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## **A “pseudosporulation” adaptation of extremely halophilic Archaea in response to prolonged salt deprivation.**

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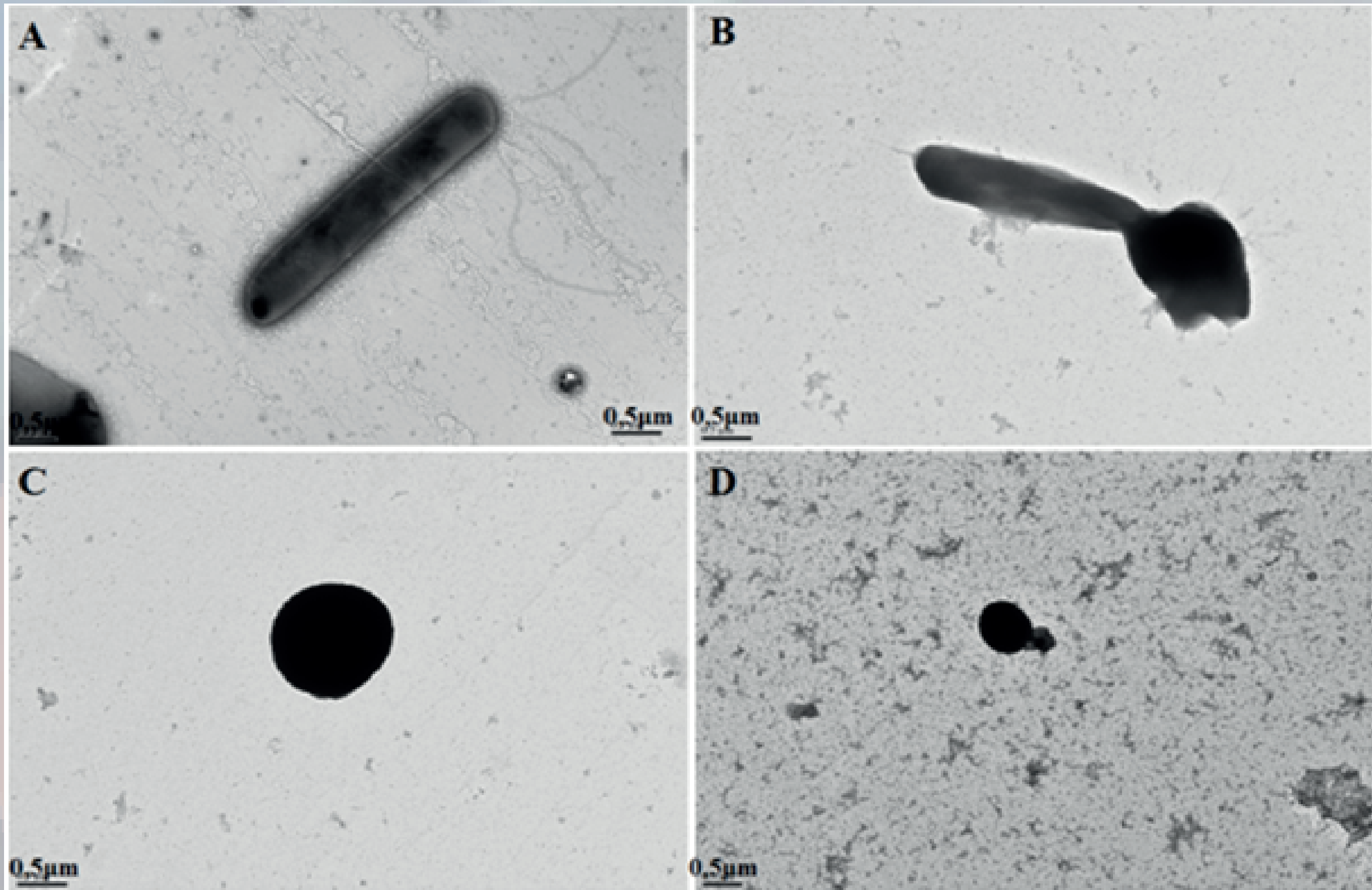


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## INTRODUCTION

Since the discovery by Woese et al. (1977) of a third kingdom in cell life classification, archaea are considered as one of the most fascinating microorganisms. Diverse and widespread distributed in the environment (soil, ice, deserts, ocean, lake, deep sea...), archaea contribute to global energy cycle. Moreover, they dominate extreme environments with harsh physico-chemical conditions: high and low temperature, high pressure, pH (acid and basic) and salt. As a consequence, archaea have evolved strategies to resist to the deleterious effect of these extreme conditions. Among these environments, salt plays a key role because one quarter of the land mass contains salt deposition. Some halophilic microorganisms require high salt concentration to live. In the ELMA group we study *Halobacterium salinarum* (HS), one of the most extreme halophilic microorganisms belonging to the domain Archaea. This microorganism requires a concentration of NaCl close to the saturation (3.4-5 mol L<sup>-1</sup> NaCl) to grow optimally and to maintain his structural integrity. When *H. salinarum* are progressively exposed to a reduction of salt concentration, most of cells survive but a deep morphological modification occurs and a perturbation in the folding state of the HS proteome was observed (§1, 2 & 3). Interestingly, when these stressed halophilic archaea are re-exposed to high salt concentration (4.2 mol L<sup>-1</sup> NaCl), a process of reactivation occurs suggesting a possible adaptation of the HS metabolism and proteome (§ 4 & 5).

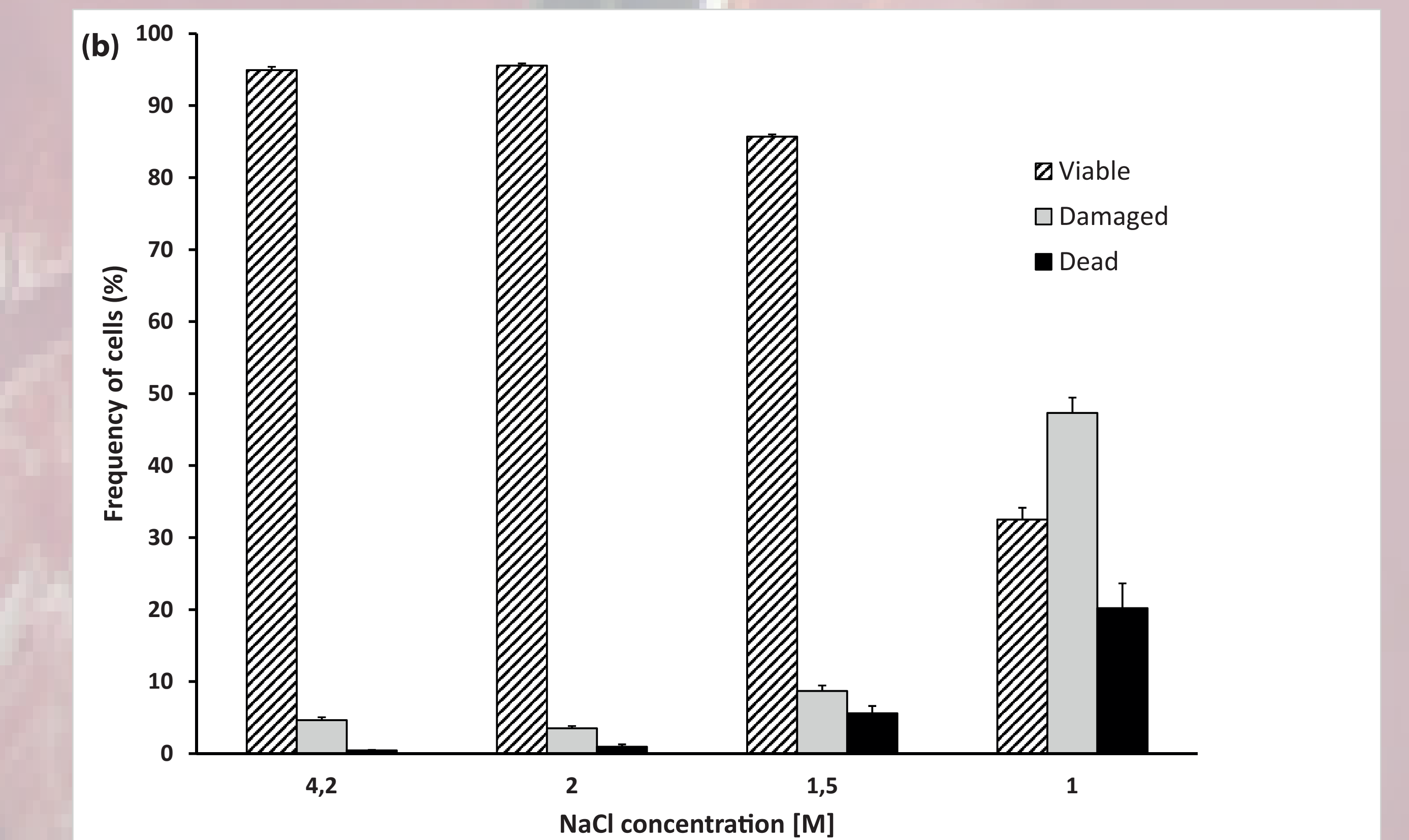
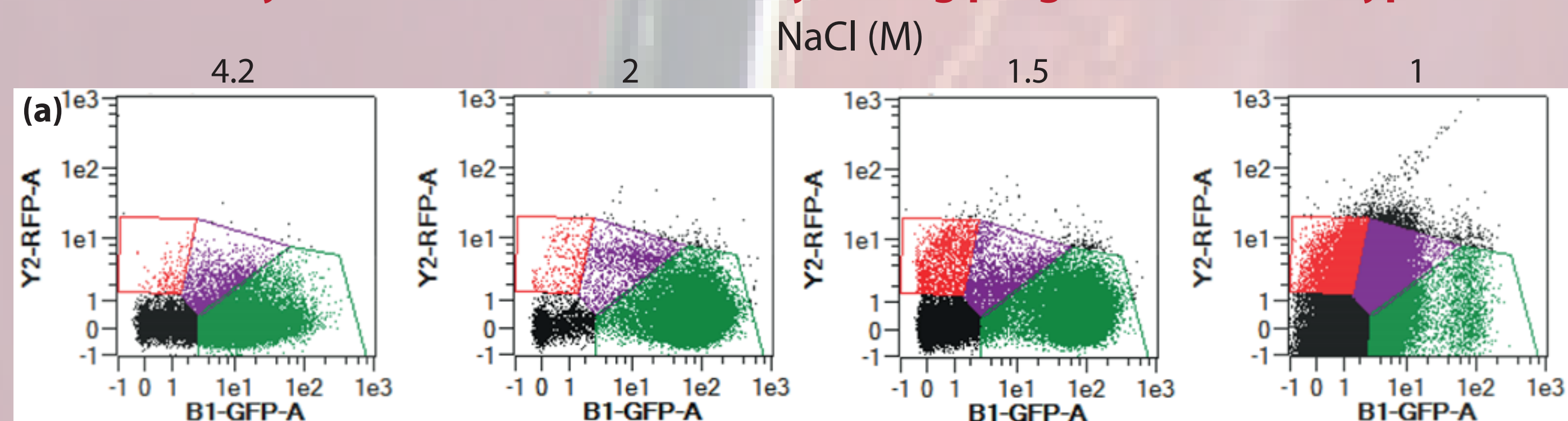
### 1 - Morphological evolution of *H. salinarum* during progressive stress hyposalin.



Pictures of negative stain electron microscopy correspond to *H. salinarum* cells taken at different NaCl concentrations. A) 4.2M; B) 2M; C) 1.5M and D) 1M of NaCl. A Transmission Electron Microscope (TEM) CM 12 was used.

We observed that a progressive decrease of salt concentration in the medium triggered morphological modifications of *H. salinarum* with no resulting lysis. At 2M of NaCl, we observed a deformation of the rod shape cells at one end, which become spherical like a pseudospore when 1.5M of NaCl was reached. Below that salt concentration, cells become smaller. Moreover, a decrease in the cytoplasm density was observed in cells stressed at 1.5M and 1M of NaCl (data not shown).

### 2 - Analysis of *H. salinarum* viability during progressive stress hyposalin.



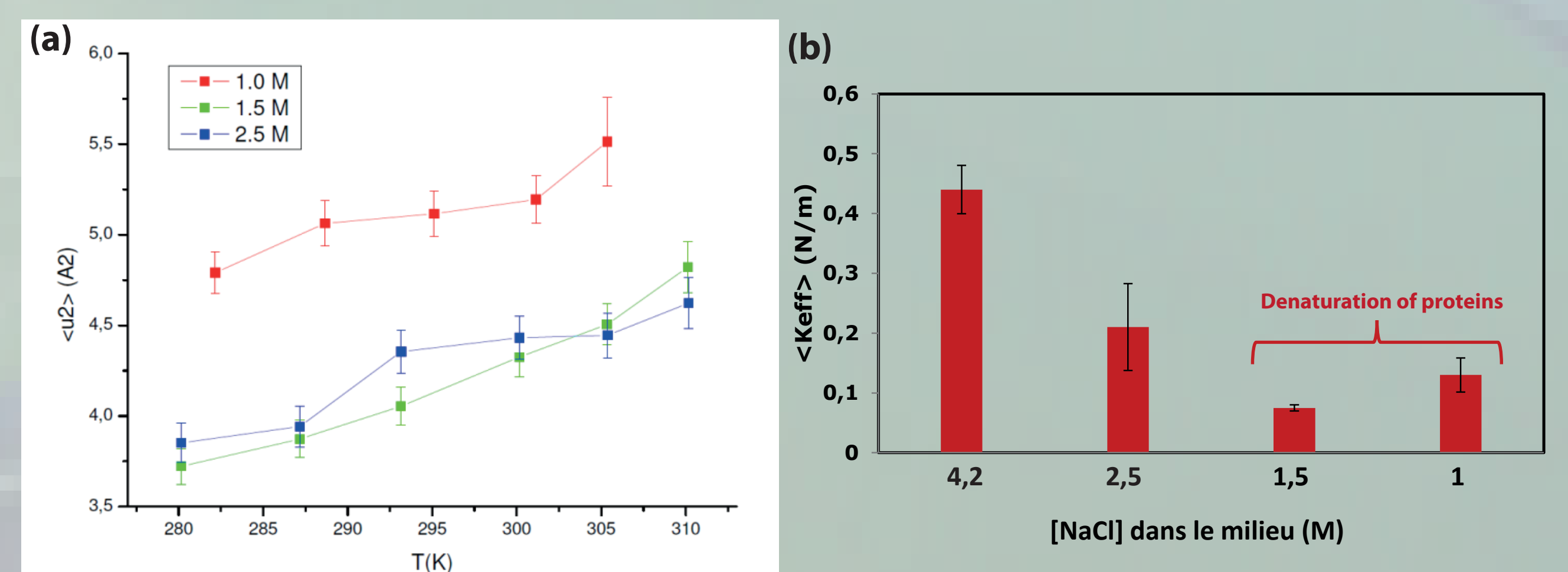
Analysis by flow cytometry of *H. salinarum* viability during progressive salt stress. Archaea were stained using the LIVE/DEAD BacLight Kit. All cells which fluoresce in green were labelled with the syto 9 stain. In contrast, red fluorescence (Propidium iodide stain) indicate non-viable cells. (a) Representative dot plots of different populations. Green: *H. salinarum* cells; Purple: damaged cells; Red: Dead cells. (b) Percentage of living, damaged and dead cells.

For the determination of viability, *H. salinarum* membrane integrity analysis using syto 9 and propidium iodide was applied. We observed that 93% and 97% of cells progressively adapted to 2 and 1.5M of NaCl were viable respectively. In contrast, the mortality rate was increased approximately by four when the cells were incubated in 1M of salt. These results suggest that despite a deep morphological modifications during hyposalin stress, most of *H. salinarum* survive.

#### Acknowledgments.

We thank F. Lacroix and R.L. Revel-Goyet (Institut de Biologie Structurale, Grenoble) for their excellent supports to the cell imaging and flow cytometer respectively (M4D, platform).

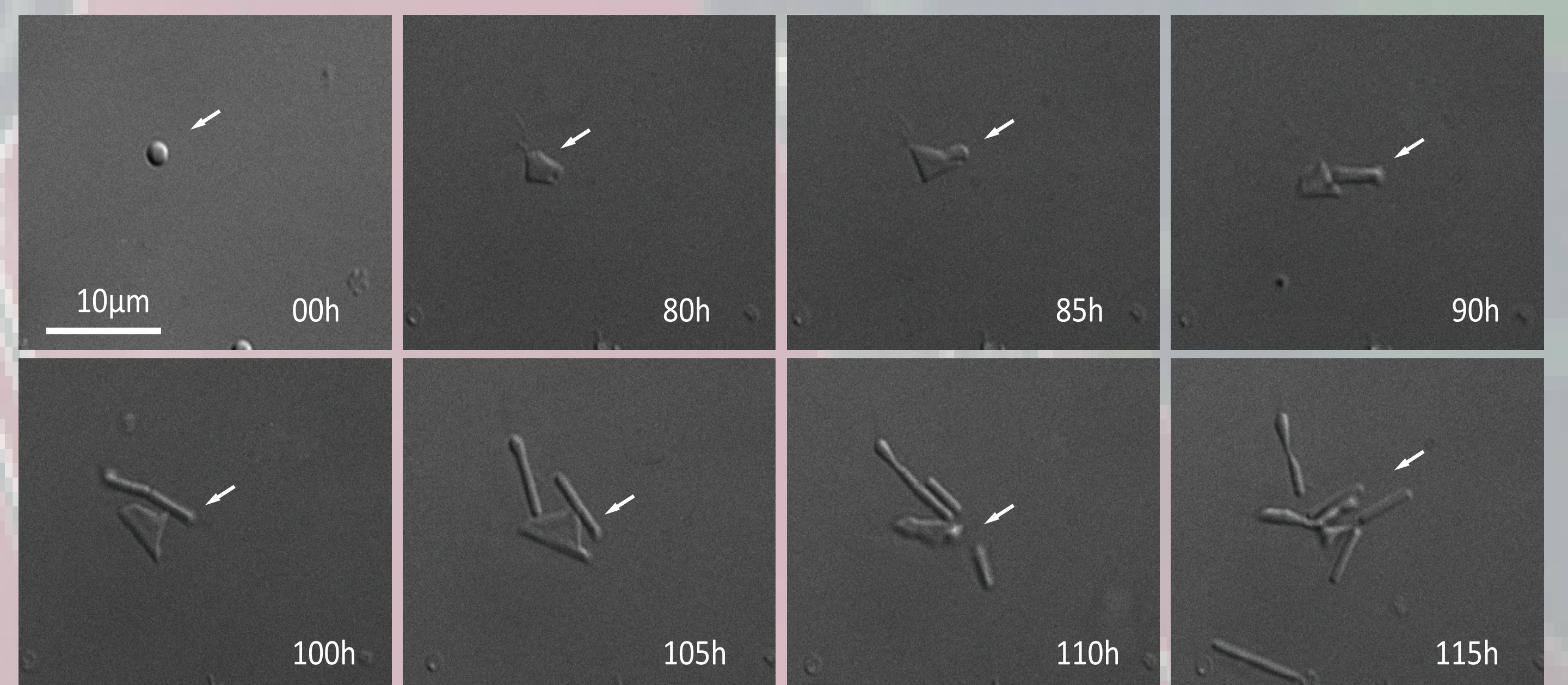
### 3 - Effect of low salinity stress on the molecular dynamics state of the in live *H. salinarum* proteome.



(a) Mean square fluctuations as a function of NaCl concentrations measured by neutron scattering. Cells cultivated in 4.2 M NaCl (triangles) and cells that have been transferred during 1 h in 2.5M (asterisks) or 2M (diamonds) NaCl. A low Q range was chosen ( $0.5 \text{ \AA}^{-1} \leq Q \leq 1.5 \text{ \AA}^{-1}$ ) (b) Histograms of the effective mean force constants ( $k'$ ) from cells subject to different NaCl concentrations (values are indicated below the histograms).

By using neutron spectroscopy, we observed an alteration in the mean molecular dynamics state of the proteome of *H. salinarum* in response to changes in salt concentrations. Values of the effective force constant ( $k'$ ) indicated less macromolecular states, which reflect an increase in the macromolecular flexibility with consequently a likely perturbation in the folding state of a substantial part of the *H. salinarum* proteome at low salinity.

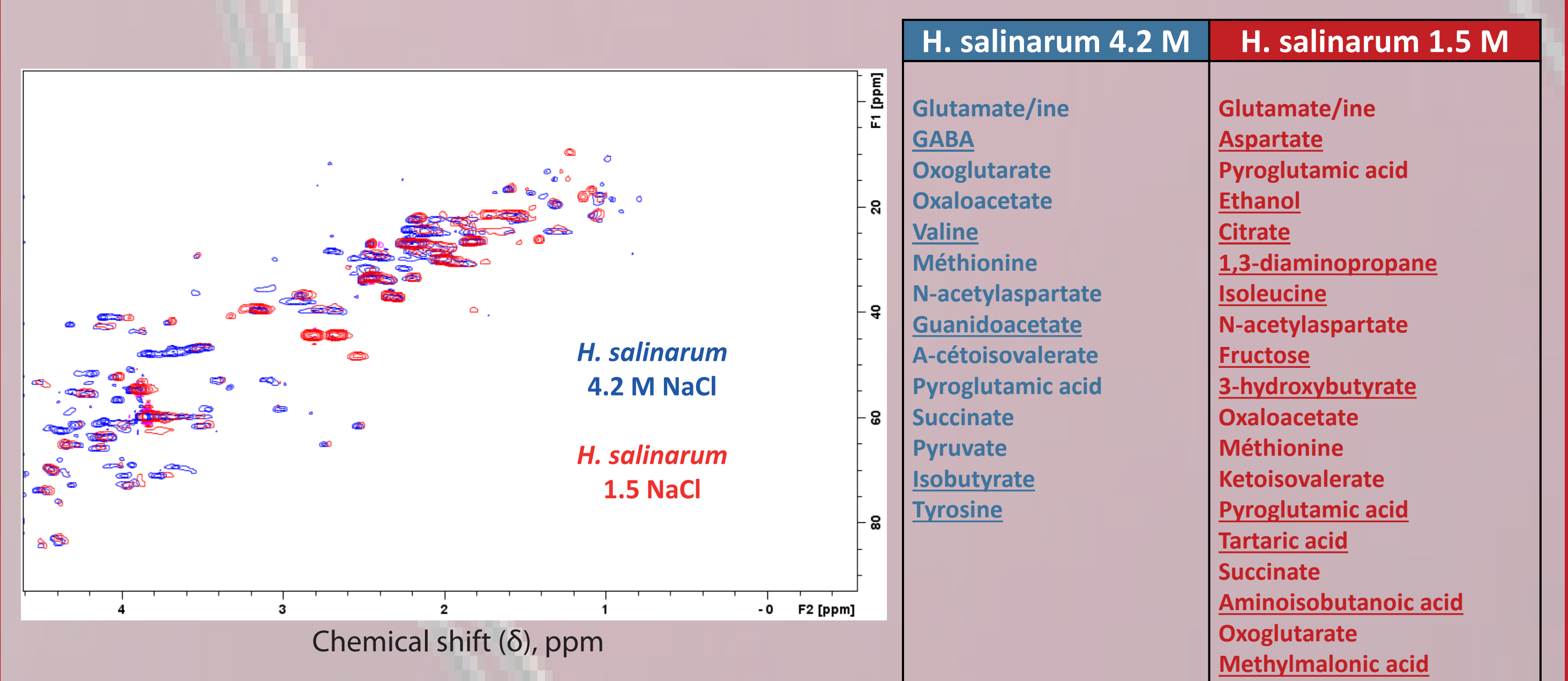
### 4 - Long term microscopy visualization of stressed *H. salinarum* reactivation.



Stressed *H. salinarum* (1.5M NaCl) incubated in medium containing 4.2M NaCl. Pictures were done by using Spinning-disk confocal, DIC imaging, 100x 1.49N.A. Oil objective; Zyla sCMOS; Bar = 10µm.

We used the platform spinning-disk confocal system for a long term experiment, which illustrates undoubtedly the recovery of the archaea *H. salinarum*. Indeed, when halophilic archaea adapted at low salinity condition (1.5M NaCl) are re-exposed to high salt concentration (4.2 M NaCl), a process of reactivation occurs. This demonstrated the large adaptability of hyperhalophile archaea to changes in environmental salinity.

### 5 - 2D-NMR (<sup>13</sup>C/<sup>1</sup>H) spectroscopy of stressed *H. salinarum* (pseudospore).



Representative in vitro <sup>13</sup>C/<sup>1</sup>H-NMR spectra of *H. salinarum* progressively adapted to 1.5M NaCl (collaboration with NMR group). Perchloric acid spectra were measured with a 700 MHz Bruker NMR spectrometer equipped with a 5-mm multinuclear probe.

By using NMR spectroscopy, we have observed that hyposalin stress induces huge differences in the composition of carbohydrates between control and stressed *H. salinarum* (underlined). This first result suggests that some specific detected metabolites could reflect a possible metabolic adaptation of *H. salinarum* during deficiency in salt.