

Identification of sites of injury in *Lactobacillus bulgaricus* during heat stress

P. Teixeira, H. Castro, C. Mohácsi-Farkas¹ and R. Kirby

Escola Superior de Biotecnologia, Porto, Portugal, and ¹University of Horticulture and Food, Department of Microbiology and Biotechnology, Budapest, Hungary

P. TEIXEIRA, H. CASTRO, C. MOHÁCSI-FARKAS AND R. KIRBY. 1997. Heat resistance of *Lactobacillus bulgaricus* in skimmed milk at 62°, 64°, 65° and 66°C was studied. The response to increasing temperatures in this range was not linear, with temperatures at 65°C and above giving a lower survival rate than would be predicted from experiments at lower temperatures. To identify sites of injury at these temperatures, chemical markers were used. Heating at 64°C and below resulted in damage to the cytoplasmic membrane. At temperatures of 65°C and above chemical markers also indicated damage in the cell wall and proteins. Using differential scanning calorimetry analysis of whole cells of *Lact. bulgaricus* seven main peaks were observed (l-51, m₁-61, m₂-73, n-80, p-89, q-100, r-112°C). Three of these peaks (l_r, m_r and p_r) were the result of reversible reactions. Analysis of cell fractions identified the cell structure involved in giving rise to each of the three reversible peaks; l_r, cell membrane lipids, m_r, ribosomes, and p_r, DNA. The evidence presented in this paper shows that irreversible reactions in the cell ribosomes are a critical site of damage in *Lact. bulgaricus* during heat stress in liquid media at 65°C and above.

INTRODUCTION

Starter cultures of lactic acid bacteria prepared by spray drying cannot be used for direct inoculation of milk for dairy fermentations. This is largely due, not to a decrease in viability, but to an increase in the lag phase before the onset of growth. Injured cells have extended lag periods before they start to grow; this means that injured cells will take longer to start their desirable activities in food fermentations (Busta 1976).

Many authors have studied the phenomena of thermal inactivation of micro-organisms (Stiles and Witter 1965; Payne and Morley 1976; Singh and Ranganathan 1980; Craven and Blankenship 1983; Andrews 1986; Mackey and Derrick 1987). Membranes, nucleic acids and certain enzymes have been identified as cellular sites of heat injury and, in some cases, information is available on the molecular nature of heat damage (Tomlins and Ordal 1976; Gould 1989). However, despite this large body of information, we still do not understand precisely how micro-organisms are killed by

heat. Moats (1971) proposed that thermal death of bacteria can be explained by assuming that death results from inactivation of a small fraction of a large number of critical sites in the cells.

When bacteria are heated a reproducible sequence of endothermic or exothermic processes occurs. These perturbations can be observed by differential scanning calorimetry (DSC), which reveals a characteristic pattern of peaks that record specific denaturation processes as a time temperature sequence (Mackey *et al.* 1991). Ribosomes, which contain ≈ 38% protein 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 and Hughes 1978), damage to ribosomes has been proposed as a possible mechanism for loss of viability. Ribosomes have been identified as the major DSC peak occurring in the 50–80°C temperature range (Verrips and Kwast 1977; Miles *et al.* 1986; Mackey *et al.* 1991). Anderson *et al.* (1991) reported that measurement of loss of viability in the calorimeter gave good correlation between cell death and the first major thermogram peak; the peaks observed in the thermogram of the ribosome cell fraction correspond to the major peak in whole cells.

The purpose of the present work was to use classical methods for the identification of stress damage to determine the physiological reasons for the observed response following heat. DSC thermograms were analysed to try to correlate loss of viability in *Lactobacillus bulgaricus* with specific denaturation events within the cell.

MATERIALS AND METHODS

Organism and growth conditions

Lactobacillus delbrueckii ssp. *bulgaricus* NCFB 1489 was used. Cultures were maintained as in Teixeira *et al.* (1994). MRS broth (Lab M) was inoculated from MRS agar (Lab M) slopes and incubated for 24 h at 42°C. This broth was then used to inoculate a second MRS broth (1% v/v). The cultures were incubated at 42°C for 24 h in a shaken water bath. Cells were harvested by centrifugation at 10 000 *g* for 10 min at 4°C and washed with sterile phosphate buffer (0.01 mol l⁻¹ K₂HPO₄ (Merck) and 0.01 mol l⁻¹ KH₂PO₄ (Merck), both dissolved in a solution of 0.15 mol l⁻¹ NaCl (Merck), adjusted to pH 7 ± 0.1 and sterilized.

Standard heat challenge

For each test three 100 ml bottles with 50 ml of skim milk, agitated by magnetic stirrer bars, were used. Two bottles were placed in a water bath at the stress temperature and the other bottle was placed in a water bath at 42°C (control temperature). After temperature equilibration, the inoculum (1 ml of 24-h-old MRS broth-grown cells) was added to one bottle at the test temperature (the other bottle at test temperature was used to monitor the temperature) and to the bottle at the control temperature (acting as control and to give an initial count).

Survival determinations

At appropriate intervals, 1 ml samples were taken from the heating menstruum and added to 9 ml of phosphate buffer. Survivors were enumerated on MRS agar, and MRS agar supplemented with a variety of additives, viz. NaCl (6.25 mg ml⁻¹) or lysozyme (50 µg ml⁻¹) or penicillin (0.016 µg ml⁻¹) or chloramphenicol (0.313 µg ml⁻¹) or rifampicin (0.065 µg ml⁻¹) or actinomycin D (0.13 µg ml⁻¹) or pyronin Y (5 µg ml⁻¹) by the drop count technique (Miles and Misra 1938). Plates were examined after incubation at 37°C for 48 h. Each selective agent was used at the minimal inhibitory concentration determined previously. All chemical additives except NaCl were obtained from Sigma and added following filter sterilization.

pH evolution in milk

The lag period in terms of acid production was evaluated by following changes in the pH of milk inoculated with (i) dilution series of unheated control cells, and (ii) heat-treated cells. Changes in the pH were determined using standard pH electrodes (Ingold U402-S7) linked to a data acquisition and automated data logging system as previously described by Lievens (1991).

Differential scanning calorimetry

Whole cell preparation. Cells of *Lact. bulgaricus* were grown to stationary phase in 15 ml of MRS broth in a shaker water bath. Cultures were centrifuged at 5000 *g* for 15 min at ambient temperature.

Crude ribosome preparation. Stationary phase cells from 3 l of MRS broth culture were harvested by centrifugation at 5000 *g* (4°C) for 20 min and resuspended in 20 ml of cold ribosome buffer (10 mmol l⁻¹ Tris/HCl (Merck), pH 7.5, containing 6 mmol l⁻¹ MgCl₂ (Merck) and 30 mmol l⁻¹ NH₄Cl (Merck). The cell suspension was disrupted using a sonifier (Labsonic U) for 7 min at 75 W with an impulse of sonification and cooling periods of 0.3 s. Deoxyribonuclease (Sigma) was added (0.2 mg ml⁻¹) and the material centrifuged at 32 500 *g* for 30 min. The supernatant liquid (cell-free extract) was centrifuged at 150 000 *g* for 3.5 h to give a pellet of crude ribosome.

Lipid extraction. Stationary phase cells from 1 l of MRS broth culture were harvested by centrifugation at 10 000 *g* (4°C) and washed twice in 10 mmol l⁻¹ Tris/HCl, pH 7.5, containing 10 mmol l⁻¹ MgCl₂. The cell pellet was finally resuspended in 4 ml of buffer and placed in a stoppered tube with 10 ml of methanol and 5 ml of chloroform. The mixture was shaken vigorously for 5 min, kept on ice for 30 min and then 4 ml of chloroform was added followed by 4 ml of water. After mixing, the suspension was spun in a bench centrifuge to separate the layers. The lower chloroform layer was removed and the chloroform evaporated using a rotary evaporator (Büchi RE 121). The resulting lipid was redissolved in a small volume of chloroform and stored at -20°C.

Cell wall isolation. Stationary phase cells from 1 l of MRS broth culture were harvested by centrifugation at 5000 *g* for 15 min and washed in 20 ml of cold saline solution (0.9% NaCl in deionized water). The cell pellet was resuspended in 20 ml of cold deionized water and the cells were disintegrated using a sonifier (Labsonic U) for 10 min at 75 W with an impulse of sonification and cooling periods of 0.3 s. After disruption of the cells, the suspension was heated to 75°C

and kept at this temperature for 15 min to inactivate autolytic enzymes. Unbroken cells were removed by centrifugation at 1000 *g* for 10 min. The sediment was discarded and the supernatant fraction was centrifuged at 22 000 *g* for 15 min. A small opaque layer of unbroken cells under a translucent layer of cell walls was obtained. The upper layer was removed by gently washing the sediment with a stream of ice-cold 0.1 mol l⁻¹ Tris buffer, pH 7, delivered from a Pasteur pipette. The upper layer was totally suspended in 15 ml of buffer and centrifuged again at 1000 *g* for 10 min. The supernatant fraction was centrifuged at 22 000 *g* for 15 min. The upper layer was resuspended in Tris buffer and these steps were repeated until a homogeneous cell wall fraction, free from intact cells, was obtained.

Calorimetry. The DSC used was a DSC-50 (Shimadzu). Samples of 2–12 mg were weighed to ±0.01 mg, sealed in high pressure aluminium pans (Shimadzu 222-01701-91) and heated at 10°C min⁻¹ from 10 to 120°C, using an empty pan as the reference. Without adjustment of the DSC or disturbance of the pans, the samples were cooled rapidly (using liquid nitrogen) to the lower limit (10°C) then reheated as before. Samples showing signs of leakage were discarded. Data for the run and rerun were collected, processed and the calorimeter was controlled by a 'Shimadzu TA-50 Work Station'. Only those peaks that were consistently present in at least three consecutive scans were selected for analysis. Temperature and power scales were calibrated according to the manufacturer's instructions using the melting of indium as standard.

Statistical analysis

The hypothesis that the viable counts on MRS agar are different from the viable counts on MRS agar supplemented with each type of additive prior to heating (average values of three replicates) was tested against the null hypothesis using a classical *t*-test (Box *et al.* 1978).

In the case of viable counts following heating, the normality of the residuals of each set of three replicates with respect to their mean, and the independence of the estimated variances *vs* the corresponding mean were tested (graphs not shown). In all cases, significant bias was detected. Hence, transformation of the data from cfu ml⁻¹ to log (cfu ml⁻¹) was performed prior to development of further statistical analyses. The transformed data were analysed via the ANOVA methodology using the StatviewTM package (Abacus Concepts, Berkeley, CA).

RESULTS AND DISCUSSION

The pH decrease after heat stress was followed with time. Results show a lag phase before pH started to decrease. The

lag phase increased with the increase in the stress temperature but the subsequent shapes of the curves were practically identical (Fig. 1). The influence of the initial number of micro-organisms on the extent of the lag phase was studied (Fig. 2). Results support the idea that the lag phase is, at least in part, due to the low initial number of viable organisms. It is also possible that due to heating the bacterial cells become injured and no longer multiply and produce lactic acid until repair has been completed. This explanation is in accordance with the work of Hurst (1977) and Ray (1986).

A bacterial cell suspension after a sublethal treatment such as heating contains dead, injured and uninjured normal cells. Sublethal injury can be taken as an inability to proliferate or survive in media containing selective agents that have no

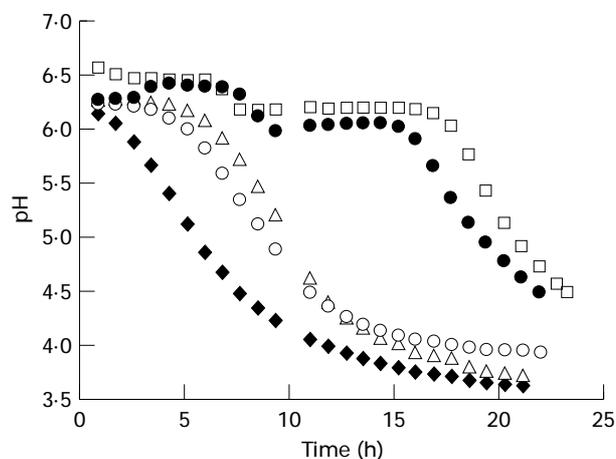


Fig. 1 pH evolution after exposure at stress temperature when recovery was attempted at 42°C. ◆, Unheated control cells; ○, stress temperature 62°C; △, stress temperature 64°C; ●, stress temperature 65°C; □, stress temperature 66°C

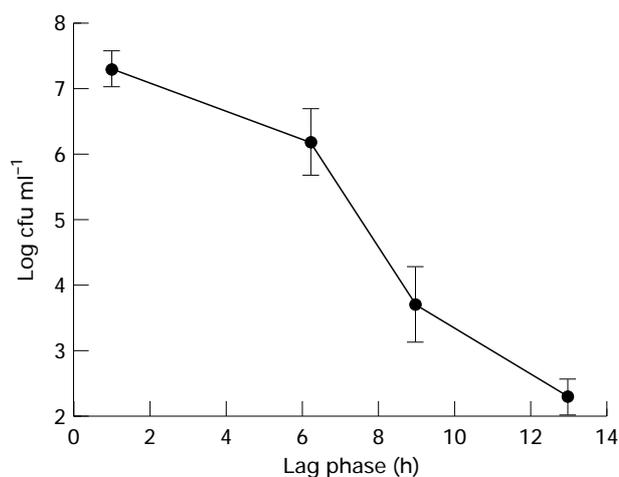


Fig. 2 Lag period before pH started decreasing as a function of the log of the number of micro-organisms inoculated in skim milk. Vertical bars represent overall standard errors of the mean

apparent inhibitory action upon unstressed cells. If the mechanism of action of these compounds is known, it is possible to identify sites of damage and mechanisms of repair by difference in counts in normal growth media and media with probes before and after stress (Kirby 1990).

Survivor curves for *Lact. bulgaricus* at 62°, 64°, 65° and 66°C show that an increase of 1°C in the heating temperature above 64°C produced a significant decline (*t*-test for paired data (Box *et al.* 1978); $P < 0.05$) in the number of survivors (Fig. 3). These results suggested that 65°C is a critical temperature for the survival of *Lact. bulgaricus* during heating in skim milk. In order to try to understand the reasons for the higher loss of viability at this temperature the effects of incorporation of chemical probes into the recovery media on the survival of cells heated at 64° and 65°C were compared.

Results from experiments on recovery of *Lact. bulgaricus* on media containing chemical probes for injury before and after heating at 64° and 65°C are shown in Tables 1 and 2, respectively. Cells not subjected to heating did not show significant sensitivity ($P < 0.05$) to any of the chemical agents tested. The differences observed following heating using the same growth media can therefore be safely attributed to damages effected by the heating process itself. Cells heated at 64°C developed sensitivity to NaCl indicating cell membrane damage (Strange and Cox 1976; Brennan *et al.* 1986; Teixeira *et al.* 1995). When heated at 65°C, *Lact. bulgaricus* become sensitive to penicillin indicating that the repair process involves peptidoglycan synthesis (Blumberg and Strominger 1974), NaCl indicating cell membrane damage (Strange and Cox 1976; Brennan *et al.* 1986; Teixeira *et al.* 1995) and chloramphenicol indicating that protein synthesis is necessary for repair (Franklin and Snow 1981).

During heating at 64°C the only structure which seems to

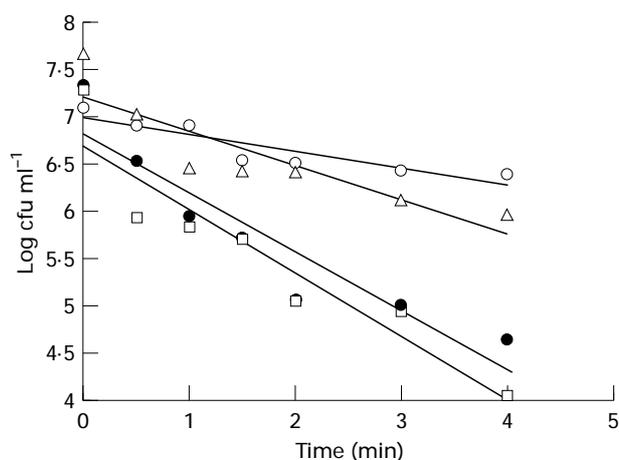


Fig. 3 Survivor curves of *Lactobacillus bulgaricus* at 62°C (○), 64°C (△), 65°C (●) and 66°C (□) as determined by plate counts in MRSA

Table 1 Experimental data for the effects of various additives on recovery of *Lactobacillus bulgaricus* heated in skim milk at 64°C

| Recovery media | Heating time (min) | | | | |
|----------------|-----------------------------|------|------|------|------|
| | 0 | 1 | 2 | 3 | 4 |
| | Log (cfu ml ⁻¹) | | | | |
| MRS | 7.61 | 6.73 | 6.62 | 6.54 | 6.34 |
| MRSP | 7.55 | 6.44 | 6.42 | 6.29 | 5.81 |
| MRSN* | 7.15 | 5.41 | 5.20 | 5.15 | 4.71 |
| MRSPI | 7.49 | 6.62 | 6.48 | 6.29 | 5.69 |
| MRSL | 7.53 | 6.69 | 6.41 | 6.13 | 6.24 |
| MRSA | 7.61 | 6.65 | 6.20 | 6.18 | 5.89 |
| MRSR | 7.60 | 6.73 | 6.51 | 6.28 | 6.22 |
| MRSC | 7.56 | 6.44 | 6.41 | 6.22 | 6.06 |

MRS, MRS agar; MRSC, MRS plus chloramphenicol (0.313 $\mu\text{g ml}^{-1}$); MRSR, MRS plus rifampicin (0.065 $\mu\text{g ml}^{-1}$); MRSA, MRS plus actinomycin D (0.13 $\mu\text{g ml}^{-1}$); MRSP, MRS plus penicillin (0.016 $\mu\text{g ml}^{-1}$); MRSL, MRS plus lysozyme (50 $\mu\text{g ml}^{-1}$); MRSPI, MRS plus pyronin Y (5 $\mu\text{g ml}^{-1}$); MRSN, MRS plus NaCl (6.25 mg ml^{-1}).

* Additives with a statistically significant effect ($P < 0.05$).

Table 2 Experimental data for the effects of various additives on recovery of *Lactobacillus bulgaricus* heated in skim milk at 65°C

| Recovery media | Heating time (min) | | | | |
|----------------|-----------------------------|------|------|------|------|
| | 0 | 1 | 2 | 3 | 4 |
| | Log (cfu ml ⁻¹) | | | | |
| MRS | 7.65 | 6.30 | 6.11 | 5.21 | 4.24 |
| MRSP* | 7.64 | 5.89 | 5.34 | 4.97 | 2.40 |
| MRSN* | 7.20 | 5.11 | 4.36 | 3.30 | 2.52 |
| MRSPI | 7.65 | 6.19 | 5.81 | 5.16 | 3.97 |
| MRSL | 7.49 | 6.20 | 5.81 | 5.21 | 3.88 |
| MRSA | 7.65 | 6.20 | 5.59 | 5.04 | 3.98 |
| MRSR | 7.58 | 6.29 | 5.96 | 5.21 | 4.16 |
| MRSC* | 7.60 | 5.91 | 5.04 | 4.08 | 3.90 |

MRS, MRS agar; MRSC, MRS plus chloramphenicol (0.313 $\mu\text{g ml}^{-1}$); MRSR, MRS plus rifampicin (0.065 $\mu\text{g ml}^{-1}$); MRSA, MRS plus actinomycin D (0.13 $\mu\text{g ml}^{-1}$); MRSP, MRS plus penicillin (0.016 $\mu\text{g ml}^{-1}$); MRSL, MRS plus lysozyme (50 $\mu\text{g ml}^{-1}$); MRSPI, MRS plus pyronin Y (5 $\mu\text{g ml}^{-1}$); MRSN, MRS plus NaCl (6.25 mg ml^{-1}).

* Additives with a statistically significant effect ($P < 0.05$).

be altered is the cytoplasmic membrane. Cell wall, cytoplasmic membrane and proteins, however, are probable target sites of damage due to heating at 65°C. In the light of these results it seems that different events occurring when cells are

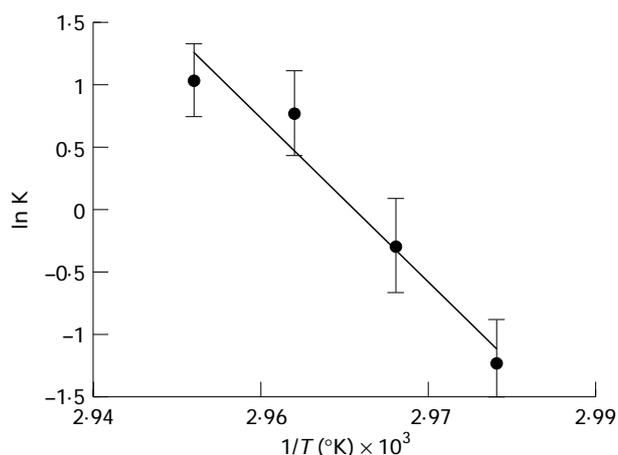


Fig. 4 Death rate constant (k) for *Lactobacillus bulgaricus* vs reciprocal heating temperature in skim milk. Vertical bars represent overall standard errors of the mean

heated at 64° or 65°C should be responsible for the different death rates observed during heating at each temperature. Great care, however, must be taken in interpreting the results obtained with the probe chloramphenicol. In healthy cells this antibiotic is toxic if present in high enough concentrations at the site of action. Results with penicillin and NaCl have already indicated the possibility that changes may occur in the cell wall and cytoplasmic membrane of heat-stressed *Lact. bulgaricus*, respectively. It is possible therefore that increased sensitization to chloramphenicol may be the result of increased penetration of the cell by the antibiotic (Kirby 1990). The fact that there are no differences in counts on the medium containing pyronin Y, actinomycin D and rifampicin may, however, mean that this is not due to a change in the permeability of the cell.

Figure 4 shows a plot of the death rate constant, k , against the reciprocal absolute heating temperature for *Lact. bulgaricus* in skim milk. The calculated activation energy (E_a) (452 kJ mol⁻¹) is within the same order of magnitude as for other micro-organisms (Elizondo and Labuza 1974). These activation energies are much greater than those for metabolic processes which are in the range of 40–80 kJ mol⁻¹ (Johnson

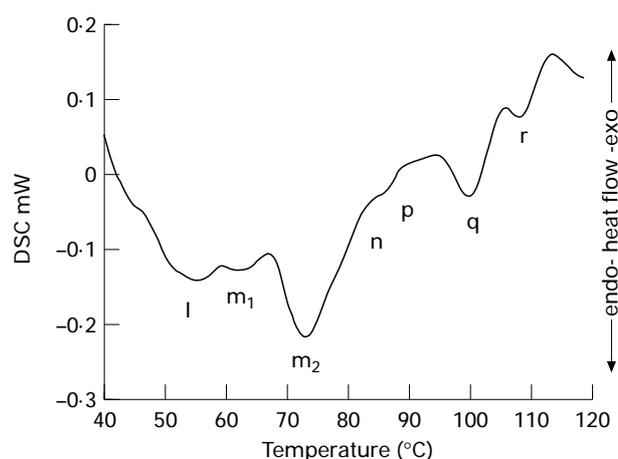


Fig. 5 Thermograms of whole cells of *Lactobacillus bulgaricus* obtained during the first heating. The letters indicate thermogram features described in the text

et al. 1974). Irreversible inactivations of most proteins have activation energies higher than 400 kJ mol⁻¹ (Joly 1965). Lepock *et al.* (1990) reported that this correlation would suggest that the rate-limiting step of cell killing may be protein denaturation. Thermodynamic quantities as determined by Daemen (1981) can be further used to elucidate the kinetics of death in terms of death mechanism. Changes in free enthalpy (ΔG^*), enthalpy (ΔH^*) and entropy (ΔS^*) were independent of the temperatures studied (Table 3). Rosenberg *et al.* (1971) concluded that virtually all proteins denature at elevated temperatures according to the compensation law of the following equation with the same value of T_c (compensation temperature) and b (constant):

$$\Delta S^* = \Delta H^*/T_c + b.$$

It was suggested that protein denaturation was the cause of thermal death in unicellular organisms since they had a specific death rate for thermal killing which followed a compensation law with constants $T_c = 329$ K and $b = -271.7$ J mol⁻¹ K⁻¹, which were in good agreement with the constants $T_c = 325$ K and $b = -276.3$ J mol⁻¹ K⁻¹ for thermal denaturation of proteins. Results presented in this work (Table 3)

Table 3 The thermodynamic parameters of death of *Lactobacillus bulgaricus* during heating in skim milk

| Temperature (K) | Reaction rate constant, k (s ⁻¹) | Changes in | | |
|-----------------|--|---|--|---|
| | | Free enthalpy, ΔG^* (KJ mol ⁻¹) | Enthalpy, ΔH^* (KJ mol ⁻¹) | Entropy, ΔS^* (KJ mol ⁻¹ K ⁻¹) |
| 335.15 | 4.5e-3 | 97.45 | 449.39 | 1.05 |
| 337.15 | 12.5e-3 | 95.21 | 449.37 | 1.05 |
| 338.15 | 35.8e-3 | 92.54 | 449.36 | 1.05 |
| 339.15 | 46.5e-3 | 92.37 | 449.34 | 1.05 |

Temperatures of peak maxima (°C)

| Sample | f | l | m ₁ | m ₂ | n | p | q | r | f _r | l _r | m _r | p _r |
|-------------|----|----|----------------|----------------|----|----|-----|-----|----------------|----------------|----------------|----------------|
| Whole cells | — | 51 | 61 | 73 | 80 | 89 | 100 | 112 | — | 44 | 70 | 90 |
| Ribosomes | — | — | — | 71 | 87 | — | — | — | — | — | 69 | — |
| Lipids | — | 50 | — | — | — | — | — | — | — | 45 | — | — |
| Cell wall | 35 | — | — | — | — | — | — | — | 32 | — | — | — |

Table 4 Temperatures of peak maxima in the thermograms of whole cells and fractions of whole cells of *Lactobacillus bulgaricus*

can also be represented by the same compensation law with a good correlation.

DSC thermograms were studied to try to identify the cellular structures responsible for loss of viability in *Lact. bulgaricus*. The thermal data are summarized in Table 4.

Whole cells exhibited a multip peaked thermogram, reproducibly containing the features identified in Fig. 5. On rescanning the sample after heating to 120°C and cooling to 10°C, three apparently reversible peaks were evident: l_r, m_r, and p_r (Fig. 6).

To try to identify the major peaks in whole cells, cellular subfractions were analysed. The same notation (as in previous publications (Miles *et al.* 1986; Mackey *et al.* 1991)) as in whole cells has been used throughout.

Thermograms of crude ribosomes (Fig. 7) showed two major peaks which resemble in shape and position peaks m₂ and n seen in whole cells. Peak m₁ identified in whole cells was not observed in ribosome samples. However, because peak m₂ is very large, it is possible that it corresponds to m₁ and m₂ which are not resolved. As in previous publications (Verrips and Kwast 1977; Anderson *et al.* 1991; Mackey *et al.* 1991), ribosomes are therefore proposed as possible contributors to the major peak observed in whole cells. On

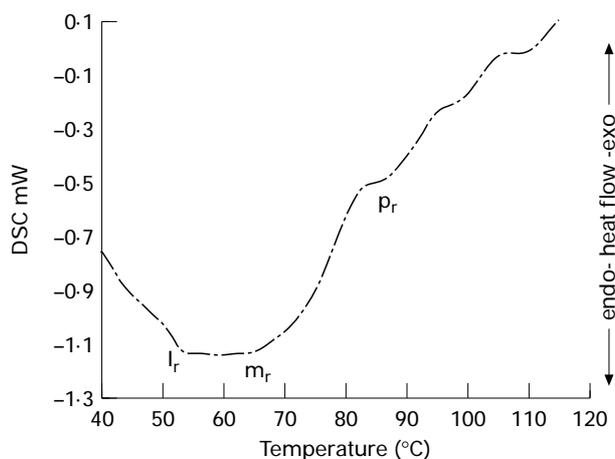


Fig. 6 Thermogram of whole cells of *Lactobacillus bulgaricus* obtained during the rescanning, after cooling. The letters indicate thermogram features described in the text

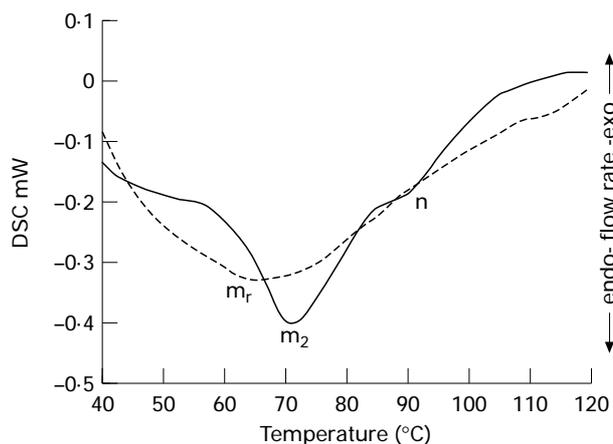


Fig. 7 Thermogram of crude ribosomes isolated from *Lactobacillus bulgaricus*. —, Initial run; ---, rerun. The letters indicate thermogram features described in the text

cooling and reheating the sample a small endotherm denoted m_r was seen, indicating that irreversible reactions occur in ribosomes due to heating. The other apparently reversible peaks, present in whole cells, were absent from ribosomes.

Extracted lipids were analysed and showed a peak at about 50°C (l) which yielded l_r on the rerun (Fig. 8). A similar endotherm was observed in whole cells both in the run and in the rerun. Previous investigators have shown that membrane lipids present the same behaviour (McElhanev 1982; Reizer *et al.* 1985). Because of its position and reversibility, transition l and l_r are identified as representing the melting and remelting of membrane lipids.

A small exothermic peak (f) was observed in cell wall preparations at about 35°C. On the rerun a broad exotherm was seen at 32°C (f_r) (Fig. 9). Mackey *et al.* (1991) examined a peptidoglycan preparation from *Escherichia coli* and found an exothermic peak in the same region which was associated with a change of state of this cellular structure.

Gasser and Mandel (1968) found that the melting temperature of DNA for *Lact. bulgaricus* was 91.7°C which is close to the value obtained for peak p (89°C). As reported for other organisms (Anderson *et al.* 1991; Mackey *et al.* 1991) peak p also yields p_r on the rerun. It is possible to conclude that peak p is associated with the 'melting' of DNA.

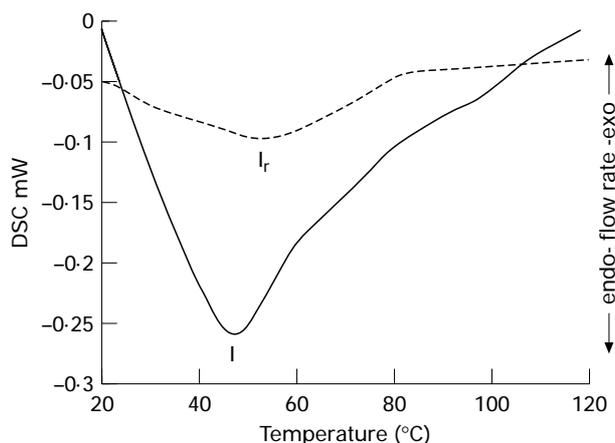


Fig. 8 Thermogram of crude lipids isolated from *Lactobacillus bulgaricus*. —, Initial run; ---, rerun. The letters indicate thermogram features described in the text

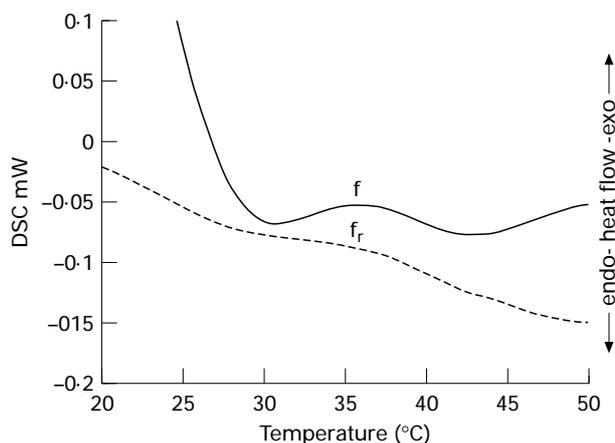


Fig. 9 Thermogram of cell wall isolated from *Lactobacillus bulgaricus*. —, Initial run; ---, rerun. The letters indicate thermogram features described in the text

Denaturation events occurring during heating can now be placed in a time/temperature sequence as follows: when *Lact. bulgaricus* cells are heated there is a broad endotherm exhibiting the maximum peak temperature around 50°C that is caused by melting of membrane lipids. The onset of this reversible reaction is, however, difficult to determine. In the same temperature range an exothermic reversible reaction occurs which is associated with a change of state of the cell wall peptidoglycan.

Ribosome melting occurs between about 60°C and 90°C and is characterized by peaks m_2 (73°C), n (80°C) and probably m_1 (61°C). This was also observed in *E. coli* by Mackey *et al.* (1991) who admitted that this process involved the sequential but overlapping denaturation of 30S, 50S and 70S particles, denaturation and/or loss of ribosomal proteins, loss of higher order structure and melting of helical regions of the

rRNA. At 90°C, peak p occurs resulting from the melting of DNA. Peak q was associated with the melting of regions of DNA (Mackey *et al.* 1991). Peak r might be associated with denaturation of thermostable envelope-associated proteins and possibly also to nucleic acids as described for *E. coli* (Mackey *et al.* 1991). Following cooling and reheating there is a broad endotherm extending from $\approx 50^\circ\text{C}$ to 85°C. This is probably caused by the reversible alterations of ribosomal RNA. At about 90°C in *Lact. bulgaricus*, peak p_r is caused by remelting of DNA. Peaks f , peptidoglycan, and l , lipids, also yielded an exothermic and endothermic process on reheating discernible only when analysing cellular subfractions.

Possible reasons for loss of viability occurring during heating can now be placed in a temperature sequence as follows: below 64°C critical sites are structures contained in or making up the cell membrane. For temperatures of 65°C and immediately above, ribosomes and/or proteins denaturation as well as cell wall damage may be responsible for thermal death. Ribosome denaturation occurs in the same temperature region as thermal inactivation. It was suggested that half the enthalpy of ribosome denaturation is associated with protein denaturation and/or disruption of higher order interactions (Mackey *et al.* 1991). Heat denaturation/melting of DNA may be responsible for death at temperatures near 90°C and above.

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