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RESEARCH ARTICLE

# Succession of the bacterial community from a spacecraft assembly clean room when enriched in brines relevant to Mars

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## Abstract

Interplanetary spacecraft are built in a spacecraft assembly facility (SAF), a clean room designed to reduce microbial contamination that could confound life detection missions or influence native ecosystems. The frigid hyperarid near-surface environment of Mars has ample hygroscopic Mg and Na salts of chloride, (per)chlorate and sulphate that may deliquesce to form dense brines, liquids with low water activity, and freezing points <0°C. The current study sought to define the climax microbial community after 6 mo of enrichment of SAF floor wipe samples in salt plains medium supplemented with 50% (w/v; ~2 M; $a_w = 0.94$) $MgSO_4$ or 20% (w/v; ~1.9 M; $a_w = 0.91$) $NaClO_3$. After 1 wk, 4 wk and 6 mo of incubation, metagenomic DNA extracts of the enriched SAF microbial community were used for high-throughput sequencing of 16S rRNA genes and subsequent phylogenetic analyses. Additionally, dozens of bacterial strains were isolated by repetitive streak-plating from the climax community after 6 mo of enrichment. Early in the enrichment, staphylococci greatly dominated and then remained abundant members of the community. However, actinobacteria succeeded the staphylococci as the dominant taxa as the cultures matured, including *Arthrobacter*, *Brachybacterium* and *Brevibacterium*. A diverse assemblage of bacilli was present, with *Oceanobacillus* being especially abundant. The SAF culture collection included representatives of *Brachybacterium conglomeratum, Brevibacterium sediminis, Oceanobacillus picturae* and *Staphylococcus sciuri*. These were characterized with biochemical and physiological tests, revealing their high salinotolerance. Shannon diversity indices were generally near 2, reflecting modest diversity at several levels of identity and the community structures were uneven throughout. However, minor members of the community seem capable of the ecosystem functions required for biogeochemical cycling. For instance, organisms capable of all the functions of the N cycle were detected. The microbial assemblage in SAFs is the most likely to be transported by spacecraft to another world. While individual microbial populations may exhibit the qualities needed for survival at the near-surface of Mars, certainly entire communities with the capacity for complete biogeochemical cycling, would have a greater chance of survival and proliferation.

## Contents

## Introduction

Robotic spacecraft that are destined to visit celestial bodies are built in clean rooms to protect against particles that could contaminate their components. Missions directed at worlds that have substantial astrobiological potential, where liquid water may exist, need to be free of levels of bioburden that are likely to cause planetary contamination. This is critical for life detection missions. Protecting the natural environment of locations that could support life increases the chance of detecting native microbial communities and recognizing these with certainty. It remains unclear whether forward contamination of Mars or the ocean worlds could lead to successful colonization by terrestrial microbes. We seek a better understanding of the microbial assemblages likely to be ensconced on robotic spacecraft and their tolerances to the extreme chemical and physical conditions of extraterrestrial environments.

The surface and near-surface of Mars is a challenging place for life to survive and proliferate (Mancinelli *et al.*, 2004; Davila *et al.*, 2010; Rummel *et al.*, 2014). While ultraviolet radiation may be avoided in the shade of rocks and soils, the aridity of Mars is unavoidable in typical near-surface locales. Liquid water is expected to be scarce near the surface of Mars. Given the low surface temperatures, only dense brines have a real possibility of persisting as liquids under conditions similar to those found on Mars today. High concentrations of salts act to lower the freezing point of water, in some cases substantially. Certain chloride, (per)chlorate and sulphate salts relevant to Mars can lower the freezing point of water to near −70°C (Nuding *et al.*, 2014; Rummel *et al.*, 2014; Fischer *et al.*, 2016; Jänchen *et al.*, 2016; Primm *et al.*, 2017; Nair and Unnikrishnan, 2020; Pál and Kereszturi, 2020; Rivera-Valentín *et al.*, 2020). However, dense brines are so salty as to also lower the water activity ($a_w$) of the solution dramatically, to levels that can inhibit microbial growth (Grant, 2004; Schneegurt, 2012). Cells need liquid water to survive, but this water also must be bioavailable. Low $a_w$ restricts growth to only those microbes physiologically adapted to these harsh chemical conditions. For instance, the vast majority of microbes cannot proliferate in seawater, with a modest salinity of 2% NaCl that lowers $a_w$ to 0.97 (with $a_w = 1.0$ representing pure water). A saturated solution of NaCl lowers $a_w$ to 0.75 and only halotolerant microbes can grow. LiCl and (per)chlorate salts can lower $a_w$ to well below 0.6, which may be nonpermissive for microbial growth of any kind (Grant, 2004; Rummel *et al.*, 2014; Hallsworth, 2019).

Using clean rooms as spacecraft assembly facilities (SAFs) greatly reduces the bioburden on spacecraft but does not eliminate biocontamination. Clean rooms can be contaminated by infiltration of outside air, the introduction of spacecraft parts, tools and test equipment, and by workers entering the SAF, despite personal protective equipment and airlocks. Previous studies of the microbial communities in SAFs during the assembly of several spacecraft have demonstrated that a diverse collection of bacteria and archaea can be cultivated from swabs of SAF surfaces (Foster and Winans, 1975; Puleo *et al.*, 1977; La Duc *et al.*, 2003; Moissl *et al.*, 2008). Molecular analyses have uncovered an even broader assemblage of microbes than cultivation campaigns (Moissl *et al.*, 2007; La Duc *et al.*, 2009, 2012; Weinmaier *et al.*, 2015; Bashir *et al.*, 2016; Danko *et al.*, 2021; Hendrickson *et al.*, 2021; Highlander *et al.*, 2023). Clean rooms are relatively low in humidity, so successful microbial colonizers may demonstrate salinotolerance, since dry environments tend to deposit evaporite minerals. Substantial salinotolerance has been demonstrated for individual microbial isolates from SAFs and more broadly across large isolate collections that appear to be enriched for salinotolerant representatives (Moissl-Eichinger *et al.*, 2013; Venkateswaran *et al.*, 2014; Smith *et al.*, 2017; Zanmuto *et al.*, 2018). This observation increases the likelihood that microbes contaminating SAFs might gain a foothold on

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Mars or a salty ocean world. However, previous studies have not examined a broad range of salts and were limited to individual microbial strains in isolation. The current study followed changes in the microbial communities derived from swabs of SAF surfaces, when enriched for months in dense brines of $MgSO_4$ and $NaClO_3$. The results show that certain genera rise to dominance during ecological succession under these extreme chemical conditions. The climax community that persists seems more likely to survive and proliferate than individual microbial strains in potential brines on Mars or the ocean worlds.

## Methods

### *Sampling of SAF*

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 aseptic assembly facility at Jet Propulsion Laboatory (JPL) during the assembly of the Mars 2020 Sample Caching System hardware (Fig. 1). The aseptic assembly facility is a certified ISO 5 clean room. All entrants into the ISO 5 clean room 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).

Three wipe samples were taken and a fourth wipe was used as a procedural control. A fresh pair of sterile gloves was worn for each sample collection. 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. The extracts were used to inoculate enrichment cultures and for direct DNA extractions.

### *Enrichment cultures*

Selective media with high concentrations of salts were used to enrich for salinotolerant microbes. Enrichment cultures 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; $CaCl_2{\cdot}H_2O$, 0.36 g; NaBr, 0.23 g; $FeCl_3{\cdot}6H_2O$, 1.0 mg; trace

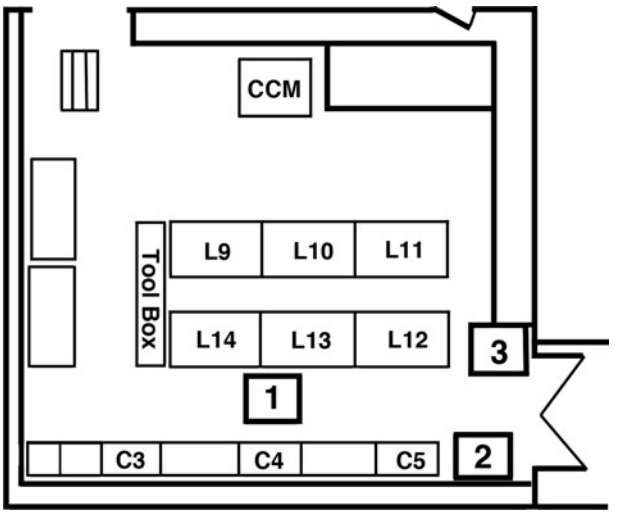


***Figure 1.*** *Map of the aseptic assembly facility in the JPL SAF showing the locations of three wipe samples of high-traffic areas of the floor.*

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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), supplemented with either 50% (w/v) $MgSO_4$ (2.0 M; $a_w = 0.94$) or 20% (w/v) $NaClO_3$ (1.9 M; $a_w = 0.91$). Flasks (100 ml) were inoculated with aliquots (1 ml) of the fluids extracted from SAF wipes and maintained on an Innova rotary shaker (125 rpm; 1-in stroke dia; New Brunswick Scientific, Edison, NJ) at room temperature for a month. Samples (6 ml) were taken weekly during this incubation for metagenomic analyses. After a month, the enrichment cultures were removed from the shaker, wrapped with parafilm to limit evaporation and stored static for an additional 5 mo, before sampling for metagenomic analyses and bacterial cultivation and isolation.

### *Bacterial isolation and characterization*

After 6 mo of enrichment, viable bacteria remaining in the cultures were isolated through serial dilution plating on SP medium supplemented to 10% NaCl. Colonies for characterization were selected by morphological characteristics and abundance. Five consecutive streak-plates of isolated colonies were used for isolation. The isolates were maintained as agar slants at room temperature and also stored at −70°C as 50% glycerol stocks. Isolates were identified by 16S rRNA gene sequencing and characterized by cell and colony morphology and a variety of biochemical tests. Gram stain was performed using Harleco reagents (Sigma–Aldrich) following the manufacturer's instructions. The endospore stain (Thermo Scientific) was carried out using the manufacturer's instructions. The SIM tests for motility, sulphide production and indole production were performed with agar stabs at 37°C using SIM medium (BBL) 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 used at 37°C and flooded with iodine solution once grown, to observe hydrolysis by amylase. Lipase enzyme was detected using Spirit Blue Agar (Difco) plates at 37°C. Gelatinase was detected using Nutrient Gelatin (Thermo Scientific) deeps that were stab inoculated and incubated at 37°C. Mannitol fermentation was observed on Mannitol salt agar plates (BBL) at 37°C. Lactose fermentation was similarly detected using MacConkey agar plates (BBL) at 37°C. Glucose and sucrose fermentation to acid and gas were observed in an assay medium (per l; 100 g NaCl, 10 g tryptone, and 0.018 g phenol red, pH 7.3) to which 0.5% (w/v) substrate and an inverted Durham tube were added.

Salinotolerance was measured in SP medium supplemented with various concentrations (all w/v) of NaCl (0.1, 10, 20 and 30%), $MgSO_4$ (30, 40, 50 and 60%), and $NaClO_3$ (5, 10, 20 and 30%). 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 4 wk. Growth was measured by absorbance spectrophotometry at 600 nm using a Genesys 10S instrument (Thermo Fisher) at 1, 3, 7, 14, 21 and 28 d after inoculation. 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 isolate and enrichment cultures 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 six 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 heated for 5 min at 80°C. Extracts were stored at −20°C before PCR amplification. Direct extracts from wipe samples before enrichment yielded insufficient DNA for reliable PCR amplification and community analyses as expected, since samples with extremely low biomass require specialized extraction methods, as previously reported (Highlander *et al.*, 2023).

Gene sequences from bacterial isolates encoding 16S rRNAs were amplified using universal bacterial primers (EUBpA: 5′-AGAGTTTGATCCTGGCTCAF-3′ and EUBpH: 5′AAGGAGGTGATCCAGCCGCA-3′) (Edwards *et al.*, 1989). Each of the 25-µl reactions contained

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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, 50°C for 1 min, and 72°C for 1 min, with a final 5-min extension at 72°C. Positive controls using *Halomonas* sp. str. HL12 (Kilmer *et al*., 2014) and negative controls with no added DNA were included with each run. PCR amplicons were visualized under ultraviolet light with ethidium bromide stain after electrophoresis on a 1.5% 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 appear in GenBank with accession numbers OP440608 to OP440641. Phylogenetic trees were constructed by maximum-likelihood analyses in MEGA v7.0 (Kumar *et al*., 2016), with control sequences selected from GenBank using BLAST, from alignments made using the SILVA v.138 database.

Crude metagenomic DNA extracts from enrichment culture samples were used for Illumina sequencing (miSeq v2 Nano PE-250bp) of 16S rRNA genes (v3/v4) by a commercial vendor (University of Kansas Center for Genomics). Each sample in the multiplex reactions produced ~ 30 000 reads and the FASTQ files were demultiplexed for forward and reverse reads. Metagenomic 16S sequence libraries were analysed on the Galaxy platform (Afgan *et al*., 2018) following the 16S microbial analysis package workflow, incorporating tools designed for mothur (Schloss *et al*., 2009). Forward and reverse reads were combined to create contigs, generating an average read length of 465 nucleotides. Chimaeras were found using chimera.vsearch and removed using remove.seqs in mothur on the Galaxy platform. These were aligned using SILVA v.138 and clustered into OTUs with a (97%) species threshold with reference to SILVA taxonomy v.138.

## Results

### *Succession of SAF bacterial communities enriched in 50% $MgSO_4$*

Microbes from three wipes of SAF floor surfaces were enriched in SP medium containing 50% (w/v) $MgSO_4$. Aliquots were withdrawn after 1 wk, 4 wk and 6 mo. Direct metagenomic DNA extracts were made from these samples and used for PCR amplification, high-throughput sequencing of 16S rRNA genes and phylogenetic analyses to describe the bacterial community. A succession of bacterial populations was observed over time within the SAF microbial community during these high-salt enrichments (Figs. 2 and 3). The harsh chemical conditions of the enrichment media were expected to greatly limit the populations of microbes present, even after a 1-wk exposure. *Staphylococcus* was the dominant taxa observed after 1 wk of $MgSO_4$ enrichment, comprising >90% of the sequences observed (grey columns of Fig. 2). After 4 wk of enrichment, the proportion of *Staphylococcus* decreased substantially in wipes 2 and 3. This trend continued, such that *Staphylococcus* was succeeded by actinobacteria in the climax community observed after 6 mo of enrichment for wipes 2 and 3 and *Staphylococcus* was greatly reduced in the wipe 1 community.

The colour columns of Fig. 2 detail the classes of bacteria observed in the $MgSO_4$ enrichments, but separately from the *Staphylococcus* results for clarity. The non-*Staphylococcus* populations after 1 or 6 mo of incubation were rich in actinobacteria. However, populations of *Alphaproteobacteria*, bacilli, clostridia and *Gammaproteobacteria* were detected in low abundance in the communities observed after 1 wk of enrichment. Only a limited number of genera were found to persist after 6 mo of enrichment in 50% $MgSO_4$. *Staphylococcus* remained a major constituent of the SAF climax communities. *Brachybacterium* and *Brevibacterium* were common and numerous across all wipes. *Glutamicibacter* (*Arthobacter*) was only a substantial portion of the community from wipe 2. The taxonomic and metabolic diversity of the microbial populations detected in the climax microbial communities after 6 mo of enrichment are discussed below.

Diversity indices were calculated for each of the bacterial communities at four levels of sequence identity (Table 1). Common identity thresholds were used to describe diversity at the taxonomic levels of division (88%), genus (94%), species (97%) and strain (99%). More than 2000 different OTUs were

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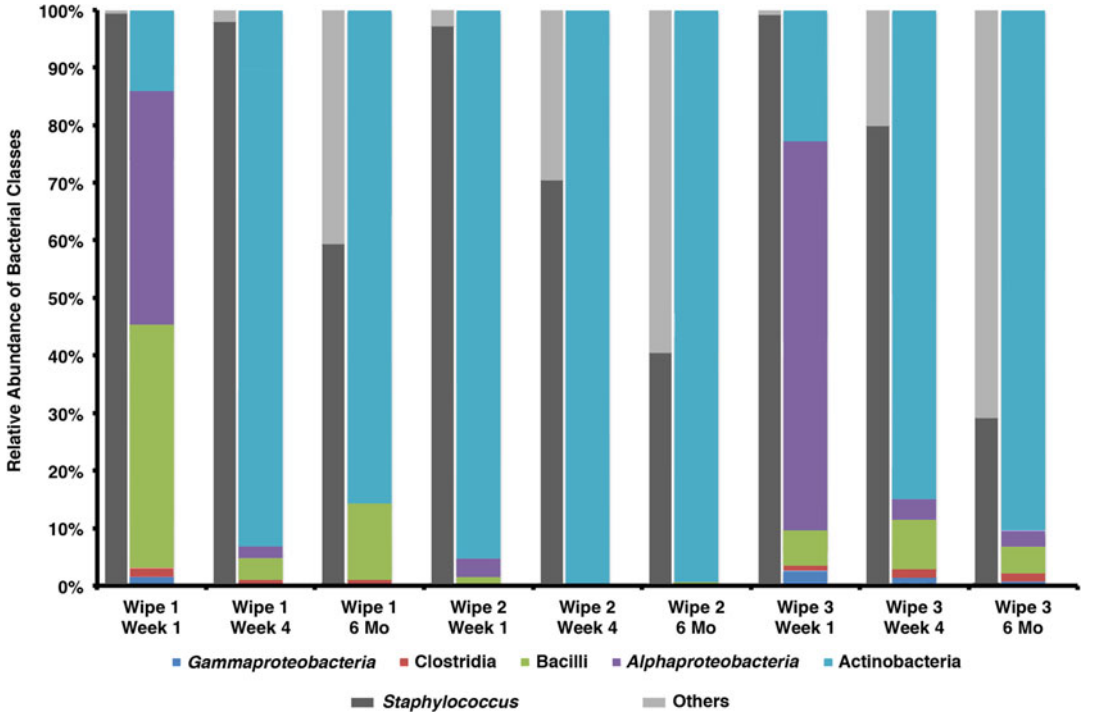


***Figure 2.*** *Relative abundance of bacterial classes observed by high throughput sequencing and phylogenetic analyses of 16S rRNA genes from metagenomic extracts of SAF wipe communities after 1 wk, 4 wk, and 6 mo of enrichment in SP medium supplemented with 50% (w/v; ~2.0 M) $MgSO_4$. The grey bars show the abundance of* Staphylococcus *relative to other members of the community. The coloured bars show the relative abundance of the bacterial classes observed without the inclusion of* Staphylococcus.

recorded at the species level, with ~1000 genera represented. Coverage overall was high, indicating that a substantial proportion of the diversity of the microbial community was observed, despite relatively small sequence libraries. Chao estimators suggest that the enriched microbial community contained ~$10^4$ species of bacteria. Non-parametric Shannon diversity and Inverse Simpson indices were generally low (~2) and increased over time, being highest after 6 mo. This likely reflects the lessening predominance of *Staphylococcus*, as evidenced by increases in the evenness of the communities by the Shannon Equitability Index. Slower growing K-strategists seemed to establish richer communities during the 6 mo of enrichment. However, the bacterial community remained uneven across all wipes, levels of identity and time points.

### *Succession of SAF bacterial communities enriched in 20% $NaClO_3$*

In a similar fashion, direct metagenomic DNA extracts were used for PCR amplification, high-throughput sequencing and phylogenetic analyses of 16S rRNA genes to describe the bacterial community developing from three wipes of SAF floor surfaces when enriched in SP medium containing 20% $NaClO_3$ (w/v). A succession of bacterial populations was observed over time within the microbial community during these harsh enrichments (Figs. 3 and 4). The chemical reactivity and lower $a_w$ of chlorate solutions were expected to limit the populations of microbes present even more than enrichments in $MgSO_4$ (Al Soudi *et al.*, 2017). Again, *Staphylococcus* was dominant at early time points, but showed signs of actinobacteria succession by 6 mo, especially apparent for wipe 3 (grey columns of

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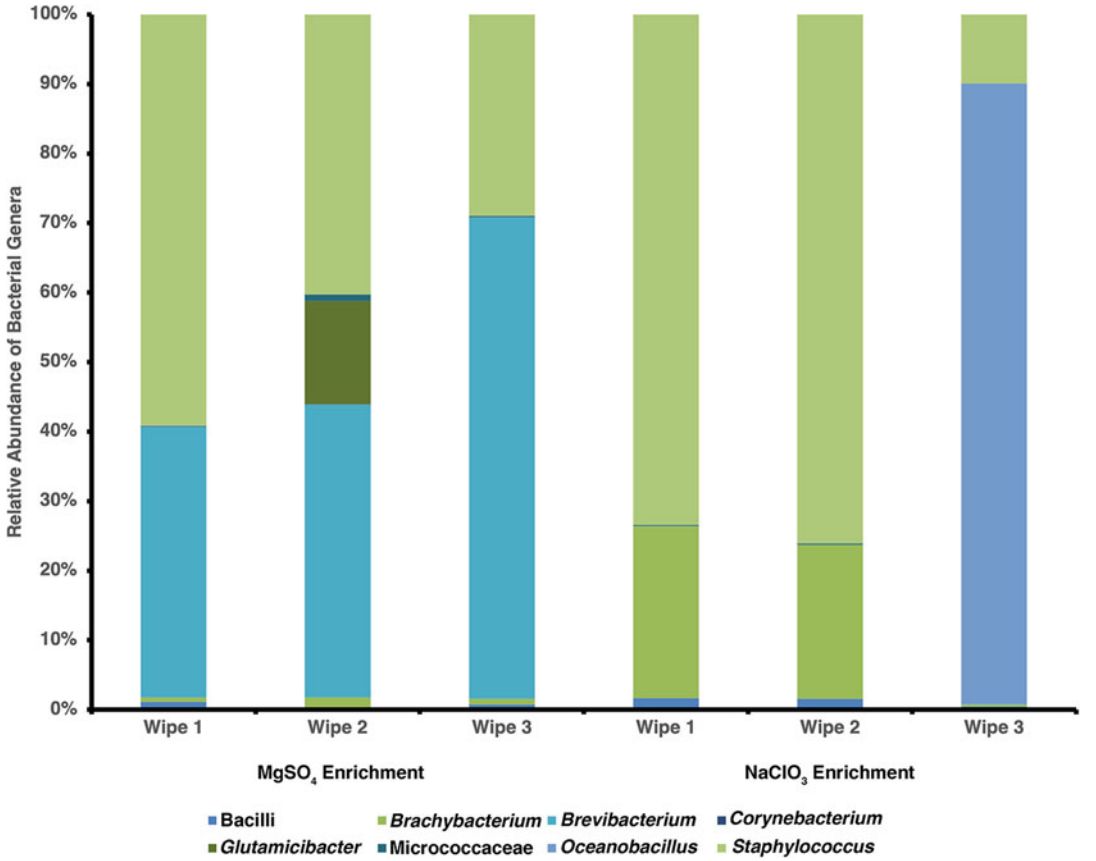


***Figure 3.*** *Relative abundance of bacterial genera observed by high-throughput sequencing and phylogenetic analyses of 16S rRNA genes from metagenomic extracts of three SAF wipe communities after 6 mo of enrichment in SP medium supplemented with 50% (w/v; ~2.0 M) $MgSO_4$ or 20% (w/v; ~1.9 M) $NaClO_3$.*

Fig. 4). The final bacterial communities enriched from wipes 1 and 2 were nearly entirely actinobacteria (colour columns of Fig. 4). The wipe 3 community retained large populations of bacilli, along with actinobacteria.

*Alphaproteobacteria* were more prominent in communities after 1 wk and 1 mo of enrichment than in the climax communities at 6 mo. *Staphylococcus* and *Brachybacterium* dominated the bacterial communities of wipes 1 and 2 after 6 mo of enrichment in 20% $NaClO_3$ (Fig. 3). Wipe 3 appeared to retain a different bacterial community, one dominated by bacilli with *Arthrobacter* and *Brachybacterium*. The diversity indices of the $NaClO_3$ enrichments followed the same trends as the $MgSO_4$ enrichments (Table 2). The number of OTUs observed and predicted for the $NaClO_3$ enrichments were similar to those of the $MgSO_4$ enrichments. Apparent diversity increased during the $NaClO_3$ enrichment across all levels of sequence identity. The evenness of the bacterial community remained low but increased substantially during the enrichment.

### *Major and minor genera detected in SAF community enrichments*

Considering the genera detected across all wipes, enrichments and time points, the dominant members of the community were all Gram-positive bacteria (Fig. 3). *Staphylococcus* was the most abundant genus detected, comprised of 24 species, with *Staphylococcus saprophyticus* (~93%) far outnumbering

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***Table 1.*** *Diversity indices for the enrichment cultures in 50% $MgSO_4$*

| Sequence identity | 99% | | | 97% | | | 94% | | | 88% | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Parameter | Wk 1 | Wk 4 | 6 Mo | Wk 1 | Wk 4 | 6 Mo | Wk 1 | Wk 4 | 6 Mo | Wk 1 | Wk 4 | 6 Mo |
| OTUs | 4339 | 5081 | 5817 | 2279 | 2615 | 2208 | 1061 | 1183 | 954 | 277 | 318 | 276 |
| Coverage | 0.95 | 0.94 | 0.93 | 0.98 | 0.97 | 0.98 | 0.99 | 0.99 | 0.99 | 1.00 | 1.00 | 1.00 |
| Chao1 | 35 825 | 38 202 | 25 178 | 9444 | 9571 | 6796 | 3568 | 4536 | 3282 | 627 | 912 | 686 |
| Lower CI | 31 670 | 34 255 | 23 339 | 8345 | 8593 | 6108 | 3059 | 3862 | 2775 | 506 | 710 | 541 |
| Upper CI | 40 613 | 42 683 | 27 209 | 10 742 | 10 711 | 7606 | 4206 | 5379 | 3930 | 812 | 1219 | 910 |
| npShannon | 1.61 | 2.56 | 3.21 | 1.27 | 2.20 | 2.64 | 0.82 | 1.72 | 2.14 | 0.27 | 0.99 | 1.37 |
| Inv Simpson | 1.34 | 2.31 | 4.29 | 1.33 | 2.30 | 4.25 | 1.25 | 2.17 | 3.95 | 1.07 | 1.71 | 2.9 |
| Shannon even | 0.15 | 0.26 | 0.33 | 0.14 | 0.26 | 0.33 | 0.11 | 0.23 | 0.30 | 0.04 | 0.17 | 0.24 |

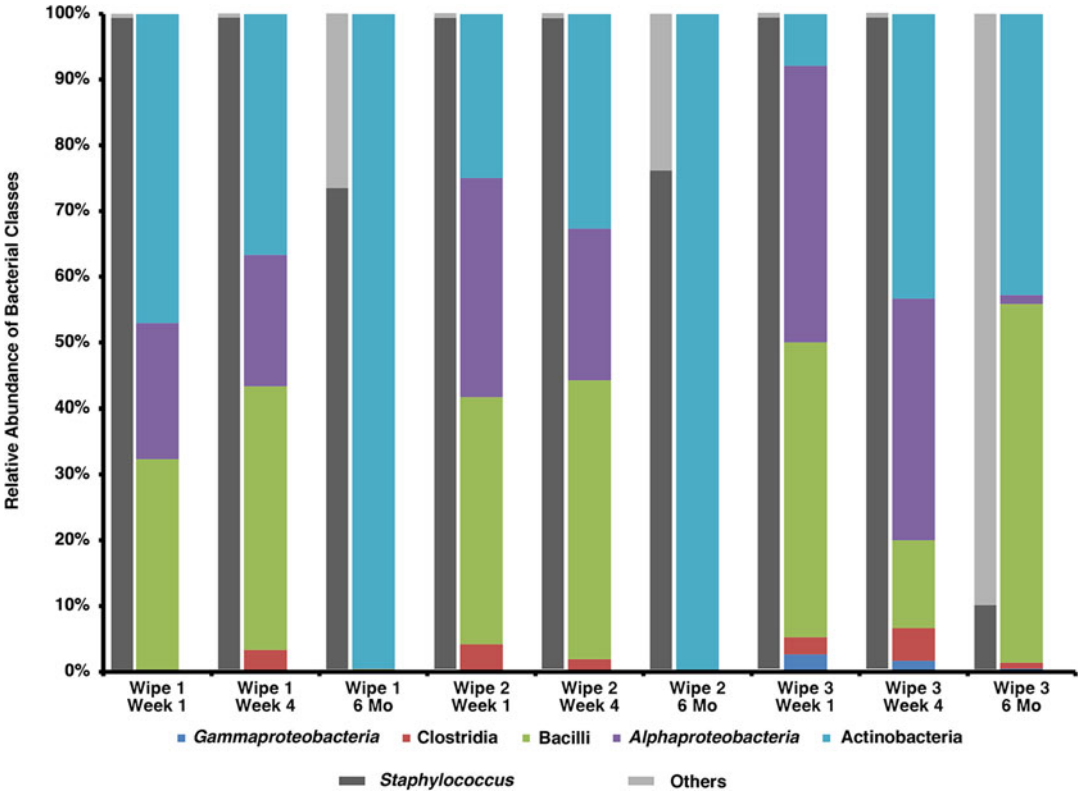


***Figure 4.*** *Relative abundance of bacterial classes observed by high-throughput sequencing and phylogenetic analyses of 16S rRNA genes from metagenomic extracts of SAF wipe communities after 1 wk, 4 wk, and 6 mo of enrichment in SP medium supplemented with 20% (w/v; ~1.9 M) $NaClO_3$. The grey bars show the abundance of* Staphylococcus *relative to other members of the community. The coloured bars show the relative abundance of the bacterial classes observed without the inclusion of* Staphylococcus.

other species, followed by *S. xylosus* (~6%) and *S. warneri* (~0.3%). Staphylococci such as *Staphylococcus saprophyticus* are halotolerant (grow in 15% NaCl), saprophytic, non-spore-forming, fermentative, facultative anaerobes that are common in foods, marine and soil environments and in the human microbiome (Scott, 1953; Kloos *et al*., 1976; La Duc *et al*., 2003; Wilson, 2005; Probst *et al*., 2010; Garza-González *et al*., 2011; Medved'ová *et al*., 2019).

As actinobacteria succeeded staphylococci in the enrichment cultures over 6 mo, *Arthrobacter, Brachybacterium* and *Brevibacterium* were the main genera observed. Among the 13 species of *Brevibacterium* detected, nearly all (~90%) were *Brevibacterium casei*, with lower abundances of *B. oceani* (~7%) and *B. permense* (~1%). *Brevibacterium casei* are non-fermentative strict anaerobes that live as saprophytes on skin and in spoiled foods (Trujillo and Goodfellow, 2012). *Brachybacterium paraconglomeratum* (~26%), *B. sacelli* (~13%) and *B. saurashtrense* (~16%) were the most common of 11 *Brachybacterium* species detected. *Brachybacterium* are non-spore-forming halotolerant bacteria (growing up to 15% NaCl) found in aerobic or microaerobic habitats in seawater, sediments, cheeses and poultry litter (Collins *et al*., 1988; Park *et al*., 2011; Buczolits and Busse, 2012). Another actinobacterium, *Arthrobacter*, observed in several enrichment samples, comprised 14 species, with the most abundant being *A. uratoxydans* (~24%). *Glutamicibacter* spp. are currently described as *Arthrobacter*, with the *A. uratoxydans* species involved in soil N cycles through ammonification and nitrate respiration (van Waasbergen *et al*., 2000; Eschbach

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***Table 2.*** *Diversity indices for the enrichment cultures in 20% $NaClO_3$*

| Sequence identity | 99% | | | 97% | | | 94% | | | 88% | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Parameter | Wk 1 | Wk 4 | 6 Mo | Wk 1 | Wk 4 | 6 Mo | Wk 1 | Wk 4 | 6 Mo | Wk 1 | Wk 4 | 6 Mo |
| OTUs | 3885 | 3581 | 5267 | 2150 | 1952 | 2242 | 991 | 906 | 993 | 241 | 263 | 285 |
| Coverage | 0.95 | 0.95 | 0.93 | 0.98 | 0.98 | 0.98 | 0.99 | 0.99 | 0.99 | 1.00 | 1.00 | 1.00 |
| Chao1 | 30 335 | 30 604 | 36 383 | 8459 | 7246 | 6902 | 3789 | 3105 | 3252 | 669 | 887 | 957 |
| Lower CI | 26 750 | 26 654 | 32 817 | 7488 | 6404 | 6223 | 3188 | 2627 | 2767 | 506 | 653 | 707 |
| Upper CI | 34 481 | 35 230 | 40 410 | 9606 | 8248 | 7697 | 4555 | 3716 | 3870 | 930 | 1261 | 1356 |
| npShannon | 1.45 | 1.37 | 3.2 | 1.14 | 1.08 | 2.71 | 0.7 | 0.69 | 2.11 | 0.19 | 0.21 | 1.56 |
| Inv Simpson | 1.26 | 1.25 | 5.71 | 1.25 | 1.25 | 5.57 | 1.18 | 1.19 | 4.7 | 1.04 | 1.05 | 3.77 |
| Shannon even | 0.13 | 0.13 | 0.33 | 0.13 | 0.12 | 0.33 | 0.09 | 0.09 | 0.30 | 0.03 | 0.03 | 0.27 |

*et al*., 2003). Of the 14 species of *Oceanobacillus* observed in certain enrichment cultures, nearly all (>99%) were *Oceanobacillus picturae*, a halotolerant (grows in 10% NaCl), fermentative, facultative anaerobe from the human gut (Lu *et al*., 2001; Lagier *et al*., 2015; Mondal *et al*., 2017). More than 25 species of *Bacillus* were detected but these were not major constituents of these enriched communities.

Minor but relatively abundant members of the communities included the actinobacteria *Plesiocystis, Pseudonocardia* and *Zhihengliuella*, the latter being a halotolerant micrococci found in marine, sediment and soil habitats (Zhang *et al*., 2007). *Plesiocystis* is a marine myxobacterium that is reported to be halophilic, requiring >1% NaCl to grow (Iizuka *et al*., 2003). Genera detected in lower abundance included *Acinetobacter, Cellulomonas, Clostridium, Compostibacillus, Corynebacterium* (seven species), *Curtobacterium, Curvibacter, Dietzia, Geodermatophilus, Geothrix, Kocuria, Limisphaera, Micropruina, Nonomuraea, Ornithinibacillus, Paenisporosarcina, Planococcus, Quadrisphaera, Saccharomonospora, Skermanella, Streptococcus* (six species), *Thermodesulfobium* and *Virgibacillus* (six species). Our sequence libraries were relatively small, so there were likely species in low abundance that were not detected. However, take note that several of the minor genera detected are known for metabolic activities central to biogeochemical cycles (*v.i.*). Even minor members of a microbial community may have important roles such as *Azorhizobium* (N fixation), *Geothrix* (Fe respiration), *Methylobrevis* (1-C metabolism) and *Synechococcus* (photosynthesis). Many of the species detected are typically associated with human microflora or soil communities, including several pathogens detected at low abundance (*v.i.*). Representatives also were detected from clearly halotolerant groups such as *Halobacillus, Haloechinothrix, Oceanicola, Salinicoccus, Salimicrobium* and *Virgibacillus* (*v.i.*).

### *Characterization of SAF bacterial isolates*

After the enrichment cultures had incubated for 6 mo, bacterial isolates were obtained from spread-plates and collected based on colony morphology and colour. Phenetic characterization was performed on 38 isolates; 23 and 15 isolates were from the 20% $NaClO_3$ and 50% $MgSO_4$ enrichments, respectively. All but three isolates were identified by 16S rRNA gene sequencing and analyzed phylogenetically (Fig. 5). Isolates SAF 1 to 23 derive from $NaClO_3$ enrichment cultures, while the rest of the isolates derive from $MgSO_4$ enrichment cultures. Biochemical and physiological characteristics of the isolates are given in Table 3.

The cultivable community was not diverse, comprising strains from only four genera, namely, *Brachybacterium, Brevibacterium, Oceanobacillus* and *Staphylococcus* (Fig. 5). Fortunately, these represent the dominant taxa detected in the enriched communities by 16S rRNA sequencing of direct metagenomic extracts, with the exception that representatives of *Arthrobacter (Glutamicibacter*) were not isolated. *Staphylococcus* was the most abundant genus recovered in both enrichment brines, with all but one isolate (SAF18) clustering with *S. sciuri*. *Oceanobacillus picturae* was recovered only from the $NaClO_3$ enrichment cultures. One isolate of *Brevibacterium sediminis* was obtained from $MgSO_4$ enrichment cultures. The *Brachybacterium* isolates recovered were in the *B. conglomeratum* cluster and all derived from the $MgSO_4$ enrichment cultures.

The isolates were a mixture of bacilli and cocci and their colonies were not highly coloured, appearing mainly cream and white. All the isolates were within Gram-positive genera, although some isolates did not retain the Gram stain well. *Oceanobacillus* were the only motile isolates and the only genus among the isolates known to form endospores. All the isolates were catalase-positive and all but three (*Brachybacterium* sp. str. SAF 30, 37 and 43) were oxidase-positive. More than half of the isolates exhibited lipase and/or gelatinase activity, but amylase activity was absent. Nearly all the isolates fermented glucose and mannitol to acid.

High salinity tolerance was observed for the isolates across three different salts. All the isolates exhibited growth tolerance to 20% NaCl (3.4 M; $a_w = 0.85$) (Table 3). All but seven isolates (82%) grew at 30% NaCl (5.1 M; $a_w = 0.76$), near saturation, showing extraordinary halotolerance. None of the isolates appeared halophilic (requiring high NaCl for growth). Epsotolerance also was high for

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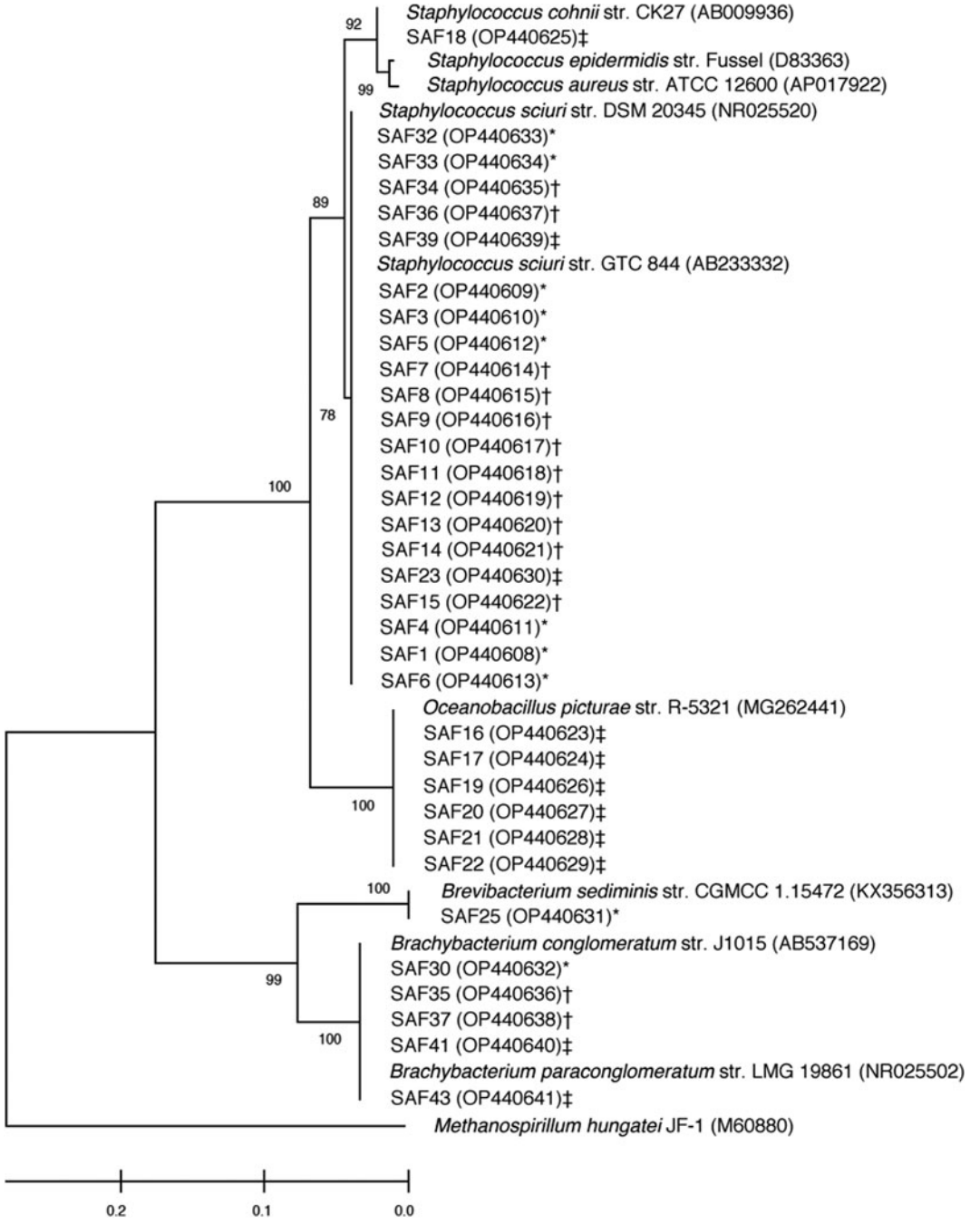


***Figure 5.*** *Phylogenetic tree based on 16S rRNA gene sequences for SAF bacterial isolates obtained by repetitive streak-plating from the climax microbial community after 6 mo of enrichment in SP medium supplemented with 50% (w/v; ~2.0 M) $MgSO_4$ or 20% (w/v; ~1.9 M) $NaClO_3$. Source of isolate: *, Wipe 1; †, Wipe 2; ‡, Wipe 3.*

the SAF isolates, with all growing at ≥40% $MgSO_4$ (1.6 M; $a_w = 0.95$) and nearly all (79%) showing growth at 50% $MgSO_4$ (Table 3). Seven isolates grew at 60% $MgSO_4$ (2.4 M; $a_w = 0.91$), near saturation (~67%). All but four of the isolates grew well at 20% $NaClO_3$ (Table 3). Three of the isolates

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***Table 3.*** *Characterization of SAF bacterial isolates*

| | SAF isolate | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Characteristic | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | |
| Gram | + | + | + | – | + | + | + | + | + | + | + | + | + | + | + | + | + | + | – | |
| Cell shape[a] | c | c | c | b | c | c | c | c | c | c | c | c | c | c | c | b | b | c | b | |
| Endospore | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | + | + | – | + | |
| Motility | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | + | + | – | + | |
| Colony Colour[b] | c | c | c | w | c | c | c | c | c | w | w | c | c | c | c | c | c | c | w | |
| Oxidase | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | |
| Catalase | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | |
| Lipase | + | – | + | – | – | + | + | + | + | – | – | + | + | + | – | + | + | + | + | |
| Amylase | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | |
| Gelatinase | + | + | + | – | + | + | + | + | + | + | + | + | + | + | + | – | – | + | – | |
| Sulphide | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | |
| Indole | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | |
| Fermentation[c] | | | | | | | | | | | | | | | | | | | | |
| Mannitol | + | + | + | + | + | + | + | + | + | + | – | + | + | + | + | + | + | + | + | |
| Lactose | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | |
| Glucose | + | + | + | + | + | + | + | + | + | – | + | + | + | + | + | + | + | + | + | |
| Sucrose | + | – | – | – | + | + | + | + | + | + | + | + | + | + | + | – | – | + | – | |
| Salinity Tolerance[d] (% w/v) | | | | | | | | | | | | | | | | | | | | |
| NaCl | 30 | 30 | 30 | 30 | 30 | 30 | 30 | 30 | 30 | 20 | 20 | 30 | 30 | 30 | 30 | 30 | 20 | 30 | 30 | |
| $MgSO_4$ | 50 | 50 | 50 | 50 | 40 | 40 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 40 | 40 | 60 | 50 | |
| $NaClO_3$ | 20 | 30 | 30 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | |
| | 20 | 21 | 22 | 23 | 25 | 26 | 27 | 28 | 30 | 32 | 33 | 34 | 35 | 36 | 37 | 39 | 40 | 41 | 43 | % + |
| Gram | – | + | + | + | + | + | – | + | + | + | + | + | + | + | + | + | + | + | + | 89 |
| Cell shape[a] | b | b | b | c | b | c | c | c | c | c | c | c | b | c | c | c | c | c | c | |
| Endospore | + | + | + | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 16 |
| Motility | + | + | + | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 16 |
| Colony Colour[b] | c | c | c | c | c | c | c | c | c | c | c | c | c | w | c | w | w | p | c | |
| Oxidase | + | + | + | + | + | + | + | + | – | + | + | + | + | + | – | + | + | + | – | 92 |
| Catalase | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | 100 |
| Lipase | + | + | + | + | + | + | + | + | – | + | + | + | – | + | – | – | + | – | – | 68 |
| Amylase | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 0 |
| Gelatinase | – | – | – | + | – | – | – | – | – | + | + | + | – | + | – | + | + | – | – | 58 |
| Sulphide | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 0 |
| Indole | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 0 |
| Fermentation[c] | | | | | | | | | | | | | | | | | | | | |
| Mannitol | + | + | + | + | + | + | + | – | + | + | + | + | – | + | + | + | + | + | + | 92 |
| Lactose | – | – | – | – | – | – | + | – | – | – | – | – | – | – | – | – | – | – | – | 3 |
| Glucose | + | + | – | + | + | + | + | + | + | + | – | + | + | + | + | + | + | + | + | 92 |
| Sucrose | – | – | – | + | – | + | + | + | + | + | + | + | – | + | + | + | + | + | + | 71 |
| Salinity Tolerance[d] (% w/v) | | | | | | | | | | | | | | | | | | | | |
| NaCl | 30 | 30 | 30 | 20 | 30 | 30 | 30 | 30 | 30 | 30 | 30 | 30 | 30 | 20 | 30 | 30 | 30 | 20 | 20 | |
| $MgSO_4$ | 40 | 40 | 40 | 40 | 50 | 60 | 50 | 60 | 50 | 50 | 60 | 60 | 50 | 60 | 50 | 60 | 50 | 50 | 50 | |
| $NaClO_3$ | 20 | 20 | 20 | 20 | 20 | 10 | 10 | 10 | 10 | 20 | 20 | 20 | 20 | 20 | 30 | 20 | 20 | 20 | 20 | |

[a]Cell shapes: b, bacillus; c, coccus.
[b]Colony colours: c, cream; p, pink; w, white.
[c]No gas observed, except for SAF27 on lactose.
[d]All isolates grew at low salt concentrations (0.1%).

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(*Brachybacterium* sp. str. SAF37 and *Staphylococcus* sp. str. SAF 2 and 3) exhibited growth at 30% $NaClO_3$ (2.8 M; $a_w = 0.89$).

## Discussion

Planetary protection protocols seek to prevent the contamination of extraterrestrial bodies by terrestrial life. For this reason, spacecraft are constructed in clean rooms certified for flight assemblies and are subject to rigorous cleaning protocols to reduce bioburden before launch. Controlled environments in SAFs exert selective pressures on indigenous microbes, where stress-tolerant species may adapt to conditions of low nutrient availability, relatively low humidity and low overall biomass. For instance, SAFs with low humidity (40 ± 5%) may select for salinotolerant microbes given that dry environments tend to retain salt evaporites. The SAF isolates appear to be relatively tolerant of high salinity, radiation and oxidants (Venkateswaran *et al*., 2001, 2003a, 2003b, 2014; La Duc *et al*., 2003; Link *et al*., 2003; Kempf *et al*., 2005; Smith *et al*., 2017; Zanmuto *et al*., 2018). The microbes isolated from SAFs closely match those isolated from spacecraft (Favero *et al*., 1966; Favero, 1971; Puleo *et al*., 1977). This suggests that SAF environments may enrich for organisms with great potential for successful survival or even colonization following forward contamination by spacecraft.

The microbial populations of SAFs have been described previously by classical cultivation and by molecular analyses to reveal complex communities (Favero *et al*., 1966; Favero, 1971; Foster and Winans, 1975; Puleo *et al*., 1977; Moissl *et al*., 2008; La Duc *et al*., 2009; Stieglmeier *et al*., 2009; Ghosh *et al*., 2010; Probst *et al*., 2010; Bashir *et al*., 2016; Hendrickson *et al*., 2017, 2021; Probst and Vaishampayan, 2020; Danko *et al*., 2021; Smith *et al*., 2022; Highlander *et al*., 2023; Lu *et al*., 2023). The microbial communities appear to be dominated by bacteria, with fewer fungi and archaea. Previously isolated representatives of *Arthrobacter, Bacillus, Exiguobacterium, Filibacter, Oceanobacillus, Sporosarcina*, *Staphylococcus* and *Streptococcus,* are bacterial genera typically associated with soils and human microbiomes (La Duc *et al*., 2003; Link *et al*., 2003; Venkateswaran *et al*., 2003a, 2003b; Kempf *et al*., 2005; Satomi *et al*., 2006; La Duc *et al*., 2007; La Duc *et al*., 2012). In molecular libraries, representatives of anaerobes and facultative strains of Firmicutes, actinobacteria and *Gammaproteobacteria* were observed. The most common Firmicutes, included the genera *Bacillus, Clostridium, Enterococcus, Lactobacillus, Paenibacillus*, and *Staphylococcus* (Moissl *et al*., 2008; Stieglmeier *et al*., 2009; La Duc *et al*., 2014; Smith *et al*., 2022; Lu *et al*., 2023). *Moraxellaceae* dominated in a cleanroom study of floor wipes that focused on human pathogens (Bashir *et al*., 2016) and were dominant, along with actinobacteria, in a recent metagenomic study (Highlander *et al*., 2023). A wide variety of media and conditions, both common and extreme, aerobic and anaerobic, were used to isolate microbes from Herschel SAF wipe samples, producing a culture collection with greater diversity than other studies (Moissl-Eichinger *et al*., 2013). Similarly, isolates from the SAF in French Guiana came from a diverse set of media enrichments, comprising nearly 50 genera (Schwendner *et al*., 2013). Several of the genera detected in fresh SAF samples were found in the microbial assemblage remaining after our extended SAF enrichments in dense brines, including actinobacteria, *Arthrobacter*, *Bacillus*, *Oceanobacillus* and *Staphylococcus*. Selective pressures appear to play a role in forming the SAF community and its enrichment of salinotolerant microbes. The microbial communities found in surgical suites and electronics clean rooms may be like those moulded by the conditions of SAFs, given the similarities of these environments.

While the enriched SAF communities of the current study included microbes typically associated with the human microbiome, the assemblage did not resemble the community found on human skin (Byrd *et al*., 2018). This supports the conclusion that our SAF samples were not simply contaminants introduced during the handling and cultivation of the samples. The absence of microbial growth in the process blank, a wipe handled as the others but never having swabbed a surface, further demonstrates that handling and cultivation did not introduce skin contaminants. Although *Staphylococcus*, common skin microbes, were observed in high abundance in enrichment cultures, *Streptococcus* and *Propionibacterium*, also common skin microbes, were in low abundance, even early in the enrichment

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cultures. Furthermore, the *Staphylococcus* detected did not include *Staphylococcus aureus* or *Staphylococcus epidermidis*, the most abundant *Staphylococcus* species on human skin. The predominant isolate from the 6-mo enrichment cultures, *Staphylococcus sciuri*, is a minor pathogen causing urinary tract infections (Kloos *et al*., 1976; Garza-González *et al*., 2011). It forms a separate taxonomic cluster (one of six *Staphylococcus* clusters) that is distinguished by being oxidase positive (Shaw *et al*., 1951). *Staphylococcus saprophyticus*, prominent in the sequence libraries, is a minor pathogen and found in foods and marine environments, often forming biofilms (Schleifer and Bell, 2009). *Oceanobacillus*, while widespread in marine and saline environments, is commonly found in the human gut and the skin-associated *Kocuria* that were detected have been found at the Atacama Desert (Lagier *et al*., 2015; Azua-Bustos *et al*., 2020). It is interesting to note that *Mycobacterium leprae*, the causative agent of leprosy, also appeared in the sequence libraries. Human pathogens detected at low abundance in previous metagenomic studies of SAF floor wipe samples included *Acinetobacter*, *Bacillus*, *Enterobacter*, *Enterococcus*, *Escherichia*, *Legionella*, *Pseudomonas* and *Staphylococcus*, with their associated virulence factors (Bashir *et al*., 2016).

Several of the bacterial genera detected in the sequence libraries from SAF enrichment cultures are well known to be halotolerant such as *Anoxybacter, Brachybacterium, Gracibacillus, Halobacillus, Haloechinothrix, Kitasatospora, Lentibacillus, Oceanobacillus, Oceanicola, Ornithinibacillus, Plesiocystis, Salinicoccus, Salimicrobium, Skermanella, Staphylococcus, Tetragenococcus, Virgibacillus* (*V. halophilus* and *V. salexigens*) and *Zhihengliuella*. Members of these genera tend to exhibit growth tolerance to 5–15% NaCl, however, certain strains may show even greater salinotolerance. For instance, *Staphylococcus* generally tolerate up to 15% NaCl, but isolates from soils of the Great Salt Plains of Oklahoma were shown to grow at >20% NaCl in culture (Maitland and Martyn, 1948; Caton *et al*., 2004; Litzner *et al*., 2006). *Halobacillus* isolates from these salt plains grew at 25% NaCl and an *Oceanobacillus* isolate appeared halophilic, growing from 10% to 25% NaCl. It is surprising that no salinotolerant Gram-negative bacterial isolates were recovered from SAF samples such as Halomonads, fast-growing polyextremophiles that are common in natural hypersaline environments (Mata *et al*., 2002; Caton *et al*., 2004; Caton and Schneegurt, 2012; Kilmer *et al*., 2014). Notably, the halotolerant bacteria isolated from a variety of common oligosaline soils were entirely Gram-positive bacilli, including *Bacillus, Halobacillus, Oceanobacillus, Staphylococcus* and *Virgibacillus*, mainly exhibiting growth tolerances of 25–30% NaCl (Howell *et al*., 2022). This supports the reasonable expectation that many of the microbes found in SAFs may derive from local soils. Archaea present in SAF enrichment cultures would not be detected by the bacterial 16S rRNA gene primers used here and the SP medium and growth conditions used do not target archaea (Edwards *et al*., 1989; Caton and Schneegurt, 2012).

The SAF enrichment cultures and the SAFs themselves are moderate indoor environments. The vast majority of the genera observed were not expected to proliferate well under environmental extremes. However, microbes known for their tolerances to temperature, pH and radiation were detected. Thermophilic bacteria were observed such as *Anoxybacter* and *Thermodsulfobium* (Mori *et al*., 2003; Zeng *et al*., 2015). Psychrophilic bacteria with growth tolerance to low temperatures (<10°C) included *Paenisporosarcina* (related to *Planococcus*) known from glaciers and soils and *Deinococcus frigens*, an isolate from Antarctica (Hirsch *et al*., 2004; Reddy *et al*., 2013). It is interesting to note that *D. frigens* is highly resistant to UV radiation, as *Deinococcus* are remarkably radiation tolerant due to superior DNA repair mechanisms (Hirsch *et al*., 2004). *Bacillus alkalinitrilicus* is a halotolerant alkaliphile that tolerates high pH conditions (Sorokin *et al*., 2008). Overall, fermentative organisms are tolerant to the low pH conditions created by acidic fermentation products such as lactic acid. Thus, microbes in SAFs have the potential to proliferate in habitats over a wide range of extreme conditions as suggested previously (Venkateswaran *et al*., 2014; Bashir *et al*., 2016; Smith *et al*., 2017; Zanmuto *et al*., 2018).

The vast majority of bacterial genera detected in sequence libraries or recovered as isolates from SAF enrichment cultures in the current study are unremarkable metabolically, being heterotrophic aerobes or facultative anaerobes. A notable aspect of the climax microbial community after 6 mo of

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enrichment was the variety of metabolic activities associated with certain populations that were detected. Although these genera may be in low abundance in this microbial community and in the natural communities of soils and waters, their biogeochemical roles are critical to ecosystem functioning. Quite a few of the genera detected are known to ferment small molecules under anaerobic conditions, often central to C recycling and the acetogen guild. Two classes of photosynthetic organisms were detected in the SAF enrichment cultures that can fix C, converting atmospheric $CO_2$ into bioavailable sugars. *Synechococcus* is a unicellular cyanobacterium found in marine environments (Castenholz, 2012). *Thiolamprovum* is a green sulphur bacterium in the *Chromatiaceae* that can perform anoxygenic photosynthesis in anoxic environments (Imhoff, 2014). *Methylobrevis* is a methylotrophic Rhizobiales that does not fix N but exhibits 1-C metabolism (Poroshina *et al*., 2015). All methanogens are strictly anaerobic archaea and hence would not have been detected here. However, the key processes of the C cycle appear to be functional in the climax SAF microbial community.

Similarly, microbes known to perform all the major processes of the N cycle appear to be present in the climax SAF microbial community. Certain organisms exhibit nitrate reduction such as *Arthrobacter, Micropruina, Oceanicola*, and *Oceanobacillus* (Hirsch *et al*., 1961; Shintani *et al*., 2000; van Waasbergen *et al*., 2000; Eschbach *et al*., 2003; Zheng *et al*., 2010; Lagier *et al*., 2015; Zeng *et al*., 2015). For instance, *Arthrobacter uratoxydans* (*Glutamicibacter uratoxydans*) from humic soils and the deep subsurface can perform denitrification through nitrate respiration and additionally produces uricase for nitrate ammonification (van Waasbergen *et al*., 2000; Eschbach *et al*., 2003). Denitrification also has been attributed to *Streptomyces* spp. (Hirsch *et al*., 1961; Shoun *et al*., 1998). Certain members of *Mycobacterium, Nocardia* and *Streptomyces* can perform nitrification by oxidizing ammonia to nitrite or nitrate (Hirsch *et al*., 1961). *Azorhizobium* can fix N, converting dinitrogen gas into bioavailable forms, while in plant root nodules or when free-living in soils (Dreyfus *et al*., 1988; Ryu *et al*., 2020). *Arthrobacter, Corynebacterium*, *Herbospirillum* and *Mycobacterium* species also are known to fix N (Gtari *et al*., 2012). Thus, a complete N cycle may be operating in the climax microbial community of the SAF enrichments of the current study, including N fixation, nitrification, denitrification and ammonification.

While microbes that perform all the processes of the S cycle were not detected, organisms known to perform the key process of dissimilatory sulphate reduction included *Desulfotomaculum, Desulfovibrio, Desufuribacillus* and *Thermodesulfobium* (Mori *et al*., 2003; Kuever *et al*., 2012). Certain actinobacteria species also are known to perform anaerobic sulphate respiration (Zeng *et al*., 2015). Finally, *Anoxybacter*, *Geothrix* and *Pelobacter* are chemoorganotrophs that can grow anaerobically by fermentation or by using Fe respiration, where oxidized Fe acts as the terminal electron acceptor (Coates *et al*., 1999; Tang *et al*., 2010; Zeng *et al*., 2015).

The succession of actinobacteria over staphylococci was the most notable shift in community structure observed during hypersaline enrichment culturing of SAF wipe samples over time. Early in the enrichment, fast-growing bacteria, with limited generalist metabolisms, dominated the microbial community. As the batch cultures matured, actinobacteria known for their metabolic versatility became dominant. This is an example of a classic secondary succession process commonly observed in natural microbial communities (Atlas and Bartha, 1987). Initially the SAF enrichment cultures were replete with the nutrients supplied by the eutrophic SP medium. Certain microbes will exploit those readily metabolized nutrients (sugars, amino acids) and rapidly proliferate. These r-strategists have the evolutionary advantage of rapid growth, but they typically have limited metabolic capabilities and are not particularly adaptive to environmental changes. The bloom of r-strategists will bust when the readily available nutrients are depleted, and their abundance then falls. The K-strategists in the community live near their carrying capacity and are more adaptive, tolerant and versatile members of the community that often form permanent biofilms. These organisms are evolutionarily successful because their versatile metabolic capabilities allow them to utilize recalcitrant nutrients of lower quality, the complex components of dead cells such as cellulose and chitin. The SAF enrichment cultures also included species that proliferate in specific niches, using electron sources and sinks associated with lithotrophy and anaerobic respiration. These tend to outlast r-strategists in stable environments, as do K-strategists in general.

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The microbes present in SAFs are the most likely to be carried by spacecraft to a planetary body. The extremely hypersaline media used for our SAF enrichment cultures are analogs for the harsh chemical conditions proposed for liquid water near the surface of Mars or in discrete locations in the icy worlds (Chevrier *et al*., 2009; Chin *et al*., 2010; Chevrier and Rivera-Valentin, 2012; Hanley *et al*., 2012; Toner *et al*., 2014). While sulphates are important on Mars, (per)chlorate brines may be the most likely sources of liquid water near the surface (Vaniman *et al*., 2004; Hecht *et al*., 2009; Kounaves *et al*., 2010; Kminek *et al*., 2017). The extraordinary chlorate tolerances observed for certain SAF isolates and enrichment cultures supports our previous conclusions that (per)chlorate tolerance is more widespread among microbes than might be expected given their chemical reactivity (Wilks *et al*., 2019). The microbial communities which develop over months in these brines from SAF surface wipes appear to comprise the functionalities central for maintaining biogeochemical cycles. In addition, many of the end members of the enrichment cultures were extremophiles and anaerobic respirers, even using Fe, which is a common oxidant on Mars.

Previous studies of microbes from SAFs mainly focused on the capabilities of individual isolates as pure cultures. However, studying microbes in the context of complex communities can enhance our understanding of natural ecosystems. When considering the forward contamination of celestial bodies, we suggest that it may be better to study the survivability and proliferation of microbial communities rather than individual microbial isolates. The climax microbial communities after enrichment of SAF samples may support fully functioning biogeochemical cycles. Subsequent experiments might begin with climax communities like these, developed over months or years, which then can be followed over long incubation periods to better mimic what may occur should the SAF microbial community be carried to another world. Sufficient biomass should be present to form biofilms, whereby microbes can retain water, recycle nutrients and find protection from harsh chemical conditions and radiation. Entire microbial communities, with every key functional niche filled, are far more likely to survive and proliferate on another world than any single remarkably resilient organism. Thus, hypertolerant microbial communities are a greater threat than microbial isolates to successfully colonize another world following forward contamination by a visiting spacecraft.

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**Competing interest.** None.

## References

Afgan E, Baker D, Batut B, van den Beek M, Bouvier D, Čech M, Chilton J, Clements D, Coraor N, Grüning BA, Guerler A, Hillman-Jackson J, Hiltemann S, Jalili V, Rasche H, Soranzo N, Goecks J, Taylor J, Nekrutenko A and Blankenberg D (2018) The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic Acids Research* **46**, W537–W544.

Al Soudi FA, Farhat O, Chen F, Clark BC and Schneegurt MA (2017) Bacterial growth tolerance to concentrations of chlorate and perchlorate salts relevant to Mars. *International Journal of Astrobiology* **16**, 229–235.

Atlas RM and Bartha R (1987) *Microbial Ecology: Fundamentals and Applications*, 2nd edn. Menlo Park: Benjamin Cummings, 533 pp.

Azua-Bustos A, Fairén AG, González Silva C, Carrizo D, Ángel Fernández-Martínez M, Arenas-Fajardo C, Fernández-Sampedro M, Gil-Lozano C, Sánchez-García L, Ascaso C, Wierzchos J and Rampe EB (2020) Inhabited subsurface wet smectites in the hyperarid core of the Atacama Desert as an analog for the search for life on Mars. *Nature Science Reports* **10**, 19183.

Bashir M, Ahmed M, Weinmaier T, Ciobanu D, Ivanova N, Pieber TR and Vaishampayan PA (2016) Functional metagenomics of spacecraft assembly cleanrooms: presence of virulence factors associated with human pathogens. *Frontiers in Microbiology* **7**, 1321.

https://doi.org/10.1017/S1473550423000277 Published online by Cambridge University Press

Buczolits S and Busse HJ (2012) *Brachybacterium*. In Goodfellow M, Kämpfer P, Busse H-J, Trujillo ME, Suzuki K, Ludwig W and Whitman WB (eds), *Bergey's Manual of Systematics of Archaea and Bacteria,* 2nd edn., vol. 5, *The Actinobacteria*. New York: Springer, pp. 730–737.

Byrd AL, Belkaid Y and Segre JA (2018) The human skin microbiome. *Nature Reviews Microbiology* **16**, 143–155.

Carte ME, Gandikota S, Chen F, Clark BC and Schneegurt MA (2020) Characterization of the microbial community in a JPL spacecraft assembly facility and its temporal development in extreme brines relevant to Mars. Abstracts and Program, 120th Annual Meeting of the American Society for Microbiology, (virtually) Los Angeles, June 2020.

Carte ME, Chen F, Clark BC and Schneegurt MA (2021) Enrichment of the microbial community of a spacecraft assembly facility in extreme brines relevant to Mars. 153rd Annual Meeting of the Kansas Academy of Science, (virtually) Fort Hays, April 2021. *Transactions of the Kansas Academy of Science* **124**, 141.

Castenholz RW (2012) Cyanobacteria. In Boone DR and Castenholz RW (eds), *Bergey's Manual of Systematics of Archaea and Bacteria,* 2nd edn., vol. 1, *The Archaea and the Deeply Branching and Phototrophic Bacteria*. New York: Springer, pp. 473–600.

Caton IR and Schneegurt MA (2012) Culture-independent analysis of the soil bacterial assemblage at the Great Salt Plains of Oklahoma. *Journal of Basic Microbiology* **52**, 16–26.

Caton TM, Witte LR, Nguyen HD, Buchheim JA, Buchheim MA and Schneegurt MA (2004) Halotolerant aerobic heterotrophic bacteria from the Great Salt Plains of Oklahoma. *Microbial Ecology* **48**, 449–462.

Chevrier VF and Rivera-Valentin EG (2012) Formation of recurring slope lineae by liquid brines on present-day Mars. *Geophysical Research Letters* **39**, L21202.

Chevrier VF, Hanley J and Altheide TS (2009) Stability of perchlorate hydrates and their liquid solutions at the Phoenix landing site, Mars. *Geophysical Research Letters* **36**, L10202.

Chin JP, Megaw J, Magill CL, Nowotarski K, Williams JP, Bhaganna P, Linton M, Patterson MF, Underwood GJC, Mswaka AY and Hallsworth JE (2010) Solutes determine the temperature windows for microbial survival and growth. *Proceedings of the National Academy of Science USA* **107**, 7835–7840.

Coates JD, Ellis DJ, Gaw CV and Lovley DR (1999) *Geothrix fermentans* gen. nov., sp. nov., a novel Fe(III)-reducing bacterium from a hydrocarbon-contaminated aquifer. *International Journal of Systematic Bacteriology* **49**, 1615–1622.

Collins MD, Brown J and Jones D (1988) *Brachybacterium faecium* gen. nov., sp. nov., a coryneform bacterium from poultry deep litter. *International Journal of Systematic Bacteriology* **38**, 45–48.

Danko DC, Sierra MA, Benardini JN, Guan L, Wood JM, Singh N, Seuylemezian A, Butler DJ, Ryon K, Kuchin K, Meleshko D, Bhattacharya C, Venkateswaran KJ and Mason CE (2021) A comprehensive metagenomics framework to characterize organisms relevant for planetary protection. *Microbiome* **9**, 82.

Davila AF, Duport LG, Melchiorri R, Jänchen J, Valea S, de los Rios A, Fairén AG, Möhlmann D, McKay CP, Ascaso C and Wierzchos J (2010) Hygroscopic salts and the potential for life on Mars. *Astrobiology* **10**, 617–628.

Dreyfus B, Garcia JL and Gillis M (1988) Characterization of *Azorhizobium caulinodans* gen. nov., sp. nov., a stem-nodulating nitrogen-fixing bacterium isolated from *Sesbania rostrata*. *International Journal of Systematic Bacteriology* **38**, 89–98.

Edwards U, Rogall H, Blöcker H, Emde M and Böttger EC (1989) Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acid Research* **17**, 7843–7853.

Eschbach M, Möbitz H, Rompf A and Jahn D (2003) Members of the genus *Arthrobacter* grow anaerobically using nitrate ammonification and fermentative processes: anaerobic adaptation of aerobic bacteria abundant in soil. *FEMS Microbiology Letters* **223**, 227–230.

Favero MS (1971) Microbiologic assay of space hardware. *Environmental Biology and Medicine* **1**, 27–36.

Favero MS, Puleo JR, Marshall JH and Oxborrow GS (1966) Comparative levels and types of microbial contamination detected in industrial clean rooms. *Applied Microbiology* **14**, 539–551.

Fischer E, Martínez GM and Rennó NO (2016) Formation and persistence of brine on Mars: experimental simulations throughout the diurnal cycle at the Phoenix landing site. *Astrobiology* **16**, 937–948.

Foster TL and Winans L (1975) Psychrophilic microorganisms from areas associated with the Viking spacecraft. *Applied Microbiology* **30**, 546–550.

Garza-González E, Morfin-Otero R, Martínez-Vázquez MA, Gonzalez-Diaz E, González-Santiago O and Rodríguez-Noriega E (2011) Microbiological and molecular characterization of human clinical isolates of *Staphylococcus cohnii*, *Staphylococcus hominis*, and *Staphylococcus sciuri*. *Scandinavian Journal of Infectious Diseases* **43**, 930–936.

Ghosh S, Osman S, Vaishampayan P and Venkateswaran K (2010) Recurrent isolation of extremotolerant bacteria from the clean room where Phoenix spacecraft components were assembled. *Astrobiology* **10**, 325–335.

Grant WD (2004) Life at low water activity. *Philosophical Transactions of the Royal Society London B* **359**, 1249–1267.

Gtari M, Ghodhbane-Gtari F, Nouioui I, Beauchemin N and Tisa LS (2012) Phylogenetic perspectives of nitrogen-fixing actinobacteria. *Archives of Microbiology* **194**, 3–11.

Hallsworth JE (2019) Microbial unknowns at the saline limits for life. *Nature Ecology and Evolution* **3**, 1503–1504.

Hanley J, Chevrier VF, Berget DJ and Adams RD (2012) Chlorate salts and solutions on Mars. *Geophysical Research Letters* **39**, L08201.

Hecht MH, Kounaves SP, Quinn RC, West SJ, Young SMM, Ming DW, Catling DC, Clark BC, Boynton WV, Hoffman J, Deflores LP, Gospodinova K, Kapit J and Smith PH (2009) Detection of perchlorate and the soluble chemistry of martian soil at the Phoenix lander site. *Science (New York, N.Y.)* **325**, 64–67.

Hendrickson R, Urbaniak C, Malli Mohan GB, Heidi A and Venkateswaran K (2017) *A Ratio of Spore to Viable Organisms: A Case Study of the JPL – SAF Cleanroom, JPL PUB 17-3*. Pasadena: Jet Propulsion Laboratory, 64 pp.

Hendrickson R, Urbaniak C, Minich JJ, Aronson HS, Martino C, Stepanauskas R, Knight R and Venkateswaran K (2021) Clean room microbiome complexity impacts planetary protection bioburden. *Microbiome* **9**, 238.

Highlander SK, Wood JM, Gillece JD, Folkerts M, Fofanov V, Furstenau T, Singh NK, Guan L, Seuylemezian A, Benardini JN, Engelthaler DM, Venkateswaran K and Keim PS (2023) Multi-faceted metagenomic analysis of spacecraft associated surfaces reveal planetary protection relevant microbial composition. *PLoS ONE* **18**, e0282428.

Hirsch P, Overrein L and Alexander M (1961) Formation of nitrite and nitrate by actinomycetes and fungi. *Journal of Bacteriology* **82**, 442–448.

Hirsch P, Gallikowski CA, Siebert J, Peissl K, Kroppenstedt R, Schumann P, Stackebrandt E and Anderson R (2004) *Deinococcus frigens* sp. nov., *Deinococcus saxicola* sp. nov., and *Deinococcus marmoris* sp. nov., low temperature and draught-tolerating, UV-resistant bacteria from continental Antarctica. *Systematic and Applied Microbiology* **27**, 636–645.

Howell SP, Kilmer BR, Porazka T and Schneegurt MA (2022) Abundance, isolation, and characterization of halotolerant microbes from common oligosaline soils. *Pedobiologia* **95**, 150827.

Iizuka T, Jojima Y, Fudou R, Hiraishi A, Ahn J-W and Yamanaka S (2003) *Plesiocystis pacifica* gen. nov., sp. nov., a marine myxobacterium that contains dihydrogenated menaquinone, isolated from the Pacific coasts of Japan. *International Journal of Systematic and Evolutionary Microbiology* **53**, 189–195.

Imhoff JF (2014) The family *Chromatiaceae*. In Rosenberg E, DeLong EF, Lory S, Stackebrandt E and Thompson F (eds), *The Prokaryotes*. Berlin/Heidelberg: Springer, pp. 151–178.

Jänchen J, Feyh N, Szewzyk U and de Vera J-PP (2016) Provision of water by halite deliquescence for *Nostoc commune* biofilms under Mars relevant surface conditions. *International Journal of Astrobiology* **15**, 107–118.

Kempf MJ, Chen F, Kern R and Venkateswaran K (2005) Recurrent isolation of hydrogen peroxide-resistant spores of *Bacillus pumilus* from a spacecraft assembly facility. *Astrobiology* **5**, 391–405.

Kilmer BR, Eberl TC, Cunderla B, Chen F, Clark BC and Schneegurt MA (2014) Molecular and phenetic characterization of the bacterial assemblage of Hot Lake, WA, an environment with high concentrations of magnesium sulphate, and its relevance to Mars. *International Journal of Astrobiology* **13**, 69–80.

Kloos WE, Schliefer KH and Smith RF (1976) Characterization of *Staphylococcus sciuri* sp. nov. and its subspecies. *International Journal of Systematic Bacteriology* **24**, 22–37.

Kminek G, Conley C, Hipkin V and Yano H (2017) COSPAR's Planetary Protection Policy. In *Review of the MEPAG Report on Mars Special Regions*. *Space Research Today*, 195, April 2017.

Kounaves SP, Hecht MH, Kapit J, Quinn RC, Catling DC, Clark BC, Ming DW, Gospodinova K, Hredzak P, McElhoney K and Shusterman J (2010) Soluble sulfate in the martian soil at the Phoenix landing site. *Geophysical Research Letters* **37**, L09201.

Kuever J, Rainey FA and Widdel F (2012) *Deltaproteobacteria*. In Brenner DJ, Krieg NR and Staley JT (eds), *Bergey's Manual of Systematics of Archaea and Bacteria,* 2nd edn., vol. 2*,* Pt. C*, The* Alpha-, Beta-, Delta-*, and* Epsilonproteobacteria. New York: Springer, pp. 922–1144.

Kumar S, Stecher G and Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* **33**, 1870–1874.

La Duc MT, Nicholson W, Kern R and Venkateswaran K (2003) Microbial characterization of the Mars Odyssey spacecraft and its encapsulation facility. *Environmental Microbiology* **5**, 977–985.

La Duc MT, Dekas A, Osman S, Moissl C, Newcombe D and Venkateswaran K (2007) Isolation and characterization of bacteria capable of tolerating the extreme conditions of clean room environments. *Applied and Environmental Microbiology* **73**, 03007–06.

La Duc MT, Osman S, Vaishampayan P, Piceno Y, Andersen G, Spry JA and Venkateswaran K (2009) Comprehensive census of bacteria in clean rooms by using DNA microarray and cloning methods. *Applied and Environmental Microbiology* **75**, 6559–6567.

La Duc MT, Vaishampayan P, Nilsson HR, Torok T and Venkateswaran K (2012) Pyrosequencing-derived bacterial, archaeal, and fungal diversity of spacecraft hardware destined for Mars. *Applied and Environmental Microbiology* **78**, 5912–5922.

La Duc MT, Venkateswaran K and Conley CA (2014) A genetic inventory of spacecraft and associated surfaces. *Astrobiology* **14**, 15–41.

Lagier J-C, Saber K, Azhar EI, Croce O, Bibi F, Jiman-Fatani AA, Yasir M, Ben Helaby H, Robert C, Fournier P-E and Raoult D (2015) Genome sequence of *Oceanobacillus picturae strain S*1, an halophilic bacterium first isolated in human gut. *Standards in Genomic Sciences* **10**, 91.

Link L, Sawyer J, Venkateswaran K and Nicholson W (2003) Extreme spore UV resistance of *Bacillus pumilus* isolates obtained from an ultraclean spacecraft assembly facility. *Microbial Ecology* **47**, 159–163.

Litzner BR, Caton TM and Schneegurt MA (2006) Carbon substrate utilization, antibiotic sensitivity, and numerical taxonomy of bacterial isolates from the Great Salt Plains of Oklahoma. *Archives of Microbiology* **185**, 286–296.

Lu J, Nogi Y and Takami H (2001) *Oceanobacillus iheyensis* gen. nov., sp. nov., a deep-sea extremely halotolerant and alkaliphilic species isolated from a depth of 1050 m on the Iheya Ridge. *FEMS Microbiology Letters* **205**, 291–297.

Lu Y, Yang J, Zhang L, Chen F, Han P and Fe Y (2023) Characteristics of bacterial community and ARG profiles in the surface and air environments in a spacecraft assembly cleanroom. *Environmental Pollution* **329**, 121613.

Maitland HB and Martyn G (1948) A selective medium for isolating *Staphylococcus* based on the differential inhibiting effect of increased concentrations of sodium chloride. *The Journal of Pathology and Bacteriology* **60**, 553–561.

Mancinelli RL, Fahlen TF, Landheim R and Klovstad MR (2004) Brines and evaporites: analogs for Martian life. *Advances in Space Research* **33**, 1244–1246.

Mata JA, Martínez-Canovas J, Quesada E and Béjar V (2002) A detailed phenotypic characterisation of the type strains of *Halomonas* species. *Systematic and Applied Microbiology* **25**, 360–375.

Medved'ová A, Havlíková A, Lehotová V and Valík L′ (2019) *Staphylococcus aureus* 2064 growth as affected by temperature and reduced water activity. *Italian Journal of Food Safety* **8**, 8287.

Moissl-Eichinger C, Pukall R, Probst AJ, Stieglmeier M, Schwendner P, Mora M, Barczyk S, Bohmeier M and Rettberg P (2013) Lessons learned from the microbial analysis of the Herschel spacecraft during assembly, integration, and test operations. *Astrobiology* **13**, 1024.

Moissl C, Osman S, La Duc MT, Dekas A, Brodie E, DeSantis T and Venkateswaran K (2007) Molecular bacterial community analysis of clean rooms where spacecraft are assembled. *FEMS Microbiology Ecology* **62**, 509–521.

Moissl C, Bruckner JC and Venkateswaran K (2008) Archaeal diversity analysis of spacecraft assembly clean rooms. *ISME Journal* **2**, 115–119.

Mondal AK, Kumar J, Pandey R, Gupta S, Kumar M, Bansal G, Mukerji M, Dash D and Chauhan NS (2017) Comparative genomics of host-symbiont and free-living *Oceanobacillus* species. *Genome Biology and Evolution* **9**, 1175–1182.

Mori K, Kim H, Kakegawa T and Hanada S (2003) A novel lineage of sulfate-reducing microorganisms: *Thermodesulfobiaceae* fam. nov., *Thermodesulfobium narugense*, gen. nov., sp. nov., a new thermophilic isolate from a hot spring. *Extremophiles* **7**, 283–290.

Nair CPR and Unnikrishnan V (2020) Stability of the liquid water phase on Mars: a thermodynamic analysis considering martian atmospheric conditions and perchlorate brine solutions. *ACS Omega* **5**, 9391–9397.

Nuding DL, Rivera-Valentin EG, Davis RD, Gough RV, Chevrier VF and Tolbert MA (2014) Deliquescence and efflorescence of calcium perchlorate: an investigation of stable aqueous solutions relevant to Mars. *Icarus* **243**, 420–428.

Pál B and Kereszturi Á (2020) Annual and daily ideal periods for deliquescence at the landing site of InSight based on GCM model calculations. *Icarus* **340**, 113639.

Park S-K, Kim M-S, Jung M-J, Nam Y-D, Park E-J, Roh SW and Bae J-W (2011) *Brachybacterium squillarum* sp. nov., isolated from salt-fermented seafood. *International Journal of Systematic and Evolutionary Microbiology* **61**, 1118–1122.

Poroshina MN, Trotsenko YA and Doronina NV (2015) *Methylobrevis pamukkalensis* gen. nov., sp. nov., a halotolerant restricted facultative methylotroph isolated from saline water. *International Journal of Systematic and Evolutionary Microbiology* **65**, 1321–1327.

Primm KM, Gough RV, Chevrier VF and Tolbert MA (2017) Freezing of perchlorate and chloride brines under Mars-relevant conditions. *Geochimica Cosmochimica Acta* **212**, 211–220.

Probst AJ and Vaishampayan P (2020) Are we there yet? Understanding interplanetary microbial hitchhikers using molecular methods. *Current Issues in Molecular Biology* **38**, 33–52.

Probst A, Vaishampayan P, Osman S, Moissl C, Anderson GL and Venkateswaran K (2010) Diversity of anaerobic microbes in spacecraft assembly clean rooms. *Applied and Environmental Microbiology* **76**, 2837–2845.

Puleo JR, Fields ND, Bergstrom SL, Oxborrow GS, Stabekis PD and Koukol R (1977) Microbiological profiles of the Viking spacecraft. *Applied and Environmental Microbiology* **33**, 379–384.

Reddy GSN, Poorna Manasa B, Singh SK, Shivaji S (2013) *Paenisporosarcina indica* sp. nov., a psychrophilic bacterium from a glacier, and reclassification of *Sporosarcina antarctica* Yu *et al*., 2008 as *Paenisporosarcina antarctica* comb. nov. and emended description of the genus *Paenisporosarcina*. *International Journal of Systematic and Evolutionary Microbiology* **63**, 2927–2933.

Rivera-Valentín EG, Chevrier VF, Soto A and Martínez G (2020) Distribution and habitability of (meta)stable brines on present-day Mars. *Nature Astronomy* **4**, 756–761.

Rummel JD, Beaty DW, Jones MA, Bakermans C, Barlow NG, Boston PJ, Chevrier VF, Clark BC, de Vera JP, Gough RV, Hallsworth JE, Head JW, Hipkin VJ, Kieft TL, McEwen AS, Mellon MT, Mikucki JA, Nicholson WL, Omelon CR, Peterson R, Roden EE, Sherwood Lollar B, Tanaka KL, Viola D and Wray JJ (2014) A new analysis of Mars "special regions": findings of the Second MEPAG Special Regions Science Analysis Group (SR-SAG2). *Astrobiology* **14**, 887–968.

Ryu M-H, Zhang J, Toh T, Khokhani D, Geddes BA, Mus F, Garcia-Costas A, Peters JW, Poole PS, Ané J-M and Voigt CA (2020) Control of nitrogen fixation in bacteria that associate with cereals. *Nature Microbiology* **5**, 314–330.

Satomi M, La Duc MT and Venkateswaran K (2006) *Bacillus safensis* sp. nov., isolated from spacecraft and assembly-facility surfaces. *International Journal of Systematic and Evolutionary Microbiology* **56**, 1735–1740.

Schleifer K-H and Bell JA (2009) *Staphylococcaceae*. In De Vos P, Garrity GM, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer K-H and Whitman WB (eds), *Bergey's Manual of Systematics of Archaea and Bacteria,* 2nd edn., vol. 3, *The Firmicutes*. New York: Springer, pp. 392–433.

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ and Weber CF (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology* **75**, 7537–7541.

Schneegurt MA (2012) Media and conditions for the growth of halophilic and halotolerant bacteria and archaea. In Vreeland RH (ed.), *Advances in Understanding the Biology of Halophilic Microorganisms*. Dordrecht: Springer, pp. 35–58.

Schwendner P, Moissl-Eichinger C, Barczyk S, Bohmeier M, Pukall R and Rettberg P (2013) Insights into the microbial diversity and bioburden in a South American spacecraft assembly clean room. *Astrobiology* **13**, 1023.

Scott WJ (1953) Water relations of *Staphylococcus aureus* at 30°C. *Australian Journal of Biological Sciences* **6**, 549–564.

Shaw C, Stitt JM and Cowan ST (1951) Staphylococci and their classification. *Journal of General Microbiology* **5**, 1010–1023.

Shintani T, Liu WT, Hanada S, Kamagata Y, Miyaoka S, Suzuki T and Nakamura K (2000) *Micropruina glycogenica* gen. nov., sp. nov., a new Gram-positive glycogen-accumulating bacterium isolated from activated sludge. *International Journal of Systematic and Evolutionary Microbiology* **50**, 201–207.

Shoun H, Kano M, Baba I, Takaya N and Matsuo M (1998) Denitrification by actinomycetes and purification of dissimilatory nitrite reductase and azurin from *Streptomyces thioluteus*. *Journal of Bacteriology* **180**, 4413–4415.

Smith SA, Benardini III JN, Anderl D, Ford M, Wear E, Schrader M, Schubert W, DeVeaux L, Paszczynski A and Childers SE (2017) Identification and characterization of early mission phase microorganisms residing on the Mars Science Laboratory and assessment of their potential to survive Mars-like conditions. *Astrobiology* **17**, 1417.

Smith LM, Lowes C, O'Driscoll NH and Lamb AJ (2022) Identification of bacterial isolates recovered from the surface of cleanroom operators' garments following wear. *European Journal of Parenteral and Pharmaceutical Sciences* **27**, 27301.

Sorokin DY, van Pelt S and Tourova TP (2008) Utilization of aliphatic nitriles under haloalkaline conditions by *Bacillus alkalinitrilicus* sp. nov. isolated from soda solonchak soil. *FEMS Microbiology Letters* **288**, 235–240.

Stieglmeier M, Wirth R, Kminek G and Moissl-Eichinger C (2009) Cultivation of anaerobic and facultatively anaerobic bacteria from spacecraft-associated clean rooms. *Applied and Environmental Microbiology* **75**, 3484–3491.

Tang SK, Zhi XY, Wang Y, Wu JY, Lee JC, Kim CJ, Lou K, Xu LH and Li WJ (2010) *Haloactinobacterium album* gen. nov., sp. nov., a halophilic actinobacterium, and proposal of *Ruaniaceae* fam. nov. *International Journal of Systematic and Evolutionary Microbiology* **60**, 2113–2119.

Toner JD, Catling DC and Light B (2014) The formation of supercooled brines, viscous liquids, and low-temperature perchlorate glasses in aqueous solutions relevant to Mars. *Icarus* **233**, 36–47.

Trujillo ME and Goodfellow M (2012) *Brevibacteriaceae*. In Goodfellow M, Kämpfer P, Busse H-J, Trujillo ME, Suzuki K, Ludwig W and Whitman WB (eds), *Bergey's Manual of Systematics of Archaea and Bacteria,* 2nd edn., vol. 5*, The Actinobacteria*. New York: Springer, pp. 685–700.

Vaniman DT, Bish DL, Chipera SJ, Fialips CI, Carey JW and Feldman WC (2004) Magnesium sulphate salts and the history of water on Mars. *Nature* **431**, 663–665.

van Waasbergen LG, Balkwill DL, Crocker FH, Bjornstad BN and Miller RV (2000) Genetic diversity among *Arthrobacter* species collected across a heterogeneous series of terrestrial deep-subsurface sediments as determined on the basis of 16S rRNA and *recA* gene sequences. *Applied and Environmental Microbiology* **66**, 3454–3463.

Venkateswaran K, Satomi M, Chung S, Kern R, Koukol R, Basic C and White D (2001) Molecular microbial diversity of a spacecraft assembly facility. *Systematic and Applied Microbiology* **24**, 311–320.

Venkateswaran K, Kempf K, Chen F, Satomi M, Nicholson W and Kern R (2003a) *Bacillus nealsonii* sp. nov., isolated from a spacecraft assembly facility, whose spores are gamma-radiation resistant. *International Journal of Systematic and Evolutionary Microbiology* **53**, 165–172.

Venkateswaran K, Hattori N, La Duc MT and Kern R (2003b) ATP as a biomarker of viable microorganisms in clean-room facilities. *Journal of Microbiological Methods* **52**, 367–377.

Venkateswaran K, Vaishampayan P, Benardini III JN, Rooney AP and Spry JA (2014) Deposition of extreme-tolerant bacterial strains isolated during different phases of Phoenix spacecraft assembly in a public culture collection. *Astrobiology* **14**, 0978.

Weinmaier T, Probst AJ, La Duc MT, Ciobanu D, Cheng J-F, Ivanova N, Rattei T and Vaishampayan P (2015) A viability-linked metagenomic analysis of cleanroom environments: eukarya, prokaryotes, and viruses. *Microbiome* **3**, 62.

Wilks J, Chen F, Clark B and Schneegurt M (2019) Bacterial growth in saturated and eutectic solutions of magnesium sulphate and potassium chlorate with relevance to Mars and the ocean worlds. *International Journal of Astrobiology* **18**, 502–509.

Wilson M (2005) *Microbial Inhabitants of Humans: Their Ecology and Role in Health and Disease*. London: Cambridge University Press, 466 pp.

Zanmuto V, Fuchs FM, Fiebrandt M, Stapelmann K, Ulrich NJ, Maugeri TL, Pukall R, Gugliandolo C and Moeller R (2018) Comparing spore resistance of *Bacillus* strains isolated from hydrothermal vents and spacecraft assembly facilities to environmental stressors and decontamination treatments. *Astrobiology* **18**, 1715.

Zeng X, Zhang Z, Li X, Zhang X, Cao J, Jebbar M, Alain K and Shao Z (2015) *Anoxybacter fermentans* gen. nov., sp. nov., a piezophilic, thermophilic, anaerobic, fermentative bacterium isolated from a deep-sea hydrothermal vent. *International Journal of Systematic and Evolutionary Microbiology* **65**, 710–715.

Zhang Y-Q, Schuman P, Yu H-Y, Zhang Y-Q, Xu L-H, Stackbrandt E, Jiang C-L and Li W-J (2007) *Zhihengliuella halotolerans* gen. nov., sp. nov., a novel member of the family *Micrococcaceae*. *International Journal of Systematic and Evolutionary Microbiology* **57**, 1018–1023.

Zheng Q, Chen C, Wang Y-N and Jiao N (2010) *Oceanicola nitratireducens* sp. nov., a marine alphaproteobacterium isolated from the South China Sea. *International Journal of Systematic and Evolutionary Microbiology* **60**, 1655–1659.