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Received: 16 January 2023 | Accepted: 28 May 2023

DOI: 10.1111/1462-2920.16451

RESEARCH ARTICLE

ENVIRONMENTAL MICROBIOLOGY Applied Microbiology International

Effects of chaotropic salts on global proteome stability in halophilic archaea: Implications for life signatures on Mars

Lorenzo Carré[1] | **Daniel Gonzalez**[2] | **Éric Girard**[1] | **Bruno Franzetti**[1]

[1]Université Grenoble Alpes, CNRS, CEA, IBS, Grenoble, France

[2]Université Grenoble Alpes, Grenoble, France

Correspondence
Bruno Franzetti, Université Grenoble Alpes, CNRS, CEA, IBS, Grenoble, France.
Email: franzetti@ibs.fr

Funding information
French National Research Agency, Grant/Award Number: ANR-15-IDEX-02; Interdisciplinary Research Institute of Grenoble (IRIG, CEA)

Abstract

Halophilic archaea thriving in hypersaline environments, such as salt lakes, offer models for putative life in extraterrestrial brines such as those found on Mars. However, little is known about the effect of the chaotropic salts that could be found in such brines, such as $MgCl_2$, $CaCl_2$ and (per)chlorate salts, on complex biological samples like cell lysates which could be expected to be more representative of biomarkers left behind putative extraterrestrial life forms. We used intrinsic fluorescence to study the salt dependence of proteomes extracted from five halophilic strains: *Haloarcula marismortui*, *Halobacterium salinarum*, *Haloferax mediterranei*, *Halorubrum sodomense* and *Haloferax volcanii*. These strains were isolated from Earth environments with different salt compositions. Among the five strains that were analysed, *H. mediterranei* stood out as a results of its high dependency on NaCl for its proteome stabilization. Interestingly, the results showed contrasting denaturation responses of the proteomes to chaotropic salts. In particular, the proteomes of strains that are most dependent or tolerant on $MgCl_2$ for growth exhibited higher tolerance towards chaotropic salts that are abundant in terrestrial and Martian brines. These experiments bridge together global protein properties and environmental adaptation and help guide the search for protein-like biomarkers in extraterrestrial briny environments.

INTRODUCTION

By shaping the range of conditions in which proteins retain their stability and solubility, amino acid substitutions and structural modifications influence the survivability of organisms in a given environment. Due to their profound long-evolved specialization, extremophiles have largely confirmed this link between molecular traits and adaptation to the environment (Ando et al., 2021; Carré et al., 2022; Reed et al., 2013). For example, *haloarchaea*, which thrive on Earth in hypersaline environments, cope with hyperosmolarity by intracellularly accumulating molar concentrations of K^+. Yet, their cytosol remains an extreme environment in which non-halophilic proteins would lose stability and solubility. Thanks to several molecular traits, including reduced hydrophobicity and increased frequency of negatively charged amino acids; proteins from halophiles have shown impressive tolerance and even dependency towards high concentrations of salts. Differences between salt-tolerance of homologous enzymes from different halophilic organisms have been pointed (Coquelle et al., 2010). In particular, the evolution of the model malate dehydrogenase (MDH) enzyme within the haloarchaeal class showed complex and non-linear trajectories leading to various adaptive strategies and responses to salt type and pH (Blanquart et al., 2021). These observations indicate that even among salt-accumulating halophiles, subtle differences in molecular traits could be related to dramatic differences in protein behaviour and salt-dependency.

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While most terrestrial halophiles, including the archaeon *Halobacterium salinarum*, which is the most halophilic organism known to date, are found in Na^+ dominated brines, it is important to note that halophiles can also be found in highly chaotropic Mg^{2+}-containing environments such as soda lakes, Dead Sea, cryobrines or deep hypersaline anoxic basins (DHABs). Chaotropicity is a concept that was first defined in 1962 (Hamaguchi & Geiduschek, 1962). It results from the activities that solutes exert on macromolecular systems, as well as from deviations of solvation water's behaviour from its bulk-like state (Ball & Hallsworth, 2015). Chaotropic cations such as Mg^{2+} or Ca^{2+} destabilize most proteins (Bye & Falconer, 2013; Tadeo et al., 2007), as predicted by Hofmeister series (Hofmeister, 1888). These ions strongly impair water structure and interact with side-chains of negatively charged amino acids, alleviating key interactions involved in protein structure (General et al., 2008; Lenton et al., 2017; Okur et al., 2017). Absolute determinations of kosmotropic/chaotropic activities have been measured for several compounds, leading to negative (kosmotropic) and similar values for NaCl and KCl, respectively -11.0 and -11.3 kJ kg^{-1} mol^{-1}, and positive (chaotropic) values for $MgCl_2$ and $CaCl_2$, respectively $+54.0$ and $+92.2$ kJ kg^{-1} mol^{-1} (Cray et al., 2013). Along with thermodynamical approaches, the chaotropic or kosmotropic nature of various types of physiological solutes was also quantified biologically, by studying how they affect microbial cell growth and metabolism (Cray et al., 2013; de Lima Alves et al., 2015). It revealed that, even at concentrations that can be found in the natural environment, chaotropic solutes can inhibit the growth of bacteria and induce the accumulation of stress proteins responsible for stabilizing proteins and membranes (Hallsworth, Heim, & Timmis, 2003; Hallsworth, Prior, et al., 2003). These observations indicate that, besides hypersalinity, chaotropicity represents a major challenge limiting the window of life. However, cellular activities and cultivable microbes were obtained from a $MgCl_2$-rich DHAB. Also, *Halorubrum sodomense* and *Haloferax volcanii*, two halophilic archaea isolated from the Dead Sea show impressively high tolerance to chaotropic ions (Oren, 1983; Pohlschroder & Schulze, 2019) and intracellular concentrations of Mg^{2+} in *H. sodomense* has been reported to be notably higher than in other halophilic archaea (Oren, 2013). Finally studies on halophilic enzymes also indicated a peculiar role of Mg in enzyme stabilization (Blanquart et al., 2021; Madern & Zaccai, 1997, 2004). These observations indicated that extreme halophiles may have evolved specific mechanisms to cope with high chaotropicity.

Besides a better definition of life window with respect to hypersaline and chaotropic environments, halophilic organisms and their related biomolecules have also been proposed as models to test the compatibility of biological processes and biomarker stability under simulated hypersaline Martian conditions (Bayles et al., 2020; Carré et al., 2022; Perl & Baxter, 2020; Sundarasami et al., 2019). Indeed, several studies have shown that metastable hypersaline liquid water bodies containing high amount of chaotropic ions could be present beneath the southern pole ice cap (Lauro et al., 2021; Orosei et al., 2018) or under the regolith in subsurface environments (Abotalib & Heggy, 2019; Clifford et al., 2010; McEwen et al., 2011; Rivera-Valentín et al., 2020). As confirmed by experimental studies (Fischer et al., 2014), such water systems would require to be hypersaline in order to remain liquid under the low temperatures of Martian environments (Brady, 1992). A notable feature of halophilic organisms is their capacity to survive for long periods when trapped in salt crystals or upon salt deliquescence (Crits-Christoph et al., 2016; Favreau, 2023; Favreau et al., 2022; Micheluz et al., 2022), some studies even suggesting a preservation over geological ages (Hallsworth, 2022; Schreder-Gomes et al., 2022). Moreover, halophilic enzymes were also shown to maintain exceptional stability when trapped in salt crystals (Tehei et al., 2002). It has also been shown that intracellular accumulation of chaotropic cations such as Mg^{2+} or Ca^{2+}, facilitates life at low temperatures as they counter the cold-induced stabilization of proteins (Chin et al., 2010; Cray et al., 2015). For these reasons, it has been proposed that traces of ancient halophilic life could be present in Martian halites (Hallsworth, 2020; Rivera-Valentín et al., 2020). Thus, the surface of the red planet should not be considered universally hostile to life (Hallsworth, 2021). In this context, extensive simulations have used members of the *Haloarchaea* class to test the effects of Martian-like simulated conditions such as UV irradiation (Fendrihan et al., 2009), desiccation-rehydration cycles (Vauclare et al., 2020), long-term desiccation and freezing cycles (Mancinelli et al., 2004) and low temperature (Laye & DasSarma, 2018; Reid et al., 2006). While such experimental approaches are useful to assess the capacity to tolerate Martian-like conditions at the organism level, they need to be complemented with studies on the biomolecules to get a complete picture of the molecular limits of life. In addition, Martian brines would display Mg^{2+} and Ca^{2+} as the dominant cations (Osterloo et al., 2008; Squyres et al., 2004) and high concentrations of chlorate and perchlorate anions (ClO_3^- and ClO_4^-), have been detected in situ (Hecht et al., 2009), especially in subsurface brines. As mentioned above, such chaotropic conditions represent a barrier to life (Hallsworth et al., 2007) and could make the Martian brines more extreme than their terrestrial analogues (Fox-Powell et al., 2016). At the molecular level, little is known about the effects of chaotropic ions on halophilic proteins and the existence of protein adaptation to chaotropic ions remains questionable. Such adaptation

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would be crucial to cope with exotic conditions such as those present in Martian brines.

Recently, the effects of perchlorate salts on the reactivity of an halophilic enzyme (Laye & DasSarma, 2018) and on the stability and reactivity of a non-halophile enzyme have been investigated (Gault & Cockell, 2021). It should, however, be stressed that the conclusions arising from isolated model biomolecules may not reflect the limiting biological edifice required to cope with extreme conditions and, in the context of astrobiology, may not indicate what biomarker resulting from a putative extraterrestrial lifeform could be searched for (Fairén et al., 2020; Sánchez-García et al., 2020). Moreover, such approaches could be biased by the choice of the enzyme. In addition to variations between organisms, even between extreme halophiles (Coquelle et al., 2010), proteins from the same organism may display drastically different responses to salts, notably as a consequence of the composition of their amino acid sequence (Becker et al., 2014). Therefore, whole-proteome approaches to study the properties of a physiologically relevant mix of proteins may offer additional insights into global biomolecule adaptation to the environment. Various techniques, including neutron scattering (Zaccai, 2013), have been successfully used to unravel global protein adaptations in thermophiles, psychrophiles (Tehei et al., 2004), piezophiles (Martinez et al., 2016; Peters et al., 2014) and halophiles (Marty et al., 2013; Vauclare et al., 2015, 2020).

By using label-free differential scanning fluorimetry (nanoDSF), a method allowing analysis of thermal denaturation and thermal aggregation of proteins, we have recently revealed a difference between the stabilizing effect of KCl and NaCl on the proteome of the halophilic archaeon *Haloarcula marismortui* (Carré et al., 2021). In this study, we used this novel approach to explore the effects of combinations between kosmotropic (KCl, NaCl) and chaotropic salts ($MgCl_2$, $CaCl_2$, $NaClO_3$, $NaClO_4$, $Mg(ClO_4)_2$ and $Ca(ClO_4)_2$) on proteome stability in five different strains of *Haloarchaea*, including Mg-tolerant strains.

EXPERIMENTAL PROCEDURES

Experimental design

This study aimed to characterize response to various salt types and combinations at the scale of the proteome through nanoDSF analysis of cell lysates of halophilic archaea. After diluting them in concentrated salt solutions to achieve desired conditions, cell lysates were loaded in capillaries and analysed with a Prometheus NT.48 (NanoTemper). This allowed monitoring of denaturation and aggregation events throughout a temperature gradient. We focused on the shifts of dominant denaturation peaks (T_{Pm}) related to changes in salt types, combinations and concentrations. Relationships between T_{Pm} and salt concentration were fitted with a linear model whose slopes represented proteome sensitivity to the given salt.

Strains and growth conditions

All strains were grown aerobically at 37°C under mild agitation by inoculating fresh medium with concentrated cell suspension stored in glycerol at −80°C. Initial stocks were purchased at the DSMZ-German Collection of Microorganisms (*H. mediterranei*, *H. sodomense*) or provided by A. Oren (*H. marismortui*, *H. salinarum*) or H. Myllykallio (*H. volcanii*). Growth medium was adapted from various references (Mevarech et al., 1976; Oesterhelt & Stoeckenius, 1974; Oren, 1983; Rodriguez-Valera et al., 1980).

Composition of *H. marismortui* medium was 208 g L^{-1} NaCl, 78 g L^{-1} $MgCl_2{\cdot}6H_2O$, 500 mg L^{-1} $CaCl_2{\cdot}2H_2O$, 125 mg L^{-1} $MnCl_2$, 5 g L^{-1}, NaBr 580 mg L^{-1}, 10 g L^{-1} yeast extract, pH adjusted to 7.5 with addition of 10 M NaOH after dissolution.

Composition of *H. salinarum* medium was 250 g L^{-1} NaCl, 33.8 g L^{-1} $MgCl_2{\cdot}6H_2O$, 2 g L^{-1} KCl, 125 mg L^{-1} $MnCl_2$, NaBr 580 mg L^{-1}, 3 g L^{-1} yeast extract, 5 g L^{-1} tryptone, pH adjusted to 7.2 with addition of 10 M NaOH after dissolution.

Composition of *H. mediterranei* medium was 195 g L^{-1} NaCl, 84 g L^{-1} $MgCl_2{\cdot}6H_2O$, 500 mg L^{-1} KCl, 1 g L^{-1} $CaCl_2{\cdot}2H_2O$, 125 mg L^{-1} $MnCl_2$, NaBr 580 mg L^{-1}, 5 g L^{-1} yeast extract, pH adjusted to 7.2 with addition of 10 M NaOH after dissolution.

Composition of *H. sodomense* medium was 125 g L^{-1} NaCl, 160 g L^{-1} $MgCl_2{\cdot}6H_2O$, 2,1 g L^{-1} KCl, 130 mg L^{-1} $CaCl_2{\cdot}2H_2O$, 125 mg L^{-1} $MnCl_2$, 5 g L^{-1}, NaBr 580 mg L^{-1}, 2 g L^{-1} yeast extract, 1 g L^{-1} peptone, 2 g L^{-1} soluble starch, pH adjusted to 7.2 with addition of 10 M NaOH after dissolution.

Composition of *H. volcanii* medium was 125 g L^{-1} NaCl, 62 g L^{-1} $MgCl_2{\cdot}6H_2O$, 3.7 g L^{-1} KCl, 500 mg L^{-1} $CaCl_2{\cdot}2H_2O$, 125 mg L^{-1} $MnCl_2$, 5 g L^{-1}, NaBr 580 mg L^{-1}, 3 g L^{-1} yeast extract, 5 g L^{-1} tryptone, pH adjusted to 7.2 with addition of 10 M NaOH after dissolution.

Proteome extracts preparation

Sixty millilitres of a culture in mid or late exponential phase (OD_{600} between 0.4 and 1.0) were centrifugated at 20,000 g for 15 min. Supernatant was discarded by aspiration and pellet was resuspend in 3 mL of 4 M KCl or 5 M NaCl and cells were then lysed by sonication. For each condition, 50 µL of cell lysate was diluted with

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water and concentrated salt solutions (4 M KCl, 5 M NaCl, 5 M $MgCl_2$, 5 M $CaCl_2$, 2 M $NaClO_3$, 2 M $NaClO_4$, 1 M $Mg(ClO_4)_2$ or 1 M $Ca(ClO_4)_2$) to the final volume of 500 µL in order to reach the final desired concentration of salts.

Characterization of effects of salts on *Haloarchaea* proteomes and *H. marismortui* MDH by differential scanning fluorimetry

Cell lysates extracted in various salt conditions were centrifugated at 20,000 g for 15 min and the supernatants (soluble fraction of cell lysate) were loaded in Prometheus™ NT.48 capillaries. Thermal denaturation curves were determined by measurements of protein intrinsic fluorescence. This analysis was performed using label-free, native differential scanning fluorimetry (nanoDSF; apparatus: Prometheus NT.48, NanoTemper). The tryptophan residues of the proteins in proteome extracts were excited at 280 nm and the fluorescence intensity was recorded at 330 and 350 nm. Excitation power was set at 80% or 100%. The temperature of the measurement compartment increased from 20 to 95°C at a rate of 2°C per minute. Light scattering was also measured as a way to detect aggregation events during heating.

To stress the inner variation of first derivative of ratio of 350/330 nm fluorescence in each sample, data were transformed using the highest and the lowest values within the 25–80°C range of temperature as boundaries by respectively setting them to 100 and 0.

Statistical analysis

All graphs and analyses were made using GraphPad Prism 9 (GraphPad software Inc, California, USA).

Temperature associated with dominant denaturation peaks were automatically determined by the PR. ThermControl software (NanoTemper). When multiple peaks were detected, we only picked the dominant one with maximum height. When no temperature could be automatically determined, we used instead the temperature associated with maximum value of first derivative of 350/330 nm fluorescence ratio. Since events associated with abnormally high fluorescence or light scattering signals occasionally happened at highest temperatures in some individual experiments, the manual assignment of denaturation peaks was limited to temperatures between 25 and 80°C. Due to low signal over noise ratio, no denaturation peak and, hence, T_{Pm}, could be determined for several experiments, mostly with lysates of *H. salinarum*.

For each condition, linear regression was made by using the automatically and manually determined T_{Pm}. Due to insufficient data, it could not be made for *H. salinarum* with the NaCl-only salt condition. For KCl-only and NaCl-only experiments, value of T_{Pm} measured at 0 M salt was excluded from the fit as this condition is actually particularly extreme for halophilic systems. This was not made with combinations since the proteome was always exposed to at least 1 M or counter-salt (KCl or NaCl). Slope of line fitting best T_{Pm} values was determined individually for each replicate. Statistical comparison of slopes in Figure 1A, was made using Brown–Forsythe and Welch ANOVA tests, comparing the mean of each column to the mean of every other column. All peak and slope values are given in Data S2–S5.

Since differences between slope values ranged over several orders of magnitude, data were transformed for Figure 2A. For each salt condition, the slope with highest absolute value was set to 100 or − 100, depending on its sign, by multiplying it by a specific number and the other slopes were multiplied by the same number. This made comparisons between strains easier for every salt condition.

RESULTS

Response of the proteome of *H. marismortui* to different salts

To investigate proteome responses to KCl, NaCl, $MgCl_2$, $CaCl_2$ and to combinations between them, we first used lysates of cells of *H. marismortui* in exponential phase. After dilution in various salt concentrations to reach the final desired composition and centrifugation, the supernatants, which contained the soluble fraction of the proteomes, were analysed using nanoDSF to derive proteome thermal denaturation and thermal aggregation curves as described in Carré et al. (2021). Untreated thermal aggregation and denaturation signals in various salt conditions are presented in supplementary material section (Figures S2–S5). Representative thermal denaturation curves for the proteome of *H. marismortui* are given in Figure 3 for solutions containing a single salt and for combinations between 1 M KCl or NaCl and chaotropic chloride or (per)chlorate salts. We first tested the effects of solutions containing one type of salt: KCl (0–4 M), NaCl (0–6 M), $MgCl_2$ (0–2 M) and $CaCl_2$ (Figure 3A). Then we studied combinations between KCl (1, 2 or 3 M) and chaotropic $MgCl_2$ or $CaCl_2$ (0–2 M) (Figure 3B) as well as combinations between NaCl (1, 2 or 3 M) and chaotropic chlorate/perchlorate salts: $NaClO_3$ (0–1 M), $NaClO_4$ (0–1 M), $Mg(ClO_4)_2$ (0–0.5 M) or $Ca(ClO_4)_2$ (0–0.5 M) (Figure 3C). The reason why we used NaCl rather than KCl with perchlorates is that immediate precipitation occurred when mixing (per)chlorate and KCl solutions, even at low concentrations.

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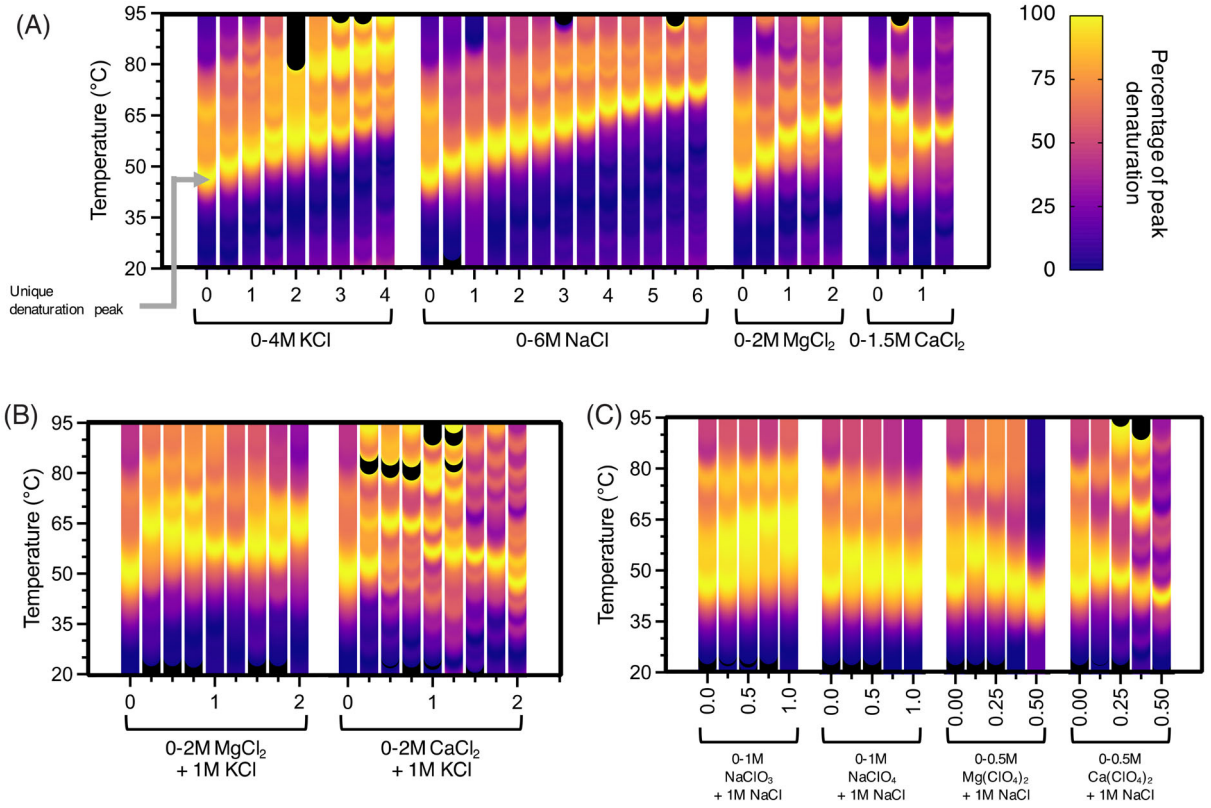


FIGURE 1 Proteome melting temperatures as a function of salt condition and proteome sensibility to various Earth-relevant salts in *Haloarchaea*. T_{Pm} values determined with KCl or NaCl only (A) and with combinations between KCl and $MgCl_2$ (D). Corresponding slopes of line fitting T_{Pm} determined with KCl (B), NaCl (C) or combinations between KCl and $MgCl_2$ (E). Statistical analysis was made using a Brown–Forsythe and Welch ANOVA test (**$p < 0.0021$; ***$p < 0.0002$; ****$p < 0.0001$). Errors bars represent standard deviation.

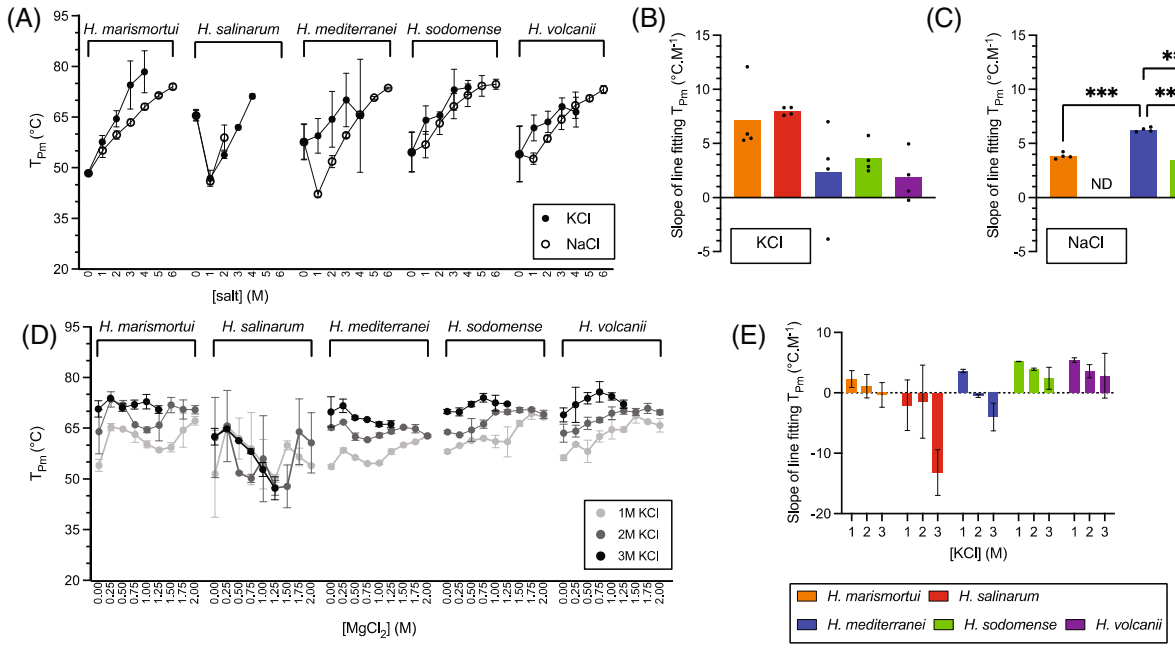


FIGURE 2 Proteome melting temperatures as a function of salt condition and proteome sensibility to various Mars-relevant salts in *Haloarchaea*. T_{Pm} values determined with combinations between NaCl and $NaClO_3$ (A), $NaClO_4$ (C), $Mg(ClO_4)_2$ (E) or $Ca(ClO_4)_2$ (G). Corresponding slopes of line fitting T_{Pm} determined with combinations between NaCl and $NaClO_3$ (B), $NaClO_4$ (D), $Mg(ClO_4)_2$ (F) or $Ca(ClO_4)_2$ (H).

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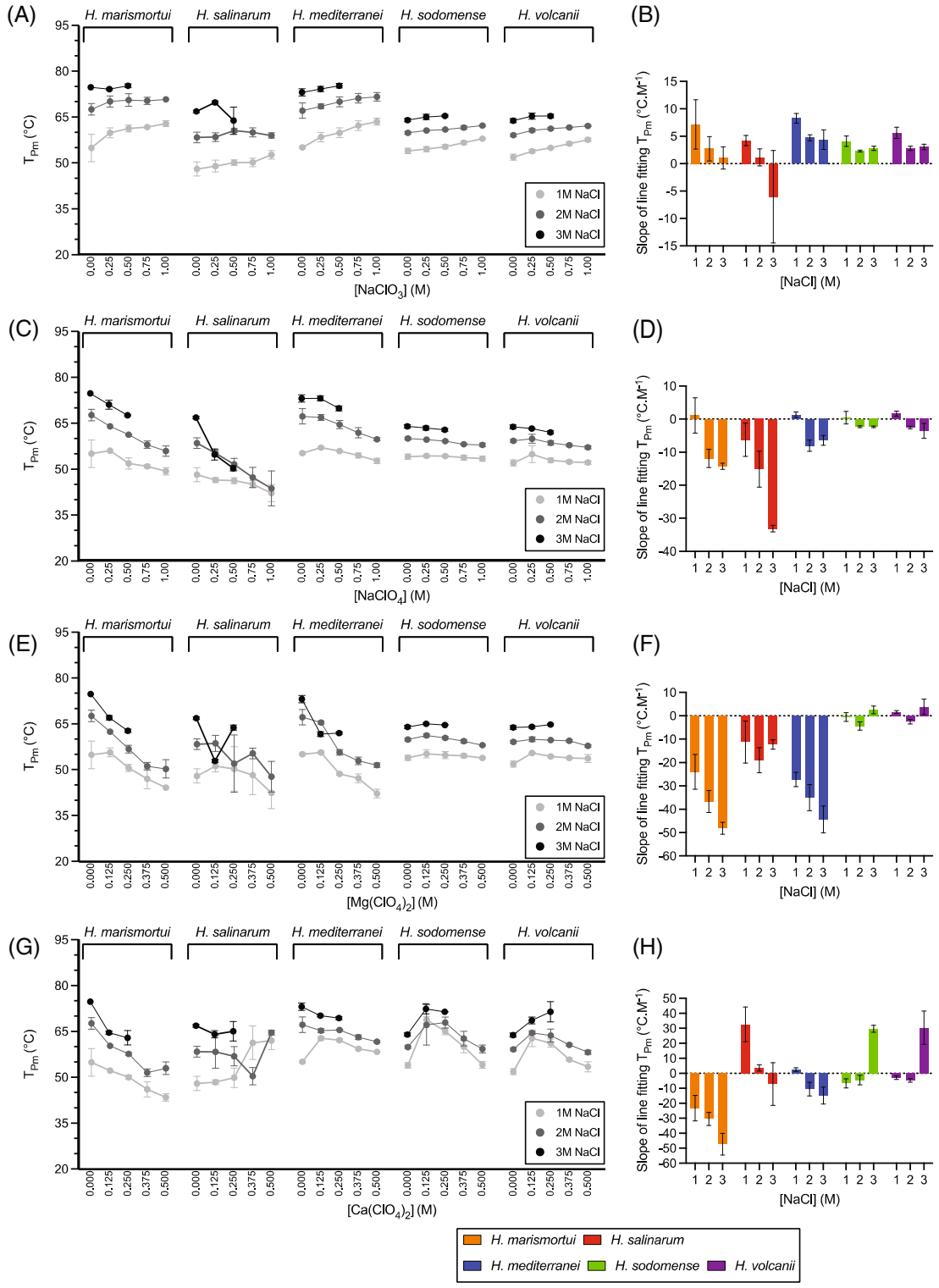


FIGURE 3 Representative thermal denaturation curves of *Haloarcula marismortui* proteome under various salt conditions. nanoDSF analysis was made with soluble fraction of lysates with only one type of salt (A), combinations between 1 M KCl and $MgCl_2$ or $CaCl_2$ (B) or with combinations between 1 M NaCl and (per)chlorate salts (C). Higher values (yellow) correspond to denaturation events. Curves were transformed to facilitate peak observation (see Experimental Procedures section). Occasionally, abnormally high signals were measured at the end of the heating ramp (in black). When signal over noise ratio was high, signal after 80°C may also appear in black due to the way colour range was set.

In the case of chaotropic salts, increasing the concentrations of both $MgCl_2$ and $CaCl_2$ delayed peak of the first derivative of the ratio of 350/330 nm fluorescence and, hence, the dominant denaturation event (denaturation peak) reflecting a stabilizing effect of these two salts on the *H. marismortui* proteome in

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spite of their chaotropicity (Figure 3A). However, the relationship between $CaCl_2$ concentration and the temperature of the denaturation peak was non-linear.

Denaturation peaks followed a non-linear trend under KCl-$MgCl_2$ or KCl-$CaCl_2$ conditions. However, the denaturation events were less resolved than in single-salt conditions (Figure 3B). Yet, one dominant denaturation peak could still be observed with most KCl-$MgCl_2$ conditions. Several KCl-$CaCl_2$ conditions led to higher values of the first derivative of the ratio of 350/330 nm fluorescence above 80°C, which was used as the superior temperature boundary for determining the peak position in low-resolved curves. When excluding the events occurring above 80°C, dominant denaturation events could be observed at 45–65°C.

Combinations between NaCl and chlorate or perchlorate salts led to less resolved denaturation peaks similar to the KCl-$MgCl_2$ or KCl-$CaCl_2$ conditions (Figure 3C). The presence of chlorate or perchlorate salts caused denaturation peaks to occur at an even lower temperature, showing a stronger destabilizing effect compared to the KCl-$MgCl_2$ or KCl-$CaCl_2$ conditions. At low concentrations, chlorate or perchlorate salts do not seem to alleviate significantly the stabilizing effect of NaCl (Figure 3A).

Since the Prometheus NT.48, which we used for the nanoDSF assay, also measures light scattering, we used peaks of first derivative of light scattering as a proxy for aggregation events. However, aggregation peaks could only be observed at high concentrations of NaCl/KCl or with magnesium or calcium-containing chloride or perchlorate salts (Figure S1). KCl-$MgCl_2$ combinations allowed observation of two separated well-resolved aggregation peaks, the first happening at $T < 75$°C and the second at higher temperatures. Both $MgCl_2$ and $CaCl_2$ promoted aggregation events since increasing their concentration lowered the temperatures at which these events were observed.

Salt responses of the proteomes from *H. salinarum*, *Haloferax mediterranei*, *H. sodomense* and *H. volcanii* (compared to the one of *H. marismortui*)

To compare proteome response to salt conditions between species, the nanoDSF experiments were extended to cell lysates of four additional haloarchaeal strains: *H. salinarum*, *H. mediterranei*, *H. sodomense* and *H. volcanii*. These strains were isolated from environments with contrasting salt conditions and displayed different requirements for salt concentrations in their growth media (see Experimental Procedures section). In particular, *H. salinarum* is the most NaCl-dependent and NaCl-tolerant known organism, *H. sodomense* is the most Mg^{2+}-tolerant organism known and *H. mediterranei* has the largest range of NaCl concentrations compatible with growth. Details about salt requirements for growth and other microbiological information about these five strains from Bergey's Manual of Systematics of Bacteria and Archaea and is given in Table S1.

Thermal aggregation curves (Figure S2) were similar between strains either when only one type of salt was present or with salt combinations. Qualitative differences could only be seen between $MgCl_2$ and $CaCl_2$, the latter promoting aggregation events at lower temperatures more drastically than $MgCl_2$. Nonetheless, aggregation peaks were smaller at higher concentrations of KCl, especially at 3 M when combined with $CaCl_2$. $MgCl_2$ favoured observation of two aggregation peaks at lower temperatures as its concentration increased. $CaCl_2$ leads to the observation of additional dominant aggregation events whose temperature was positively correlated with $CaCl_2$ concentration. Interestingly, this was not observed with proteomes of *H. marismortui* and *H. salinarum* the two most NaCl-dependent strains.

In contrast, the denaturation curves largely differed between strains. Figure S3 shows representative denaturation curves under one-salt conditions for all tested strains. Overall, proteomes showed similar behaviour, all being gradually stabilized by higher KCl or NaCl concentrations. Stabilization by $MgCl_2$ and $CaCl_2$ was also clearly observed with the proteomes of *H. mediterranei* and *H. sodomense* and, to a lesser extent, with *H. volcanii*. Cell lysates of *H. salinarum* were associated with the least resolved denaturation peaks, making their observation difficult or even impossible, especially when only $MgCl_2$ or $CaCl_2$ was present. Interestingly, thermal denaturation curves of lysates of *H. mediterranei* showed two clearly separated dominant denaturation events.

With salt combination experiments, increasing the concentration of chaotropic salt promoted observation of more than one noticeable denaturation event (Figure S4). As a reminder, variation of fluorescence signal is a function of exposure of aromatic amino acids to solvents upon protein denaturation.

This was observed with proteomes of *H. sodomense* and *H. volcanii* at 1 and 2 M KCl with increasing $MgCl_2$ concentrations. The first event to occur, which was observed at 1 and 2 M and unseen at 3 M KCl, was shifted towards low temperatures when $MgCl_2$ concentration increased. On the contrary, the second event to occur happened at a slightly higher temperature as $MgCl_2$ concentration increased from 0 to 2 M. This showed that destabilizing as well as stabilizing effects of $MgCl_2$ on both proteomes could be observed in the same conditions.

Denaturation peaks were less resolved with KCl-$MgCl_2$ combinations for proteomes of *H. marismortui* and *H. salinarum*. However, it could be confirmed for these strains that increasing concentration of $MgCl_2$

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lowered temperatures at which denaturation peaks could be observed at most KCl concentrations, showing an overall destabilizing effect of $MgCl_2$. Use of $CaCl_2$ as the chaotropic salt reduced resolution of denaturation peaks for all strains, with the notable exception of *H. mediterranei* which maintained well-resolved denaturation peaks.

Effects of (per)chlorate-containing salts on proteome stability were similar between strains (Figure S5). $NaClO_3$ was nearly always stabilizing while $NaClO_4$ and $Mg(ClO_4)_2$ had destabilizing effects. However, these effects were reduced by increasing the concentration of NaCl. $Ca(ClO_4)_2$ had a complex non-linear effect on temperature associated with dominant denaturation events and generally reduced signal over noise ratio. As previously noted, denaturation curves obtained with proteomes of *H. marismortui* and *H. salinarum* were less resolved and, as with single-salt experiments, denaturation curves of proteome of *H. mediterranei* (Figure S5C) presented two distinct dominant denaturation events in most conditions.

Statistical analyses to compare proteome melting temperatures and sensitivity

To enable statistical comparisons, experiments with KCl or NaCl only and with salt combinations (except KCl-$CaCl_2$) were independently replicated for the five strains: three additional experimental points with fewer salt concentrations for KCl or NaCl only and two additional experimental points with the same conditions for KCl-$MgCl_2$ and NaCl-(per)chlorate combinations. The temperatures associated with the peak value of the first derivative of ratio of 350/330 nm fluorescence associated with the main dominant denaturation events, which we previously defined as proteome melting temperatures (T_{Pm}) (Carré et al., 2021), were retrieved. The slope of lines fitting T_{Pm} as a function of the concentration of the salt were also calculated. T_{Pm} and slope values are given in Figures 1 and 2.

The reproducibility of the measured T_{Pm} was good in spite of the complexity of the samples as shown by the low standard deviation values (Figures 1 and 2) except for *H. salinarum* for which it was not always possible to derive a T_{Pm} value. Variations in T_{Pm} values could be observed and were dependent on the strain and the salt type. The previously observed trends showed to be robust and reproducible. Increasing KCl or NaCl concentrations nearly always increased T_{Pm} in salt combinations experiments (Figures 1A,D and 2A,C,E,G). At every KCl concentration, the relationship between $MgCl_2$ and T_{Pm} was complex and non-linear (Figure 1D). $NaClO_3$ reproducibly stabilized proteomes and this effects linearly increased with its concentration (Figure 2A). In contrast, $NaClO_4$ gradually decreased T_{Pm} in most of the conditions (Figure 2C). Effects of $Mg(ClO_4)_2$ were similar to those due to $NaClO_4$ (Figure 2E). Like $MgCl_2$ in the presence of KCl, $Ca(ClO_4)_2$ had complex non-linear effects on T_{Pm}, increasing stability of proteomes of *H. sodomense* and *H. volcanii* at low concentrations and then destabilizing them at higher concentrations (Figure 2G).

While not all salts lead to linear relationships between their concentration and the T_{Pm}, the slopes of the fitting line enabled the comparison of global proteome sensitivity to a given salt type, regardless of its intrinsic thermostability. No statistically significant differences in proteome stabilization by KCl were observed between the five tested strains (Figure 1B). In contrast, the proteome of *H. mediterranei* presented a statistically significant higher stabilization response to NaCl (Figure 1C), being more efficiently stabilized by this salt than the other strains (with the exclusion of *H. salinarum* whose proteome did not offer enough well-resolved denaturation peaks). In addition, the proteome of *H. salinarum* was more destabilized by $MgCl_2$ (Figure 1E), less stabilized by $NaClO_3$ (Figure 2B) and more destabilized by $NaClO_4$ (Figure 2D). *H. sodomense* and *H. volcanii* presented strikingly similar proteome responses to all salt types, being the least sensitive to $NaClO_4$ or $Mg(ClO_4)_2$ and the only ones being stabilized by $Mg(ClO_4)_2$ (Figure 2F) or $Ca(ClO_4)_2$ (Figure 2H) in the presence of 3 M NaCl. Proteomes of *H. marismortui* and *H. mediterranei* showed intermediary behaviours between those observed for *H. salinarum* on the one hand and *H. volcanii*/*sodomense* on the other hand. In addition, both were very sensitive to $Mg(ClO_4)_2$ (Figure 2F).

To assess possible correlations between sensitivity to the tested salt conditions, Pearson correlation coefficients were calculated using slope values from the five tested strains and the corresponding correlation matrix determined (Figure 4A). Sensitivities to $MgCl_2$, $NaClO_3$ and $NaClO_4$ were positively correlated as were sensitivities to $Mg(ClO_4)_2$ and $Ca(ClO_4)_2$. However, positive and negative correlations were dependent on the counter-salt (NaCl or KCl) concentration.

Our results clearly show that the proteome response to chaotropic salts differs between strains. This is summarized in Figure 4B, highlighting distinct behaviours, such as high sensitivity to NaCl in the case of *H. mediterranei* proteome and its peculiar response to Martian or Earth-relevant salts.

DISCUSSION

Cellular proteomes contain different types of proteins with different biochemical and biophysical properties thus, studies related to single proteins cannot properly reflect the adaptative nature of the whole proteome. With this respect, nanoDSF is a valuable approach to

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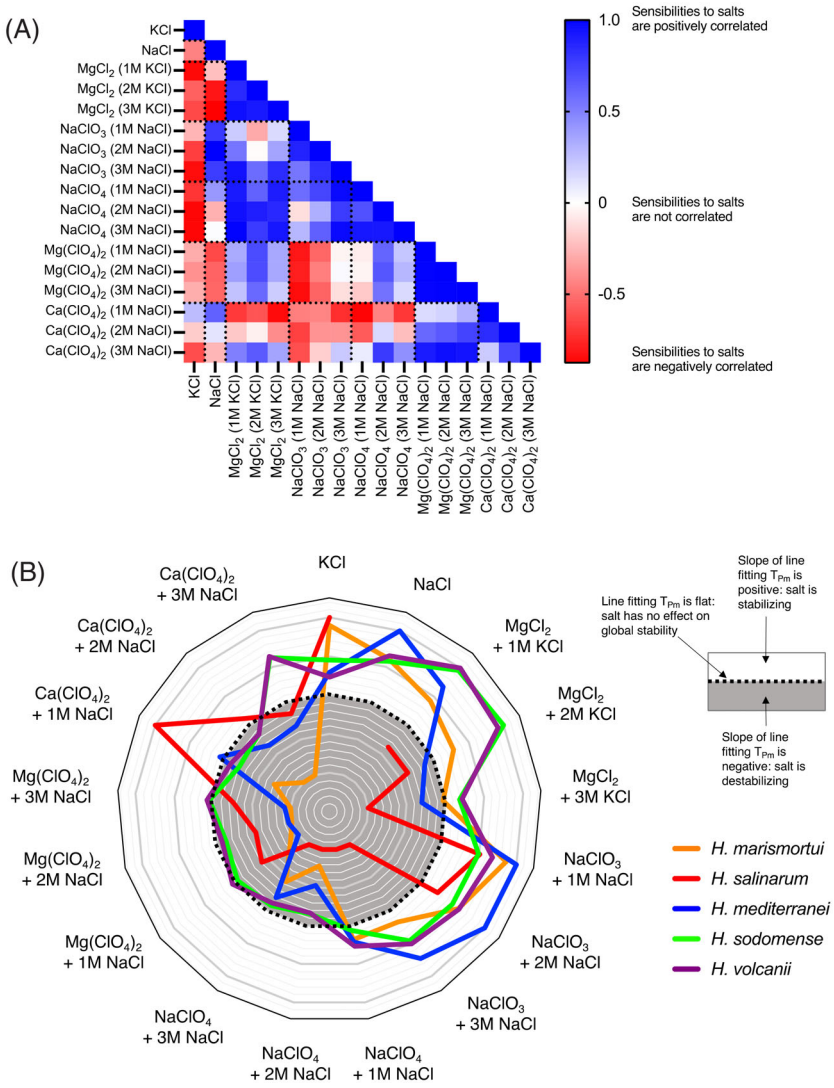


FIGURE 4 Correlation between strains and sensitivities to salt. (A) Correlation matrix of sensitivities to salt over the five strains. Dotted lines separate conditions with same main salt with, when applicable, the conditions of counter-salt concentrations. (B) Radar chart of the normalized slopes of line fitting T_{Pm} values of proteomes of each haloarchaeal strain for every tested salt condition. Grey zone corresponds to negative slopes and, hence, destabilizing effects of salts whereas white zone corresponds to positive slopes and stabilizing effects.

probe molecular response to chemical conditions at the proteome scale. In the present work, we extended our proof of concept experiment (Carré et al., 2021) to the comparison of several halophilic extremophiles. Obviously, the intrinsic fluorescence signal depends on the existence of aromatic residues within the sample and comparison between strains may be biased if aromatic residue content differed. Thus, we compared frequencies of Phe/Tyr/Trp residues in the proteomes of the five strains and within a set of 93 representative proteins listed in Data S1, distribution being represented in Figure S6. While the genomes of both strains encode on average similar frequencies of the aromatic amino acids, *H. sodomense* showed a reproducibly strong nanoDSF signal in our experiments, unlike *H. salinarum*. Nonetheless, the frequency of the aromatic amino acids in *H. salinarum* within the 93 representative proteins set is also close to the frequencies found in the genomes with *H. marismortui* and *H. sodomense*. Another aspect that may influence nanoDSF signal interpretation is the relative abundancy of certain protein families within the proteome. However, our study relies only on *haloarchaea* strains, limiting the differences in proteome complexity and relative composition. Our exploration of the halophilic proteome response revealed the existence of limited protein denaturation and/or aggregation events happening at clearly distinct temperatures in several salt conditions, depending on the strain. This observation could be related to the fact that distinct protein populations behave differently with respect to salt nature and concentration.

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The first important information provided by the nanoDSF experiments performed is that the proteome stability, with respect to salt, differed between the considered strains in spite of the fact that they are all strictly hyperhalophilic *Haloarchaea* accumulating molar concentrations of potassium ions in their cytosol. Of the five tested strains, *H. salinarum* and *H. mediterranei* showed the most distinctive behaviours. The proteome of *H. salinarum* gave the least resolved denaturation curves. This happened with every tested salt type, including the most chaotropic. This could be explained by a reduced frequency of aromatic residues contributing to the measured intrinsic protein fluorescence, hence reducing the peak height. As discussed, frequency of aromatic amino acids in the five considered strains is close. Therefore, scarcity of aromatic amino acids does not offer a reasonable explanation for the low-resolution denaturation peaks obtained with lysates of *H. salinarum*. Our observations and the similar aromatic residue frequency may indicate that proteins composing the cell lysates of *H. salinarum* may denature on a broader range of temperatures, thus limiting peak sharpness.

H. mediterranei stands out in another way: its proteome stability displayed significantly higher dependency on Na^+ than the other strains. While all haloarchaea, including *H. mediterranei* (Pérez-Fillol & Rodriguez-Valera, 1986), intracellularly accumulate K^+ rather than Na^+, the latter remains the dominant cation in most hypersaline environments. Most studies about intracellular salt composition in haloarchaea and its dependency on extracellular environment have essentially investigated intracellular K^+ associated to medium with modified NaCl concentrations at constant K^+ supply. In spite of the fact that no study has, to our knowledge, measured the intracellular concentration of Na^+ as a function of K^+ concentration in the growth medium, some studies with *H. marismortui* (Ginzburg et al., 1970; Jensen et al., 2015) and in *H. salinarum* (Lanyi & Silverman, 1972; Matheson et al., 1976) presented evidences of, respectively, decreased and increased accumulation of K^+ and Na^+ when K^+ is scarce in the medium. This indicates that haloarchaea may accumulate Na^+ instead when K^+ is scarce. In such conditions, proteome of *H. mediterranei* could therefore benefit from K^+ scarcity by having a proteome that can still benefit from Na^+ abundancy. It should also be pointed out that *H. mediterranei* possess fewer carotenoid pigments and lacks both bacteriorhodopsin and halorhodopsin (Oren, 2017; Oren & Hallsworth, 2014), which are photodependent H^+ and Cl^- pumps essential for K^+ accumulation and for intracellular ion homeostasis in *Haloarchaea* (Oren, 1999). Moreover, *H. mediterranei* is the only strain among the five tested whose genome encodes the mechanosensitive transporter MscS protein, which promotes K^+ effusion out of the cell, and all known haloarchaeal Mg^{2+} transporter whereas genome of *H. volcanii* encodes only two of them (MgtA and MgtB) and *H. salinarum* and *H. marismortui* none (Table S2) (Becker et al., 2014).

Our study shows a positive correlation between the NaCl requirement for growth and proteome sensitivity to chaotropicity nature. Indeed, among the five studied strains, *H. salinarum* is the one requiring the highest amount of NaCl for optimal growth and is also the one that tolerates the highest NaCl concentration in its environment (Oren & Ventosa, 2017). Interestingly, *H. salinarum* proteome was destabilized by chaotropic salts, $MgCl_2$ as well as (per)chlorate salts. By comparison, *H. sodomense* and *H. volcanii* optimally grow at lower NaCl concentrations (McGenity & Grant, 2015; Ventosa et al., 2015) and showed proteomes that are the least destabilized and even distinctively stabilized in some occasions by chaotropic salts. Moreover, *H. mediterranei* has the largest NaCl concentration range compatible with growth and tolerates both high and low NaCl concentrations (Oren, 2017; Oren & Hallsworth, 2014) and showed a proteome with intermediary behaviour in our measurements. Therefore, growth requirement for NaCl appears to be correlated with proteome sensitivity to $MgCl_2$ and (per)chlorate salts, underlying the link between proteome intrinsic properties and adaptation to the environment. Moreover, our results indicate that proteome tolerance to $MgCl_2$ is correlated to tolerance to perchlorate salts, suggesting the existence of common proteome traits allowing tolerance to general salt-induced chaotropicity. Whether this tolerance resulted from primary structure of proteins, compatible solutes accumulation, intracellular salinity, or expression of specific proteins such as chaperones or ion channels remains to be determined.

In cell lysates of *H. mediterranei*, two subpopulations of proteins denaturing at different temperatures were observed in NaCl or KCl single-salt conditions. One explanation could be that the proteins of this organism are, on average, more soluble over a larger range of KCl/NaCl conditions and, hence, more diverse with respect to the salt concentration effect. As this organism has one of the largest range of NaCl concentrations compatible with growth among haloarchaea (Oren, 2017; Oren & Hallsworth, 2014), this could reflect an adaptative trait allowing proteins to function over a broader range of environmental salt conditions. With *H. sodomense* and *H. volcanii*, which are two strains whose growths are exceptionally tolerant to $MgCl_2$ (Mullakhanbhai & Larsen, 1975; Oren, 1983), increasing $MgCl_2$ concentration at constant KCl concentration also segregated two subpopulations of proteins denaturing at different temperatures with one subpopulation stabilized by this divalent salt while the second is destabilized. These results further stress the complexity of the proteomes, which contains proteins with various response to high concentrations of Mg^{2+}.

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Besides denaturation, multiple aggregation events were observed with all halophilic strains at reproducibly similar temperatures with combinations of KCl-$MgCl_2$ and NaCl-$Mg(ClO_4)_2$. Both populations of aggregates formed at lower temperatures as the concentration of Mg-containing salt increased. Mg^{2+} hence segregates protein populations during thermal aggregation in a similar manner across strains. Interestingly, Hofmeister series predict instead that this salt should increase solubility and, hence, could limit aggregation. However, these predictions are mostly based on observations made with non-halophilic proteins, at a constant temperature, lower concentrations of chaotropic salts and generally for folded proteins. The thermal aggregation which happened during the experiments is made through hydrophobic interactions between residues exposed to solvent by the thermal unfolding. Since we observed, at least with some of the strains, two peaks in both denaturation and aggregation curves, it could be hypothesized that they are both formed by the same two distinct protein subpopulations. For example, a subpopulation more sensitive to Mg^{2+} could denature and aggregate before the other, hence producing the first peaks, followed by the other subpopulation forming the latter peaks.

High concentrations of chaotropic salts such as $MgCl_2$ correspond to complex extreme conditions as they combine high salinity, high ionic strength, low water activity and high chaotropicity. This is especially true with magnesium/calcium perchlorate salts which also add oxidative stress. Hence, it is commonly accepted that the effects of such salts on biomolecules are not well predictable and depend on the system. While the Hoffmeister series predict some of their effects, $MgCl_2$ and $CaCl_2$ increasing protein solubility and reducing protein stability, they do not indicate precisely the concentrations at which these salts become deleterious to biological systems.

We showed that increasing concentration of $MgCl_2$ reproducibly increased average thermostability of the proteomes, especially when the medium contained low concentrations of KCl. This effect continued to be observable at the highest $MgCl_2$ concentration values in *H. sodomense* and *H. volcanii*. This observation is astonishing as the concentrations of $MgCl_2$ or $CaCl_2$ tested in this study are considered to be particularly extreme at scales of both cellular life and biomolecular systems (Hallsworth et al., 2007; Yakimov et al., 2015), especially in the context of astrobiology and Martian brines (Fox-Powell et al., 2016; Hallsworth, 2021). For example, 2 M of $CaCl_2$ reduces by 20°C the T_m of bovine pancreatic ribonuclease (Von Hippel & Wong, 1965) and the model MDH enzyme from *H. marismortui* has previously shown to be fully denatured by 2 M $CaCl_2$.

Interestingly, in spite of being more chaotropic and oxidizing than chloride salts, Mars-relevant salts had contrasting effects on proteome stability as illustrated by the T_{Pm} values (Figure 4). $NaClO_3$, was always stabilizing all halophilic proteomes, except for *H. salinarum* at 3 M NaCl. $NaClO_4$ had a destabilizing effect, especially for *H. salinarum*, but only marginally for *H. sodomense* and *H. volcanii*. The trend remained the same for $Mg(ClO_4)_2$ with even more destabilized proteomes of *H. marismortui*, *H. salinarum* and *H. mediterranei*, and again significantly less effect on *H. sodomense* and *H. volcanii*. In contrast, concentrations of $Ca(ClO_4)_2$ below 0.5 M induced noticeable proteome stabilization with *H. sodomense* and *H. volcanii* at every NaCl concentration. The proteome of *H. mediterranei* was also stabilized by $Ca(ClO_4)_2$ but only at 1 M NaCl. Since adding $Ca(ClO_4)_2$ drastically diminished peak resolution with *H. salinarum*, T_{Pm} determination may be even more biased with this strain. Interestingly, a recent study showed, with a similar thermostability-based approach, that the bovine α-chymotrypsin was stabilized by $MgSO_4$, slightly stabilized by low concentrations of $MgCl_2$ and significantly destabilized by $NaClO_4$, $Mg(ClO_4)_2$ and $Ca(ClO_4)_2$ (Gault & Cockell, 2021). Another recent study considered instead the effects of perchlorate salts on the ß-galactosidase enzyme from *Halorubrum lacusprofundi*, an extremely cold-adapted halophilic archaeon (Laye & DasSarma, 2018), which is phylogenetically close to *H. sodomense* (Becker et al., 2014; Kamekura & Dyall-Smith, 1995). It has been found that the ClO_4^- anion acted as a very weak non-competitive inhibitor at concentrations above 0.88 M $NaClO_4$. Unlike the study on bovine α-chymotrypsin, the effects on stability were not investigated. In summary, over the five considered strains, *H. sodomense* and *H. volcanii* proteomes stand out with an outstanding tolerance to perchlorate salts such as those encountered on Mars, while *H. salinarum* proteome displays a high sensibility to these salts.

This work stresses the fact that simpler systems made with purified proteins may not represent the behaviour of the whole proteome. It has been previously shown that model MDH enzyme from *H. marismortui* and other *Haloarchaea* can be stabilized by low concentrations of $MgCl_2$ and $CaCl_2$, which only become destabilizing above 0.5–1 M (Blanquart et al., 2021; Madern & Zaccai, 1997). However, each enzyme from a single organism may display a specific salt response. For example, various enzymes (Bräsen & Schönheit, 2005; Camacho et al., 2009; Cendrin et al., 1994; Goldman et al., 1990) and large molecular assemblies (Franzetti et al., 2002; Shevack et al., 1985) from *H. marismortui* are stable below 2 M KCl. Since these proteins may be significantly abundant in the proteomes, highly salt-sensitive enzymes like MDH may not represent properly the proteome as a whole. This is especially critical in the context of the search for biomarkers. Indeed, putative extraterrestrial

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microorganisms would probably not leave behind purified systems but rather complex mixtures of their molecular components, based on their individual stability and eventual interactions. Hence, study of the biophysical effects of extraterrestrial-like simulated conditions on proteomes provides additional insights into how such conditions would shape habitability and the chances of finding biomarkers.

As pointed out by this study, halophilism, defined as NaCl-dependency, does not necessarily make the most relevant criterion for choosing a halophilic model for all astrobiological studies. Instead, organisms whose biomolecules present higher tolerance to $MgCl_2$, such as *H. sodomense* and *H. volcanii*, should be considered as more relevant in the context of chaotropic extraterrestrial brines.

The fact that $NaClO_3$ stabilized proteomes of all tested *Haloarchaea* and that some displayed low sensitivity to $NaClO_4$ and $Mg(ClO_4)_2$ and even some stabilization by $MgCl_2$ and $Ca(ClO_4)_2$ in low concentrations of KCl and NaCl may also increase the chances of finding salt-adapted biomarkers in Martian brines where these two monovalent salts are scarcer. In particular, low amounts of NaCl/KCl, which are not expected to be abundant in Martian brines, are not a limit per se for halophilic proteome stability which can be increased by various other salts.

Further studies could also consider other factors which may compensate effects of Martian-relevant salts on halophilic proteomes. For example, high pressure, which reduces perchlorate enzymatic inhibition on bovine α-chymotrypsin (Gault et al., 2020) and deleterious effects of $MgCl_2$, $MgSO_4$ and $Mg(ClO_4)_2$ on ligand biding by tRNA (Jahmidi-Azizi et al., 2021). Moreover, low temperatures of the surface and upper subsurface of Mars would also counteract chaotropic effects of Martian salts (Chin et al., 2010; Cray et al., 2015). Other factors compensating the effects of Martian-relevant salts include trimethylamine N-oxide (TMAO), a common intracellular osmolyte, which mitigates chaotropic effect of $Mg(ClO_4)_2$ on water structure (Laurent et al., 2020). Similarly, other kosmotropic salts, solutes and osmolytes counteract chaotropicity of chaotropic salts (de Lima Alves et al., 2015; Hallsworth et al., 2007; Stevenson et al., 2015; Yakimov et al., 2015).

Another important implication may arise from this work. While effects of pure water solutions on halophilic systems were out of the scope of this study, the denaturation curves obtained with only one type of salt show that all tested salts increased average thermostability in comparison to pure water conditions. This was seen with proteomes of all tested strains, except *H. salinarum* which should not always be considered as the most-used halophilic model for astrobiology. Indeed, sample returns are challenging goals of future Mars missions. Therefore, we stress the fact that extreme caution should be taken when analysing samples from brines or salt-containing rocks. In particular, special attention should be paid to the composition of the hypersaline buffers that should be used to resuspend the Mars samples.

AUTHOR CONTRIBUTIONS

Lorenzo Carré: Conceptualization (equal); data curation (equal); investigation (equal); methodology (lead); writing – original draft (equal). **Daniel Gonzalez:** Investigation (equal). **Éric Girard:** Conceptualization (equal); methodology (equal); supervision (equal). **Bruno Franzetti:** Conceptualization (lead); data curation (equal); funding acquisition (lead); investigation (equal); methodology (equal); project administration (lead); supervision (lead); validation (lead); writing – original draft (equal); writing – review and editing (equal).

ACKNOWLEDGEMENTS

This work is supported by the French National Research Agency in the framework of the Investissements d'Avenir programme (ANR-15-IDEX-02), through the funding of the 'Origin of Life' project of the Université Grenoble-Alpes. IBS acknowledges integration into the Interdisciplinary Research Institute of Grenoble (IRIG, CEA).

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

Datasets related to this article are available upon request.

ORCID

Bruno Franzetti https://orcid.org/0000-0001-5323-0510

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How to cite this article: Carré, L., Gonzalez, D., Girard, É. & Franzetti, B. (2023) Effects of chaotropic salts on global proteome stability in halophilic archaea: Implications for life signatures on Mars. *Environmental Microbiology*, 25(11), 2216–2230. Available from: https://doi.org/10.1111/1462-2920.16451

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