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Monitoring the live to dead transition of bacteria during thermal stress by a multi-method approach

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ABSTRACT

Rapid microbiological methods to assess cell physiological properties of microorganisms are gaining interest in the elucidation of the effect of antimicrobial agents or physical inactivation. Fluorescent probes combined with flow cytometry or microplate assays provide information about cellular targets of chemical or physical stressors and help to clarify the underlying mode of action. In this work we exemplarily monitored the bacterial response of *Listeria innocua*, *Staphylococcus aureus*, *Salmonella enterica* and *Escherichia coli* to a mild thermal treatment by applying various methods to illustrate bacterial vital functions like the redox activity, membrane potential, esterase activity, efflux activity, glucose uptake, membrane integrity and plate counts. It was observed that some cellular properties are affected earlier than others. Respiration, glucose-uptake and pump activity were the most sensitive parameters, followed by the loss of membrane potential and membrane integrity. Unspecific esterase was found to be relatively resistant to mild heat exposure. This study shows that such a multi-method approach is a suitable tool to monitor the impact of inactivation treatments on bacteria, providing information about the mode of action, the heterogeneity of populations, species-specific differences to stressors and valuable insight in vital functions beyond pure culturability.

1. Introduction

Thermal treatments have been applied for microbial inactivation for a long time in various areas, especially for food preservation. Heat is generally assumed to affect several cellular components like proteins, membranes, RNA and DNA. Bioactive molecules like enzymes, as well as structurally important proteins and elements such as phospholipids which determine membrane integrity, a crucial requirement for functioning homeostasis and energy generation, are typical targets. Conclusively, a multi-target process is responsible for inactivation. The heat-shock response with associated heat shock proteins (HSP) and various inducible genes is a cellular attempt to cope with thermal stress (Wesche et al., 2009). Heat sensitivity generally varies in dependence of specific adaption capabilities and structural properties of microorganisms (Russell, 2003). The success of a decontamination processes is usually determined by classic microbiological culture based plate counting methods. This is a robust, easy and cheap approach, accounting the reproducibility of cells as the only criterion for viability. This is certainly sufficient for treatments targeting complete sterilization. However damaged or stressed, non-reproducing cells, which may arise from gentle or insufficient treatments, remain undetected and no information about the cells functional or structural breakdown as well as the heterogeneity of bacterial populations may be attained. Furthermore, colony counting implies the sole occurrence of single cells, cell clusters, initially present or formed during stress exposure, are not considered. Culture independent, rapid microbiological methods, on the other hand, are a powerful tool to reveal changes in the vitality of bacterial cells. Especially flow cytometric investigations coupled with fluorescent probes represent a fast way to determine the physiological state of microorganisms and give insight into microbial vitality beyond culturability. Novel decontamination processes like the application of high hydrostatic pressure, pulsed light, or gas plasma as well as novel antimicrobials e.g. plant extracts are currently gaining interest in the field of food microbiology. The elucidation of their impact on microbial structures and physiology is a key factor regarding their implementation. Therefore knowledge about cellular targets and microbial responses is required in order to detect differences among microorganisms, the occurrence of sublethally damaged or stressed cells or resistance development. The impact of several physical or chemical sterilization processes on microbial cells has been studied by flow cytometry (Berney et al., 2006; Cronin and Wilkinson, 2008, Paparella et al., 2012; Kennedy et al., 2011; Wang et al., 2010) but often only fluorescent probes to detect esterase activity and membrane integrity are used (Paparella et al., 2008; Ananta and Knorr, 2009; Khan et al., 2010). Few studies have applied a comprehensive approach including various rapid microbiological methods to characterize bacterial inactivation and to our knowledge, there is no comprehensive report on the shift in bacterial functional and structural properties during mild heat stress to date. Therefore, the objective of this study was to exemplarily assess changes in vitality

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of different food relevant bacteria during the exposure to mild heat (50–64 °C) by flow cytometry in combination with fluorescent stains. The bacterial viability was furthermore assessed by measuring the current respiration activity based on a microplate assay as well as by traditional plate counting. In this way it is possible to monitor population dynamics, their heterogeneity and cellular targets during exposure to stressors. This work presents a multi method approach to monitor the shift of bacteria from live to dead under thermal treatment and intends to critically emphasize the potential and methodical drawbacks regarding the assessment of bacterial physiology. The presented set of culture independent methods may be a suitable instrument to elucidate the mechanisms of inactivation produced by novel decontamination processes or new antimicrobials.

2. Material and methods

2.1. Bacterial strains, growth conditions and sample preparation

Listeria innocua DSM 20649, which is often used as a non-pathogenic surrogate for *Listeria monocytogenes*, was used as a model organism in this study, as well as *Escherichia coli* DSM 498, *Staphylococcus aureus* DSM 346 and *Salmonella enterica* ATCC BAA-1045. All test strains were obtained from the Leibniz Institute DSMZ — German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), except for *S. enterica* ATCC BAA-1045, which was obtained from the American Type Culture Collection (ATCC, Mansassas, Virginia, US). Bacteria were initially grown in 100 ml tryptic soy broth (Oxoid, Hampshire, UK) at 37 °C for 16–18 h in a shaking bath. Tryptic soy agar (Oxoid, Hampshire, UK) was subsequently inoculated with the bacterial suspension using an inoculating loop. The stock cultures were incubated for 24 h at 37 °C and stored at 5 °C. Working cultures were made by inoculating 100 ml of tryptic soy broth with cell material from the agar surface and following incubation for 16–18 h at 37 °C in a shaking bath until early stationary phase. 25 ml of the cell culture were subsequently centrifuged at 9000 g for 10 min and washed twice with phosphate buffer solution.

2.2. Heat treatment conditions

Heat exposure of bacteria was performed in closed 2 ml containers. 1 ml of undiluted bacterial suspension was transferred into each container and subsequently heated in a Thermomix comfort (Eppedorf, Hamburg, Germany). The temperature was recorded with a Thermologger 309 (Voltcraft, Wollerau, Switzerland). After 1, 2, 3.5, 5, 7, and 10 min, three samples were removed, immediately cooled down on ice and subsequently analyzed according to the methods described below. All trials were performed at least twice on different days with three independent parallel samples within each experiment ($n \geq 6$).

2.3. Determination of colony forming units (cfu)

The culturability of the heat treated bacteria was determined by measuring the number of colony forming units of each sample. 50 µl of appropriate dilutions in ringer solution (Oxoid, Hampshire, UK) or undiluted sample suspension were spread onto tryptic soy agar with an Eddy-Jet spiral plater (IUL instruments, Königswinter, Germany). The plates were subsequently incubated for 48 h at 37 °C and the number of colony forming units per sample determined with the Countermat Flash and Grow (IUL instruments, Königswinter, Germany).

2.4. Determination of metabolic activity

The current metabolic activity of all test bacteria after the heat treatment was determined with the Microbial Viability Assay kit (Dojindo Molecular Technologies, Kumamoto, Japan) according to a method reported by Kramer and Muranyi (2014). This kit contains the tetrazolium salt WST-8 and the electron mediator 2-methyl-1.4-naphthoquinone. WST-8 is cleaved to a water soluble formazan dye by active dehydrogenases, indicating metabolic activity of microbial cells. This reaction leads to the formation of a yellow color at which the intensity is proportional to the number of viable cells (Tsukatani et al., 2008). 100 µl of the sample suspensions were transferred to a 96-well microplate and mixed with 100 µl two-fold concentrated mueller hinton broth. 10 µl of a 1:10 (for *E. coli* and *S. enterica*) or 1:80 (for *L. innocua* and *S. aureus*) mixture of the electron mediator and WST-8 was added immediately. Each sample was measured in triplicates and blanks (only PBS) as well as untreated controls were included in every assay. The absorption was measured at 450 nm and recorded every minute in a microplate reader (Spectramax 190, Molecular Devices, Sunnyvale, USA) for one hour at 37 °C. Within this timeframe a linear increase in absorption could be observed. A linear relationship ($R^2 > 0.99$) between the number of inoculated cells and the absorption after 60 min was found for all bacteria (data not shown). The relative metabolic activity after the heat treatments was determined by calculating the ratio of absorbance between the treated samples and the untreated controls after 60 min.

2.5. Investigation of membrane integrity, membrane potential, esterase activity and glucose uptake by flow cytometry

Sample preparations for flow cytometric investigations were performed according to the methods described by Kramer and Muranyi (2014). The impact of the mild heat treatment on the cells membrane integrity was investigated on the basis of their staining characteristics using the fluorescent dye propidium iodide (PI, Dojindo Molecular Technologies, Kumamoto, Japan). Samples were diluted 1:100 in filtered (0.22 µm) PBS and incubated with 30 µmol l^{-1} PI for 15 min at room temperature in the dark before the flow cytometric analysis.

Depolarization of cell membranes was assessed with Bis (1,3-dibutylbarbituric acid) trimethine oxonol, sodium salt ($DIBAC_4$, Dojindo Molecular Technologies, Kumamoto, Japan). Samples were diluted 1:100 in filtered PBS and incubated with 2 µmol l^{-1} (*L. innocua*, *S. aureus*) or 5 µmol l^{-1} (*E. coli*, *S. enterica*) $DIBAC_4$ for 15 min at room temperature in the dark before the flow cytometric analysis.

The esterase activity of the heat treated bacteria was assessed using 5(6)-Carboxyfluorescein diacetate (CFDA, Dojindo Molecular Technologies, Kumamoto, Japan). Samples were diluted 1:100 in filtered PBS and incubated with 100 µmol l^{-1} CFDA for 15 min at 37 °C in the dark. In case of *E. coli* and *S. enterica*, 1 mmol l^{-1} EDTA was added during the incubation period in order to facilitate the uptake of CFDA into the cells. The bacterial suspensions were subsequently centrifuged at 9000 g for 5 min and the cell pellets were resuspended in filtered PBS before the flow cytometric analysis.

Glucose uptake was investigated according to the method reported by Kramer et al. (2015). The fluorescently labeled glucose analog 2-NBDG (2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose) (Life Technologies, Carlsbad, CA) was used as substrate. NBDG is a moderately hydrophilic compound, which is, like unlabelled glucose, specifically taken up by the phosphoenolpyruvate phosphotransferase system (PEP-PTS; Sträuber and Müller, 2010). The mean values of the fluorescent signals of 10,000 bacterial cells were recorded. Bacteria were separated from the background noise by regulation of Forward scatter (FSC) and Side scatter (SSC) amplification. Uptake kinetics over a period of 15 min was initially recorded in duplicate (data not shown). An incubation period of 2 min was then chosen for both bacteria in order to ensure linear increase in the fluorescent signal within all uptake assays. Fluorescence of the cells was measured directly after adjustment of 5 µmol l^{-1} 2-NBDG in the sample suspension and again after 2 min. Results are presented as relative uptake rate by calculating the ratio of relative fluorescence units (RFU) of each sample and the mean RFU of all untreated reference samples.