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Special Issue Article

Microbiology of a NaCl stalactite 'salticle' in Triassic halite

Thomas P. Thompson,[1] **Stephen A. Kelly**,[1] **Timofey Skvortsov**,[1] **Gill Plunkett**,[2] **Alastair Ruffell**,[2] **John E. Hallsworth**,[3] **Jason Hopps**[4] **and Brendan F. Gilmore**[1,3*]

[1]*Biofilm Research Group, School of Pharmacy, Queen's University Belfast, Medical Biology Centre, Belfast, BT9 7BL, UK.*

[2]*School of Natural and Built Environment, Department of Archaeology, Geography and Palaeoecology, Queen's University Belfast, Belfast, BT7 1NN, UK.*

[3]*Institute for Global Food Security, School of Biological Sciences, Queen's University Belfast, Belfast, BT9 5DL, UK.*

[4]*Irish Salt Mining & Exploration Company Ltd., Carrickfergus, BT38 9BT, UK.*

Summary

Large regions of Earth's surface are underlain by salt deposits that evaporated from ancient oceans and are populated by extreme halophilic microbes. Some of these halophiles may have been preserved over geological timescales within hypersaline fluid inclusions, but ingresses of water and/or anthropogenic activities can lead to the formation of alternative habitats, including NaCl stalactites or other speleothems. While the microbiology of ancient evaporites has been well studied, the ecology of these recently formed structures is less-well understood. Here, the microbiology of a NaCl stalactite ('salticle') in a Triassic halite mine is characterized. The specific aims were to determine the presence of fluid inclusions, determine the microbial structure of the salticle compared with a nearby brine-pool and surficial soil, and characterize the ecophysiological capabilities of this unique ecosystem. The salticle contained fluid inclusions, and their microbiome was composed of Euryarchaetota, Proteobacteria, and Actinobacteria, with Haloarchaea in greater abundance than brine-pool or soil microbiomes. The salticle metagenome exhibited a greater abundance of genes involved in osmoregulation, anaerobic respiration, UV resistance, oxidative stress, and stress-protein synthesis relative to the soil microbiome. We discuss the potential astrobiological implications of salticles as enclosed salt-saturated habitats that are protected from ionizing radiation and have a stable water activity.

Introduction

Well-perserved, unaltered evaporite deposits (including unaltered bedded halite) are distributed throughout much of the Earth and are known from Proterozoic until modern times (Sankaranarayanan *et al*., 2011). The rapid precipitation of halite leads to entrapment of microorganisms in the-parent water and they continue to subsist *in situ*, so these deposits represent massive subsurface repositories for microbial life. During halite crystallization, hypersaline fluid containing halophilic microbes (of each domain of life) become trapped within; they have repeatedly been photographed, retrieved, and cultured (McGenity *et al*., 2000; Benison, 2019). A recent study by Huby *et al*. demonstrated that artificially halite-entombed archaeal communities were resilient to entombment for 21 weeks (Huby *et al*., 2020). In nature, fluid inclusions in halite, gypsum, and other salts can preserve both living cells and DNA, thus providing genetic records of the microbial diversity and environmental conditions of over almost 150 million years (Sankaranarayanan *et al*., 2011).

Within halite, fluid inclusions that contain both *Dunaliella salina* and Haloarchaea, darkness-induced programmed cell death of the former can release glycerol and other organic substances, which can be utilized as nutrients by Haloarchaea (Orellana *et al*., 2013), and according to one study, would enable survival of Haloarchaea for up to 30, 000 years (Schubert *et al*., 2009). Their longevity is a result of low-level metabolism as described in deep marine

Received 2 January, 2021; revised 6 April, 2021; accepted 8 April, 2021. *For correspondence. E-mail b.gilmore@qub.ac.uk; Tel. (+44) 0 28 9097 2305; Fax (+44) 0 28 9024 7794.

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sediments (Parkes *et al*., 2014; Morono *et al*., 2020) and possibly also phenotypic plasticity. While there is no evidence of Haloarchaea forming spores, the presence of cyst-like structures has been observed in some species (Grant *et al*., 1998), and the stress-induced formation of a persister-cell phenotype has been described in *Haloferax volcanii* (Megaw and Gilmore, 2017). A series of insightful studies have elucidated aspects of the microbiology of ancient halite deposits (Jaakkola *et al*., 2016; Megaw *et al*., 2020), the geology of salt-based speleothems (a geological structure formed of salt precipitate) (Filippi *et al*., 2011), the microbial ecology of hypersaline fluid inclusions (Schubert *et al*., 2009; Benison, 2019), and the precipitation of minerals in anthropogenically perturbed subsurface environments (Borgonie *et al*., 2015). Such studies also have implications for the preservation of life in other hypersaline environments (e.g., Bension, 2019; Sankaranarayanan *et al*., 2011).

Borgonie *et al*. (2015) identified microorganisms present in stalactites ($CaCO_3$) that had formed in South African gold mines (Borgonie *et al*., 2015). Here, we also seek insights into the microbial ecology of newly formed stalactites, but this time within a 220 million-year-old deposit of Triassic halite, located in the northern hemisphere and known as the Larne Basin deposit. This halite deposit spans from the northeast of Europe beneath the north of England and into County Antrim of Northern Ireland. This system has previously been studied to identify some of the resident microbes (Norton *et al*., 1993; Megaw *et al*., 2020). The current study focused on the microbial ecology of a newly identified habitat within this ancient system; a recently formed (10–15 year old) NaCl-stalactite that we refer to as a 'salticle'. The specific aims were to analyse salticle architecture and determine whether there are fluid inclusions; characterize the ecosystem's microbial structure; compare the salticle microbiome with those of nearby (salt-saturated brine-pool) and surficial soil; and determine the ecophysiological capacity of the salticle's microbiome. Elemental analysis and petrographic microscopy were used to characterize the salticle's structure and locate any fluid inclusions. Metagenomic analysis was used to elucidate the microbiomes of salticle, brine-pool, and soil, and then characterize and quantify genes as copies per million (CPM), related to osmoregulation, anaerobic respiration, resistance to UV, oxidative stress, stress proteins, and possible persister-cell biology. The findings are discussed in the context of the other terrestrial halophile systems, and the implications potential for extraterrestrial habitats such as the Mars subsurface.

Results and discussion

Habitat characterization: a NaCl stalactite (or 'salticle')

The salticle has a thin, pointed morphology, with a central tube that feeds brine and thus facilitates the growth of the salticle (Fig. 1A). Generally, speleothems formed from saturated-brine dripwater grow by the precipitation of salts (in this case largely NaCl). The slow-moving saturated brine remains liquid even at low temperatures and remains at a constant water activity (Winston and Bates, 1960) thereby providing a biophysically stable

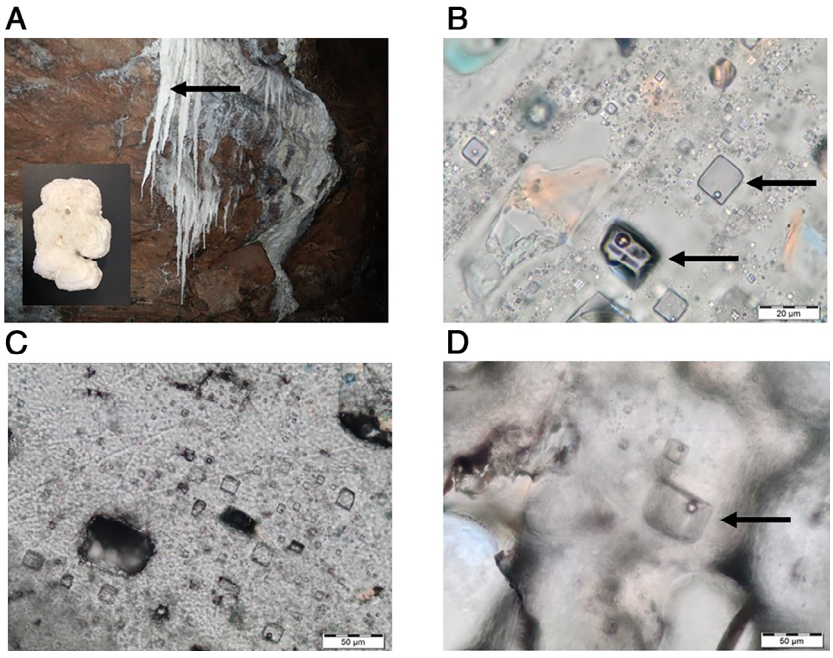


Fig 1. Images of halite "salticle". (A) View of salticle (white), growing on the walls of bedded halite (brown) of the Triassic Mercia Mudstone Group in Kilroot ISME Salt Mine (~ 130 m - 200 m depth below surface), Carrickfergus, Country Antrim, Northern Ireland. The salticles were approximately 10 cm in diameter and 45 cm in height. Inset image shows cross-sectional view with central feeding tube. (B-D) Primary fluid inclusions in salticle halite. [Color figure can be viewed at wileyonlinelibrary.com]

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habitat for halophiles. The age of these salticles is estimated to be between 10 and 15 years (Irish Salt Mining & Exploration Company, Estimate, 2018), and a number have formed within the halite mine at a depth 130–200 m below ground level, and similar structures have been reported previously (Filippi *et al*., 2011). We carried out an elemental analysis of the salticle as well as a nearby sample of primary halite to determine whether the salticle was composed of near-pure NaCl, as the bedrock is known to be. This analysis confirmed that both halite and salticle are abundant in high-grade halite (NaCl) (>99% w/w) with minor quantities of gypsum (<0.55% w/w) (Table 1).

As shown in Fig. 1B–D, fluid inclusions are present in the salticle (also visible in supplementray Video S1); all of these contain brine that may have been trapped during the initial growth of the crystalline structure (Goldstein and Reynolds, 1994). Saturated NaCl has a water activity of about 0.755 and is known to select for extremophiles (halophiles); yet this salt concentration (and water activity) can support biomass dense and biodiverse ecosystems (Lee *et al*., 2018).

Microbial life of the salticle: insights from nearby habitats

We took a three-pronged approach to study the microbiology of the NaCl stalactite. Firstly, we determined the taxa present in the fluid inclusions using a metagenomics-based approach; secondly, we compared the salticle community with those of a nearby, comparable habitat (a brine-pool) and nearby non-saline soil (a stagnosol); and thirdly, we carried out functional analyses of metagenomic data to seek evidence of glycerol metabolism that can be implicated in longevity and/or persister cell formation. The habitats selected for the comparison were a NaCl-saturated brine-pool within the same salt mine (i.e., another type of hypersaline and stable water-activity habitat, but one that is bulk liquid) and a soil ecosystem (a low-NaCl and highly biodiverse surface habitat that overlies the halite deposit and is spatially and temporally highly variable in relation to water activity). The culturome and overall microbial diversity (as determined by culture-independent studies) of Kilroot Salt Mine has been described by Megaw *et al*. (Megaw *et al*., 2020). Therefore, the next step that we took was to characterize the higher-rank taxa and species-level taxa that give rise to the salticle's ecology.

The microbiomes of the salticle, brine-pool and soil exhibited clear differences (Fig. 2A–C). For the salticle sample, 10.15% of reads were *Bacteria* and 89.33% of reads were *Archaea*. For the brine-pool, it was determined that 24.70% of reads were *Bacteria* and 76.40% were *Archaea*. For both salticle and brine-pool, *Archaea* were more abundant than *Bacteria*, but the ratio of

Table 1. Metagenome-assembled genome (MAG) with >90% completeness and <5% contamination.

Bin ID	Completeness (%)	Contamination (%)	Taxonomy (contained)	Taxonomy (sister lineage)	GC	Genome size (Mbp)	Gene count
Saltcle_maxbin_15	95.76	1.71	o__*Halobacteriales*; f__*Halobacteriaceae*	g__*Halorubrum*	65.604	3.444	3508
Saltcle_metabat_01	93.2	3.83	o__*Halobacteriales*; f__*Halobacteriaceae*	s__*Natronomonas_pharaonis*	65.342	3.561	3671
Saltcle_metabat_02	98.41	1.65	o__*Halobacteriales*; f__*Halobacteriaceae*	s__*Haloplanus_natans*	64.811	2.247	2423
Saltcle_metabat_06	96.08	3.76	o__*Halobacteriales*; f__*Halobacteriaceae*	s__*Natronomonas_pharaonis*	63.621	2.783	3102
Saltcle_metabat_10	91.61	2.6	p__*Proteobacteria*; c__*Deltaproteobacteria*	s__*Anaeromyxobacter_dehalogenans*	64.611	5.656	4117
Saltcle_metabat_16	97.11	2.34	o__*Halobacteriales*; f__*Halobacteriaceae*	s_*Natronomonas_pharaonis*	64.175	2.625	2745
Saltcle_metabat_32	92.25	3.17	f__*Halobacteriaceae*; g__*Halorubrum*	s__*Halorubrum_saccharovorum*	68.072	2.561	2752
Saltcle_metabat_40	91.5	0.69	o__*Halobacteriales*; f__*Halobacteriaceae*	s__*Natronomonas_pharaonis*	66.106	2.109	2229
Brine_maxbin_04	95.09	2.53	o__*Halobacteriales*; f__*Halobacteriaceae*	g__*Halorubrum*	65.837	3.17	3361
Brine_metabat_01	97.2	1.45	c__*Gammaproteabacteria*; o__*Oceanospirillales*	g__*Marinobacter*	63.089	2.713	2639

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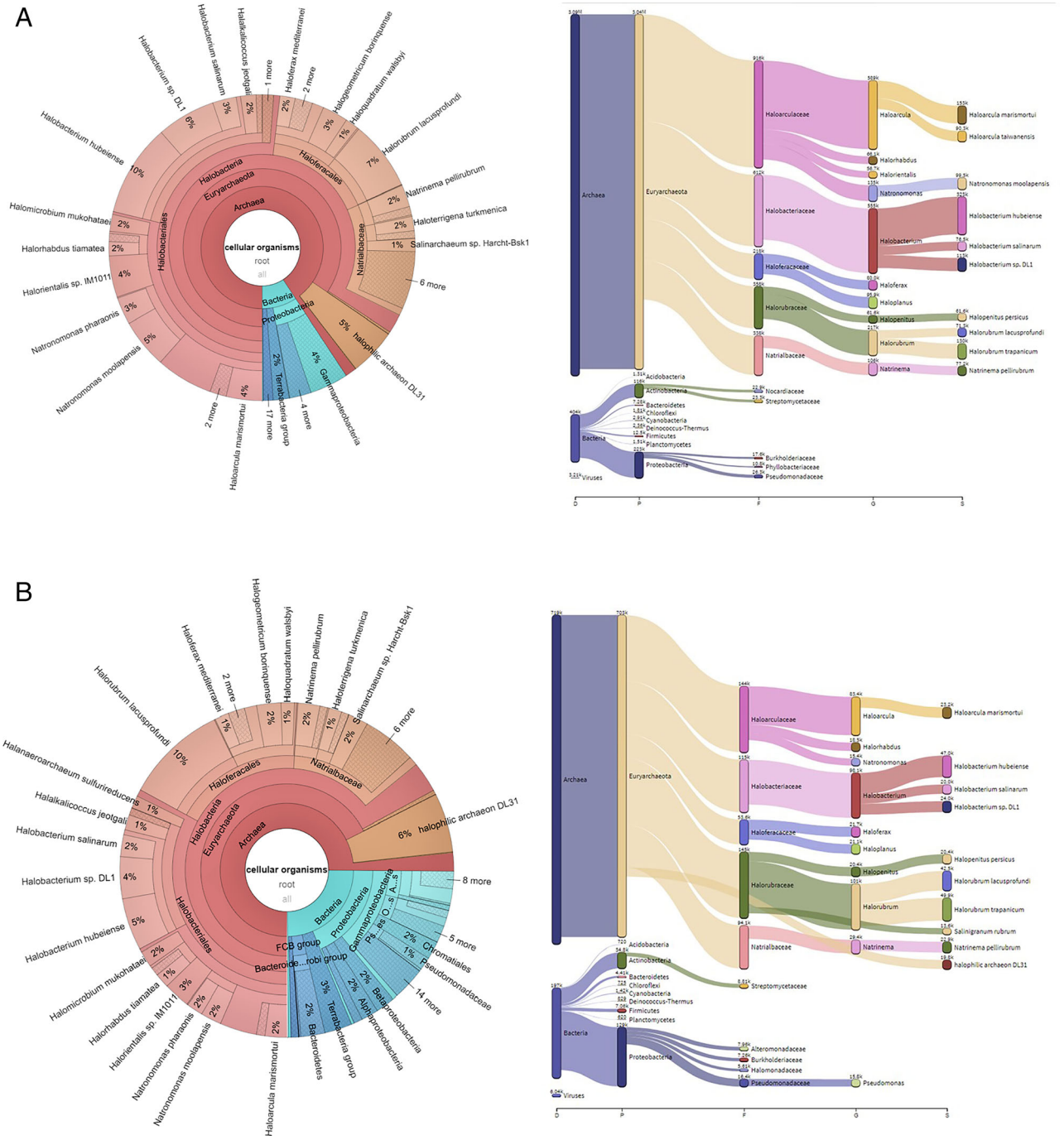


Fig 2. Identification of microorganisms from (A) salticle metagenome, (B) brine-pool metagenome, and (C) soil metagenome. The Sankey diagrams (left) show the flow of reads from the root of the taxonomic tree to more specific levels, summarizing both taxonomic diversity and abundance based on annotation from Kraken 2; the Krona charts (right) are based on Kaiju annotation. [Color figure can be viewed at wileyonlinelibrary.com]

Archaea to *Bacteria* was greater in salticle metagenome, suggesting that the salticle hypersaline fluid inclusions select for archaeal taxa. In the soil metagenome, 99.12% of reads were classified as *Bacteria*, and only 0.59% were *Archaea*. Taxonomic analysis and reconstruction of metagenome-assembled genomes (MAGs) (Table 2) confirmed the high prevalence of halotolerant *Archaea* in the salticle- and brine-pool samples. Usually, high-quality MAGs can be successfully assembled from the most abundant and/or genomically distinct microorganisms. Two MAGs of bacterial origin identified in this study were classified as *Anaeromyxobacter dehalogenans* and

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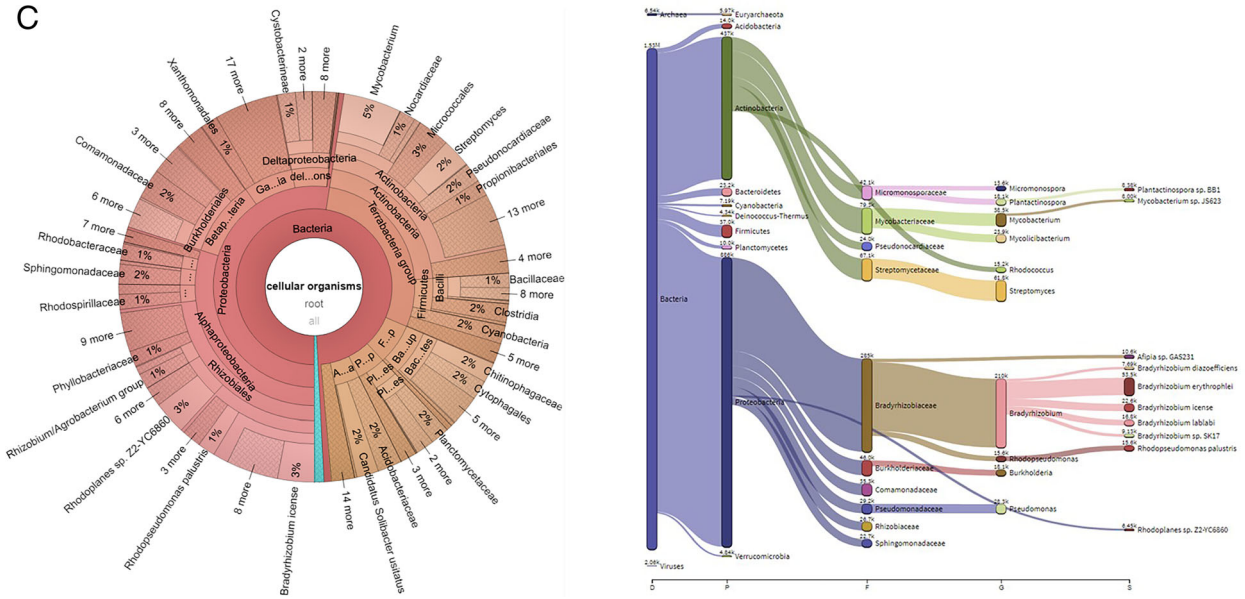


Fig 2. (Continued) [Color figure can be viewed at wileyonlinelibrary.com]

Table 2. Chemical characterization of salticle and primary halite samples obtained from Kilroot Salt Mine. Average values are calculated from repeated measurements are shown, reported as weight (%, w/w).

Element	Salticle	Primary halite
Na	26.770	27.304
Mg	0.140	0.113
Al	0.011	0.030
Si	0.030	0.160
P	-	0.004
S	0.130	0.153
Cl	72.286	71.938
K	0.059	0.047
Ca	0.440	0.400
Fe	0.027	0.034
Zn	0.005	-
Sr	0.006	-
Ge	-	0.004
Br	0.015	0.022
Nd	0.034	0.024

(-) Below detection limits.

Marinobacter sp.. Some species of *Marinobacter* can degrade hydrocarbons, while *A. dehalogenans* can utilize a wide spectrum of electron acceptors for anaerobic respiration including halogenated aryls, which suggests a potential connection between these taxa in terms of energy metabolism (Sanford *et al*., 2002; Xu *et al*., 2018).

Ecophysiological functions of the salticle microbiome

There have been numerous studies investigating the adaptive strategies of halophiles (Gunde-Cimerman *et al*., 2018). In relation to the survival of Haloarchaea within fluid inclusions, it is known that *H. volcanii* can grow on non-carbohydrate sources, by utilizing organic acids excreted by *Microcoleus chthonoplastes* (Zviagintseva *et al*., 1995) and the glycerol excreted by *Dunaliella* sp. (Elevi Bardavid *et al*., 2008). Haloarchaea have numerous strategies which enable them to survive in the presence of multiple stressors including osmotic challenges such as salinity and temperature fluctuation, anaerobic conditions, high-dose ionizing and UV radiation, oxidative stress (Jones and Baxter, 2017), and they possess numerous stress-related proteins to protect the cell (Macario *et al*., 1999). The presence of fluid inclusions in the salticle enabled our investigation of genes that could enable microbial subsistence here. Functional analysis was used to quantify the abundance of genes related to salt tolerance in the salticle microbiome, compared with the brine-pool and soil microbiomes.

Capacity for osmoregulation in NaCl-saturated solutions

Halophiles and extreme halophiles can survive high salt, osmotic stress, low water-activity, freeze–thaw cycles, and desiccation-rehydration cycles. Hyper-osmotic stress is tolerated using two types of osmotic adjustment (Fig. 3) (Gunde-Cimerman *et al*., 2018). The first approach, known as the ‘salt-in’ approach, is used primarily by Haloarchaea and involves the accumulation of molar concentrations of inorganic ions (largely K^+), thereby preventing water efflux in hypersaline environments. The second approach, the ‘salt-out’ strategy, entails the biosynthesis of energetically more-costly

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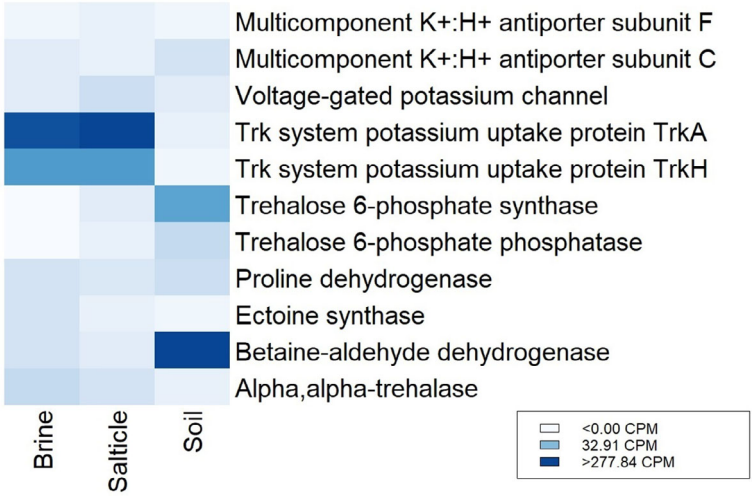


Fig 3. Heatmap representation based on the relative abundance of enzymes related to osmotic adjustment from the salticle-, brine-pool, and soil metagenomes. The colour coding indicates relative abundance, ranging from white (0.00 CPM) to dark blue high abundance (>227.84 CPM). [Color figure can be viewed at wileyonlinelibrary.com]

compatible solutes to increase internal osmolality (Roeßler and Müller, 2001). The most-commonly utilized organic compatible solutes that are produced by most *Bacteria* include trehalose, ectoine, proline, glycine betaine, glucosylglycerol, and dimethylsulfoniopropionate (León *et al*., 2018). There is evidence that some bacterial species may also utilize glycerol for osmotic adjustment and other stress-protection roles (Pavankumar *et al*., 2021).

During high osmotic pressure, the energy source required for the extrusion of Na^+ and accumulation of K^+ during 'salt-in' osmotic adjustment is generally provided through bacteriorhodopsin or anaerobic respiration (Becker *et al*., 2014). The low level of illumination within the Kilroot Salt Mine that is provided by artificial lighting suggests that Haloarchaea are not dependant on bacteriorhodopsin for ATP synthesis. Therefore, the cellular energy required for the 'salt-in' strategy is likely obtained by respiration via nitrate reductase as illustrated in Fig. 4A.

According to the analysis of genes (Fig. 3), a greater abundance of genes related to TrK system potassium uptake protein and voltage-gated potassium channels was observed in the salticle- and brine-pool metagenomes, suggesting that the 'salt-in' strategy is common within the microbiome. The relative abundance of these genes is higher in the salticle metagenome than in the brine-pool microbiome, likely due to the higher ratio of Haloarchaea to bacteria in the former. Metagenomic analysis also revealed a higher abundance of H^+/K^+ antiporters in both salticle- and brine-pool samples; these are known to play a role in potassium efflux during osmotic downshock. For the 'salt-out' strategy, genes related to proline (for proline dehydrogenase synthesis) and ectoine metabolism (for ectoine synthase synthesis) have higher abundance only in the brine-pool metagenome, likely due to the higher proportion of halophilic bacteria than in the salticle metagenome. Somewhat, unexpectedly, the genes involved in the glycine betaine biosynthesis pathway (betaine-aldehyde dehydrogenase) were found in the highest abundance in the soil metagenome. Betaine-aldehyde dehydrogenase is also involved in glycine, serine and threonine metabolism, which may explain this high abundance. Overall, the genes involved in osmoregulation in both salticle, and brine-pool environments are representative of the ratios of halophilic bacteria to Haloarchaea within them. 'Salt-in' Haloarchaea can accumulate potassium ions up to at least 4 M (Mevarech *et al*., 2000) but likely higher concentrations given the lower water-activity limits for growth of some extreme halophiles (Stevenson *et al*., 2015). At such high concentrations, potassium can prevent freezing of the cytosol at low temperatures. The lowest temperatures recorded on Earth are in the range of −60 to −89°C and those typical of the Mars surface are in the range of −40 to −80°C (Carr, 1979).

The abundance of trehalose-6-phosphate synthase- and trehalose-6-phosphatase genes in all three metagenomes suggests insubstantial capacity for production of trehalose in soil, whereby the two hypersaline microbiomes (from salticle and brine-pool) have a greater capacity for the breakdown of trehalose, with a higher abundance of alpha, alpha-trehalase. While trehalose has been thought to be used as an organic solute for osmotic adjustment (Leuko *et al*., 2015), it has a high-molecular weight and low solubility (compared with compounds such as glycerol), so it is not effective for osmotic regulation at water activities below about 0.975 (de Lima Alves *et al*., 2015). However, trehalose also plays a role in tolerance to other stresses, including dehydration-rehydration cycles, oxidative stress, and protecting DNA from radiation damage, and preventing protein

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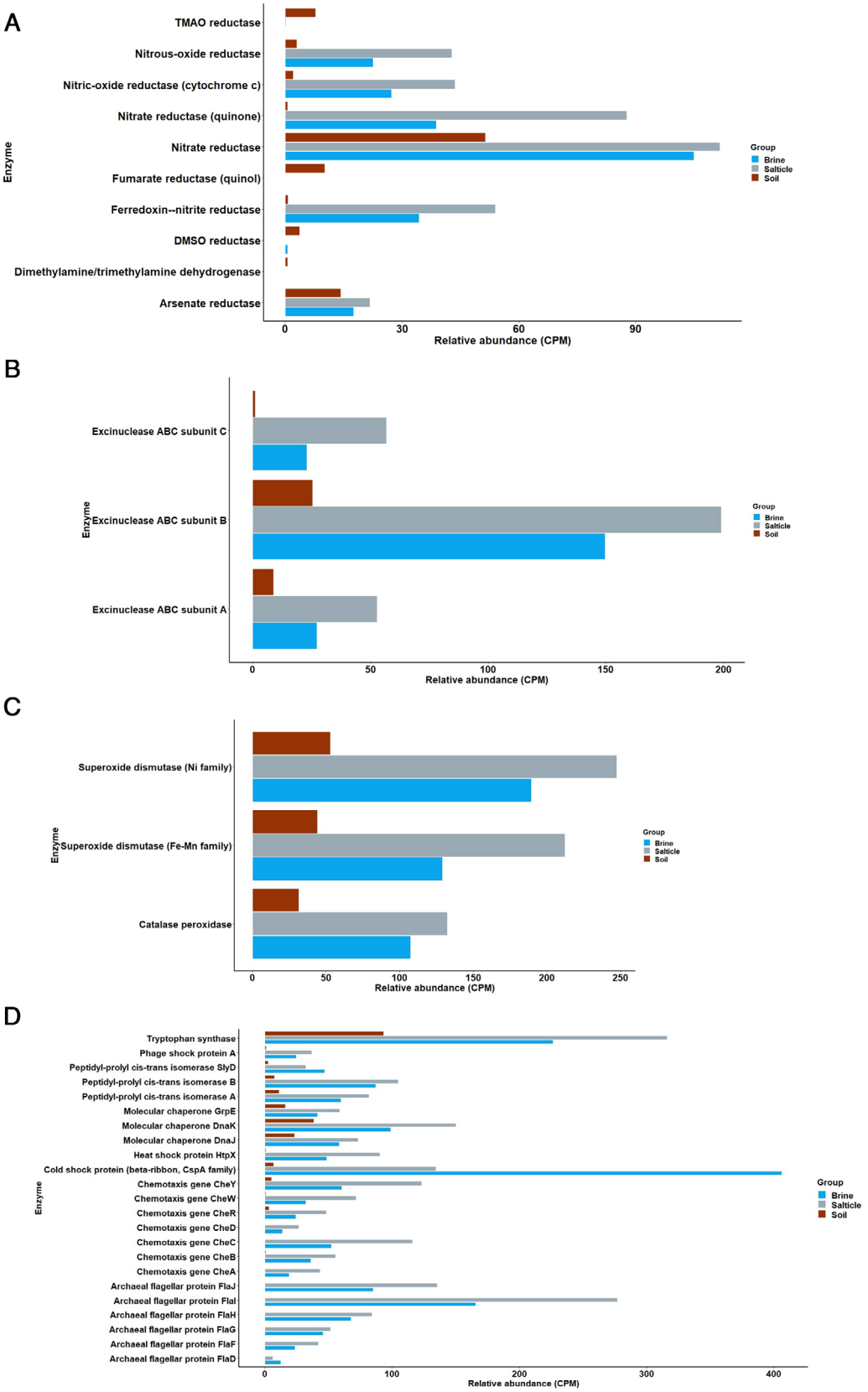


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denaturation (Yoshinaga *et al*., 1997; Carroll *et al*., 2003). Trehalase could facilitate the recycling of bacterial trehalose.

Energy production under salt stress and in the absence of molecular oxygen

Metabolism in high-salt environments, or indeed under any kind of stress, requires considerable energy generation (Hallsworth, 2018). Haloarchaea generally grow as aerobic chemoheterotrophs, but there is limited solubility of oxygen in brines. Some species, such as *Haloferax mediterranei* have developed strategies to enable survival and growth in the absence of oxygen (Matarredona *et al*., 2020). Other halophile species can also use alternative electron acceptors for anaerobic respiration, these include nitrate, (NO_3^-), nitrite (NO_2^-), fumarate ($C_4H_4O_4$), dimethyl sulfoxide (DMSO), and/or trimethylamine N-oxide (TMAO) (Hartmann *et al*., 1980; Oren, 2013; Cherak and Turner, 2017). In the current study, functional analyses of salticle-, brine-pool, and soil metagenomes were used to determine the abundance of genes related to anaerobic respiration, many of which belong within the iron–sulfur molybdoenzyme (CISM) family (Fig. 4A). These analyses revealed that the primary mode of anaerobic respiration in all three ecosystems was via NO_3^- reduction to nitrogen gas (N_2). The core genes involved (coding for nitrate reductase, nitrite reductase, nitric-oxide reductase, and nitrous-oxide reductase) were present at higher abundance than the other CISM-family genes arsenate reductase, DMSO reductase, and TMAO reductase. The functional analyses did not reveal the presence of genes coding for formate dehydrogenase, arsenite oxidase, or polysulfide reductase. To quantify the relative abundance of genes related to N_2 metabolism, the CPM of genes were determined. For nitrate reductase, its abundance was twofold higher in salticle- (111.8 CPM) and brine-pool (105.1 CPM) metagenomes than in the soil metagenome (51.5 CPM). A similar trend was evident for nitrate reductase (quinone); it had a relative abundance of 87.8 CPM in salticle, 38.9 CPM in brine-pool, and 0.6 CPM in the soil metagenomes. For ferredoxin-nitrite reductase, the relative abundance was 54.1 CPM in salticle, 34.4 CPM in brine-pool, and 0.7 CPM in soil. The relative abundance for nitric oxide reductase was 43.7 CPM in salticle, 27.4 CPM in brine-pool, and 2.1 CPM in soil. Finally, nitrous-oxide reductase had a relative abundance of 42.9 CPM in salticle-, 22.6 CPM in brine-pool, and 2.9 CPM in soil. While nitrogen fixation commonly occurs in soils, the higher abundance in the salticle and brine-pool hints at the metabolic versatility of halophiles that can use alternative terminal electron accepters when required.

Paradoxical evidence for UV tolerance of the salticle microbiome

Cyanobacteria, known widely as primary producers, can live heterotrophically in the darkness of the subsurface (Puente-Sánchez *et al*., 2018; Cirigliano *et al*., 2021). Conversely, the salticle microbiome exhibits a considerable capacity for tolerance to high-energy radiation damage. Haloarchaea protect their DNA from intense UV radiation and ionizing radiation via the synthesis of carotenoids, the benefits of polyploidy, and a high GC content in their genomes (McCready *et al*., 2005). They also use several DNA-repair proteins, the excinucleases, which repair DNA after damage caused by UV, osmotic stress, low water-activity, and desiccation (Jones and Baxter, 2017) and can have highly efficient energy-generation systems, which provide the energy to support adaptation and damage repair (Cray *et al*., 2013). Our analysis, (Fig. 4B) revealed a higher abundance of the three UvrABC excinucleases in the hypersaline environments (salticle and brine-pool) than in the soil metagenome. For excinuclease ABC subunit B was 7.8-fold greater in salticle- (199.1 CPM) and 5.8-fold greater in brine-pool (149.7 CPM) metagenomes than in the soil (25.5 CPM). Kilroot Salt Mine has been excavated within an entirely subterranean halite deposit extending to a depth of >300 m. Given the absence of UV radiation in the subsurface, it is likely that the high excinuclease abundances are a product of osmotic stress, indicating a putative role for these enzymes in a more general stress response.

Ability to resist oxidative stresses induced by growth and/or stress

Cellular metabolism can generate considerable amounts of reactive oxygen species (ROS), and stress (including osmotic stress) exacerbates this (Hallsworth, 2018). Therefore, we assessed the abundance of genes involved in the removal of ROS. Genes involved in oxidative-stress response were evident in all three microbiomes (Fig. 4C). There was a considerably higher abundance of catalase, which breaks down hydrogen

Fig 4. Relative abundance of predicted functional enzymes based on the annotation of KO and EC numbers in Uniref 90 and Uniref 50 mode. All data were normalized to show abundance as CPM reads. (A) Anaerobic respiration, (B) UV protection, (C) oxidative stress, (D) stress proteins. The metagenomes for salticle- (dark blue), brine-pool (light blue), and soil (brown) are shown. [Color figure can be viewed at wileyonlinelibrary.com]

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peroxide, and superoxide dismutase, which catalyses the dismutation of superoxide (O_2^-) into molecular oxygen (O_2) in both the salticle- and brine-pool metagenomes. The relative abundance of catalase genes was 4.2-fold higher in the salticle- (132.4 CPM) and 3.4-fold higher in the brine-pool metagenomes (107.5 CPM) than in the soil metagenome (31.7 CPM). Superoxide dismutase (Cu-Zn family) and superoxide dismutase (Fe-Mn family) were 4.67- and 4.8-fold higher in the salticle metagenome (247.7 and 212.5 CPM, respectively), and 3.5- and 2.9-fold higher in the brine-pool metagenome (189.7 and 129.1 CPM, respectively) than in the soil metagenome (53 and 44.2 CPM, respectively).

Proteins involved in responses and adaptations to stress

Haloarcheal genes encoding stress tolerance allows enables subsistence and proliferation in brines (Macario *et al*., 1999; Matarredona *et al*., 2020). The stress-related genes identified in the salticle-, brine-pool, and soil microbiomes are shown in Fig. 4D. There was a considerable increase in the abundance of different types of shock-response protein. The salticle metagenome exhibited much higher abundances of the following proteins compared with the soil metagenome: phage-shock protein (29.6 times higher), heat-shock protein (78.6 times higher), cold-shock protein (18.3 times higher), peptidyl-propyl cis-trans isomerase (PPIase) SlyD (10.7 times higher), PPIase A (5.3 times higher), and PPIase B (10.8 times higher). The brine-pool metagenome also had higher abundances of stress-related genes relative to the soil metagenome; these included phage-shock proteins (19.9 times higher), heat-shock proteins (42.5 times higher), cold-shock proteins (53.3 times higher), SlyD (15.7 times higher), PPIase A (7.2 times higher), and PPIase B (13.1 times higher). For the other PPIases (SlpA, FkpA, FkIB, SurA, PPIase C, PPIase D, and PPIase F), there was no difference in abundance for the three microbiomes under study (data not shown). The physiological role(s) of phage-shock protein is broader than the name suggests and include the regulation of inner-membrane permeability (Flores-Kim and Darwin, 2016). The high abundance of both heat-shock proteins and cold-shock proteins in the salticle metagenome indicates that members of the salticle's microbiome can tolerate biophysical challenges even though there is a steady temperature in the salt mine (approximately 17°C).

Tryptophan synthase is associated with biofilm formation in bacteria (Hamilton *et al*., 2009; Ghosh *et al*., 2019; Harrison *et al*., 2019), and archaeal flagellar proteins, and chemotaxis genes (CheA, CheB, CheC, CheD, CheR, CheW, and CheY) play important roles in cell adhesion and social mobility within biofilms according to studies of *H. volcanii* (Fröls, 2013; Chimileski *et al*., 2014). Biofilms provide a source of energy, and the hydrated extracellular polymeric substances (EPSs) prevent desiccation and protects against other stresses (Fröls, 2013). In addition, there is spatial heterogeneity of metabolic profiles, growth rates, and tolerance to antimicrobial challenges, i.e., a level of complexity that can enhance overall robustness. Biofilm-forming Archaea have been observed previously; in single species such as *Halorubrum lacusprofundia* (Liao *et al*., 2016) and within bacteria-dominated mixed (communities according to studies of surface colonization of glass that was immersed in seawater; (Webster and Negri, 2006). The higher abundance of tryptophan synthase in the hypersaline environments (salticle 3.3-fold higher than soil and brine-pool 2.4-fold higher than soil) suggests the presence of, or ability to form, biofilms or types of aggregates of cells (Simon *et al*., 2002). This is further confirmed by the high abundance of genes encoding archaeal flagellar proteins and chemotaxis response regulator in both the salticle- and brine-pool metagenomes (Fig. 4D).

Dormancy of persister cells versus low-level metabolism of subsisting cells

Glycerol can play key roles in microbial stress tolerance and longevity of subsisting communities (see above). Genes involved in glycerol metabolism are known to be present in the Haloarchaea according to a genomic survey carried out by Williams *et al*. (Williams *et al*., 2017). Among the Haloarchaea, glycerol is metabolized either by the sn-glycerol-3-phosphate pathway (G3P) or the dihydroxyacetone (DHA) pathway. We, therefore, carried out a functional analysis of genes involved in energy metabolism, focusing on glycerol utilization (Fig. 5).

The higher abundance of glycerol kinase in the hypersaline habitats (181.6 CPM in salticle and 112 CPM in brine-pool, versus 15.9 CPM in soil), glycerol-3-phosphate dehydrogenase (G3PDH) (266.4 CPM in salticle and 137.3 CPM in brine-pool versus 9.44 CPM in soil), and fructose-bisphosphate aldolase (225.7 CPM in salticle and 187.8 CPM brine-pool, versus 93.8 CPM in soil) suggest that the G3P metabolic pathway is important for, and likely being utilized by, microbes within the salticle and brine-pool. This is consistent with the low abundance of the gene coding for glycerol dehydrogenase in salticle-, bine-pool-, and soil-metagenomes (Fig. 5), which is the primary enzyme used in the DHA pathway, whereby it oxidizes glycerol to DHA, which is then phosphorylated by DHA kinase to dihydroxyacetone phosphate (DHAP). Within the G3P pathway, glycerol is phosphorylated by glycerol kinase to glycerol-3-phosphate which is then oxidized by G3PDH to DHAP. The metabolic intermediate DHAP is produced within the

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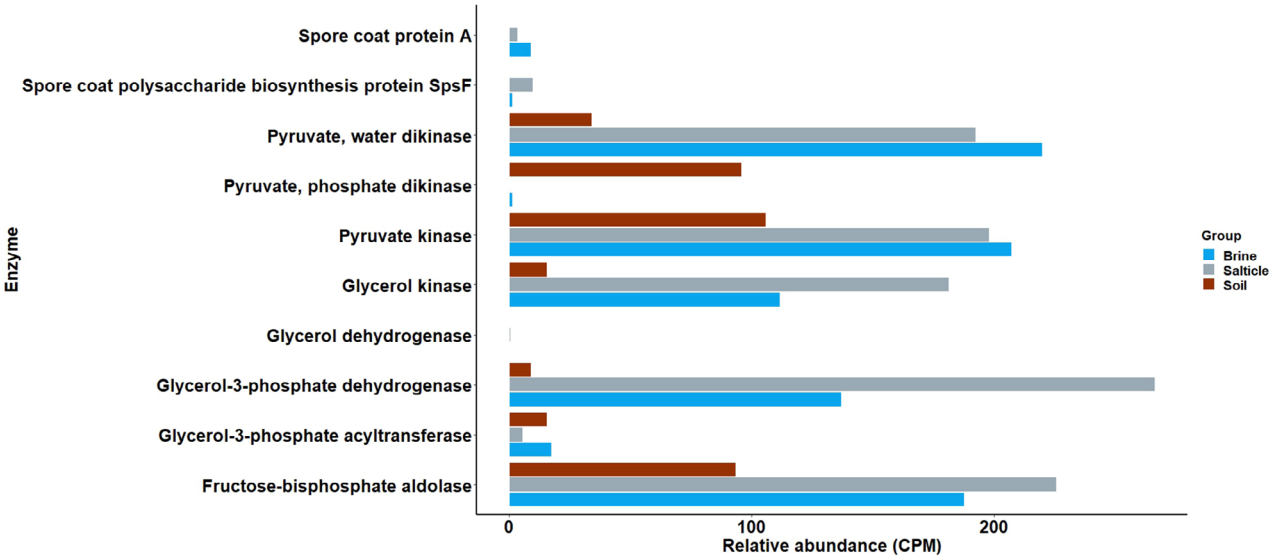


Fig 5. Relative abundance of predicted functional enzymes based on the annotation of KO and EC numbers in Uniref 90 and Uniref 50 mode. All data were normalized to show CPM reads. Genes are related to glycerol utilization and spore coat proteins. The metagenomes for brine (light blue), salticle (dark blue), and soil (brown) are shown. [Color figure can be viewed at wileyonlinelibrary.com]

glycolytic cycle and is reversibly metabolized by fructose-bisphosphate aldolase into fructose 1,6-bisphosphate. Fructose-bisphosphate aldolase is involved in glycolysis, gluconeogenesis, and in the Calvin cycle, which enables downstream ATP production.

Notably, glycerol is one of the only compatible solutes/osmolytes that has a sufficiently low-molecular weight and high solubility to reduce water activity to a level at or below that of saturated NaCl (de Lima Alves *et al*., 2015). Furthermore, the lowest water-activity values at which microbes have been reported capable of biotic activity (differentiation and cell division) was in the presence of high intracellular glycerol concentrations, according to studies of xerophilic/halophilic fungi (Stevenson *et al*., 2017a, 2017b).

Interestingly, Fig. 5 also reveals that pyruvate metabolism differs greatly between the salticle- and brine-pool metagenomes compared with the soil metagenome. While both systems utilize pyruvate kinase, which catalyses the irreversible conversion of pyruvate to phosphoenolpyruvate, there is a stark difference in preference for the use of pyruvate dikinase that carries out the reversible conversion of pyruvate to phosphoenolpyruvate. Within the soil metagenome, there is a much higher abundance of pyruvate phosphate dikinase (96.1 CPM) compared to the salticle- (0.4 CPM) and brine-pool (1.7 CPM) metagenomes. Whereas within the salticle- (192.7 CPM) and brine-pool (219.9 CPM) metagenomes, there is a much greater abundance of pyruvate, water dikinase compared to the soil metagenome (34.4 CPM). The preference for water dikinase may lead to the metabolism-mediated production of water, thus mitigating some effects of osmotic stress.

Other potential indicators of dormancy were also investigated, such as the presence of spore-coat proteins. Their abundance was only marginally higher in the salticle-and brine-pool metagenomes relative to the soil metagenome (data not shown). Glycerol-3-phosphate dehydrogenase and glycerol-3-phosphate acyltransferase have been reported to participate in persister-cell formation in the mesophile *Escherichia coli* (Spoering *et al*., 2006). In the current study, there was no significant difference between the three metagenomes in the abundance of glycerol-3-phosphate acyltransferase, but a higher abundance of glycerol-3-phosphate dehydrogenase was observed in the salticle- and brine-pool metagenomes. Although induction of persister cells mediated via glycerol-3-phosphate dehydrogenase has not been demonstrated in Haloarchaea, the demonstration of persister cell formation in *H. volcanii* as a stress response (Megaw and Gilmore, 2017) alongside the high abundances of this gene within the salticle- and brine-pool metagenomes suggests a similar mechanism for induction of a persister phenotype could exist within this domain. Within fluid inclusions, persister cells could maintain the viability of Haloarchaea and thus promote longevity and long-term survival under stressful conditions. Previous results from Fig. 4D regarding biofilm formation may also aid in persister cell development.

The detection of small amounts of halite in several meteorites, some of which originate from Mars (Gooding, 1992; Treiman *et al*., 2000), has supported the

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notion that Martian microbial life could still be present on Mars given that Earth and Mars have a similar geological past (Cabrol, 2021). Leuko *et al*. (Leuko *et al*., 2010) proposed that the search for life on Mars should involve looking for shelters of Haloarchaea such as stromatolite formations since Haloarchaeal strains have been isolated from modern terrestrial stromatolites and ancient terrestrial halite. The capacity of Haloarchaea to survive indefinite timescales within fluid inclusions makes them popular as models for astrobiology. The possibility of salticles in the (warm) subsurface of Mars raises intriguing questions about the possible habitat types for putative Martian life.

Experimental procedures

Site description

The ISME (Irish Salt Mining and Exploration, Ltd) Kilroot salt mine in Carrickfergus, County Antrim, Northern Ireland (54.7331° N, 5.7509° W), is a drive-in, underground mine in the Larne Halite Member of the early Triassic Mercia Mudstone Group. The bedded salt mined here has been interpreted as deposits of shallow acid salt lakes that existed in northeastern Pangaea approximately 250 million years ago (Andeskie *et al*. 2018). Primary fluid inclusions in the Larne Halite Member are well-preserved remnants of these Triassic acid (pH values ~1-3), brine lake waters. In addition to acid brines, these inclusions also preserve Triassic air bubbles, sulphate minerals, and suspect microorganisms, including Dunaliella algae (Eichenlaub, 2016; Eichenlaub *et al*., 2016). The salticles of this current study are a product of partial dissolution and reprecipitation of the bedded halite of the Larne Halite.

Petrography of fluid inclusions

A 45-cm long (10-cm diameter) salticle and a 1-kg block of primary halite were collected from the salt mine in July 2018. For the current study, the salticle was collected from depth 130 m to 200 m below ground level range from the ceiling of the mine. The primary halite was collected from 60 m to 450 m below ground level. Salticle and primary halite were used to carry out petrographic determinations of elemental composition (see below). Thin cross sections of each were prepared by cutting along the marked lines using an oil-based lubricant (by private contractor John Turner, Bridport, Dorset, UK). The flat surfaces of these sections were embedded in epoxy resin and mounted on to 2-mm glass block. These sections were then ground in oil-based lubricant to 40 μm, the thickness being checked by micrometre and halite birefringence. These sections were then examined and photographed using an Olympus SC30 stereo microscope, at 40× and 100× magnification.

Determination of elemental composition

Qualitative and quantitative analyses were carried out using wavelength dispersive X-ray fluorescence (Panalytical Axios mAX WDXRF spectrometer, ITAlabs) to determine the elemental composition of the salticle and primary halite. These analyses were conducted by ITA Labs Materials Testing Solutions of the International Tin Association (St Albans, UK).

Sample collection and DNA extraction

Metagenomic DNA was extracted from the salticle and brine-pool sample. The salticle used for DNA extraction was approximately 10 cm in diameter, 45 cm in height, and weighed 0.9 kg. It was placed into a sterile container and transported immediately to the laboratory. The entire salticle was dissolved in nuclease-free water and passed through a 0.22-μm nitrocellulose-membrane filter (Merck, UK) to collect any cells and insoluble particles onto the filter surface. Total DNA was extracted from the salticle using a modified version of the method described by Radax *et al*. (2001). Following storage at −80°C for several days, filters were thawed and cut into small (approx. 1 × 1 cm sizes) pieces, which were vortexed in 5 ml Tris–HCl EDTA (TE) buffer [100 mM Tris–HCl, 10 mM ethylenediaminetetraacetic acid (EDTA)], pH 8.0 and containing 1% w/v SDS. Acid-washed glass beads were then added, along with 50 μl lysozyme solution (Sigma-Aldrich, UK; 1% w/v in TE buffer), samples were vortexed for 2 min and then incubated at 37°C for 1 h. Following boiling for 10 min and then vortexing for a further 2 min, 1 ml lysis buffer (4% w/v SDS in 50 mM Tris–HCl, 100 mM EDTA, pH 8.0) and 40 μl proteinase K solution (taken from a 10 mg/ml stock solution) were added. Thermal shock was performed by freezing at −80°C for 5 min and then boiling at −100°C for 5 min, samples were then vortexed for 1 min and incubated at 56°C for 1 h. Lysis was followed by RNase A (10 mg/ml) (Sigma-Aldrich) depletion with incubation for 2 min at 18°C. The DNA purification was carried by following the 1:1 phenol/chloroform method (Marmur 1961) on the lysate solution. The solution was centrifuged for a few seconds, and the aqueous phase was carefully removed; 0.1 volume of 2 M sodium acetate, pH 4.8, and 3 volumes of EtOH (96%) were added, and the sample was kept overnight at −20°C. After centrifuging at 12 800*g* for 45 min at 4°C and removal of the supernatant, pellets were air dried. DNA was then purified using the GeneJET PCR Purification Kit (Thermo Scientific™, UK) as per the manufacturer's instructions.

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The brine-pool sample (each 4 l) was collected in sterile bottles from a saturated-brine pool inside the salt mine, and the cells from the brine were concentrated onto the surface of a 0.22-μm nitrocellulose membrane by filtering the 4 l of brine through the membrane under vacuum. This membrane was then cut into several pieces with a sterile scalpel, and DNA was extracted using GenElute™ Bacterial Genomic DNA Extraction kit (Sigma-Aldrich) following the manufacturer's instructions. The soil metagenome (ERX1184011) was accessed from the NCBI database (https://www.ncbi.nlm.nih.gov/bioproject/PRJEB11634) and is from slightly acidic stagnosol soil cores taken from agricultural (pasture) fields located in the vicinity of the salt mine (54.077286° N, 6.003420° W).

Metagenomic sequencing

Quality control was performed on the metagenomic datasets using FastQC (Simon Andrews, 2020), and reads were processed with BBMap v 38.73 (Bushnell, 2014). Reads were trimmed of adapters, and all reads with an average Q-score < 10 or containing Ns were discarded. The raw nucleotide sequences of the metagenomic data for the brine and the salticle samples were deposited as fastQ files at Zenodo.

Taxonomic analysis and functional characterization

After application of read quality filters, the profile of microbial taxonomic composition of the metagenomes was performed with Kraken 2 (Wood *et al*., 2019), using the minikraken2 version2 database. The results from the Kraken 2 were analysed and visualized by Pavian version 1.0.0 (Breitwieser and Salzberg, 2019). Kaiju, a web-based server (http://kaiju.binf.ku.dk), was utilized with the RefSeq Genomes selected as the reference database. 'Greedy' run mode was applied with a minimum match score of 75, and an allowance of five mismatches (Menzel *et al*., 2016). Taxonomic comparisons of the salticle-, brine-pool, and soil samples were made using Krona charts (Ondov *et al*., 2011) with cellular organisms placed at the root. To understand the functional profile of each environment, functionally profiling of all metagenomes was conducted using v3.0 of HUMAnN (Franzosa *et al*., 2018). The abundances of gene families were summed according to Enzyme Commission (EC) number annotation in UniRef 90 and UniRef 50 modes. The reads were also annotated according to KEGG Orthology (KO) in UniRef 90 and UniRef 50 modes. Per sample gene abundances were normalized to 'copies per million' (CPM) units and stratified according to known/unclassified community contributions. Data analyses were performed using R Studio 1.2.5033 platform (RStudio Team, 2015), and graphs generated using ggplot2 (Valero-Mora, 2010).

The agricultural soil metagenome was used as a comparison to understand the basal abundance of genes in a nearby environment that is not hypersaline. This was then used as a comparison against the salticle and brine-pool metagenomes from the salt mine, to identify genes with higher relative abundances that may be responsible for support of microbial life in saturated NaCl solutions within fluid inclusions of the salticle and within the brine-pool.

Metagenome binning

Metagenome assembly and binning was performed using ATLAS (Kieser *et al*., 2019) using the default parameter settings. The completeness and contamination levels of metagenomically assembled genomes (MAGs) were assessed using Anvi'o (Eren *et al*., 2015).

Conclusion

The current study provides insights into the microbiology of inclusions within a NaCl salticle, and the functional analysis revealed that many genes, such as those related to glycerol metabolism, can enable longevity within these inclusions are in high abundance with respect to the mesophilic environment. Whether microbes trapped within these fluid inclusions are inactive and in a persister state or whether they remain active (albeit at low metabolic rates) has yet to be resolved.

Notably, the microbiology of the salticle differed from that of the nearby brine-pool. This study suggests that salt speleothems (e.g. salticle structures) can provide habitats for putative life beyond Earth, including the Martian subsurface. The Mars 2020 Perseverance Rover has four main goals: assess past habitability of an astrobiologically relevant site on Mars, search for potential biosignatures and signs of ancient microbial life, cache samples from the shallow subsurface, and prepare for human exploration (NASA, 2020). We look forward to these findings, as well as future missions designed to access and assess habitability of the deep Martian subsurface.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Video S1A Supplementary Movie S1(A –B) Confirmation of fluid inclusions is provided by observation of the movement of included bodies within the inclusion by Brownian motion. The recording was made using an Olympus SC30 stereo microscope.

Video S2B Supporting information

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