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RESEARCH ARTICLE

Abundance, isolation and characterization of salinotolerant bacteria in a spacecraft assembly facility

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Abstract

Spacecraft assembly facilities (SAFs) house clean rooms where interplanetary spacecraft are built, thereby reducing the bioburden on spacecraft to protect planetary environments from terrestrial microbes that may interfere with the search for life or disturb potential native ecosystems. The most plausible environments for living systems on celestial bodies involve brines with depressed freezing points. Here, we specifically measure the abundance of salinotolerant microbes on SAF surfaces. Most probable number analyses performed with salty liquid media were applied to washes of SAF floor wipes. Microbial abundance was measured using Salt Plains medium at low salt or supplemented with (all w/v) 10% NaCl (1.7 M; $a_w = 0.92$), 50% $MgSO_4$ (2.0 M as epsomite; $a_w = 0.94$), 5% $NaClO_3$ (0.5 M; $a_w = 0.98$), or 5% $NaClO_4$ (0.4 M; $a_w = 0.98$). The abundance of salinotolerant microbes was generally 1 to 10% (10^2 to 10^4 cells m^{-2}) of the total population of microbes observed in low-salt medium (10^5 cells m^{-2}). Microbes were isolated by repetitive streak-plating of positive enrichment cultures and then characterized. All of the 38 isolates were Gram-positive bacteria, mainly spore-forming Bacillaceae, with some *Staphylococcus*. The isolate collection showed strong tolerance to high concentrations of NaCl (to 30%), $MgSO_4$ (to 50%) and sucrose (to 70%). There also was substantial tolerance to pH (5 to 10) and temperature (4 to 60 °C). Taken together, these SAF isolates are polyextremophiles that are in substantial abundance in the clean rooms where spacecraft are assembled.

Contents

Introduction

Spacecraft assembly facilities (SAFs) are clean rooms with stringent protocols to prevent dust and microbes from contaminating robotic probes as much as possible. The microbes found in SAFs are the most likely to be carried to celestial bodies, having the potential for contamination that may confound the search for life (Favero *et al.*, 1966; Favero, 1971; Puleo *et al.*, 1977). Diverse assemblages of microbes have been detected in SAFs and described through cultivation campaigns and molecular genetic analyses (Favero *et al.*, 1966; Favero, 1971; Foster and Winans 1975; Puleo *et al.*, 1977; Moissl *et al.*, 2008; Probst *et al.*, 2010; La Duc *et al.*, 2009; Stieglmeier *et al.*, 2009; Ghosh *et al.*, 2010; Bashir *et al.*, 2016; Hendrickson *et al.*, 2017, 2021; Probst and Vaishampayan 2020; Danko *et al.*, 2021; Carte *et al.*, 2024a,b). The SAF bacterial assemblage has been reported to be rich in *Arthrobacter, Bacillus, Exiguobacterium, Filibacter, Oceanobacillus, Sporosarcina*, *Staphylococcus* and *Streptococcus*, all associated with the human microbiome or soils (La Duc *et al.*, 2003, 2009, 2012; Link *et al.*, 2003; Venkateswaran *et al.*, 2003; Kempf *et al.*, 2005; Satomi *et al.*, 2006). Greater diversity is apparent in genetic libraries, including anaerobes that are not typically cultivated (Moissl *et al.*, 2008; Stieglmeier *et al.*, 2009; La Duc *et al.*, 2014; Carte *et al.*, 2024a). Actinobacteria, Firmicutes and *Gammaproteobacteria* were observed, including the genera *Bacillus, Clostridium, Enterococcus, Paenibacillus* and *Staphylococcus*.

Clean rooms apply selective pressures on microbial assemblages, with low humidity, low nutrient availability and extremely low total biomass. The dry conditions in clean rooms may enrich the microbial assemblage for salinotolerant microbes, since aerosols when dried can leave salt evaporites. Bacterial isolates from SAFs have been shown to be salinotolerant, with substantial radiation resistance and tolerance to oxidants (Venkateswaran *et al.*, 2001, 2003, 2014; La Duc *et al.*, 2003; Link *et al.*, 2003; Kempf *et al.*, 2005; Smith *et al.*, 2017; Zanmuto *et al.*, 2018; Carte *et al.*, 2024a). Long-term enrichment cultures at high salinity resulted in a diverse bacterial community from SAF wipes that included representatives of each biogeochemical functional guild required for the C, N and S cycles (Carte *et al.*, 2024a). Salinotolerant microbial communities, capable of biogeochemical cycling, are more likely to persist on celestial bodies than individual polyextremophile isolates. This becomes important in the context of the chemical conditions on Mars. All of the most attractive environments to search for life on Mars are (potentially) hypersaline, including ices and their brine channels, caves, subsurface and evaporite minerals (Carrier *et al.*, 2020). Environments on ocean worlds also may be salty, from their oceans to briny sills in their icy crusts, to evaporite deposits on their surfaces. There is practical value in understanding the salinotolerant microbial assemblages in SAFs, which are most likely to be transported to the hypersaline environments of solar system bodies.

The current study collected wipe samples of SAF surfaces to measure the abundance of viable salinotolerant microbes using most probable number (MPN) analyses. Enrichment cultures at high salinity were used to isolate microbes from the SAF wipes. Only Gram-positive bacteria were recovered, and these were characterized with respect to their growth tolerances to NaCl, $MgSO_4$ and sucrose at high concentrations and their range of growth tolerances to pH and temperature.

Methods

Sampling of SAFs

Sterile polyester wipes (Texwipe; Kernersville, NC), moistened with 15 ml of sterile water, were used to swab 1-m^2 surfaces of high-traffic floors of the NASA Jet Propulsion Laboratory (JPL) Aseptic Assembly Facility or the main assembly bay, certified ISO 5 clean rooms. All entrants into the ISO 5 clean rooms donned sterile gowning and gloves. The environment was monitored for biological cleanliness by surface sampling, air sampling and utilization of an instantaneous detection system for airborne particles (microbial and inert).

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Several wipe samples and a procedural blank were taken using fresh pairs of sterile gloves. The wipes were packaged in sterile polypropylene tubes with screw caps and shipped overnight in a cool container from JPL to Wichita State University. Upon arrival, the wipes were wetted with 30 ml of a sterile chaotropic solution (0.1% Na pyrophosphate) to dislodge microbes. After 10 min, the liquid was squeezed from the wipes in the tubes with a sterile syringe plunger.

Abundance of salinotolerant microbes by MPN

Liquids from SAF wipe samples were used directly to inoculate tubes for most probable number (MPN) analyses. The arrays were designed with five repetitions of six fivefold or tenfold serial dilutions in 13 × 100-mm culture tubes with a volume of 2 ml and then maintained on an orbital shaker (150 rpm) at room temperature. MPN analyses were performed in Salt Plains (SP) medium containing (per liter): NaCl, 1 g; KCl, 2.0 g; $MgSO_4 \cdot 7H_2O$, 1.0 g; $CaCl_2 \cdot 2H_2O$, 0.36 g; NaBr, 0.23 g; $FeCl_3 \cdot 6H_2O$, 1.0 mg; trace minerals, 0.5 ml; yeast extract, 10.0 g; tryptone, 5.0 g; glucose, 1.0 g; and brought to a final pH of 7.0 (Caton *et al.*, 2004), unsupplemented or supplemented (all w/v) with 10% NaCl (1.7 M; $a_w = 0.92$), 50% $MgSO_4$ (2.0 M as epsomite; $a_w = 0.94$), 5% $NaClO_3$ (0.5 M; $a_w = 0.98$), or 5% $NaClO_4$ (0.4 M; $a_w = 0.98$). Positive tubes were scored after 2 wk, visually or by measuring turbidity at 600 nm using a Genesys 10S spectrophotometer (ThermoFisher). A threshold value of 0.2 OD units was used to score positive growth. Scores were compared to a statistical table to determine the MPN and the 95% confidence interval factor was 3.3 (Woomer 1994).

Bacterial isolation and characterization

Positive MPN cultures containing 50% $MgSO_4$ were spread plated on SP medium supplemented with 25% $MgSO_4$. Colonies were haphazardly collected and re-streaked on SP medium supplemented with 10% NaCl, selecting isolated colonies, six times to purify bacterial strains. The isolates were maintained on SP medium supplemented with 10% NaCl and their physiology and biochemistry were characterized.

Gram stain was performed using Harleco reagents (Sigma-Aldrich) following the manufacturer's instructions. The endospore stain (Thermo Scientific) was performed following the manufacturer's instructions. The presence of catalase was determined by applying 3% hydrogen peroxide solution to smears of culture on microscope slides. The presence of oxidase was determined using DrySlides (BBL). Starch agar plates (Difco) were inoculated and incubated at 37 °C and then flooded with iodine solution once grown to observe hydrolysis by amylase.

Solute tolerance was measured in SP medium supplemented with various concentrations (all w/v) of NaCl (1, 10, 20 and 30%), $MgSO_4$ (20, 30, 40 and 50%) and sucrose (30, 50 and 70%). Shake-tubes (2 ml in 13 × 100-mm tubes) were lightly inoculated (to below 0.05 OD units at 600 nm) and incubated at room temperature for 2 wk. Growth was measured by absorbance spectrophotometry at 600 nm using a Genesys 10S instrument (ThermoFisher) at 1, 3, 7 and 14 d after inoculation. Similarly, growth was measured in shake-tubes using SP medium with 10% NaCl at various pHs (4 to 9) and temperatures (4 to 60 °C). The threshold for positive growth was 0.2 OD units.

DNA extraction and molecular analyses

Crude DNA extracts were made from aliquots (6 ml) of a liquid culture of each isolate using a freeze-thaw technique (Caton *et al.*, 2004). Cells were collected by serial microcentrifugation for 5 min at 14,000 × *g*. Pellets were resuspended in 300 µL of sterile water before 6 cycles of freezing in liquid N_2 and thawing at 80 °C, with vigorous vortex mixing every other cycle. Homogenates were clarified by microcentrifugation for 10 min at 14,000 × *g* and the final supernatant was heated for 5 min at 80 °C. Extracts were stored at –20 °C before PCR amplification.

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Gene sequences encoding 16S rRNA from bacterial isolates were amplified using universal bacterial primers (EUBpA: 5'-AGAGTTTGATCCTGGCTCAF-3' and EUBpH: 5'AAGGAGGTGAT CCAGCCGCA-3') (Edwards *et al.*, 1989). Each of the 25-µL reactions contained 2.5 µL of each primer (0.2 µM), 1 U of DreamTaq DNA polymerase in master mix (Thermo Scientific) and 5 µL of DNA extract. A thermal cycler (Eppendorf Mastercycler) denatured the DNA at 95 °C for 2 min, followed by 40 cycles of 95 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min, with a final 5-min extension at 72 °C. PCR amplicons were visualized under ultraviolet light with ethidium bromide stain after electrophoresis on a 2% agarose gel to confirm amplicon size and purity. Single-pass Sanger sequencing was performed by a commercial vendor (Eurofins Genomics, Louisville, KY) using the EUBpA primer. Isolate sequences (≥634 bp) appear in GenBank with accession numbers PQ895309 to PQ895335. Phylogenetic trees were constructed by maximum-likelihood analyses using Jukes-Cantor rules and 100 bootstrap repetitions in MEGAX (Kumar *et al.*, 2016), from alignments made using SINA v1.2.12 and the SILVA v.138 database, with control sequences selected from GenBank using BLAST.

Results

Microbial abundance in SAFs by MPN

The abundance of microbes tolerant to high and low salinity was measured in washes from wipe samples of the JPL SAFs on two occasions. Microbial abundances by MPN analyses in media supplemented with 50% $MgSO_4$ (2.0 M; $a_w = 0.94$) or 10% NaCl (1.7 M; $a_w = 0.92$) are presented in Table 1 for five wipe samples of SAF surfaces in the main assembly bay. Unfortunately data from the assays of low-salt control cultures were lost. The highest values were from an area near a trash can, reaching 3.6×10^5 cells m^{-2} for both brines. The floor entrance showed particularly high abundance of microbes tolerant to $MgSO_4$ (2.4×10^5 cells ml^{-2}), much more than the abundance of microbes tolerant to NaCl. The remaining samples ranged from 3.3×10^3 to 4.2×10^4 cells m^{-2}.

A second experiment (Table 2) used the same wipe samples from the Aseptic Assembly Facility as our previous study on long-term hypersaline cultivation, microbial isolations and molecular community analyses of the end members of the enrichment (Carte *et al.*, 2024a,b). A low-salt control medium was included to determine the relative abundance of tolerant microbes in the SAF assemblage. The abundance of microbes tolerant to 50% $MgSO_4$ was a log unit lower than those tolerant of 5% (per)chlorates and somewhat lower than the abundance of epsotolerant microbes observed in the main assembly bay (Table 1). The microbial abundance of microbes tolerant to 5% $NaClO_3$ (0.5 M; $a_w = 0.98$) (3.4×10^5 cells m^{-2}) was ~ 5-fold greater than the abundance of microbes tolerant to 5% $NaClO_4$ (0.4 M; $a_w = 0.98$) (7.1×10^4 cells m^{-2}). Overall growth tolerance to

Table 1. *Microbial abundance by MPN of wipes from surfaces in the JPL SAF main assembly bay.*

Sample location	Microbial abundance (10^4 cells m^{-2})	
	50% $MgSO_4$	10% NaCl
Floor, entrance	24.0	0.50
CI	7.2–79.2	0.15–1.7
Floor, near trash can	36.0	36.0
CI	11.0–118.8	11.0–118.8
Floor, metal panel	1.41	4.20
CI	0.42–4.7	1.3–13.8
Ladder, steps	0.51	0.33
CI	0.15–1.7	0.11–1.1
Ladder, wheels	1.65	0.35
CI	0.50–5.4	0.11–1.1

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Table 2. *Microbial abundance by MPN of wipes from surfaces in the JPL SAF Aseptic Assembly Facility.*

Salt	Microbial abundance (cells m^{-2})			
	Wipe 1*	Wipe 2	Wipe 3	% Control
0.1% NaCl	1.0×10^6	7.4×10^4	3.5×10^5	100.0
CI	3.2×10^5–3.5×10^6	2.3×10^4–2.4×10^5	1.1×10^5–1.1×10^6	
5% $NaClO_3$	1.7×10^4	1.2×10^4	7.4 × 104	7.0
CI	4.8×10^3–5.3×10^4	3.6×10^3–3.9×10^4	2.3×10^4–2.4×10^5	
5% $NaClO_4$	1.2×10^4	nd	2.6×10^3	1.5
CI	3.6×10^3–3.9×10^4	nd	7.7×10^2–8.9×10^3	
50% $MgSO_4$	3.5×10^2	2.0×10^2	7.4×10^2	0.1
CI	1.1×10^2–1.1×10^3	5.6×10^1–6.5×10^2	2.3×10^2–2.4×10^3	

*The locations of these floor samples within the room are shown in Figure 1 of Carte *et al.*, 2024a,b.

perchlorates has been observed previously to be substantially lower than tolerance to chlorates (and chlorides and sulfates), so this result is not unexpected (Al Soudi *et al.*, 2016). The high percentage of microbes tolerant to $NaClO_3$ at 5% is not surprising given that bacteria in our previous study were shown to tolerate >25% (2.75 M; $a_w = 0.89$).

Note that the MPN technique does not distinguish between bacterial, archaeal and fungal growth; however, these enrichment cultures are likely to be dominated by bacteria, as observed previously (Moissl *et al.,* 2007; Plemenitaš *et al.,* 2014; Venkateswaran *et al.,* 2014; Checinska *et al.,* 2015; Weinmaier *et al.,* 2015; Hendrickson *et al.,* 2017; Carte *et al.*, 2024a,b). Archaea and fungi are nearly absent in SAFs. The media used here are designed for bacteria, having salinities too low for haloarchaea, which typically contain >20% NaCl. Furthermore, the cultures were not grown at 37 °C, which is the best choice for haloarchaea. The vast majority of archaea would not grow under the conditions used in the current study. Similarly, the media did not contain enough sugar to support strong growth of common fungi, which grow best with a 10-fold higher concentration of sugar, as in Sabouraud medium (Sabouraud 1892; Emmons 1963). In addition, we did not observe the heavy pellicle or clump of mycellium common for molds, nor did we notice the common odor of yeasts. It is possible that a portion of the turbidity in certain tubes was attributable to yeasts, molds, or haloarchaea, but the growth conditions selected against these, while favoring bacteria. Note that the medium selected for aerobic heterotrophic bacteria that could grow at moderately high salinity and mesophilic temperatures. Therefore, anaerobes and lithotrophs, for instance, would not have substantially contributed to the turbidity observed.

Characterization of SAF isolates

Microbial isolates were obtained by repetitive streak-plating from the first set of SAF samples after a short enrichment in saline media. Identification of the isolates by 16S rRNA sequence analysis showed that all of the isolates were Gram-positive in the low G + C group (Figure 1). Most of the 38 isolates were in the Bacillaceae, with representatives clustering with the genera *Bacillus, Cytobacillus, Halobacillus* and *Virgibacillus*. The other cluster included *Staphylococcus* and the related *Jeotgalicoccus*. All of these are known from previous studies as having members that are salinotolerant.

Halotolerance of the isolates was measured, with all growing in medium supplemented with 10% NaCl (Figure 2). Ten of the isolates (JPL 4, 7, 15, 23, 24, 31-35) spread across the taxa (Figure 1) did not appear to grow at 1% NaCl (0.17 M; $a_w = 0.95$) and may be halophilic, requiring high salt for growth. Measurements were not made in media containing between 1 and 10% NaCl. The majority of isolates (26) grew at 20% NaCl (3.4 M; $a_w = 0.85$) and 9 isolates (JPL 2, 3, 6, 8, 13, 22, 24, 25, 26 and 36) grew in 30% NaCl (5.2 M; $a_w = 0.76$), near saturation. Epsotolerance was prevalent among the isolates, with

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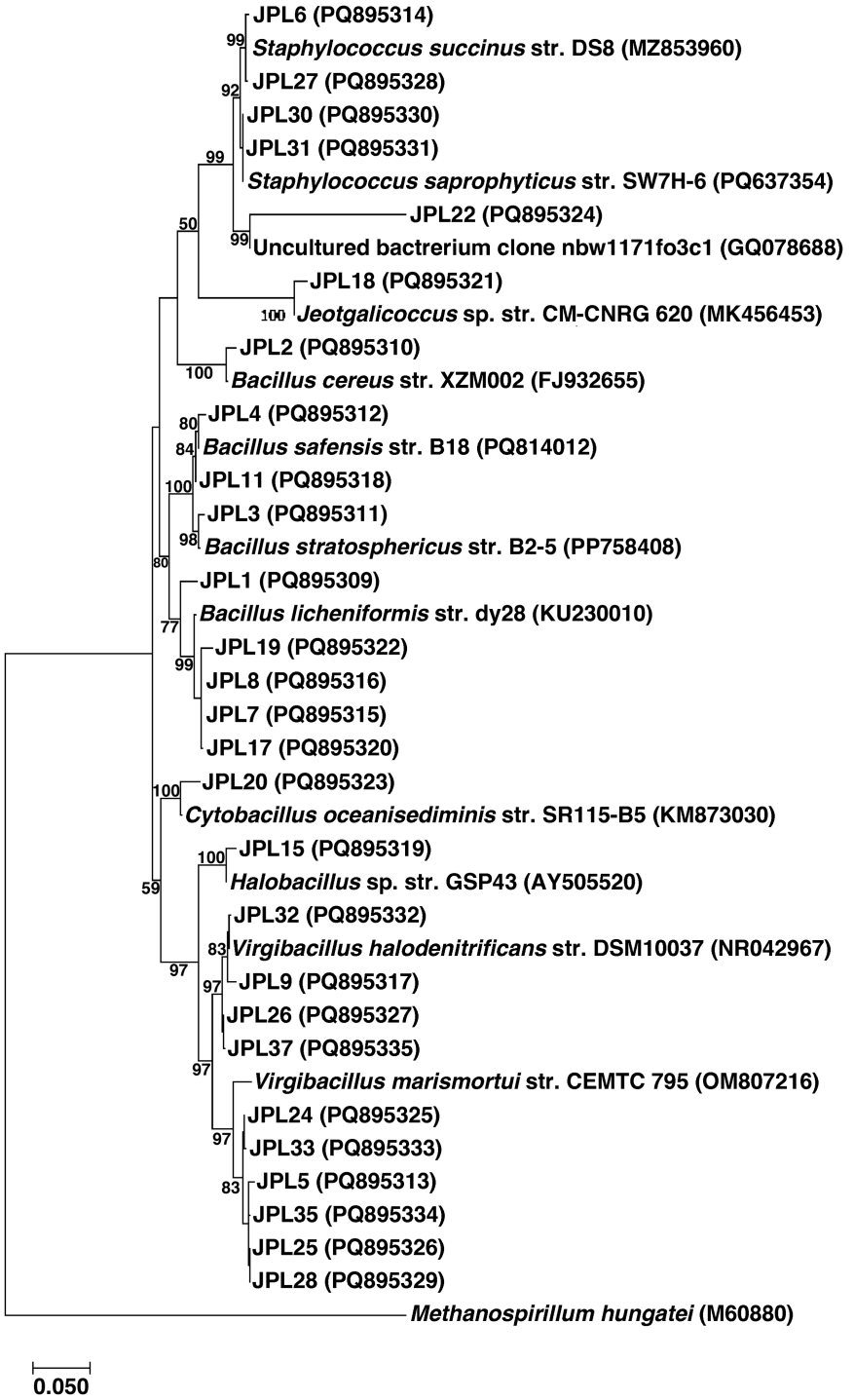

Figure 1. *Phylogenetic tree based on 16S rRNA gene sequences from SAF bacterial isolates obtained by repetitive streak-plating of saline enrichment cultures of wipe eluates from the main assembly bay.*

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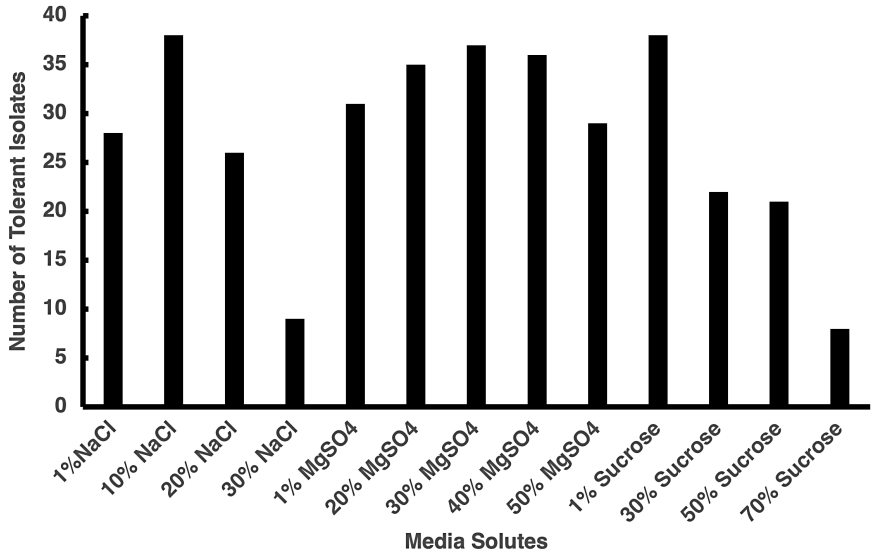

Figure 2. *Growth tolerances of SAF bacterial isolates to NaCl, $MgSO_4$ and sucrose.*

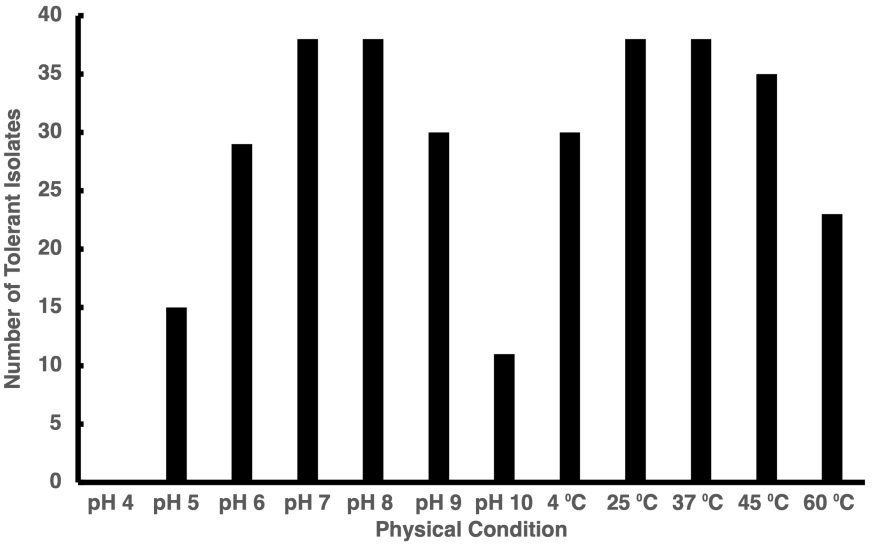

Figure 3. *Growth tolerances of SAF bacterial isolates to pH and temperature.*

all but two isolates, growing at 40% $MgSO_4$ (1.6 M; $a_w = 0.95$). The majority of isolates (29) grew at 50% $MgSO_4$, the highest concentration tested. Substantial sucretolerance was observed, using a nonionic solute. More than half of the isolates (21) grew at 50% sucrose (1.5 M; $a_w = 0.91$), while 8 isolates (JPL 2, 3, 6, 13, 24, 25, 26 and 36) grew at 70% sucrose (2.0 M; $a_w = 0.90$), near saturation. Note that all of the isolates grew at 1% sucrose, despite a few not exhibiting growth at 1% NaCl. All of the isolates that grew at 70% sucrose also grew at 30% NaCl and 50% $MgSO_4$. All of the isolates that grew at 30% NaCl also grew at 50% $MgSO_4$.

The isolates exhibited a wide range of tolerances to temperature (Figure 3). All of the isolates grew at 37 °C, with all but three growing at 45 °C. More than half (23) grew at 60 °C and 80% (30) grew at 4 °C. There were 15 isolates that grew at both 4 and 60 °C. The isolates preferred neutral and basic pH media

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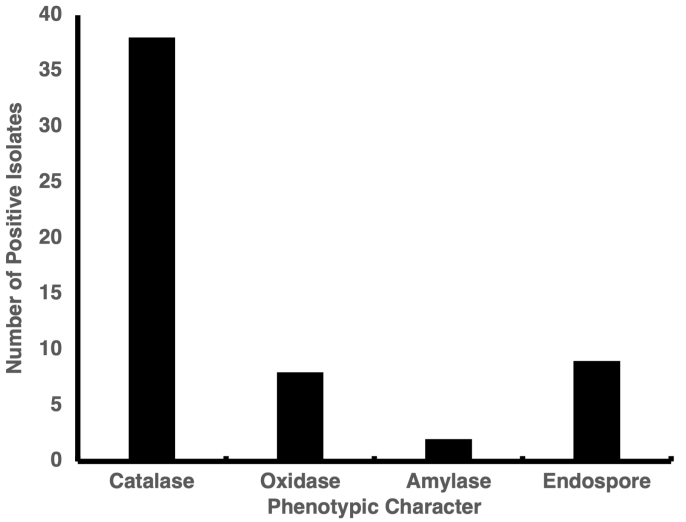

Figure 4. *Occurrence of enzyme activities in SAF bacterial isolates.*

(Figure 3). All of the isolates grew at pH 8, with 80% (30) growing at pH 9. Eleven isolates grew at pH 10. Only 15 isolates grew at pH 5, while none grew at pH 4. All but one of the isolates that grew at pH 10 also grew at pH 5. All of the isolates were catalase positive but only eight were positive for oxidase (Figure 4). Nine of the isolates positively stained for endospores under the conditions tested. Only two isolates (JPL 9 and 21) were positive for amylase, a characteristic observed to be in low prevalence in previous studies of salinotolerant bacteria (Caton *et al.*, 2004; Litzner *et al.*, 2006).

Discussion

Concern about microbial bioburden on spacecraft and their assembly facilities has continued since the early days of spaceflight (Favero *et al.*, 1966; Favero, 1971; Foster and Winans 1975; Puleo *et al.*, 1977). Over time, the cleanliness of assembly facilities has improved, and the techniques used for spacecraft sterilization are more effective today. The measurement of bioburden in SAFs has taken several approaches. A number of studies have examined airborne microbes in assembly facilities (Newcombe *et al.*, 2008; Checinska *et al.,* 2015; Lu *et al.*, 2023). Fallout particles from the air are particulate materials that have been shown to carry microbes such as *Bacillus* spp. (Mohan *et al.*, 2019). There are several published studies using cultivation to measure microbial abundance from wipe samples of SAF surfaces (v.i.). Other studies have used alternative estimators of microbial abundance such as the prevalence of rRNA genes. The results consistently show that the microbial assemblage in SAFs is nearly entirely bacteria, often with fewer than 1% being fungi or archaea (Moissl *et al.,* 2007; Plemenitaš *et al.,* 2014; Venkateswaran *et al.,* 2014; Checinska *et al.,* 2015; Weinmaier *et al.,* 2015; Hendrickson *et al.,* 2017; Carte *et al.*, 2024b). It is not unusual to find the assemblage enriched for organisms that exhibit high tolerance to environmental factors such as salinity, pH, or temperature.

An extensive study of 26 SAF clean room environments determined microbial abundance from surface wipe samples using ATP luminescence, cultivation and qPCR. Cultivable bacteria (in a single heterotrophic medium) ranged over three orders of magnitude from 10^3 to 10^6 CFU m^{-2} (La Duc *et al.*, 2007). Total bacteria determined by qPCR of 16S rRNA genes also ranged greatly, from 10^6 to 10^8 copies m^{-2}. There was no agreement across the three methods on microbial abundance, due to factors such as ATP content per cell, gene copies per cell and cultivable vegetative cells versus spores. The cultivated bacterial collection was predominantly Gram-positive *Bacillus* and *Staphylococcus*. Proteobacteria were isolated in lower numbers. In another study, cultivable bacteria from ~100 SAF

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wipe samples averaged 1.2×10^1 to 6.6×10^3 CFU m^{-2} (mean of 4.4×10^2), while another set of samples assayed by ATP content averaged 2.8×10^4 CFU m^{-2} (Hendrickson *et al.*, 2017). Heat-shocked isolates were nearly all Bacilli, with *Virgibacillus* being most abundant, and related *Brevibacillus, Oceanobacillus*, and *Paenibacillus*, and *Streptomyces* were observed. Vaishampayan *et al.* (2013) estimated bacterial abundance from floor swabs of an active SAF by qPCR of 16S rRNA genes. The observed abundance ranged from 5×10^4 to 2×10^6 copies m^{-2}. Phylochip analyses found as few as 3 genera in the least populated samples and as many as 411 genera in the most populated samples. Viable microbes in another study, as measured by cultivation from wipe samples, found mesophile abundance ranged from 3×10^2 to 8×10^4 CFU m^{-2}, with most samples towards the lower end of the range (Ghosh *et al.*, 2010). Bacillaceae were observed, but Proteobacteria were more common, and a few Actinomycetes were seen. An early study (Puelo *et al.*, 1977) reported that the abundance of cultivable aerobic microbes detected on surfaces of the Viking spacecraft were low (mainly 10^2, but as high as 10^4 CFU m^{-2}) relative to floor swabs in previous studies and consisted mainly of *Bacillus*, *Micrococcus* and *Staphylococcus*.

Previous studies have measured the abundance of mesophilic heterotrophic aerobic bacteria. The low-salt controls in the current study, estimating microbial abundance to be between 4.9×10^4 to 6.9×10^6 cells m^{-2}, certainly fall within the wide range observed in previous studies. Our experiments were primarily directed at organisms with greater salinotolerance, which are generally 1 to 10% of the total cultivable bacteria. A previous study reported that SAF floor swabs yielded as many at 1.4×10^3 CFU (3.9×10^3 CFU m^{-2}; calculated based on 0.36 m^{-2} area, as in Stieglmeier *et al.*, 2012) on medium supplemented with 10% NaCl, with as many as 8.1×10^3 CFU (2.25×10^4 CFU m^{-2}) at 3.5% NaCl (Moissl *et al.*, 2013). These levels are similar to the abundances observed in the current study. At 4 °C, a range of 5.6×10^2 to $>2.0 \times 10^4$ CFU (1.6×10^3 to $>5.6 \times 10^4$ CFU m^{-2}) were observed in different SAFs, and a similar range was observed at pH 11. A lower abundance of 0.0 to 1.9×10^2 CFU (0.0 to 5.3×10^2 CFU m^{-2}) was observed for microbes tolerant to 50 °C. *Bacillus, Micrococcus, Paenibacillus* and *Staphylococcus* were isolated that grew at 10% NaCl and pH 11, with some also growing at 50 °C. In contrast, none of the isolates from the largest study SAF microbial abundance were substantially halotolerant (La Duc *et al.*, 2007). In the current study, for instance, *Bacillus cereus* str. JPL2 and *Bacillus stratosphericus* str. JPL3 showed growth from pH 4 to 10, from 4 to 60 °C, and over a wide range of salt and sugar concentrations. Overall, more than half of our isolates were tolerant to 20% NaCl (69%) or 50% sucrose (55%), and nearly all (76%) were tolerant to 50% $MgSO_4$. Nearly all (84%) of the collection was tolerant to pH 9, with 39% growing at pH 5. Nearly all (79%) of our isolates grew at 4 °C, with 61% growing at 60 °C. Taken together, these results demonstrate that the SAF isolates from the current study are remarkably capable polyextremophiles.

It is not surprising that SAF isolate collections tend to be dominated by Gram-positive bacteria, particularly those that form endospores, given the dry, oligotrophic conditions in clean rooms. We previously isolated only Gram-positive bacteria from long-term hypersaline enrichment cultures from SAF floor swabs (Carte *et al.*, 2024a). While isolates from genera known to produce endospores predominated the collection characterized in the current study, some isolates do not produce endospores, such as *Jeotgalicoccus* and *Staphylococcus*. Our cultures were not specifically designed to promote sporulation, however, 9 isolates were positive for endospores by staining. The growth observed in MPN experiments may well have arisen from endospores in the floor wipe samples, since the SAF environment is so dry and oligotrophic, as to be unfavorable for vegetative cells. It is interesting to note our related study, where we measured the abundance of salinotolerant microbes in common soils, with subsequent isolation and characterization (Howell *et al.*, 2022). The major isolates were similar to those of the current study, namely, *Bacillus, Halobacillus, Staphylococcus* and *Virgibacillus*. When in these natural soils, the organisms were found as vegetative cells, with ~0.1% of viable cells being endospores that survived boiling. Surprisingly, neither study recovered Gram-negative bacteria, despite species such as *Halomonas* being in high relative abundance in natural hypersaline environments (Caton *et al.*, 2004).

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Our finding that salinotolerant microbes represent a substantial portion of the total microbial community on surfaces in SAFs has implications for planetary protection and the search for life, since the microbes within SAFs are the most likely to be transported to another world by spacecraft. Observing a substantial abundance of microbes tolerant to high concentrations of sulfate and (per) chlorate salts has relevance to Mars regolith, which is richer in sulfate and (per)chlorate salts than soils on Earth. Note that the 5% (per)chlorate used in the current study is much greater, and more inhibitory to microbial growth, than the 0.6% (per)chlorate salts detected on Mars (Hecht *et al.*, 2009; Kounaves *et al.*, 2010; Clark and Kounaves, 2015). While (per)chlorate salts would only be relevant to arid worlds, sulfate and chloride salts are found on both arid and ocean worlds.

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