Solutes determine the temperature windows for microbial survival and growth

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Microbial cells, and ultimately the Earth's biosphere, function within a narrow range of physicochemical conditions. For the majority of ecosystems, productivity is cold-limited, and it is microbes that represent the failure point. This study was carried out to determine if naturally occurring solutes can extend the temperature windows for activity of microorganisms. We found that substances known to disorder cellular macromolecules (chaotropes) did expand microbial growth windows, fungi preferentially accumulated chaotropic metabolites at low temperature, and chemical activities of solutes determined microbial survival at extremes of temperature as well as pressure. This information can enhance the precision of models used to predict if extraterrestrial and other hostile environments are able to support life; furthermore, chaotropes may be used to extend the growth windows for key microbes, such as saprotrophs, in cold ecosystems and manmade biomes.

biosphere function | chaotropic agents | fungal ecology and limits of life | osmotic stress | psychrophilic bacteria

Collectively, microorganisms are responsible for moderating the composition of the Earth's atmosphere, including biogeochemical cycling of nutrients and minerals, soil formation and fertility, plant health, and ecosystem development and sustainability. A large proportion of the Earth's terrestrial surface is either permanently frozen or at temperatures (i.e., < 10–15 °C) that are below the growth optimum for the vast majority of microbes; this includes the Arctic, Alaska, Canada, most of the European Continent, the Himalayas, the Andes, Argentina, New Zealand, and Antarctica (1, 2). The temperatures of the Earth's oceans at ≤500 m (a major microbial habitat) (3) and those of the sea surface in temperate and polar regions typically range from 12 °C downward. Low-temperature environments have a limited microbial biomass and diversity, and even cold-tolerant microbes able to grow at ≤4 °C can have relatively high-temperature optima for growth (≥20 °C) (4).

For one species of bacterium, *Escherichia coli*, we know that an individual gene coding for chaperonin production can determine the limit of low-temperature tolerance (5). Indeed, it is the interactions between environmental parameters such as temperature and water activity on the one hand and the structure and function of cellular macromolecules on the other that define the physicochemical windows of life, their mechanistic bases, and the ecophysiological adaptive responses that they induce. In other words, it is ultimately the cellular phenotype that lies at the core of approaches to understanding life processes, their ecology, and evolution. A number of studies have characterized the parameters that determine the limits of microbial life at high temperature (e.g. 6). Although there have been numerous reports of microbial growth below 0 °C (indeed, down to temperatures between −20 °C and −10 °C) (Table S1), the mechanistic basis of cell failure below these temperatures has not yet been elucidated for most microbial species. We carried out a series of studies to investigate the cross-talk between different stress parameters, focusing on the way in which the solute activities of environmentally relevant substances can modify the growth windows and survival capabilities of microbial cells at low temperatures. Structural interactions within and between cellular macromolecules are dependent, either directly or otherwise, on water molecules (7–9). Generally low temperatures promote noncovalent interactions and, thereby, rigidify cellular macromolecules and membranes (2). Conversely, chaotropic solutes are known to disorder macromolecular systems, inducing a potent form of cellular stress (10), and they are known to limit the functional biosphere in some locations on Earth (11–13).* We formulated the hypothesis that chaotropic substances which under many circumstances act as stressors can, nevertheless, enhance cellular activity at suboptimal growth temperatures and thereby extend the biotic windows of microbial cells in cold environments. To test our hypothesis, we recruited microbes from two classes of extremophile; those that are cold-adapted (psychrophilic) and those that are highly solute-tolerant (xerophiles, including those that are halophilic and osmophilic). A range of extremely psychrophilic microbes from diverse taxonomic groups were screened for solute tolerance on the basis of these data (Fig. 1 and Table S2), the most solute-tolerant of these—the yeast *Mrakia frigida* (Fig. 1*A*)—was selected for further study. One hundred sixty-one of the most xerophilic microbes known to science (all fungi) (13) were screened for low-temperature tolerance at 15 °C, and the seven most cold-tolerant strains were selected for further study. These extremophiles were used as model systems to investigate the interplay between the chemical activities of solutes and the temperature windows for microbial growth.

Results and Discussion

Chaotropicity Extends the Window for Life at Low Temperature. Radial growth rates of four xerophiles found to be the most cold-tolerant reached ≥1 mm day^{-1}, and strains grew equally well on media supplemented with kosmotropic or chaotropic solutes at 30 °C (Fig. 2 *A*, *E*, *I*, and *M*). At low temperatures (+5 °C and +1.7 °C), microbial activity was not only diminished, but in contrast to higher temperatures, growth rates were optimal on media supplemented with fructose (Fig. 2 *D*, *H*, *L*, and *P*), which is chaotropic. Furthermore, rates were relatively low on media supplemented with kosmotropic solutes; indeed, there was no

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*Chaotropic activity is a catch-all expression used to describe the behaviors of chemically diverse substances that disrupt noncovalent interactions and/or hydrophobic forces within and between organic macromolecules and their structures (14); kosmotropic activity describes the behavior of substances that promote interactions within and between macromolecular structures (15). Chaotropic and kosmotropic activities (*i*) are macromolecule-level phenomena and not properties of pure chaotrope/kosmotrope solutions (16), (*ii*) are solvent-specific, (*iii*) may not correlate absolutely with Hofmeister effects, and (*iv*) are neither restricted to ionic solutes nor to macromolecular structures that are proteinaceous.

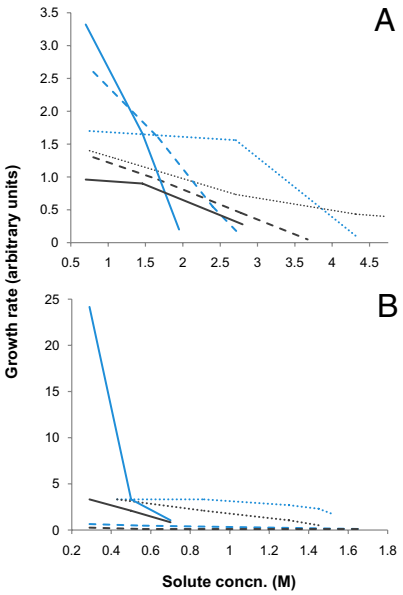

Fig. 1. Growth profiles for two model psychrophiles. (*A*) *M. frigida* and (*B*) *Psychrobacter urativorans* on media supplemented with sucrose (continuous line), glucose (dashed line), and glycerol (dotted line) at either +1.7 °C (blue) or −5 °C (gray). Plots were constructed using Microsoft Office Excel 2007 based on growth-rate data shown in Table S2. Growth-rate units were derived by calculating the inverse of log (time taken for cultures to attain maximum colony density in weeks) (Table S2). Relative to the optimum activity of *P. urativorans* (*B*), growth rates were reduced by ≥85% under solute stress. By contrast, *M. frigida* (*A*) was able to retain high levels of activity even as solute concentrations increased.

growth on some kosmotrope-supplemented media at low temperatures for all four strains (Fig. 2 *C*, *D*, *H*, *L*, and *P*). Collectively, these data demonstrated an apparently potent promotion of growth at low temperature by a chaotropic solute, irrespective of fungal species.

To establish if this phenomenon was particular to xerophilic ascomycetes, fructose-supplemented media, and/or temperatures over 0 °C, we carried out a Spot-Test growth assay (17). The assay was used to determine the biotic window for the psychrophile *M. frigida*, a basidiomycete with a planktonic growth form, on media containing either the kosmotrope sucrose (Fig. 2*Q*) or one of a range of chemically diverse chaotropes (i.e., methanol, $MgCl_2$, and glycerol) (Fig. 2 *R*–*T*). Although the mechanisms by which diverse solutes exert chaotropic activity are not yet well-understood, the net effects of ionic, nonionic, and hydrophobic chaotropes (9, 10, 18) may be indistinguishable to the cell (13).[†] Our data show that *M. frigida* growth was rapid at the highest incubation temperature (+1.7 °C), regardless of medium type, but that growth rates were greatly enhanced on all chaotrope-supplemented media relative to that on the kosmotropic medium at subzero temperatures (Fig. 2 *Q*–*T*). Generally, sucrose is highly permissive for growth of *M. frigida* (Fig. 1*A* and Table S2); furthermore, the water activity of some chaotrope-supplemented media (e.g., 1.1 M glycerol; 0.978 a_w at +1.7 °C) is marginally more stressful for this yeast than that of the sucrose medium (0.73 M sucrose; 0.982 a_w at +1.7 °C). Collectively, therefore, these data support the hypothesis that exogenously supplied chaotropic substances can enhance cellular function in microbial habitats. We were, therefore, curious to see if microbes were able to synthesize and/or accumulate chaotropic metabolites intracellularly at low temperatures.

Fungi Preferentially Accumulate Chaotropic Metabolites at Low Temperature. A xerophile strain able to grow well at temperatures close to 0 °C (Fig. 2*P*) was used as a model organism to study intracellular stress metabolites (compatible solutes) during growth on fructose- and sucrose-supplemented media over a range of incubation temperatures (Fig. 3). Whereas the growth rate was more than 200% higher on the kosmotropic sucrose medium (Fig. 3*B*) relative to that observed on the chaotropic fructose medium at 30 °C (Fig. 3*A*), the converse was true at low temperatures (+5 °C and +1.7 °C). On fructose-supplemented media, fungal hyphae were able to accumulate higher concentrations of fructose (up to 126 μg mg^{-1}) (Fig. 3*A*), suggesting that intracellular fructose, a known compatible solute, may facilitate osmotic adjustment (20). The possibility remains that the intracellular fructose accumulated at +5 °C and +1.7 °C (up to 20 μg mg^{-1}) may counter the macromolecular rigidification induced by low temperature through its chaotropic activity, thereby facilitating sustained growth down to 1.7 °C (Fig. 3*A*). Fungi grown on the sucrose-supplemented medium did not accumulate sucrose nor did they synthesize a kosmotropic-compatible solute such as trehalose, mannitol, arabitol, or erythritol. In accordance with our hypothesis (but nevertheless, to our surprise), cells preferentially synthesized and accumulated a chaotropic-compatible solute, glycerol (Fig. 3*B*) (13). It is energy expensive to both synthesize compatible solutes de novo and to retain them in the cell, and indeed, the cost imposed to the cellular system is known to determine the limits of microbial growth windows under stressful conditions (e.g., low water activity or ethanol stress) (21, 22). The energy expenditure required for the retention of glycerol, in particular, under stressful conditions may explain why the levels of this compatible solute decreased sharply at low temperatures and why the failure of the growth window at ≤1.7 °C corresponded with a loss of this intracellular chaotrope (Fig. 3*B*).

Solute Activities Determine Survival of Spores Exposed to Extreme Temperatures and Pressures. Having obtained evidence that the physicochemical activities of extra- and intracellular solutes can modify microbial growth windows at low temperature, we asked if chaotropic/kosmotropic activity can enhance survival of microbial cells under extreme conditions. Microbial propagules not only represent a key stage in the life cycles of many species (reproduction and dispersal), but they are also strategically important for their ecology and evolution (genetic variation, survival, and stress resistance). To answer our scientific question, we exposed microbial propagules, conidia of xerophilic fungi, to temperature extremes (freezing down to −80 °C and heating up to +65 °C) and high pressures (up to 750 MPa). Fungi were cultivated on chaotrope- or kosmotrope-supplemented media, and conidia were harvested in chaotrope- or kosmotrope-supplemented solutions. Then, the resulting spore suspensions were exposed to extreme temperatures and pressures (Fig. 4). Whereas conidia subjected to chaotrope treatments lost between 30% and 93% of their viability at high temperatures and high pressures (regardless of fungal species), there was virtually no loss of viability (≤5%) after a 24-h period of exposure to temperatures of −20 °C or −80 °C (Fig. 4 *A*, *C*, and *E*). The converse trend was observed for kosmotrope-treated conidia, which lost up to 60% viability after exposure to low temperatures but survived relatively well (up to 96%) after exposure to high temperatures and pressures, regardless of the solute (Fig. 4 *B*, *D*, and *F*).

Concluding Remarks. In conclusion, the solute activities of environmentally relevant substances determined the temperature windows for both survival and growth of microbial cells, and the

[†]There is increasing evidence that chaotropic substances disorder macromolecular structures through diverse mechanisms, and it is likely that a number of mechanistic frameworks will be developed within the current decade to further elucidate their modes of action, particularly for ions (18, 19) and hydrocarbons (9).

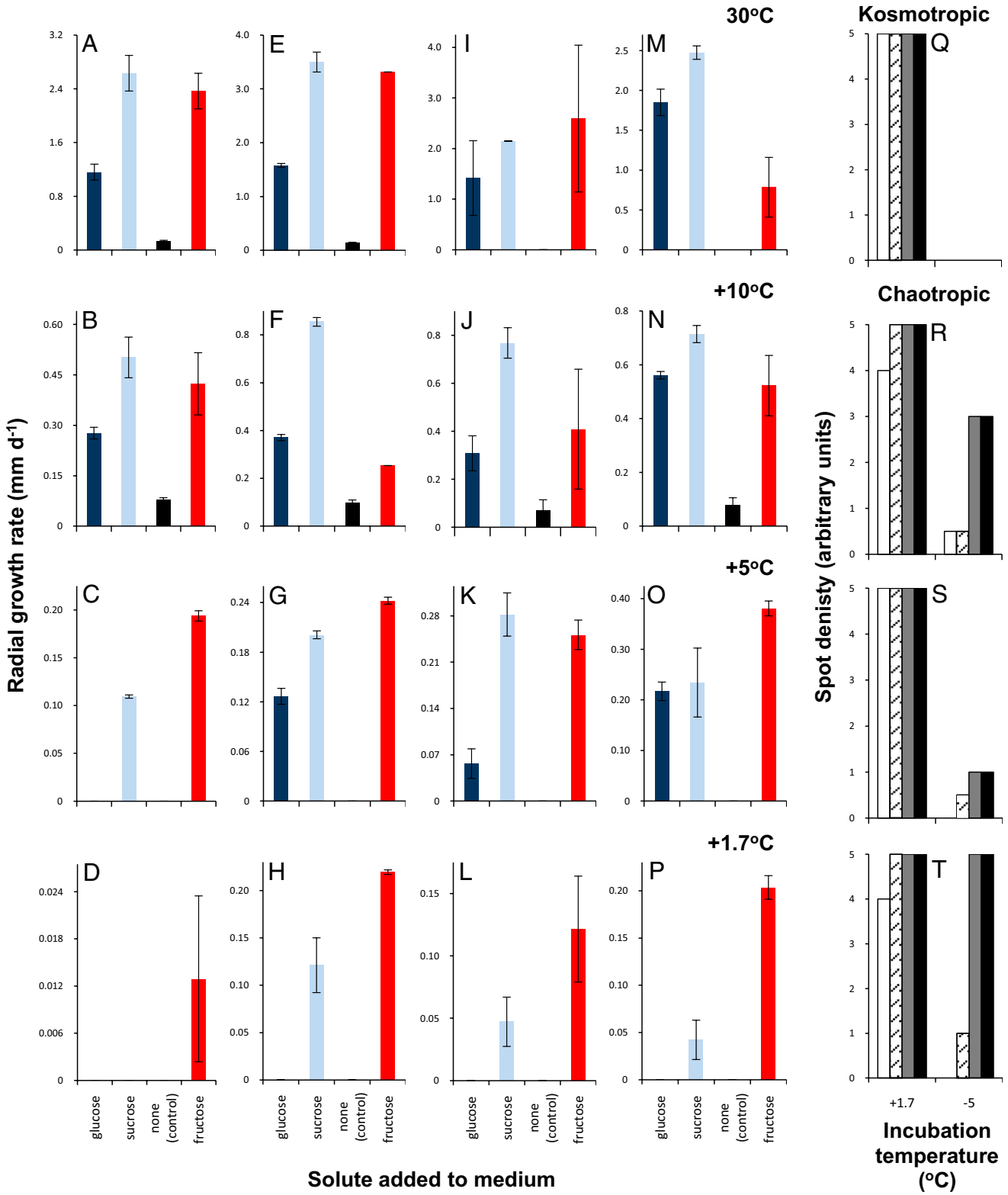

Fig. 2. Growth of ecophysiologically diverse microbes on kosmotrope- and chaotrope-supplemented malt-extract, yeast-extract phosphate agar (MYPiA) nutrient media over a range of incubation temperatures. Radial growth rates of four xerophilic fungi [*Eurotium herbariorum* FRR 2418 (*A–D*), isolate JH06IN47 (*E–H*), *E. herbariorum* FRR 5354 (*I–L*), and isolate JW07JP13 (*M–P*)] on media with no added solute (control medium at 0.997 a_w shown in black), and media supplemented with kosmotropic (glucose at 3.8 M and 0.851 a_w shown in blue–black; sucrose at 2.2 M and 0.880 a_w shown in sky blue) or chaotropic solute(s) (fructose at 2.6 M and 0.929 a_w shown in red) at 30 °C (*A*, *E*, *I*, and *M*), 10 °C (*B*, *F*, *J*, and *N*), 5 °C (*C*, *G*, *K*, and *O*), and 1.7 °C (*D*, *H*, *L*, and *P*). Colony density of the psychrophile *M. frigida* DSM 70883 after incubation for 17 days (white), 24 days (hashed), 45 days (gray), and 59 days (black) at +1.7 °C and −5 °C on MYPiA supplemented with (*Q*) the kosmotropic solute sucrose (0.73 M and 0.982 a_w), and the chaotropic solutes (*R*) methanol (1.56 M and a_w not determined; *SI Materials and Methods*), (*S*) $MgCl_2$ (1.0 M and 0.934 a_w), and (*T*) glycerol (1.1 M and 0.978 a_w). Mean growth rates, calculated from three independent triplicate treatments, were plotted, and bars indicate SEM (*A–P*).

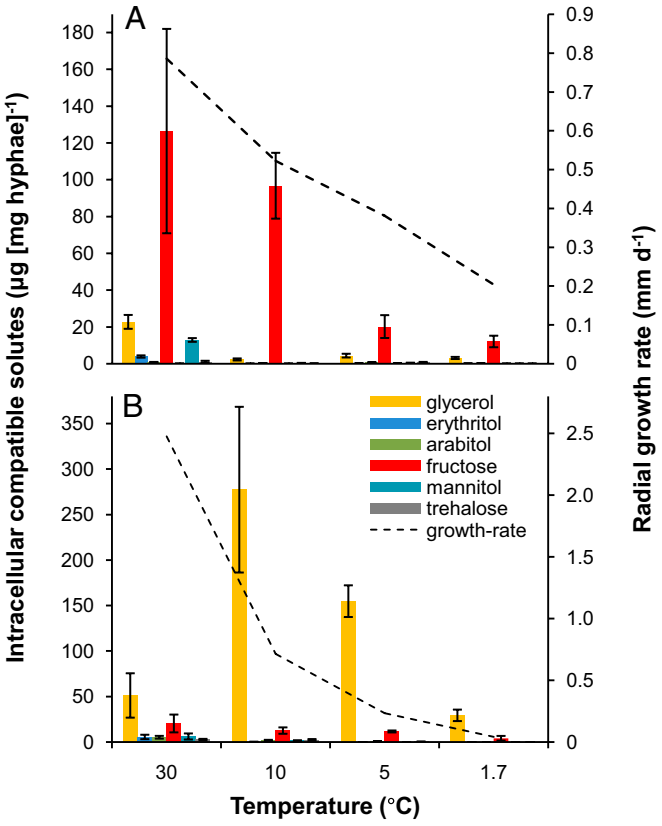

Fig. 3. Intracellular compatible solutes and radial growth rates of xerophile isolate JW07JP13 on MYPiA media supplemented with (*A*) a chaotropic (fructose at 2.6 M) or (*B*) a kosmotropic solute (sucrose at 2.2 M) over a range of incubation temperatures (+30 to +1.7 °C). Compatible solute concentrations are mean values calculated from extractions and analyses of three independent triplicate treatments, and the bars indicate SEM.

mechanistic basis of this phenomenon correlates with the way in which physicochemically diverse stress parameters influence the structural interactions of cellular macromolecules. Whereas high temperatures and chaotropic activity disrupt interactions (10), factors such as desiccation, kosmotropic solutes, and low temperature promote interactions (15, 23, 24). High pressures are frequently associated with increased temperatures that can disrupt macromolecular interactions, and high pressures alone can induce protein denaturation (25); our results suggest that kosmotropes counter these effects. On the one hand, there is evidence that high pressures stabilize microbial cell membranes (26), and on the other hand, data from diverse sources are consistent with our findings. (*i*) High-temperature treatment exacerbates the lethality of high pressures for microbial spores (27). (*ii*) Sucrose and NaCl (both kosmotropic) protect *Lactococcus lactis* against potentially lethal high-pressure treatments (28). (*iii*) Low temperatures enhance pressure tolerance of stationary phase *E. coli* cells (29). (*iv*) Enzyme and membrane assays show metabolic activities enhanced by glycerol at low temperature (10), and (*v*) studies show ethanol-induced cold tolerance of yeasts and bacteria (11, 24).

Whereas the vast majority of compatible solutes are kosmotropic, it is remarkable to consider that microbial cells may be genetically hardwired to preferentially produce and/or accumulate chaotropic metabolites, such as glycerol and fructose, under conditions that promote macromolecular interactions to an extent that limits metabolic activity and cell division (e.g., temperatures close to and below 0 °C). Environmental parameters and intracellular stress metabolites that determine microbial growth windows ultimately determine the extent of the functional biosphere (5, 12, 13, 20, 30–32). The finding that chaotropicity can intervene in potentially lethal processes has implications for microbial activity in the Earth's biosphere (many microbial habitats contain chaotropes: $MgCl_2$, $CaCl_2$, glycerol, fructose, urea, aromatics such as phenol, etc.) as well as for other planetary bodies. Recent analyses of extraterrestrial environments indicate that planetary bodies such as Mars, Europa, and the Earth's moon have both water and high concentrations of chaotropic ions/salts including $CaCl_2$ and ClO_4^- (33–37). The findings of our study have implications for the feasibility of cellular function in such environments (13, 38, 39). For instance, chaotropic ions in the Mars regolith might favor the growth of putative early microbial life on Mars at low temperature; it is intriguing to ask what conditions microbial saprotrophs would require to create an agriculturally productive, self-sustaining human base on the moon (40). Knowledge-based approaches to facilitating the activity of saprotrophic microbes that are known to fail in cold-limited ecosystems (41–43) could lead to the use of environments that have hitherto remained hostile to life (44). For example, environmentally innocuous soil fertilizers such as ammonium nitrate and urea not only reduce the freezing point of water, but they are biologically permissive for microbial growth and yet, moderately chaotropic (10). Experimental microcosms and mathematical models used to predict potential biosphere function in extraterrestrial and other hostile environments (31, 45) should in the future incorporate physicochemical activities of environmentally prevalent solutes such as chaotropes.

Materials and Methods

Identification of Solute-Tolerant Psychrophilic and Cold-Tolerant Xerophilic Microbes. A range of taxonomically diverse extremophilic microbes were screened for stress tolerance on solute-supplemented nutrient media (10, 46) over a range of temperatures; these included algae, fungi and yeasts, bacteria and Archaea (*SI Materials and Methods*). Water-activity values were quantified using a Novasina IC-II water-activity machine fitted with an alcohol-resistant humidity sensor and eVALC alcohol filter (Novasina), as described previously (47). The most solute-tolerant psychrophiles and most cold-tolerant xerophiles were selected for further study, as described in *SI Materials and Methods*.

Low-Temperature Tolerance of Diverse Extremophiles on Solute-Supplemented Media. Four ecophysiologically distinct xerophilic fungi and the psychrophilic yeast *M. frigida* were used to test if chaotropic and kosmotropic activities of solutes affect cold-tolerance; details of the experimental approach are described in *SI Materials and Methods*.

Determination of Intracellular-Compatible Solutes in Xerophilic Fungi over a Range of Temperatures. Compatible-solute determinations were carried out using a ICS-3000 Dionex Ion Chromatography System (Dionex) fitted with a CarboPac MA1 plus guard column (Dionex), and they were quantified by pulsed electrochemical detection based on the methods reported in ref. 48 (*SI Materials and Methods*).

Survival of Conidia of Xerophilic Fungi Exposed to Extremes of Temperature and Pressure. Conidia of three species of xerophilic fungi that were obtained (30) from cultures exposed to chaotropic and kosmotropic solutes were harvested in solutions of the same chao- or kosmotrope, and they were assayed for tolerance to low temperature (−20 °C and −80 °C), high temperature (+55 °C and +65 °C), and high pressure (300 MPa and 750 MPa), as described in *SI Materials and Methods*.

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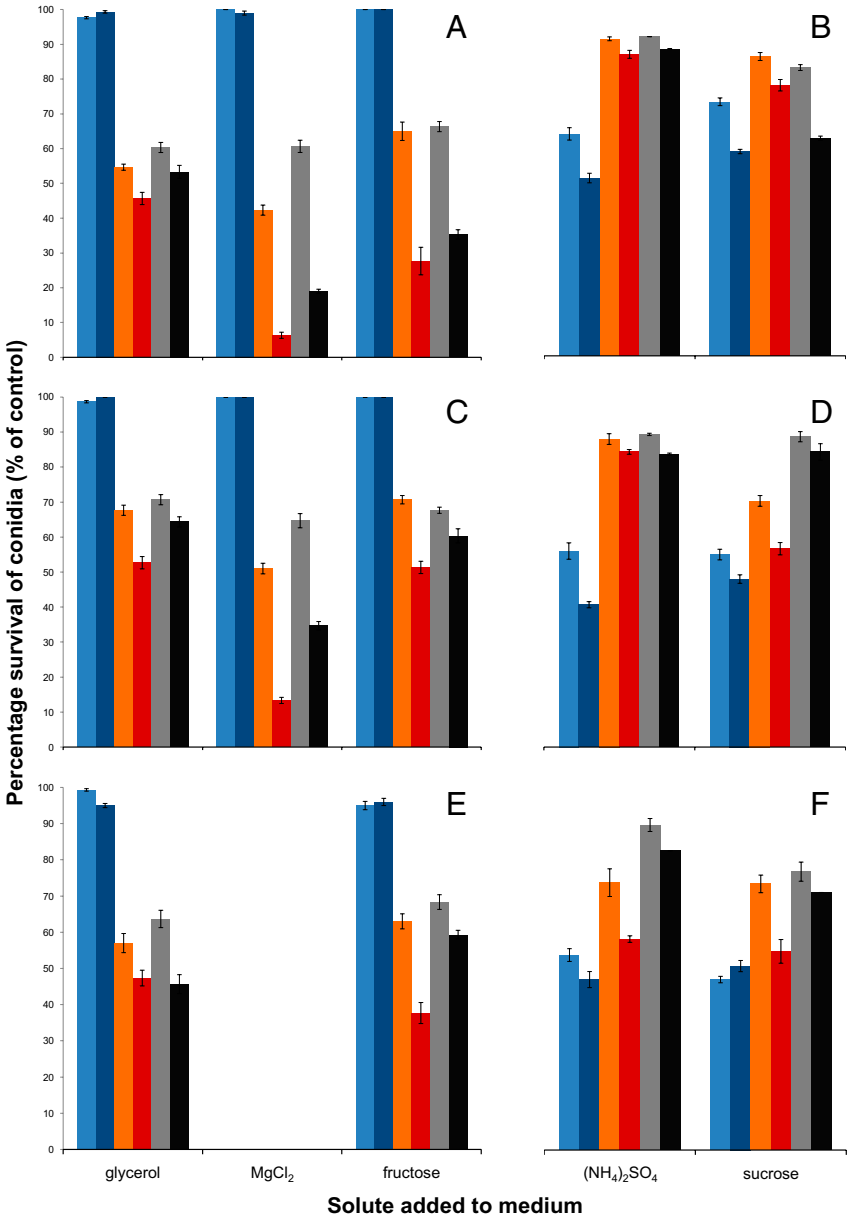

Fig. 4. Percentage survival of xerophile conidia for (*A* and *B*) isolate JH06GBM, (*C* and *D*) isolate JH06JPD, and (*E* and *F*) *Xeromyces bisporus* FRR 2347 that had been produced on MYPiA nutrient media and were harvested in liquid media, both of which had been supplemented with a chaotropic (*A*, *C*, and *E*; 4.5 M glycerol, 1.0 M $MgCl_2$, and 3.0 M fructose) or kosmotropic solute [*B*, *D*, and *F*; 2.9 M $(NH_4)_2SO_4$ and 2.2 M sucrose] and then exposed to low-temperature (−20 °C, light blue; −80 °C, dark blue), high-temperature (+55 °C, orange; +65 °C, red), or high-pressure treatments (300 MPa, gray; 750 MPa, black). One hundred conidia from each replicate spore suspension (i.e., 300 in total) were examined to determine percentage survival; plotted values are the means of these independent triplicates, and bars show SEM.

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