Weak organic acid stress in Bacillus subtilis

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Chapter 6

On the Origin of Heterogeneity in (Preservation) Resistance of *Bacillus* spores: Input for a ‘Systems’ Analysis Approach of Bacterial Spore Outgrowth

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6.1. Abstract

Bacterial spores are the ultimate (stress) ‘survival capsules’. They allow strains from the *Bacillus* and *Clostridium* species to survive harsh environmental conditions. In addition to the decision to enter sporulation the decision to do the reverse (germinate) is also a decisive event after which there is no return. Generally it is observed that the behaviour of spores towards the environment is not homogeneous. In fact in many cases it is even quite heterogeneous, certainly upon subjecting the spores to a thermal stress treatment. Genome information coupled to high resolution single-cell analysis techniques allow us currently to analyse signalling events of individual cells. In the area of food preservation the next challenge is to couple the newly acquired mechanistic data to the physiologically observed heterogeneity in spore behaviour.

The current paper will introduce the background of physiological heterogeneity while discussing the molecular processes that likely contribute to the observed heterogeneity in outgrowth. The discussion is set in the framework of contemporary and future needs for single-cell data integration in order to enhance the mechanistic basis of food preservation and spoilage models targeting bacterial spores.
6.2. On bacterial spores and heterogeneity

Bacterial sporeformers are agents of prime concern to human health for a number of reasons. Some are highly pathogenic such as *Bacillus anthracis* and *Clostridium botulinum*. While the former aerobic spore former is not common in most foods but rather notorious as bioterrorism agent, the latter anaerobic spore former is THE oldest concern to the food industry due to its ubiquitous occurrence in foods, thermal stress resistance and extreme danger of the toxin produced by its vegetative cells (Esty & Meyer, 1922, Peck, 2006, Stringer et al., 2005). Other Clostridia of importance include *C. perfringens*, *C. tyrobutyricum*, and *C. sporogenes*. *Clostridium perfringens* is an important anaerobic pathogen causing food-borne gastrointestinal diseases in humans and animals (Rahmati & Labbe, 2008). The food poisoning *C. perfringens* isolates typically carry the enterotoxin gene (*cpe*) on a plasmid or in their chromosome (Miki et al., 2008). *C. sporogenes* and *C. tyrobutyricum* are organisms commonly associated with the spoilage of cheese during its manufacturing. In a near to anaerobic environment the cells may grow and cause ‘blowing’ of the cheese due to an excessive production of gas (Le Bourhis et al., 2007). Noticeably, in the second half of the previous century attention focussed more and more on the aerobic Bacilli. A prime reason for this shift in attention was that these organisms form extremely heat resistant spores. Examples of such heat resistant Bacilli include wild-type isolates of *Bacillus subtilis* and strains from *Bacillus sporothermodurans* (Oomes et al., 2007, Scheldeman et al., 2006). In order to fulfil the quest of contemporary producers to meet consumer demands for nutritious products that are subjected to a milder thermal processing, but are still microbiologically stable, a thorough understanding of the often significant heterogeneity in behaviour of individual cells and spores with respect to the survival efficiency of (thermal) preservation stress of such Bacilli is paramount. Thus, chances of outgrowth and product spoilage can be predicted more precisely herewith preventing unnecessary over processing with consequent loss of product quality. As processing temperatures come down, the data also become of value and interest to those studying the behaviour of the less heat tolerant spores from toxigenic Bacilli (*e.g.* *B. cereus*) and the previously discussed *Clostridia*. A detailed account of heterogeneity in the various phases of germination of non-proteolytic *Clostridium botulinum* has been described by Stringer et al. (2005). Typical assays include those where wild-type thermally treated spores are sorted individually in a 96 wells plate and tested for growth under various product relevant conditions for periods up to several weeks (Smelt & Brul, 2007). In addition to assessing growth, individual spores may also be followed mechanistically through an analysis of the behaviour of reporter proteins specific for cellular signalling pathways that regulate their response to the environment. An integration of the physiological and molecular data at the single-cell level will be instrumental in predicting, with the necessary level of confidence, the behaviour of the spores even outside the direct boundaries of the tested conditions. Such approaches will facilitate the choice of the appropriate preservation strategy.
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Fig. 6.1. Theoretical impact of bi- or multi-modal (heterogeneous) and monomodal (homogeneous) responses on spore characteristics. A genetic response within an isogenic population may show variable expression patterns: the response of a population may be monomodal (homogeneous) – showing a normal distribution (red or green) – or bi- or multi-modal (heterogeneous) – giving rise to two (green) or more (not shown) different sub-populations. The dashed line indicates an arbitrary cut-off: cells that induce a response with intensity above the cut-off will form spores with altered resistant properties and/or germination rates. AU, arbitrary units.

by food technologists (Brul & Westerhoff, 2007, McMeekin et al., 2008). The molecular basis of most of the processes governing heterogeneous behaviour of bacterial spores is, however, still enigmatic and topic of discussion. Even the convention on what to call heterogeneity is not fully unambiguous. Many biological responses follow a normal distribution and are thus often called homogeneous. Still, differential biological behaviour at the edges of the normal distribution (noise) might be relevant (Elowitz et al., 2002, discussed in Veening et al., 2008b).

For us this is especially relevant when the specific response of the cell determines or influences the heat resistance of an emerging endo-spore and/or its germination rates. In this paper we describe spore heterogeneity as simply the different resistance characteristics and/or germination rates of spores observed within a spore crop. We do not define here whether the spore crop consists of either distinct (bi- or multi-stable) populations or just one single (broad) homogeneous population. In theory, the observed heterogeneity in spores might be caused by monomodal (homogeneous) and/or bi- or multi-modal (heterogeneous)
Fig. 6.2. A schematic overview of factors influencing spore properties during sporulation, the subsequent dormant state and germination. Upon nutrient limitation, some cells decide to initiate sporulation, while others do not. It is speculated that some of these return to the decision point (dashed line; Veening et al., 2008c). Both the decision to sporulate and the sporulation process may be influenced by environmental conditions or applied preservatives.

responses. Fig. 6.1 gives a conceptual representation of the above. 'Extremes' in a homogeneous response (i.e. cells that respond with an intensity far outside the standard deviation of the normal distribution) may have a significant effect on the properties of the mature spore. This can be caused by changed external conditions which may lead to a shift and possible 'broadening' of the response (from red to green in Fig. 6.1). For the spore physiology this may have as consequence an increase in heterogeneity in resistant properties and/or germination rates.

An example of 'intrinsic' noise leading to heterogeneity in spore thermal behaviour may be the expression of germination receptors. If the set of germination receptors genes (up to seven different ones in the genomes of Bacilli) is expressed at various levels, this might lead to a great numerical variance of receptor proteins per spore. This is more so as the actual number of Ger receptors in a spore is likely to be low (estimated at up to 20 per type of Ger receptor per spore) (Paidhungat & Setlow, 2001). The outcome of such events would in all likelihood be a measurable difference in the germination response of individual spores in a population.
Furthermore we will discuss extrinsic factors contributing to heterogeneity in spore behaviour whilst using *B. subtilis* as a model bacterial spore former. We will consider heterogeneity in spore (preservation) stress resistance due to a non-homogeneous (bistable) initiation of spore development as well as heterogeneity in spore behaviour due to conditions during spore formation and maturation. The sporulation phases and conditions that may occur during these stages are indicated schematically in Fig. 6.2. Intrinsic and extrinsic noise in the decision to sporulate and the sporulation process may influence the variability in spore resistance characteristics. Upon release of the endospore from the mother cell into the environment, its properties are further influenced by maturation, ageing and accumulated damage. These factors in combination with the germination conditions will determine the level of outgrowth heterogeneity of the spore population.

Below we will discuss the state of the art information on these parameters in relation to the resulting spore physiology. The data will be instrumental in generating mechanistic models for bacterial spore outgrowth, a basis for a ‘systems’ approach in food preservation (Brul & Westerhoff, 2007, Marthi *et al.*, 2004; the ETP Food for Life at http://etp.ciaa.be).

### 6.3. Factors influencing a cell’s decision to sporulate and spore stress resistance

It has been shown previously using liquid cultures that the decision to sporulate upon nutrient limitation within a population is a heterogeneous, so called bistable, process (Veening *et al.*, 2005). This reflects the notion that within one isogenic culture some cells reach a certain threshold level of phosphorylated Spo0A (Spo0A~P), the key sporulation regulator, and activate the sporulation program, while other cells do not reach the high levels of Spo0A~P and consequently, do not switch on sporulation (Fujita & Losick, 2005). The positive feedback of Spo0A~P on *spo0A* transcription plays an important (but not critical) role in the emergence of distinct sub-populations (Veening *et al.*, 2008c). The faith of the non-sporulating cells is not entirely clear but the data of Veening *et al.* (2008c) suggest that some may return to the decision point and sporulate later. Recently, Tracy *et al.* (2008) corroborated this notion by showing that Clostridia cultures may undergo multiple rounds of sporulation. Interestingly, recently Veening *et al.* (2008a) have shown, using buoyant density centrifugation to separate endospore containing cells from vegetative cells, that vegetative cells produce extracellular proteases also in a bistable fashion. This expression is indirectly under control of Spo0A~P and induced by the DegS-DegU two-component system. Heterogeneity in the induction of spore formation is evidently observed in multicellular communities such as biofilms and biofilm-like structures. Spores of *B. subtilis* are predominantly formed in elevated structures within the biofilm (Branda *et al.*, 2001, Veening *et al.*, 2006). Spo0A~P is also the master regulator involved in activating (a small fraction of) the cells in a *B. subtilis* biofilm to produce extracellular matrix compounds (Chai *et al.*, 2008, Vlamakis *et al.*, 2008). Activated *spo0A* gene-expression, in combination with low levels of...
Spo0A-P, seems to be needed for the expression of the genes responsible for the extracellular matrix. Veening et al. (2008c) have also shown recently using image analysis on growing single layer cell colonies that the actual decision to sporulate is made several generations before the sporulation cascades commences. The inheritance of the decision seems to be via epigenetic mechanisms of which the molecular basis is still enigmatic.

The general stress response (GSR), mediated by SigB is another important pathway Bacillus cells can induce upon nutrient (glucose) depletion (see for a review on SigB e.g. Hecker et al., 2007). Whether sporulating cells also induce the GSR is until now unclear. Our recent data show that under glucose deprived conditions B. subtilis induces initially both the GSR and sporulation, but in distinct cells (Fig. 6.3). A strain harbouring fluorescent reporters for both sporulation as well as the GSR (amyE::PspolA-cfp Pctc-yfp) was grown into the stationary phase initiated by the depletion of glucose. Clearly, some cells induced sporulation (green cells) while other cells induced the GSR (red cells). Yet other cells did not show a distinct red or green fluorescence indicating that they did not respond or induced another response under these conditions. Hardly any ‘yellow’ cells were observed, meaning that the GSR and the sporulation signalling cascade were, for the majority of the population, not induced in the
same cells at the same time. It seems that both responses are under these conditions mutually exclusive. Although the fluorescent signals have not yet been quantified by flowcytometry, this observation suggests also a possible bistable switch with the GSR mediated by SigB being somehow dependent on the onset of the sporulation cascade or vice versa. The organization of the operon containing the sigB gene, encoding SigB provides some clues in support of this hypothesis (Fig. 6.4). The last four genes including sigB of the eight-gene operon are preceded by an internal SigB promoter (Kalman et al., 1990, Wise & Price, 1995). Consequently, SigB increases its own transcription and thereby this positive feedback loop may induce bistability. It remains to be elucidated whether the cells that induce the GSR in the stationary phase are still able to sporulate and if these cells subsequently form spores with altered resistant and germination properties thus contributing to the observed heterogeneous behaviour in spore-crops.

Finally, we have recently shown that a mild treatment of vegetative B. subtilis cells with the commonly used food preservative sorbic acid causes a unique stress response and does not induce sporulation (Ter Beek et al., 2008). Our more recent data show that the formation of spores upon glucose depletion in these sorbic acid stressed cells is postponed when compared to an untreated culture (Fig. 6.5). The culture treated with potassium sorbate in the exponential phase (at OD_{600} = 0.2) entered the stationary phase, caused by glucose depletion, approximately half an hour later than the untreated control culture. The emergence of spores in the preservative treated culture was around 3 h later than in the control culture. This is significantly later (~2.5 h) than the initial 30 min delay upon entry into the stationary phase. After 24h both cultures contained predominately heat resistant spores (> 80%) (our unpublished observations). We now have preliminary indications that spore crops formed under sorbic acid stress conditions show increased germination rates in the presence of the originally added concentration of the preservative compound when compared to a control spore crop (Van Beilen et al., unpublished observations).

In summary, many environmental stress conditions, including some relevant to food manufacturing, can influence a cell’s timing to sporulate and thus the conditions under which the spores are formed. The data is currently extended to include mechanistic studies and single spore germination conditions to be able to analyse possible effects on spore crop heterogeneity.
Fig. 6.5. Sorbic acid stress in the exponential phase delays the formation of spores induced by glucose depletion in the stationary phase. *B. subtilis* was grown exponentially in a defined minimal (MOPS based) medium of pH 6.4 (Ter Beek et al., 2008) in Erlenmeyer shake-flasks. The optical density at 600 nm (OD<sub>600</sub>) was followed in time for 11 h (diamonds). At an OD<sub>600</sub> of 0.2 (t = 0 h) one culture was stressed with 3 mM potassium sorbate (KS) (open diamonds). The closed diamonds indicate the growth of the control culture (no addition of KS). The percentage of heat resistant spores was calculated in the control (closed triangles) and the stressed culture (open diamonds) by determining the colony forming units before and after a 20 min 80°C heat treatment.

6.4. Sporulation conditions have major impact on spore properties

6.4.1. Temperature

Numerous reports have shown that sporulation conditions have a major impact on the final properties of the spore, mostly reported by variance in heat resistance or germination capacities. One of these conditions is sporulation temperature which, under laboratory conditions, can be kept constant. Generally, an increase in sporulation temperature results in increased heat resistance of the spores, likely due to a lower core water content (Gerhardt & Marquis, 1989, Melly et al., 2002). The effects of temperature variation during sporulation on germination properties however, are less studied. For *Bacillus cereus* spores, an increase in sporulation temperature results in a decrease in germination rate, while a similar but less pronounced response was described for *B. subtilis* when germinated with L-alanine (Cortezzo & Setlow, 2005, Gounina-Allouane et al., 2008). Some of our own recent data (Fig. 6.6) showed, in contrast, a significantly increased germination rate of spores produced at higher temperatures. These spores were, however, formed on defined minimal (MOPS based)
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Germination in:

Fig. 6.6. Germination properties of *B. subtilis* spores sporulated in defined minimal (MOPS based) medium at 21°C (dashed bars), 30°C (dotted bars) and 37°C (white bars). The spores were germinated in MOPS based medium (left) and TSB medium (right) and both media were supplemented with a mixture of 10 mM L-alanine and AGFK. The % OD fall of the spore suspension was measured after 30 min. A fall in OD of 55% represents a fully germinated spore population.

Medium (details on the sporulation conditions described in Oomes *et al.*, 2007) and germinated in the same medium or TSB, both supplemented with a mixture of L-asparagine, glucose, fructose and potassium, (AGFK). The measurement used, (decrease in the optical density), does not allow us to discriminate between a homogeneous enhanced germination rate of all 37°C formed spores or a heterogeneous response in which some spores germinated much faster than others.

6.4.2. Other physicochemical factors

Well known parameters significantly affecting spore properties include pH, nutrient and/or mineral composition of the sporulation medium (Hornstra *et al.*, 2006, Lee *et al.*, 2003, Oomes *et al.*, 2007). Contrary to temperature, these parameters are, however, not constant during sporulation as nutrients and metals are being consumed and the culture pH increases upon progression of sporulation (de Vries *et al.*, 2005). They may thus contribute strongly to observed heterogeneity in germination rates and thermal resistance of the resulting spores.

The presence of certain mineral ions during sporulation, specifically potassium and manganese (probably indirectly by stimulating the uptake of potassium), has a clear effect on the spore's heat resistance properties (Cazemier *et al.*, 2001, Eisenstadt, 1972, Oomes *et al.*, 2007).
Our own experiments, in which we have sporulated *B. subtilis* 168 cells in minimal defined (MOPS based) medium with an increasing amount of Ca\(^{2+}\), and subsequently determined the heat resistance of these spores, are in agreement with previous data (Fig. 6.7). However, the mechanism behind this observation remains to be elucidated, and current experiments in our lab aim to illuminate the genes involved during sporulation under increased Ca\(^{2+}\) concentration conditions. Oomes & Brul (2004) showed in initial experiments that genes coding for the two small acid soluble proteins (SASPs A and B) were induced earlier during sporulation in media that contained higher mineral concentrations and produced more thermal-resistant spores. The temporal resolution of these studies was however low and there were only morphological means of assessing sporulation stages. The latter precluded a detailed analysis of sporulation progression. Recently, a study at a much higher time resolution showed that specific genes involved in the synthesis of the spore coat polysaccharides (sps) are all significantly induced in cells undergoing sporulation under high calcium conditions (Oomes *et al*., submitted). Confirmatory experiments in which functional heat resistance tests on sps knock-outs are currently being planned.

Beside ion concentration, nutrients present in the sporulation medium influence final properties of the spore. Sporulation in different media can influence the expression of the germination operons, and by this deliver spores with a unique composition of receptors. These spores differ in their germination responses to nutrients (Hornstra *et al*., 2006). Finally,

![Fig. 6.7. The effect of the Ca\(^{2+}\) concentration present in the sporulation medium on the heat resistance of *B. subtilis* spores. Spores were prepared in defined minimal (MOPS based) medium containing 0.014 mM Ca\(^{2+}\) (dashed bars), 0.14 mM Ca\(^{2+}\) (dotted bars) and 1.4 mM Ca\(^{2+}\) (white bars), while 0.14 mM Ca\(^{2+}\) is the normal Ca\(^{2+}\) concentration in the medium used. Sporulation proceeded normally in all three media up to more than 99%. Spores were heated at 90°C for 0 min, 10 min and 20 min in which the spores of the 0 min sample were heated until 90°C and immediately transferred to ice after reaching this temperature. 100% survivors is represented by germination and outgrowth of the non-heated (although heat-activated) spores.](image-url)
it has been reported that spores formed on solid media have increased resistance properties when compared to spores prepared on the same liquid medium (Rose et al., 2007). Obviously, cells within colonies will individually face different ‘local’ nutrient environments, likely contributing to the different physiological characteristics of the resulting spore population.

For now, the molecular basis of most discussed environmental effects remains to be discovered. It may be that fluctuations in the intracellular pH, a crucial physiological indicator, play an important role. To address this issue, the cytosolic expression of pHluorin (a pH sensitive GFP variant originally constructed by Miesenbock et al. (1998) and recently expressed in our lab in Saccharomyces cerevisiae (Orij et al., 2009) has been adapted for use in B. subtilis cells (Ter Beek et al., manuscript in preparation). This will allow for an assessment of the intracellular pH in individual sporulating (and stressed) cells.

6.5. Processes affecting spore properties after spore release.

Upon completion of sporulation, the spores are released from the mother cell late. Spores just released from the mother cell undergo what is called a maturation process that may last from days up to weeks (Atrihi et al., 1996). It is often seen that in this period their heat resistance still gradually increases (Oomes & Brul, unpublished observations). Although it is assumed that the maturation process involves adaptations of the cortex, the exact mechanism is not yet known. It may be envisaged that local (micro)heterogeneity in the physicochemical conditions during maturation can lead to further heterogeneity in spore properties.

6.5.1. Spore damage and heterogeneity

Although spores are well-known for their formidable resistance capacities, stressful environmental conditions may damage the spore. In the environment this may be due to exposure to solar radiation (Nicholson et al., 2000, Setlow, 2006). As spores may remain in their dormant state in nature for an incredible period of time, they may gradually collect radiation that will damage the DNA. DNA repair mechanisms, however, are capable of repairing DNA damage in one of the first stages of germination (Keijser et al., 2007, Moeller et al., 2008). It has not been quantitatively investigated yet whether the quantity of collected DNA damage and subsequent repair influences the outgrowth time of the damaged spore.

Although heat is still to date the most used food preservation method, the effect of wet heat and the damage it inflicts upon the spore is less well understood (Setlow, 2006). A slight rise in temperature results in activation of the spore population, while further increasing temperature eventually starts damaging spores and perturbing their germination efficiency. Ultimately, spores will be unable to germinate at all upon exposure to very high temperatures.

Generally, significant heterogeneity is seen in the germination and outgrowth behaviour of individual spores after their exposure to the conditions indicated above. This makes, as was
already indicated earlier, knowledge of the behaviour of heat damaged spores very relevant to the food industry. Common industrial heat treatments are known to not only increase the (average) length of the lag, but also its variance. Outgrowth analysis of single heat damaged spores, measured by sorting them in single wells and subsequent incubation in TSB at 30°C showed spores with enormous variance in outgrowth (Smelt et al., 2008, Smelt & Brul, 2007). In fact, spores might be capable of growing out months after application of the heat treatment. Processes occurring in these spores are largely unknown, but the heat treatment may have damaged a number of essential proteins or enzymes (Coleman et al., 2007). A normal distribution of such damage may already lead to large variation in outgrowth efficiency (see also the discussion pertaining to Fig. 6.1 of the current paper and Mallidis & Drizou, 1991, Webb et al., 2007). Additionally, possible repair processes may contribute to (heterogeneity in) spore recovery. Once all hurdles have been overcome, the germinating spore will proceed quickly to normal cell metabolism and division.

6.5.2. Towards the molecular basis of repair of thermal damage

A mild thermal treatment likely results in three types of spores; non-damaged spores, damaged spores and dead spores. In a proper preservation treatment the first population is absent while the second and third will be present at unknown levels. The enormous variance in cell types in subsequent stages of development precludes population based “omics” techniques including micro-arrays to study the response of heat damaged spores. A similar observation has been described by Morohashi and coworkers, where they advice careful interpretation of data obtained from cells yielding population heterogeneity (Morohashi et al., 2007). New, single cell-based, approaches are needed to shed light on processes occurring in individual germinating and outgrowing spores.

Germination and outgrowth are both (tightly) regulated cascades of molecular processes ultimately leading to a vegetative growing cell. A few parameters are indicative for the progression of the spore through the early phases of germination such as the change from phase bright to phase dark, or the moment of DPA release (Chen et al., 2006). Outgrowth is difficult to detect as this involves many processes without any morphological changes of the germinating spores. In order to identify the different developmental stages of outgrowing spores a current approach is to use fluorescent reporter proteins specific for the various outgrowth phases. Such reporters are based on the data by Keijser et al. (2007) on a genome-wide expression analysis of B. subtilis spore germination under optimal conditions. Our laboratory has recently constructed a range of strains containing outgrowth-phase specific GFP reporter genes (Fig. 6.8). The system will allow one to determine the time from germination to the GFP signal for individual heat treated spores. Herewith it will be possible to look in more detail at which part of the cascade of processes during germination, the inflicted heat damage is causing a significant delay and thus the molecular targets of heat damage are
to be sought. Furthermore, these germination “clock” markers can be used to determine the amount of heterogeneity in a population by determining the time between initiation of germination (time after addition of the germinants) and the detection of GFP in individual spores germinating in micro-well systems. In order to be able to see these processes in the germinating spores, systems such as those developed by Ingham et al. (2007) combined with sophisticated fluorescence analysis tools (Hoebe et al., 2007) that should allow for single spore GFP detection will have to be developed.

6.6. Outlook

From the above it may easily be inferred that noise levels in gene-expression and related phenomena contribute significantly to spore formation and spore preservation stress responses, mostly heat resistance. It is interesting to speculate about a regulation of noise levels in cells under different environmental conditions, giving a cell population the possibility to regulate the level of heterogeneity in a spore population depending on environmental conditions (Webb et al., 2007). Veening et al. (2008b) already discussed the advantage of having (physiological) heterogeneity in bacterial populations as it allows for more flexibility in dealing with (rapidly) changing environmental conditions. They touched upon spores in this context as an alternative to vegetative stress responses. Indeed, our data on the heterogeneity with respect to cells inducing the SigB dependent stress response and
sporulation, within one population of *B. subtilis* cells reaching stationary phase, corroborate their notion.

Heterogeneity of the spore characteristics themselves in terms of survival of preservation stress conditions as discussed throughout this paper seems to represent a next layer of diversification of the cellular responses to adverse environmental conditions. Heterogeneity in thermal resistance and outgrowth efficiency is obviously relevant to the food industry (Cronin & Wilkinson, 2008, Oomes *et al.*, 2007). Whether or not there is an intentional diversification of spore characteristics through the use of noise in gene-expression or whether it remains a more fortuitous process remains to be discovered.

In closing, the study of heterogeneous populations urgently needs integrated molecular and physiological analyses. Such ‘systems approaches’ to biological problems are getting more and more common in (micro)biology and may soon find application in the area of food microbiology (discussed in Brul *et al.*, 2008). The experiments should be set-up and analysed with in mind the ultimate goal of transforming mechanistic insight into the basis for models ultimately predicting the likelihood and efficiency of spore outgrowth in foods.

### 6.7. Acknowledgements

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