Live-imaging of Bacillus subtilis spore germination and outgrowth

Pandey, R.

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Chapter 7

General discussion and future perspectives
7.1 Discussion

The food industry experiences huge economic losses due to food spoilage caused by spores that may survive under food preservation conditions. Hence efforts are being made to eliminate or inactivate these bacterial spores in foods. Complete thermal inactivation of microorganisms in food products has become less popular due to the adverse effects on food quality and flavour (Hornstra et al., 2009; Leistner et al., 1995). To fulfil the consumers demand and safety of the food product “hurdles preservation” is needed that controls the outgrowth of these bacteria in food. The spore germination and outgrowth progression are often very heterogeneous. Heterogeneity makes it difficult to pinpoint which phases of germination and outgrowth are specifically affected upon a given preservation treatment. Therefore it is necessary to analyse the behaviour of single spores/cells to quantify the effect and degree of heterogeneity in each phase of the spore’s life cycle. In order to examine the heterogeneity at spore germination and outgrowth level, single cell analysis is a preferred method. The studies described in this thesis are to assess the impact of weak organic acids (sorbic acid and acetic acid), natural compounds (focussed on tea compounds) and heat on germination and (out)growth of *B. subtilis* spores and cells at the single cell level. The project focused on three main aspects; (1) To develop an image analysis tools for the measurement of germination and outgrowth of spores as well as the internal pH of the cells at single spore/cell level; (2) To develop a closed air-containing chamber for actual experimentation with aerobic bacterial spore formers; (3) To study the inhibitory effect of weak organic acid, heat and tea compounds on different phases of germination and outgrowth of the spores as well as the effect of sorbic acid and acetic acid on the internal pH of *B. subtilis* cells at single cell level.

Chapter 2 describes the analysis tools (SporeTracker and Multichannel-SporeTracker) that were used in chapter 3 to chapter 6 for the analysis of different phases of germination and outgrowth of spores and internal pH measurement of cell at single spore/cell level. Chapter 3 describes the design of an air-containing chamber that is used for growth of aerobic bacteria and the analysis of the germination and outgrowth of its spores. The chamber has sufficient oxygen as well as sufficient cell culture medium to maintain optimal growth conditions of aerobic bacteria during at least 24 h of culture. In this chamber the cells/spores are sandwiched between an agarose-medium pad and a glass coverslip. This chamber was used as basic slide preparation method for all live-imaging microscopy experiments presented in this thesis. This chapter also highlights the heterogeneity in growth behaviour when un-treated spores were germinated and cultured in minimal defined medium.
Weak-acids are commonly used natural preservatives in the food industry. Sorbic acid is used as a preservative, well known to inhibit the growth of different microorganisms including bacteria, yeast and fungi. The compound is lipophilic in nature and can diffuse into and over the membrane. Inside the membrane, sorbic acid can upset normal membrane functions of the cell, while in the cytosol the compound can cause a decrease in pH and therefore also in this way affect normal cellular function. Its effect on spore germination and outgrowth has not been studied well and thus gained limited attention. In this thesis, chapter 5 describes the study of the effect of sorbic acid on germination and outgrowth of *Bacillus subtilis* 1A700 spores at single spore level using phase contrast microscopy. This provides a frame work for future studies of the effects seen in vegetative cells. In this study the spores were stressed with 3 mM sorbic acid (final concentrations) at pH 6.4. The results showed that sorbic acid primarily affected the outgrowth and generation time of the bacteria emerging from spores. Besides sorbic acid, the effect of heat (85°C/10min) and combined stresses of heat and sorbic acid were also analysed. By using Raman spectroscopic techniques Coleman et al., (2007) showed that wet heat causes protein denaturation in a nonspecific way. From our results it is concluded that the heat stress primarily affected the germination process. This is corroborated by the results described in chapter 3. There the data shows that the heat treatment delays the time to start of germination and increases the germination time, i.e. the time from phase bright to phase dark. However, there was no effect on both outgrowth and generation time of vegetative cells that emerged from the spores. The combination of both a thermal treatment and sorbic acid showed a synergistic effect as it further reduced the number of germinating spores, reduced the outgrowth rate and increased the time from end of germination until the burst time. an important objective for the future will be to extend the possible incubation times without compromising the physicochemical characteristics of the incubation chamber(see furtheron)

Ter Beek et al., (2008) showed that in the cytosol sorbic acid could cause a decrease in pH and therefore affect normal cellular function. In order to study this effect in single cells chapter 6 describes the study of the effect of weak acids on the intracellular pH dynamics in *Bacillus subtilis* vegetative cells using the set-up described in chapter 3. In this study, an improved version of the genetically encoded ratiometric, IpHluorin was used along with live-imaging. The protein was expressed from the native *B. subtilis* promoter that is specifically active during vegetative growth on glucose ($P_{pts}$G). Dual wavelength excitation ratio imaging was set up and allowed us to resolve the population data at single cell level. Weak organic acid such as sorbic acid and acetic acid caused concentration-dependent intracellular acidification. The result illustrates that sorbic and acetic acid both lower the internal pH of the cell compared to the control condition.
Interestingly, more acetic acid is needed to decrease the internal pH of the cell than sorbic acid. To make the system suitable for monitoring spore germination pH dynamics, expression of IpHluorin from spore specific promoters such as sspE may be preferred. Van Beilen et al., (2013) have shown the feasibility of such approach.

The antimicrobial effect of plant extracts has been used for many applications such as pharmaceuticals and to some extends in food preservation. The effect of tea plant extracts on germination, outgrowth and growth of vegetative cells emerging from spores was tested. Tea is already known for long for its antimicrobial activity against many microorganisms. Studies have shown that tea polyphenols can inhibit the growth of a wide range of Gram-positive bacteria. Still, the effect of these compounds on germination and outgrowth of bacterial spores has gained limited attention. In this regard chapter 4 describes at single spore resolution level a study of the effect of selected tea compounds on B. subtilis spore germination and outgrowth. We tested galloallocatein gallate and Teavigo (>90% epigallocatechin-3-gallate) which is a type of catechin (flavan-3-ol monomer), theaflavin 3,3’-digallate, a type of theaflavin (flavan-3-ol dimer), and gallic acid which is a phenolic weak acid with pKa of 4.5. We concluded from the results that in general, the tested compounds had a significant effect on most stages of germination and outgrowth. However, germination efficiency (ability of the spores to take up water and become phase dark) was not affected. Gallic acid most strongly reduced the ability to grow out. Additionally, all compounds, in particular theaflavin 3,3’-digallate, clearly affected the growth of emerging vegetative cells.

7.2 Future perspectives

Bacteria exhibit natural heterogeneity that complicates the prediction of bacterial growth, especially if they are present in low numbers. In the case of bacterial spore formers heterogeneous behaviour is evident in the germination and/or outgrowth phase of the bacterial spores. Den Besten et al. (2012) showed in microtiter plate experiments (population level) that the heterogeneity increases during the outgrowth phase of Bacillus cereus spores when they are germinated in the presence of 0.75 mM undissociated sorbic acid. However, this measurement includes the germination, outgrowth and vegetative growth phase and thus does not provide information about variation within each of these phases, or about heterogeneity in behaviour of individual spores within the population. The analysis of the germination and outgrowth behaviour of spores at single spore level should provide better insight in the individual spore/cell behaviour underlying the observations
made on the population as a whole. It also will provide information on the query whether any correlation exists between the duration of each of the different phases. It may for instance be that the lag time before the spores are triggered to germinate (using the glucose, fructose, potassium, amino acids as germinants) varies greatly as it depends on the type and concentration of germinant (Zhang et al., 2010). In addition the efficiency of the germination process itself is thought to depend on the amount of germination receptors present in the inner membrane of the spore. On average, spores with higher levels of germination receptors germinate faster, though this is not the primary cause for heterogeneity observed at the start of germination (Zhang et al., 2013). Furthermore, the outgrowth phase that commences after spore germination can also be quite heterogeneous. Smelt et al., (2008) performed experiments in microtiter plates and showed that outgrowth of spore occurred even after 150 hrs. Stringer et al., (2005) described the analysis of germination and outgrowth of Clostridium botulinum at single cell level (using microscopy). Previous research showed that the common preservation strategies increase the heterogeneity in spore germination and outgrowth. While, for example a mild heat activation treatment reduces heterogeneity in lag times of B. subtilis spores (Smelt et al., 2008), more severe heat treatments increase heterogeneity and average duration of lag times both in B. subtilis and C. botulinum (Smelt et al., 2008; Stringer et al., 2011).

The results described in this thesis link phenotypic observations of B. subtilis spore germination and outgrowth under weak-acid (sorbic and acetic acid), natural compounds and heat stress at the single cell/spore level. This provides more detailed insight in the impact of these stresses on germination and (out)growth heterogeneity of B. subtilis spores. In addition we also checked B. cereus spores at single cell level. The data are of similar quality allowing us to dissect the process of spore germination and outgrowth into its underlying basic cellular events (data not shown). The thesis describes the use of an air-contaning chamber for growth of aerobic bacteria and spores under microscope condition. Its efficiency was tested for a maximum of 10 hrs. The future challenge would be to use the chamber for a long period of time (24 hrs. or more). This is necessary to approach more real life realistic time frames. After all, the food industry uses long preservation times.

Finally, we can extend our finding by translating the current knowledge to different strains and species including anaerobic Clostridia. This would quantify and give information on the diversity in stress response and in turn may contribute to improved prediction of outgrowth under preservation stress.

In the food industry the bacteria in food-processing environments come across more than one effect simultaneously such as heat and acid. But many studies on heterogeneity in spore germination and outgrowth have focused on the effect of
single stresses such as either heat or acid (Smelt et al., 2008; Stringer et al., 2005; Wang et al., 2011). Thus in chapter 5, we quantified the effect of preservation stress (heat) combined with a second preservative stress (sorbic acid) at single spore level with respect to the germination and outgrowth phases of the spores. Besides the sorbic acid other weak organic acid preservatives such as acetic and lactic acid are used by the food industry to preserve the food. Thus future research at this level of stress resolution ought to focus broader on the use of different weak organic acid preservatives such as acetic acid and lactic acid along with an analysis of effects of varying the concentrations of acid. Besides this, the thermal stress, time-temperature combination or combinations of heat and weak organic acid stress can be explored. Important and additional challenges are to extend the analysis of the heterogeneity in germination and outgrowth under mild preservation stress, to more complex environments such as those present in an actual food product.

An internal pH measurement over time can be a direct method to investigate the physiological state of individual cells or germinating spores when stress is applied. This can be done with fluorescence microscopy using the pH sensitive green fluorescent protein pHluorin (Miesenböck et al., 1998). pHluorin expression in the cells allows single cell live-imaging ratiometric $pH_i$ assessment thus facilitating a time resolved measurement of the internal pH during growth of bacteria. Chapter 6 describes the study of the effect of sorbic and acetic acid on the internal pH of growing B. subtilis cells using fluorescence microscopy. Similar approaches can be used for the measurement of the internal pH behavior in spores during different stages of germination and outgrowth. Preliminary population based data have been reported in Van Beilen and Brul (2013). Such single cell live-imaging approaches will give relevant information about the heterogeneity in different phases of germination and outgrowth of spores, which leads to a better understanding of the behaviour of microorganisms in food products and should facilitate the development of more accurate models for bacterial growth under preservative stress as well as allow appropriate combinations of preservation strategies to be optimized. Thus contributing to improved microbial food stability and food safety (McMeekin et al., 2010). This knowledge can also be extended to other bacterial groups such as Clostridium spp. However, GFP needs oxygen for the maturation of its chromophore composed of threonine, tyrosine and glycine. Hence GFP cannot be used in anaerobic bacteria but another fluorescent protein called flavin-binding fluorescent protein (FbFPs) could be used instead. It is a recently developed new class of genetically encoded probes that have small size and do not require oxygen for maturation of their fluorophores (Cui et al., 2012; Drepper et al., 2007). The fluorescent microscope set-up for live-imaging that we developed here has as limitation that due to the fully closed nature of the
incubation environment it prevents the long term monitoring of germination and outgrowth that is needed for analyses that aim at approaching real-life conditions of food preservation. The use of microfluidics (Ducret et al., 2009) might be an avenue that could offer a way to face this challenge. Such tool should allow for the study of the dynamic behaviour of cells/spores exposed to changing environmental conditions of interest to the industry. In conclusion, the study of the effect of sorbic acid, heat and the combination of both stresses and tea compounds at single spore and cell level described in this thesis provide better insight in the population heterogeneity and mode of action of common preservation methods on single spore forming bacteria. Thereby the results obtained in this thesis contribute to improve or design new preservation concepts of microbial food stability and food safety.

7.3 References


Chapter 7: General discussion and future perspectives


