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Quantification of the impact of single and multiple mild stresses on outgrowth heterogeneity of *Bacillus cereus* spores

C.C.J. van Melis [a,b], H.M.W. den Besten [b], M.N. Nierop Groot [a,c], T. Abee [a,b,*]

[a] Top Institute Food and Nutrition, Nieuwe Kanaal 9A, 6709 PA, Wageningen, The Netherlands
[b] Food Microbiology Laboratory, Wageningen University, Bornse Weilanden 9, 6708 WG, Wageningen, The Netherlands
[c] Food and Biobased Research, Wageningen University & Research Centre, Bornse Weilanden 9, 6708 WG, Wageningen, The Netherlands

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ABSTRACT

Outgrowth heterogeneity of bacterial spore populations complicates both prediction and efficient control of spore outgrowth. In this study, the impact of mild preservation stresses on outgrowth of *Bacillus cereus* ATCC 14579 spores was quantified during the first stages of outgrowth. Heterogeneity in outgrowth of heat-treated (90 °C for 10 min) and non-heat-treated germinated single spores to the maximum micro-colony stage of 256 cells was assessed by direct imaging on Anopore strips, placed on BHI plates at pH 7 and pH 5.5, without and with added NaCl or sorbic acid (HSA). At pH 7 non-heated and heat-treated germinated spores required 6 h to reach the maximum microcolony stage with limited heterogeneity, and these parameters were only slightly affected with both types of spores when incubated at pH 7 with added NaCl. Notably, the most pronounced effects were observed during outgrowth of spores at pH 5.5 without and with added NaCl or HSA. Non-heat-treated germinated spores showed again efficient outgrowth with limited heterogeneity reaching the maximum microcolony stage after 6 h at pH 5.5, which increased to 12 h and 16 h with added NaCl and HSA, respectively. In contrast, heat-treated spores displayed a strong delay between initial germination and swelling and further outgrowth at pH 5.5, resulting in large heterogeneity and low numbers of fastest growers reaching the maximum microcolony stage after 10, 12 and 24 h, without and with added NaCl or HSA, respectively. This work shows that Anopore technology provides quantitative information on the impact of combined preservation stresses on outgrowth of single spores, showing that outgrowth of germinated heat-treated spores is significantly affected at pH 5.5 with a large fraction of spores arrested in the early outgrowth stage, and with outgrowing cells showing large heterogeneity with only a small fraction committed to relatively fast outgrowth.

1. Introduction

The food industry's current trend of processing under milder conditions and reduced use of preservatives may provide opportunities for spore forming bacteria, such as the food pathogen and spoilage bacterium *Bacillus cereus* (Logan, 2012; Stenfors Arnesen et al., 2008; Ehling-Schulz et al., 2004; Andersson et al., 1995). Dormant spores are resistant to a variety of environmental stress factors, but if appropriate nutrient or non-nutrient germinants are present or applied, they can return to life through germination followed by outgrowth (Setlow, 2003; Abee et al., 2011). Germination of a dormant spore population often occurs as a heterogeneous process (Eijlander et al., 2011; Stringer et al., 2011; Barker et al., 2005), i.e., not all spores will germinate at the same time, nor will transition to vegetative cells and subsequent outgrowth occur at the same rate. Variability in spore behavior (Yi and Setlow, 2010; Stringer et al., 2005) complicates the prediction of outgrowth considerably, since it requires extensive knowledge on the responses of individual spores. Spoilage of products may arise from just a few spores that survived processing and therefore the presence of just a few individual spores in the population with enhanced survival properties can be a challenge to the industry. At the population level, this variability will remain unnoticed and therefore this information cannot be obtained by conventional plate counts or optical density based methods. It is therefore important to study heterogeneity by quantifying outgrowth at the single spore level.

Heterogeneity in germination and outgrowth may be further increased by the application of (mild) preservation methods, such as milder heating regimes that do not fully inactivate the spore population, leaving surviving (partially damaged) spores. Thus, less intensive heating should therefore be combined with other preservation 'hurdles', including for example the addition of organic acids, low concentrations of salts, or combinations thereof, in order to control germination and outgrowth of surviving spores in food products. Quantification of the impact of stress exposure on population heterogeneity has been presented for heat treatment of *B. cereus* spores (Cronin and Wilkinson, 2008), *Bacillus subtilis* (Smelt et al., 2008; Pandey et al., 2013) and

* Corresponding author at: Food Microbiology Laboratory,Wageningen University, Bornse Weilanden 9, 6708 WG, Wageningen, The Netherlands. Tel.: +31 317 484981.
E-mail address: tjakko.abee@wur.nl (T. Abee).

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Clostridium botulinum (Stringer et al., 2011) and heat and/or salt-stressed *C. botulinum* spores (Webb et al., 2007). We previously used flow cytometry sorting to monitor germination and outgrowth of single dormant spores and quantified acid-induced population heterogeneity at pH 5.5 without and with added sorbic acid. Evidence was presented that *B. cereus* ATCC14579 spore germination efficiency was not a good predictor for heterogeneity in final outgrowth (den Besten et al., 2012).

This underlines the importance to quantify the first stages of (stressed) outgrowth, directly following the germination phase. Anopore technology was first described by Ingham et al. (2005), and has been used to quantify *B. cereus* ATCC 14579 population heterogeneity at a low incubation temperature and under salt stress conditions (den Besten et al., 2007, 2010). We now used Anopore to monitor the outgrowth of individual non-stressed and heat-treated spores at neutral and slightly acidic pH (pH 7 and pH 5.5) without and with added NaCl (2.5% *w*/*v*) or HSA (0.25 mM undissociated sorbic acid). Behavior of germinated spores was followed up to the microcolony stage (maximum 256 cells), enabling quantification of heterogeneous outgrowth of *B. cereus* spores in the absence and presence of preservation stresses.

2. Materials and methods

2.1. Strain and culture conditions

B. cereus strain ATCC 14579 was obtained from the American Type Culture Collection (ATCC) and stored in brain heart infusion (BHI) broth, supplemented with 50% glycerol at −80 °C. *B. cereus* cells were cultivated at 30 °C with aeration at 200 rpm (Innova 4335 Incubator Shaker, New Brunswick Scientific, United States). Overnight cultures were used to inoculate defined minimal sporulation medium (De Vries et al., 2004) and spores were produced and harvested as described previously (Van Melis et al., 2011) with the following modification: the Tween 80 content was reduced from 0.1% to 0% by daily washing steps over a period of 5 days. Pure spore crops devoid of vegetative cells and debris were stored in phosphate-buffered saline (100 mM PBS, pH7) at 4 °C for not more than 8 weeks until use.

2.2. Outgrowth percentages on agar plates

To select a heating regime leading to sub-lethally damaged spores, a stock of dormant spores (2×10^8 spores/ml) was ten times diluted in MES buffer (100 mM) and exposed to various time/temperature combinations in Eppendorf tubes at 85, 90, 95 and 100 °C for either 10 or 20 min. After treatment, the spore solutions were further diluted and plated on BHI plates that were buffered at pH 7 and contained either 0 or 1.5% (*w*/*v*) of added NaCl to determine the fraction of sub-lethally damaged spores, since these will not produce colonies in the latter condition whereas they will in the absence of added NaCl. The heat treatment of 90 °C for 10 min was selected for further experiments since this heat treatment induced the highest fraction of sub-lethally damaged spores (data not shown).

To assess the colony forming capacity of heated and non-heated spores in the presence of mild stresses, dilutions were made in MES buffer (100 mM, pH 7 or pH 5.5, depending on the test conditions), and plated on buffered BHI plates containing 0 or 2.5% (*w*/*v*) NaCl (at both pH 7 and pH 5.5), or 0.25 mM undissociated sorbic acid (HSA, only at pH 5.5). Colonies were counted after an incubation period of 24 h at 30 °C, and again after 48 h. Quantification of colony forming spores was performed in three individual experiments (biological triplicates), each of which consisted of technical duplicates, resulting in a total number of six determinations per condition. The numbers of colonies formed by non-heated spores at pH 7 were averaged, and this value was set as 100%, and the number of colonies of the other stress-conditions is expressed relative to those observed for non-heated spores.

2.3. Growth on Anopore strips

Anopore strips (Whatman, The Netherlands) were prepared as described previously (den Besten et al., 2007, 2010). Sterile Anopore strips were placed on pre-warmed (30 °C) BHI plates (buffered at either pH 7 or pH 5.5 with 100 mM MES), supplemented with 2.5% NaCl (*w*/*v*) or, in case of plates with pH 5.5, with 0.25 mM undissociated sorbic acid (HSA, total concentration SA of 1.63 mM). Anopore strips were inoculated with non-heated and heat-treated spores derived from a dormant spore stock (2×10^8 spores/ml) appropriately diluted in MES buffer (either pH 7 or pH 5.5, depending of the test conditions) in order to ensure that dormant spores and the subsequent microcolonies would arise from a single spore and would not merge during the incubation times of the experiments. The plates were closed and then incubated at 30 °C in a moist chamber.

For each imaging time point, one Anopore strip was transferred right side up to a microscope slide ($50 \times 76 \times 1$ mm) covered with a 1 mm thick film of 1% (*w*/*v*) solidified low-melting-point agarose (Invitrogen, The Netherlands). The agarose was dissolved in MES, buffered at the appropriate pH, and 2.5 μM SYTO-9 (Invitrogen, The Netherlands) was added to stain the micro-colonies on the Anopore strip. The micro-colonies were then incubated for 15 min at room temperature in the dark before imaging.

Anopore strips were imaged directly (i.e. without the use of a coverslip and immersion oil) using an Axioskop fluorescence microscope equipped with an LD-Plan Neofluar 63× 0.75Ph 2 Corr lens (Zeiss, The Netherlands) and Zeiss type 38 filters. An Olympus XC30 camera, controlled by Olympus Cell B imaging software, was used to capture the images. The images were analyzed as described previously (den Besten et al., 2007, 2010). The fluorescence-based approach of Anopore allowed fluorescence to be monitored in spores as soon as germination started and the moment when spores turned phase dark is therefore the start of visualization. This step was reached after 20 to 30 min under all test conditions (data not shown), and 30 min was therefore chosen as the starting time point for all subsequent measurements. The intervals of imaging were chosen in such a way that for each stress condition the process of outgrowth and subsequent vegetative growth could be followed, starting from germinated single spores up to microcolonies of 256 cells, after which 2D-based area measurements were no longer possible due to multiple layers of cells being formed in the microcolonies. At each imaging time point, the number of images that were processed covered an average of 280 (250–300) spores/microcolonies per sampling point.

2.4. Quantification of heterogeneity

Microsoft Excel was used to calculate the distribution of microcolony areas per imaging time point for each experimental condition, and the observed frequency distributions were presented in histograms. To optimally envisage the increase of microcolony area over time, the binning of the histograms was based on the number of cells per microcolony, calculated by dividing the areas of the individual microcolonies (excluding the intercellular area) by the area of an average single cell, determined by the surface area of 300 individual cells, just after division. This approach proved adequate for microcolonies consisting of vegetative cells, but not for representing the different stages of outgrowing spores. Therefore, all areas that were smaller than that of one cell were grouped together into one bin (represented by the letter S in the figures), representing both germinated and outgrowing spores. The bins were named '1, 2, 4, etc' for illustrative purposes only. The population heterogeneity was expressed by calculating the variance per sampling time-point for each condition, based on the ^{2}log values of area of each microcolony.