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Original article

SPECTROFLUORIMETRIC STUDY OF THE MEMBRANE PERMEABILITY DISTURBANCE IN *LISTERIA MONOCYTOGENES* AT HYPERTHERMIC TEMPERATURES

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Summary

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Thermal resistance of pathogenic bacteria, determined by the colony forming ability after thermal stress, is important for the microbial inactivation by heat. It is related to the ion permeability barrier disturbance at a high temperature (T_g). The mechanism of this membrane disturbance is not clear. Our goal was to study this disturbance in the plasma membrane of *L. monocytogenes*. After cultivation the bacteria were washed in a low-salt medium and heated to T_g. Hyperthermia induced out leakage of cytosolic electrolytes with sigmoid temperature profile, centered at T_g, as detected by conductometry (10 kHz). The T_g temperature correlated the thermal resistance of bacteria, acclimated at various temperatures of growth. To this end, we used fluorescent probes Pyrene and N-(3-Pyrenyl) maleimide (PyM). While Pyrene intercalates in the lipid region of plasma membranes PyM binds covalently the SH-groups of membrane proteins. The excimerisation of Pyrene reporting on the microfluidity of lipid bilayer did not change at T_g, while excimerisation of PyM raised indicating immobilisation and association of integral proteins. Overall, the ion permeability rise at T_g could be ascribed to changes in the tertiary and quaternary structure of integral proteins.

Key words: heat killing, ion leakage, microbial pathogen, overcritical fluidisation

INTRODUCTION

The intrinsic resistance against thermal inactivation of pathogenic bacteria is most frequently described in terms of the *D* value (decimal reduction time in minutes required for the heat destruction of one log of bacteria population through expo-

sure to specified temperature) and in the *Z* value (the change in temperature necessary to cause a tenfold change in the *D* value).

Besides the intrinsic thermal resistance, the thermotolerance and ability for temperature acclimatisation of pathogenic bacteria are also important factors which should be taken into account in modelling and quantifying microbial inactivation during thermal processing of foods. Therefore, a more mechanistic approach is needed for more accurate modelling of thermal inactivation (Sergelidis & Abrahim, 2009).

The permeability barrier function of cells is a vital requisite and the ability to maintain it emphasises resistance to deleterious conditions including heat stress. Generally, the membranes of cells undergo an ethanol facilitated permeabilisation at a specific high temperature, T_g, which in some cases has been related to the thermal resistance of cells (Wu & Walner, 1984; Ivanov & Boytcheva, 1994). The mechanism leading to disturbance of the permeability barrier at T_g, however, is not well understood. Based on above considerations our goal was to study the permeability barrier disturbance at T_g in the plasma membrane of *L. monocytogenes* using proper fluorescent probes.

L. monocytogenes bacteria strain is associated with illness in humans (Ward *et al.*, 2004) and farm animals (Hitchins, 1998). Cytoplasmic membrane of the cell envelope of *L. monocytogenes* (Volk, 1992) contains a lipid bilayer with intercalated integral proteins.

The thermal resistance of bacteria was determined by the classical colony forming test of heat challenged bacteria. The thermal stability of plasma membranes was assessed by the temperature, T_g, for half leakage of ions, determined by conductometry. The thermostability of integral proteins was expressed by the peak temperature of excimerisation of the fluorescent dye Pyrene maleimide (PyM) which binds covalently to integral proteins.

MATERIALS AND METHODS

Test microorganisms

L. monocytogenes was obtained from the National Reference Center of Food Safety at the National Diagnostic and Research Veterinary Institute, Sofia, Bulgaria. Prior to growth, stock cultures of bacteria were maintained in liquid nutrient medium with glycerol, placed in criovials under liquid nitrogen (–196 °C). The bacteria were considered old when kept at – 196 °C for more than 5 years. The following strains were used: N4 – *Listeria monocytogenes* 60 (an isolate of sausage); N5 – *Listeria monocytogenes* (MB IMCC 8632) – reference strain; N6 – *Listeria innocua* 27.

Preparation of bacteria cells

The stock suspension of indicated bacterial strain was prepared inoculating bacteria in Caso broth TSAYE (Merck, cat No. 1.05459.500) at the optimal temperature of 30 °C. The maximal cell concentration was obtained after 18–20 h growth as determined by spectrophotometry at 600 nm.

Temperature acclimatisation of bacteria

Fifty μL of the obtained stock bacterial suspension were inoculated into 5 mL of the same Caso broth TSAYE. The bacteria were allowed to grow at the indicated growth temperature, controlled by a thermostat (Dry thermostatic cell chamber TLSO 80, ETNA producer, Bulgaria). To determine the cellular resistance to heat, only cells harvested from cultures in the

early stationary phase were used. The stationary phase was attained after 14 days growth at 2.5 °C, 7 days growth at 6 °C, 18–20 hours growth at 30 °C, and so on.

Hyperthermic killing of cells and determination of survival fraction

Thermal resistance of acclimated bacteria was determined against a stress temperature (54 °C), which was significantly higher than the maximum temperature of growth (43 °C) for these bacterial strains. Ten mL of cell culture were placed in water bath at 54 °C. During the heating (the warm up time was excluded), aliquots of 1 mL were taken at the indicated time intervals and duplicate probes of 0.1 mL were plated on agar, supplemented with Caso broth TSAYE, to determine the fraction of cells (in CFU/mL) remaining intact. After 48 h growth at 30 °C, the colonies formed were counted and the survival fraction determined (Konings *et al.*, 1984). Survivor curves were obtained by plotting mean counts (log N) from duplicate plate samples against treatment time at 54 °C.

Test of the thermal stability of plasma membranes

The thermal stability test was conducted as per Ivanov & Boytcheva (1994) and Ivanov *et al.* (2010). Test bacteria were harvested in Caso broth TSAYE at the indicated growth temperature, isolated and once washed in 0.5 mL medium of 3 mM NaCl and 70 mM sucrose. The washed cells were resuspended in 0.1 mL of the same medium to impose strong transmembrane gradient of ion concentration. The latter suspension, with cytocrit about 0.05, was placed in a thin glass tube (ϕ 3 mm and L 100 mm) that contained two electrodes of thin platinum wire distanced at 10 mm under alternating voltage of 100 mV, 10 kHz. The glass tube was tightly incorporated within an aluminum block whose temperature was measured by a thermocouple. The aluminum block and suspension were heated at a constant heating rate (3 °C/min) from 20 to 85 °C. The hyperthermia induced out leakage of cellular electrolytes with sigmoid temperature profile with midpoint temperature, T_g. The ion leakage was detected by the increase in low frequency (10 kHz) conductivity, K_s, of suspension.

During the heating, the suspension conductivity, K_s, was continuously measured by a Solarton 1260 impedance/gain-phase Analyser (Farnborough, UK) and the output signal of the impedance meter was transmitted into a personal computer. Similar to the conductivity of each electrolyte solution, K_s exhibits a Boltzmann type of dependence on temperature (t, °C). In a narrow temperature interval, Δt, this dependence is reduced to closely linear one: $K_s = K_{so} (1 + \alpha\Delta t)$, where K_{so} is the value of K_s at the initial temperature and α is the temperature coefficient of K_s. It was not informative to this study and was circumvented using the temperature-derivative of K_s that could be expressed as $dK_s/dt = K_{so}.\alpha$. During the heating, only α is variable sensing, at this frequency (10 kHz), the changes in the volume of cells and conductivity of outside medium. The volume of cells remains constant, hence, the extracellular concentration of ions appears as the major determinant of K_s and α, sensing the out leakage of ions. At a steady-rate heating, the temperature profile (thermogram) of dK_s/dt appeared as a horizontal line, unless α changed causing a sharp peak around the inducing temperature, T_g (Ivanov *et al.,* 2010). The reproducibility of determining T_g in repeated experiments was within ± 0.3 °C.

Fluorescence measurements

For fluorescence monitoring of the molecular dynamics of bacterial plasma membrane during heating, we used two UV-excitable fluorescent probes, Pyrene and N-(3-Pyrenyl) maleimide (PyM), both forming excimers (short for exited dimer). The fluorescent dye PyM (Fluka, Switzerland) was freshly prepared as 0.9 mg/mL stock solution in DMSO (Merck, Germany). Prior to the addition of bacteria 5 µl of this solution was added to 3 mL deaerated (100 °C, 5 min) suspension medium containing 20 mM Tris buffer, pH 7.0. The final concentrations of DMSO and PyM were 0.17 % (v/v) and 5 µM, respectively. Fluorescence was excited at 336 nm and the intensities, I, of the emission peaks at 376 nm and 416 nm were measured. The first peak of spectra with intensity I_m, corresponds to the emission of monomer, while the second peak with intensity I_e, depicts the emission of excimer. In a separate experiment Pyrene (Merck, Germany) was dissolved in ethanol to the stock concentration of 1.5 mM. Prior to the addition of bacteria 5 µL of the latter solution was added to the same suspension medium to a final concentration of 3 µM Pyrene. Fluorescence was excited at 323.5 nm and the intensities of the emission peaks at 373 nm, I_m, for the monomer and 473 nm, I_e, for the excimer components of spectra, respectively, were measured. The excimerisation coefficient of each dye, i.e., the number of excimers to number of monomers ratio was estimated by the ratio of I_e/I_m.

The source bacterial suspensions were diluted 5 times in 20 mM Tris-HCl buffer, pH 7. Aliquots of 5 µL of this suspension were added to 3 mL of the dye containing buffer and the final concentration of 0.67 mg protein/mL (about 0.02 mg/mL plasma membrane protein) was obtained as measured by spectrophotometry with Beckman DU-50 (Germany) UV-VIS spectrometer. Spectra of the dyes were scanned between 300 and 500 nm at different temperatures increased by steps (3 ºC, 10 min) from 20 to 95 ºC. Data for the obtained spectra were collected by DAQ USB-6008 device (National Instruments Corp., USA) and processed with Origin software (Origin Lab Corp. USA).

The fluorescence measurements were carried out in a heated quartz fluorescence cuvette. The core instrument was a spectrofluorimeter Jobin Yvon, model JY3 D, equipped with magnetic stirrer and cuvette holder, heated by a U10 water-flow thermostat (Medingen, Germany). The temperature of the measuring cuvette was measured by Cu-constantan Thermocouple Meter, Omega, Newport Electronics, Inc. Model IDT-1 (Deckenpfronn, Germany), resolution 0.1 ^oC. Fluorescence spectra were corrected by the blank fluorescence in the absence of fluorescent label. Due to the strong temperature variations in the intensity of membrane fluorescence, different apparatus magnifications were used and calibrated to the reference fluorescence intensity of the PyM label, dissolved in DMSO and kept at room temperature.

RESULTS

Fig. 1 demonstrates that the survivor curves of *L. monocytogenes* submitted to heat stress (54 °C) depended on the temperature of growth within the entire temperature interval of growth. Cells grown at the temperature extremes (6 °C and 40 °C) exhibited maximum resistance to consequent heat stress while cells acclimated to moderate and optimum temperatures (30 °C) were far more sensitive to heat challenge.

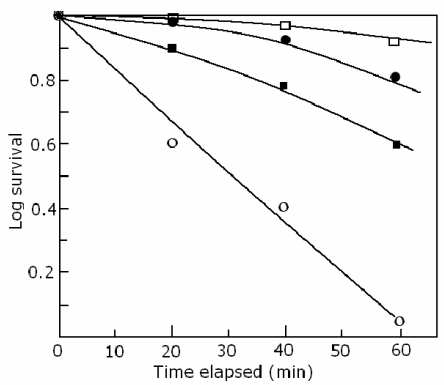

Fig. 1. Survivor curves of *L. monocytogenes* depending on the growth temperature. The cells were acclimated at the indicated temperatures, exposed to 54 °C for the specified time intervals and the survival fractions were counted. The cells of strain N4 were acclimated at 30 °C (○) and 6 °C (■), and the cells of strain N 5 (●) and N 6 (□) were acclimated at 40 °C.

Fig. 2 shows the particular changes in the temperature derivative of suspension conductivity, dK_s/dt, encountered in this study during the heating of *L. monocytogenes*, strain N4 suspension. Similar thermograms were obtained with the other strains, N5 and N6 (not shown). Two sharp, irreversible peaks within the interval of hyperthermic temperatures indicated thermally induced ion leakage reminiscent for specific disturbance of permeability barrier and structural alteration in plasma membranes. The top temperature of these peaks, T_{g1} and T_{g2}, are considered representative of the ability of plasma membranes to retain their cytosolic ions at hyperthermia, i.e., thermal stability of plasma membranes.

Fig. 3 shows the temperature profile of the excimerisation of PyM bound to the plasma membranes of *L. monocytogenes*, strain N4. With increasing the temperature and thermal motion of molecules the excimerisation of PyM, covalently bound to the integral proteins of membranes, smoothly decreased as expected. The excimerisation of PyM sharply increased at the T_{g1} and T_{g2} temperatures indicating a fall in intramolecular mobility and convergence of integral proteins. By contrast,

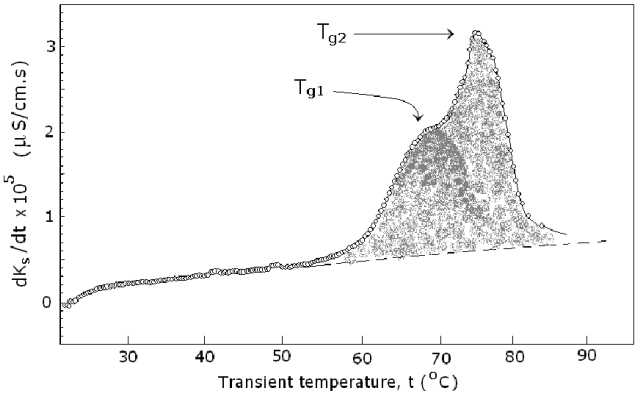

Fig. 2. Temperature profile of the leakage of cytosolic ions from *L. monocytogenes*, strain N4, during heating. The bacteria were harvested at 30 °C, isolated, washed once in a low-salt medium of 3 mM NaCl/50 mM sucrose, and resuspended in the same medium, cytocrit 0.05. The suspension was heated at 3.0 °C/min and the rate of ion loss was detected by the changes in the temperature derivative of suspension conductivity, dK_s/dt, assayed at 10 kHz. T_{g1} and T_{g2} are the peak temperatures for maximum ion loss.

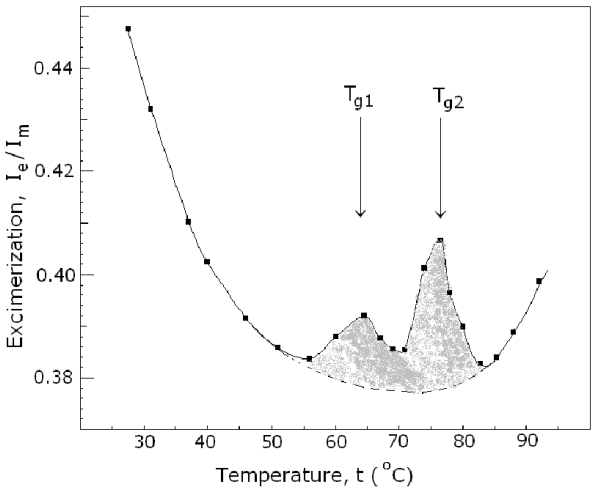

Fig. 3. Temperature dependence of the excimerisation (I_e/I_m) of PyM, covalently bound to the integral proteins of *L. monocytogenes*, strain N4. The PyM excimerisation, i.e., the number of excimers to monomers ratio was calculated as ratio of the emission peak intensities at 416 nm and 376 nm, respectively.

the excimerisation of pyrene did not change about the T_{g1} and T_{g2} temperatures (not shown). These results indicate that not the lipids but the integral proteins sustained change in their conformation leading to their association and immobilisation during the membrane disturbance and related ion leakage at the T_g temperature.

DISCUSSION

In this study the temperature acclimatisation of *L. monocytogenes* was ascertained testing the intrinsic thermal resistance of bacteria, cultivated in the whole temperature interval of growth. The same dependence on the growth temperature was previously reported for the midpoint temperature of ion leakage, T_g, the so called thermal stability parameter of plasma membrane (Ivanov *et al.,* 2010). The T_g had higher values with bacteria cultivated at the low and high temperature extremes of the growth temperature interval and considerably (~8 °C) lower values with bacteria grown at the center of this interval. Overall, recent and previous results altogether indicate that the T_g temperature could be a reliable parameter in predicting the intrinsic thermal resistance of bacteria, as dependent on their growth temperature.

The relevance of the T_g, i.e., the ion leakage peak, in predicting the intrinsic resistance of *L. monocytogenes* to heat was now supported by another finding. In recent experiments we frequently encountered the case when the temperature profile of suspension conductivity derivative exhibited two T_g peaks (Fig. 2), instead of a single one as previously reported (Ivanov *et al.*, 2010).

According to this result two different populations of the same bacterial strain were present whose plasma membranes had different thermal stability. This adaptive response of *L. monocytogenes* to heat could be responsible for the frequent occurrence of deviations (tails and shoulders) during heat treatments that were are observed in the exponential model of mi-

crobial inactivation (Sergelidis & Abrahim, 2009). These deviations from log-linear kinetic are interpreted in terms that in addition to the *D* and *Z* values the prediction of food safety needs another parameter (Sergelidis & Abrahim, 2009), for example the shape of the ion leakage, or T_g, peak(s).

The thermally induced structural alteration in bacterial plasma membranes about the T_g temperature was further investigated with the excimer-forming, fluorescence probes, Pyrene and PyM. Excimers form and are apparent in the fluorescence spectrum of probes when two Pyrene moieties come in close proximity stack on each other. Due to their high hydrophobicity these probes intercalate into the plasma membranes. The first probe resides in the lipid bilayer reporting the changes in its viscosity. At the chosen pH 7.0 the PyM probe binds covalently the SH-groups of integral proteins monitoring changes in the dynamics, tertiary and quaternary structure of the latter. PyM exists as monomer when bound to a spatially isolated SH-group of a membrane protein. When two or more PyM-marked SH-groups come within a 4-10 Å proximity distance to each other their PyM monomers form an excimer, i.e., a combination of an excited singlet dye molecule with another unexcited one. Emission of the PyM excimers, bound to the integral proteins of bacterial plasma membranes was used to assess the intramolecular dynamics and spatial convergence of these proteins (Chiti, 2010).

Similar convergence and immobilisation of integral proteins, without denaturation of their secondary structure, have been recently established in the plasma membrane of human erythrocytes about their T_g temperature (Ivanov *et al.*, 2011).

In conclusion, the presented data provide structural information about the thermally induced alteration in the plasma membrane of *L. monocytogenes* about the T_g temperature, relevant for the heat killing of bacteria. The ion permeability rise at T_g accompanies and possibly could be ascribed to the thermally induced changes in the tertiary and quaternary structure of integral proteins.

REFERENCES

Chiti, F., 2010. Investigation of the structure of toxic protein oligomers associated with protein deposition diseases. Bioscience 2010 Forschungszentrum Jülich. Invited Talks. Page 5.

Hitchins, A. D., 1998. *Listeria monocytogenes*. In: *Food and Drug Administration Bacteriological Analytical Manual*, 8th edn (revision A), ed R. L. Merker, *AOAC International*, Gaithersburg, MD, pp. 1001–1013.

Ivanov, I. T. & S. Boytcheva, 1994. Relationship between the hyperthermia induced killing of *Streptococcus faecalis* bacteria cells and their permeability barrier disturbance event at high temperature. *Journal of Thermal Biology*, **19**, 199–206.

Ivanov, I. T., T. Stoyanchev & H. Daskalov, 2010. Temperature acclimatization of *Listeria monocytogenes* according to the changes in their plasmatic membrane stability. *Trakia Journal of Sciences*, **8**, Suppl. 2, 25–31.

Ivanov, I. T., A. Zheleva & I. Zlatanov, 2011. Anion exchanger and the resistance against thermal haemolysis. *Internaltional Journal of Hyperthermia*, **27**, 286–296.

Konings, A. W. T., J. J. Gripp & M. B. Yatvin, 1984. Radio- and thermosensitivity of *E. coli* K1060 after thiol depletion by diethyl maleate. *Radiation and Environment Biophysics*, **23**, 245–253.

Sergelidis, D. & A. Abrahim, 2009. Adaptive response of *Listeria monocytogenes* to

heat and its impact on food safety. *Food Control*, **20**, 1–10.

Volk, W. A., 1992. Basic Microbiology, 7th edn, Harper Collins, New York.

Ward, T. J., L. Gorski, M. K. Borucki, R. E. Mandrell, J. Hutchins & K. Pupedis, 2004. Intraspecific phylogeny and lineage group identification based on the prfA virulence gene cluster of *Listeria monocytogenes*. *Journal of Bacteriology*, **186**, 4994–5002.

Wu, M. T. & S. Y. Walner, 1984. Heat stress responses in cultured plant cells. Heat tolerance induced by heat shock versus elevated growing temperature. *Plant Physiology*, **75**, 778–780.

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