubes and stored at -80 °C. In the event that a microbe was unable to grow in liquid culture, colonies from the TSA plates were scraped with a sterile loop into a 1:1 mixture of TSB:25% glycerol and frozen. All frozen cultures were then restreaked on TSA with a sterile loop and grown at 25 °C or 32 °C until growth was seen. If no growth, a mixed culture, or a culture with unmatched description was observed, the culture was discarded and renewed attempts were made to obtain a pure culture from the original plates. It was only after a stock culture was frozen and the growth was verified that the original plates were discarded. All strains were classified as either suspected fungi or suspected bacteria based on plate morphology.

DNA EXTRACTION

The DNA extraction procedure was conducted using a Zymo Quick-DNA Fungal/Bacterial Miniprep Kit (D6005). To collect pellets, pure cultures were scraped from TSA plates using sterile loops and deposited into a weighed microcentrifuge tube. The tubes were centrifuged at maximum speed for 1 min in a Fisher Scientific accuSpin Micro 17 Microcentrifuge (13-100-675). The microcentrifuge tubes were weighed and the pellet weight was recorded. For the kit used, the ideal wet weight of the pellet is between 50–100 mg. In most cases, the pellets fell within this range and wherever possible we endeavored not to use pellets below 25 mg. In some cases, it was necessary to grow multiple plates of the microbe in order to collect enough cell material. Pellets were stored in a -20 °C freezer until ready for processing. For DNA extraction, pellets were thawed in a BSL2 hood and the manufacturer's instructions were followed for isolating genomic DNA. Following DNA elution, the resulting DNA concentration was measured using a Thermo Scientific Nanodrop Lite under the dsDNA setting. All DNA concentrations were recorded.

DNA AMPLIFICATION

PCR was conducted using an Applied Biosystems SimpliAmp Thermalcycler (A41192). Primers were ordered from Integrated DNA Technologies (IDT) and resuspended using the Dilution Calculator on the IDT website. Following PCR, sample tubes were stored at 4 °C until further processed.

For bacterial 16S rRNA gene amplification, the mixture included 1 µl of 10 mM Deoxynucleotide (dNTP) Solution Mix (N0447S NEB), 1 µl of 20 µM 16S rRNA For primer (IDT ReadyMade primer 51-01-19-06), 1 µl of 20 µM 16S rRNA Rev primer (IDT ReadyMade primer 51-01-19-07), template DNA to a concentration of approximately 100 ng, 10 µl of 5X One*Taq* Standard Reaction Buffer (NEB), 0.2 µl of One*Taq* DNA polymerase (M0480L NEB), and sterile water up to 50 µl final volume. The settings used were as suggested by NEB and IDT and are as follows:

1) Initial denaturation: 94 °C for 30 seconds
2) 35 cycles:
    a. Denaturation: 94 °C for 30 seconds
    b. Annealing: 52 °C for 1 minute
    c. Elongation: 68 °C for 1 minute

3) Final extension: 68 °C for 5 minutes
4) Hold: 4 °C for ∞

For amplification of the fungal ITS region, the mixture included 1 µl of 10 mM dNTP Solution Mix (N0447S NEB), 1 µl of 20 µM ITS5 forward primer (IDT) [7], 1 µl of 20 µM ITS4 reverse primer (IDT) [7], template DNA to a concentration of approximately 100 ng, 10 µl of 5X One*Taq* Standard Reaction Buffer (NEB), and 0.2 µl of One*Taq* DNA polymerase (M0480L NEB), and sterile water up to 50 µl final volume. The settings used were as described in [7].

1) Initial denaturation: 95 °C for 10 minutes
2) 35 cycles:
    a. Denaturation: 95 °C for 15 seconds
    b. Annealing: 52 °C for 30 seconds
    c. Elongation: 72 °C for 1.5 minutes
3) Final extension: 72 °C for 7 minutes
4) Hold: 4 °C for ∞

DNA VISUALIZATION

PCR products were visualized using an Invitrogen E-Gel Powersnap System (G8352ST). In short, 2% agarose E-Gels with SYBR Safe (Invitrogen) were loaded into the system. 5 µl of E-Gel 1 KB Plus DNA Ladder (Invitrogen) was loaded into the corresponding well. Then 2 µl of E-Gel Sample Loading Buffer (Invitrogen) was mixed with 5 µl of PCR product and loaded into designated wells. The gel was run under the pre-set condition for 0.8–2% E-Gels for 26 minutes. Gels were then visualized with UV light and images were captured of each. The 16S rRNA amplification product was approximately 1500 bp, while the ITS amplification product was approximately 500 bp. Samples that failed to show bands at expected sizes underwent a repeat PCR. Only samples with clear bands of expected size were purified in the following step.

DNA PURIFICATION

DNA purification was conducted with the Zymo Research DNA Clean & Concentrator -100 Kit (Cat No. D4030). Zymo-Spin collection reservoirs were discarded and the remaining column was placed into a labeled collection tube. For 50 µl PCR samples, the PCR product was mixed with 250 µl of DNA Binding Buffer and gently pipetted up and down to mix. Material was transferred into a column and centrifuged at maximum speed for 1 minute. 600 µl of DNA Wash Buffer was added to the columns and centrifuged at maximum speed for 1 minute. Centrifugation was repeated to clear the column of residual fluid. The columns were placed into new Eppendorf tubes and then 50 µl of water was added directly onto the column. Samples were allowed to sit for 1 minute to ensure DNA on the column dissolved into the water. Tubes were then centrifuged at maximum speed for 1 minute before the column was discarded. Finally, DNA concentration was measured using the nanodrop as described previously. All purified DNA was stored at 4 °C. Three blank columns were carried through the procedure and used as negative controls.

SANGER SEQUENCING AND DNA ANALYSIS

For sequencing of PCR products, samples were diluted to approximately 10 ng/µl concentration, and 10 µl was mixed with 5 µl of 5 µM 16S rRNA For primer or 5 µM ITS5 primer. The resulting 15 µl samples were arrayed into a 96 well plate. These samples included three water negative controls, and three blank DNA purification kit negative controls. The plate was packaged securely and then shipped to Azenta Life Sciences-Genewiz for Sanger sequencing. Following the sequencing procedure, raw data was returned. The quality of the DNA was assessed using the metrics provided by Azenta Life Sciences-Genewiz, including quality score (QS), contiguous read length (CRL), and the DNA traces. FASTA sequences were downloaded and trimmed, then searched against the 16S or ITS entries in the National Center for Biotechnology Information (NCBI) free nucleotide Basic Local Alignment Search Tool (BLAST). The

resulting identifications were assessed using the Query Cover and Percent Identity. To assign identity of the isolates, the following guidelines were used:

1) ≥ 99% identity = genus and species ID
    i. Need 0.2% difference between IDs to call it a certain species
2) ≥ 97% identity = genus only ID
3) < 97% identity = unidentified

MICROSCOPY

Smears were prepared from a single colony of a streak for isolation. A drop of sterile water was placed on a glass slide and mixed with the colony using a sterile metal inoculating loop. The smears were allowed to dry for at least an hour and heat fixed by three passes over an open flame. The slides were then stained for 30 seconds with either crystal violet (Fisher) or safranin (Fisher). Additionally, some smears were Gram stained using the American Society for Microbiology Gram Stain Protocol [8]. Slides were rinsed with water, dried using bibulous paper, and then examined under all magnifications of a Zeiss Primostar 3 Microscope. Images were captured using a cell phone.

RESULTS AND DISCUSSION

In total, 95 microbial isolates were collected from cleanrooms or beach-exposed panels. Based on plate morphology, 11 were classified as fungi while 84 were considered bacteria. As expected, cleanrooms demonstrated fewer microbes than the uncontrolled areas, with the exception of Location F which is uncontrolled but geographically isolated and infrequently visited. Air from all cleanrooms tested yielded no microbes. Furthermore, the surfaces and floors that were sampled within the cleanrooms were in highly trafficked locations (Table 1).

Of the 95 isolates collected, we amplified PCR products from 77 strains. The remaining 18 strains were streaked fresh on TSA, packaged and sent to Johnson Space Center (JSC) in Houston, TX. With funding and support from NASA's Planetary Protection Officer (PPO) at Headquarters (HQ), we traveled to JSC and aided in the DNA isolation, purification, amplification, and sequencing of these strains which resulted in eventual identification for all 18, at least to a genus level (Table 2, blue).

Of the remaining 77 DNA samples submitted for DNA sequencing, 66 DNA samples yielded high quality DNA traces while 11 demonstrated sequencing problems. Most of these were due to either high background, indicating potentially contaminating DNA, or poor quality, indicating that the DNA was insufficient to be sequenced. Of the 11, six were identified at least to a genus level using the NCBI database, but these have all been flagged as potentially contaminated or unreliable identifications (Table 2, yellow). Finally, the remaining five showed no significant similarity within the NCBI database (Table 2, red). Of the 66 DNA samples that yielded high quality DNA traces, only one sample showed no significant similarity found within the NCBI database (Table 2, green), while one other sample pulled up no matches above 97% identity (Table 2, gray). The remaining 64 were identified to at least a genus level specificity. Combining the results from JSC with our work, we achieved a 93% success rate with highly confident identifications.

Table 2: Molecular Identification of Microbial Isolates

NAME	ORIGIN	Identities
PPS11	KSC beach panel swab	***Aureobasidium pullulans*** and maybe contaminant bacteria *Calidifontibacillus/Bacillus* sp.
PPS12	KSC beach panel swab	*Bacillus* sp.
PPS13	KSC beach panel swab	***Nigrospora sphaerica***
PPS14	KSC beach panel swab	***Toxicocladosporium* sp.**
PPS19	KSC beach panel swab	***Cladosporium* sp.**
PPS20	KSC beach panel swab	***Alternaria alternata***
PPS21	KSC beach panel swab	***Pestalotiopsis* sp.**

PPS22	KSC beach panel swab	***Nigrospora osmanthi***
PPS23	KSC beach panel swab	***Toxicocladosporium irritans***
PPS24	KSC beach panel swab	*Sphingomonas roseiflava*
PPS25	KSC beach panel swab	*Bacillus* sp.
PPS26	KSC beach panel swab	***Aureobasidium pullulans***
PPS27	KSC beach panel swab	***Nigrospora vesicularifera***
PPS47	Location A air sample	*Staphylococcus* sp.
PPS48	Location A air sample	*Neomicrococcus lactis*
PPS49	Location A air sample	*Fictibacillus* sp.
PPS50	Location A air sample	*Metabacillus idriensis*
PPS51	Location A air sample	*Knoellia flava*
PPS52	Location A air sample	*Brevibacillus borstelensis*
PPS53	Location A air sample	*Deinococcus ficus*
PPS54	Location A air sample	*Knoellia flava*
PPS55	Location E wet swab floor	*Alkalihalobacillus gibsonii*
PPS56	Location E wet swab floor	*Paenibacillus periandrae*
PPS57	Location A	No significant similarity found
PPS58	Location D wet swab table	*Priestia megaterium*
PPS59	Location D wet swab table	*Lederbergia* sp.
PPS60	Location D wet swab table	*Pseudarthrobacter* sp. (high background)
PPS61	Location D wet swab table	No ID >97%
PPS62	Location D wet swab table	*Bacillus* sp.
PPS63	Location D wet swab table	*Psychrobacillus vulpis*
PPS64	Location D wet swab table	No ID >97% (poor quality)
PPS65	Location D wet swab table	*Streptomyces* sp.
PPS66	Location D wet swab floor	*Micrococcus* sp.
PPS67	Location D wet swab floor	*Brevundimonas vesicularis/nasdae*
PPS68	Location D wet swab floor	*Arthrobacter koreensis*
PPS69	Location D wet swab floor	*Acinetobacter lwoffii* or *Prolinoborus fasciculus*
PPS70	Location D wet swab floor	*Microbacterium pumilum* (non-specific)
PPS71	Location D wet swab floor	*Pseudomonas glycinis/koreensis* (non-specific)
PPS72	Location D wet swab floor	*Paenarthrobacter nitroguajacolicus*
PPS73	Location D wet swab floor	*Staphylococcus hominis*
PPS74	Location D wet swab floor	*Brevundimonas* sp.
PPS75	Location D wet swab floor	*Pseudogracilibacillus endophyticus*
PPS76	Location D wet swab floor	*Acinetobacter lwoffii* or *Prolinoborus fasciculus*
PPS77	Location D wet swab floor	*Janibacter hoylei*
PPS78	Location C wet swab floor	*Bacillus* sp.
PPS79	Location C wet swab floor	*Bacillus* sp.
PPS80	Location C wet swab floor	*Stenotrophomonas nematodicola* (high background)
PPS81	Location C wet swab floor	*Acinetobacter lwoffii* or *Prolinoborus fasciculus*
PPS82	Location A dry swab table	*Bacillus atrophaeus*
PPS83	Location A dry swab table	*Bacillus atrophaeus/vallismortis*

PPS84	Location A dry swab table	*Staphylococcus hominis*
PPS85	Location A dry swab table	*Paenibacillus* sp.
PPS86	Location A dry swab table	*Staphylococcus capitis/caprae*
PPS87	Location A dry swab table	*Staphylococcus epidermidis*
PPS88	Location A wet swab table	*Staphylococcus* sp.
PPS89	Location A wet swab table	*Staphylococcus auricularis*
PPS90	Location A wet swab table	*Bacillus* sp.
PPS91	Location A wet swab table	*Staphylococcus* sp.
PPS92	Location A wet swab table	*Bacillus* sp.
PPS99	Location A wet wipe table	*Okibacterium* sp. (poor quality)
PPS100	Location A wet wipe table	*Bacillus* sp.
PPS101	Location A wet wipe table	*Bacillus atrophaeus*
PPS102	Location A wet wipe table	*Curtobacterium allii*
PPS103	Location A wet wipe table	No significant similarity found (high background)
PPS104	Location A wet wipe table	*Bacillus atrophaeus*
PPS105	Location A wet wipe table	*Pseudomonas* sp.
PPS106	Location A wet wipe table	***Rhodotorula mucilaginosa***
PPS107	Location C wet wipe table	*Bacillus atrophaeus*
PPS108	Location C wet wipe table	*Bacillus atrophaeus*
PPS109	Location C wet wipe table	No significant similarity found (non-specific)
PPS110	Location E wet wipe table	***Cryptococcus albidus***
PPS111	Location E wet wipe table	*Bacillus licheniformis*
PPS112	Location E wet wipe table	*Curtobacterium* sp.
PPS113	Location E wet wipe table	No significant similarity found (non-specific)
PPS114	Location D wet wipe table	*Bacillus atrophaeus*
PPS115	Location D wet wipe table	No significant similarity found (high background)
PPS116	Location D wet wipe table	***Rhodotorula* sp.**
PPS117	Location D wet wipe table	*Mycetocola manganoxydans*
PPS118	Location D wet wipe table	***Aureobasidium pullulans***
PPS119	Location D wet wipe table	*Arthrobacter ginsengisoli/Pseudoarthrobacter psychrotolerans*
PPS120	Location D wet wipe table	*Erwinia* sp.
PPS121	Location D wet wipe table	*Frigoribacterium faeni*
PPS122	Location D wet wipe table	***Cladosporium* sp.**
PPS123	Location D wet wipe table	*Rhodococcus fascians*
PPS124	Location D wet wipe table	***Penicillium crustosum***
PPS125	Location D wet wipe table	***Neocylindroseptoria* sp.**
PPS126	Location D wet wipe table	Likely ***Rhodotorula* yeast sp.** and maybe contaminant bacteria *Arthrobacter oryzae* (high background)
PPS 221	Location F wet wipe floor	*Sphingomonas desiccabilis*
PPS222	Location F wet wipe floor	***Cryptococcus albidus***
PPS223	Location F wet swab floor	*Bacillus toyonensis/thuringiensis*
PPS224	Location F wet swab floor	*Bacillus* sp.

PPS225	Location F wet swab floor	*Brevibacillus* sp.
PPS226	Location F wet swab floor	*Bacillus acidiceler*
PPS229	Location F wet swab floor	*Bacillus acidiceler*
PPS230	Location F wet swab floor	*Bacillus* sp.

A key cause of DNA amplification problems within the PP Lab at MSFC was misidentification of many yeast/fungal strains as bacteria (i.e. yeast will not have a 16S rRNA gene and therefore no DNA should be amplified in the 16S PCR). Of the 18 sent to JSC, we misidentified seven yeasts as bacteria. Furthermore, for the 11 strains that demonstrated sequencing problems, we re-streaked the strains and performed simple light microscopy. We found that five demonstrated morphology consistent with yeast. This was also the case for PPS57 (Figure 2A) which, surprisingly, had high quality 16S rDNA amplification. In total, 24 of the 95 strains, or approximately 25%, were identified as fungi either using DNA sequencing or microscopy (Table 2, bold). These findings indicate that there was likely contaminating DNA in some samples, or potentially mixed cultures. To address this, we re-streaked these strains for isolation twice, then froze fresh stocks. While this project is now complete, a careful repeat of the procedure in Figure 1 would likely yield in high quality sequences and identification for most, or all, of the 11.

However, another possible area of contamination may be the DNA kits themselves. Of the six negative controls submitted for sequencing, one DNA purification kit blank resulted in any sequencing data, which when compared against the NCBI 16S rRNA database, resulted in no significant similarity. While ideally we would see no DNA sequence resulting from negative control samples, it is well documented that commercial DNA extraction and purification kit reagents harbor contaminant DNA [9]. For positive controls, we saw correct identification as expected.

To minimize amplification or sequencing problems, we have implemented additional steps in our molecular identification procedure (Figure 1). This includes a second streak from isolation to ensure pure isolates (a repeat of step 2). It also includes a microscopy step to aid in the classification of strains as bacteria or fungi (following step 2). This would help determine the type of PCR performed for the sample (i.e. ITS or 16S), and therefore increase chances of successfully identifying the organism.

Of particular interest are the two strains that yielded no significant results above 97% identity, PPS61 and PPS64. Microscopy indicated that PPS61 to be short, stubby rods, while PPS64 was highly filamentous (Figure 2 B and C). In both cases, Gram stains were inconclusive. Therefore, deeper sequencing would be necessary to determine if these microbes have been characterized before or are truly novel species.

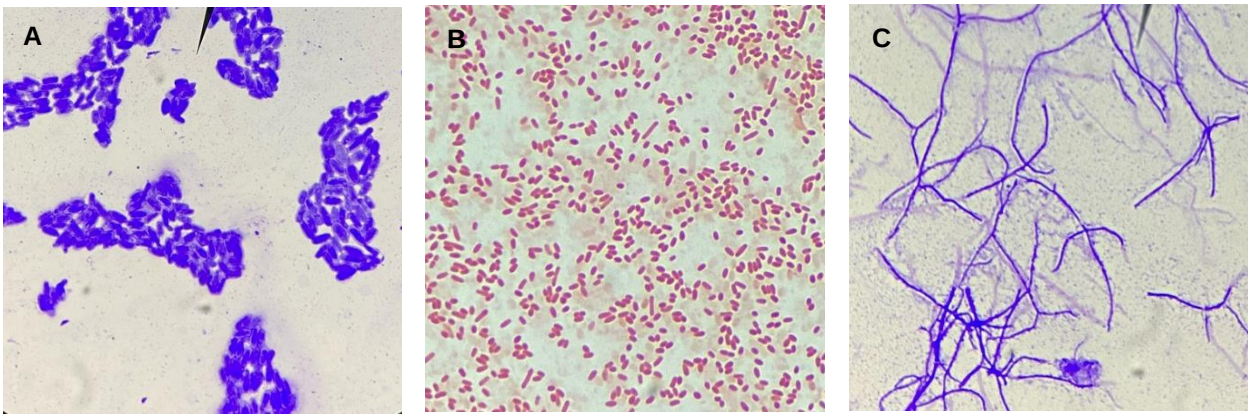

Figure 2: Microscopy of Unidentified Isolates
Smears of unknown strains A) PPS57, B) PPS61, and C) PPS64 were created and stained with either crystal violet or safranin. Images are at 1000x magnification.

While many of the identified microbes are associated with soil or water, some of them are commonly associated with skin. This is unsurprising since the majority of these microbes were isolated from either a beach environment or floors and surfaces of human-used, built environments. Although many of the microbes are poorly understood, some of them are known as extremophiles. Specifically, we found that species of the genus *Brevundimonas* and *Kocuria* have demonstrated the ability to survive in simulated Martian conditions [10, 11]. Furthermore, some identified genera, including *Deinococcus* and *Janibacter*, have been associated with resistance to certain stressors like ultraviolet radiation within the space environment [12, 13]. Finally, some of the genera identified, including *Alkalihalobacillus*, *Bacillus*, *Brevibacillus*, *Calidifontibacillus*, *Fictibacillus*, *Lederbergia*, *Paenibacillus*, *Priestia*, *Psychrobacillus*, *Pseudogracilibacillus*, and *Streptomyces*, are known to form endospores which are hardy and capable of withstanding extreme environments [14 – 21]. These findings underly the importance of performing molecular identification of microbial contaminants to understand the project risk associated with the contamination as well as the best method of mitigation. Specifically, these findings indicate that cleanrooms house many microbes that could be of potential risk to NASA missions as they relate to PP.

SUMMARY AND CONCLUSIONS

The work described in this white paper has achieved multiple different objectives. First, we developed and equipped the MSFC PP Lab with molecular capabilities. This included procurement of instrumentation as well as the completion of a standard operating procedure and the training of personnel to complete the lab operations. The functional procedures established now bring MSFC closer in line with other NASA centers by offering the capability of microbial identification through advanced molecular techniques. Furthermore, we were able to develop working connections with HQ, JSC, and Goddard Space Flight Center (GSFC) microbiologists also attempting to use molecular approaches to advance PP. Finally, we have created a microbial library of PP-relevant strains with known identities which is of direct interest to the PPO as well as other centers and can be the foundation for follow-on proposals.

FUTURE WORK

Additional work will include continued review of the published scientific literature on the genera associated with the identifications listed in Table 2. Already, these findings lay the groundwork for future research and proposal applications. An area of increasing interest to NASA's PPO is the effect of the space environment on microbes. Specifically, PP is interested in whether the space environment might be able to provide sterilizing effects for spacecraft. To this end, the Space Environmental Effects (SEE) team at MSFC is interested in harnessing the instrumentation available onsite to answer these questions. Of interest is whether microbes from cleanroom environments or associated with spacecraft materials exhibit increased ability to survive simulated space conditions. The microbes identified in this study represent an excellent pool from which to base future experimental plans and space environment exposures. Furthermore, the success of this work lays the foundation for advanced molecular methods, including next generation sequencing and whole genome sequencing to both identify undocumented microbes and assess alterations in genomes due to space environmental simulations.

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