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Relationship between membrane fatty acid composition and heat resistance of acid and cold stressed *Salmonella senftenberg* CECT 4384

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ABSTRACT

This study evaluates the adaptative response to heat (63 °C) and the modifications in membrane fatty acid composition of *Salmonella senftenberg* after its growth in an acidified medium and after its exposure to combinations of acid and cold stresses. Cells were grown in Brain Heart Infusion (BHI) buffered at pH 7.0 and acidified up to pH 4.5 (fresh cultures) and kept at refrigeration temperature (4 °C) for 7 days (refrigerated cultures). The results indicate that previous adaptation to a low pH increased the bacterial heat resistance, but combinations of sublethal stresses reduced *S. senftenberg* heat tolerance, specially when the growth medium pH was decreased. Acid-adapted cells showed D_{63}-values ranging from 3.10 to 6.27 min, while non-acid-adapted cells showed D_{63}-values of 1.07 min. As pH decreased, over the pH range studied (7.4–4.5), D_{63}-values of the resulting cells increased. However, refrigerated acid-adapted cells showed lower D_{63}-values, which ranged from 0.95 to 0.49 min. A linear relationship between the thermotolerance of *S. senftenberg* cells and the previous growth medium pH was found in both fresh and refrigerated cultures, which allowed us to predict changes in heat resistance of *S. senftenberg* that occur at any pH value within the range used in the present study in which most foodstuffs are included.
Both acidification of the growth medium and refrigeration storage of cells induced modifications in membrane fatty acid composition, which were clearly linked to their heat resistance. Acid-adapted cells, regardless of the pH value of the growth medium, showed the lowest UFA/SFA ratio and a CFA content 1.5–2-fold higher than that observed for non-acid-adapted cells. On the other hand, the UFA/SFA ratio found for *S. senftenberg* cells exposed to a cold stress was 1.2–1.8-fold higher than that observed for non-refrigerated cultures. This increase in the UFA/SFA ratio was specially high for acid-adapted cells. The highest thermotolerance was observed for cells with low UFA/SFA ratio, and, consequently, having a low membrane fluidity. However, changes observed in CFA content did not explain the great heat sensitivity of refrigerated acid-adapted cells.

1. Introduction

Salmonella is one of the most prevalent pathogens linked to outbreaks in foodborne disease (D'Aoust, 2000). Although *Salmonella enteritidis* and *Salmonella typhimurium* are the most frequent causative agents of human salmonellosis, other serovars, such as *Salmonella senftenberg*, have been recently implicated in foodborne gastroenteritis (L'Ecuyer et al., 1996; Kumar and Kumar, 2003). This fact is of great importance since *S. senftenberg* is a persistent contaminant in slaughterhouses (Sogaard and Nielsen, 1979; Liebana et al., 2001) and it has also been revealed as one of the predominant serovars isolated from marine environments and seafood (Martínez-Urtaza et al., 2004).

Heat treatment is one of the principal methods used in food industry to eliminate pathogen microorganisms from food products. For this reason, extensive investigation in this area focused on the effects of environmental factors on the bacterial heat resistance has been performed. As was shown, microorganisms show modifications in their heat resistance after exposure to certain environmental and preservation stresses, such as acid and cold stresses (Annous and Kozempel, 1998; Rowan and Anderson, 1998; Martínez et al., 2003; Álvarez-Ordóñez et al., 2008). In the particular case of *Salmonella* spp. there are several studies on the influence of acid stress on their heat resistance and there seems to exist an accordance with the fact that these microorganisms exhibit a "heat induced tolerance" after exposure to moderate acidic environments (Leyer and Johnson, 1993; Wilde et al., 2000; Mazzota, 2001; Bacon et al., 2003; Tosun and Gonul, 2003). The effect of cold stress has been less studied, although from data in literature for *S. typhimurium* and *S. enteritidis* it can be deduced that their exposure to low temperatures is accompanied by a decrease in

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the subsequent heat resistance (Humphrey, 1990; Mañas et al., 2003; Álvarez-Ordóñez et al., 2008). However, as far as we know little attention has been paid to the combined effects of both stresses on the bacterial thermal resistance in spite of its great practical interest in food preservation where multiple stresses are often used.

The mechanisms involved in the bacterial thermal tolerance are not fully understood and the majority of studies are focused on the role of stress proteins (heat shock proteins) and the regulation of gene expression in response to environmental changes (Foster and Spector, 1995; Foster, 2000; Dodd and Aldsworth, 2002). However, a link between the membrane fatty acid composition and the bacterial heat resistance has also been found (Annous et al., 1999; Sampathkumar et al., 2004; Álvarez-Ordóñez et al., 2008). In general, these authors have shown that cells with a decreased concentration of unsaturated fatty acids or with an increased content of saturated fatty acids have a decreased membrane fluidity, which is linked to a higher heat resistance. Furthermore, in a previous study performed in our laboratory we found that the formation of cyclic fatty acids plays an important role in protecting acid-adapted *S. typhimurium* cells from heat inactivation (Álvarez-Ordóñez et al., 2008).

To the best of our knowledge, there is no information available either on the behaviour against heat of acid and cold stressed cells or on the changes in membrane fatty acid composition under these stressful conditions for *S. senftenberg*, even though this microorganism not only represents a potential concern for food safety but also is frequently used as a biological indicator to assess the lethality of heat treatments with regard to *Salmonella* spp. because of its greater heat resistance. Thus, the aim of this study is to evaluate the subsequent thermal resistance of *S. senftenberg* following its exposure to combinations of acid and cold stresses. A further aim is to check the effect of acid adaptation and cold storage on *S. senftenberg* membrane fatty acid profile in an attempt to clarify the role of membrane composition and fluidity in its heat resistance.

2. Material and methods

2.1. Bacterial strain and culture conditions

The *Salmonella enterica* serovar Senftenberg strain CECT 4384 was obtained from Colección Española de Cultivos Tipo (CECT) (Spanish Type Culture Collection). Revitalized cultures were stored on Brain Heart Infusion Agar (BHIA; Oxoid) plates at 4 °C and then were activated by transferring an isolated colony from BHIA to Brain Heart Infusion (BHI; Oxoid) and incubated at 37 °C for 24 h to give a stock suspension of 10^9 cfu/mL. Non-acidified and acid-adapted cells were obtained by inoculating a portion of the activated culture into flasks containing 50 mL of BHI (pH 7.4) non-acidified and acidified at pH values of 6.4, 5.4 and 4.5 with hydrochloric acid (Panreac), respectively. In order to obtain non-acid-adapted cells, BHI was buffered at pH 7.0 using a Sorensen buffer 0.2 M (bisodium (Merck)–monopotassium (Panreac) phosphate). All cultures were then incubated at 37 °C until a late stationary phase of growth was reached (36 h) and were used to determine the thermotolerance and membrane fatty acid composition.

Cold adaptation consisted of maintaining cultures at 4 °C for 7 days. Preliminary studies confirmed that in all cases cell viability remained constant throughout the cold storage period.

2.2. Growth curves and calculation of growth parameters

Samples (1 mL) of the cultures obtained at different assayed conditions were decimally diluted in sterile 0.1% (w/v) peptone solution (Oxoid) and appropriate dilutions were plated in duplicate on BHIA. Plates were incubated at 37 °C for 48 h, and the number of colonies enumerated. Viable counts were converted to $\log_{10}$ values. Growth curves generated by fitting the data to the Gompertz equation (Buchanan et al., 1997) were used to calculate lag phase duration, exponential growth rate, generation time and maximum population density, as well as the time needed to reach the stationary phase.

2.3. Determinations of thermoresistance

Heat resistance experiments (63 °C) were performed on 0.2 mL aliquots of cell cultures heated in 350 mL of BHI in a thermoresistometer TR-SC (Condón et al., 1993). After different heating times, 0.2 mL samples were collected and immediately pour-plated in BHIA. Plates were then incubated at 37 °C for 48 h and survivors were counted by means of an automatic cfu counter (Protos Analytical Measuring Systems, Cambridge, UK) following the specifications described by Ibarz et al. (1991).

D-values (time in min for survival count to drop 1 log cycle) for three different cultures were calculated as the negative reciprocals of the slopes of the regression lines obtained by plotting the log number of survivors against time (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA). The statistical significance ($p \leq 0.05$) of the differences between the *D*-values was tested using the Student's *t* test as described by Steel and Torrie (1986) (Statistica for Windows version 4.5, Statsoft, Inc., Tulsa, OK, USA).

2.4. Fatty acid analysis

The membrane fatty acid composition was determined using a chromatographic method as described elsewhere (Álvarez-Ordóñez et al., 2008). Lipids were extracted with chloroform and methanol from 1 L of each bacterial culture obtained as described above, following the Bligh and Dyer (1959) method. Afterwards, the chloroform rich phase obtained containing the cellular lipids was removed and the solvent was evaporated. Lipids were methylated in an alkaline medium using $NaOCH_3$ (Merck). The resulting fatty acid methyl esters (FAME) were analyzed on a gas chromatographer (HP 6890, Hewlett Packard, Avondale, PA) equipped with a mass selective detector (HP 5973) using a capillary column (60 m, 0.25 mm, 0.20 μM). The FAME were identified by comparing their retention times to those corresponding to known standards (Supelco) and by comparing their mass spectra to the data bank (Hewlett Packard). The results obtained from a single experiment are expressed as relative percentages of each fatty acid.

3. Results

3.1. Influence of acid adaptation and cold storage on the heat resistance of S. senftenberg

In order to study the effects of acid and cold adaptation on the subsequent heat resistance of *S. senftenberg*, cells were grown at 37 °C in non-acidified BHI pH 7.4 (non-acidified cells), buffered BHI pH 7.0 (non-acid-adapted cells) and BHI acidified with hydrochloric acid at pH values of 6.4, 5.4 and 4.5 (acid-adapted cells). Fig. 1 shows an example of growth curves obtained in all conditions assayed. It is worth noting that *S. senftenberg* grew well at all pH values tested. The effects of pH on kinetic parameters were relatively small at pH values $\geq$ 5.4. The acidification of the growth medium at pH 4.5 doubled the generation time (0.57 h) and the time needed to reach the stationary phase (16 h), tripled the lag phase duration (6.61 h) and decreased the maximum population density about a half log cycle.