

Rates of chilling to 0°C: implications for the survival of microorganisms and relationship with membrane fluidity modifications

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Abstract The effects of slow chilling ($2^{\circ}\text{C min}^{-1}$) and rapid chilling ($2,000^{\circ}\text{C min}^{-1}$) were investigated on the survival and membrane fluidity of *Escherichia coli*, of *Bacillus subtilis*, and of *Saccharomyces cerevisiae*. Cell death was found to be dependent on the physiological state of cell cultures and on the rate of temperature downshift. Slow temperature decrease allowed cell stabilization, whereas the rapid chilling induced an immediate loss of viability of up to more than 90 and 70% for the exponentially growing cells of *E. coli* and *B. subtilis*, respectively. To relate the results of viability with changes in membrane physical state, membrane anisotropy variation was monitored during thermal stress using the fluorescence probe 1,6-diphenyl-1,3,5-hexatriene. No variation in the membrane fluidity of all the three microorganisms was found after the slow chilling. It is interesting to note that fluorescence measurements showed an irreversible rigidification of the membrane of exponentially growing cells of *E. coli* and *B. subtilis* after the instantaneous cold shock, which was not observed with *S. cerevisiae*. This irreversible effect of the rapid cold shock on the membrane correlated well with high rates of cell inactivation. Thus, membrane alteration seems to be the principal cause of the cold shock injury.

Keywords Chilling rate · Rapid cold shock · Cell inactivation · Membrane fluidity · Membrane modifications

Introduction

Variations in the surrounding temperature are probably the most common stress for all living organisms. Microorganisms respond to this stress in a variety of ways. A downshift in temperature leads to a reduction in membrane fluidity, impaired protein biosynthesis, and stabilization of secondary structures of deoxyribonucleic acid and ribonucleic acid (Panadero et al. 2006; Russell et al. 1995; Thieringer et al. 1998). Consequently, the adaptive response to cold shock in most organisms includes a change in the lipid composition of membranes and the remodeling of the transcriptional and translational machinery. These changes are mainly triggered by a drastic variation in the gene expression program, which leads to both survival and adaptation to the new environmental conditions. Temperatures above 0°C are usually associated with an adaptive response of microorganisms, typically the synthesis of specific proteins (Jones and Inouye 1994), whereas subzero temperatures are well known to inhibit metabolic activity and cell growth for most organisms (Panoff et al. 1998). However, several studies have demonstrated that chilling in the range of temperature above 0°C can also be lethal to cell survival (Hagen et al. 1976; Leder 1972; Meynell 1958; Ring 1965; Sato and Takahashi 1968). It was noted that using cold washing medium or diluting cells rapidly into cold medium during sampling induced the loss of accumulated substrates (Leder 1972). Hagen et al. (1976) showed an important loss in viability of *Bacteroides fragilis* during exposure to chilling at 4°C. More recently, much work has been

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undertaken on cold shock response and adaptation of microorganisms at temperatures above 0°C (Aguilera et al. 2007; Jones and Inouye 1994; Kandror et al. 2004; Kandror et al. 2002; Phadtare 2004; Thieringer et al. 1998; Weber and Marahiel 2003). Nevertheless, the chilling rate in this temperature range has often been neglected, contrary to the cooling rate used during freezing, which has been well documented (Dumont et al. 2004; Koshimoto and Mazur 2002; Mazur et al. 1993; Steponkus and Lynch 1989). This parameter should be taken into more careful consideration, as such nonfreezing temperatures are widely used in the food industry, in laboratories (e.g., for cell preservation, cell dilution, or enzyme extraction) and in combination with other physical parameters like osmotic pressure (Beney et al. 2001) or chemical agents like alcohol and nisin (Boziaris and Adams 2001; Chu-Ky et al. 2005). The application of a cold shock or cold acclimation may increase heat sensitivity in *Listeria monocytogenes* (Miller et al. 2000). Transient sensitivity to nisin has also been reported in Gram-negative cells subjected to chilling (Boziaris and Adams 2001). Thus, understanding the effects of different chilling rates on diverse ranges of cell types could help to reveal strategies to increase microbial sensitivity to treatments, enhancing microbiological safety and stability.

Because it is important to emphasize the impact on microorganisms of the chilling rate in the positive temperature range, our objective was to investigate the effect of the chilling rates from the growth temperature (37 or 30°C) to 0°C on the viability of several microorganisms. Two bacteria: a Gram-negative, the TG1 mutant of *Escherichia coli* K-12, a Gram-positive *Bacillus subtilis* ATCC 31324, and a yeast, *Saccharomyces cerevisiae* CBS 1171 were chosen in this study for their difference in cell envelope characteristics and also because their responses to cold shock were rather well documented (Aguilera et al. 2007; Al-Fageeh and Smales 2006; Graumann and Marahiel 1999; Inouye and Phadtare 2004; Jones and Inouye 1994; Kaul et al. 1992; Weber and Marahiel 2003). The relationships between cold shock-induced changes in membrane fluidity and cell survival were also explored by using the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH), which is widely used to study the structure and the physical state of biological membranes (Chu-Ky et al. 2005; Konopasek et al. 2000).

Materials and methods

Microbial strain and culture conditions

Escherichia coli strain TG1 (K12 *supE hsdΔ5 thi Δ (lac-proAB) F'[traD36 proAB⁺ lacI^qlacZΔM15]*) was from the Microbiology Laboratory Culture Collection, ENSBANA,

Dijon, France. *Bacillus subtilis* ATCC 31324 was supplied from the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany. *Saccharomyces cerevisiae* CBS 1171 was obtained from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

E. coli was maintained on Luria Broth (LB) agar plates (Sigma-Aldrich, St Louis, MO) at 4°C. The subculture was prepared by transferring a colony from each plate into 10 ml of LB broth, followed by incubation for 18 h at 37°C. A culture was then prepared by injecting 0.1 ml of bacterial suspension into 10 ml of the respective growth medium and incubated statically until the desired growth phase (exponential or stationary) was obtained.

B. subtilis was maintained on Petri dishes with Columbia agar (Biokar Diagnostics, Allonne, Beauvais, France) at 4°C. Cultures were grown in 250-ml conical flasks containing 100 ml of brain–heart infusion (BHI) broth (Biokar Diagnostics) at 37°C and stirred at 115 rpm. Inoculation was carried out by using 1 ml of bacterial suspension from a 24-h subculture grown under analogous conditions.

Cultures of the yeast *S. cerevisiae* CBS 1171 were grown in 250-ml conical flasks containing 100 ml of aerated modified Malt Wickerham medium at 30°C and 250 rpm. This medium is composed of glucose, 10 g l⁻¹ (Sigma-Aldrich), peptone pancreatic, 3 g l⁻¹ (VWR International, Fontenay sous Bois, France), yeast extract, 3 g l⁻¹, and NaH₂PO₄, 1.5 g l⁻¹ (Sigma-Aldrich). The pH of the medium was adjusted to 5.35 by adding orthophosphoric acid (Sigma-Aldrich). After 48 h of incubation, an aliquot (1 ml) of culture was transferred into a conical flask containing the same medium and was grown under the same conditions. The growth phases of the microbial cultures were estimated from the absorbance at 600 nm (*A*₆₀₀).

Achieving various chilling rates to 0°C

Chilling was conducted in phosphate-buffered saline (PBS). Cells were collected by centrifugation at 2,800×g for 10 min. The pellets were then washed in saline, resuspended in PBS to a cell concentration of about 10⁸ Colony-forming units (CFU) ml⁻¹. One-milliliter aliquots of cell suspension were then transferred to 1.5 ml cryotubes (Sarstedt, Nümbrecht, Germany) for experiments. One tube was held at initial temperature *T*_i (*T*_i=37 or 30°C, depending on the growth temperature of the strains) throughout all experiments as a control. All materials were held at these temperatures initially. Two chilling rates were investigated: a low rate of 2°C min⁻¹ and an instantaneous rate of 2,000°C min⁻¹. For the low chilling rate, one tube was placed in a cryostat at *T*_i (Julabo-F81, SeelBach, Germany). Temperature changes were planned and monitored using a program to

obtain a linear and progressive temperature decrease from T_i to 0°C . The rapid chilling rate ($2,000^\circ\text{C min}^{-1}$) was achieved by plunging 0.1 ml of cell suspension at initial temperature into 9.9 ml of PBS at -0.5°C to obtain a final temperature of 0°C . In this case, the cell suspension reached 0°C nearly instantaneously, from which we estimated a chilling rate of $2,000^\circ\text{C min}^{-1}$.

The kinetics of chilling was followed using a T-thermocouple (TCSA, Dardilly, France) inserted into an uninoculated tube (blank) that was placed into the cryostat along with the inoculated samples. This thermocouple was connected to an InstruNet 100 acquisition card (GWI, Somerville, USA). A maximum of 60,000 measurements per second were recorded with this system, which could then be evaluated using a spreadsheet. The chilling rates were calculated from these measurements. When the target temperature of 0°C was obtained, samples were stored at this temperature for 24 h or were transferred directly to a water bath at T_i for warming at 2°C min^{-1} ; this rate did not influence cell viability (results not shown). The experimental design is represented schematically in Fig. 1a and b.

Labeling of microorganisms with fluorescent probe

DPH from Molecular Probes (Invitrogen SARL, Cergy Pontoise, France) was used to investigate the effect of the cold stress on the microbial membranes. This probe was dissolved in tetrahydrofuran (Sigma-Aldrich) and was added directly to exponentially or stationary growing cells at different concentrations, depending the cell type. The

protocol for the DPH-labeled cells was optimized as follows: cells were harvested by centrifugation ($2,800\times g$, 10 min) and were washed twice in PBS. The cell pellets were resuspended in the same buffer, and the cell concentration was adjusted to $A_{600}=0.6$ for bacterial cells and 0.7 for yeast cells. Cells were submitted to a slow chilling of 2°C min^{-1} and an instantaneous cold shock of $2,000^\circ\text{C min}^{-1}$ from T_i to 0°C , during which fluorescence changes were recorded continuously.

For the slow kinetics, 3 ml of cell suspension were placed in a stirred and thermostat-equipped quartz cuvette, then an appropriate volume of 1 mM DPH solution was added to the cell suspension (9 μl for bacterial cell suspension and 4 μl for yeast cell suspension). The temperature was then decreased linearly from T_i to 0°C using the Peltier system (Wavelength Electronics, Bozeman, USA). Sample temperatures were monitored by a T-thermocouple placed in the sample. Temperature variations were recorded every 8 s using a PicoLog Recorder (Pico Technology, Cambridgeshire, USA). To achieve the rapid temperature downshift, 9 (for bacterial cells) or 4 μl (for *S. cerevisiae*) of the DPH solution were added into 2.7 ml of PBS buffer, and then the mixture was brought to -3.4°C in a quartz cuvette. A volume of 0.3 ml of the tenfold concentrated cell suspension was incubated with DPH (9 μl in the case of bacterial cells and 4 μl in the case of yeast cells) in the dark for 5 min at T_i before the treatment to ensure incorporation into the cell membrane. This was then rapidly injected into the cuvette at -3.4°C . This manipulation allowed an abrupt temperature drop of $2,000^\circ\text{C min}^{-1}$ from T_i to 0°C .

Steady-state fluorescence anisotropy measurements during thermal treatment

Steady-state fluorescence anisotropy measurements were performed with a spectrofluorometer (Fluorolog-3, Jobin Yvon, USA) in a T format. Indeed, in this format, one emission polarizer is fixed in the H position (polarization vector lying in the optical plane) and the other in the V position (polarization vector normal to the optical plane), while the excitation polarizer is rotated between the V and H positions. Fluorescence intensity and anisotropy of DPH were measured at 430 nm with excitation at 360 nm. Intensities both parallel and perpendicular to the vertically polarized excitation beam were measured. Fluorescence anisotropy was measured continuously every 8 s during the treatment. This parameter is inversely proportional to membrane fluidity: The higher the value of anisotropy, the more rigid is the membrane. The steady-state anisotropy (r) was calculated according to Lakowicz (1983) from the equation:

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}}$$

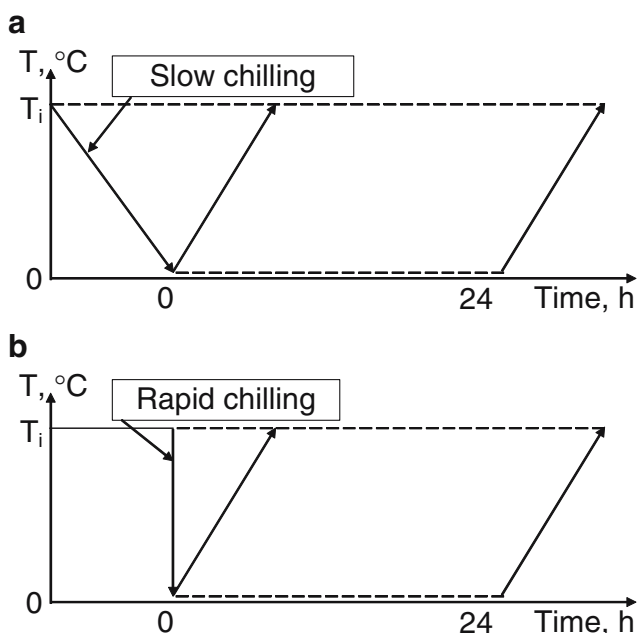


Fig. 1 a, b Schematic representation of the experimental design

where I_{VV} and I_{VH} represent the intensities measured with polarizers parallel and perpendicular to the vertically polarized exciting beam, respectively. G is a factor used to correct the instrument's polarization and is given by the ratio of vertically to horizontally polarized emission components when the excitation light is polarized in the horizontal direction.

Changes in membrane fluidity were expressed as the percentage of anisotropy based on initial anisotropy values. All fluorescence anisotropy measurements were performed at least in triplicate from independent cultures. Means and 95% confidence intervals were calculated.

Viability determinations

Cell viabilities were determined by the CFU method. Cell suspensions taken after treatment were diluted serially and then plated simultaneously onto nutritive media and incubated at 37°C for 24 h before counting for *E. coli* and *B. subtilis* or 30°C for 48 h for *S. cerevisiae*. Viabilities were determined by comparing the experimental and control CFU counts. Each experiment was repeated at least three times.

Results

Effects of different chilling rates on cell survivals

The effect of the chilling rate (2°C and 2,000°C min⁻¹) and of the cell growth phase (exponential or stationary phase) were assessed on the survival of the two bacterial species *E. coli* and *B. subtilis* and of the yeast *S. cerevisiae* (Fig. 2a, b,c). Cells in both exponential and stationary phases of growth appeared resistant to the slow chilling at 2°C min⁻¹. A maximal population reduction of 16% was observed with exponentially growing cells of *E. coli* (Fig. 2a). Maintaining cells after slow chilling at 0°C for 24 h did not induce any significant increase in population reduction. The instantaneous cold shock resulted in a reduction of 26% of the stationary-phase cell population for *E. coli* (Fig. 2a) and only 9% for *B. subtilis* in the same growth phase (Fig. 2b). It is interesting to note that exponentially growing cells of *E. coli* and *B. subtilis* were more sensitive to the rapid cold shock with a drastic decrease of more than 91 and 73% in CFU counts, respectively (Fig. 2a and b), whereas the viability of *S. cerevisiae* only decreased slightly to 89 and 83% after the rapid temperature downshift for exponentially and stationary growing cells, respectively (Fig. 2c).

While maintaining cells at 0°C for 24 h after slow chilling had little or no effect on the cell survival (results not shown), the rapid cold shock followed by a 24-h incubation at

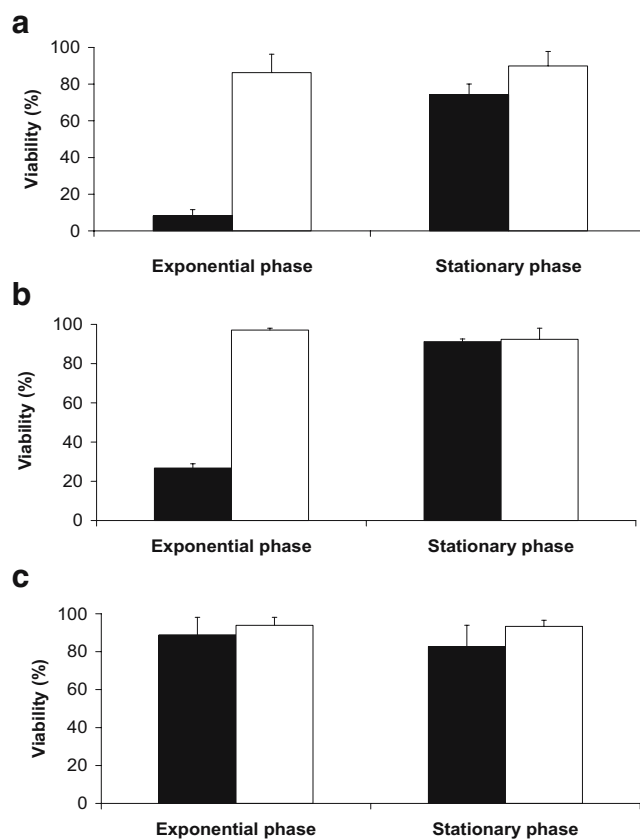


Fig. 2 Viability of **a** *E. coli*, **b** *B. subtilis*, and **c** *S. cerevisiae* as a function of growth phase and chilling rate. Empty squares, chilling at 2°C min⁻¹; filled squares, chilling at 2,000°C min⁻¹ (error bars represent standard deviations)

0°C resulted in an important increase of population reduction for all the three microorganisms (Table 1). Again, the yeast *S. cerevisiae* seemed to be less sensitive to the rapid cold treatment than the two bacteria, about 0.34 log and 0.13 log CFU₁₀ reduction in the number of survivors were observed with yeast cells in exponential and stationary growth phases, respectively. Concerning the two bacteria, the rapid cold shock at 0°C for 24 h induced a reduction of about 2 and 0.3 log CFU₁₀ in the initial population of exponentially and stationary growing cells, respectively (Table 1).

Effect of chilling on the membrane physical state

To study the impact of chilling on the cell membrane fluidity, cells in exponential and stationary growth phases were submitted either to the slow chilling of 2°C min⁻¹ or to the rapid chilling of 2,000°C min⁻¹ from T_i to 0°C in PBS buffer, as described above. The membrane physical state was monitored by steady-state fluorescence anisotropy measurements of the DPH-labeled cells. Measurements showed that the degree of anisotropy of DPH in membrane of exponentially growing cells was lower than that of stationary growing cells at physiological temperature (e.g.,

Table 1 Effect of cold shock duration on survival of microorganisms

Microorganisms	Population reduction ^a (log CFU ml ⁻¹)			
	Exponential phase		Stationary phase	
	Immediate cold shock	24-h cold shock ^b	Immediate cold shock	24-h cold shock ^b
<i>B. subtilis</i>	0.57±0.03	2.15±0.34	0.04±0.01	0.35±0.12
<i>E. coli</i>	1.09±0.15	1.92±0.21	0.13±0.03	0.31±0.08
<i>S. cerevisiae</i>	0.05±0.04	0.34±0.19	0.08±0.06	0.13±0.04

^a Log reduction in cell viability±standard deviations

^b Rapid cold shock followed by a 24-h incubation at 0°C

0.145±0.003 for *B. subtilis* in exponential phase compared with 0.162±0.003 for *B. subtilis* in stationary phase). This indicates that exponentially growing cells have a more fluid membrane than stationary growing cells.

Effect of the slow chilling on plasma membrane fluidity of microbial cells

Changes in membrane anisotropy of cells of *E. coli* and *B. subtilis* in relation to slow temperature changes are displayed in Fig. 3a and b, respectively. Membrane rigidification was observed for both cells in stationary and exponential growth phases; it occurred progressively with the decrease in temperature. Membrane of bacterial cells in the stationary growth phase was less affected by the chilling than that of cells in the exponential growth phase. At 0°C, the DPH anisotropy value was 140% compared with its initial value for both *E. coli* and *B. subtilis* in stationary growth phase, while it was higher for cells in exponential growth phase (155% for *E. coli* and 191% for *B. subtilis*. Fig. 3a and b, respectively), indicating that the membranes of exponentially growing cells were more susceptible to temperature changes. Upon warming, the degree of fluorescence anisotropy returned to the baseline level regardless of the cell type and the anisotropy level at 0°C.

Effect of the rapid chilling on plasma membrane fluidity of microbial cells

Figures 4a and b show the changes in DPH anisotropy of *E. coli* and *B. subtilis* cells submitted to the rapid chilling, respectively. The rapid cold shock induced an abrupt increase in anisotropy between 140 and 197% of the initial anisotropy value, depending on the cell type and the cell growth phase; this corresponds to an instantaneous decrease in membrane fluidity (Fig. 4a and b). Nevertheless, the increase in anisotropy values induced by the rapid cold shock of exponentially growing cells was higher than that of stationary growing cells (the steady-state anisotropy at 0°C was 160 vs 140% for *E. coli* and 197 vs 152% for *B. subtilis*).

During warming, DPH anisotropy of cells in stationary growth phase behaved in a similar way to those submitted to the slow chilling (Fig. 4a and b), reaching back the initial values. Surprisingly, anisotropies of both *E. coli* and *B. subtilis* cells growing exponentially did not return to their initial level but were about 10% higher than anisotropy of control cells (Fig. 4a and b, respectively). This level of

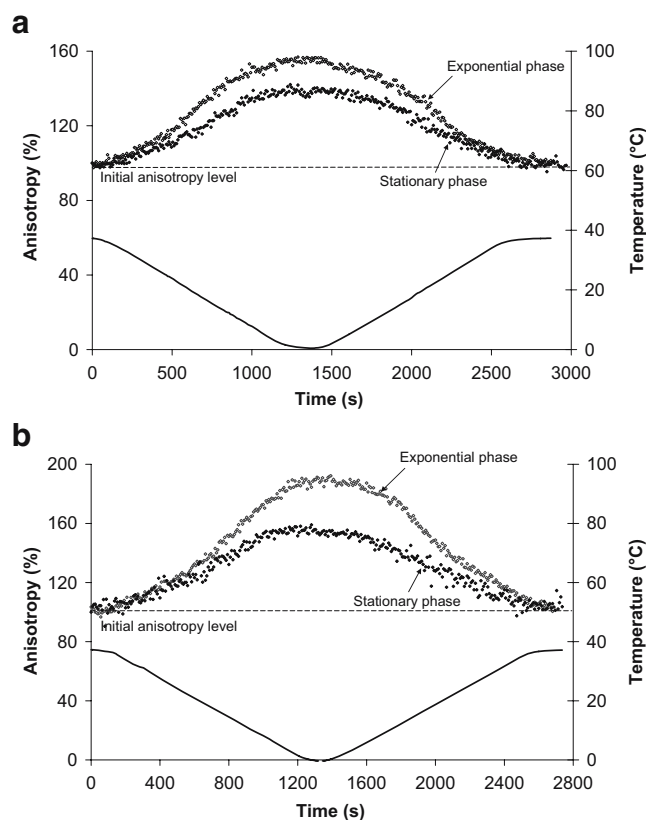


Fig. 3 Changes in membrane fluidity (percentage anisotropy) of **a** *E. coli* and **b** *B. subtilis* in exponential (empty diamonds) and stationary (filled diamonds) growth phases during chilling at 2°C min⁻¹ from 37 to 0°C followed by warming to 37°C. Solid line, temperature variation. The initial fluorescence anisotropy value (100%) was 0.184±0.005 (stationary growth phase) or 0.176±0.008 (exponential growth phase) for *E. coli* and 0.162±0.003 (stationary growth phase) or 0.144±0.003 (exponential growth phase) for *B. subtilis*

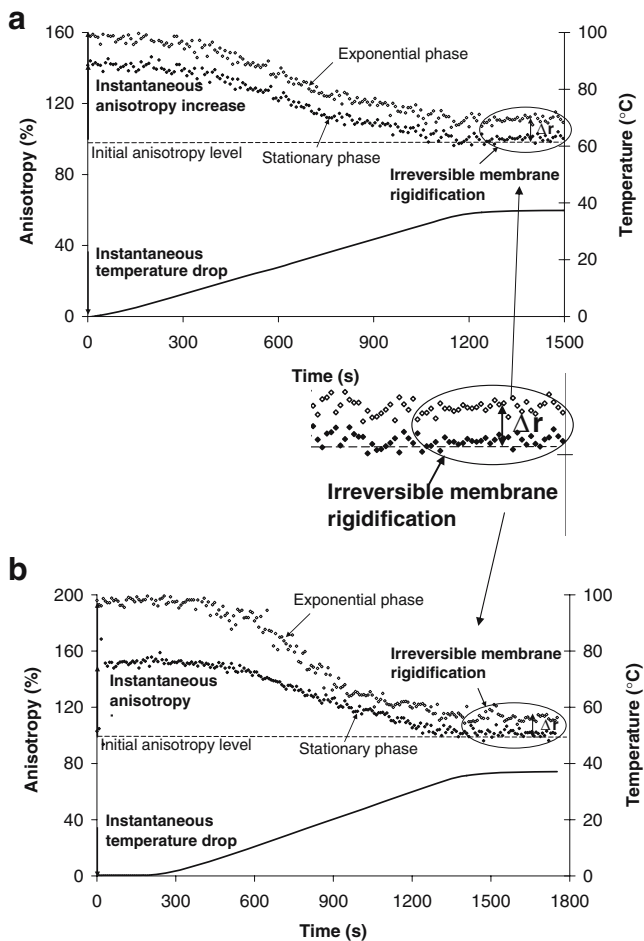


Fig. 4 Changes in membrane fluidity (percentage anisotropy) of **a** *E. coli* and **b** *B. subtilis* in exponential (empty diamonds) and stationary (filled diamonds) growth phases after a rapid cold shock from 37 to 0°C followed by warming to 37°C. Solid line, temperature variation. $\Delta r = r_{\text{final}} - r_{\text{initial}}$: the difference between final and initial anisotropy levels. The initial fluorescence anisotropy value (100%) was 0.184 ± 0.005 (stationary growth phase) or 0.176 ± 0.008 (exponential growth phase) for *E. coli* and 0.162 ± 0.003 (stationary growth phase) or 0.144 ± 0.003 (exponential growth phase) for *B. subtilis*

anisotropy was maintained even after more than 3 h at 37°C. Thus, this effect caused by the rapid cold shock may reflect a permanent loss of membrane integrity of bacterial cells growing exponentially.

Concerning *S. cerevisiae*, the steady-state anisotropy measurements showed no difference between cold-shocked cells and control cells after the treatment. DPH anisotropies of yeast membrane were of 0.164 ± 0.004 with stationary-growing cells and 0.157 ± 0.001 with exponentially growing cells. These increased slowly (during the cold slope) or rapidly (upon the instantaneous cold shock) and reached 143% at 0°C compared with their initial values in both cases, regardless of the cell growth phase. After warming, anisotropies of cold-shocked cells reached back their initial value, whatever the chilling rate and the cell growth phase. Therefore, the membrane irreversible alteration of expo-

nentially growing bacterial cells that resulted from rapid temperature downshift was not observed with cells of *S. cerevisiae*.

Discussion

In all microorganisms, an abrupt temperature drop can have a profound impact on cell functions at various levels. This does not necessarily require the formation of extracellular or intracellular ice crystals, and strong effect can also be detected at positive temperatures (Weber and Marahiel 2003). Although much work has been done in an effort to demonstrate the microorganism cold shock responses, comparatively, little has been done to relate the rates of temperature variation to cell inactivation in positive temperature ranges. In this study, we found that rapid chilling produced greater inactivation than slow chilling, and the inactivation kinetics was different between Gram-negative and Gram-positive bacteria and yeast. Yeast cells seemed to be less affected by cold shock than bacteria. *E. coli* showed higher inactivation rates than *B. subtilis* immediately after the rapid cold shock (1.09 log CFU₁₀ reduction compared with 0.57 log CFU₁₀ reduction, respectively). However, the incubation at 0°C after the rapid cold shock seemed to affect *B. subtilis* more than *E. coli*, as its reduction in viability after a 24-h incubation was slightly greater compared with *E. coli* (Table 1). Further experiments confirmed also that *B. subtilis* was more susceptible to incubation at reduced temperature than *E. coli* (results not shown). These findings are in agreement with those of Ingram and Mackey (1976), who reported that Gram-positive bacteria were more susceptible to cold shock than Gram-negative ones. Concerning yeast cells, it has been shown that they are adapted to cooler temperatures than bacteria (Inouye and Phadtare 2004). Membranes of yeast cells contain sterols, which participate in the proper functioning of the yeast membrane and in particular in the resistance to different types of stress. They interact with phospholipids during a temperature downshift and thus prevent them from the conformational transition. Consequently, the higher the quantity of sterols in the yeast membrane, the less important the number of phospholipids participating in the lipid phase transition (Schechter 1997). This suggests that yeast cells are more resistant to chilling than bacteria, thus the viability remained high after the cold treatment applied.

In particular, in the present work, there was a good correlation between membrane anisotropy changes and loss of viability after the rapid cold shock treatment. Bacterial cells in the exponential growth phase that were cold shocked rapidly showed a higher level of anisotropy while returning to initial conditions, compared with anisotropy of

control cells (Fig. 4a and b). For these cells, high rates of cell inactivation were observed ($0.57 \log \text{CFU}_{10}$ with *B. subtilis* and $1.59 \log \text{CFU}_{10}$ with *E. coli* in exponential growth phase; Table 1). In all other cases, no change in the steady-state anisotropy of the membrane was observed, and rates of cell inactivation were low. Thus, the rapid cold shock seemed to have a critical effect on the cell membrane. This and the fact that the rate of cell death increased for high chilling rate suggest that the cell membrane is differently destabilized by rapid or slow chilling and that the instantaneous chilling induces strong membrane rigidification and highly disorganized state of the cell membrane. Compared with the rapid cold shock at $2,000^\circ\text{C min}^{-1}$ to 0°C , the rate of 2°C min^{-1} gave a 19-min delay to cells to adapt themselves to the temperature change. Cell membranes, which are in a liquid crystalline form at physiological temperature, undergo a transition to a gel phase state when cooled. Passing through membrane phase transition can result in cell death if the cells are chilled very rapidly. Upon rapid chilling, all the phospholipids species may undergo lipid phase transition at the same time. Such rapid chilling prevents the lateral phase separation of phospholipids and protein domains that occurs during slow chilling (Leder 1972), inducing high level of membrane permeabilization as a result of phase separation within the plane of the lipid bilayer (Drobnis et al. 1993). This loss in membrane integrity would be unfavorable to the maintenance of homeostasis, gives rise to discontinuities in the gelled membrane, and creates hydrophilic channels facilitating the outflow of cell components and consequently leads to the cell death. Besides, by using propidium iodide (PI), a nucleotide-binding fluorescent probe impermeant to intact cells and which thus is a good indicator of membrane integrity and/or permeability (Wouters et al. 2001), a far greater PI uptake was seen in bacterial cells in the exponential growth phase upon the rapid chilling than during slow chilling (results not shown). This indicates that the membrane of exponentially growing cells was highly permeabilized after the rapid cold treatment, which correlates well with results obtained from the measurements of membrane fluidity. Additionally, rapid cold shock-induced irreversible alteration in membrane fluidity was sustained, even after returning the cells to initial conditions for several hours. This suggests that the cell damage persisted and the effect of the instantaneous chilling on cell membrane is not transient. Thus, the irreversible membrane rigidification induced by the rapid chilling could have caused the abrupt decrease in cell viability. Furthermore, incubating cells at 0°C does not permit their recovery but increases cell mortality, as shown in Table 1, suggesting that there is no reparation process and cells continued to die from the effect of cold shock injury.

It has been shown that microorganisms respond to cold stress by overexpression of a small group of proteins, the so-termed cold shock proteins, or induction of trehalose synthesis (Al-Fageeh and Smales 2006; Thieringer et al. 1998; Weber and Marahiel 2003). Nevertheless, these mechanisms require some nutrients (Laroche and Gervais 2003). In our experiment, cells were cold shocked and maintained in buffer, thus did not have any nutrients available to repair damage caused by the rapid chilling. Furthermore, no loss of viability was observed when cells were incubated before the slow chilling with chloramphenicol (at $100 \mu\text{g ml}^{-1}$ for 15 min), a protein synthesis inhibitor (results not shown). This indicates that instantaneous chilling-induced cell mortalities is due to a physical effect, which is the rapidity of the temperature downshift. In other words, loss of viability appeared to be directly related to a permanent loss of physical integrity in exponential growing cells.

The results obtained in this present work support also observations reported previously that stationary-phase cells possess maximum thermal tolerance (Miller et al. 2000; Pagan and Mackey 2000). The membrane of exponentially growing cells is more fluid to promote exchange between cells and growth medium and to support cell division. This contributes to the increased fragility of these cells (Casadei et al. 2002). Our measurements of fluorescence anisotropies showed that cells in the stationary growth phase had higher initial anisotropy values than cells in exponential growth phase, indicating that the membrane of the former cells is more rigid (Figs. 3a,b and 4a,b). Besides, anisotropies at 0°C of the exponential-phase cells were higher than those of stationary-phase cells at any chilling rate, suggesting that membranes of exponentially growing cells were more destabilized by chilling. Entry into the stationary phase or starvation is accompanied by profound structural and physiological changes that result in increased resistance to heat shock and oxidative, osmotic, and acid stresses (Kolter et al. 1993). Moreover, stationary-phase cell membranes have a higher amount of protein and thus a higher protein-to-lipid ratio than that of exponential-phase cell membranes (Souzu 1986). An augmentation of the protein-to-lipid ratio in the stationary-phase cell membrane also suggests a reinforcement of lipid-protein interactions, which may stabilize the membranes. Therefore, the resistance of stationary-growing cells to chilling may be attributed to the stable membrane structures.

Recently, Simonin et al. (2007) have related the cell death induced by osmotic shock to transient plasma membrane modifications. We have described for the first time the relationship between irreversible membrane damage and loss of viability after the rapid chilling in exponentially growing cells. Further studies are needed to emphasize the role of the membrane in determining the

chilling sensitivity of exponential and stationary growth-phase cells. Bacteria were found to be more susceptible to the temperature downshifts at near freezing temperatures than yeast. This in turn implies that different groups of microorganisms exhibit distinct types of responses to cold shock. We can expect also an increase in the cold shock sensitivity of cells in the stationary phase of growth by producing more lethal cold shock or increasing the chilling magnitude, as the terms “cold shock sensitive” and “cold shock resistant” are not absolute but rather valid only if the rates of chilling and the final temperatures attained are defined (Morris and Clarke 1987). A better understanding of the influence of thermal kinetics will allow the optimization of current techniques of food product preservation with a view to its industrial exploitation. Finally, as the widespread uses of cold temperatures in the food-processing industry are currently food conservation and stabilization, the present work may encourage new methods by which cooling could be used for microbial destruction.

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References

- Aguilera J, Randez-Gil F, Prieto JA (2007) Cold response in *Saccharomyces cerevisiae*: new functions for old mechanisms. *FEMS Microbiol Rev* 31:327–341
- Al-Fageeh MB, Smales CM (2006) Control and regulation of the cellular responses to cold shock: the responses in yeast and mammalian systems. *Biochem J* 397:247–259
- Beney L, Marechal PA, Gervais P (2001) Coupling effects of osmotic pressure and temperature on the viability of *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 56:513–516
- Bozariar IS, Adams MR (2001) Temperature shock, injury and transient sensitivity to nisin in Gram negatives. *J Appl Microbiol* 91:715–724
- Casadei MA, Manas P, Niven G, Needs E, Mackey BM (2002) Role of membrane fluidity in pressure resistance of *Escherichia coli* NCTC 8164. *Appl Environ Microbiol* 68:5965–5972
- Chu-Ky S, Tourdot-Marechal R, Marechal PA, Guzzo J (2005) Combined cold, acid, ethanol shocks in *Oenococcus oeni*: effects on membrane fluidity and cell viability. *Biochim Biophys Acta* 1717:118–124
- Drobnis EZ, Crowe LM, Berger T, Anchordoguy TJ, Overstreet JW, Crowe JH (1993) Cold shock damage is due to lipid phase transitions in cell membranes: a demonstration using sperm as a model. *J Exp Zool* 265:432–437
- Dumont F, Marechal PA, Gervais P (2004) Cell size and water permeability as determining factors for cell viability after freezing at different cooling rates. *Appl Environ Microbiol* 70:268–272
- Graumann PL, Marahiel MA (1999) Cold shock response in *Bacillus subtilis*. *J Mol Microbiol Biotechnol* 1:203–209
- Hagen JC, Wood WS, Hashimoto T (1976) Effect of chilling on the survival of *Bacteroides fragilis*. *J Clin Microbiol* 4:432–436
- Ingram JM, Mackey BM (1976) Inactivation by cold. In: Skinner FA, Hugo WB (eds) *Inhibition and inactivation of vegetative microbes*. Academic, New York, pp 111–151
- Inouye M, Phadtare S (2004) Cold shock response and adaptation at near-freezing temperature in microorganisms. *Sci STKE* 237:pe26
- Jones PG, Inouye M (1994) The cold-shock response—a hot topic. *Mol Microbiol* 11:811–818
- Kandror O, DeLeon A, Goldberg AL (2002) Trehalose synthesis is induced upon exposure of *Escherichia coli* to cold and is essential for viability at low temperatures. *Proc Natl Acad Sci USA* 99:9727–9732
- Kandror O, Bretschneider N, Kreydin E, Cavalieri D, Goldberg AL (2004) Yeast adapt to near-freezing temperatures by *STRE/Msn2,4*-dependent induction of trehalose synthesis and certain molecular chaperones. *Mol. Cell.* 13:771–781
- Kaul SC, Obuchi K, Komatsu Y (1992) Cold shock response of yeast cells: induction of a 33 kDa protein and protection against freezing injury. *Cell Mol Biol (Noisy-le-grand)* 38:553–559
- Kolter R, Siegele DA, Tormo A (1993) The stationary phase of the bacterial life cycle. *Annu Rev Microbiol* 47:855–874
- Konopasek I, Strzalka K, Svobodova J (2000) Cold shock in *Bacillus subtilis*: different effects of benzyl alcohol and ethanol on the membrane organisation and cell adaptation. *Biochim Biophys Acta* 1464:18–26
- Koshimoto C, Mazur P (2002) Effects of cooling and warming rate to and from -70 degrees C, and effect of further cooling from -70 to -196 degrees C on the motility of mouse spermatozoa. *Biol Reprod* 66:1477–1484
- Lakowicz JR (1983) *Principles of fluorescence spectroscopy*. Plenum, New York
- Laroche C, Gervais P (2003) Unexpected thermal destruction of dried, glass bead-immobilized microorganisms as a function of water activity. *Appl Environ Microbiol* 69:3015–3019
- Leder IG (1972) Interrelated effects of cold shock and osmotic pressure on the permeability of the *Escherichia coli* membrane to permease accumulated substrates. *J Bacteriol* 111:211–219
- Mazur P, Cole KW, Schreuders PD, Mahowald AP (1993) Contributions of cooling and warming rate and developmental stage to the survival of *Drosophila* embryos cooled to -205 degrees C. *Cryobiology* 30:45–73
- Meynell GG (1958) The effect of sudden chilling on *Escherichia coli*. *J Gen Microbiol* 19:380–389
- Miller AJ, Bayles DO, Eblen BS (2000) Cold shock induction of thermal sensitivity in *Listeria monocytogenes*. *Appl Environ Microbiol* 66:4345–4350
- Morris GJ, Clarke A (1987) Cells at low temperatures. In: Grout BWW, Morris GJ (eds) *The effects of low temperatures on biological systems*. Edward Arnold, Bedford Square, London, pp 72–119
- Pagan R, Mackey B (2000) Relationship between membrane damage and cell death in pressure-treated *Escherichia coli* cells: differences between exponential- and stationary-phase cells and variation among strains. *Appl Environ Microbiol* 66:2829–2834
- Panadero J, Pallotti C, Rodriguez-Vargas S, Randez-Gil F, Prieto JA (2006) A Downshift in temperature activates the high osmolarity glycerol (HOG) pathway, which determines freeze tolerance in *Saccharomyces cerevisiae*. *J Biol Chem* 281:4638–4645
- Panoff J-M, Thammavongs B, Gueguen M, Boutibonnes P (1998) Cold stress responses in mesophilic bacteria. *Cryobiology* 36:75–83
- Phadtare S (2004) Recent developments in bacterial cold-shock response. *Curr Issues Mol Biol* 6:125–136
- Ring K (1965) The effect of low temperatures of permeability in *Streptomyces hydrogenans*. *Biochim Biophys Res Commun* 19:576–581

- Russell NJ, Evans RI, ter Steeg PF, Hellemons J, Verheul A, Abee T (1995) Membranes as a target for stress adaptation. *Int J Food Microbiol* 28:255–261
- Sato M, Takahashi H (1968) Cold shock of bacteria. I. General features of cold shock in *Escherichia coli*. *J Gen Appl Microbiol* 14:417–428
- Schechter E (1997) Fluidité membranaire. In: Schechter E (ed) *Biochimie et biophysique des membranes. Aspects structuraux et fonctionnels*. Masson, Paris Cedex 06, pp 95–110
- Simonin H, Beney L, Gervais P (2007) Cell death induced by mild physical perturbations could be related to transient plasma membrane modifications. *J Membr Biol* 216:37–47
- Souzu H (1986) Fluorescence polarization studies on *Escherichia coli* membrane stability and its relation to the resistance of the cell to freeze-thawing. I. Membrane stability in cells of differing growth phase. *Biochim Biophys Acta* 861:353–360
- Steponkus PL, Lynch DV (1989) Freeze/thaw-induced destabilization of the plasma membrane and the effects of cold acclimation. *J Bioenerg Biomembr* 21:21–41
- Thieringer HA, Jones PG, Inouye M (1998) Cold shock and adaptation. *Bioessays* 20:49–57
- Weber MH, Marahiel MA (2003) Bacterial cold shock responses. *Sci Prog* 86:9–75
- Wouters PC, Bos AP, Ueckert J (2001) Membrane permeabilization in relation to inactivation kinetics of *Lactobacillus* species due to pulsed electric fields. *Appl Environ Microbiol* 67:30923101