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# *Escherichia coli* and *Lactobacillus plantarum* responses to osmotic stress

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**Abstract** *Escherichia coli* and *Lactobacillus plantarum* were subjected to final water potentials of −5.6 MPa and −11.5 MPa with three solutes: glycerol, sorbitol and NaCl. The water potential decrease was realized either rapidly (osmotic shock) or slowly (20 min) and a difference in cell viability between these conditions was only observed when the solute was NaCl. The cell mortality during osmotic shocks induced by NaCl cannot be explained by a critical volume decrease or by the intensity of the water flow across the cell membrane. When the osmotic stress is realized with NaCl as the solute, in a medium in which osmoregulation cannot take place, the application of a slow decrease in water potential resulted in the significant maintenance of cell viability (about 70–90%) with regard to the corresponding viability observed after a sudden step change to same final water potential (14–40%). This viability difference can be explained by the existence of a critical internal free $Na^+$ concentration.

## Introduction

Water potential, which is equivalent to osmotic pressure for liquid media, is a thermodynamic parameter. It acts on the energy balance of microorganisms and thus variations in the water potential of the medium induce perturbations in the metabolism within microorganisms, such as a decrease in growth rate, and cell mortality for large variations of water potential (Scott 1957; Esener et al. 1981).

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When the water potential of the external medium is decreased, two cell response mechanisms must be considered.

Firstly, a stage of passive water exit, which can lead to cell death. This response is characterized by a rapid decrease in cell volume. The final volume of the cell depends on the stress intensity (Munns et al. 1983).

Secondly, only when the decrease in water potential is not too great, an active biological response occurs due to the active osmoregulatory system of the cell. In *Escherichia coli*, the primary event in volume and turgor regulation is the controlled accumulation of potassium ions ($K^+$) and the subsequent accumulation of small organic compounds called compatible solutes (Dinnbier et al. 1988; Galinski and Trüper 1994).

On the basis of the nature of the cell's response to a hydric shift, i.e. an increase in the rate of water exit, the kinetics of the decrease in the external water potential were found to be very important for cell survival (Maréchal and Gervais 1994; Poirier et al. 1997). The application of slow decreases in water potential allows the maintenance of a high cell viability (80–100%) with regard to the corresponding viability observed after a sudden step change to the same final water potential (15–57%) (Maréchal and Gervais 1994; Poirier et al. 1997). The water flow is related to the water potential gradient between the cell and the external medium. During a rapidly applied and large osmotic stress, cell mortality can be attributed, according to these authors, to a significant and instantaneous flow of water across the cell membrane.

In order to analyse the response of the cell to hydric perturbations, and to understand the difference in viability observed following application of osmotic shock and a slow decrease in water potential, two parameters were measured: cell viability after the hydric stress and the corresponding change in cell volume of two bacteria, *E. coli* and *Lactobacillus plantarum*. In order to allow measurement of the cell volume, bacteria were subjected to osmotic stresses of low intensity, i.e. less than 12 MPa. In order to discriminate between passive and

active responses, cells were stressed in a binary medium (water/solute) or in a culture medium (culture medium/solute).

The role of the solute was also investigated through the use of two polyols and one electrolyte.

## Materials and methods

### Bacterial strains and medium composition

Two bacteria strains were used:

1. *L. plantarum* strain L-73 (Hansen CHR, St. Germain les Arpajon-France), and *E. coli* K-12 strain TG1 [*supE hsd*Δ5 *thi* Δ*(lac-proAB)* F′ (*traD36 proA*$^+$ *B*$^+$ *lacI*$^q$ *lacZ*ΔM15)] (Gibson 1984). *L. plantarum* was maintained by monthly subcultures on MRS agar plates (Merck, Germany) as described by De Man et al. (1960). The water activity of this medium is equal to 0.992.
2. *E. coli* was maintained by monthly subcultures on Luria-broth agar plates (Miller 1972) (Sigma, USA) with a water activity value equal to 0.992.

For experiments, cultures were placed in test tubes containing 9 ml MRS medium for *L. plantarum* and 9 ml Luria-broth medium for *E. coli*.

Inoculation was carried out with 0.1 ml bacteria suspension taken from a 12-h subculture. All samples were taken at the end of the exponential growth phase.

### Experimental design used to achieve a decrease in the water potential of the medium

The cells were harvested by centrifugation and suspended in 9 ml of a solute/water solution (binary solution) or a solute/culture medium solution with the same water potential as the growth medium. The decrease in the water potential of the inoculated solution was then realized by the addition of the solute (water depressor).

Three solutes were used to decrease the water potential: one electrolyte,

i.e. NaCl (Prolabo, France; 99.5%); and two polyols,

i.e. glycerol (Sigma; 99%) and D-sorbitol (Sigma; 98%). Sorbitol was used only with *E. coli*.

Glycerol is a freely permeant polyol, whereas sorbitol is a very slow permeant solute (Berner and Gervais 1994). Two final water potentials were tested: −5.6 MPa and −11.5 MPa. For each experiment, the solute was added rapidly (within about 1 s) to realize an osmotic shock, and slowly (20 min) to realize a slow water potential decrease (slope). The total volume of solute necessary to reach the final water potential was calculated using the Norrish equation (Norrish 1966) and controlled with an osmometer Aqualab-CX2 (Decagon, Devices, USA).

During and/or after the addition of the solute, the cell viability and the cell volume were measured. The slow addition of the solute was realized using a syringe pump system (Melsungen, Germany).

### Viability measurement

Cells were maintained at the final water potential for 30 min before a viability measurement was made, using the dilution method, as reported by Gervais et al. (1992) and Poirier et al. (1997).

### Cell volume estimation through optical density measurement

After exposing cells to osmotic stress (for about 15 s), changes in optical density were measured using a spectrophotometer spectronic 20D (Bioblock scientific, Paris, France). The absorbance of the bacterial suspension was determined with a 1-cm path length at 570 nm.

In order to evaluate the correct changes in cell volume from the measured changes in absorbance, it was necessary to take into account the dilution of the cell suspension due to the addition of the solute (NaCl, glycerol or sorbitol). The effect of the dilution was determined by adding to the initial cell suspension the same volume of solute/water or solute/culture medium solution made up to the same water potential as that of the initial suspension.

A second correction to consider is the effect of the increased refractive index (RI) of the medium due to the addition of NaCl, glycerol and sorbitol. This was done by independently measuring the variation in absorbance of the cell suspension following the addition of a solution of Ficoll, a high-molecular-mass polymer of sucrose (Whatmore and Reed 1990). Ficoll alters RI, but, because of its size, it has no appreciable osmotic effect.

Changes in cell volume due to the addition of this solute were calculated from the measured variations in absorbance by assuming that the absorbance is inversely proportional to the volume at the 3/2 power (Koch 1984).

### Cell volume measurement by direct visualization under the microscope

The volume of the cells submitted to osmotic stress was measured using a phase-contrast microscope (×100) (Leica DMLB, USA), a camera (×1.5) (CCD 6710 model, COHU, USA) and an image analysis system (serie 151, Imaging-Technology, USA). The cells were analysed individually by determination of their area ($S$). Assuming cells of *L. plantarum* and *E. coli* to be spherical, the volume ($V$) was then calculated from the measured area.

## Results

### Influence of the kinetics of a decrease in water potential on cell viability

#### *Hydric perturbations realized in binary solution*

When *E. coli* was submitted to a final water potential of −5.6 MPa with glycerol or sorbitol, the observed viability was not different whether the change in water potential was realized by osmotic shock or by slow decrease (slope) (Table 1). In all cases, cell viability was greater than 80%. The same results were observed for *L. plantarum* with glycerol (Table 1). In contrast, when *E. coli* was submitted to a final water potential of −5.6 MPa with NaCl as the water depressor, the viability was found to depend on the kinetics of the water potential decrease (Table 1). The viability was 60% and 100%, respectively, after a shock and after a slower decrease (slope of 20 min).

When *L. plantarum* was submitted to a final water potential of −5.6 MPa with NaCl as the water depressor, the viability was not significantly affected by the kinetics, i.e. 79% following a shock and 82% following a slower decrease.

When *E. coli* and *L. plantarum* were submitted to a final water potential of −11.5 MPa, a difference in cell viability following application of osmotic shock and a slower decrease of water potential was only observed with NaCl (Table 1). With glycerol and sorbitol as solutes, the cell viability was always greater than 85%. With NaCl, the cell viability was about 92% for *E. coli*

**Table 1** Viability and cell volume of *E. coli* and *L. plantarum* after an osmotic shock and a slow decrease in water potential (slope of 20 min). Solutes used: glycerol, NaCl, sorbitol. Initial water potential: −1.1 MPa. Final water potentials: −5.6 MPa and −11.5 MPa. Temperature = 25 °C. These experiments were realized using a solute/water medium (binary medium). Cell volume was measured by optical density

| | *E. coli* | | | | | | *L. plantarum* | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Glycerol | | NaCl | | Sorbitol | | Glycerol | | NaCl | |
| | shock | slope | shock | slope | shock | slope | shock | slope | shock | slope |
| Final water potential: −5.6 MPa | | | | | | | | | | |
| Viability (% related to a control) | 96 ± 7 | 94 ± 9 | 60 ± 1 | 100 ± 1 | 88 ± 5 | 85 ± 1.5 | 87.5 ± 3.5 | 93.5 ± 0.7 | 78.5 ± 1.5 | 82 ± 17 |
| Cell volume (% of the initial volume) | 43 ± 1 | 43.7 ± 0.6 | 35.5 ± 0.7 | 36.5 ± 0.7 | 11.3 ± 0.6 | 11 | 67 ± 1 | 64.5 ± 0.7 | 67 ± 1.4 | 66.5 ± 0.7 |
| Final water potential: −11.5 MPa | | | | | | | | | | |
| Viability (% related to a control) | 85 ± 5 | 91 ± 8.5 | 42 ± 2.5 | 92 ± 2.5 | 89 ± 8.6 | 81.7 ± 4 | 92.5 ± 3.5 | 95 ± 7 | 13.7 ± 6.3 | 66 ± 7 |
| Cell volume (% of the initial volume) | 14.7 ± 0.6 | 15.5 ± 0.7 | 21.5 ± 2.1 | 22.5 ± 0.7 | 10.3 ± 0.4 | 10.5 ± 0.7 | 36 ± 1.4 | 34.5 ± 0.7 | 44 ± 1.4 | 43.5 ± 0.7 |

and 60% for *L. plantarum* for a slow water potential decrease, whereas the shock preserved only 42% of cells for *E. coli* and 14% for *L. plantarum* (Table 1).

### *Hydric perturbations realized in culture medium*

When *E. coli* and *L. plantarum* were submitted, in culture medium, to a final water potential of −5.6 MPa with NaCl as the solute, no differences in cell viability were observed whether the change in water potential was realized by osmotic shock or slowly. The viability of *E. coli* and *L. plantarum* after the shock was 85% and 100% respectively (Fig. 1A).

## Influence of the osmotic stress on cell volume variation

### *Hydric perturbations realized in binary medium*

*Measurement of the volume by optical density* When *E. coli* and *L. plantarum* were subjected to a final water potential of −5.6 MPa, for each water depressor, the final volume observed was the same whatever the rate of variation of water potential (shock or slower kinetics) (Table 1). For *E. coli*, the final volume was equal to 36% of the initial volume with NaCl, to 43% with glycerol and to 11% with sorbitol. For *L. plantarum*, the final




**Fig. 1** Viability (**A**) and cell volume (**B**) of *E. coli* and *L. plantarum* after an osmotic shock (■) and a slow water potential decrease (20 min) (■). Solute used: NaCl. Initial water potential: −1.1 MPa. Final water potential: −5.6 MPa. Temperature: 25 °C. These experiments were realized using a solute/culture medium solution. Cell volume was measured by image analysis

volume was the same with NaCl or glycerol (about 60% of the initial volume).

When these two strains were submitted to a final water potential of −11.5 MPa, whatever the water depressor used, the final volumes observed after the shock and the slower kinetics were the same. For *E. coli*, the final volume was equal to 15% of the initial volume with glycerol, to 21% with NaCl and to 10% with sorbitol. For *L. plantarum*, the final volume was equal to 35% with glycerol and 44% with NaCl (Table 1).

When cells were maintained at the final water potential (−5.6 MPa and −11.5 MPa) for several hours (8 h), no recovery of the cell volume was observed.

*Measurement of volume by direct observation under the microscope* Results presented in Fig. 2 show that the measured volumes by direct observation under microscope were similar to those measured by optical density.

*Hydric perturbations realized in culture medium*

When *E. coli* and *L. plantarum* were subjected to a final water potential of −5.6 MPa with NaCl as the solute, the cell volume decrease, measured by direct visualization under a microscope, was found to be greater following osmotic shock (62% and 32%) than following a gradual change in water potential (48% and 20%) as shown in Fig. 1B.

After the slow change in water potential, the cell volume of *E. coli* immediately increased (Fig. 3) and after 150 min morphological changes were observed (Fig. 4).

For *L. plantarum*, an immediate increase of the cell volume was observed, reaching 100% of the initial volume after 60 min (Fig. 3). In culture medium, the osmoregulation system acts to restore the cell volume.

With spectrophotometric analysis, cell division interfered with the measurements of volume regulation.

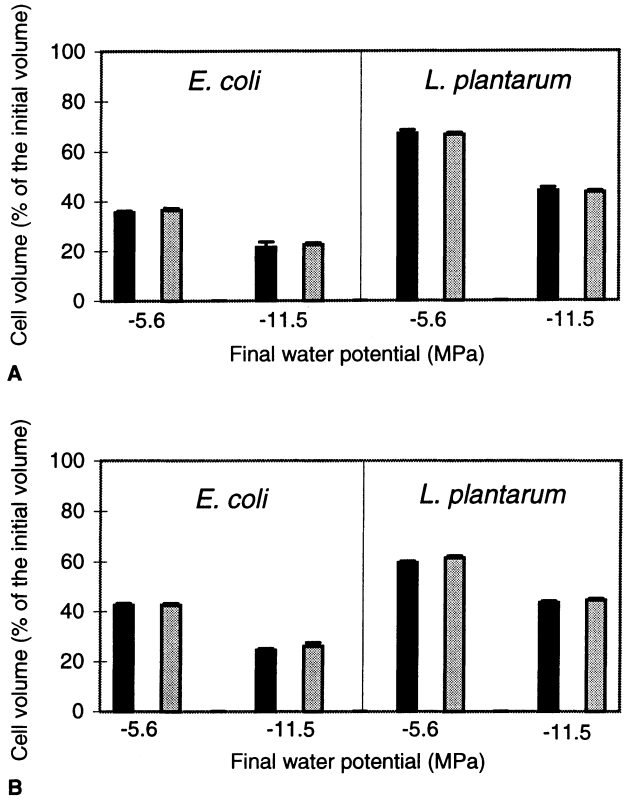


**Fig. 2** Cell volume of *E. coli* and *L. plantarum* measured by image analysis (**A**) and optical density (**B**) in relation to the kinetics of water potential decrease: (■) osmotic shock; (▩) slow kinetics (20 min). Solute used: NaCl. Initial water potential: −1.1 MPa. Final water potentials: −5.6 MPa and −11.5 MPa. Temperature: 25 °C. Experiments were realized using solute/water solutions

## Discussion

### Osmotic stresses realized in binary medium

The extent of the cell volume decrease is related to the solute used. Sorbitol is a very slowly permeant solute (Berner and Gervais 1994). It could not diffuse across the plasma membrane and the decrease of the cell volume was strictly dependent on the water transfer due to the osmotic gradient.

Glycerol is a permeant solute which crosses the cytoplasmic membrane rapidly by diffusion and facilitated diffusion (Heller et al. 1980; Roth et al. 1985) and it limits the cell volume decrease.

Following exposure to NaCl, the cell volume decrease was similar to that observed following application of glycerol (Table 1). This result can be explained by the incorporation of $Na^+$ and $Cl^-$ into the cell in order to counterbalance the extracellular water potential, and to limit water exit from the cell. Recently, the accumulation of $Na^+$ during an osmotic stress was observed to occur in *Listeria monocytogenes* (Gerhardt et al. 1996). In order to prove whether $Na^+$ enters the cell or not, flame photometry, atomic absorption spectroscopy, or Na-NMR analysis should be used.

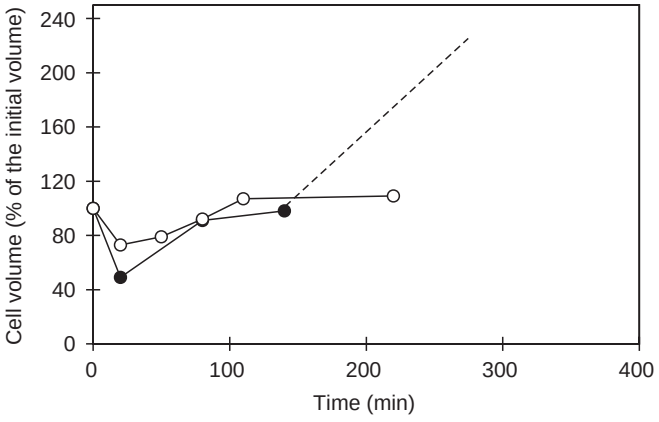


**Fig. 3** Evolution of cell volume of *E. coli* (●) and *L. plantarum* (○) measured by image analysis after a slow water potential decrease (20 min). Water depressor used: NaCl. Initial water potential: −1.1 MPa. Final water potential: −5.6 MPa

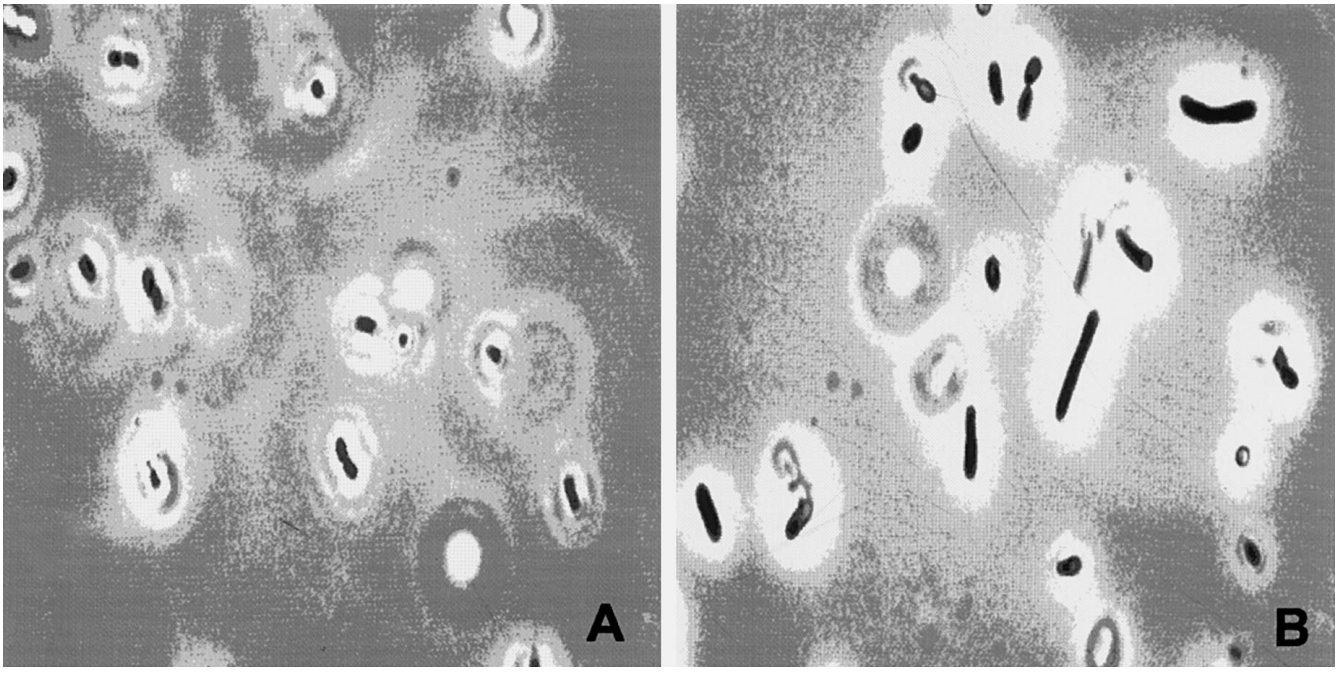


**Fig. 4A, B** Changes in cell morphology of *E. coli* after osmotic stress realized with NaCl. **A** Morphology of cells 1 min after the stress. **B** Morphology cell 310 min after the stress

In this study, a difference in the viability of cells caused by osmotic shock and by gradual changes in water potential was only observed when NaCl was the solute. This result shows that the cell mortality observed in the presence of NaCl is not related to the intensity of water flow across the cell membrane or to a critical final volume. Effectively, for an osmotic shock of the same intensity, the volume decrease (and so the intensity of the water flow) was more significant with sorbitol, whereas the mortality was more significant with NaCl.

Previous studies have that when glycerol is used to change to a final water potential of less that −100 MPa, cell viability depends on whether this change is induced quickly or slowly (Maréchal and Gervais 1994; Poirier et al. 1997). In these cases, the flow of water during the osmotic shock, which is related to the water potential gradient between the cell and the external medium (Kedem and Katchalsky 1958), was very important and could account for the cell mortality observed.

For the osmotic stresses applied in this study, the cell mortality observed following exposure to NaCl could be explained by the toxic effect of the $Na^+$ ions in the cell (Gaxiola et al. 1992). During the slow decrease in water potential, the cell seems to be able to respond to the progressive increase of the $Na^+$ intracellular concentration to preserve cell viability.

In binary medium, the $K^+$ concentration appears to be insufficient to allow osmoregulation and no restoration of cell volume was observed. Moreover, this low $K^+$ concentration could explain the incorporation of $Na^+$ into the cell.

Osmotic stress realized in culture medium

When *E. coli* and *L. plantarum* were subjected to a final water potential of −5.6 MPa in culture medium using NaCl, the viability was more important than in the binary solution (Fig. 1). This viability increase could be explained by a rapid accumulation of $K^+$ ions and amino acids, and by the maintenance of the $Na^+$ ions outside the cell.

The final volume was more important after the gradual change in water potential than after the shock (Fig. 1), which means that during the former osmoregulation can occur to restore cell volume.

For *E. coli*, the increase of the internal concentration of NaCl resulted in the formation of markedly elongated cells (Fig. 4). This phenomenon was also observed by Jorgensen et al. (1995) in a study of *Listeria monocytogenes*.

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