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Viability of *Escherichia coli* after combined osmotic and thermal treatment: a plasma membrane implication

Yannick Mille, Laurent Beney, Patrick Gervais*

Laboratoire de Génie des Procédés Alimentaires et Biotechnologiques, Ecole Nationale Supérieure de Biologie Appliquée à la Nutrition et à l'Alimentation, 1 Esplanade Erasme, Dijon 21000, France

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Abstract

This study investigates the influence of temperature (T) and osmotic pressure (Π) on the viability of *Escherichia coli* K12 during an osmotic treatment. Osmotic shock (dehydration and rehydration within 1 s) in liquid media at different temperatures (4, 10, 30 and 37 °C) and different levels of osmotic pressure (26, 30, 35, 40, 82 and 133 MPa) were realized.

Results show that a sudden dehydration, below 40 MPa, destroyed up to 80% of the bacterial population for each tested temperature, whereas viability was greater than 90% for an osmotic pressure less than 26 MPa. The influence of T and Π on the membrane's physical structure is finally considered to explain the results in light of FTIR and electron microscopy study of the influence of temperature and osmotic pressure on *E. coli* membrane phospholipids conformation.

Keywords: Viability; *Escherichia coli*; Membrane

1. Introduction

Because biological membranes are much more permeable to water than to most solutes, living cells are affected by changes in the total solute concentration of their environment [25]. When cells are submitted to hydric stress, such as an increase in the osmotic pressure of the medium, two response mechanisms are observed.

First, a passive stage of water exit occurs. This is related to the osmotic pressure gradient ($\Delta\Pi$) between the cell and the external medium, and can subsequently lead to cell death [29]. This response results in a cell volume decrease corresponding to a cytoplasmic volume reduction in bacteria such as *Escherichia coli* [13]. The osmotic flow that crosses the membrane is proportional to $\Delta\Pi$ [12].

Second, only when the decrease in water potential is not too high, an active biological response occurs through the active osmoregulatory system of the cell. This metabolic process consists of the biosynthesis of solutes and of a rapid modification of the membrane permeability that allows cells to import external water. In *E. coli*, the primary event in volume and turgor regulation is the controlled accumulation of potassium ions (K^+) [28] and the subsequent accumulation or production of small organic compounds such as proline and/or glycine betaine. This active response enables the cell to restore its internal volume when the membrane has not been damaged by the passive phase of the osmotic response. Many studies have demonstrated that cell membranes are a primary site of injury during an osmotic stress [4,9], and others have shown the influence of environmental variables such as temperature and osmotic pressure on the viability of several organisms like yeasts [2] or bacteria [23]. Several reports [8,23] emphasized the importance of the kinetics of dehydration and rehydration for the prevention of cell death. Bacterial viability is better when dehydration and/or rehydration occurs slowly whereas osmotic shock represents a dramatic treatment for cells. Some authors have investigated the relationship between the survival of microorganisms and the structural change of membrane phospholipids [18]. Recently, Laroche et al. [17] demonstrated that the death of *Saccharomyces cerevisiae* during osmotic shock could be related to a membrane phase transition. So, with regard to the thermotropic nature of

* Corresponding author. Tel.: +33-3-80-39-66-54; fax: +33-3-80-39-66-11.

E-mail address: gervais@satie.u-bourgogne.fr (P. Gervais).

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phospholipids in *E. coli* [11], both temperature and hydration levels are expected to influence cell resistance to osmotic treatment. In this study, we investigated the influence of increasing osmotic shock, achieved at various temperatures, on the recovery of *E. coli*. The potential roles of the membrane structural changes and cytoplasmic volume variations in cell death are then discussed in light of our observations through a FTIR study and electron micrographs of stressed *E. coli*.

2. Material and methods

2.1. Microorganisms and growth conditions

E. coli K12 strain TG 1 [sup E hsd Δ5 thi Δ (lac-proAB)F′ (traD 36 pro A^+ B^+ $lacI^q$ lac Z Δ M15)] was maintained on Luria-Bertani (LB) agar plates (Miller, 1972) (Sigma, USA) at 4 °C. For experiments, liquid cultures were prepared by inoculation with colonies into test tubes containing 9 ml of LB broth at 37 °C for 12 h. A subculture was then prepared by injecting 0.1 ml of bacteria suspension into 9 ml of LB broth, and this was grown under the same temperature as described above. Cells were harvested after 24 h of growth (stationary phase) and used immediately in the experiments.

2.2. Osmotic treatment

Cells were harvested by centrifugation ($120 \times g$ for 5 min) and suspended in 10 ml of a glycerol/water solution with the same water potential as the growth medium. The final concentration in the sample generally ranged from 9×10^9 to 2×10^{10} CFU/ml. An aliquot of this suspension (approximately 1 ml) was thermally equilibrated (10 min in a thermally controlled water bath). Four temperatures were tested: 4, 10, 30 and 37 °C.

2.2.1. Dehydration mode

The increase in osmotic pressure was then realized by the addition of a freely permeant polyol, glycerol (Sigma-Aldrich; 99% w/v) as a water activity depressor. This solute was added to water with the following weights, for 1000 g of water: 903, 1040, 1204, 1376, 2865, and 5015 g of glycerol to produce, respectively, 26, 30, 35, 40, 82 and 133 MPa, shocks solutions. The weight of solute necessary to reach the final osmotic pressure for each level was calculated using the Norrish equation [21], and verified with an Aqualab-CX2 osmometer (Decagon Devices, USA). Osmotic shock was then realized by injecting 0.5 ml of the thermally equilibrated *E. coli* suspension in 10 ml of thermally equilibrated solution of glycerol.

2.2.2. Rehydration mode

After a 30-min maintainance in the hyperosmotic solution, bacteria were rehydrated by quickly injecting 1 ml of the cell suspension into 9 ml of adequate glycerol solution in order to return it to the initial osmotic pressure (1.38 MPa).

2.3. Viability measurement

Viability assays were performed essentially as described by Gervais et al. [8] and Poirier et al. [23]. Cell viability was measured by comparing plate counts with a control that was thermally equilibrated, but did not undergo osmotic challenge. After rehydration, cells were applied on LB agar plates (in triplicate) and were incubated at 37 °C for 24 h. Each experiment was repeated three times.

2.4. FTIR study

2.4.1. Sample preparation

We used an *E. coli* total extract purchased from Avanti Polar lipids which was dissolved in chloroform (UV grade) to reach final concentration of 20 mg/ml. Films of these extracts were prepared by casting the chloroformic solution (150 μl) on IR transparent ZnSe window and completely evaporating the solvent under gentle dry N_2 flow for 2 h at 55 °C. Rehydration were performed in situ by eluate water/glycerol solutions until equilibration to the wanted osmotic pressure (26, 40 and 82 MPa) at 55 °C.

2.4.2. Spectra

In order to measure CH_2 symmetric stretching, spectra were recorded between 2800 and 2900 cm^{-1} for each osmotic pressure step adjustment by means of a Vector 22 FTIR spectrometer from Brucker (Karlsruhe, Germany), which was fitted with an ATR cell. Temperature of ZnSe window was decreased to 7 °C and measures were realised from 7 to 44 °C. Temperature was monitored by a thermocouple located against the edge of the cell window. Ten scans (at a resolution of 4 cm^{-1}) were co-added for one spectrum. Data processing was done by the OPUS software package (Brucker).

2.5. Electron microscopy

To determine the effects of osmotic challenge on bacterial membrane, ultrathin sections of osmotically shocked *E. coli* K12 were observed. In order to correlate bacterial viability, three osmotic pressures were tested: 26, 40 and 82 MPa. After 24 h of culture, cells were pelleted at $120 \times g$ for 5 min and osmotically challenged with 26, 40 or 80 MPa water/glycerol solutions. After being maintained for 30 min in shock solution, the cells were centrifuged at $120 \times g$ for 5 min and immediately fixed in glutaraldehyde (Merck, France). The sample was then washed in 0.1 M cacodylate buffer (Rectapur, Prolabo, France) and postfixed with osmium tetroxide (Euromedex,

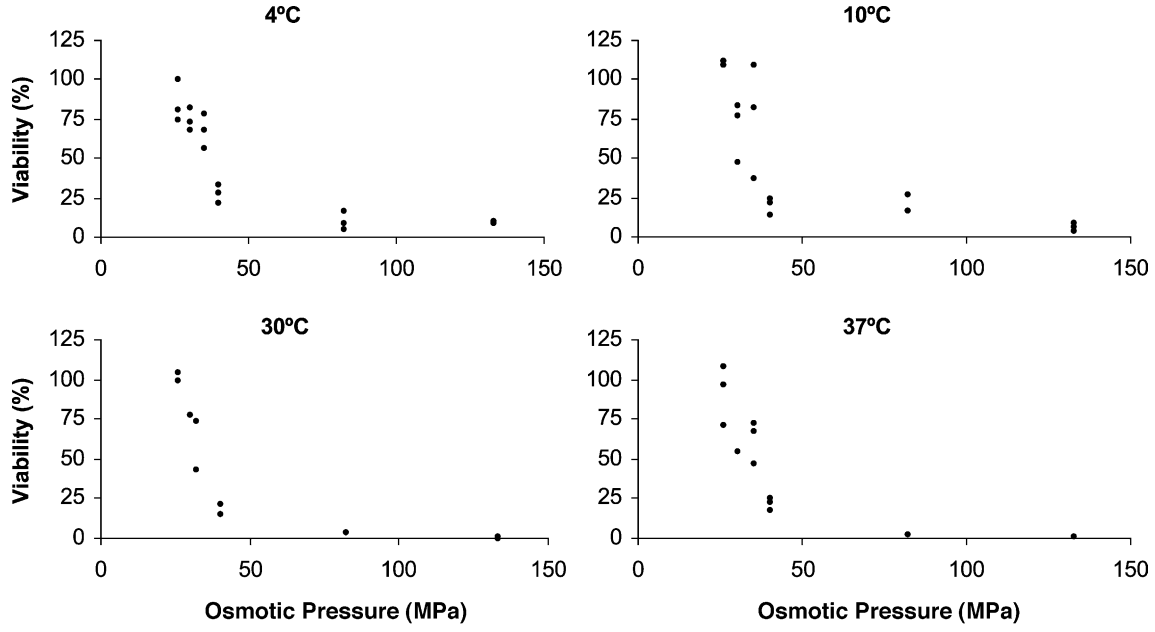

Fig. 1. Influence of exposure to increasing level of osmotic pressure in glycerol solutions on *E. coli* K12 viability at 4, 10, 30 and 37 °C.

France) for 60 min. After dehydration with increasing concentrations of ethanol, cells were embedded with EPOX 812 and finally polymerized at 60 °C for 48 h. To improve contrasts, samples were stained with uranyl acetate (Euromedex). Ultrathin sections were deposited on a copper grid and viewed in a HITACHI H600 electron microscope at 75 kV using a 30-μm objective aperture.

3. Results

3.1. Effects of osmotic level and temperature of the osmotic challenge

E. coli K12 viability was found to be dependent upon the osmotic pressure level of the osmotic treatment

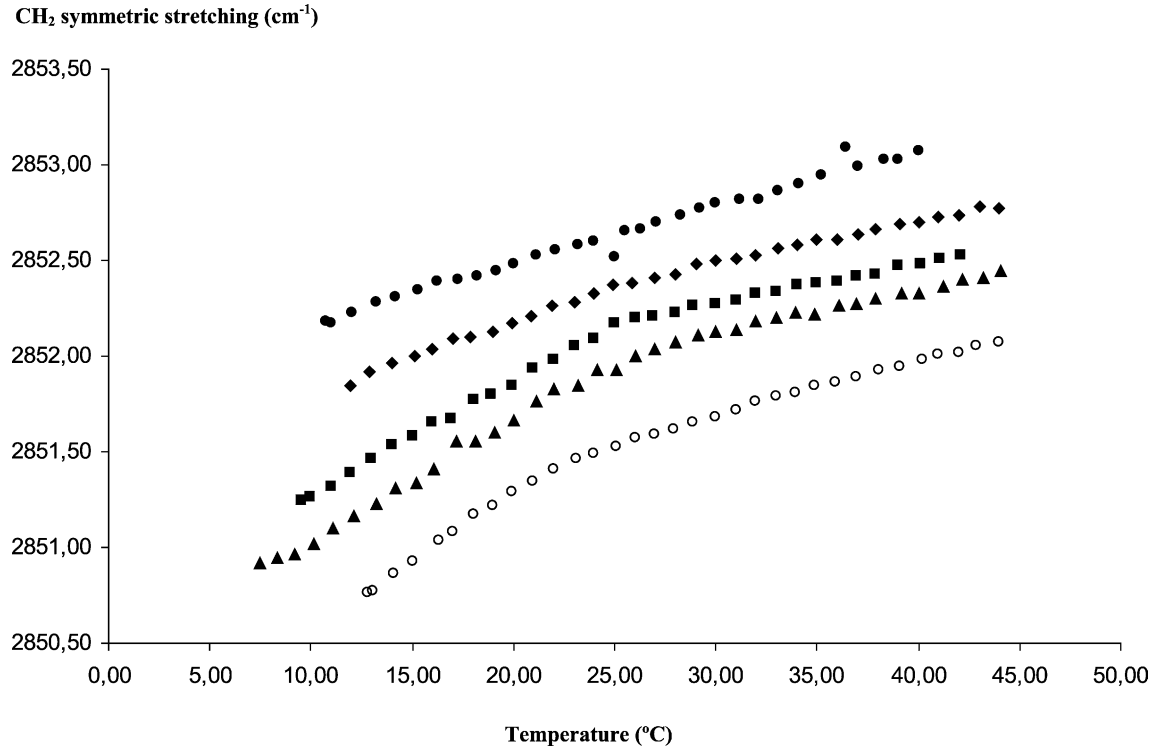

Fig. 2. Temperature dependence of the CH_2 symmetric stretching band in the infrared spectra of phospholipids extract from *E. coli*, in the fully hydrated state (●), in 26 MPa glycerol solution (♦), in 40 MPa glycerol solution (■), in 82 MPa glycerol solution (▲) and in totally dried state (○).

(Fig. 1). When bacteria were shocked at a final osmotic pressure of 26 MPa, the observed viability was greater than 80%, but the viability decreased to below 10% when the final osmotic pressure of the treatment was greater than 133 MPa. The range of osmotic shifts between 1.38 and 35 MPa involved high viability levels (up to 60%)

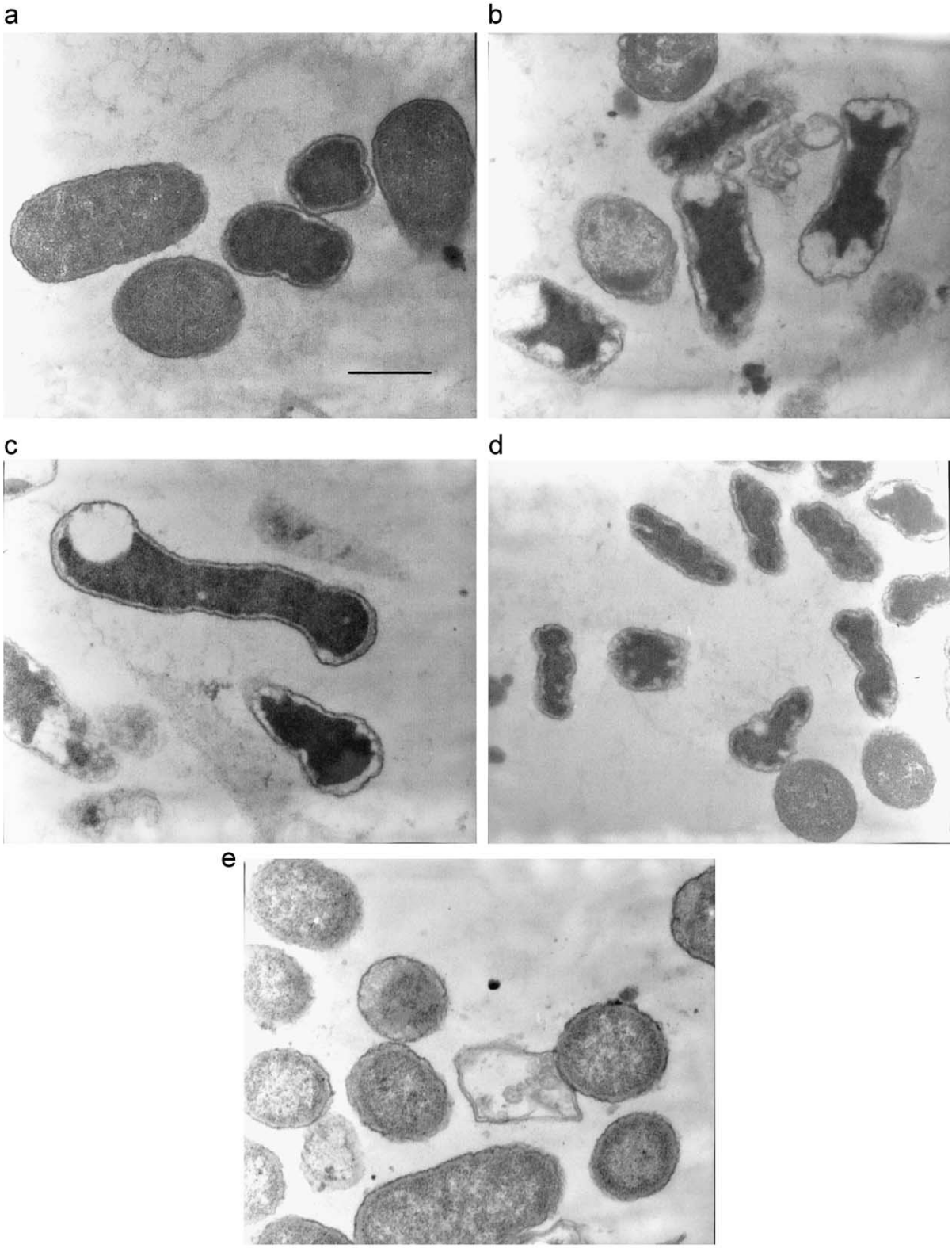

Fig. 3. Electron micrographs of *E. coli* K12 in isoosmotic medium (a), and of osmotically shocked cells at 26 MPa (b, c), 40 MPa (d) and 82 MPa (e) in glycerol solutions. Bar represents 1 μm.

and, in this range, the temperature appeared not to influence the final viability. For instance, at 26 MPa and 4 °C (Fig. 1a) the viability (85%) was about the same as that for 26 MPa and 37 °C (92%; Fig. 1d), and the same viability values were observed for 10 °C (111%; Fig. 1b) and 30 °C (101.9%; Fig. 1c). For hydric shifts between 30 and 35 MPa, the viability level of *E. coli* slowly started to decrease. This fall seems to be more important at 37 °C, for which the viability was 54.8% on average, whereas it was 69.2% at 10 °C and 74.7% at 4 °C. The data show a dramatic decrease in viability when the osmotic pressure was near 40 MPa. This loss of viability (which occurred for all experimental temperatures) corresponds to a viability drop of 40% with regard to the values obtained at 35 MPa; that is, viability decreased from 62.4% to 22% at 37 °C and from 67.6% to 28.1% at 4 °C. Likewise, a critical range of osmotic pressures compatible with cell recovery was reached at about 40 MPa.

In the second range, above 40 MPa, where the viability was low, temperature had a greater influence on *E. coli* viability. At 82 and 133 MPa, two ranges of temperature could be distinguished: 30–37 and 4–10 °C. Bacterial viability was strongly and equally decreased by an osmotic shock of 40 MPa regardless of temperature, whereas, at 133 MPa, there was a greater decrease in viability which was found to be related to the temperature of the medium. Low temperatures allowed the viability to be maintained above 10%, with the viability falling to less than 5% for 30 °C and 37 °C. At 133 MPa, the lowest level of water potential, viability was 0.7% at 30 °C and 0.84% at 37 C, whereas it was 9.1% at 4 °C and 6.5% at 10 °C.

It appears that viability of *E. coli* K12 is mainly influenced by the osmotic pressure during the osmotic treatment. Viability suddenly decreased with an osmotic shock of 40 MPa for each tested temperature. However, low temperatures seem to improve bacterial survival for very high osmotic pressures (82 and 133 MPa).

3.2. Study of the influence of temperature and osmotic pressure on E. coli membranes phospholipids by FTIR

Fig. 2 presents the variations of the symmetric CH_2 stretching ($\upsilon_s CH_2$) for different moistures in front of temperature. As ever described, $\upsilon_s CH_2$ bands are recorded in wave numbers range of 2850 to 2853 cm^{-1}. Results show that the full hydrated extract was different from dried extract. As $\upsilon_s CH_2$ frequency varied between 2852.1 and 2852.9 cm^{-1} for the hydrated sample, this one fell down from 2851.9 to 2850.6 cm^{-1} for dried sample. Three intermediate moistures were tested by flowing water/glycerol solutions whose osmotic pressures were fixed at 26, 40 and 80 MPa. Data have shown that an increasing input of glycerol, after equilibration, makes the signal to achieve a frequency near to the dried sample frequency. So at 37 °C we can see that $\upsilon_s CH_2$ bands were near 2852.8 cm^{-1} for full hydrated sample, 2852.5 cm^{-1} at 26 MPa, 2852.3 cm^{-1} at 40 MPa, 2852.2 cm^{-1} at 80 MPa and 2851.7 cm^{-1} for totally dried sample.

3.3. Electron microscopy

Electron micrographs of *E. coli* K12 sections are presented in Fig. 3. The first (3a) shows *E. coli* at steady state and isoosmotic conditions (1.38 MPa). The cytoplasm was thin and regularly distributed and the three layers (outer membrane, murein wall and cytoplasmic membrane) were closely apposed. No small endocytotic vesicles were observed. The following photographs show osmotic challenged cells in glycerol until 26 MPa (Fig. 3b,c), 40 MPa (Fig. 3d) and 80 MPa (Fig. 3e). At 26 MPa, bacteria were largely plasmolysed as shown in Fig. 3b: large periplasmic spaces were denoted. First, small endocytotic vesicles were visible at this osmotic pressure as shown in Fig. 3c. Different results were obtained at 40 MPa where plasmolysis was less pronounced (periplasmic spaces were smaller) than at 26 MPa but endocytotic vesicles were still visible. Lastly, at 80 MPa plasmolysis was not observed. Cells seemed to be identical to the control. However, layers could not be clearly identified and the cytoplasm appeared less dense than cytoplasm control.

4. Discussion

All of the viability data of *E. coli* obtained for each tested couple of temperature and osmotic pressure are presented in Table 1. Many reasons, which could be advanced to explain cell mortality in front of osmotic treatment, deal with bacterial membranes damages. Several authors link cell death to the conformational disorder of membranes phospholipids induced by temperature and hydration conditions modifications. This hypothesis deals with the thermotropic behavior of membrane phospholi-

Table 1
Mean viability (SD) of *E. coli* K12 (in percent) after osmotic treatment achieved at different temperatures in glycerol solutions of various osmotic pressures

	26 MPa	30 MPa	35 MPa	40 MPa	82 MPa	133 MPa
4 °C	85 (13.3)	74.7 (7.5)	67.6 (10.8)	28.1 (5.7)	10.5 (6)	9.1 (0.9)
10 °C	111 (2)	69.2 (19.1)	76.6 (3.6)	20.2 (5.2)	20.2 (5.6)	6.5 (2.1)
30 °C	101.9 (3.3)	78 (0.3)	68.3 (22.1)	20.7 (4.7)	3.6 (0)	0.7 (0.3)
37 °C	92 (19)	54.8 (0.3)	62.4 (10.4)	22 (0)	2 (0)	0.84 (0)

pids. The reorganization of the membrane components (such as proteins and phospholipids) induced by temperature and osmotic pressure variation could prevent cell mortality, as shown by the high viability obtained with slow changes in osmotic pressure [14]. The increase of the area-to-volume ratio could explain the cell deformation, but is certainly not sufficient to explain the cell vesiculation.

4.1. Membrane phospholipid phase modification

According to the composition of *E. coli* membranes (25% phospholipids, 78% of which is phosphatidylethanolamine, 20% is phosphatidyl glycerol and about 2% is cardiolipin), the possible role of the membrane phase transition must be discussed. At physiological temperatures and levels of hydration, the phospholipids bilayer of biological membranes is in a fluid, lamellar, liquid–crystalline phase. The phospholipid phase transition temperature (T_m) varies with the osmotic pressure of the surrounding medium and with membrane composition. When cells are dehydrated, membrane phase transition occurs at a higher temperature than for fully hydrated cells. Leslie et al. [18] determined that *E. coli*, after freeze drying, had a T_m of 50 °C when hydrated cells exhibited a T_m of 10 °C. Crowe et al. [5] showed that when the dry cell is rehydrated, it undergoes a phase transition from the gel back to the liquid–crystalline phase, during which leakage of solutes may occur. Our results have pointed out the existence of a threshold range of osmotic pressure, between 35 and 40 MPa, where the viability of *E. coli* is dramatically decreased after sudden dehydration and rehydration. It could be assumed, according to cited authors, that viability of *E. coli* has fallen because of the phase transition, which would occur between 35 and 40 MPa at all the observed temperatures.

Results proposed in Fig. 2 have shown that the frequency of $\upsilon_s CH_2$ bands of the phospholipidic extract was significantly varying with the hydration level. Table 2 presents different frequencies of CH_2 bands recorded in different pure phospholipids during their phase transition. Data show that pure phospholipids undergo phase transition with only a difference of 2 cm^{-1} in $\upsilon_s CH_2$ bands vibrations. So, as the results given in Fig. 2 showed that there are more than this difference of 2 cm^{-1} between hydrated and dried phospholipids extracted from *E. coli*, it could be assumed that these phospholipids undergo phase transition by increasing osmotic pressure. Accordingly, glycerol addition induced a decrease in $\upsilon_s CH_2$ frequency from the hydrated sample to the dried one. In the representation of $\upsilon_s CH_2$ frequency versus temperature, the corresponding T_m of the extract for each level of hydration has not been precisely determined because of the progressive character of the phenomenon (which is probably due to the fact that the extract was composed of a mix of lipids).

4.2. Membrane vesiculation

The second major effect of osmotic treatment is the volume variation. When subjected to an osmotic shock, a cell may behave like an osmometer in order to reach an osmotic equilibrium with its environment [1]. This involves movement of water and solutes, and thus a change in cellular volume, which has been shown by previous authors [20,23]. The volume of the cell decreases as soon as the osmotic pressure increased and appears to be related to the osmotic stress intensity [10]. This volume variation has different consequences on cell physiology.

The first concerns membrane vesiculation. High osmotic pressures induce high osmotic shrinkage and then cause severe damage to the cytoplasmic membrane [29]. Alemohammad and Knowles [1], Schwarz and Koch [26] and, more recently, Delamarche et al. [6] have shown the appearance of small membrane-bound vesicles in the cytoplasm of *E. coli* when submitted to increased osmotic pressure. Our electron micrographs (Fig. 3) show that the *E. coli* K12 strain produced such vesicles when submitted to hyperosmotic shock led at 26 and 40 MPa. The drastic decrease in cell volume could induce a mechanical effect on the membrane, which is deformed by cell shrinkage and can be disrupted under such a constraint. The increase in the area-to-volume ratio of the cytoplasmic membrane leads to irreversible surface changes, such as membrane vesiculation, which induces surface depletion. When subjected to fast rehydration, cells cannot recover

Table 2
Wave numbers corresponding to symmetric stretching bands of different phospholipids

Phospholipids	Lα wavenumbers (cm^{-1})	Lβ wavenumbers (cm^{-1})	H_{II} wavenumbers (cm^{-1})	Reference
POPC	2851.5–2852.5	2850.8–2851.5	×	Pohl et al. [22]
DLPC	2850.4–2852.3	2850.1–2850.4	×	Gauger et al. [7]
DMPC	2850.4–2852.2	2849.9–2850.4	×	Gauger et al. [7]
DPPC	2852.2–2853.5	2850.8–2852.2	×	Kota et al. [16]
OPPC	2851.4–2852.5	2850.8–2851.4	×	Lobau et al. [19]
DOPE	2851.6–2852.5	2850.6–2851.6	2852.5–2853	Chia and Mendelsohn [3]

POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), DLPC (1,2-dilauroyl-*sn*-glycero-3-phosphocholine), DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine), DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine), OPPC (1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine) and DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine) in Lα (liquid-crystalline phase), Lβ (lamellar gel phase) and H_{II} (inverted hexagonal phase) conformation.

their initial internal volume, and rupture. The influence of kinetics in an osmotic treatment was shown by Gervais et al. [8] and Poirier et al. [24], who have noted the importance of the rehydration kinetics towards cell preservation. After a severe increase in osmotic pressure, rapid rehydration was found to be more harmful than a slow rehydration, which allowed recovery of the initial area-to-volume ratio. Thus, the dramatic fall in viability observed for *E. coli* can be assigned to the sudden variation of cell volume according to the weakening of the membrane for a particular threshold of osmotic pressure (35 to 40 MPa). Many studies have examined the effects of osmotic shock using water depressors that are not permeant like sucrose [26], and have shown that vesicles appear at low osmotic pressures (e.g., 6 MPa for a 16% (w/v) sucrose solution). According to Schwarz and Koch, this vesiculation could be related to the cell plasmolysis. Korber et al. [15] have shown that plasmolysis could be used as an indicator of bacterial viability: only viable cells could plasmolyse. This assumption was verified here as shown by compare results of Table 1 and Fig. 3: no plasmolysis was observed at 82 MPa where viability was only 0.7%. This cell membrane vesiculation could perhaps be facilitated by phase transition, as proposed by Steponkus [27] and so could be related to the previous observations concerning the membrane phospholipids disorder.

These propositions concerning the correlation between the membrane modifications (lipid reorganization and phase transition) or volume variations (vesiculation, etc.) and cell death need to be verified through different complementary experiments. Further experiments will be based on an investigation of the *E. coli* membrane structure in different osmotic conditions by measurement using fluorescent probes and IR spectroscopy.

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