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Cryopreservation of *Escherichia coli* K12TG1: Protection from the damaging effects of supercooling by freezing

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ABSTRACT

Injuries in living cells caused by water freezing during a freeze-thaw process have been extensively reported. In particular, intracellular water freezing has long been incriminated in cell death caused by a high cooling rate, but this supposition could not always be demonstrated. This work aims to discriminate the role of water freezing, dehydration and cold-induced injuries in cellular damage occuring during cryopreservation. For this purpose, *Escherichia coli* K12TG1 suspensions were maintained in a supercooled or frozen state at −20 °C for times ranging from 10 min to 5 h. The supercooled state was maintained for a long period at −20 °C by applying a non-injurious isostatic pressure ($P < 40$ MPa). Next, viability and membrane damage were determined by agar plating and fluorescence staining with propidium iodide and bis-oxonol. It was clear that keeping the cell suspensions in the supercooled state had a detrimental effect on both viability and plasma membrane permeability. Conversely, when cells were subjected to cold stress by freezing, the survival rate remained high throughout the experiment, and the cell membranes suffered little damage. Moreover, cells subjected to 5 h of osmotic treatments at −20 °C, conditions that mimic cryoconcentration upon freezing, and subsequently diluted and thawed suffered little damage. Dehydration due to cryoconcentration upon freezing protects the cells against the deleterious effects of supercooling, especially in the plasma membranes. The decrease in membrane leakage upon dehydration at low temperatures could be linked to differences in the gel state of the membrane revealed by a higher Laurdan general polarization (GP) value.

Introduction

Because glycerol's ability to serve as a cryoprotectant was first discovered in 1949 [26], considerable progress has been made in the field of cryopreservation. Today, cryopreservation is commonly used for the long-term preservation of the biological functions of a variety of cells, including spermatozoa or frozen cell stocks. However, despite ongoing research to improve our understanding of cryopreservation and to develop new cryopreservation techniques, numerous bottlenecks persist: the cryopreservation of composite tissues remains impossible [2]; numerous plant germplasms cannot be cryopreserved [10]; oocytes are also highly susceptible to physical stresses, and their cryopreservation is still considered as experimental [29]; and even the oldest applications require improvement for optimal preservation of biological functions, as stated by Fonseca [6] for lactic acid bacteria.

Several physico-chemical phenomena are involved in cell damage during the freeze-thaw process. The role of water crystallization has been extensively reported [5,17,36]. It is now commonly accepted that at low freezing rates, extracellular ice formation leads to cell dehydration due to cryoconcentration of the extracellular medium. All the solutes and materials in suspension, including cells, concentrate in unfrozen compartments. The cells are thus exposed to an increasingly concentrated solution while the temperature decreases. This process continues until the cryoconcentrated solution crystallizes as a eutectic system, forms glass, or persists as an unfrozen solution depending on the final temperature. If cells are cooled more rapidly (200–5000 °C min^{-1}), they will undergo lethal intracellular ice-formation because the intracellular water does not flow out fast enough to prevent extensive supercooling, and eventual nucleation of the supercooled water *in situ*. The cooling rates that cause extracellular or intracellular crystallization depend on the cell type and the cryoprotectant concentration. Finally, at very high cooling rates and high cryoprotectant concentrations, it is possible to prevent water freezing. This phenomenon is due to the vitrification of the whole system. Recent

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developments in vitrification protocols have significantly improved the cryopreservation of various cell types, particularly germplasms [9,29]. Whatever the cooling protocol, the thawing and storage conditions must also be strictly controlled to decrease the chances of ice nucleation or recrystallization [6,18,29].

However, in addition to reports of injuries occurring during freezing, some studies report additional phenomena that may result in cell damage [6,22]. These studies attribute unexpected cell injury to the osmotic imbalance occurring during cell thawing.

In addition to mechanical and osmotic stresses due to freezing, cells are also subjected to a cold stress when cryopreserved [25], and cold stress can be very harmful for two reasons: first, cooling rates are often high, which according to our knowledge, can be considered as injurious cold shocks [4]; second, cells remain at subzero temperatures while the medium is in liquid state before ice formation. In fact, water and aqueous solutions tend to cool to a temperature significantly below their melting point before ice nucleation occurs. It has been shown that supercooling is a critical step in the cryopreservation process [21].

Thus, there is still a need to determine the role of freezing, dehydration and cold-induced injuries in cell damage that occurs during cryopreservation. In this way, we will improve not only our understanding of the mechanisms of cryopreservation but also improve cryopreservation protocols.

The objective of this work was to distinguish the damage to *Escherichia coli* caused by freezing from the damage caused by the cold. For this purpose, bacterial suspensions were supercooled for several hours. Supercooling is a metastable liquid state below the freezing point that can theoretically be obtained down to −40 °C in pure water [7]. In practice, the extent of supercooling depends mainly on the sample volume, its composition, and the cooling rate applied. For example, Searles et al. reported supercooling ranging from −8 °C to −18 °C in 2 mL of 10% (w/v) starch solutions in a freeze-dryer [30]. In the present work, supercooling was maintained for up to 5 h at −10 °C and −20 °C. For the experiments at −20 °C, the cell suspensions were subjected to an isostatic pressure of up to 40 MPa. At 40 MPa, the supercooling state was more stable than at ambient pressure because the pressure induced a decrease in the freezing point of water by −3.2 °C. The supercooling range can also be extended using this technique (down to −92 °C at 200 MPa). In a previous study, this pressure level was shown to have no effect on *E. coli* viability, even at −20 °C [24].

Our team has been studying the cell membrane's responses to physical perturbations for over a decade and has demonstrated in many studies that it is a key target of osmotic, mechanical and thermal stresses [4,8,14,32]. Cell membrane integrity, polarity, and phase changes were thus chosen to investigate the mechanisms of cell injury.

Material and methods

Cell culture

Escherichia coli K12TG1 (PAM, Agrosup Dijon, France) was grown statically in 20 mL test tubes containing 10 mL of Luria Bertani broth (LB, Sigma Aldrich) at 37 °C for 24 h. Liquid cultures were then prepared by injecting 0.2 mL of subculture into 30 mL test tubes containing 20 mL of LB broth. The cultures were grown statically at 37 °C for 24 h until the early stationary phase was reached. Final cell concentrations ranged from 2×10^8 to 5×10^8 CFU/mL. Each cell culture was centrifuged at 2880*g* for 5 min and washed twice in the same volume of a sterile water–glycerol solution with an a_w (water activity) of 0.992 (4% w/w glycerol in water).

Freezing and supercooling at −10 °C

Freezing and supercooling at −10 °C were achieved using the protocol described by Moussa et al. [23]. Aliquots (1 mL) of cell suspensions were transferred aseptically to cryotubes. The samples were then cooled by immersing the cryotubes in a cryostat that was kept at −10 °C. The temperature was measured using K-type thermocouples (NiCr/NiAl, response time 70 ms; Thermocoax, Suresnes, France) inserted through the plugs in the cryotubes and positioned in the centre of the cell suspensions. The mean cooling rate estimated from the temperature profiles was 8 °C/min. For the frozen samples, freezing was triggered by tapping the tubes as soon as the temperature reached −5 °C. Samples were then maintained at −10 °C for times ranging from 10 min to 5 h. Before warming up the supercooled samples, the supercooled state was visually checked to ensure that the samples were liquid by turning the tubes upside down.

Before analysis, the samples were warmed at a rate of 16 °C/min by immersion in a water-bath at 27 °C.

Freezing and supercooling at −20 °C

Supercooling at −20 °C was achieved using a high-pressure device as described by Moussa et al. [23]. Briefly, aliquots (0.5 mL) of cell suspensions were transferred aseptically to polyethylene bags (Sarstedt), which were heat-sealed after removal of air bubbles. Samples were then placed in a small high hydrostatic pressure vessel (Top Industrie, France; 80 mm external diameter, 10 mm internal diameter, 100 mm high, and 55 mm deep, 4 mL inner volume) connected to a hand-operated pump (Novaswiss, Cesson, France). The inner chamber was then filled with 97% ethanol, which acted as a pressure-transmitting fluid. For supercooled samples, the pressure was first increased to 10 MPa (samples maintained for 10 min or 1 h at −20 °C) or 40 MPa (samples maintained for 5 h at −20 °C). The temperature was then lowered first by immersing the high-pressure vessel in a cryostat (F81-HP, Julabo, Seelbach, Germany) maintained at −60 °C to accelerate heat transfer, and then by transferring it to a second cryostat (RC6CP, Lauda, Germany) containing ethanol at −20 °C to maintain a constant temperature during the holding time. The mean cooling rate was 7.5 °C/min. After the holding time, the vessel was warmed via immersion in a water bath maintained at 27 °C. As described previously [22], the small volume of the vessel and the static conditions ensured that no crystallization occurred in the liquid even though the temperature was below the liquid's theoretical freezing point. Changes in pressure and temperature in the treatment vessel were used to ensure the onset of freezing. Frozen samples were first crystallized by immersing the high-pressure vessel in the cryostat before increasing the pressure to 10 or 40 MPa.

Osmotic treatment in cold conditions

The cells were subjected to cold osmotic shocks to simulate the osmotic stress they experience during freezing at −10 °C or −20 °C: 0.1 mL aliquots of cell suspensions were transferred aseptically to cryotubes and cooled to −5 °C to mimic the supercooling before freezing. Samples were then mixed at a constant temperature with 0.9 mL of a water–glycerol solution to a final a_w of 0.908 or 0.822, thermostated at −5 °C to mimic cryoconcentration due to freezing. The water was considered to freeze until the concentration of the unfrozen solution reached a thermodynamic equilibrium with an a_w of 0.908 at −10 °C and 0.822 at −20 °C. The a_w of the theoretical cryoconcentrated solution was deduced from the phase diagram of water–glycerol solutions [12]. Samples were then cooled to −10 °C or −20 °C and maintained at this temperature for times ranging from 10 min to 5 h. Finally, the samples