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Heat resistance, membrane fluidity and sublethal damage in *Staphylococcus aureus* cells grown at different temperatures

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ABSTRACT

In this work the influence of growth temperature (10–42 °C) on *Staphylococcus aureus* heat resistance was studied, and its relationship with the ability of cells to repair sublethal damages and with membrane fluidity was evaluated. Non-linear, convex from above survival curves were obtained, and therefore a special case of the Baranyi model was used to fit them. For exponential phase cells, heat resistance did not change with growth temperature in the range between 10 and 37 °C, but cells grown at 42 °C were significantly more resistant, showing D_{58} and *shoulder length* (sl_{58}) values 2.5 and 4 times greater than the others, respectively. For stationary growth phase cells, an increase in growth temperature above 20 °C resulted in an increase in D_{58} values, and cells grown at 42 °C also displayed the highest D_{58} and sl_{58} values. The increased heat resistance at 58 °C of stationary growth phase cells grown at higher temperatures was coincident with the appearance of a higher proportion of sublethally damaged cells capable of recovery and outgrowth in non-selective medium. Membrane fluidity was measured at treatment temperatures, and it was observed that those cells with more rigid membranes displayed greater heat resistance (Pearson coefficient = 0.969***). Additionally, *S. aureus* cells whose membrane was fluidized through exposure to benzyl alcohol were notably sensitized against the action of heat, in a concentration-dependent manner. Results obtained in this research indicate that membrane physical state could be an important factor determining the survival capacity of bacterial cells to a heat treatment.

1. Introduction

Despite the widespread use and proven efficacy of thermal treatments for the inactivation of pathogen and spoilage microorganisms from foods, there is still a lack of knowledge regarding the factors influencing bacterial inactivation by heat, their mode of action, the rationale behind the kinetics of inactivation, and the responses that bacteria may develop to increase their tolerance to heat, among other aspects. This is still more relevant now, since consumer requirements for fresher and less processed foods are currently leading to a decrease in the intensity of treatments, thus requiring improved knowledge of all these factors in order to design processes that ensure food safety (Abee and Wouters, 1999; Raso and Barbosa-Cánovas, 2003).

Growth temperature is generally acknowledged as one of the main factors influencing bacterial resistance to heat (Cebrián et al., 2017; Jay, 1992; Russell, 1984). It is assumed that bacteria display greater heat resistance when grown at higher temperatures (Cebrián et al., 2008; Elliker and Frazier, 1938; Knabel et al., 1990; Mañas et al., 2003; Pagán et al., 1999). Nevertheless, it should be noted that the degree of influence of growth temperature varies widely among species (Pagán et al., 1999). Although several theories have been proposed to explain this behavior, studies specifically designed to elucidate the mechanisms leading to the increased resistance of cells grown at higher temperatures are scarce.

One controversial question is whether cells grown at higher temperatures are more heat-resistant because they have more stable membranes. The cytoplasmic membrane is considered to be one of the targets of heat treatment (Hurst et al., 1973; Mackey, 2000). However, it has hitherto not been possible to establish a direct relationship between membrane damage and bacterial inactivation by heat. It has been suggested that the membrane could play an indirect role in inactivation, since its damage might result in a loss of homeostasis and a loss of cellular components that would lead to cell death (Coote et al., 1994; Kramer and Thielmann, 2016; Mackey et al., 1991; Marcén et al., 2017; Teixeira et al., 1997). Therefore, it is reasonable to suppose that changes in membranes − for instance in their structure, composition, or mechanical behavior − may influence cell survival to a variety of stressing agents, including heat. Beuchat and Worthington (1976) proposed that the increased heat tolerance of cells grown at higher temperatures might be related to a higher degree of saturation of the

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fatty acids of their membranes. Such increased saturation would lead to less fluid and thus more thermo-stable membranes reduce their fluidity, thus making membranes more thermo-stable. However, it should be noted that fatty acid composition is not the only factor that determines membrane fluidity; other components should be considered in order to determine how membrane fluidity varies with growth temperature (Denich et al., 2003; Stintzi, 2003). Furthermore, to validate this hypothesis, it would be necessary to carry out measurements of membrane fluidity at lethal temperatures, since the complex composition of bacterial membranes makes it difficult to predict a membrane's degree of fluidity at a given temperature.

On the other hand, it has also been observed that variations in growth temperature induce proteomic changes. When bacterial cells are grown close to their maximum growth temperature, a number of so-called Heat-Shock Proteins (HSPs), including chaperones and proteases, are induced (Lim and Gross, 2010; Schumann, 2007). Various authors have hypothesized that these proteins might be responsible for the increased resistance of bacterial cells grown at higher temperatures, which could be attributed to their greater ability to repair damages caused by heat exposure at different cellular levels (Herendeen et al., 1979; Schumann, 2007; Smith et al., 1991). Moreover, it is known that several heat shock proteins also exert direct stabilizing effects on cellular structures including membranes (Coucheney et al., 2005; Török et al., 1997; Tsvetkova et al., 2002).

Staphylococcus aureus is a well-known foodborne pathogen (Baird-Parker, 2000). However, in spite of its frequent isolation as a causative agent of food poisoning in almost every part of the world, little is known about the influence of physiological factors on the susceptibility of this microorganism to heat treatments. In this study we attempt to describe and characterize the influence of growth temperature on heat resistance, along with the occurrence of repairable cellular injuries, in *S. aureus* cells in both exponential and stationary growth phases. Our ultimate aim was to examine the role that membrane fluidity plays in cell survival, and to gain fundamental knowledge about the physiology of bacterial inactivation by heat treatments.

2. Materials and methods

2.1. Bacterial culture and media

Staphylococcus aureus CECT 4459 was used in this investigation. The bacterial culture was maintained frozen at −80 °C in cryovials. Stationary-phase cultures were prepared by inoculating 10 mL of tryptone soya broth (Biolife, Milan, Italy) supplemented with 0.6% yeast extract (Biolife) (TSB-YE) with a loopful of growth from tryptone soy agar supplemented with 0.6% yeast extract (TSA-YE) (Biolife) and incubating the resulting culture for 12 h at 37 °C in a shaking incubator. 50 μL of this culture were inoculated into 50 mL of fresh TSB-YE at 42, 37, 30, 20 and 10 °C. For each culture temperature, samples were taken after different incubation times to construct growth curves and to determine the time needed to reach stationary growth phase. In order to obtain stationary phase cultures (containing approximately 6–8 × 10^8 cells/mL; data not shown), flasks were incubated for 18, 24, 24, 48 and 344 h (14 days) at 42, 37, 30, 20 and 10 °C, respectively. Exponential-phase cells were prepared by inoculating 50 μL of the stationary-phase culture into 50 mL of fresh TSB-YE and incubating at the different temperatures listed above until optical density at 600 nm reached 0.8, which corresponded to approximately 8 × 10^7 CFU/mL.

Cell suspensions were examined under a phase contrast microscope in order to evaluate the presence of cell aggregates (Nikon Eclipse E-400, Japan). It was determined that cell suspensions contained aggregates with a different number of cells which corresponded, on average, to 4.3 cells for exponential phase cell suspensions grown at 10–37 °C, to 2.0 cells for exponential phase cells grown at 42 °C and for stationary phase cells grown at 10–37 °C, and to 3.1 cells for stationary phase cells grown at 42 °C.

2.2. Heat treatments

Heat treatments were carried out in a specially designed resistometer (Condón et al., 1993). Briefly, this instrument consists in a 350-mL vessel provided with an electrical heater for thermostation, an agitation device to ensure inoculum distribution and temperature homogeneity, and ports for the injection of microbial suspension and for the extraction of samples. Once treatment temperature had attained stability (± 0.1 °C), 0.2 mL of an appropriately diluted cell suspension was injected into the main chamber containing the treatment media. After inoculation, samples were collected at different treatment times and immediately pour-plated (Condón et al., 1996). The treatment medium was McIlvaine citrate-phosphate buffer of pH 7.0 (Dawson et al., 1974).

In some experiments, benzyl alcohol (BA) (Sigma, St. Louis, USA) was added as a membrane fluidizer to the cell suspensions and to the treatment medium at concentrations ranging from 5 mM to 20 mM (Cebrián et al., 2016; Shigapova et al., 2005).

2.3. Incubation of heated samples and survival counting

The recovery medium was TSA-YE. After treatments, plates were incubated for 24 h at 37 °C and, after incubation, colony-forming units (CFU) were counted. It was checked that longer incubation times did not influence survival counts (data not shown).

To estimate the percentage of sublethally injured cells, a selective recovery medium consisting in the addition of NaCl to TSA-YE was used. The loss of tolerance to the presence of NaCl is attributed to damage to the functionality and/or integrity of the cytoplasmic membrane (Mackey, 2000). The sodium chloride concentration employed was chosen in previous experiments as the maximum non-inhibitory concentration (MNIC) for untreated cells, and corresponded to 13% w/v (Cebrián et al., 2014). It was ascertained that the MNIC was the same for all the cells regardless of growth temperature and phase. When cells were recovered in selective medium containing NaCl, incubation times of 48 h were required for macroscopic colony growth. It was also checked that longer incubation times did not influence survival counts in NaCl-added medium (data not shown).

2.4. Resistance parameters

Survival curves were obtained by plotting the logarithm of the fraction of survivors *versus* treatment time. Survival curves were fitted to a special case of the Baranyi and Roberts model (Baranyi and Roberts, 1994), which considers a survival curve as a mirror image of a growth curve (Baranyi et al., 1996). Since survival curves did not show tails, this model was defined by only two parameters: the maximum inactivation rate (μ, min^{-1}), and the shoulder length (*sl*, min), that corresponds with the duration of the shoulder phase in the survival curve. For practical reasons, the maximum inactivation rate parameter (μ) was transformed into D value ($D = 1/\mu$, decimal reduction time value, minutes).

Microsoft Office Excel 2002 (Microsoft Co., Redmond, Washington, U.S.A.) with the special DMFit package (http://www.ifr.ac.uk/safety/DMFit, IFR, Norwich, U.K.) was used to fit the data. Standard deviations, statistical significance of differences ($p < 0.05$) and Pearson's correlation coefficient were calculated using the GraphPad PRISM® statistical software package (Graphpad Software, Inc., San Diego, CA, USA).

All resistance determinations were performed at least three times on separate working days. Error bars in figures correspond to the mean standard deviation.

2.5. Measurement of fluorescence anisotropy

Fluorescence anisotropy of the probe DPH (1,6-diphenyl