

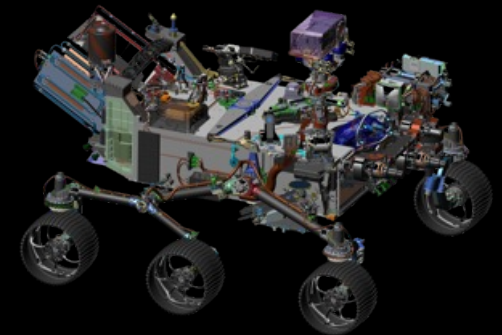
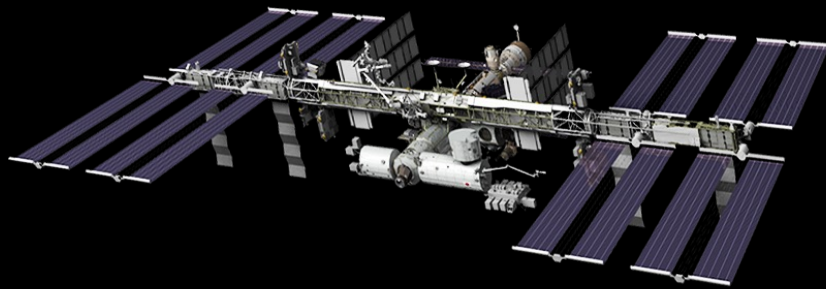
Developments in Planetary Protection Science—Clean-Room Extremophiles, Metagenomics, and Space Stations

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Venkat



George



Alex



Jack



Rob



Shoban



Anna



Annis



Lorraine



Asif



Fumito



Sivapriya



Ugur



Jason



Atul



Fathi



Dani



Rekha



Satish



Steran



Vijay



Phil



Chris

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METAGENOMIC INSIGHTS INTO SPACE MICROBIOME & PLANETARY PROTECTION FOR MOON-TO-MARS EXPLORATION

THE SPACE MICROBIOME (ISS & Spacecraft)

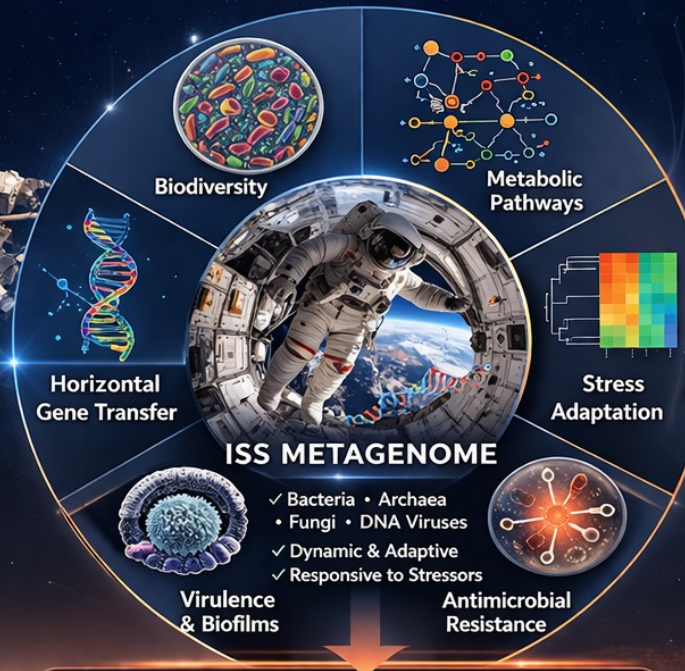


Microbial Diversity in ISS & Spacecraft

- Low Biomass
- Desiccation
- Radiation
- Microgravity
- Oligotrophie

METAGENOMIC & BIOMOLECULAR TOOLS

- Shotgun Metagenomics
- Amplicon Sequencing
- Single-Cell Genomics
- Metatranscriptomics
- Proteomics / Metabolomics
- Culturomics + AI/ML



MOON-TO-MARS: ROLE OF METAGENOMICS



PLANETARY PROTECTION APPLICATIONS

Forward Protection (Earth → Mars)

- Pre-launch Bioburden Assessment
- Cleanroom Monitoring (Sequencing-Based)
- Real-Time Onboard Surveillance
- Sterilization Validation (Biomolecular)

Backward Protection (Mars → Earth)

- Detect Martian Microbial Contamination
- Quarantine & Sample Handling
- Metagenomic Screening of Crew & Habitat
- Biosecurity Decision Support

EXPECTED MICROBIOME CHANGES (2-3 Year Crewed Mars Mission)

Reduced Diversity	Oligotrophic & closed habitat
Selection for Stress-Tolerant Taxa	Spore-formers, radiation-resistant species
Increased ARGs (Antimicrobial Resistance Genes)	Due to antibiotic use & stress
Biofilm-Forming & Surface-Associated Taxa	On habitat walls, air filters, tools
Fungal Increase	<i>Aspergillus</i> , <i>Penicillium</i> , etc.

KEY MESSAGE: "Metagenomics is the Next Frontier for Planetary Protection in the Era of Human Space Exploration"

THE SPACE MICROBIOME (ISS & Spacecraft)



Low
Biomass



Desiccation



Radiation



Microgravity



Oligotrophs

Table 2. Microbial burden of Spacecraft Assembly Facility floors by date.

Sampling Date	Viable microbes* (RLU/m ²) [B1]	Viable microbes* (16s/m ²) [B2]	Cultivable* (CFU/m ²) [C]	Cultivable Spores* (CFU/m ²) [D]	Cultivable ATP % [†] [C/B1] x 100	Cultivable qPCR % [†] [C/B2] x 100	Spore % [†] [D/C] x 100	Spore to VO (ATP) ^{‡§} [B1/D]	Spore to VO (qPCR) [‡] [B2/D]
3/1/16	5.7 x 10 ⁴	1.3 x 10 ⁴	6.9 x 10 ¹	6.4 x 10 ¹	0.7	0.5	55.8	708	206
3/15/16	6.3 x 10 ⁴	2.1 x 10 ⁴	4.8 x 10 ²	3.0 x 10 ¹	1.3	4.9	22.9	1,923	718
3/30/16	1.3 x 10 ⁵	7.4 x 10 ⁴	3.0 x 10 ²	2.4 x 10 ¹	0.6	2.4	17.1	5,261	3,036
4/12/16	9.4 x 10 ³	1.1 x 10 ⁵	9.6 x 10 ²	3.1 x 10 ¹	11.4	1.1	6.9	306	3,484
5/17/16	5.7 x 10 ³	5.1 x 10 ⁴	6.9 x 10 ²	8.3 x 10 ¹	14.5	2.3	15.0	68	618
6/1/16	1.0 x 10 ⁴	2.9 x 10 ⁴	1.1 x 10 ³	4.2 x 10 ¹	11.7	8.6	18.2	241	683
6/14/16	4.3 x 10 ³	2.2 x 10 ⁴	2.2 x 10 ²	2.0 x 10 ¹	7.2	1.7	21.5	211	1,071
6/28/16	6.3 x 10 ³	1.2 x 10 ⁵	1.9 x 10 ²	1.7 x 10 ¹	3.5	0.3	14.6	326	7,043
7/12/16	8.0 x 10 ³	7.8 x 10 ⁴	2.7 x 10 ²	7.2 x 10 ¹	7.2	0.7	40.6	110	1,080
7/26/16	1.5 x 10 ³	2.7 x 10 ⁵	1.0 x 10 ²	2.9 x 10 ¹	9.2	0.04	35.5	52	9,217
8/15/16	3.6 x 10 ³	1.0 x 10 ⁵	4.4 x 10 ²	2.8 x 10 ¹	19.4	0.5	6.2	158	3,866
Average ^{††}	2.8 x 10 ⁴	5.3 x 10 ⁶	4.4 x 10 ²	3.6 x 10 ¹	7.7	2.2	22.9	746	2,176

SAF environmental surface microbial population

Intra-ATP

PMA-qPCR

NSA (non-HS)

NSA (HS)

Viable
microbes*
(RLU/m²)
[B1]

Viable
microbes*
(16s/m²)
[B2]

Cultivable*
(CFU/m²)
[C]

Cultivable
Spores*
(CFU/m²)
[D]

Cultivable
ATP %[†]
[C/B1] x
100

Cultivable
qPCR %[†]
[C/B2] x
100

Spore
%[†]
[D/C]
x 100

2.8 x 10⁴

5.3 x 10⁶

4.4 x 10²

3.6 x 10¹

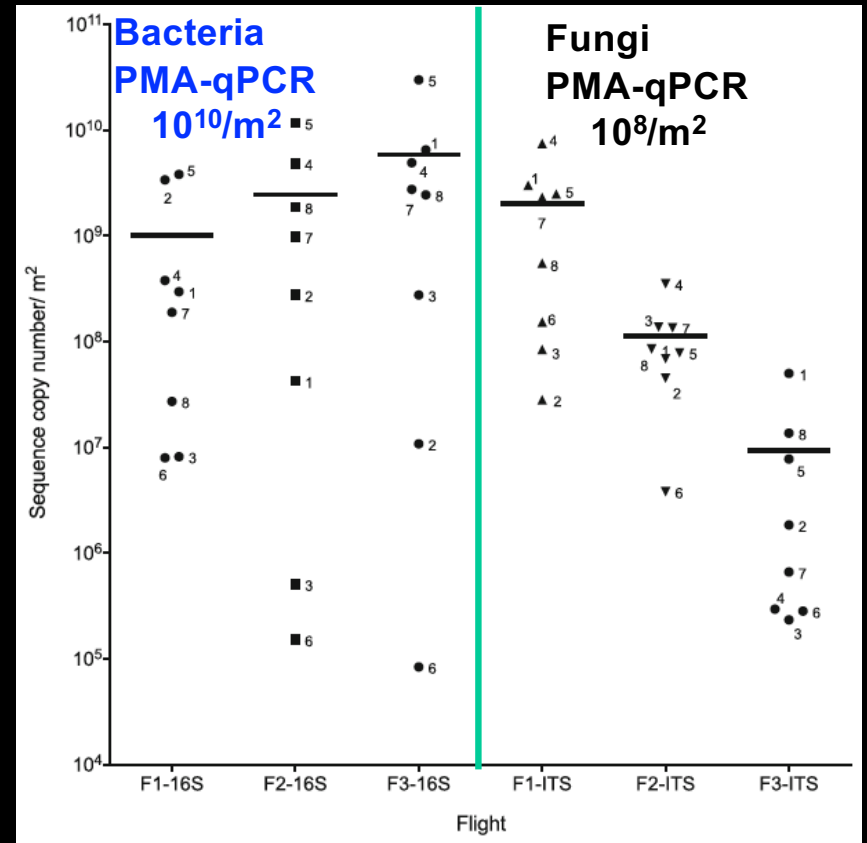
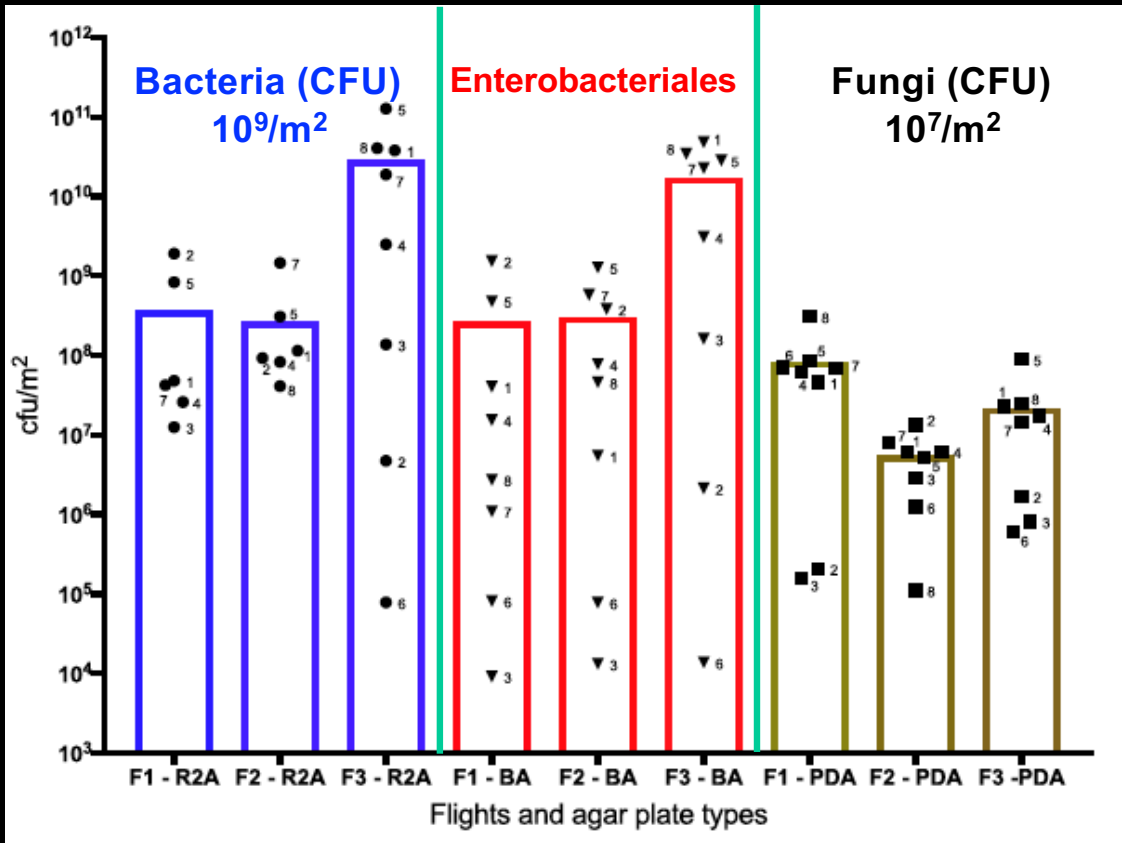
1.6%

0.008%

22.9



ISS environmental surface microbial population (MT-1)



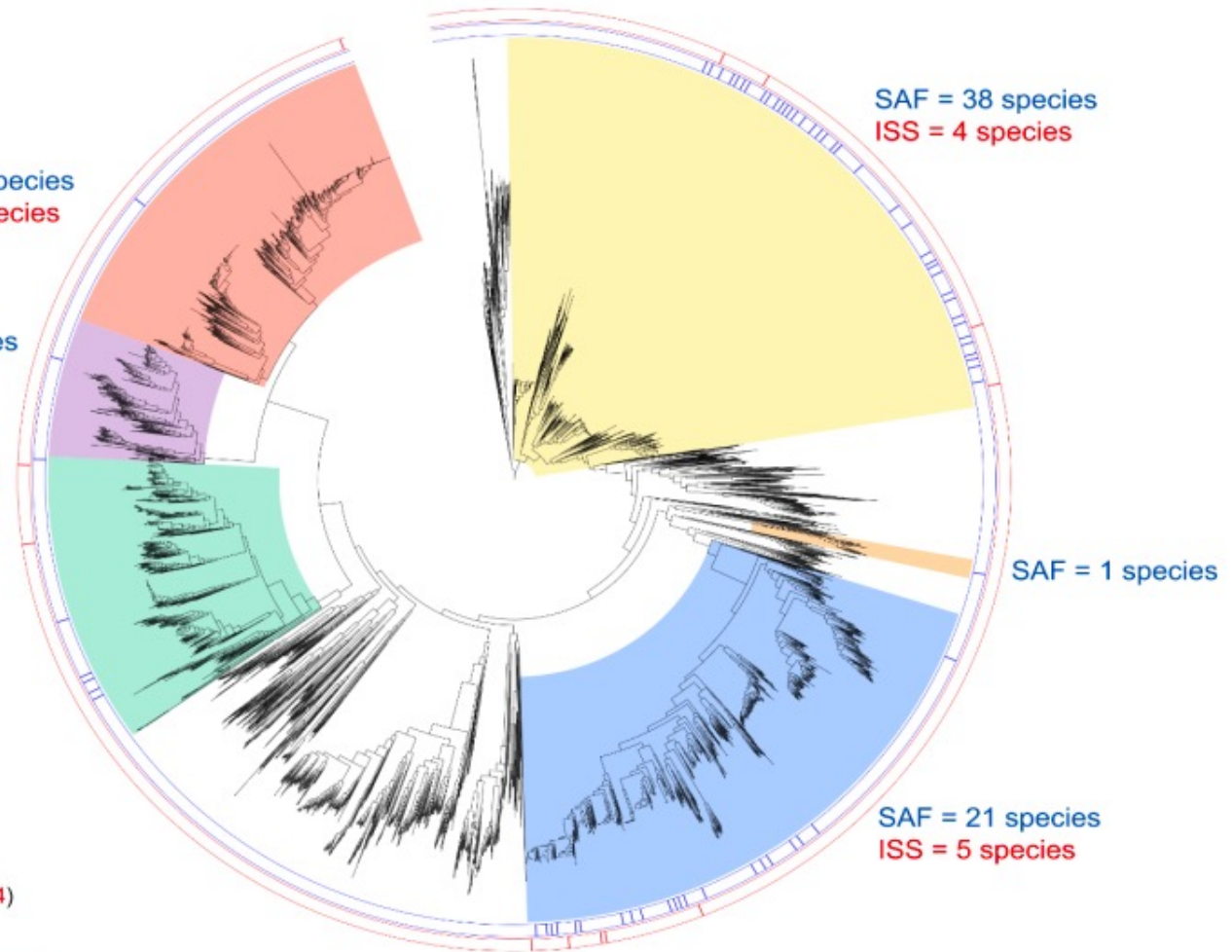
A.

Type strain of Novel Species
and sampling location

- SAF Cleanrooms
- ISS

Classes and number of
nov. sp. genomes (SAF:ISS)

- Bacilli (319; 201)
- Deinococci (1)
- Actinomycetes (89; 29)
- Sphingobacteriia (1)
- Alphaproteobacteria (66; 14)
- Betaproteobacteria (2)
- Gammaproteobacteria (36; 123)



Tree scale: 1

Influence of crew on the microbial diversity

Taxa	WGS strains	SAF	ISS	Novel sp. SAF	Novel sp. ISS
Bacilli	520	319	201	38	4
Deinococci	1	1		1	
Actinobacteria	118	89	29	21	5
Sphingo	1	1			
Alphaproteo	80	66	14	6	2
Betaproteo	2	2		1	
Gammaproteo	159	36	123	1	1
<i>Candidatus</i>	6			6	
Total	887	514	367	74	12
# of species		123	51	60%	24%

Can metagenome capture PP-relevant taxa that are low in abundance?



Microbiology
Spectrum

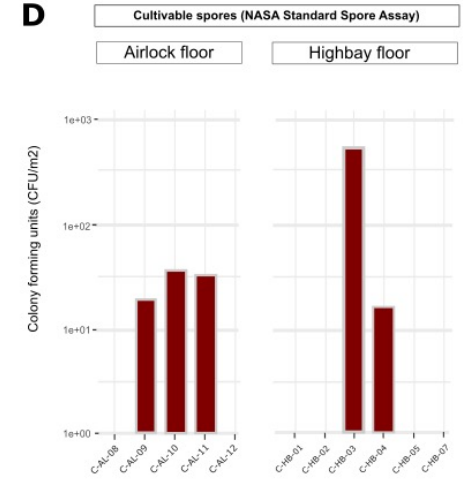
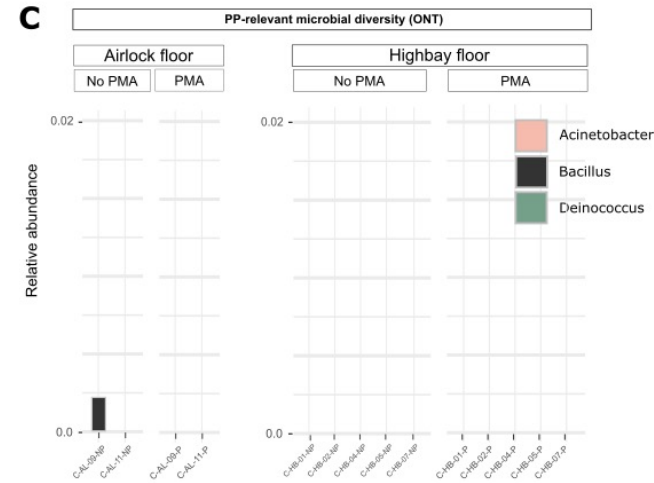
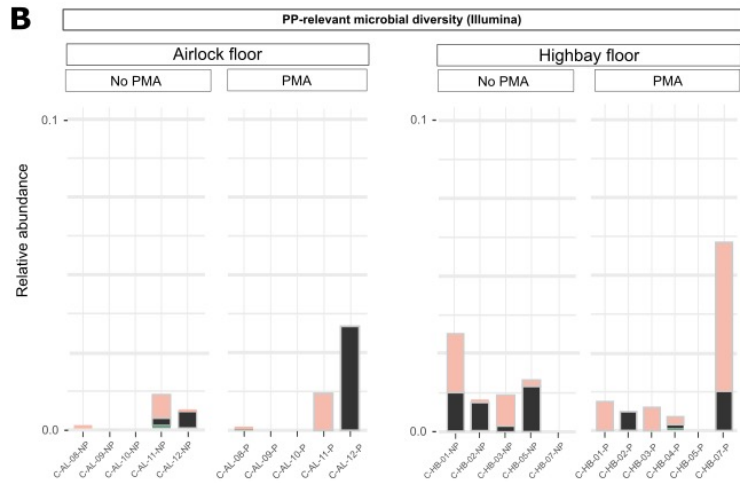
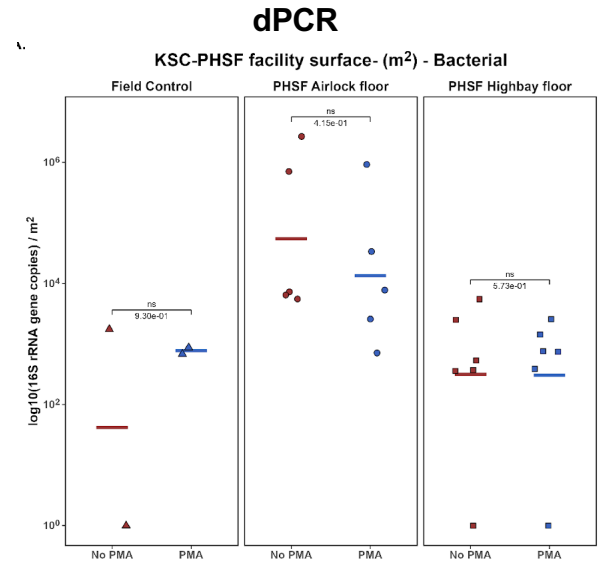
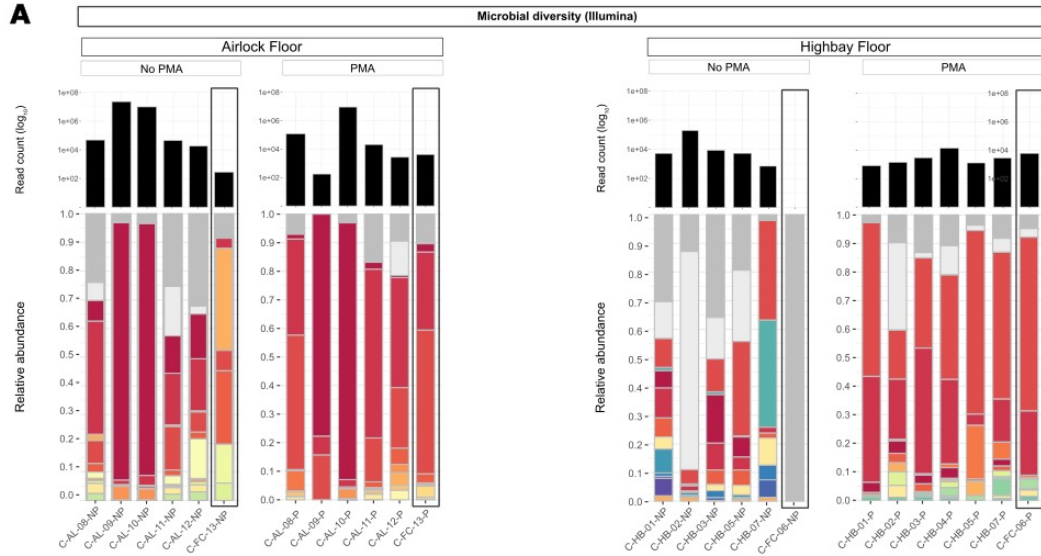


Open Peer Review | Environmental Microbiology | Research Article

Unveiling hidden microbial diversity in Mars 2020 mission assembly cleanroom: molecular insights into the persistence and emergence of novel species defying metagenome sequencing

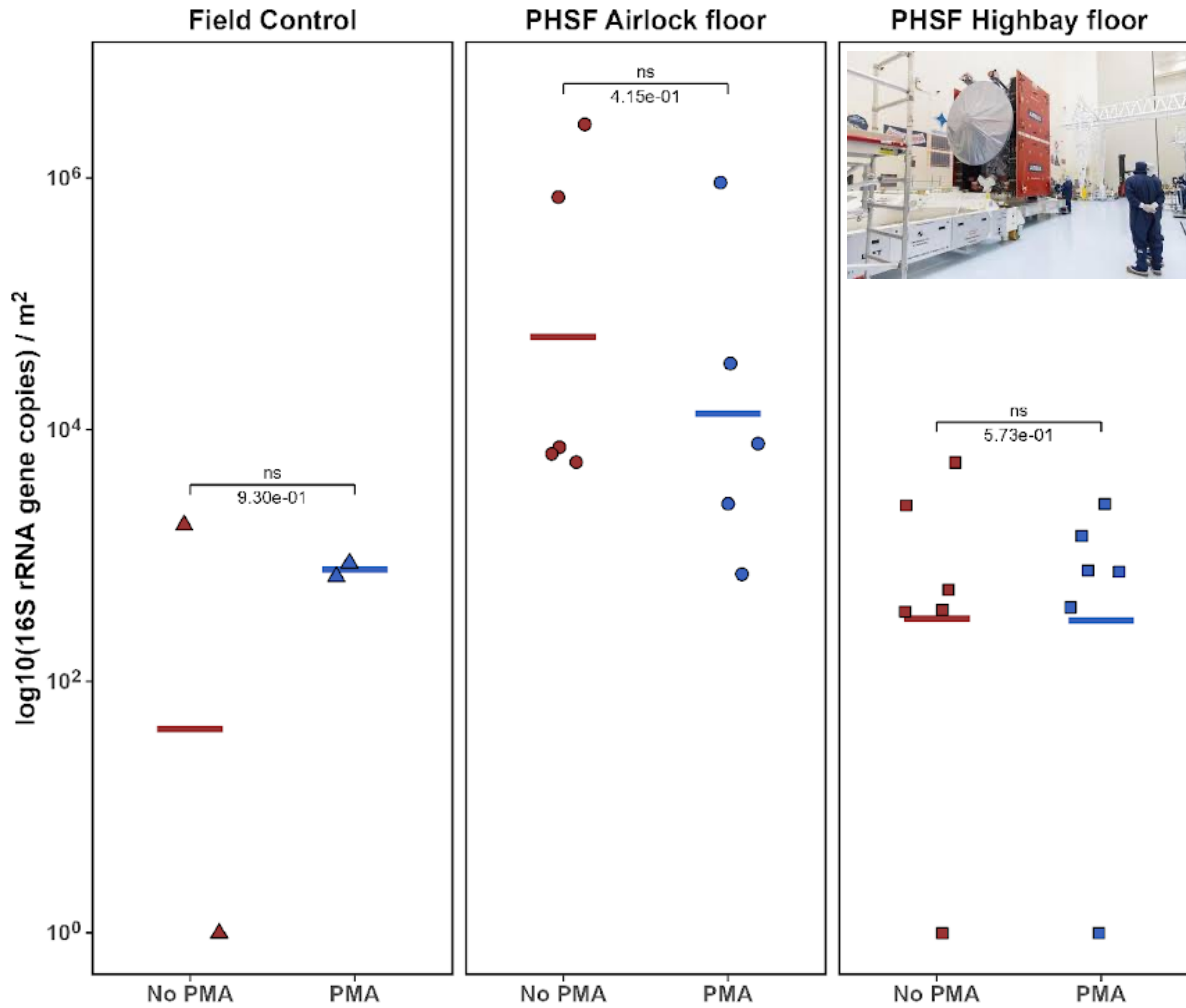
The answer is NO

Shobhan Karthick Muthamilselvi Sivabalan,¹ Varsha Vijayakumar,¹ Pratyay Sengupta,^{1,2,3} Siddhakam Palmal,⁴ Srinivasan Krishnamurthi,⁴ Nitin Kumar Singh,⁵ Nikos C. Kyrpides,^{6,7} Karthik Raman,^{2,3} Kasthuri Venkateswaran⁵

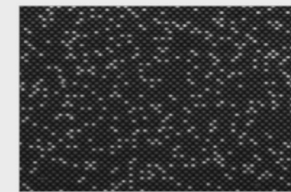


dPCR

KSC-PHSF facility surface- (m²) - Bacterial

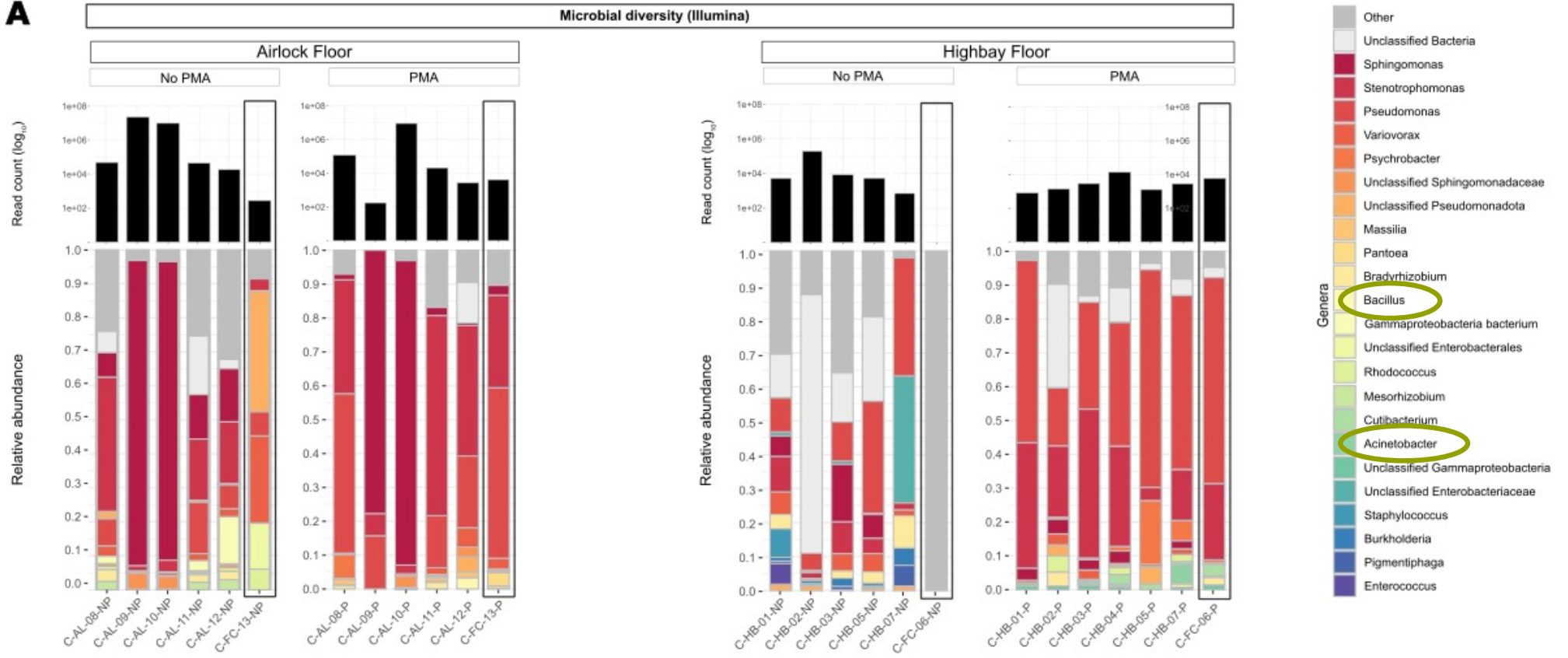


QIAcuity Four Platform



Absolute quantification

- No standard curves
- Higher precision and reproducibility
- Low sensitivity to inhibitors
- End-point detection

A

B**PP-relevant microbial diversity (Illumina)****Airlock floor**

No PMA

PMA

Highbay floor

No PMA

PMA

Relative abundance

0.1

0.1

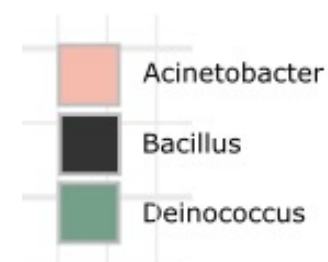
0.0

0.0

Can't get MAGs

C-AL-08-NP
C-AL-09-NP
C-AL-10-NP
C-AL-11-NP
C-AL-12-NP
C-AL-08-P
C-AL-09-P
C-AL-10-P
C-AL-11-P
C-AL-12-P

C-HB-01-NP
C-HB-02-NP
C-HB-03-NP
C-HB-05-NP
C-HB-07-NP
C-HB-01-P
C-HB-02-P
C-HB-03-P
C-HB-04-P
C-HB-05-P
C-HB-07-P



C**PP-relevant microbial diversity (ONT)**

Airlock floor

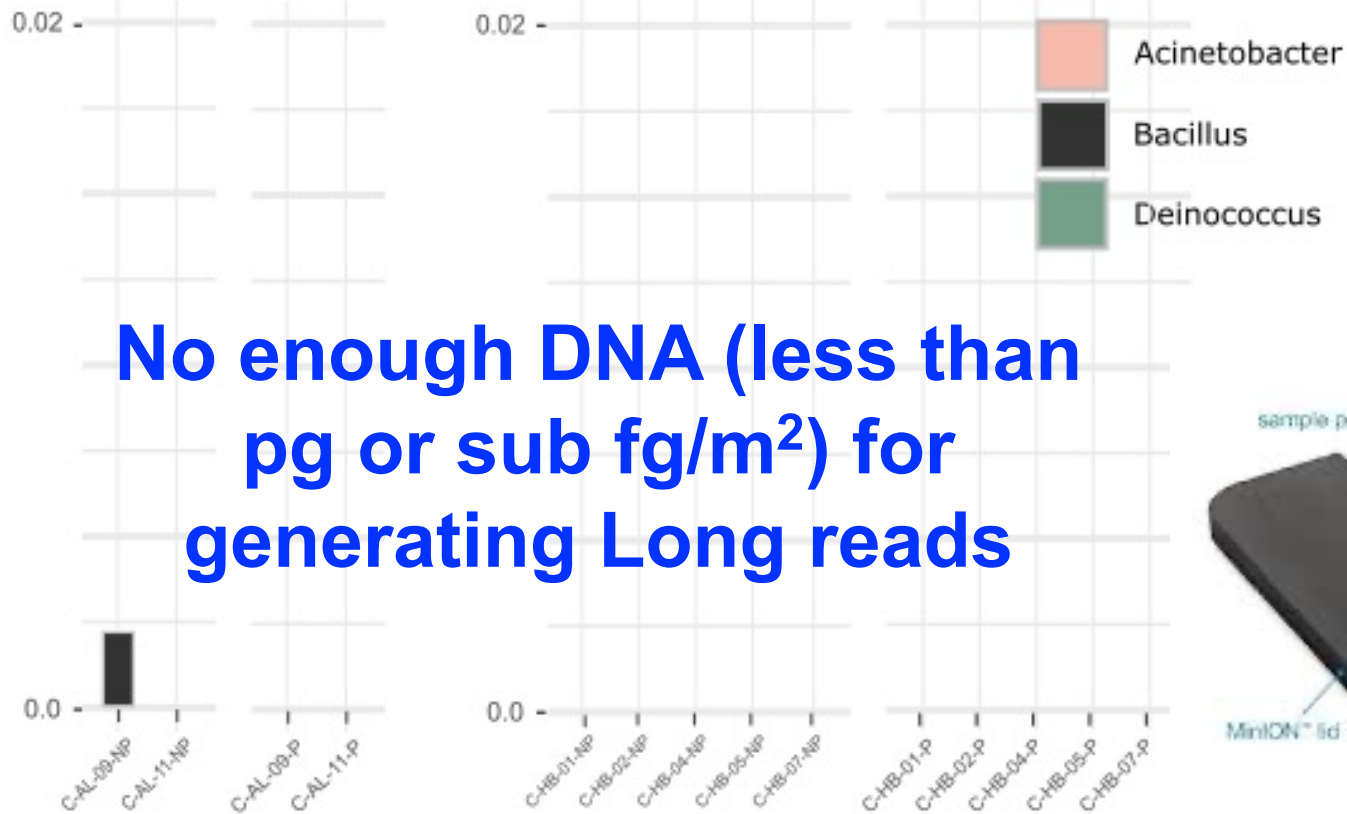
Highbay floor

No PMA

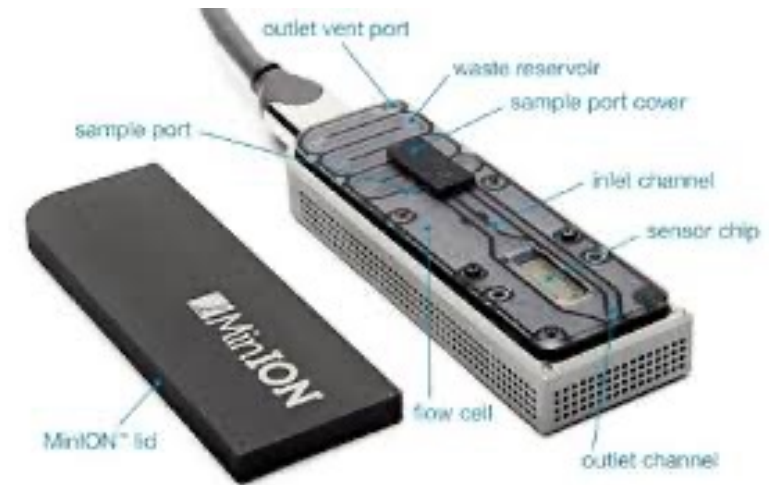
PMA

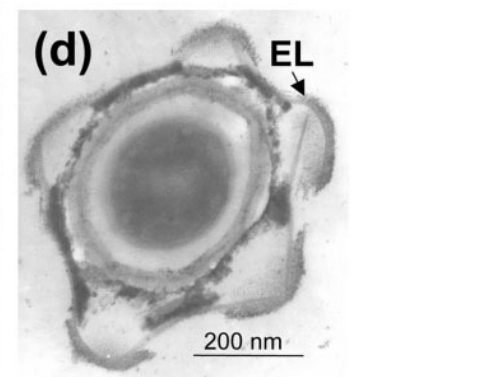
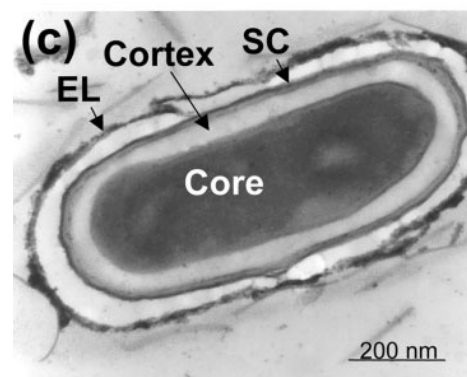
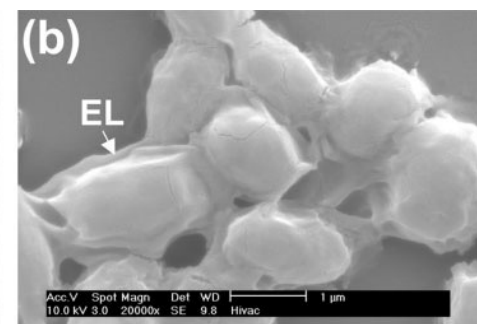
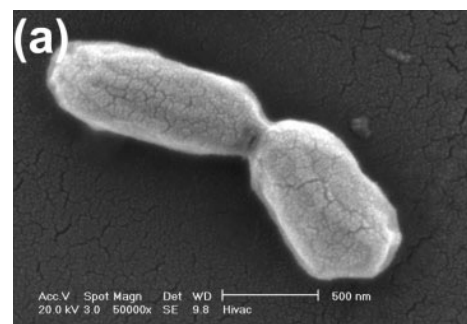
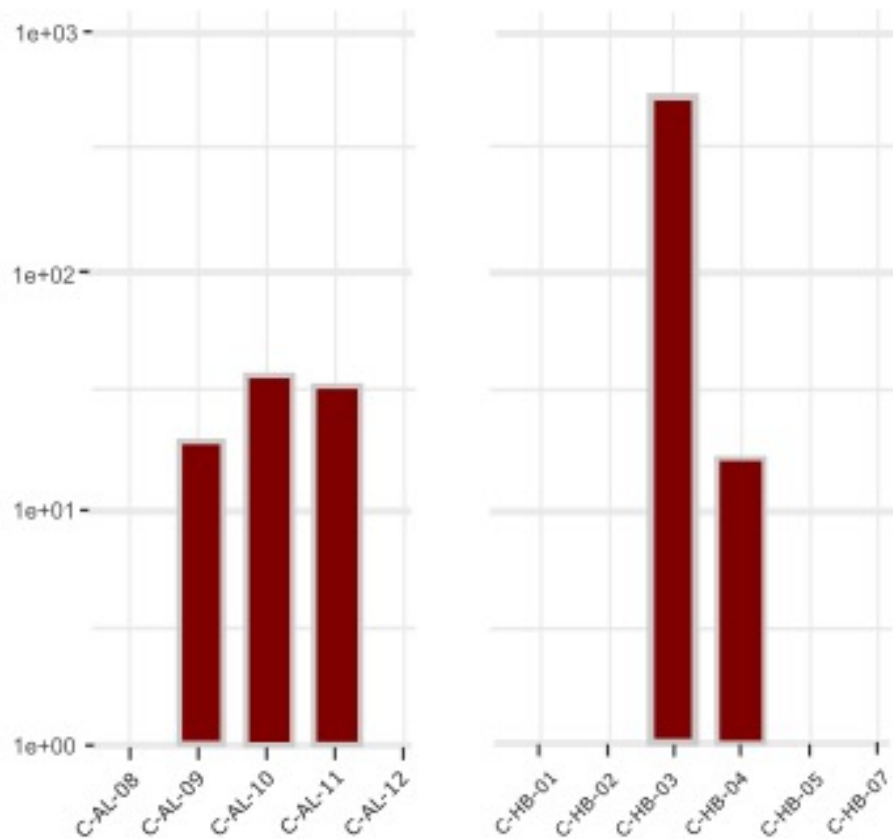
No PMA

PMA



**No enough DNA (less than
pg or sub fg/m²) for
generating Long reads**

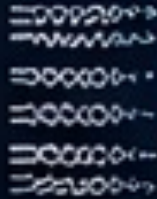


D**Cultivable spores (NASA Standard Spore Assay)****Airlock floor****Highbay floor**Colony forming units (CFU/m²)

METAGENOMIC & BIOMOLECULAR TOOLS



**Shotgun
Metagenomics**



**Amplicon
Sequencing**



**Single-Cell
Genomics**



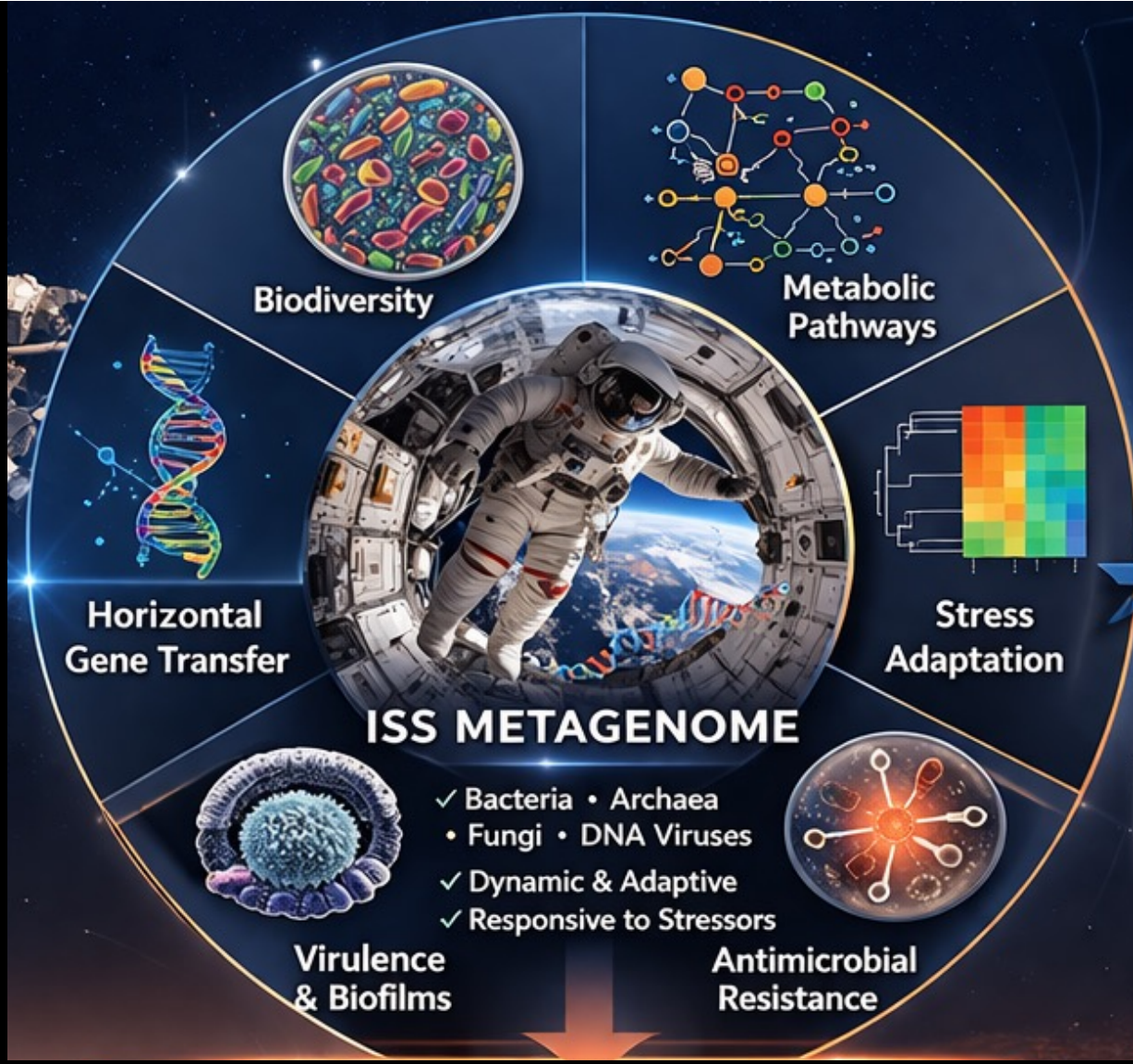
Metatranscriptomics



**Proteomics /
Metabolomics**



**Culturomics
+ AI/ML**

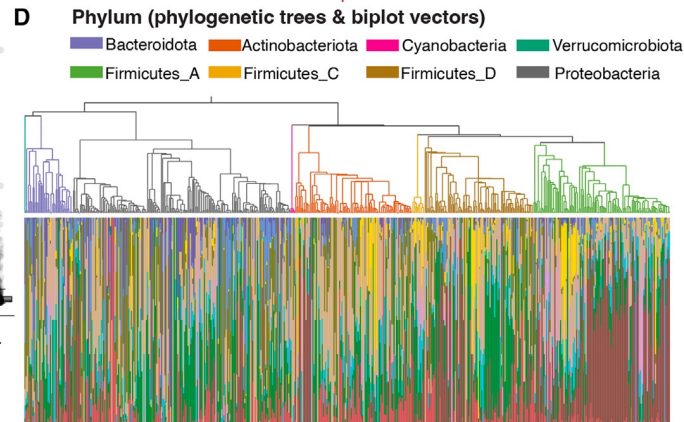
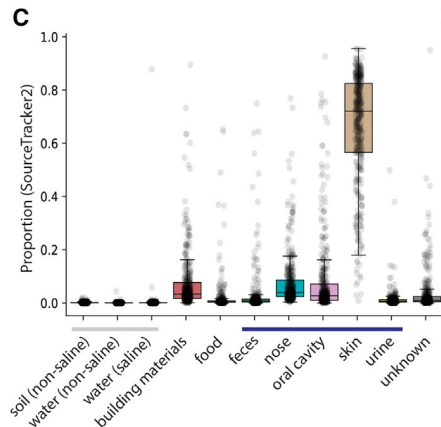
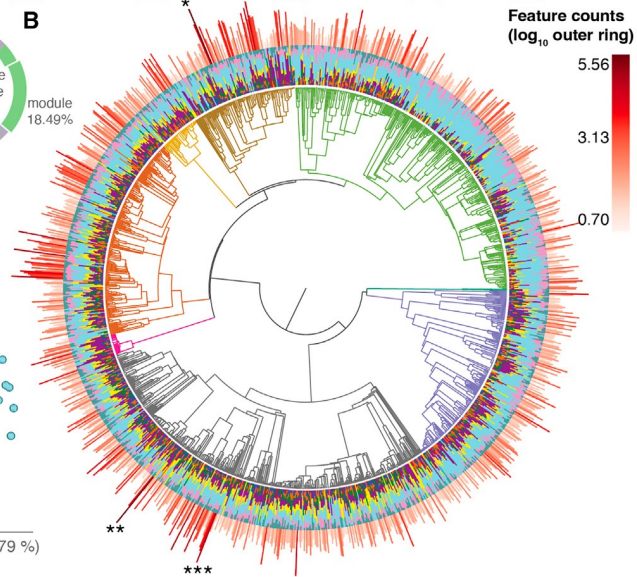
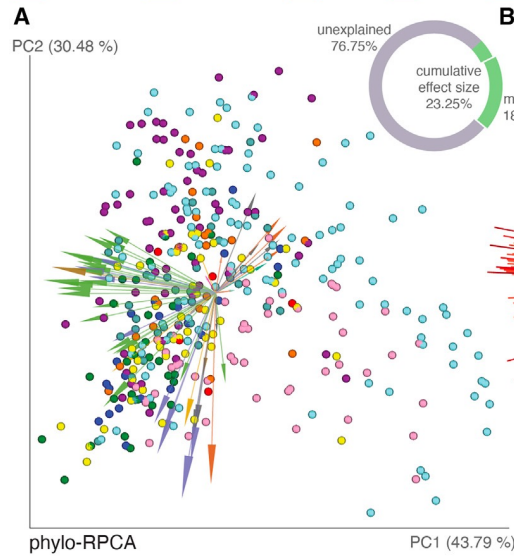
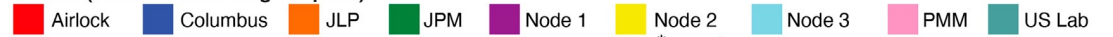




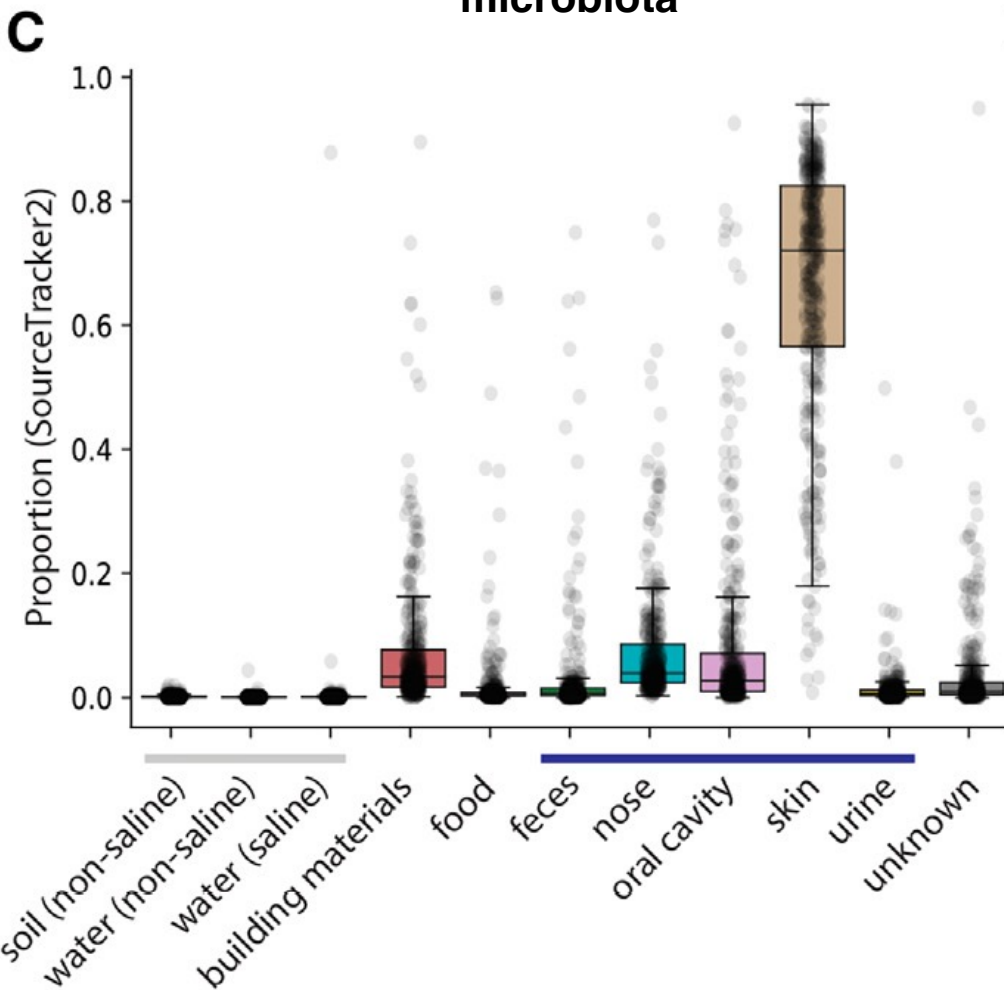
Biodiversity

K. Venkateswaran

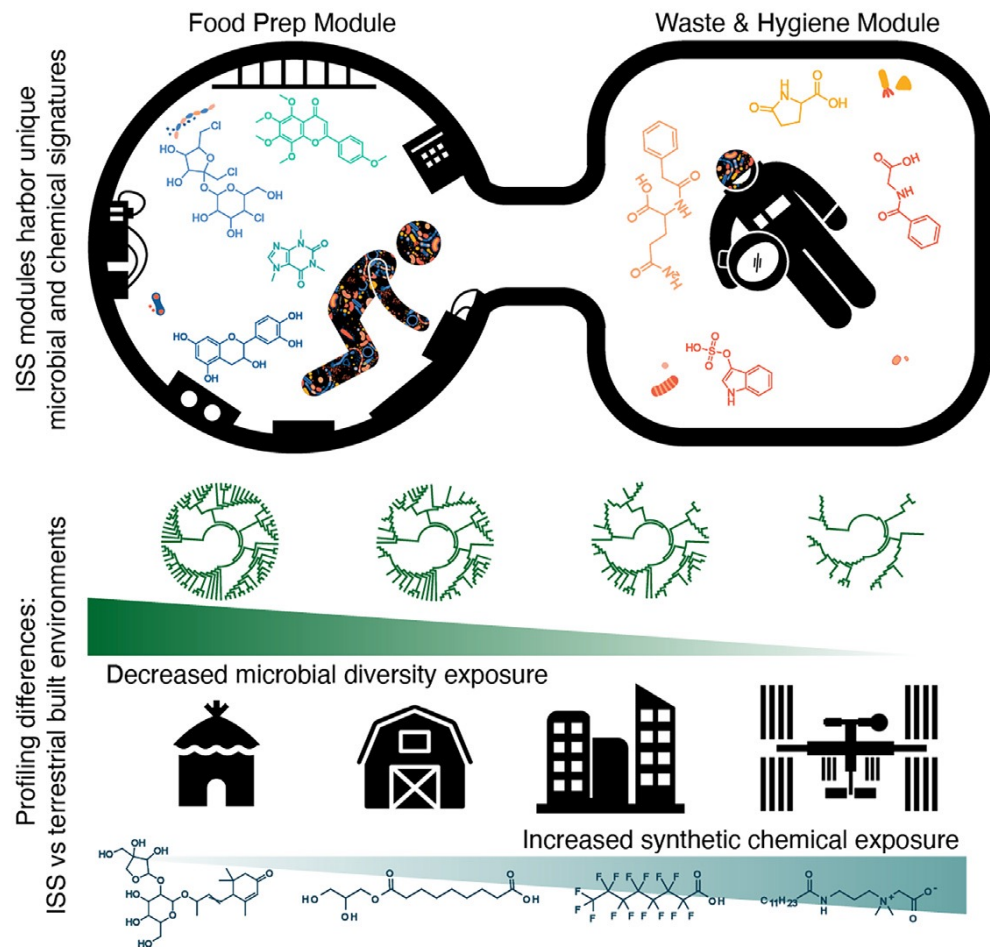
Module (scatter & inner ring bar plots)

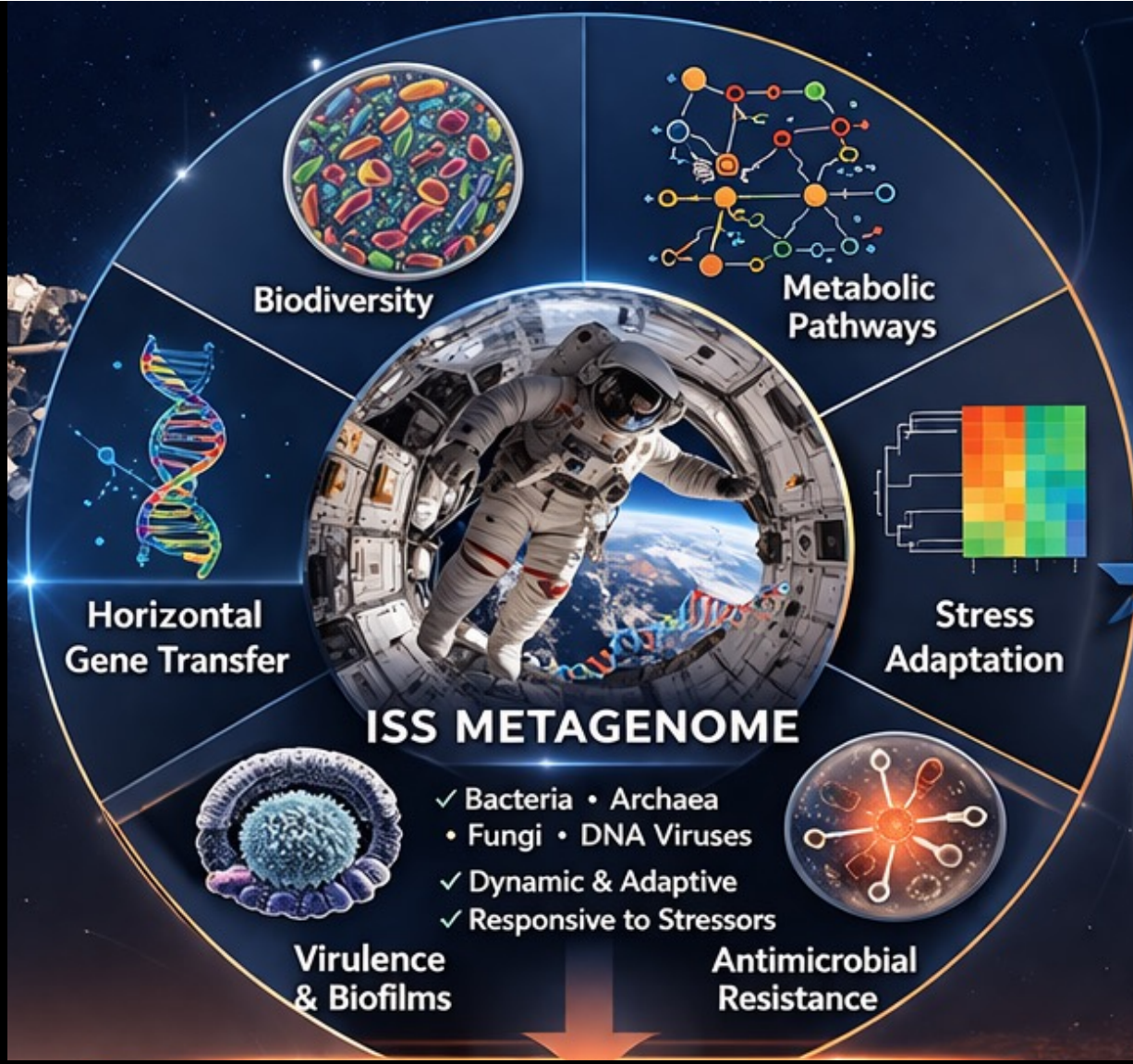


Boxplots of SourceTracker estimates for microbial source contributions to ISS microbiota

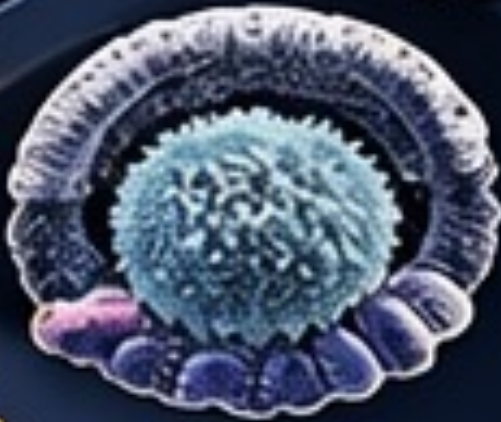


Microbial and chemical characterization of the International Space Station (ISS)





ISS METAGENOME



**Virulence
& Biofilms**

- ✓ Bacteria • Archaea
- Fungi • DNA Viruses
- ✓ Dynamic & Adaptive
- ✓ Responsive to Stressors

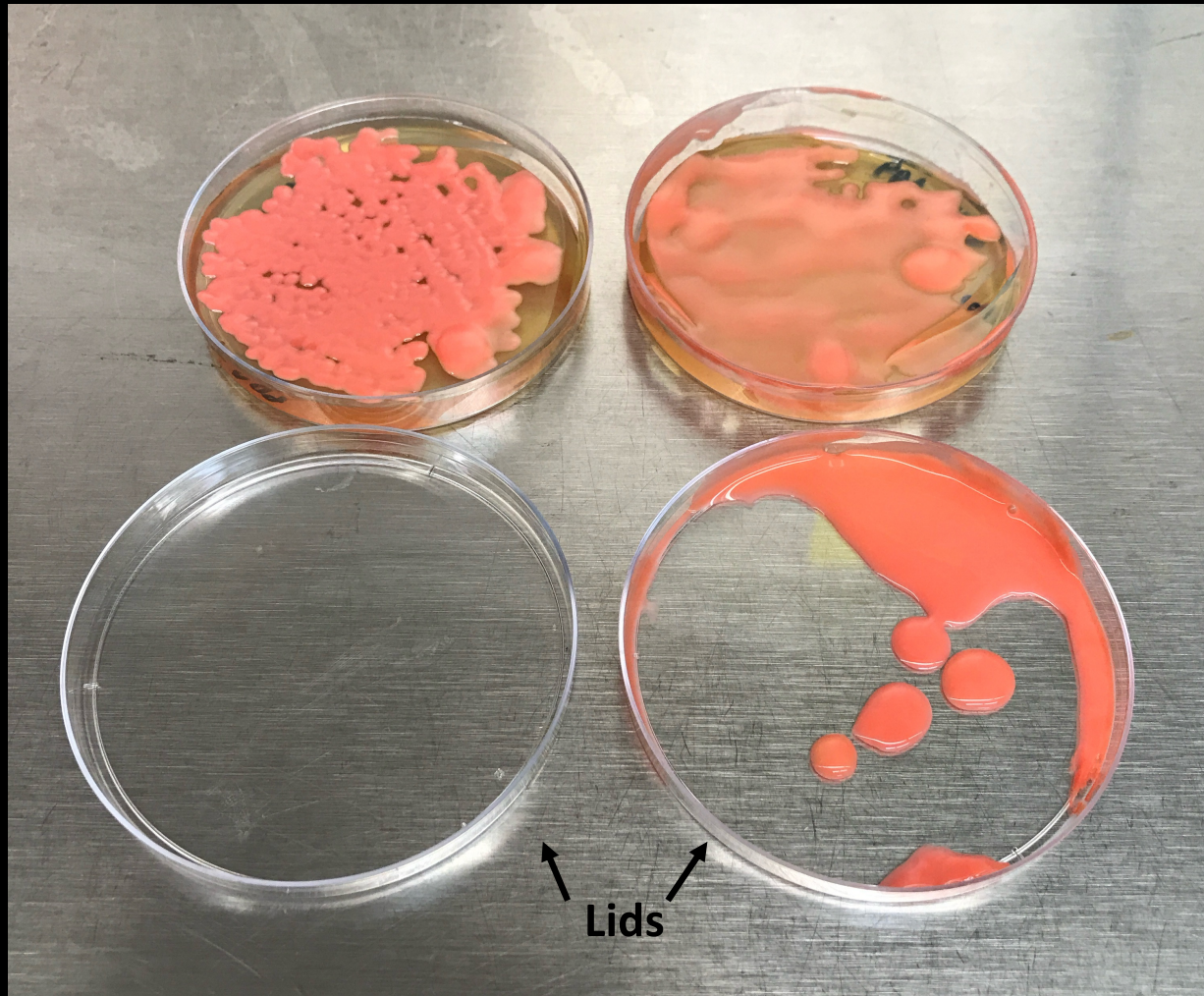


**Antimicrobial
Resistance**

Issues and concerns about biofilm on ISS system

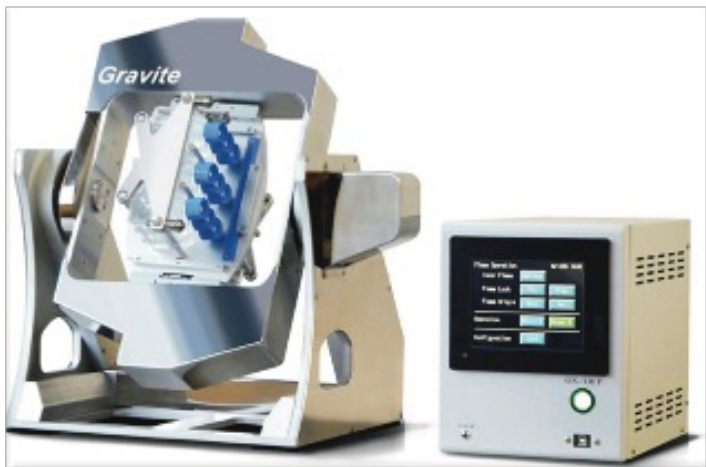


Rhodotorula mucilaginosa Dimorphic Pigmentation



KSC Microgravity Simulator Support Facility (MSSF)

Gravitee for tubes (Ground)



In collaboration with Dr. Ye Zhang and Dr. Jeff Richards from KSC



Rhodotorula mucilaginosa Biofilm formation Under Simulated μ Gravity

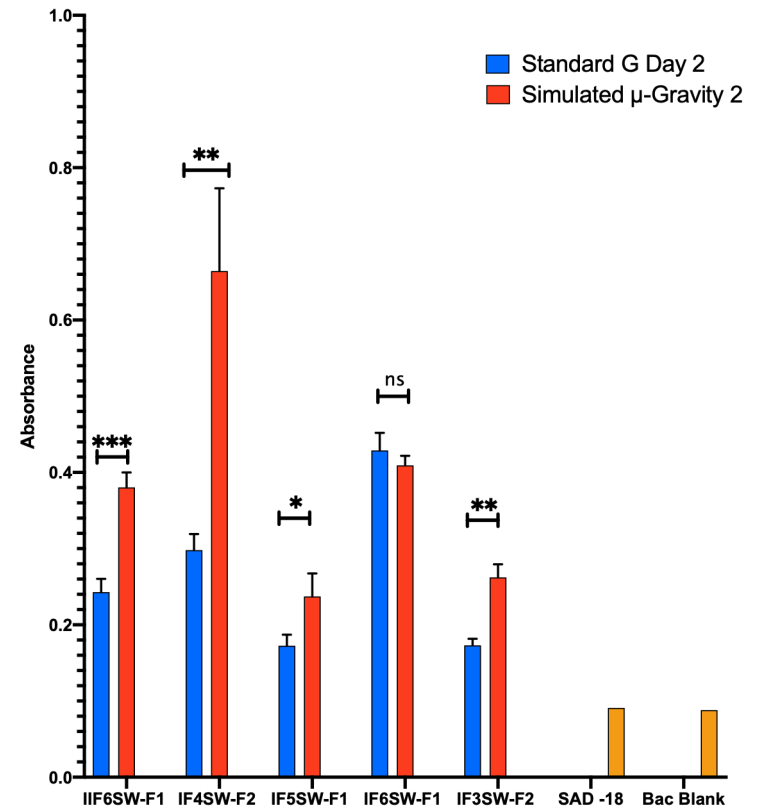
- KSC MSSF allows for simulated μ -gravity on a variety of 2D and 3D simulators (including the 3D Gravite).
- Data is indicating that specific yeast isolates from *R. mucilaginosa* produce larger biofilms when grown in simulated microgravity.

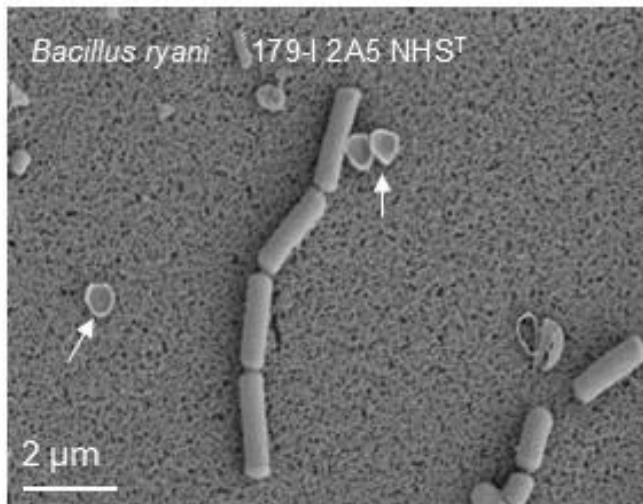
ns = > 0.05

** = 0.0049 - 0.0005

*** = < 0.00049

* = 0.05 - 0.0049



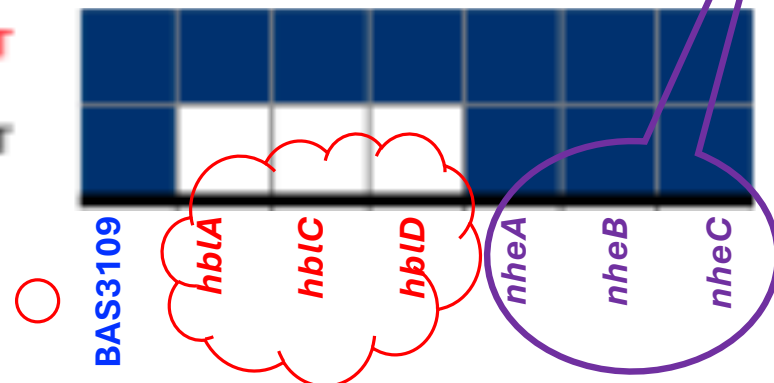


Bacillus ryani 179-I 2A5 NHST

Bacillus luti TD41^T

The *hblACD* genes (*hblA*, *hblC*, *hblD*) are significant because they encode the three-component **hemolysin BL (HBL)**, one of the primary diarrheal enterotoxins produced by the *Bacillus cereus* group, which includes *B. cereus* and *B. thuringiensis*.

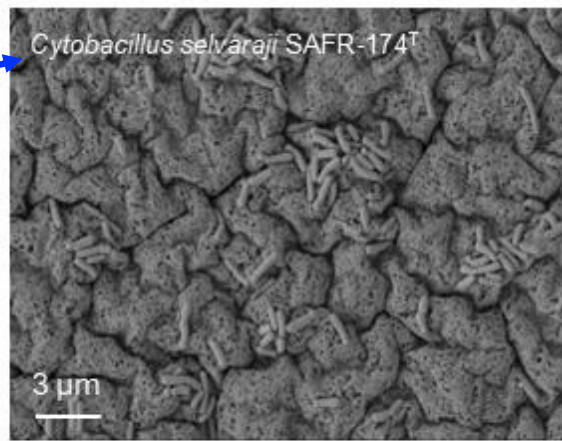
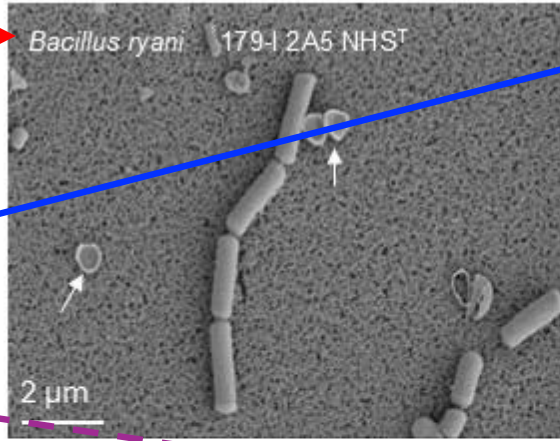
The *nheABC* genes encode the Three-Component Non-Hemolytic Enterotoxin (Nhe), a major virulence factor in *Bacillus cereus* responsible for severe food poisoning and diarrheal syndrome.



The **BAS3109** gene encodes a **thiol-activated cytolyisin** that plays a significant role in the virulence of *Bacillus anthracis*, the bacterium responsible for anthrax.

Stress response genes (clp proteases)

- Bacillus ryani* 179-I 2A5 NHST^T
- Bacillus luti* TD41^T
- Sphingomonas jepii* 179-A 2A2 NHST^T
- Sphingomonas panaciterrae* KCTC 42346^T
- Sphingomonas kannani* SAFR-052^T
- Sphingomonas lacusdianchi* JXJ CY 53^T
- Rhizobium mayilrajii* SAFR-030^T
- Rhizobium rhizoryzae* DSM 29514^T
- Pseudobacillus manavalani* 179-B 2D1 NHST^T
- Pseudobacillus wudalianchiensis* FJAT-27215^T
- Cytobacillus selvaraji* SAFR-174^T
- Cytobacillus firmus* NCTC10335^T
- Dietzia jepii* 179-F 9C3 NHST^T
- Dietzia psychrocaliphila* ILA 1^T
- Arthrobacter hugenholzii* SAFR-179^T
- Arthrobacter woykaea* SAFR-044^T
- Arthrobacter liuii* CGMCC 1 12778^T
- Arthrobacter kypidesii* SAFR-014^T
- Arthrobacter ipis* IA7^T
- Paenibacillus sekari* SAF-068^T
- Paenibacillus xylanexedens* DSM 21292^T
- Paenibacillus sivalingami* SAF-054^T
- Paenibacillus chibensis* NBRC 15958^T
- Ureibacillus jepii* 179-F W5.1 NHST^T
- Ureibacillus galli* Re31^T
- Ureibacillus foxi* SAFN-010a^T
- Ureibacillus acetophenoni* JC23^T
- Curtobacterium jepii* 179-B 9B NHST^T
- Curtobacterium subtropicum* RHC-IP20^T
- Curtobacterium pasadenensis* SAFR-003^T
- Curtobacterium citreum* DSM 20528^T
- Candidatus Brevibacillus galwayanus* SAFN-007a^T
- Brevibacillus gelatini* DSM 100115^T
- Candidatus Ferdinandocohnia californiensis* SAFN-114^T
- Ferdinandocohnia onubensis* 0911MAR22V3^T
- Candidatus Pedobacter galwayanus* SAFR-022^T
- Pedobacter quisquiliarum* CGMCC 1 15343^T
- Candidatus Solibacillus galwayanus* SAF-065^T
- Solibacillus ferritrahens* YIM B08730^T
- Candidatus Sporosarcina californiensis* SAFN-010^T
- Sporosarcina jeotgali* B20-11^T
- Candidatus Sporosarcina galwayana* SAFN-015^T
- Sporosarcina koreensis* KACC 1129^T



Clp proteases are crucial, ATP-dependent protein-degrading molecular machines that maintain protein homeostasis by removing misfolded or damaged proteins during environmental stress. They function as essential survival mechanisms against heat, oxidative, and osmotic stress, while regulating virulence and biofilm formation in bacteria.

MOON-TO-MARS: ROLE OF METAGENOMICS



Baseline Microbiome

Pre-Launch
(Astronauts & Spacecraft)



In-Transit Changes

(Months)



Mars Surface / Habitat

(Years)



Return to Earth

(Monitoring & Quarantine)

PLANETARY PROTECTION APPLICATIONS

Forward Protection (Earth → Mars)



Pre-launch Bioburden
• Assessment



Cleanroom Monitoring
(Sequencing-Based)



Real-Time Onboard
Surveillance



Sterilization Validation
(Biomolecular)

Backward Protection (Mars → Earth)



Detect Earth-based but
modified microbial
signatures (HGT/SNP...)



Quarantine &
Sample Handling








Metagenomic Screening
of Crew & Habitat



Biosecurity Decision
Support

EXPECTED MICROBIOME CHANGES (2–3 Year Crewed Mars Mission)

	Reduced Diversity	Oligotrophic & closed habitat
	Selection for Stress-Tolerant Taxa	Spore-formers, radiation-resistant species
	Increased ARGs (Antimicrobial Resistance Genes)	Due to antibiotic use & stress
	Biofilm-Forming & Surface-Associated Taxa	On habitat walls, air filters, tools
	Fungal Increase	<i>Aspergillus, Penicillium, etc.</i>

How can metagenomics and other biomolecular techniques be incorporated effectively into planning for forward and backward planetary protection during human exploration of Mars?

- Metagenomics and related biomolecular methods should be incorporated as part of a **multi-layer monitoring architecture** spanning pre-launch, transit, surface operations, sample handling, and Earth return.
- They are most valuable when used not as a stand-alone technology, but as part of a decision system that links **who is there, what they can do, whether they are viable, where they are moving, and what operational risk they pose.**

Is it time for biomolecular data to replace the spore standard for evaluation of biological cleanliness of robotic and crewed spacecraft?

- Not yet as a full replacement—but yes as the basis for the next-generation standard.
- The spore assay is too narrow to represent the real biological burden of modern spacecraft, but biomolecular methods are not yet standardized enough, by themselves, to serve as the only compliance metric across agencies and mission classes.

Are metagenomic studies of orbiting and landed lunar spacecraft a critical part of the Moon-to-Mars architecture for planetary protection?

- Yes—primarily as an operational testbed, not because the Moon poses the same biological risk as Mars.
- The Moon is not Mars, but cislunar and lunar missions are the most realistic place to validate the tools, workflows, contamination models, and crew-operational protocols that human Mars planetary protection will require.

Assuming a duration of two to three years for the first crewed mission to Mars, what changes can we anticipate in the microbiota of crewed spacecraft between departure from and return to Earth?

- We should expect the spacecraft microbiota to become more human-associated, more habitat-selected, more spatially differentiated, and functionally enriched for stress survival, biofilm formation, and antimicrobial resistance determinants, even if total diversity does not collapse completely.
- That expectation is based on ISS and astronaut longitudinal studies, though a Mars mission would likely amplify some trends because of longer duration, tighter closure, radiation exposure, constrained resupply, and repeated habitat reuse.

Developments in Planetary Protection Science—Clean-Room Extremophiles, Metagenomics, and Space Stations

1. Metagenomics should be integrated now into Mars PP planning, but as part of a layered framework with dPCR, viability assays, cultivation, and transport modeling.
2. The spore standard should no longer be stand alone, but biomolecular data are not yet mature enough to be the sole compliance standard.
3. Lunar and cislunar missions are essential rehearsal space for Moon-to-Mars planetary-protection metagenomics.
4. Mars transit and surface missions will drive microbiome succession, making longitudinal molecular surveillance a mission requirement, not a research luxury.

Key Message

**Metagenomics is the Next Frontier
for Planetary Protection in the Era
of Human Space Exploration**