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Reduction of the Temperature Sensitivity of *Halomonas hydrothermalis* by Iron Starvation Combined with Microaerobic Conditions

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The limits to biological processes on Earth are determined by physicochemical parameters, such as extremes of temperature and low water availability. Research into microbial extremophiles has enhanced our understanding of the biophysical boundaries which define the biosphere. However, there remains a paucity of information on the degree to which rates of microbial multiplication within extreme environments are determined by the availability of specific chemical elements. Here, we show that iron availability and the composition of the gaseous phase (aerobic versus microaerobic) determine the susceptibility of a marine bacterium, *Halomonas hydrothermalis*, to suboptimal and elevated temperature and salinity by impacting rates of cell division (but not viability). In particular, iron starvation combined with microaerobic conditions (5% [vol/vol] O_2, 10% [vol/vol] CO_2, reduced pH) reduced sensitivity to temperature across the 13°C range tested. These data demonstrate that nutrient limitation interacts with physicochemical parameters to determine biological permissiveness for extreme environments. The interplay between resource availability and stress tolerance, therefore, may shape the distribution and ecology of microorganisms within Earth's biosphere.

Knowledge of the physical and/or chemical parameters that can limit cell division and metabolic activity is of critical importance in fields such as ecology, agriculture, food preservation, biotechnology, and astrobiology (1–7). The physicochemical boundaries beyond which multiplication of all microorganisms is prevented are imposed by low water activity, extremes of temperature (approximately −20 to 120°C), and other situations, including high pH (>12), chaotropicity, and oxidative damage (e.g., due to UV radiation) (8–10). The stress mechanism for a number of these parameters involves changes in the entropic status of lipid bilayers and other macromolecular systems (see reference 9 and citations therein). Others, including the reactive oxygen species generated by UV radiation, act by inducing alterations in the primary structure of cellular macromolecules (11). One of the primary effects of extreme pH is the breakdown of electrochemical (and other) gradients across the plasma membrane (12).

An increasing body of evidence suggests that it is frequently the net effect of diverse stress parameters that defines the boundary space for life (8, 13–18). The sea ice bacterium *Shewanella gelidimarina*, for example, has been shown to exhibit an increased temperature range for cell division when cultured at high (NaCl) salinity, with increases in membrane lipid packing and fatty acid content conferring tolerance of both conditions (13). Other solutes (including $MgCl_2$) have also been found to influence the temperature limits for microbial multiplication (15, 17). Moreover, adaptation to high hydrostatic pressure has been proposed as a mechanism that enables bacterial multiplication within hypersaline deep-sea environments (14). Such interactions between stress parameters are frequently likely to be multifactorial. Indeed, since no microbial isolates to date have been found to exhibit cell division under a combination of extremely high salinity, temperature (>60°C), and pH (>8), these conditions may be collectively prohibitive to life (8, 16). Other combinations of stress parameters may also determine the limits for cell division within extreme environments. For example, there is an absence of prokaryotic strains that are able to multiply under combinations of elevated (>10 MPa) hydrostatic pressure and extremely low (<4) or high (>9) pH (8).

Despite our knowledge of the physicochemical boundaries for microbial cell division having been greatly advanced over the past three decades, we are only beginning to understand how interactions between different stressors define biological permissiveness for natural ecosystems (8, 13–18). In particular, while several studies have identified minimal and maximal limits for microbial life in response to the surrounding environment (1, 5, 19–21), there is a paucity of information on how rates of cell division are impacted by multiple stress parameters within extreme habitats. Detailed investigations of this topic are required to determine the limits of the biosphere under conditions that account for the physicochemical complexity of extreme environments on Earth, not only with reference to the minimal and maximal boundaries for life but also within the entire range for cell division (8). Moreover, we know relatively little about the impacts of small fluctuations in

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environmental conditions on rates of microbial multiplication and on the level of cellular sensitivity to water activity and other fundamental parameters (10).

Frequently, those chemical elements that can strongly affect rates of cell division are also known to play essential roles in cellular metabolism and microbially mediated redox processes. For example, iron is required for protein structure and function, and oxygen acts as the final electron acceptor during aerobic respiration. These and other elements can be scarce in both biologically hostile and biologically permissive environments. Yet, despite the fact that the majority of microorganisms on Earth are located in oligotrophic and/or oxygen-poor habitats (22–27), research into the physicochemical limits for life has most commonly relied on data obtained using resource-rich, aerobic media. Here, we sought to determine how iron (Fe^{3+}) starvation and microaerobic conditions impact temperature and salt tolerance of the marine bacterium *Halomonas hydrothermalis* (28). This strain was employed as a model organism due to its ability to proliferate under a broad range of temperatures and salinities (14, 28). Rates of cell division and levels of viability were determined for 36 distinct culture conditions created from permutations of incubation temperature, NaCl concentration, Fe^{3+} availability, and (aerobic or microaerobic) atmospheric composition. This approach enabled us to obtain detailed insights into the growth phenotype of *H. hydrothermalis* under multiple-stress conditions that are permissible to cell division.

MATERIALS AND METHODS

Bacterial strain. *Halomonas hydrothermalis* DSM 15725^T (28) was obtained from the German Collection of Microorganisms (DSMZ, Braunschweig, Germany). This strain exhibits cell division across temperatures of 2 to 40°C (optimal growth reported at 30°C), total salt concentrations of 0.5% to 22% (wt/vol) (optimal range of 4% to 7% [wt/vol]), and pH values of 5 to 12 (optimal range of 7 to 8) (28). An exponential-phase culture was obtained by culturing the bacterium in marine broth (DSMZ medium no. 514) with 3.5% (wt/vol) NaCl (pH 7.5; incubation at 30°C), and aliquots were prepared (25% [vol/vol] glycerol) for storage at −80°C. The stored cultures were used to inoculate marine agar (3.5% [wt/vol] NaCl), which was incubated at 30°C for 48 h and stored at 4°C until use.

Growth rate assays. Starter cultures were prepared by transferring cells to 6 ml of marine broth (3.5% [wt/vol] NaCl and pH 7.5) and to a loosely capped 10-ml Falcon tube. The cultures were kept overnight in a shaking incubator (30°C, 100 rpm) and diluted using fresh marine broth to give a cell density equivalent to an optical density at 600 nm (OD_{600}) of 0.3. Optical density measurements for the starter cultures were performed for volumes of 2.5 ml, using a Helios Gamma spectrophotometer (Thermo Spectronic, Cambridge, United Kingdom).

Growth assays were initiated by adding starter culture to fresh marine broth at a ratio of 1:100 (vol/vol). Cultures (each 100 μl; $n = 5$) were incubated in 96-well microplates sealed with gas-permeable optical seals (4titude Ltd., Surrey, United Kingdom). Six variations of culture media based on marine broth were initially used, with 1%, 3.5%, or 11.8% (wt/vol) NaCl prepared in the presence or absence of Fe(III) citrate. The iron-depleted media were spiked with 100 μM diethylenetriaminepentaacetic acid (DETAPAC), an extracellular ferric chelator that has previously been employed to induce bacterial iron starvation (29–31). The media with and without Fe(III) citrate contained ~0.8 mg liter^{-1} and ~0.1 mg liter^{-1} of Fe, respectively. Complex media spiked with an iron chelator have occasionally been used for studies of iron starvation in bacteria (31, 32). Marine broth is an undefined medium that has been successfully employed as a model system and a proxy for seawater for *in vitro* studies of bacteria under conditions of various salinity and nutrient regimens (33–35).

All culture types were incubated at 22, 30, or 35°C. As with the salinities employed in our study, these temperatures have been previously reported to represent suboptimal, optimal, and supraoptimal values for cell division by *H. hydrothermalis* under growth conditions that were otherwise optimal (28). Although this strain can actively grow at 2°C, its rates of cell division at this temperature are very low (28), and therefore a higher minimum temperature was selected. The cells were kept under conditions of either aerobic or microaerobic (5% [vol/vol] O_2, 10% [vol/vol] CO_2, and 85% [vol/vol] N_2) atmospheres by placing the microplates within a Synergy 2 microplate reader (BioTek Instruments Inc., Vermont, USA) or inside sealed 2.5-liter jars containing an Oxoid CampyGen envelope (36). For microaerobic treatments, all media types were equilibrated to microaerobic conditions prior to inoculation. CampyGen envelopes have previously been used to compare bacterial survival mechanisms in complex media incubated under either aerobic or microaerobic atmospheres (37). Cultures within both the microplate reader and the jars were shaken continuously (at 1,080 rpm and 100 rpm, respectively). Cell density was monitored for up to 7 days by OD_{600} measurements using the microplate reader, with medium-only controls ($n = 3$) included for each culture condition. In order to determine whether cells incubated under a combination of iron-deprived and microaerobic conditions remained metabolically active, a separate growth assay was performed for iron-depleted media with 18% (wt/vol) NaCl under a microaerobic atmosphere, with OD_{600} values monitored over 9 days. With the exception of this medium type, pH values of both aerobic and microaerobic media (incubated at 30°C) were monitored by duplicate daily measurements.

Determination of cell viability. To assess cell viability, all culture types (see "Growth rate assays" above) were sampled on two consecutive days during the stationary-growth phase (measurements performed on days 3 and 4 following the onset of the growth assays at 22 and 30°C and on days 2 and 3 for 35°C). Earlier sampling times were employed for cultures incubated at 35°C due to cells frequently entering the death phase following incubation at this temperature for 4 days. Stationary-phase cultures were selected for analyses of cell viability to account for the net effect of different stress parameters on bacterial survival during earlier growth stages (i.e., both the lag and log phases).

Viability was measured using a LIVE/DEAD BacLight staining kit (Life Technologies, Paisley, United Kingdom) (38, 39) in combination with flow cytometry. Aliquots of both the cultures ($n = 3$) and negative controls ($n = 1$) were transferred at a ratio of 1:100 (vol/vol) to sterile 1.5% (wt/vol) NaCl solution and stained in a total volume of 100 μl according to the manufacturer's instructions. The stained samples were analyzed using a FACSCanto II flow cytometer (BD Biosciences, California, USA) equipped with a 488-nm laser and FACSDiva software (Version 6.1.3; BD Biosciences). Syto 9 fluorescence and propidium iodide fluorescence were detected using a 525/30-nm-wavelength band-pass filter (fluorescein isothiocyanate [FITC] channel) and a 670-nm-wavelength long-pass filter (peridinin chlorophyll protein [PerCP] channel), respectively. A minimum of 35,000 events were measured for each replicate.

Prior to the analysis of experimental samples, gates corresponding to intact cells, compromised cells, and nonspecific fluorescence signals were defined, using negative-control samples and overnight cultures stained with either a single dye or both dyes. Both untreated ("live") and isopropanol-treated ("dead") cultures were stained to facilitate the adjustment of gating settings (see Fig. S1 in the supplemental material). Additionally, mixtures of these live and dead cultures were analyzed to confirm that a 1:1 (vol/vol) ratio of the two dye components produced reliable staining results for *H. hydrothermalis* (linear regression, $r^2 = 0.99$), following the manufacturer's instructions.

Data analysis and statistics. Specific growth rates (h^{-1}) were calculated using OD_{600} measurements corresponding to either the entire exponential portion of the growth curve or a region of maximal slope on the curve when exponential growth was absent (14). Growth rates were calculated using the following formula (40):

$$\mu = \frac{\ln 2}{t_d}$$

TABLE 1 Two-way ANOVA results for bacterial growth rates[a]

Culture condition	Effect	Df	*F* value	*P* value
Fe^+, Aer^+	Temperature	2	18.79	<0.001
	Salinity	2	43.09	<0.001
	Temperature × salinity	4	0.99	0.426
Fe^-, Aer^+	Temperature	2	68.81	<0.001
	Salinity	2	68.02	<0.001
	Temperature × salinity	4	6.47	<0.001
Fe^+, Aer^-	Temperature	2	186.86	<0.001
	Salinity	2	105.90	<0.001
	Temperature × salinity	4	33.53	<0.001
Fe^-, Aer^-	Temperature	2	1.89	0.168
	Salinity	2	38.18	<0.001
	Temperature × salinity	4	7.12	<0.001

[a] The data correspond to the main effects and interactions between the factors "temperature" and "salinity," as shown for four distinct culture conditions. Cultures were incubated under iron-rich conditions (Fe^+) or iron-starved conditions (Fe^-), in the presence of either an aerobic atmosphere (Aer^+) or a microaerobic atmosphere (Aer^-). There were 36 residual degrees of freedom (Df) for each test. Results for *post hoc* Tukey's HSD tests are shown in Fig. 1. Data for the culture condition "Fe^+, Aer^+" were Box-Cox transformed to alleviate heteroscedasticity.

where μ = growth rate (h^{-1}) and t_d = population doubling time (h^{-1}).

Proportions of viable bacteria (percentages of total fluorescence events) were calculated using the FlowJo software package (version X.0.6; TreeStar Inc., California, USA), following gating (see "Determination of cell viability" above).

For comparisons of growth rates, 2-way analyses of variance (ANOVA) were performed with salinity (1% to 11.8% [wt/vol] NaCl) and temperature (22 to 35°C) as the factors. Analyses were performed for each combination of iron availability and type of atmosphere (see "Growth rate assays" above), followed by *post hoc* Tukey's honestly significant difference (HSD) tests. Measurements obtained for cells incubated under aerobic and iron-rich conditions were Box-Cox transformed ($\lambda = -0.05$) in order to alleviate heteroscedasticity (41). This transformation has been previously employed in several microbiological studies (see, e.g., references 42, 43, and 44). For comparisons of the proportions of viable cells across each combination of iron availability and atmospheric composition, a one-way ANOVA and Box-Cox-transformed data ($\lambda = 2.8$) were used in conjunction with a Tukey's HSD test. All statistical analyses were performed using R v3.0.2 (45).

RESULTS AND DISCUSSION

Under aerobic conditions with plentiful iron, bacterial growth rates were susceptible to moderate changes in temperature and salinity (Table 1 and Fig. 1A). For example, the mean growth rates of cultures incubated at a temperature-salinity combination of 35°C and 11.8% (wt/vol) NaCl were 43% and 44% lower than those at 35°C or 30°C and 3.5% (wt/vol) NaCl, respectively (Fig. 1A). Moreover, mean growth rates at 22°C and 11.8% (wt/vol) NaCl were 67% lower than those at 30°C and 3.5% (wt/vol) NaCl (Fig. 1A). Under these aerobic and iron-rich conditions, there was no evidence of an interaction between temperature and salinity in terms of their impacts on bacterial growth rates (Table 1). However, some *Halomonas* species incubated in aerobic high-salinity media have exhibited enhanced growth rates at temperatures below 22°C (14).

Under iron starvation and aerobic conditions, *H. hydrothermalis* cultures generally exhibited reduced growth rates (Fig. 1B) in comparison with nonstarved aerobic cultures at equivalent temperatures and salinities (Fig. 1A). Both temperature and salinity significantly impacted these rates (Table 1), with iron starvation suppressing bacterial sensitivity to sub- and supraoptimal salinities at 22°C but not at higher temperatures, as shown by a Tukey's HSD test (Fig. 1B). The combined impacts of low oxygen availability and high CO_2 concentrations on bacterial growth rates differed, with high rates maintained at most salinities at 30 or 35°C, despite an overall reduction in growth rates at 22°C (Fig. 1C). Under these microaerobic conditions, temperature and salinity, both as individual parameters and in combination, remained key determinants for rates of cell division (Table 1). Interestingly, the most pronounced impact of microaerobic culture conditions involved the growth rate limitation of cultures at suboptimal salinities and/or temperatures (Fig. 1A to C).

When *H. hydrothermalis* was cultured under conditions of both iron starvation and a microaerobic atmosphere (Fig. 1D), mean growth rates were reduced by as much as 83% relative to those of nonstarved cells (Fig. 1A). In fact, cell division was suppressed across all culture conditions to the extent that bacterial growth rates no longer varied as a function of temperature (Table 1). As maximal OD_{600} values of >0.2 were observed under all the examined incubation conditions (corresponding to mean CFU counts ranging from 2.2×10^8 ml^{-1} to 5.6×10^8 ml^{-1} in aerobic cultures incubated at 30°C [$n = 3$]; data not shown), this result was not attributable to low biomass or to optical density values residing below the detection limit for turbidimetry. Therefore, to assess whether this phenomenon was mediated by a decline in cell viability (46), we calculated the proportions of surviving stationary-phase cells under each set of culture conditions (see Materials and Methods; see also Fig. S1 in the supplemental material). The greatest difference between the different conditions was minimal (8.4%; Fig. 2) and did not correlate with growth rates (Fig. 1). Moreover, no significant differences ($P > 0.05$) in viability were observed across the majority of the culture conditions (Fig. 2), indicating that the growth rate values were accurate (and were not an artifact occurring due to differential rates of cell death). In addition, bacteria were able to proliferate under a combination of iron starvation and microaerobic conditions and a NaCl concentration of 18% (wt/vol) (see Fig. S2 in the supplemental material), which is close to the upper limit of salinity for cell division in *H. hydrothermalis* (28). Collectively, these data evidence the continued metabolic activity of the cultures despite their reduced sensitivity to temperature and suggest that the incubation conditions used in our study did not influence the upper limit of salinity for cell division in *H. hydrothermalis* (47).

Compounded impacts of iron availability and atmospheric gas composition on microbial temperature and salt sensitivities have not been previously reported, despite observations that nutrient-starved cells frequently exhibit increased tolerance of heat, UV-B radiation, antibiotics, and oxidative stress (48–53). Interactions between starvation conditions, and the ability of abiotic parameters to influence bacterial cell division, have implications for the habitability and productivity of natural systems, including marine waters (14, 22, 24, 27, 54) and nutrient-poor rocks (55, 56), and are also likely to modulate the growth and persistence of microorganisms within nutrient-limited artificial environments (57). Such interactions may additionally influence the distribution and composition of microbial communities within and between habitats. Rates of microbial cell division within biologically hostile habitats (2, 8–10, 16, 18), therefore, can potentially be impacted

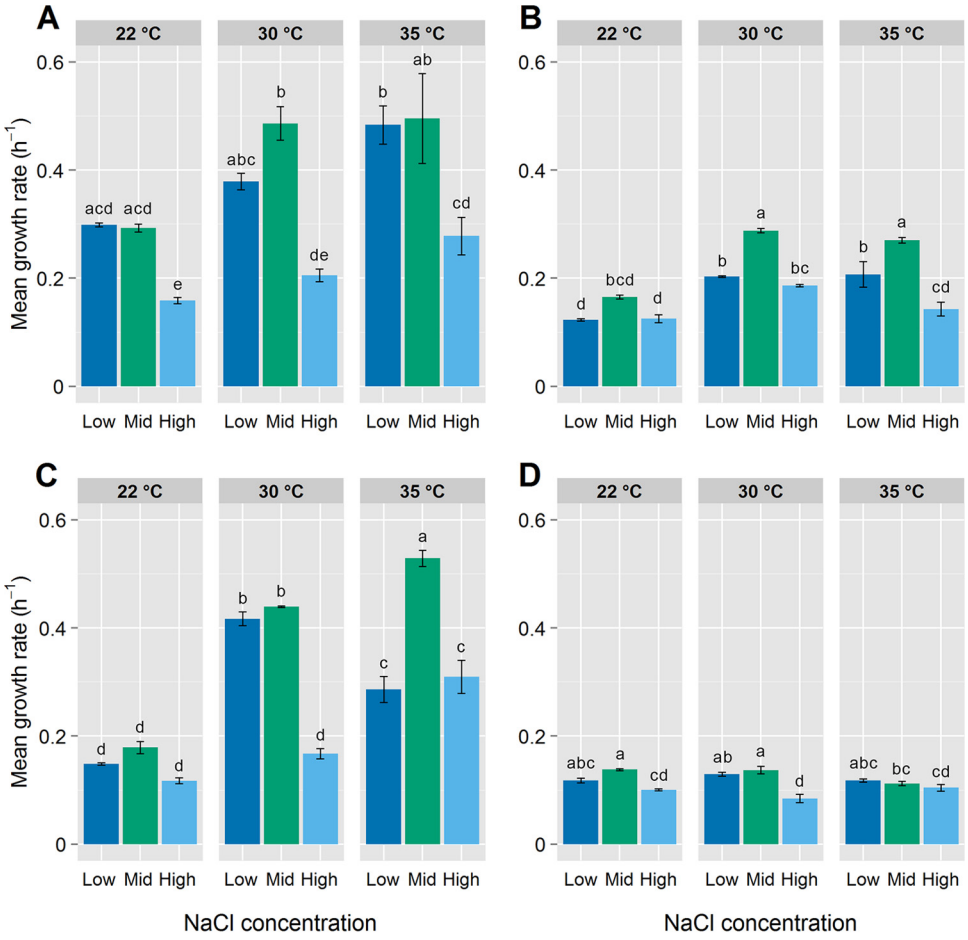

FIG 1 Growth rates of *Halomonas hydrothermalis* under different culture conditions. (A) Cells grown under conditions of plentiful iron and oxygen. (B) Cells grown in iron-deprived media under aerobic conditions. (C) Cells grown in the presence of freely available iron under microaerobic conditions. (D) Cells grown in iron-deprived media under microaerobic conditions. Low, Mid, and High, 1%, 3.5%, and 11.8% (wt/vol) NaCl, respectively. Growth rates were calculated from growth curves as described in Materials and Methods. Data are presented as untransformed means ± standard errors of the means (SE) ($n = 5$). Different letters above the bars indicate significant differences between samples within a given combination of conditions of iron availability and type of atmosphere (aerobic or microaerobic) ($P < 0.05$ [Tukey's HSD test following 2-way ANOVA; see Table 1 for ANOVA results]).

by effects of resource limitation on microbial sensitivity to extremes, which in turn are likely to depend on other environmental conditions.

Microbial responses to individual stress parameters such as supraoptimal salinities and temperatures are well characterized, involving mechanisms that include the regulation of intracellular ion (e.g., Na^+ and K^+) concentrations, solute accumulation, and alterations in membrane fluidity (58, 59). However, the biophysical and/or metabolic mechanism(s) by which responses to iron starvation and microaerobic conditions can determine tolerance of specific stresses has yet to be fully characterized. It is known that several general-stress proteins can be triggered in response to nutrient starvation and are additionally induced by oxygen limitation as well as other physicochemical challenges, such as heat shock, acid stress, hydrophobic stressors, reactive oxygen species, and osmotic stress (60–62). It is plausible that, in the present study, iron starvation and microaerobic conditions exerted a compound influence on *H. hydrothermalis* tolerance of differences in temperature and salinities either directly or as a consequence of secondary stress responses (such as those induced by a reduction in pH under CO_2-enriched conditions; see Table S1 in the supplemental material). Indeed, exposure to a given extreme can frequently confer cross-protection against other environmental stressors in both prokaryotes and eukaryotes, due to overlapping physiological adaptations and stress-response networks (8, 60, 62–65).

In summary, the findings of the current study suggest that both the cellular stress mechanisms and stress responses of microbial extremophiles can be fully understood only when related to the wider metabolic energy budget of a given habitat. Moreover, the specific impacts of nutrient availability on rates of microbial cell division are likely to depend on other environmental factors, including the ambient gas composition. Since the majority of microbial taxa within the biosphere are adapted to nutrient-poor conditions, the ability of microorganisms to exhibit differential responses to nutrient limitation is likely to impact population sizes within specific habitats (66, 67) and the biological permissiveness of extreme environments on Earth. The current study focused on characterizing the phenotype-level impacts of multiple stress parameters on the growth rates and viability of *H. hydrother-*

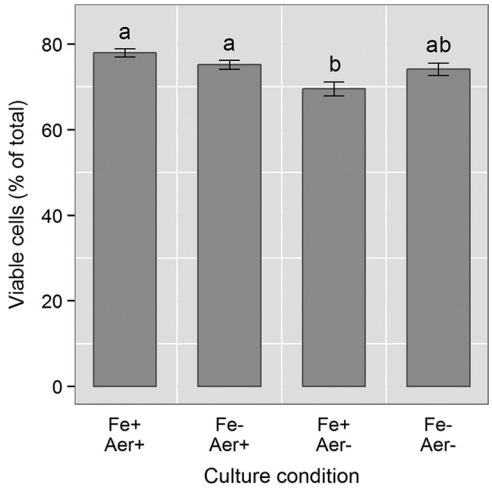

FIG 2 Viability of stationary-phase *Halomonas hydrothermalis* cells under different culture conditions. Cultures were incubated under iron-rich (Fe+) or iron-starved (Fe−) conditions, in the presence of an aerobic atmosphere (Aer+) or a microaerobic atmosphere (Aer−). Data are presented as untransformed means ± SE ($n = 54$). Different letters above the bars indicate significant differences between samples within a given combination of conditions of iron availability and type of atmosphere (aerobic or microaerobic) ($P < 0.05$ [Tukey's HSD test]), determined following a one-way ANOVA using Box-Cox-transformed data ($P < 0.001$) ($F_{3,212} = 6.13$). The measurements are based on LIVE/DEAD staining of cells followed by flow cytometry, with data pooled from two consecutive days. For details on the experimental protocol and instrumental calibration, see Materials and Methods and Fig. S1 in the supplemental material.

malis under conditions that enable cell division. However, it is possible that interactions between nutrient availability and stress sensitivity can affect the physicochemical growth limits of diverse microbial taxa (1, 5, 13, 19–21, 47). The findings of our study, therefore, suggest that the habitability of extreme environments (including hypersaline habitats) is often defined by the interplay between several physicochemical and biological factors rather than by one parameter alone.

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