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NOTE

# High-pressure tolerance in *Halobacterium salinarum* NRC-1 and other non-piezophilic prokaryotes

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**Abstract** In this study, we examined the high-pressure survival of a range of prokaryotes not found in high-pressure environments to determine the effects of adaptations to osmotic and oxidative stresses on piezo-resistance. The pressure survivals of *Halobacterium salinarum* NRC-1, *Deinococcus radiodurans* R1, and *Chromohalobacter salexigens* were compared to that of *Escherichia coli* MG1655. *C. salexigens*, which uses the compatible solute ectoine as an osmolyte, was as piezo-sensitive as *E. coli* MG1655, suggesting that ectoine is not a piezolyte. *D. radiodurans* R1 and *H. salinarum* NRC-1, both resistant to oxidative stress, were found to be highly piezo-resistant. *H. salinarum* NRC-1 showed nearly full survival after pressurization up to 400 MPa; a survival 3.5 log units higher than *E. coli* MG1655. This piezo-resistance was maintained in *H. salinarum* NRC-1 for pressurizations up to 1 h. We hypothesize that the high-pressure resistance of *H. salinarum* NRC-1 is due to a combination of factors including cell envelope structure and the presence of intracellular salts.

**Keywords** High-pressure · Prokaryote · Halophile · Adaptation · Osmotic stress · Oxidative stress · Membrane composition

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## Introduction

The survival of microbial species under high-pressure conditions has been gaining attention in recent years due to isolation and characterization of piezo-tolerant and piezophilic species, as well as the use of high-pressure for sterilization. Discoveries during the past decade suggest that the biomass of microorganisms living deep in the Earth's crust may outweigh that on the surface (Daniel et al. 2006; Pfiffner et al. 2006). How organisms survive pressure extremes has yet to be fully understood.

The isolation and study of several naturally occurring piezo-tolerant and piezophilic microorganisms from deep hydrothermal vents and surrounding sediments has provided insights into obligate piezophily. Archaea such as *Pyrococcus* and *Thermococcus* strains were isolated and found to tolerate pressures of 100 MPa at 100°C (Edgcomb et al. 2007). Studies of *Methanococcus jannaschii* sp., another isolate from the deep sea, have demonstrated cell envelope rupture upon rapid decompression (Park and Clark 2002) and the lipid-ordering effect of pressure on archaeal cell membranes (Kaneshiro and Clark 1995). The accumulation of intracellular solutes such as beta-hyroxybutyrate was found to aid the pressure resistance of the moderately piezophilic bacterium *Photobacterium profundum* SS9 (Martin et al. 2002).

Alternatively, microorganisms not typically associated with piezo-tolerance, such as *Escherichia coli* (Griffin et al. 2011; Hauben et al. 1997; Koseki and Yamamoto 2006; Malone et al. 2006; Pagán and Mackey 2000; Van Opstal et al. 2003; Van Opstal et al. 2005; Welch et al. 1993; Wuytack et al. 2002) and *Saccharomyces* species (Palhano et al. 2004; Perrier-Cornet et al. 2005; Perrier-Cornet et al. 1999; Sato et al. 1996), have been employed to elucidate the underlying mechanisms of pressure resistance under a

range of laboratory conditions. Experiments studying the metabolically diverse mesophile *Shewanella oneidensis* MR-1 conducted at very high pressure (1.2 GPa) using diamond anvil cell techniques provided observations on metabolic activity (specifically formate oxidation) at these gigapascal high pressures (Sharma et al. 2002, 2005). Owing to the microliter volumes of these pressure cells, however, Sharma et al. (2002) were not able to confirm cell division or quantify viability after pressure release. *E. coli* cultures were also shown to survive to gigapascal pressures (Sharma et al. 2002), a result that has been recently confirmed through directed evolution experiments (Vanlint et al. 2011). Variations in high-pressure survival described within a single *E. coli* strain (Griffin et al. 2011) highlight the inherent potential for non-piezophilic cells to adapt to high-pressure conditions. Variations in piezo-tolerance have also been linked to the growth phase and the particular strain examined (Pagán and Mackey 2000).

High-pressure has many effects on cellular structure and function (see reviews Abe 2007; Bartlett 2002; Oger and Jebbar 2010; Simonato et al. 2006). Exposure of organisms to high pressure has been shown to disrupt metabolism, nucleic acid synthesis, protein synthesis, enzymatic activity, and cell membrane structure leading to cell death (see review in Abe 2007). In *E. coli*, pressures less than 100 MPa can produce all these effects (see review in Oger and Jebbar 2010). There are, however, adaptive strategies that can reduce the cytotoxic effects of high-pressure. Identifying and understanding these mechanisms is important for the efficacy of high-pressure sterilization, in particular in the food industry where pressures of 200–800 MPa are routinely used for sterilization (Balasubramaniam et al. 2008). We focused on the effects of osmotic stress adaptations, oxidative stress adaptations, and membrane composition on piezo-resistance in prokaryotic organisms.

Previous studies have suggested a link between osmotic stress and pressure-tolerance. Increasing the osmolarity of the growth medium increased the piezo-tolerance of *Lactococcus lactis* due to increased accumulation of intracellular disaccharides (Molina-Höppner et al. 2004); a similar strategy to that employed by the piezophilic *P. profundum* SS9 for pressure resistance (Martin et al. 2002), showing that some osmolytes can function as piezolytes. Decreased water activity can also have a stabilizing effect on cellular proteins under high-pressure conditions (Oliveira et al. 1994). Oxidative stress has been shown to play a role in the lethal effects of high-pressure. *E. coli* mutants lacking reactive oxygen species scavengers were more pressure-sensitive than wild type strains (Aertsen et al. 2005). The same study showed also evidence of disulfide bond formation in the cytoplasm, a marker for intracellular oxidative stress, at elevated pressures. Finally, in both archaea and bacteria, pressure has been shown to have a membrane-ordering effect resulting in a shift in lipid composition to increase membrane fluidity at higher pressures. Piezophilic bacterium such as *P. profundum* SS9 and *Shewanella* sp. DB21MT-2 increase their concentration of monounsaturated and polyunsaturated fatty acids under increased pressures (Simonato et al. 2006). Archaea, including *M. jannaschii*, lack ester-linked fatty acids in their cell membranes, using instead isopranyl chains ether-linked to glycerol molecules. Like in the Bacteria, however, pressure was shown to have a membrane-ordering effect in *M. jannaschii* and result in a shift in lipid composition to increase membrane fluidity at higher pressures (Kaneshiro and Clark 1995).

In this study, we investigate the piezo-tolerance at the cellular and molecular level of the extremely halophilic archaeon *Halobacterium salinarum* NRC-1, the mesophilic bacterium *E. coli* MG1655, the halotolerant bacterium *Chromohalobacter salexigens*, and the multi-resistant bacterium *Deinococcus radiodurans* R1. None of these species is naturally found in a high-pressure environment. *E. coli* MG1655 was used as a piezo-sensitive control. The other prokaryotic species were selected based on cellular adaptations previously hypothesized to aid in piezo-tolerance. Both *H. salinarum* NRC-1 and *C. salexigens* are halophilic species inhabiting hypersaline environments. Microorganisms inhabiting these types of environments are obliged to adopt one of two strategies for preventing osmotic shock: 'salt-in' in which intracellular salts are stored in equimolar amounts to the extracellular environment, or 'salt-out' in which compatible solutes are used in the place of intracellular salts. We compared the high-pressure survival of the halophilic archaeon *H. salinarum* NRC-1, which employs a 'salt-in' strategy against osmotic stress accumulating high intracellular concentrations of chloride and potassium ions (Engel and Catchpole 2005), to the halophilic bacterium *C. salexigens*, which accumulates ectoine as an osmolyte in a 'salt-out' strategy (Arahal et al. 2001). The intracellular accumulation of compatible solutes has been shown to increase piezo-tolerance in mesophilic and moderately halophilic species by aiding in the maintenance of membrane fluidity in the presence of increased concentrations of sucrose or sodium chloride in the growth media (Molina-Höppner et al. 2004; Tanaka et al. 2001). The role of intracellular salts in piezo-tolerance has not previously been tested. *D. radiodurans* R1 was used, along with *H. salinarum* NRC-1, to test the correlation between oxidative stress resistance and piezo-resistance, as both species have been shown to be highly resistant to conditions such as ionizing radiation, which produce oxidative stress (Kottemann et al. 2005; Mattimore and Battista 1996). The organisms selected also represent a broad range of membrane compositions, from Gram-negative (*E. coli* MG1655 and *C. salexigens*) to Gram-positive (*D. radiodurans* R1) and archaeal (*H. salinarum* NRC-1)

cell membranes. The goals of this study were to determine the survival of a broad range of prokaryotic species to identify the cellular adaptations relevant to piezo-tolerance, and generate hypotheses about the mechanisms underlying piezo-resistance.

## Materials and methods

*H. salinarum* NRC-1, *E. coli* MG1655, *C. salexigens*, and *D. radiodurans* R1 were cultured in the appropriate growth medium at the appropriate temperature (see Table 1 for details) with shaking at 220 rpm. Growth media used were GN101 (250 g/L NaCl, 20 g/L $MgSO_4$-$7H_20$, 2 g/L KCl, 3 g/L Na citrate, 10 g/L Oxoid peptone; pH 7.2), LB (10 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract), TGY (5 g/L tryptone, 1 g/L glucose, and 3 g/L yeast extract), ATCC Medium 87 (100 g/L NaCl, 8 g/L BD nutrient broth). Buffers used for dilutions prior to plating were BSS (250 g/L NaCl, 20 g/L $MgSO_4$-$7H_20$, 2 g/L KCl, 3 g/L Na citrate; pH 7), PBS (8 g/L NaCl, 0.20 g/L KCl, 1.44 g/L $Na_2HPO_4$, 0.24 g/L of $KH_2PO_4$; pH 7.4), and 1.7 M PBS (PBS + 100 g/L NaCl). Both stationary and exponential phase cultures were incubated aerobically in triplicate in liquid media at optimal temperatures. For the former, stationary phase cultures were diluted in media to $OD_{600nm}$ of 1.0 and immediately transferred to pre-sterilized straws. All exponential phase cultures were grown to an $OD_{600nm}$ of 0.6 prior to pressurization. For the fast-growing cultures, *E. coli* MG1655, *C. salexigens*, and *D. radiodurans* R1, overnight cultures were diluted to an $OD_{600nm}$ of 0.1 in the appropriate growth medium, incubated at optimal temperature with shaking until they reached the desired optical density ($OD_{600nm}$ 0.6). *H. salinarum* NRC-1, which grows more slowly, was monitored in exponential phase until $OD_{600nm}$ reached 0.6, and then transferred to experimental straws.

Aliquots of 1 mL of culture in growth medium were loaded into pre-sterilized straws and heat sealed on both ends leaving approximately 0.2–0.4 mL of headspace (Griffin et al. 2011). Straws for experimental samples were individually loaded into the cold seal apparatus (Kerrick 1987; Sterner 1994) and exposed to hydrostatic pressure at room temperature, while straws for control samples were kept at atmospheric pressure for the same time duration. Survival assays were performed at 100, 200, 300, or 400 MPa for 10 min. High-pressure timed assays were performed in the same manner for exponential phase culture of both *E. coli* MG1655 and *H. salinarum* NRC-1 at 175 MPa over a range of exposure times (10, 30, and 60 min). *H. salinarum* NRC-1 cultures were also pressurized to 400 MPa for 10, 30, and 60 min. In all cases, the pressure was then released in a controlled manner at a rate of approximately 20 MPa/s.

Both control and experimental samples were then aspirated out of the straw using a needle and syringe, diluted using the buffers listed in Table 1 and plated onto the appropriate agar medium. Dilutions were optimized to yield approximately 20–200 colonies per plate, and five replicate plates were used for each replicate culture at each pressure investigated. Plates were incubated at the appropriate temperatures (see Table 1) and times for each organism (plate incubations were 16 h for *E. coli* MG1655, 36–48 h for both *C. salexigens* and *D. radiodurans* R1, and 7 days for *H. salinarum* NRC-1 due to variations in doubling times between organisms), after which time microbial survival was enumerated by counting colony-forming units for both pressurized samples and the unpressurized control sample. Survival was calculated as the average ratio of the survival ($N/N_o$) of number of colonies from the experimental sample ($N$) and a control sample ($N_o$), representing at least 3 full replicates. Paired $T$ tests were used to determine the statistical significance of any change in survival between control and experimental samples with $P < 0.01$ considered as significant.

## Results and discussion

The pressure resistance of each prokaryotic species in this study was measured over pressures from 0.1–400 MPa (see Fig. 1a, b). A number of studies have focused on the role of ionic strength of the surrounding milieu on piezo-tolerance

**Table 1** Strains and growth conditions used in this study

| Organism | Source | Taxonomy | Growth medium | Dilution buffer | Incubation temperature (°C) |
|---|---|---|---|---|---|
| *Halobacterium salinarum* NRC-1 | Gift from Dr. Jocelyne DiRuggiero | Archaea (Euryarchaeota) | GN101 | BSS | 45 |
| *Chromohalobacter salexigens* | ATCC BAA-138 | Bacteria (Proteobacteria; Gram-negative) | ATCC medium 87 | 1.7 M PBS | 37 |
| *Escherichia coli* MG1655 | ATCC 47076 | Bacteria (Proteobacteria; Gram-negative) | LB | PBS | 37 |
| *Deinococcus radiodurans* R1 | ATCC 13939 | Bacteria (Deinococcus-Thermus; Gram-positive) | TGY | PBS | 30 |

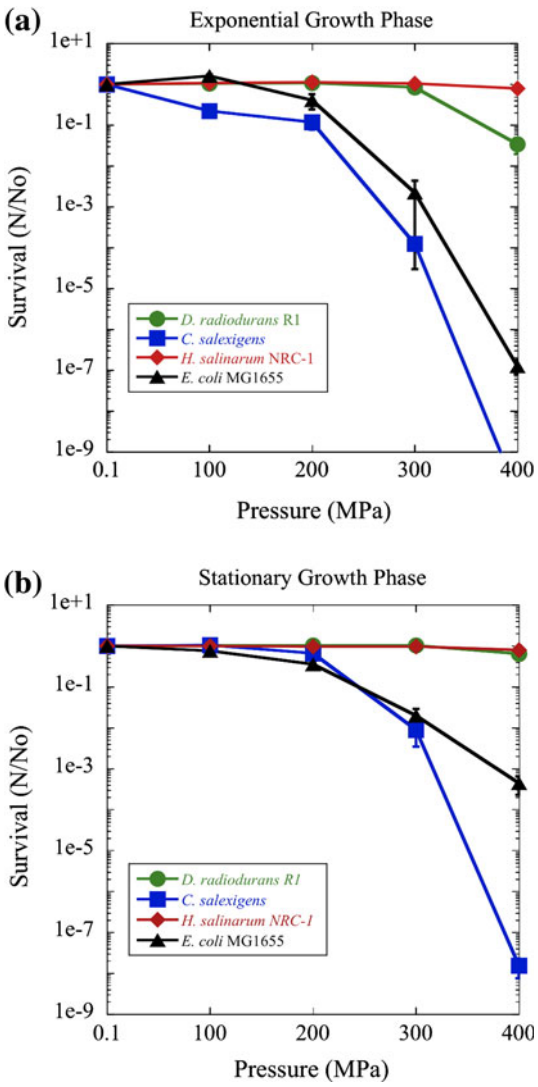


**Fig. 1** High-pressure survival after 10 min exposures to pressurization at 100, 200, 300 and 400 MPa compared to unpressurized controls. Survival was measured as the ratio of survival of the experimental ($N$) samples to the control ($N_o$). Data shown are the averages of at least three independent replicate experiments, with the exception of *D. radiodurans* R1 survival in stationary phase at 200 MPa, which is based on two independent replicates. **a** Pressure survival in exponential growth phase. Note that for *C. salexigens*, no surviving colony-forming units were observed after plating undiluted exponential growth phase cultures pressurized to 400 MPa for 10 min. **b** Pressure survival in stationary growth phase

in the Bacteria (Kaye and Baross 2004; Martin et al. 2002; Molina-Höppner et al. 2004; Tanaka et al. 2001), but prior to our work, little was known about how halophilic archaea would respond to pressure. In this study, *H. salinarum* NRC-1 showed the greatest piezo-resistance, with full survival up to the limit of the cold seal apparatus used for these experiments (400 MPa) in both exponential and stationary growth phases. *C. salexigens* cultures in both exponential and stationary phases showed the lowest levels of survival after pressurization, with a nearly fourfold reduced survival in exponential phase and a twofold reduced survival in stationary phase after exposure to 300 MPa compared to *H. salinarum* NRC-1. The piezo-tolerance of *C. salexigens* more closely matched that of its bacterial counterpart *E. coli* MG1655 rather than that of the other halophilic *H. salinarum* NRC-1. The fact that the survival of *C. salexigens* under high-pressure stress was significantly lower than *H. salinarum* NRC-1 is consistent with the hypothesis that intracellular chloride and potassium provide greater protection against the effects of high-pressure than the compatible solute ectoine used by *C. salexigens*. The piezophilic bacterium *P. profundum* SS9 also accumulates compatible solutes for use as both osmolytes and piezolyte, but the authors note that while the piezolytes identified also function as osmolytes, not all the osmolytes in *P. profundum* SS9 act as piezolytes (Martin et al. 2002). Thus, we hypothesize that the ectoine accumulated in *C. salexigens* acts only as an osmolyte and not as a piezolyte. In addition, the proteins in *H. salinarum* NRC-1 are adapted to functioning in a high-salt environment due to the presence of high concentrations of intracellular salts as a protective strategy against osmotic stress in hypersaline environments. The presence of high concentrations of intracellular salts in halophilic archaea promotes the formation of hydrophobic bonds that serve to increase protein stability (Lanyi 1974). In thermophilic microorganisms, hydrophobic interactions were found to increase the stability of proteins under high-pressure conditions, regardless of whether or not the organism was piezophilic (Hei and Clark 1994; Michels and Clark 1997; Robb and Clark 1999). The high salt concentration within haloarchaeal cells reduces water activity, which, in experiments using increasing concentrations of glycerol to reduce water activity, has also been shown to stabilize proteins under high-pressure conditions (Hayman et al. 2008; Smolin and Winter 2008; Oliveira et al. 1994). Therefore, the presence of intracellular salts may aid the piezo-tolerance of *H. salinarum* NRC-1 by stabilizing protein structures and activities. The findings presented in this study, however, do not exclude the roles of other factors such as membrane composition and adaptations to oxidative stress in piezo-tolerance as high-pressure tolerance is likely the result of a combination of factors.

Both *H. salinarum* NRC-1 (Kaur et al. 2006; Kish et al. 2009; Kottemann et al. 2005; Shahmohammadi et al. 1997; Whitehead et al. 2006) and *D. radiodurans* R1 (Daly et al. 2007; Ghosal et al. 2005; Slade and Radman 2011) have been shown to be highly resistant to a range of conditions resulting in oxidative stress. The relationship between pressure and oxidative stresses was first established using *E. coli* MG1655 as a model system (Aertsen et al. 2005). Analyses of the transcriptional response to high-pressure exposure for both *E. coli* MG1655 and *S. cerevisiae*

showed up-regulation of redox homeostasis proteins and free radical scavengers (Fernandes et al. 2004; Malone et al. 2006) indicating oxidative stress after pressurization in both organisms. As shown in Fig. 1a, b, the survival of *D. radiodurans* R1 matched that of *H. salinarum* NRC-1, with the sole exception that in exponential phase *D. radiodurans* R1 cultures showed an approximately 1-log lower survival at 400 MPa than cultures of *H. salinarum* NRC-1. *D. radiodurans* R1 showed statistically significant reductions in survival in comparison with control samples after pressurization at both 300 and 400 MPa ($P$ value $<0.0001$ for both), but still maintained survival ratios several logs greater than that of the other two bacterial species in this study (Fig. 1a, b). Neither *D. radiodurans* R1 nor *H. salinarum* NRC-1 are exposed to high-pressure conditions in their native environments. Thus, we hypothesize that in both species adaptations to oxidative stress play a strong role in piezo-resistance.

*H. salinarum* NRC-1 displayed a high level of piezo-resistance in both exponential and stationary growth phases, whereas *E. coli* MG1655 samples showed a 3-log difference in survival at 400 MPa ($P$ value $<0.0001$) between cultures in exponential phase and stationary phase. In stationary phase, *H. salinarum* NRC-1 cultures showed only a minor, but statistically significant ($P$ value $= 0.0017$) drop in survival at 400 MPa to roughly 80% from nearly 100% survival up to 300 MPa (Fig. 1b). Growth phase has been shown to affect a number of factors in pressure stress survival, including membrane fluidity (Casadei et al. 2002; Pagán and Mackey 2000). Membrane fluidity has been proposed to be the overriding factor influencing high-pressure survival for *E. coli* MG1655 cultures in exponential growth phase, but the relative impact of membrane fluidity on cytotoxicity after pressure stress in stationary phase cultures is reduced (Casadei et al. 2002). Maintenance of membrane integrity has been shown to be a significant factor for cell survival, both for mesophiles under pressure (Pagán and Mackey 2000) and for piezophiles during decompression (Park and Clark 2002). In the Bacteria, membrane fluidity is reduced at high pressure due to increased crowding of fatty acyl chains. Changes in lipid composition with increased pressure have also been noted in the Archaea (Kaneshiro and Clark 1995), with the difference that archaeal cell membranes lack fatty acids and have instead ether-linked lipids with isoprenoid acyl chains bound to glycerol moieties. These structural features render archaeal lipids more stable than their bacterial or eukaryotic counterparts do. Amongst the bacteria, the increased structural rigidity given by the thick peptidoglycan layer in the cell envelope of Gram-positive bacteria, such as *D. radiodurans* R1, does not necessarily aid in piezo-resistance compared to Gram-negative bacteria such as *C. salexigens* and *E. coli* MG1655 that have a thinner peptidoglycan layer surrounded by an outer membrane. One study comparing the piezo-tolerances of a variety of Gram-positive and Gram-negative bacteria found that there was considerable overlap in the survival of the two types of bacteria, especially over 300 MPa (Wuytack et al. 2002). This suggests the cell membrane composition is not the sole predicting factor of pressure-resistance. Beyond the cell membrane, the presence of a proteinaceous surface S-layer as part of the cell envelope has been shown to increase the pressure resistance of cell membranes (Schuster and Sleytr 2002). Both *H. salinarum* NRC-1 and *D. radiodurans* R1 possess such S-layers, which likely contribute to their piezo-resistance.

To determine the effects of time on pressure resistance, we measured the survival of both *E. coli* MG1655 and *H. salinarum* NRC-1 cultures in exponential phase at 10, 30, and 60 min at 175 MPa [the pressure at which the survival curve of *E. coli* MG1655 begins to show a slight decrease (data not shown)]. The survival of *E. coli* MG1655 decreased to less than 20% after 60 min at 175 MPa, whereas *H. salinarum* NRC-1 cultures maintained 85% survival ($P$ value $= 0.0002$) (Fig. 2). This result has implications for sterilization practices in the food industry, indicating that longer pressure-sterilization times may be required depending on the types of prokaryotic species found in the food product. We further examined the survival of *H. salinarum* NRC-1 cultures over increased intervals of pressurization at 400 MPa, as *H. salinarum* NRC-1 showed full survival after 10 min exposures to this pressure. The halophilic bacterium *Micrococcus roseus*

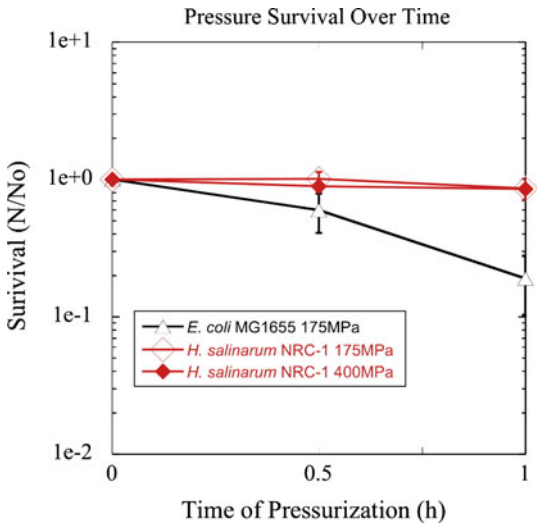


**Fig. 2** Pressure survival over time for *E. coli* MG1655 and *H. salinarum* NRC-1 at 175 MPa, and *H. salinarum* NRC-1 at 400 MPa. 175 MPa is the pressure at which *E. coli* MG1655 begins to show reduced survival (data not shown). *H. salinarum* NRC-1 cultures were additionally tested at 400 MPa to test the survival over time at the maximum pressure achieved by the cold seal apparatus used in these experiments

(Tanaka et al. 2001) has been shown to have nearly full survival up to 2 h at what was shown to be a sublethal pressure for *M. roseus* under the experimental conditions employed (138 MPa). In comparison, the decreased piezo-resistance over time displayed by *H. salinarum* NRC-1 at 1 h may have resulted from the markedly higher pressures used in our study (400 MPa versus 138 MPa for *M. roseus*). In addition, differences in cell physiology and inactivation kinetics after high-pressure exposure between these two species may have contributed to the different results observed for piezo-resistance over time.

The broad range of new findings about the effects of pressure on microorganisms necessitates the direct study of a wide variety of model prokaryotic organisms. This work generated a number of hypotheses for the mechanisms underlying piezo-resistance, particularly in *H. salinarum* NRC-1. Firstly, that the presence of intracellular salts potentially can act to increase the stability of cellular proteins and act as piezolytes to help maintain cellular integrity. Secondly, adaptations to oxidative stress can increase piezo-tolerance. Finally, the presence of an S-layer around the cell membrane can increase membrane integrity under high-pressure conditions. Previous studies have demonstrated the adaptive capacity of non-piezophilic species such as *E. coli* to high-pressure conditions through directed evolution (Vanlint et al. 2011). In this work, we show that even without directed evolution, non-piezophilic species such as *H. salinarum* NRC-1 may be piezo-resistant based on adaptations to other environmental stresses.

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