Thermal inactivation of *Listeria monocytogenes* studied by differential scanning calorimetry

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(Received 3 September 1990; revised 23 January 1991; accepted 25 February 1991)

The effect of NaCl on the thermal inactivation of *Listeria monocytogenes* has been investigated by conventional microbiological techniques and by using differential scanning calorimetry (DSC). Addition of 1.5 M-NaCl to cells grown at lower NaCl concentrations significantly increases the tolerance of cells to mild heat stress (56–62 °C). DSC thermograms show five main peaks which are shifted to higher temperatures in the presence of 1.5 M-NaCl. Measurement of loss of viability in the calorimeter gave good correlation between cell death and the first major thermogram peak at two NaCl concentrations. The time course of the loss of this first peak when cells were heated and held at 60 °C in the calorimeter matched the loss of viability, whereas the peak attributable to DNA showed little change during this process. The use of DSC to investigate the mechanisms involved in thermal inactivation is discussed.

Introduction

The thermal resistance of the food-borne pathogenic bacterium *Listeria monocytogenes* has been investigated in a variety of foods and model systems including milk (Bunning *et al.*, 1988), meat and vegetables (Conner *et al.*, 1986; Gaze *et al.*, 1989), and in relation to microwave-heated foods (Coote *et al.*, 1990). These studies indicate that environmental conditions during heating can have significant effects on thermal inactivation. A number of these conditions have been quantified in other bacteria, for example, the effect of reduced water activity in increasing the heat resistance of *Salmonella* spp. (McDonough & Hargrove, 1968; Goepfert *et al.*, 1970; Corry, 1974). In the majority of these studies the underlying molecular mechanisms have not been investigated.

The primary site of damage in the cell may vary with the severity of the stress. Hence, with mild heat, freezing or osmotic shock, membrane damage has been frequently observed and leakage of potassium and other cellular solutes correlates with loss of viability (Lambert & Hammond, 1976). At high temperatures, denaturation of proteins might be a major factor in cell death. Release of magnesium from the outer membrane of Gram-negative bacteria and from cell-wall teichoic acids of

Gram-positive bacteria, damage to ribosomes, and inactivation of specific enzymes or transport systems are other reported consequences of thermal or freeze injury (Hurst, 1984; Ray, 1984, 1986). Ray (1986) has also speculated that some repairable injury may involve conformational changes in macromolecules and that recovery involves reversal rather than *de novo* synthesis. Such events may be more difficult to investigate experimentally.

Ribosomal damage and degradation have been observed in several Gram-negative bacteria following thermal stress (Lee & Goepfert, 1975; McCoy & Ordal, 1979). In *Staphylococcus aureus*, sub-lethal heating causes both release of wall magnesium and destabilization of the ribosomes (Hurst & Hughes, 1978, 1981). Hurst (1984) has argued that ribosome damage is a result of magnesium depletion, as heating in a Tris/MgCl₂ buffer does not result in dissociation of the ribosomes into 30S and 50S particles, whereas heating in a magnesium-chelating buffer does. In both buffer systems membrane damage occurs and increased salt sensitivity of the surviving cells is similar (Hurst & Hughes, 1978). The primary sites of thermal damage in Gram-positive bacteria and consequences of these on other cellular functions require further study.

The technique of differential scanning calorimetry (DSC) has been used to compare the thermal denaturation curves (thermograms) of bacteria (Verrips & Kwast, 1977; Miles *et al.*, 1986) and to determine the base

Abbreviations: DSC, differential scanning calorimetry; TPB, tryptic phosphate broth.

composition of bacterial DNA (Mackey *et al.*, 1988). The instrument has also been used to accurately apply heat to bacteria and determine thermal death times (Grieme & Barbano, 1983). However, the two uses of the instrument have not been combined to compare thermograms concurrently with the reduction of bacterial numbers due to heat, nor has the technique been used to define the sequence of denaturation in whole cells that occurs during thermal inactivation of bacteria under different environmental conditions.

In the present study we have investigated the effects of NaCl on the thermal inactivation of *L. monocytogenes* and, using DSC, have attempted to correlate loss of viability with specific denaturation events within the cell.

Methods

Organisms and media. *Listeria monocytogenes* ATCC 19115 and *L. monocytogenes* Scott A were obtained from J. S. Crowther and H. Seliger, respectively. Cultures were grown at 30 °C overnight in tryptic phosphate broth (TPB) at pH 7.0 (Conner *et al.*, 1986) and maintained on slopes of tryptone soy agar (TSA) (Oxoid) at 4 °C.

Thermal inactivation studies. An overnight culture was resuspended in 10 ml TPB or TPB plus NaCl at a cell density of approximately 10^9 ml⁻¹. After 15 min incubation at 20 °C the suspension was heated in a submerged-coil heating apparatus (Cole and Jones, 1990). Samples (0.2 ml) were removed at predetermined intervals and rapidly cooled in 5 ml TPB. After 90 min resuscitation, to allow for recovery of heat-injured cells, survivors were counted following serial dilution in TPB and plating on TSA.

Differential scanning calorimetry (DSC). (i) Sample preparation. An overnight culture was centrifuged at 8000 g for 15 min and resuspended in 10 ml citrate/phosphate buffer (100 mM, pH 7.0) with or without NaCl. After incubation for 15 min at 20 °C, cells were repelleted and weighed samples (10–13 mg wet wt) were hermetically sealed in 20 µl aluminium DSC pans. Reference pans contained 10 µl of suspension buffer.

(ii) Calorimetry. Samples were heated in a Perkin Elmer DSC-7 instrument at 10 °C min⁻¹ from 0 to 110 °C. After heating, samples were rapidly cooled in the machine and selected samples were rescanned to investigate the reversibility of thermograms. The calorimeter was calibrated using a scan rate of 10 °C min⁻¹ with indium metal (99.99% pure) for both temperature and enthalpy calibration (m.p. = 156.6 °C, ΔH = 28.45 J g⁻¹). A second temperature standard, β -naphthyl ethyl ether (m.p. = 35 °C), was used as a check of temperature calibration at lower temperatures. The accuracy of temperature and enthalpy calibration was greater than ± 0.5 °C. In selected experiments, samples were heated at 10 °C min⁻¹ to 60 °C and held for various times before cooling to 20 °C at 20 °C min⁻¹. These were then rescanned at 10 °C min⁻¹ up to a temperature of 110 °C.

(iii) Measurement of viability. Samples prepared as above were heated using the calorimeter. Pans were removed at predetermined temperatures, cooled on ice, split using a sterile scalpel and vortex-mixed in 9 ml TPB for 15 s. After resuscitation for 90 min, survivors were counted as before.

Ribosome preparation. *L. monocytogenes* Scott A was grown with shaking at 30 °C in TPB for 16 h to give a cell density of approximately 10^9 ml⁻¹. The 16 h culture (2 l) was centrifuged at 5000 g (4 °C) for

20 min. The pellets were resuspended in 20 ml cold ribosome buffer [10 mM-Tris/HCl, pH 7.5, 6 mM-MgCl₂, 30 mM-NH₄Cl (Rheinberger *et al.*, 1988)] and stomached for 10 min in reinforced plastic bags (Colworth Stomacher, Stewart Labs) to remove flagella, which were then separated from the whole cells by centrifugation for 15 min at 5000g. After discarding the supernatant, the cells were resuspended in 20 ml cold (4 °C) ribosome buffer.

A French press (Aminco) was used for cell disruption. The piston/chamber was precooled to 0 °C and washed with cold ribosome buffer before being filled with the cold (4 °C) cell suspension. Cell breakage was achieved by passage of the cell suspension four times using a pressure of 20000 p.s.i. (1.38 MPa). The disrupted cell suspension was collected in a chilled glass vessel and centrifuged for 30 min at 25000 g (4 °C). The cell debris (pellet) was discarded and the supernatant was then centrifuged at 4 °C for 2 h at 100000 g in an Europa 65 M ultracentrifuge (MSE). The supernatant was discarded and the pellets containing the ribosomes stored at -20 °C for less than 28 d.

Results

Effect of NaCl on thermal inactivation

A high concentration of NaCl (1.6 M) significantly increased the heat resistance of *L. monocytogenes* at temperatures between 56 and 62 °C (Fig. 1). After 30 min at 56 °C in TPB (which contains 0.1 M-NaCl), the number of surviving cells had been reduced by a factor of greater than 10^4 (Fig. 1), whereas in the presence of NaCl (added to give a concentration of 1.6 M-NaCl in TPB), after the same time period, the number of viable cells had only been reduced by a factor of 10. A concentration of 0.6 M-NaCl in TPB afforded a much lower level of protection. These effects have also been observed at a range of pH values (4.4–7.0) (unpublished observations).

DSC thermograms in the presence and absence of NaCl

Both strains of *L. monocytogenes* examined exhibited very similar patterns of endothermic transitions when scanned in the calorimeter at 10 °C min⁻¹. The thermogram of *L. monocytogenes* ATCC 19115 between 10 and 110 °C in the absence of NaCl showed five distinct peaks (labelled A–E, Table 1). The first peak (A) was the largest, with a maximum at 68.6 °C. Peak D (92–93 °C) was the only peak to reappear following rapid cooling and reheating (Fig. 2). Conversely, when the sample was cooled slowly and reheated, this peak did not appear. In the presence of 0.5 M-NaCl, peak D moved from 93.1 to 96.3 °C, whereas the major peak (A) remained at approximately 68 °C. An increase to 1.5 M-NaCl caused further movement in peak D, this time to 103.3 °C. These results are consistent with peak D corresponding to DNA (Miles *et al.*, 1986). However, at the higher NaCl concentration a shift was also apparent in the major peak (A) by 6 °C to 74.3 °C. A general decrease in peak

Fig. 1. Effect of NaCl on the thermal inactivation of *L. monocytogenes* ATCC 19115. Cells were heated at (a) 56 °C, (b) 60 °C and (c) 62 °C in TPB, pH 7.0, containing 0.1 M- (▲), 0.6 M- (■) or 1.6 M- (◆) NaCl.

Table 1. Effect of NaCl on the thermograms of *L. monocytogenes*

Cells were grown either in TPB (0.1 M-NaCl) or in TPB with NaCl added to give 1.6 M-NaCl and resuspended in citrate/phosphate buffer, pH 7.0, containing 0, 0.5 M or 1.5 M-NaCl. Values represent the mean peak positions (°C) independently determined from two trials. The single peak obtained after a second scan is shown as D'.

Concn of NaCl (M)		Thermogram peak positions (°C)					
Growth	DSC	A	B	C	D	E	D'
0.1	0	68.6	75.3	81.5	93.1	105.3	92.0
0.1	0.5	68.4	—	—	96.3	—	95.6
0.1	1.5	74.3	—	—	103.3	—	102.7
1.6	0	65.4	76.7	84.5	94.5	—	92.2
1.6	1.5	71.2	—	—	91.4	—	—

Fig. 2. Thermogram of *L. monocytogenes* ATCC 19115. Samples (approx. 11 mg wet wt) were heated at 10 °C min⁻¹ from 0 °C to 110 °C (—). After heating the sample was cooled to its starting temperature and reheated as before (---). Letters indicate the major peaks as described in Table 1. Bar, 30 J kg⁻¹ °C⁻¹.

resolution was observed with the addition of NaCl (Fig. 3).

When *L. monocytogenes* was grown with added NaCl (to 1.6 M in TPB) and resuspended in NaCl-free buffer, the thermogram showed four peaks (A–D, Table 1). The major peak (A) had shifted to a lower temperature (65.4 °C) with little change in the position of the DNA peak (D). Resuspension in 1.5 M-NaCl buffer caused the major peak (A) to shift to 71.2 °C. This was 3 °C lower than the position observed with bacteria grown in TPB without additional NaCl and subjected to identical resuspension conditions. The DNA peak (D) also shifted by 3 °C to a lower temperature of 91.4 °C (Table 1).

Effect of NaCl on the viability of *L. monocytogenes* during heating in the calorimeter.

Viability of cells heated in the calorimeter in the absence of added NaCl showed a sudden and rapid decrease over the range 60 to 70 °C (Fig. 4a). This reduction coincided with the endothermic reaction which resulted in the major peak (A) of the DSC thermogram. No viable cells were detected at temperatures above 80 °C, which is lower than the DNA melting temperature (93 °C). Viability of cells heated in the calorimeter in the presence of 1.5 M-NaCl showed similar death kinetics to

Fig. 3. Effect of NaCl on the thermogram of *L. monocytogenes* ATCC 19115. Vegetative cells were resuspended in citrate/phosphate buffer, pH 7.0, containing no NaCl (A), 0.5 M- (B) or 1.5 M- (C) NaCl. Bar, 30 J kg⁻¹ °C⁻¹.

Fig. 4. Viability of *L. monocytogenes* ATCC 19115 in the presence of 0 and 1.5 M-NaCl. Cells were grown in TPB and resuspended in citrate/phosphate buffer, pH 7.0, containing (a) no NaCl or (b) 1.5 M-NaCl. The thermogram (—) was determined as in Fig. 2. Viability (---) was determined from cell suspensions heated in aluminium pans in the calorimeter. Bar, 30 J kg⁻¹ °C⁻¹.

those heated in the absence of salt. However, here the reduction in viable-cell numbers occurred over the range 70 to 80 °C. This increase in heat resistance corresponded to the 6 °C shift in the major peak (A) in response to heating in 1.5 M-NaCl (Fig. 4b).

Fig. 5. Effect of holding time at 60 °C on the thermogram of *L. monocytogenes* Scott A. Cells, in buffer without added NaCl, were heated in the calorimeter pans to 60 °C and held for 0 min (B), 2 min (C) and 5 min (D). These were then cooled to 20 °C at 20 °C min⁻¹ followed by reheating to 110 °C at 10 °C min⁻¹. These are compared to a control thermogram (A) for a cell suspension heated from 0 to 110 °C without any intermediate cooling and reheating. Bar, 30 J kg⁻¹ °C⁻¹.

Changes in thermogram peak height and position in response to holding at 60 °C

In view of the good correlation between loss of viability and peak A (Fig. 4), it seemed pertinent to examine the time course of peak loss during heating in the calorimeter. Heating cells in buffer without added NaCl to 60 °C, with no holding time, caused this peak to broaden and decrease in height from approximately 180 to 60 J kg⁻¹ °C⁻¹ (Fig. 5, A and B). Holding at 60 °C for 2 min resulted in a further reduction in major peak height to <30 J kg⁻¹ °C⁻¹ (Fig. 5, C) and holding at 60 °C for 5 min culminated in its disappearance (Fig. 5,

Fig. 6. Thermogram of a ribosomal fraction. A sample (10.8 mg wet wt) in Tris/HCl/MgCl₂/NH₄Cl buffer, pH 7.5, was heated at 10 °C min⁻¹ from 0 to 110 °C in the calorimeter. Bar, 30 J kg⁻¹ °C⁻¹.

D). Holding for 2 and 5 min at 60 °C would have reduced viability by about 90 and 99.5%, respectively (Fig. 1 *b*). The DNA melting endotherm (peak D) was largely unaffected by holding times up to 5 min at 60 °C and was visible on all thermograms.

Thermogram of a ribosome-enriched cytoplasmic fraction

A ribosome-containing cell fraction resuspended in Tris/HCl/MgCl₂/NH₄Cl buffer (Rheinberger *et al.*, 1988) gave a double peak between 68 and 82 °C when heated in the calorimeter at 10 °C min⁻¹. The position of the major peak is comparable to that of whole cells when resuspended in 1.5 M-NaCl.

Discussion

The effects of the environment in modulating the response of bacteria to stress have been frequently described in general terms, but the underlying molecular mechanisms are poorly understood. DSC allows whole cells to be studied whilst applying a heat stress. Thermograms of several bacterial species have been published (Verrips & Kwast, 1977; Miles *et al.*, 1986) and in general the scans of *L. monocytogenes* resemble other mesophilic bacteria with respect to the number and position of the major peaks.

DSC scans of whole cells show only a few peaks but these can be due to a larger number of transitions which are not resolved from each other. The peaks in the region of the thermogram at which loss of viability was observed are irreversible and most probably due to protein unfolding and denaturation. Contributions from other macromolecules cannot be ruled out but the irreversible component of DNA and RNA melting has

been considered to be only a minor fraction of total enthalpy (Lepock *et al.*, 1988). Peak area is a function of protein concentration and the specific calorimetric enthalpy of the protein (Privalov & Knechninashvili, 1974). Hence, large peaks in thermograms of whole cells will either be due to proteins present at high concentrations or, more probably, to combinations of several proteins with peaks at the same transition temperature. The disadvantage of being unable to resolve contributions from individual proteins is offset by the reproducibility of the method and the very significant gain of the ability to investigate changes in whole-cells concurrently with measuring viability.

The temperature tolerance of *L. monocytogenes* was increased by the addition of NaCl to cell suspensions and, at the same time, the major thermogram peaks were shifted to a higher temperature. A similar change has been observed in mammalian cells subjected to heat shocks where glycerol protects the cells from hyperthermia and causes an upward shift in both the temperature at which denaturation occurs and the major thermogram peaks (Lepock *et al.*, 1988). In *L. monocytogenes*, loss of viability of cells heated in the calorimeter was correlated with the first major peak which was at 68 °C in low-salt (0.1 M) and at 74 °C in high-salt (1.6 M) solutions. A shift of about 5 °C in the major DSC peak was observed in *Citrobacter freundii* when resuspended in 1.5 M-sucrose, but, in contrast to *L. monocytogenes*, not with salt (Verrips & Kwast, 1977). Addition of NaCl will, in the short term, cause water loss from the cells by osmosis and a consequent increase in internal solute concentration. Cells with dehydrated cytoplasm such as *Bacillus* endospores have well-known heat resistance but, in addition, thermal protection of a variety of cytoplasmic enzymes by NaCl or other ions has been shown for vegetative cells of *Bacillus coagulans* (Crabb *et al.*, 1975; Jones & Spencer, 1985) and ionic protection has been proposed as the thermal-tolerance mechanism employed by facultative thermophilic bacteria (Amelunxen & Murdock, 1978). When *L. monocytogenes* was grown in media containing high levels of NaCl, osmotic adaptation occurred and thermogram peak A was shifted from 68.6 °C to only 71.2 °C, compared with 74.3 °C when cells were first resuspended in high-salt solution (Table 1). Resuspension of cells grown in high-salt medium in NaCl-free buffer reduced the thermogram peak A transition temperature to 65.4 °C, which is 3 °C below that in 'normal cells'. These results indicate that the thermogram peaks respond rapidly and in a predictable way to the internal osmotic status of the cell. We would also suggest that the internal solute composition and concentration, and/or water content, are major factors in the thermal tolerance of *Listeria* and other mesophilic bacteria.

Heating bacteria at 60 °C in the calorimeter for different times indicated that the time course for denaturation of peak A closely followed the death kinetics. It will be of considerable interest, therefore, to try to identify the major cellular components which contribute to this first peak. Ribosomes, which contain approx. 38% protein (Noller & Nomura, 1987), comprise a major part of the dry weight of the bacterial cell. Since there is some evidence that mild heat stress can damage ribosomes directly (Hurst, 1984) or indirectly through magnesium loss (Hurst & Hughes, 1978), ribosomes have been proposed as possible contributors to the major DSC peak (Verris & Kwast, 1977; Miles *et al.*, 1986). A ribosomal fraction from *L. monocytogenes* has a DSC thermogram with a single or double peak between 64 and 70 °C. The exact position and shape of the peak is dependent upon the ionic composition and concentration of the suspension buffer used. Under ionic conditions which stabilize the ribosome (Rheinberger *et al.*, 1988), the peaks observed in the thermogram of the ribosome cell fraction (Fig. 6) correspond to the major peak in whole cells resuspended in high ionic strength medium and thermally most resistant (Fig. 4). Further work is in progress to elucidate the exact relationship between ionic environment, ribosomal stability and thermal resistance of *Listeria*.

The authors wish to thank Grahame Gould and Peter Wilding for their helpful discussions and support during the course of this work. Peter Newham is thanked for his invaluable technical assistance in the preparation of ribosomes.

References

- AMELUNXEN, R. E. & MURDOCK, A. L. (1978). Mechanisms of thermophily. *CRC Critical Reviews in Microbiology* **6**, 343–393.
- BUNNING, V. K., DONNELLY, C. W., PEELER, J. T., BRIGGS, E. H., BRADSHAW, J. G., CRAWFORD, R. G., BELIVEAU, C. M. & TIERNEY, J. T. (1988). Thermal inactivation of *Listeria monocytogenes* within bovine milk phagocytes. *Applied and Environmental Microbiology* **54**, 364–370.
- COLE, M. B. & JONES, M. V. (1990). A submerged-coil heating apparatus for investigating the thermal inactivation of bacteria. *Letters in Applied Microbiology* **11**, 233–235.
- CONNOR, D. E., BRACKETT, R. E. & BEUCHAT, L. R. (1986). Effect of temperature, NaCl and pH on growth of *Listeria monocytogenes* in cabbage juice. *Applied and Environmental Microbiology* **52**, 59–63.
- COOTE, P. J., HOLYOAK, C. D. & COLE, M. B. (1991). Thermal inactivation of *Listeria monocytogenes* during simulated microwave heating. *Journal of Applied Bacteriology* (in the Press).
- CORRY, J. E. L. (1974). Effects of sugars and polyols on the heat resistance of salmonellae. *Journal of Applied Bacteriology* **37**, 31–43.
- CRABB, J. W., MURDOCK, A. L. & AMELUNXEN, R. E. (1975). A proposed mechanism of thermophily in facultative thermophiles. *Biochemical and Biophysical Research Communications* **62**, 627–633.
- GAZE, J. E., BROWN, G. D., GASKELL, D. E. & BANKS, J. G. (1989). Heat resistance of *Listeria monocytogenes* in homogenates of chicken, beef steak and carrot. *Food Microbiology* **6**, 251–259.
- GOEPFERT, J. M., ISKANDER, I. K. & AMUNDSON, C. H. (1970). Relation of the heat resistance of Salmonellae to the a_w of the environment. *Applied Microbiology* **19**, 429–433.
- GRIEME, L. E. & BARBANO, D. M. (1983). Method for use of a differential scanning calorimeter for determination of bacterial thermal death times. *Journal of Food Protection* **46**, 797–801.
- HURST, A. (1984). Reversible heat damage. In *Repairable Lesions in Microorganisms*, pp. 303–318. Edited by A. Hurst & A. Nasim. London: Academic Press.
- HURST, A. & HUGHES, A. (1978). Stability of ribosomes of *Staphylococcus aureus* S6 sublethally heated in different buffers. *Journal of Bacteriology* **133**, 564–568.
- HURST, A. & HUGHES, A. (1981). Repair of salt tolerance and recovery of lost D-alanine and magnesium following sublethal heating of *Staphylococcus aureus* are independent events. *Canadian Journal of Microbiology* **27**, 627–632.
- JONES, M. V. & SPENCER, W. N. (1985). Thermostability of the enzymes of the tricarboxylic acid cycle of *Bacillus coagulans*. *Antonie van Leeuwenhoek* **51**, 193–201.
- LAMBERT, P. A. & HAMMOND, S. M. (1973). Potassium fluxes, first indication of membrane damage in microorganisms. *Biochemical and Biophysical Research Communications* **54**, 796–799.
- LEE, A. C. & GOEPFERT, J. M. (1975). Influence of selected solutes on thermally induced death and injury of *Salmonella typhimurium*. *Journal of Milk and Food Technology* **38**, 175–200.
- LEPOCK, J. R., FREY, H. E., RODAHL, M. & KRUV, J. (1988). Thermal analysis of CHL V79 cells using differential scanning calorimetry: implications for hyperthermic cell killing and heat shock response. *Journal of Cellular Physiology* **137**, 14–24.
- MACKEY, B. M., PARSONS, S. E., MILES, C. A. & OWEN, R. J. (1988). The relationship between base composition of bacterial DNA and its intracellular melting temperature as determined by differential scanning calorimetry. *Journal of General Microbiology* **134**, 1185–1195.
- MCCOY, D. R. & ORDAL, Z. J. (1979). Thermal stress of *Pseudomonas fluorescens* in complex media. *Applied and Environmental Microbiology* **37**, 443–448.
- MCDONOUGH, F. E. & HARGROVE, R. E. (1968). Heat resistance of Salmonella in dried milk. *Journal of Dairy Science* **51**, 1587–1591.
- MILES, C. A., MACKEY, B. M. & PARSONS, S. E. (1986). Differential scanning calorimetry of bacteria. *Journal of General Microbiology* **132**, 939–952.
- NOLLER, H. F. & NOMURA, M. (1987). Ribosomes. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, pp. 104–125. Edited by F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter & H. E. Umbarger. Washington, DC: American Society for Microbiology.
- PRIVALOV, P. L. & KNECHNINASHVILI, N. N. (1974). A thermodynamic approach to the stabilization of globular protein structure: a calorimetric study. *Journal of Molecular Biology* **86**, 665–654.
- RAY, B. (1984). Reversible freeze injury. In *Repairable Lesions in Microorganisms*, pp. 237–271. Edited by A. Hurst & A. Nasim. London: Academic Press.
- RAY, B. (1986). Impact of bacterial injury and repair in food microbiology: its past, present and future. *Journal of Food Protection* **49**, 651–655.
- RHEINBERGER, H., GEIGENMULLER, U., WEDDE, M. & NEIRHAUS, K. H. (1988). Parameters for preparation of *E. coli* ribosomes and ribosomal sub-units active in tRNA binding. *Methods in Enzymology* **164**, 658–662.
- VERRIS, C. T. & KWAST, R. H. (1977). Heat resistance of *Citrobacter freundii* in media with various water activities. *European Journal of Applied Microbiology* **4**, 225–231.