Osmolyte protection of *Escherichia coli* and *Salmonella enterica* against inactivation by acetic acid

by

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Abstract

The combination of acid and osmolytes (salts, sugars) is a common application of ‘hurdle technology’ used to preserve many foods. Implicit in the hurdle approach is the assumption that as each hurdle is made ‘higher’, microbiological safety and shelf life will be improved. However, under certain conditions, osmolytes appear to protect *Escherichia coli* and *Salmonella enterica* against inimical acetic acid challenge. This thesis: 1) describes the apparent protection of *E. coli* and *S. enterica* by moderate levels of NaCl and sucrose against inactivation by acetic acid under non-growing conditions; 2) places those observations of protection in context with the published literature, international industry guidelines and Australian contemporary manufacturing processes for cold-filled sauces, dressings and mayonnaises; and 3) investigates possible mechanisms of this protection involving changes in the cell envelope.

The time to a 3-log$_{10}$ reduction of *E. coli* O157 strain SERL 2, in the presence of acetic acid, was observed to be non-monotonic, i.e. initially decreasing, but then increasing, in response to increasing NaCl concentrations but also to be dependent on pH / acid concentration. Further study demonstrated osmolyte protection against acetic acid inactivation to be conserved among different strains of acid resistant *E. coli* and *S. enterica*, and to be largely independent of osmolyte type (NaCl, sucrose). In addition, simple descriptive models were developed from data available in the published literature. From those models, an increase in time-to-3-log$_{10}$ reduction with increasing sucrose concentration was predicted for *E. coli*. The models also predicted an increase in the time-to-3-log$_{10}$ reduction with increasing NaCl for *S. enterica*. From the published data the concentration of NaCl accounted for little of the variability. pH and 1/ absolute temperature were the most important predictor variables, collectively accounting for at least 50% of the variability in the models. Conditions under which osmolyte protection of *E. coli* and *S. enterica* were observed fall within the recommendations of international industry guidelines (the “CIMSCEE Code”) for the manufacture of microbiologically-safe cold-filled acid sauces, dressings and mayonnaises. A survey of Australian manufacturers of cold-filled acid products confirmed that contemporary formulation practices included conditions for which osmolyte protection against acetic acid could be expected.
The antibacterial activity of acetic acid is usually explained by weak acid theory, involving diffusion of the undissociated moiety followed by dissociation and cytoplasm acidification, and acetate anion accumulation and toxicity. These proposed mechanisms are ultimately mediated at the level of the cell envelope and, in particular, the cytoplasmic membrane. The most obvious changes due to osmotic stress affect the structure and composition of the cell envelope, suggesting a possible mechanism of protection against acetic acid inactivation, i.e., that non-monotonic inactivation in response to increasing osmolarity in the presence of acetic acid arises from damage to, or changes in, the cell envelope. Improved survival at ‘intermediate’ (hypertonic) osmolarities could arise from: 1) one type of damage increasing with osmolarity above or below the optima, or 2) one type of damage increasing with increasing osmolarity and a second type of damage increasing with decreasing (hypotonic) osmolarity.

Studies of E. coli and S. enterica recovery in the presence of bile salts and crystal violet suggested that damage to the outer membrane in the presence of acetic, but not hydrochloric, acid is non-monotonic with increasing osmolarity. Flow cytometry and Three Dimensional Structured Illumination Microscopy (3D-SIM) were used to assess membrane changes in the total population of E. coli and S. enterica. Substantial outer membrane damage by acetic acid was confirmed in this manner using the fluorescent dyes, hexidium iodide and SYTO® 9, with the latter changing non-monotonically with increasing osmolarity. Outer membrane damage to E. coli and S. enterica by acetic acid has not previously been reported. Outer membrane damage to E. coli was also observed to be substantially slowed by storage at 5°C, correlating with improved survival.

Substantial loss of cytoplasmic membrane integrity, as assessed by flow cytometry with propidium iodide, was not observed. However 3D-SIM showed the development of distinct, brightly SYTO® 9- stained membrane domains in response to increasing exposure time, osmolarity and acidity, suggesting that changes in cytoplasmic membrane structure, if not integrity, did occur. Using 3D-SIM and staining with nonyl acridine orange (NAO), it was shown that the membrane domains were enriched with cardiolipin. Cardiolipin has previously been shown to be involved in the response of E. coli to osmotic stress, but production in response to acid, and acetic acid, stress.
has not previously been reported for *E. coli* or *S. enterica*. The proportion of cells exhibiting cardiolipin-enrichment increased with increasing exposure time, and responded non-monotonically to increasing osmolarity. The point of minimum cardiolipin-enrichment typically occurred at a lower osmolarity than that for cell inactivation and outer membrane damage, thus correlating minimum inactivation in the presence of acetic acid in response to osmolarity with some cardiolipin enrichment.

Cardiolipin enrichment has been previously shown to alter membrane permeability, membrane potential (Δψ) and aggregation of membrane proteins. In this study membrane fluidity, assessed using fluorescence polarisation of the probes DPH and TMA-DPH revealed a complex, dynamic, bimodal response to increasing osmolarity in the presence of acetic acid for both *E. coli* and *S. enterica*. Similarly, assessment of Δψ changes suggested that any advantage of cardiolipin production to cell survival in the presence of acetic acid is complex. Among *E. coli* and *S. enterica* populations exposed to inimical acid conditions for 72h, fewer of the cells enriched in cardiolipin were depolarised. However, at the shortest exposure time of 6h more cardiolipin-enriched than non-cardiolipin-enriched cells were depolarised at higher osmolarities, while more non-cardiolipin-enriched than cardiolipin-enriched cells were depolarised at lower osmolarities. Therefore, determination of the significance of cardiolipin enrichment in the presence of acetic acid on membrane function remains unclear at this point, and requires further investigation.

In conclusion, this study generated novel insights regarding cell envelope changes that accompany exposure to combined acetic acid and osmotic stress, and how these responses are modified by pH and temperature. *E. coli* and *S. enterica* are protected against inactivation by acetic acid by osmolytes, and this protection appears coincident with maintenance of outer membrane integrity and with some increase in the cardiolipin content of the cytoplasmic membrane. It remains a compelling hypothesis that such changes in the cell envelope could contribute to osmolyte protection of *E. coli* and *S. enterica* against acetic acid inactivation.
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