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Acid adaptation sensitizes *Salmonella enterica* serovar Typhimurium to osmotic and oxidative stresses

Säure-Anpassung sensibilisiert Salmonella enterica serovar Typhimurium für osmotischen und oxidativen Stress

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Summary

The influence of acid adaptation on the subsequent sensitivity of *S.* Typhimurium to osmotic and oxidative stresses was assessed using cells grown in buffered BHI (pH 7.0) and BHI acidified up to pH 4.5 with acetic, citric, lactic and hydrochloric acids and treated in (i) BHI-2.5 M NaCl and (ii) BHI-30 mM H_2O_2. The growth of *S.* Typhimurium in the presence of organic acids resulted in an increased vulnerability to the toxicity of salt and hydrogen peroxide, especially when acetic acid was used to obtain acid adapted cells. Non-acid adapted cells showed D-values of 493.45 and 31.85 min in BHI-2.5 M NaCl and BHI-30 mM H_2O_2, respectively, which turned out to be about 1.5-2 and 1.5-2.5 times higher than those observed for acid adapted cells. These findings may show an impact on food preservation regimes, as the synergistic effect of the combination of acid and osmotic and oxidative stresses could help in controlling pathogen contamination or survival in several food products, such as cheese and other fermented foods.

Keywords: *Salmonella* Typhimurium, acid adaptation, salt, oxidative stress, food safety

Zusammenfassung

Der Einfluss der Säure-Anpassung auf die Empfindlichkeit von *Salmonella* Typhimurium gegenüber osmotischem und oxidativem Stress wurde untersucht. Zu diesem Zweck wurden Zellen in gepuffertem BHI (pH 7,0) und angesäuertem BHI (bis pH 4,5 mit Essigsäure, Zitronensäure, Milchsäure und Salzsäure) angezüchtet und anschließend in (i) BHI-2,5 M NaCl und (ii) BHI-30 mM H_2O_2 überführt. Das Wachstum von S. Typhimurium in Gegenwart von organischen Säuren führte dabei zu einer erhöhten Anfälligkeit gegenüber der Wirkung von Salz und H_2O_2. Dies zeigte sich vor allem, wenn Essigsäure verwendet wurde, um Säure-angepasste Zellen zu erhalten. Nicht-angepasste Zellen zeigten D-Werte von 493,45 und 31,85 min in BHI-2,5 M NaCl und BHI-30 mM H_2O_2. Diese Werte lagen 1,5-2 und 1,5-2,5-mal höher als jene, die für Säure-angepasste Zellen beobachtet wurden. Diese Ergebnisse haben eine Bedeutung für die Entwicklung von Strategien zur Haltbarmachung von Lebensmitteln, da die synergistische Wirkung von Stressoren (Kombination von Säure und osmotischen und oxidativen Stress) genutzt werden kann, um Krankheitserreger in Lebensmitteln wie Käse und anderen fermentierten Lebensmittel zu kontrollieren.

Schlüsselwörter: *Salmonella* Typhimurium, Säure-Anpassung, osmotischer Stress, oxidativer Stress, Lebensmittelsicherheit

Introduction

Salmonella Typhimurium is a gram-negative pathogenic microorganism capable of withstanding a variety of hostile environmental conditions, including the many stresses encountered during the production, preparation and storage of food (Abee and Wouters, 1999; D'Aoust, 2000). One of the most known strategies followed by *S.* Typhimurium to overcome harsh conditions is the Acid Tolerance Response (ATR), which protects it against severe acid stress, increasing the risk of survival in the extreme conditions of the human gastrointestinal tract (Bacon et al., 2003a; Greenacre et al., 2003; Yuk and Schneider, 2006; Álvarez-Ordóñez et al., 2009a,b, 2010a,b). Furthermore, in addition to protect against extreme acid environments, the ATR also confers protection to other food-related stresses. Therefore, it is well known that acid adaptation of *S.* Typhimurium induces an increase in its subsequent heat resistance (Mazzota, 2001; Bacon, et al., 2003b; Tosun and Gonul, 2003; Álvarez-Ordóñez et al., 2008, 2009b,c). However, little effort has been made to study the influence of acid adaptation on S. Typhimurium resistance to other stressful conditions prevailing during food processing (e. g. osmotic and oxidative stress), information which would be very useful to improve food processing and preservation methods in order to control this pathogenic microorganism in foods. The influence of acid adaptation on *S.* Typhimurium resistance to osmotic and oxidative stress has been investigated on some occasions but with contradictory results. Thus, whereas Greenacre and Brocklehurst (2006) and Greenacre et al. (2006) have found that acid adaptation sensitizes *S.* Typhimurium to osmotic or oxidative damage, other authors (Foster and Hall, 1990; Leyer and Johnson, 1993; Lee et al., 1995; Kwon et al., 2000) have identified an ATR-linked tolerance to both adverse conditions.

Recent studies carried out in our laboratory established accurate conditions to induce an ATR in *S.* Typhimurium CECT 443 in response to several organic acids commonly used in the food industry (Álvarez-Ordóñez et al., 2009a), and showed that this adaptive response also confers protection to heat treatments (Álvarez-Ordóñez et al., 2008). The aim of this work was to study, under the same experimental conditions, the effect of acid adaptation on the resistance of *S.* Typhimurium CECT 443 to osmotic and oxidative stresses, using as challenge medium Brain Heart Infusion (BHI) supplemented with 2.5 M sodium chloride and 30 mM hydrogen peroxide, respectively.

Material and methods

Bacterial strain and culture conditions

Salmonella enterica serovar Typhimurium strain (CECT 443) used in this study was obtained from Colección Española de Cultivos Tipo (CECT) (Spanish Type Culture Collection). The lyophilized cultures were revived in BHI (Oxoid) and incubated for 24 hours at 37 ºC. Pure cultures were maintained on BHI agar plates at 4 ºC. Subcultures were prepared by transferring an isolated colony from a plate into a test tube containing 10 mL of sterile BHI followed by incubation at 37 ºC for 24 h. These fresh subcultures were used to produce acid adapted and non-acid adapted cells.

Flasks containing 50 mL of sterile BHI (pH 7.4) non-acidified and acidified at pH values of 6.4, 5.4 and 4.5 with acetic (Prolab), citric (Sigma), lactic (Merck), and hydrochloric acids (Panreac) were inoculated with the subculture to a final concentration of 10^3 cells/mL. So as to obtain non-acid adapted control samples, buffered BHI adjusted to pH 7.0 by addition of Sorensen buffer 0.2 M (bisodium monopotassium phosphate (Panreac)) was used. Afterwards, cultures obtained as described above were incubated at 37 ºC during the time needed to reach the late stationary-phase of growth. When acidified BHI was used as growth medium the inactivation experiments were carried out, for each acid, only at the highest and the lowest pH values which allowed for *S.* Typhimurium growth.

Osmotic and oxidative treatments

Aliquots of 5 mL of stationary-phase cells were harvested by centrifugation at 8000 G for 5 min at 4 ºC (Eppendorf centrifuge 5804R). The supernatant liquid was discarded and cells were resuspended in 50 mL of BHI supplemented with (i) 2.5 M NaCl or (ii) 30 mM H_2O_2. Then, after incubation at room temperature, survival was monitored periodically. Samples (0.1 mL) were collected after different treatment times, ten-fold serial dilutions were produced in sterile 0.1 % (w/v) peptone solution (Oxoid) and suitable dilutions were plated in duplicate on BHI agar. Viable cell densities at each point in time were enumerated following incubation of the plates at 37 ºC for 48 h (longer incubation times did not have any influence on the counting). Survivors were counted with a modified Image Analyser Automatic Counter (Protos Analytical Measuring Systems, Cambridge, UK) as described elsewhere (Ibarz et al., 1991). All experiments were performed in triplicate using three different fresh cultures.

Survivor curves and statistical analysis

D-values (min) were determined by plotting the log number of survivors against time for each culture. The line that best fits survivor plots was determined by linear regression (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA), and the negative reciprocal of the slope was used for the D-value.

D-values were compared using Student's *t*-test (Steel and Torrie, 1986) (Statistica for Windows v. 7.0 program, Statsoft Inc., Tulsa, Okla).

Results

The subsequent resistance of *S.* Typhimurium CECT 443 to osmotic and oxidative stresses was assessed using cells grown in non-acidified BHI (pH 7.4), buffered BHI (pH 7.0) and BHI acidified with acetic (pH 6.4), citric (pH 6.4 and 4.5), lactic (pH 6.4 and 5.4) and hydrochloric (pH 6.4 and 4.5) acids and challenged in BHI containing 2.5 M NaCl or 30 mM H_2O_2. In all cases, survival curves obtained fitted properly into a first order kinetic (R^2 ranging from 0.92 to 0.99; data not shown). An example of inactivation curves obtained under all challenge conditions for cells grown in buffered BHI and BHI acidified with the different acids is shown in Figure 1. D-values calculated are shown in Table 1, expressed as mean values of three independent experiments ± standard deviations.

Under all culture conditions tested *S.* Typhimurium resulted to be extremely resistant to salt, as shown by the fact that only 1–3 log reductions were achieved after a 12 hours treatment. D-values obtained for *S.* Typhimurium

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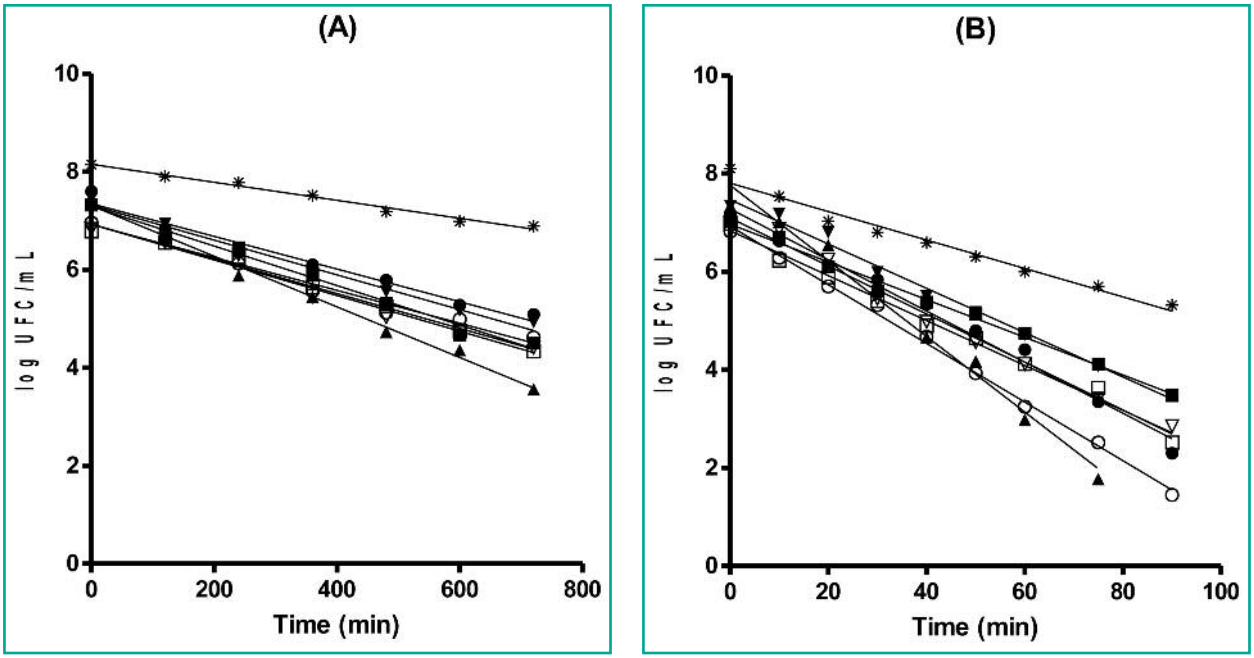

FIGURE 1: *Survival curves in BHI containing (A) 2.5 M NaCl or (B) 30 mM H_2O_2 for S. Typhimurium (CECT 443) cells grown in buffered BHI (*) and acidified BHI with acetic acid at pH 6.4 (▲), citric acid at pH 6.4 (▼) and 4.5 (▽), lactic acid at pH 6.4 (●) and 5.4 (○), and hydrochloric acid at pH 6.4 (■) and 4.5 (□).*

cells grown in buffered BHI (493.45 min) and non-acidified BHI (461.05 min) were significantly higher than those found for acid adapted cells, with D-values ranging from 245.20 to 330.25 min. It is important to note that, for acid adapted cells, neither the pH value of the culture medium nor the type of acidulant significantly modified the response found.

With regard to oxidative stress, cells grown in buffered BHI (D-value of 31.85 min) and non-acidified BHI (D-value of 28.55 min) also showed a significantly higher resistance to hydrogen peroxide than acid adapted cells, with D-values ranging from 12.10 to 20.90 min. In this case, although the pH value of the adaptation medium did not cause significant modifications in the oxidative resistance observed, the type of acid used showed a marked influence, with acid adapted cells grown in the presence of acetic acid being significantly more sensitive than acid adapted cells produced in the presence of the rest of acidulants.

TABLE 1: *D-values (min) for cells of S. Typhimurium (CECT 443) grown at 37 ºC in buffered BHI (non-acid adapted control cells), non-acidified BHI and acidified BHI at different pH values with several acids and treated in BHI containing 2.5 M NaCl or 30 mM H_2O_2*

Growth medium	2.5 M NaCl	30 mM H_2O_2
B-BHI	493.45 ± 70.07^{a}	31.85 ± 3.67^{a}
NA-BHI	461.05 ± 64.42^{a}	28.55 ± 3.70^{ad}
A-BHI$_{\text{acetic pH 6.4}}$	245.20 ± 36.49^{b}	12.10 ± 1.25^{c}
A-BHI$_{\text{citric pH 6.4}}$	311.65 ± 42.36^{b}	20.45 ± 2.36^{b}
A-BHI$_{\text{citric pH 4.5}}$	309.07 ± 32.14^{b}	20.06 ± 2.16^{b}
A-BHI$_{\text{lactic pH 6.4}}$	330.25 ± 42.92^{b}	17.12 ± 2.66^{b}
A-BHI$_{\text{lactic pH 5.4}}$	325.25 ± 38.96^{b}	16.10 ± 1.81^{b}
A-BHI$_{\text{HCl pH 6.4}}$	281.25 ± 47.02^{b}	20.90 ± 3.08^{bd}
A-BHI$_{\text{HCl pH 4.5}}$	300.85 ± 27.65^{b}	18.75 ± 3.40^{b}

B-BHI: Buffered BHI pH 7.0; NA-BHI: non-acidified BHI (pH 7.4); A-BHI: acidified BHI with different acids: acetic, citric, lactic and HCl at different pH values; $^{a-d}$: D-values (mean of three experiments ± SD) with different superscript in the same column are significantly different ($P < 0.05$)

Discussion

Although much is known about *S.* Typhimurium response to several environmental stresses, the significance of these responses for its survival in foods has received little attention. It is important to note that the physiological state of the test microorganism could influence its survival in foods and its resistance to food processing preservatives. Therefore, non-stressed cells, grown in rich media in the laboratory, probably do not accurately represent the physiology of cells found in the food environment (Leyer and Johnson, 1993). In this work we studied whether *S.* Typhimurium acid adaptation caused a cross-protection response against osmotic and oxidative stresses, commonly found in the food industry environment.

Sodium chloride is a component that regulates water activity of several foods, and survival of *S.* Typhimurium at very high salt content has been previously reported (Leyer and Johnson, 1993; Greenacre and Brocklehurst, 2006). In our case, only 1–3 log reductions were achieved after a 12 hours treatment in BHI-2.5 M NaCl, a concentration significantly above the concentration used in most food products. Acid adaptation did not protect against osmotic stress in the form of NaCl. In fact, the growth of *S.* Typhimurium in the presence of organic acids resulted in an increased vulnerability to the toxicity of NaCl. These findings may show an impact on food preservation regimes, as the synergistic effect of the combination of acid and salt could help in controlling pathogen contamination or survival in several food products, such as cheese and other fermented foods, where a number of stresses, including acidification, salt addition and decreased water activity

contribute to achieve the bacterial inactivation. Our results agree with those reported by Kwon et al. (2000), who found that a mixture of short-chain fatty acids was unable to provide cross-protection to 2.5 M NaCl. Also Greenacre and Brocklehurst (2006) showed for S. Typhimurium that acid adaptation in the presence of lactic acid rendered cells hypersensitive to NaCl. However, these latter authors showed that acid adaptation in the presence of acetic acid provided cells with protection against NaCl, and Leyer and Johnson (1993) have previously reported an increase in salt tolerance for *S.* Typhimurium cells previously exposed to hydrochloric acid. These apparent contradictory results available in literature could be due to the different methodological approach used to produce acid adapted cells or to the possible fact that cross-protection responses were a strain-dependent process, as previously shown by Faleiro et al. (2003). Thus, it is important to note that the previously mentioned studies not only used a different *S.* Typhimurium collection strain, but also achieved the acid adaptation by means of exposure of the bacterial cultures to a mild acidic environment for short time periods, whereas in our study cells were acid adapted by means of bacterial growth in media acidified with organic acids. The mechanism of bacterial response to osmotic stress is not fully understood but involves the accumulation of ions and osmoprotectant compounds in which uptake the OmpC and OmpF membrane porins are involved (Bremer and Krämer, 2000). The levels of these porins vary in response to different demands and stresses, including acid stress (Leyer and Johnson, 1993; Bremer and Krämer, 2000). Leyer and Johnson (1993) have previously reported for *S.* Typhimurium that under mildly acidic conditions the expression of OmpC was enhanced and OmpF was repressed, and, although OmpC seems to have a greater role in osmoprotectant permeation (Bremer and Krämer, 2000), the repression of OmpF could be responsible for the lower salt resistance observed for acid adapted cells in our study. In any case, it seems reasonable to suggest that both stresses act on the same cellular target, the bacterial membrane, as it has been previously reported a great influence of acid adaptation on the membrane composition and physical fluidity (Brown et al., 1997; Álvarez-Ordóñez et al., 2008).

Hydrogen peroxide is an antimicrobial agent frequently found by microorganisms in the food environment from different origin. This chemical compound is produced not only by starter bacteria added during the manufacturing of several fermented products but also during the metabolic burst that occurs in the phagolysosome. Our study shows that *S.* Typhimurium acid adaptation causes an increase in its sensitivity to hydrogen peroxide, a phenotype that could be exploited in preservation regimes. The type of acidulant used to produce acid adapted cells was an important factor influencing the response found, with the lowest oxidative resistance being observed when cells were grown in the presence of acetic acid, probably due to its greater ability to enter the cell. Our results agree with those previously reported by Greenacre et al. (2006), who described that acid adapted cells in the presence of lactic acid were vulnerable to oxidative damage and displayed a hypersensitive phenotype compared with unadapted cells. Similar results were obtained by Foster and Hall (1990), who reported that cross-protection between acid adaptation and hydrogen peroxide treatments was negligible for *S.* Typhimurium. On the contrary, several studies have identified an ATR-linked resistance to hydrogen peroxide for this microorganism (Lee et al., 1995; Kwon et al., 2000). In these latter studies the authors found that adaptation of *S.* Typhimurium with HCl (Lee et al., 1995) or mixtures of short-chain fatty acids (Kwon et al., 2000) induced resistance to hydrogen peroxide. The different methodology followed to produce acid adapted cells could be again responsible for the divergent results observed. It is important to bear in mind that hydrogen peroxide mode of action includes the formation of oxidative radicals which damage the DNA and the cytoplasmic proteins (Storz and Zheng, 2000). Once inside the cell, its action is partly counteracted by several defensive proteins, such as catalase, alkyl hydroperoxidase, glutathione reductase, and the DNA-binding protein Dps, most of them under *oxyR* regulation (Dukan and Touati, 1996; Storz and Zheng, 2000). Therefore, the lower resistance showed by acid adapted cells could be attributed to a lower amount of protective enzymes. Results obtained by Greenacre et al. (2006) support this statement, as these authors have reported that five members of the *oxyR* regulon were down-regulated during *S.* Typhimurium acid adaptation in the presence of lactic acid.

To sum up, we have shown that acid adaptation sensitizes *S.* Typhimurium to osmotic and oxidative stresses, finding which suggests that an effective preservation of

foods is likely if the preservation measures are based on the intelligent selection and combination of hurdles. Evidences provided in this study may prove useful to food manufacturers, as in certain fermented dairy and meat products, such as cheese and dry sausages, where *Salmonella* species represent a food safety concern, this pathogenic microorganism needs to overcome a set of hurdles including low pH, high sodium chloride concentrations and exposure to oxidative stress conditions, in order to finally cause the illness. An understanding of the mode of action of the antimicrobial compounds and technologies used is therefore critical for the design of suitable combinations of hurdles in the food industry. Further studies are needed in order to elucidate the targets of different preservative factors within the microbial cells and this should definitely help to improve the application of the hurdle technology.

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