Bacterial Spores in Food: Survival, Emergence, and Outgrowth

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Abstract

Spore-forming bacteria are ubiquitous in nature. The resistance properties of bacterial spores lie at the heart of their widespread occurrence in food ingredients and foods. The efficacy of inactivation by food-processing conditions is largely determined by the characteristics of the different types of spores, whereas food composition and storage conditions determine the eventual germination and outgrowth of surviving spores. Here, we review the current knowledge on variation in spore resistance, in germination, and in the outgrowth capacity of spores relevant to foods. This includes novel findings on key parameters in spore survival and outgrowth obtained by gene-trait matching approaches using genome-sequenced *Bacillus* spp. food isolates, which represent notorious food spoilage and pathogenic species. Additionally, the impact of strain diversity on heat inactivation of spores and the variability therein is discussed. Knowledge and quantification of factors that influence variability can be applied to improve predictive models, ultimately supporting effective control of spore-forming bacteria in foods.

INTRODUCTION

Foodborne Disease and Spoilage by Sporeformers

Spore-forming bacteria play an important role in food spoilage and foodborne disease, and food industries actively employ strategies to ensure adequate inactivation of spores and control outgrowth. Sporeformers have the extraordinary ability to enter sporulation as an adaptive strategy to survive conditions encountered in their natural habitat, for instance, in soil, in aquatic environments, or in the gut of insects and animals (Carlin 2011, Heyndrickx 2011, Markland et al. 2013, Nicholson 2002). This complex regulatory process transforms the bacterial cell into a dormant endospore (Al-Hinai et al. 2015, Eijlander et al. 2014, Higgins & Dworkin 2012, Hilbert & Piggot 2004, Talukdar et al. 2015), a state in which it can reside for undefined periods of time. Dormant endospores are resistant to environmental stress conditions, including heat, salinity, acidity, radiation, oxygen and/or water depletion, and low availability of nutrients (McKenney et al. 2013, Nicholson et al. 2000, Setlow 2006). Their resistance properties lie at the heart of their ubiquitous presence in the environment, and as a consequence, it is inevitable that spores enter the food chain. Given the robustness of spores, they are generally quite resistant to processing and preservation treatments used in food manufacturing (Checinska et al. 2015, Stecchini et al. 2013). Yet spores can sense changes in their direct surroundings (e.g., the availability of nutrients), which can trigger the process of germination (Setlow 2013). This may occur in foods because they are rich in nutrients. Once germination is initiated, the spore can return to its vegetative cell form and once again start exponential cell division.

Spore germination in a final food product, followed by growth of vegetative cells (possibly even followed by sporulation), can lead to food spoilage. In the case of foodborne pathogens, foodborne illness may occur upon the intake of foods that contain spores of pathogenic species that may germinate and grow in the gut, or upon intake of foods in which spores have already germinated and grown to high numbers. In the latter case, foodborne illness may result from the intake of toxins produced in the food (food poisoning) or the intake of vegetative cells of the pathogen that subsequently form toxins in the gut, leading to diarrhea (foodborne infection) (Anderson et al. 1995, Eijlander et al. 2011, Logan 2012, Stecchini et al. 2013).

Two major bacterial spore-forming species, *Bacillus cereus* and *Clostridium perfringens* (Anderson et al. 1995), account for an estimated 1.3% and 4.0% of cases of bacterial foodborne disease, respectively (EFSA 2005). Another important sporeformer able to cause food poisoning is *Clostridium botulinum* (Carter & Peck 2015). Although the impact of pathogenic sporeformers is apparent, nonpathogenic species also pose major challenges to the food industry. A plethora of sporeformers—sometimes with very distinct characteristics with respect to spore resistance and/or requirements for growth—may be responsible for product defects or spoilage in a variety of food products, leading to substantial economic losses in the food chain and to substantial food waste (Checinska et al. 2015, Postollec et al. 2012, Stecchini et al. 2013).

Selective Pressure Applied by Processing Conditions and Food Characteristics

Strategies to preserve food often include an inactivation treatment (e.g., heat) to reduce microbial loads in combination with factors that control the growth of microbes throughout shelf life (Gould 1996). This includes intrinsic factors in foods (e.g., water activity, pH, organic acids, and other antimicrobial factors) and extrinsic factors such as temperature and the (un)availability of oxygen (Gould 1996). There is an increased demand for healthy, nutritious, yet easy-to-prepare foods with increased freshness and shelf life. As a result, milder processing techniques and fewer preservatives

(e.g., salt, sugar, and acids such as sorbate) are being employed in food processing (Pasha et al. 2014). This poses new challenges in assuring the stability and safety of a variety of foods.

In products that undergo only minimal processing, both vegetative cells and spores survive, and bacterial outgrowth must be controlled. This can be accomplished in various ways, such as by cooling, by lowering pH, and/or by applying (natural) preservatives. In products undergoing inactivation treatments aimed at killing the vegetative microflora but that are insufficient to kill spores (often pasteurization for at least 15 s at 72°C or the equivalent), sporeformers may then be a prominent concern, especially those species that can subsequently grow in the food product (Logan 2012, Postollec et al. 2012). In low-acid (below pH 4.6) pasteurized food products, such as vegetable (e.g., tomato) and fruit products (e.g., apple, citrus fruits) as well as dairy products (e.g., yogurt), a low pH puts selective pressure on the surviving microflora, and if spoilage occurs, it is generally the result of the growth of molds, yeasts, or thermoresistant lactic acid bacteria (Shearer et al. 2002). However, acidophilic sporeformers can also grow out in such products; these include various *Bacillus* species (e.g., *Bacillus coagulans*) and *Alicyclobacillus acidoterrestris* (Chang & Kang 2004, Oomes et al. 2007, Silva & Gibbs 2004, Steyn et al. 2011).

In pasteurized products with higher pHs, including a range of refrigerated processed foods of extended durability (REPFEDs) or pasteurized milk and dairy products, storage at refrigeration temperatures is generally key in controlling microbial growth and to ensuring food quality and safety throughout shelf life. Species of concern with respect to the safety of such pasteurized refrigerated products include psychrotrophic pathogenic sporeformers such as *Bacillus weihenstephanensis*, certain *B. cereus* strains (Anderson Borge et al. 2001), nonproteolytic *C. botulinum* (Carter & Peck 2015), and certain strains of *C. perfringens* (Xiao et al. 2015). However, such products may also be prone to spoilage by nonpathogenic species that require control; some examples of this are *Clostridium* species in cold-stored vacuum-packaged meat products (Kalinowski & Tompkin 1999, Moschonas et al. 2011), *Clostridium tyrobutyricum* in cheese (Doyle et al. 2015), and *Bacillus* or *Paenibacillus* species in REPFEDS (Samapundo et al. 2014a) and pasteurized milk (Ivy et al. 2012, Scheldeman et al. 2004).

High-heat treatments are applied to produce commercially sterile foods with a long shelf life, such as sterilized canned foods (e.g., meats, fish, and vegetables) and ultra-high-temperature (UHT)-treated liquids (e.g., juices and milk). In canned, bottled, or vacuum-packed products with a pH high enough to allow for growth of *C. botulinum*, heat treatments must exceed 3 min at 121°C (or the equivalent) to inactivate spores of this organism (Anderson et al. 2011). Highly heat-resistant spores can survive such heat treatments, and depending on the product characteristics and storage temperatures during shelf life, different species may cause spoilage (Logan 2012). These may include *Bacillus* spp. and *Paenibacillus* spp. (Burgess et al. 2010; Scheldeman et al. 2004, 2006) but also obligate thermophilic species that require temperatures greater than 45°C for growth, such as *Geobacillus* spp. (André et al. 2013, Burgess et al. 2010, Durand et al. 2015). Anaerobic sporeformers producing highly heat-resistant spores may also play a role in spoilage of commercially sterile foods; the most heat-resistant spores described to date are produced by *Moorella thermoacetica* (Byrer et al. 2000), which belongs to the class Clostridia (phylum Firmicutes), which is naturally associated with anaerobic warm waters. The spores can easily survive sterilization, with a reported decimal reduction value (*D*-value) of 111 min at 121°C (Byrer et al. 2000).

Challenges for the Food Industry

There is an increasing need to understand and predict which spores can survive certain inactivation methods and whether they can subsequently germinate and grow out, ultimately threatening food

quality and/or safety. Clearly, this is a challenge, given the fact that different spore-forming species show enormous diversity with respect to their spore resistance properties and their specific growth characteristics. Although conditions that allow for sporulation and germination are quite well understood for a number of model organisms, in particular for type strains of *Bacillus subtilis* and *B. cereus* (Eijlander et al. 2014, Higgins & Dworkin 2012, Setlow 2013), this is generally not the case for spores of strains that are encountered in food products. Conditions of spore formation in their natural habitat are often unknown but nevertheless may affect properties of spores that ultimately end up in raw food ingredients or in processing lines. Moreover, conditions that trigger spore germination and requirements for recovery and growth are not always known. This complicates enumeration and detection on standard laboratory media, where outgrowth to a visible colony is required. In addition, it makes it hard to predict whether germination and outgrowth will occur across food products with different compositions. The application of heat may introduce further variation in the potential of surviving spores to germinate and to grow in food matrices (Augustin 2011, Eijlander et al. 2011, van Melis et al. 2014).

Improved detection and enumeration of species of concern may be required for track-and-trace purposes and to enable reduction in the food chain. This requires further insights into the (molecular) mechanisms involved in spore formation of diverse species and in the (regulatory) mechanisms that determine spore properties, such as resistance, germination, and outgrowth capacity. The use of omics technologies can help to characterize strains that occur in the food chain, and their genetic traits can be linked with phenotypic traits. In addition, more knowledge is needed about environmental factors that influence spore formation, spore maturation, dormancy, germination, emergence, and outgrowth in the food chain (**Figure 1**). Such multidisciplinary approaches will allow for targeted intervention strategies to control sporeformers in foods.

The focus of this review (see sidebar, Sporeformers: Friends and Foes) is bacterial spores that are commonly associated with foods, with a particular focus on survival of inactivation treatments, and variation in germination, emergence, and outgrowth of spores in foods. In addition, current techniques that allow for the analysis of spore diversity at individual and population levels are described, and the need for improved predictive models to assess spore survival and outgrowth of the bacterium in foods are discussed.

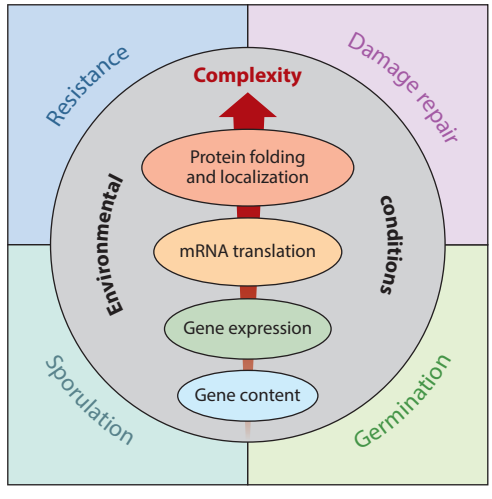

Figure 1

Levels of complexity influencing spore properties and behavior.

SPOREFORMERS: FRIENDS AND FOES

Sporeformers play an important role not only in food safety and quality but also in human and animal health and disease (e.g., probiotic species improve health in animal husbandry, and *Clostridium difficile*, *Clostridium botulinum*, *Clostridium perfringens*, *Bacillus cereus*, and *Bacillus anthracis*, for example, cause disease in humans and animals) and in many applications such as food fermentations (e.g., *Bacillus subtilis* in soy fermentations), crop protection (e.g., *Bacillus thuringiensis* insecticides), crop yield improvement owing to the presence of sporeformers in the rizosphere (*Paenibacillus* spp. and *Bacillus* spp.), and industrial fermentations (e.g., *Clostridium* spp. in biofuel production). Sporeformers are also rich sources of natural antimicrobials and industrial enzymes. When applying spores widely in the environment (crops, probiotics in food and feed), care must be taken that counterselection takes place against undesirable properties (e.g., high heat resistance, antibiotic resistance) to avoid possible dissemination of such genetic traits into the food chain.

SPORE RESISTANCE

The Composition of a Spore

The formation of a bacterial endospore is the result of a complex regulatory process that is reviewed elsewhere (Al-Hinai et al. 2015, Eijlander et al. 2014, Higgins & Dworkin 2012, Hilbert & Piggot 2004, Talukdar et al. 2015). Bacterial spores have various layered structures (**Figure 2**) that provide resistance against environmental insults and thus against several food-processing conditions. The way in which different structures contribute to spore resistance has been extensively reviewed elsewhere (Leggett et al. 2012, Setlow 2006) and is only briefly discussed here. First and foremost, the spore core is strongly dehydrated, which largely rotationally immobilizes the proteins present in the spore (Sunde et al. 2009). A specific type of protein present in spores is α/β-type small acid-soluble protein (SASP), which protects the genetic material against DNA damage. In addition, the core contains the spore-specific chemical pyridine-2,6-carboxylic acid, also known as dipicolinic acid (DPA), which is chelated with divalent cations (mostly Ca^{2+}) (Setlow 2006). Surrounding the core is the inner membrane, which provides protection against chemicals (Griffiths & Setlow 2009, Setlow 2003) and contains proteins required for germination (further discussed in the section Germination and Recovery). Outside the inner membrane lie the germ cell wall, which becomes the cell wall after germination, and the cortex, which consists of spore-specific peptidoglycan, characterized by the muramic-δ-lactam moiety and low peptide cross-linking (Popham 2002). The cortex is required for the development of full resistance toward wet heat (discussed in more detail below). The cortex is surrounded by an outer membrane for which no clear role in resistance is known (reviewed in Leggett et al. 2012). Finally, the outermost layers make up the spore coat, which contains various proteins that provide protection against lysozyme, toxic chemicals, and grazing protozoa, among others (extensively reviewed in McKenney et al. 2013). For certain species (e.g., *B. cereus*, *Bacillus thuringiensis*, *Bacillus anthracis*, and *Clostridium* spp.), the spore coat is further surrounded by an exosporium, which is in direct contact with the environment and is potentially involved in pathogenicity (reviewed in Henriques & Moran 2007, Paredes-Sabja et al. 2014).

Food-Processing Techniques to Inactivate Spores

The composition of spores provides resistance against conditions that are commonly used in the food industry to reduce bacterial loads, such as heat, acid, salt, and oxidizing agents (McKenney

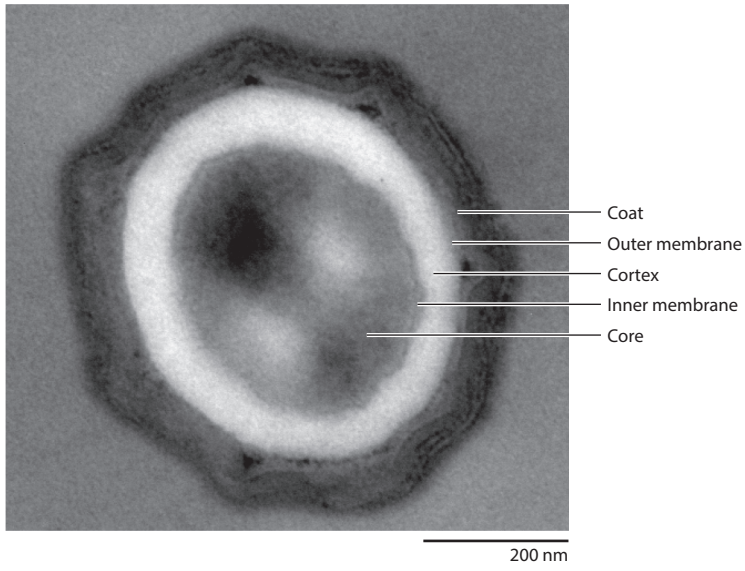

Figure 2

Transmission electron microscopy (TEM) cross section of a spore of *Bacillus subtilis* 168 (E.M. Berendsen, unpublished results). The ultrastructure's coat, cortex, inner membrane, and core are labeled.

et al. 2013, Setlow 2013). Heat application is an important process used to inactivate microbes. Pasteurization is applied to inactivate vegetative microorganisms, but higher-heat treatments, such as sterilization or UHT treatments, are needed to inactivate spores in food products (see Introduction). Such treatments are usually effective to reduce spores to sufficiently low numbers; however, various species of sporeformers, such as *Bacillus sporothermodurans*, *M. thermoacetica*, and *Geobacillus* spp. are able to survive commercial wet heat sterilization (Byrer et al. 2000, Durand et al. 2015, Scheldeman et al. 2006).

The exact mechanisms that lead to inactivation of spores after application of wet heat are still not clear, but there is evidence that after exposure to wet heat at 87°C to 90°C at various time points, heat denatures certain proteins that are required for germination, and DPA is released from the spore core (Coleman et al. 2007, 2010; Wang et al. 2011). Heat treatment is still a dominant process in the food industry to inactivate spores, but over the past decade, various alternative or combination treatments have been developed to achieve the same. One example is tyndallization, or the application of high hydrostatic pressure (HHP) processing combined with heat. First, modest pressures (400–600 MPa) are applied so that spores germinate and lose their resistance properties. Subsequent application of moderate heat, similar to what is needed to inactivate vegetative cells, is then sufficient to inactivate spores (Ahn et al. 2007). However, not all spores will be triggered to germinate by the pressure treatment, and consequently, the subsequent application of moderate heat may not kill all spores. Therefore, tyndallization is not likely to be applied in a commercial setting. Effective spore inactivation can, however, be achieved through pressure-assisted thermal processing (PATP), which is the application of pressure (500–600 MPa) in combination with higher heat (typically 90–120°C) (Balasubramaniam et al. 2015). Pressure-induced spore inactivation is thought to result from a loss of resistance properties upon germination, damage to germination-specific proteins present in the inner membrane, and/or remodeling of the inner membrane (Reineke et al. 2013, Sarker et al. 2015). PATP techniques pose

new challenges for modeling of spore inactivation kinetics because characteristic tailing is observed in the inactivation curves owing to variation in resistance properties within a spore population (Ahn et al. 2007, Margosch et al. 2006). Alternative methods for spore inactivation include the use of cold atmospheric plasma (van Bokhorst-van de Veen et al. 2015) and exposure to UV light on surfaces of foods. These techniques can also be applied to sterilize equipment surfaces, although it has been reported that many spores are resistant to high doses of UV (Setlow 2006).

Wet heat application remains the most common treatment applied in the food industry to decrease bacterial spore loads from food ingredients and products. Mechanisms involved in spore resistance against wet heat are further discussed in the next section.

Differences in Spore Wet Heat Resistance: Genetic Factors

As discussed above, spores encountered in food ingredients and in foods may have a large natural diversity, including variation in heat-resistance properties. The optimal growth temperature of species often correlates with the thermal resistance of their spores; thermophilic species generally produce spores with higher thermoresistance than mesophilic species, which in turn produce spores with higher heat resistance than psychrotrophic species (Gerhardt & Marquis 1989, Nicholson et al. 2000, Warth 1978), although this is not always the case. Marked differences in spore heat resistance are found not only between different species, but also between different strains of the same species (Berendsen et al. 2015b, Luu-Thi et al. 2014, Orsburn et al. 2008, Peck et al. 2011, van Asselt & Zwietering 2006, Xiao et al. 2015). This may be attributed partly to sporulation and maturation conditions, which can influence spore resistance properties (see section Environmental Factors Influencing Spore Wet Heat Resistance). However, it is also clear that different strains of the same species are able to produce spores with vastly different heat-resistance properties, even when cultured under identical conditions (Berendsen et al. 2015b, Luu-Thi et al. 2014, Peck et al. 2011, Xiao et al. 2015). For instance, it is known that food-associated *C. perfringens* strains that produce the enterotoxin CPE and that carry the *cpe* gene on the chromosome (C-*cpe*) form spores with much higher heat resistance than their counterparts without *cpe* or with *cpe* on a plasmid (P-*cpe*) (Orsburn et al. 2008, Xiao et al. 2015). The C-*cpe* food isolates were shown to belong to a distinct multilocus sequence-typing (MLST) group (Xiao et al. 2012) and may carry mutated or specific genes or possess specific regulatory processes that affect spore formation and result in higher spore heat resistance. A known genomic determinant for *C. perfringens* spore wet heat resistance is the SASP *ssp4* gene, which upon deletion, reduces spore heat resistance from a *D*-value of 59.1 to 8.7 min at 100°C (Li et al. 2009).

In a recent study by Berendsen et al. (2015b), two distinct groups of *B. subtilis* strains could be identified based on their spore wet heat resistances, with one group showing, on average, 0.1 log reduction after 1 h exposure to 100°C, whereas the other group showed inactivation of approximately 10 log units using the treatment. Assuming a relatively homogeneous genetic background in these strains, such findings pointed to specific genetic features or regulatory processes during sporulation that determine spore heat resistance. To investigate this further, genome sequences were determined of 9 strains that produced highly heat-resistant spores and 9 strains that produced spores with lower heat resistant. Gene absence or presence was evaluated at a whole-genome level for all strains versus the phenotypic trait spore heat resistance (see sidebar, Next-Generation Sequencing and Integrative Bioinformatics). Using this so-called gene-trait matching approach (Bayjanov et al. 2012), a transposon was uniquely identified in the *B. subtilis* isolates that showed high wet heat resistance (E.M. Berendsen, J. Boekhorst, O.P. Kuipers, M.H.J. Wells-Bennik, unpublished results). Further characterization of this transposon revealed a critical role of a specific operon via a yet unknown mechanism that was shown to influence DPA levels and core

NEXT-GENERATION SEQUENCING AND INTEGRATIVE BIOINFORMATICS

Advances in both next-generation sequencing (NGS) technologies and bioinformatics techniques allow for the combined analysis of NGS data and the results of high-throughput phenotypic screenings. This has provided powerful tools for unraveling the molecular mechanisms underlying spore formation, outgrowth, and resistance. The combination of cell-sorting techniques (discussed in the section Germination and Recovery) with single-cell transcriptome sequencing (reviewed in Wu et al. 2014) allows for studying population heterogeneity, whereas genome sequences from bacteria that are not amenable to growth under laboratory conditions can be reconstructed from shotgun metagenomics on environmental samples (Albertsen et al. 2013). The resulting omics data can be integrated with phenotypic data using advanced classification techniques (e.g., Random Forest analysis; reviewed in Touw et al. 2013). Although the principle role of these techniques is explorative (i.e., in the lead generation), they allow us to address questions that are very difficult to study with more traditional techniques.

water content (E.M. Berendsen, J. Boekhorst, O.P. Kuipers, M.H.J. Wells-Bennik, unpublished results). Furthermore, genomic analysis revealed that this transposon most likely originated from plasmids in *B. cereus* strains and entered *B. subtilis* cells via horizontal gene transfer (E.M. Berendsen, J. Boekhorst, O.P. Kuipers, M.H.J. Wells-Bennik, unpublished results). Strikingly, this transposon was also found in food isolates of *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, and *Bacillus sporothermodurans*, but only in those strains that produce spores with high heat resistance (E.M. Berendsen, J. Boekhorst, O.P. Kuipers, M.H.J. Wells-Bennik, unpublished results). Whether the element also confers increased wet heat resistance of spores of *B. cereus* or other *Bacillus* or *Clostridium* spp. remains to be elucidated. It is conceivable that different mechanisms play a role in the high heat resistance of other species (e.g., Clostridia). Despite similarities in the core genetic properties involved in sporulation of Bacilli and Clostridia, important variation exists between the two classes (Al-Hinai et al. 2015, Galperin et al. 2012, Paredes et al. 2005, Talukdar et al. 2015), and the roles of different genes in resistance phenotypes remain to be established.

The findings described above indicate that horizontal transfer of specific elements can contribute to large variation in spore heat resistance (and possibly other traits). Such genetic fluxes between species and strains can occur via phage transduction, conjugation, or uptake of external DNA via natural competence (Ochman et al. 2000). Insight into conditions that trigger transfer of genetic elements with resistance traits will allow for preventive measures to limit the occurrence of highly heat-resistant spores in the food chain.

Environmental Factors Influencing Spore Wet Heat Resistance

It is also well known that in addition to the intrinsic properties of a strain, the environmental factors present during sporulation can influence properties of spores, including wet heat resistance (Abbas et al. 2014, Lenz & Vogel 2015, Nguyen Thi Minh et al. 2011). The temperature during the sporulation process has been reported to play a role in the wet heat resistance of the spores that were formed. Melly et al. (2002) reported that increased sporulation temperatures resulted in increased wet heat resistance of *B. subtilis* spores, which correlated with a lower core water content and a higher degree of muramic acid cross-linking in the cortex. Other reports describe increased wet heat resistance of *B. subtilis* and *Bacillus megaterium* spores upon heat shock (45°C or 48°C for 30 min) during sporulation (Melly & Setlow 2001, Movahedi & Waites 2000, Sedlak et al. 1993). It is not clear how this increase in resistance is mediated; despite their presence, heat shock proteins appear not to be involved in increased spore heat resistance (Melly & Setlow 2001).

The presence of cations in the growth medium (in particular, calcium, potassium, magnesium, and manganese) can also contribute to an increased wet heat resistance of spores (Cazemier et al. 2001, Oomes & Brul 2004). Interestingly, such effects may be species-specific, as Ghosh et al. (2011) observed an increased wet heat resistance of *B. megaterium* spores with higher concentrations of manganese in the sporulation medium, whereas Granger et al. (2011) did not see this effect for spores of *B. subtilis*.

To achieve maximal wet heat–resistance properties, spores require a maturation period after release from the sporulating cell (Sanchez-Salas et al. 2011). The presence of divalent cations, such as Ca^{2+}, are required for this maturation, because addition of ethylenediaminetetraacetic acid (EDTA) abolished the maturation and thereby acquisition of maximal wet heat resistance (Sanchez-Salas et al. 2011). This maturation and subsequent increase in wet heat resistance is most likely mediated by chemical cross-linking of proteins in the outer coat layer and crust (Abhyankar et al. 2015).

Conditions encountered during sporulation in the natural habitat of sporeformers undoubtedly play a role in resistance properties of spores that occur in foods and ingredients (Heyndrickx 2011, Lima et al. 2011, van Zuijlen et al. 2010). However, often these conditions are not known or are hard to mimic in a laboratory setting. When preparing spores in a laboratory, conditions used to prepare the spores (e.g., medium type, incubation temperatures, liquid broth, or agar surface) can greatly influence spore wet heat resistance. This is a factor to be reckoned with, and such conditions may require optimization when studying the mechanisms that determine the wet heat–resistance properties of spores.

GERMINATION AND RECOVERY

Spore Germination: Differences Between Species

Germination of spores in food is a critical step in food spoilage and foodborne illness by sporeformers, especially when conditions in the product allow for outgrowth. In general, spore germination in foods is triggered by small molecule germinants, including amino acids, sugars, nucleosides, and salts that are sensed by specific germinant receptors (GRs). Spore germination can also be triggered by non-nutrient agents, such as Ca-DPA and cationic surfactants, or by the application of moderate pressure. A moderate heat–activation step may furthermore aid germination (Setlow 2003, 2014). Moreover, peptidoglycan fragments have been shown to induce germination (Shah et al. 2008). The initial germination events are accompanied by a loss of spore heat resistance and dormancy and can be recognized microscopically by the transition of spores from phase bright to phase dark. Once the spore is triggered to germinate, a series of events follows, including loss of Ca-DPA and cortex hydrolysis, after which a vegetative cell emerges that can resume exponential growth upon recovery (Setlow 2003, 2014).

The molecular details of the initiation of spore germination are still not fully understood, but recent studies involving *Bacillus* spp. and *Clostridium* spp. have provided more insight into the specific roles of several components, including (a)biotic germination triggers, GRs, and cortex-lytic enzymes (CLEs) (reviewed in Abee et al. 2011, Brunt et al. 2014, McKenney et al. 2013, Olguín-Araneda et al. 2015, Setlow 2014). This also revealed interesting differences between species of both genera; for example, cortex degradation precedes Ca-DPA release in *Clostridium* spp., whereas in *Bacillus* spp., release of Ca-DPA via SpoVA proteins (Setlow 2014, Velásquez et al. 2014) is first required to activate CLEs. In *B. subtilis*, all three functional GRs, SpoVA proteins, and GerD (a lipoprotein involved in GR-dependent germination) colocalize in the inner membrane of the spore in a structure termed the germinosome (Setlow 2014). The numbers and types of GRs

can vary significantly in other *Bacillus* spp. and *Clostridium* spp. and the presence and functionality of germinosomes in other species and strains remains to be elucidated (Olguín-Araneda et al. 2015, Xiao et al. 2011).

The impact of processing and preservation conditions on spore germination responses and outgrowth capacity in (model) foods can be characterized and quantified using single-spore or single-cell approaches (see below). In combination with studies that provide insight in the diversity and functionality of germination complexes in *Bacillus* and *Clostridium* spp., new or improved concepts for enhanced control of spore-forming bacteria in foods can be developed.

Germination of Natural Isolates in Food

Although spore germination has been studied extensively, most germination studies have been performed with mutants of *Bacillus* and *Clostridium* spp. that contain single or multiple deletions in genes encoding GRs, CLEs, and/or SpoVA proteins, and experiments have generally been conducted in laboratory media, often using high (20 to 100 mM) concentrations of germinants. Assessment of the contribution of different components in the spore that play a role during germination and outgrowth in (model) foods is generally lacking. Moreover, in many cases, germination and outgrowth behavior in foods could not be predicted on the basis of germination data generated in laboratory media using buffers and broths (van der Voort & Abee 2013, van der Voort et al. 2010, Løvdal et al. 2011, Samapundo et al. 2014b). Further studies are thus required to investigate the occurrence and roles of GRs and other relevant cellular components in germination and outgrowth in different strains as well as in different (model) foods.

An effective approach that can be taken to obtain new insights into the role of (novel) genes in germination is a comparison of the gene content of strains with distinctly different phenotypes (using a gene-trait matching approach; see sidebar, Next-Generation Sequencing and Integrative Bioinformatics). For instance, recent studies that included reference strains and food isolates from *B. subtilis* (A.O. Krawczyk, R.T. Eijlander, J. Omony, S. Holsappel, A. de Jong, M.H.J. Wells-Bennik, O.P. Kuipers, unpublished results) and *B. cereus* (A.K. Warda, Y. Xiao, J. Boekhorst, M.H.J. Wells-Bennik, M.N. Nierop Groot, T. Abee, unpublished results) provided new insights into the role of GRs in nutrient-induced germination of non-heated and heat-treated spores. Spores encountered in foods may thus have very different germination properties than well-studied laboratory strains and hence different germination requirements, which are not always known. The composition of foods may determine the ability of spores to germinate, possibly followed by outgrowth. Variation in germination properties may furthermore compromise enumeration of spores when cultivation conditions do not trigger germination.

Spore counts may be grossly underestimated when germination does not occur in response to known nutrients or on rich media. This was very clearly demonstrated in a recent study in which only a fraction (maximum 5%) of phase-bright spores of *Bacillus thermoamylovorans* germinated in the presence of rich laboratory medium and common germinants, despite the fact that the genomes of the strains encoded GRs. The recovery of spores improved drastically (greater than 95%) upon non-nutrient germination (using Ca-DPA) prior to plating. Counts of spores after heat inactivation treatments (at temperatures greater than 100°C) were ~2 log units higher after Ca-DPA exposure than after direct plating on rich medium. Upon improved germination, spores showed *D*-values of 1.9 to 1.3 min at 120°C, in line with observed survival of these spores in sterilization processes in the food industry (Berendsen et al. 2015a). Spores that did not germinate on rich medium might be considered dormant, but this may be because of a lack of knowledge regarding optimal germination conditions. In practice, a strong positive correlation appears to exist between high spore heat resistance and dormancy (Berendsen et al. 2015a, Kort et al. 2005,

Lima et al. 2011), which suggests that the most heat-resistant spores are the hardest to retrieve on cultivation media owing to their impaired germination. In such cases, proper germination and cultivation conditions are needed to avoid underestimation of viable spore numbers in foods or food ingredients (Leguerinel et al. 2000, Warda et al. 2015).

Variation in Germination of Pure Strains and Superdormancy

Although large variations exist in the germination responses of different strains or species owing to differences in germination machinery, single spores in one population of a pure strain may also display significantly different germination responses. A small fraction of spores in one population can, for instance, exhibit extremely long lag periods between exposure to germinants and ultimate germination, or not respond to known germination triggers at all (Berendsen et al. 2015a; Stringer et al. 2005, 2011). Such spores have been termed superdormant (Setlow 2013) and are notorious for their unpredictable germination and outgrowth responses. In addition, superdormant spores generally display elevated heat resistance, which is likely the result of reduced core water content (Ghosh & Setlow 2009). The underlying molecular mechanisms for such differences within a population are still not fully understood but may result from heterogeneity in gene and protein expression during sporulation (de Jong et al. 2010, Veening et al. 2005). The number of GRs in the dormant spore and the levels of cations available during sporulation have been hypothesized to play an important role (Ghosh et al. 2012), although there is recent evidence that this is not the primary cause of germination heterogeneity (Zhang et al. 2013).

A further understanding of heterogeneity in spore germination requires the ability to study spore formation, germination, and outgrowth processes at the single-cell level. Recent advances in suitable single-cell technologies have already significantly contributed to new insights (Chen et al. 2006, Pandey et al. 2013, van Melis et al. 2014, Zhang et al. 2012), some of which are discussed in more detail in the section Recent Techniques to Study Heterogeneity in Spore Germination, Emergence, and Outgrowth.

Damaged but Viable Spores: Repair Prior to Outgrowth

Differences in germination are the result of the genetic makeup of sporeformers and conditions during sporulation. Further variation in the ability of spores to germinate can be introduced by exposure to chemical or physical treatments aimed at inactivating dormant spores in foods or on food contact materials (e.g., heat, UV, pulsed light, HHP, and disinfectants). Spores may survive such treatments but sustain damage to DNA and proteins, and it is generally assumed that repair cannot take place during long lasting dormancy owing to lack of (or very low) metabolic activity in the spore (Campos et al. 2014, Ghosh et al. 2015, Setlow et al. 2014). Consequently, damage accumulates until repair processes become active once metabolic activity resumes.

Molecular mechanisms involved in repair of damaged DNA in spores directly upon germination have been studied, capitalizing on knowledge available for vegetative cells (reviewed in Gioia et al. 2007, Lenhart et al. 2012). Apurinic/apyrimidinic (AP) endonucleases (Nfo and ExoA) are thought to play a role, as their absence in *B. subtilis* severely delayed spore revival (Campos et al. 2014). DNA repair and outgrowth processes appear to be aligned, as the DNA integrity scanning protein (DisA) was reported to delay *B. subtilis* spore outgrowth until oxidative damage repair of DNA was completed (Campos et al. 2014). *B. cereus* also expresses *disA* upon spore outgrowth, which also suggests a role of DisA in DNA repair in this species (A.K. Warda, M.H. Tempelaars, J. Boekhorst, T. Abee, M.N. Nierop Groot, unpublished results). Exposure of *B. cereus* spores to DNA-damaging treatments such as pulsed light or irradiation resulted not only in longer lag

times to spore outgrowth but also in reduced growth rates, possibly owing to permanent mutations (Aguirre et al. 2015). DNA damage in *Bacillus* spores has furthermore been reported to result from UV exposure or application of dry heat at moderately high temperatures of 80 to 100°C (Setlow et al. 2014). At temperatures greater than 200°C, dry heat also appears to lead to protein damage, in particular damage to the spore core proteins, which points to the temperature-dependent damaging effects of dry heat (Setlow et al. 2014). Wet heat, on the other hand, is thought to cause protein damage by particularly targeting the germination machinery of spores. More detailed information on protein damage and repair is provided in reviews by Chondrogianni et al. (2014) and Visick & Clarke (1995).

Although most repair processes are thought to be activated upon germination, spores may already be equipped with repair enzymes (produced during endospore formation) that allow for quick repair upon germination. Recently, it was suggested that protein synthesis already occurs during the early stages of germination (Sinai et al. 2015), although it remains to be elucidated if this serves a role in repair processes.

Spore Emergence and Outgrowth in Foods

Minimal heat treatments of foods meet the desire for mildly processed food. This may eventually lead to spores in food products that are not completely inactivated but are instead sublethally damaged. In addition, highly heat-resistant spores may survive commercial sterilization processes (Postollec et al. 2012). If (sufficient) inactivation of undesirable spores in foods is not achieved by processing, blocking germination or preventing outgrowth of emerged cells are effective alternative or complementary strategies. The ultimate ability of (damaged) spores to recover, emerge, and grow out is largely determined by environmental conditions such as the food matrix composition (water activity, nutrients, pH, antimicrobials) and storage conditions (oxygen availability and humidity) (Mafart 2000, Samapundo et al. 2014b, Smelt et al. 2008, Warda et al. 2015).

Small lipophilic compounds such as undissociated sorbic acid (HSA) and low concentrations of corresponding alcohols such as hexanol and pentanol have been shown to block germination of *B. cereus* spores almost completely (van Melis et al. 2011, 2012). Conceivably, this results from disturbance of the organization and presumed protein-protein interactions in the germinosome. It is known that HSA molecules are embedded near the surface of the outer phospholipid layer of the cell membrane; this suggests that interactions between head groups of phospholipids and/or membrane proteins can be affected (Chu et al. 2009). In this scenario, germination signaling could be blocked even when the GRs are activated by nutrients (van Melis et al. 2014). Some natural antimicrobials also have potential to inhibit spore-forming bacteria. Nisin, for instance, is a well-known food additive that prevents the formation of a membrane potential in the outgrowing spore (Gut et al. 2011). Sorbic acid also severely inhibits outgrowth of germinated spores at pH 5.5 (van Melis et al. 2014).

Spore survival, germination, and outgrowth are also influenced by stresses that spores undergo during food processing and storage (Mafart 2000, Samapundo et al. 2014b, Smelt et al. 2008, Warda et al. 2015). By applying Anopore technology, it was found that spores that had undergone heat stress showed more heterogeneous germination and outgrowth than non-heat-stressed spores (van Melis et al. 2014). Interestingly, heterogeneity in outgrowth of heat-damaged spores was also observed in different food matrices, such as broccoli and rice-based media as well as mildly acidified (pH 5.5) meat-based brain heart infusion (BHI) medium (Warda et al. 2015). Spores that were subjected to (and survived) wet heat for 1 min at 95°C showed delayed outgrowth on these media compared with non-heated spores. For heat-treated *B. cereus* spores, outgrowth of spores was higher on BHI pH 7.4 than on rice, broccoli, and BHI pH 5.5. Such findings highlight the

impact of different (model) food recovery media on outgrowth efficiency and heterogeneity of non-heat-treated and heat-damaged *B. cereus* spores.

Resistance and germination properties, damage-repair mechanisms of spores, recovery of emerging spores, and ultimate outgrowth abilities are important aspects in the design of preservation strategies to control sporeformers in foods. Assessment of germination and outgrowth performance in (model) foods with and without added preservatives using single-spore/-cell approaches (described in more detail below) will provide further understanding and thus greater ability to predict spore germination and outgrowth behavior.

RECENT TECHNIQUES TO STUDY HETEROGENEITY IN SPORE GERMINATION, EMERGENCE, AND OUTGROWTH

Time-Lapse Phase-Contrast and Fluorescence Microscopy

Time-lapse phase-contrast microscopy is a powerful tool to follow the development of one single cell or spore in time and allows for simultaneous monitoring of hundreds of cells (or spores) during their development (de Jong et al. 2011). By making use of changes in the refractive index (Zernike 1955) in phase-bright dormant spores (low water and high Ca-DPA content) and phase-dark germinated spores [release of Ca-DPA and uptake of water during germination (Setlow 2006)], sporulation, spore dormancy, and spore germination can be followed in time using specific microscopic equipment and accompanying software. Time-lapse phase-contrast microscopy has been widely used to study the kinetics of sporulation and germination, especially the length of the lag time (i.e., the time between the first exposure of a spore to a germinant and actual germination) of multiple individual spores of Bacilli and Clostridia (Billon et al. 1997, Pandey et al. 2013, Stringer et al. 2005, Wang et al. 2011). Furthermore, the use of molecular labels, such as fluorophores, allows for the identification of genetic or epigenetic inherited traits during sporulation (e.g., gene expression dynamics) (de Jong et al. 2010, Eijlander & Kuipers 2013, Young et al. 2012).

Specifically developed software programs that tag single spores and quantify their phase-contrast intensities enable (semi)automatic analysis of the images obtained (Kong et al. 2011, Pandey et al. 2013). Such advances have allowed for simultaneous investigation of the effect of wet heat on spore germination, outgrowth, and vegetative growth of cells that emerged from heat-stressed spores (Pandey et al. 2013). Using these techniques, these authors demonstrated that wet heat treatment decreased the number of *B. subtilis* spores that germinated and grew out and increased the time needed for spore germination. However, once a cell emerged, the outgrowth time and generation times were not affected by the heat treatment to which the spores were exposed (Pandey et al. 2013).

Challenges for the future lie in molecular imaging approaches to obtain a more complete understanding of the molecular mechanisms that determine the observed differences in spore germination and/or outgrowth kinetics. Ideally, germination and outgrowth behavior of individual spores can then be linked with the history of gene expression and/or protein content or protein localization in the same spore. Further development of the microscope automated fluidic systems (Ducret et al. 2009) can potentially play a crucial role.

Surface-Enhanced Raman Spectroscopy

Surface-enhanced Raman spectroscopy is another powerful technique that allows for analysis of spore germination at the single-spore level. Raman spectroscopy measures inelastic (Raman) scattering of laser light by the illuminated sample. The intensity of Raman scattering depends on

the amount of various molecular components of the sample (Peng et al. 2009). The technique can be applied to measure levels of Ca-DPA in individual spores and therefore the release of Ca-DPA from spores during germination (Daniels et al. 2006, Evanoff et al. 2006). Hereby, it was shown that germination of spore populations occurs faster and is less heterogeneous at higher temperatures (up to 55°C) and at higher nutrient concentrations (Daniels et al. 2006). Raman spectroscopy is commonly used together with laser tweezers [laser tweezers Raman spectroscopy (LTRS)], which enables quantitative measurements of Ca-DPA and the kinetics of its release from single spores trapped at the focus of a laser beam (Chen et al. 2006, Ghosh et al. 2009, Kong et al. 2011). Analysis of the Ca-DPA-specific Raman spectral peaks with LTRS indicates differences between dormant and superdormant spores in the Ca-DPA environment in the spore core; such differences can be used to predict the presence of superdormant spores in spore populations (Ghosh et al. 2009).

When Raman spectroscopy is combined with methods that provide additional information about the shape of the spore, its size, and its refractive index (e.g., phase-contrast microscopy), this technique can be applied not only to the optically trapped spores but also to the single spores adhered to a microscope coverslip, which gives essentially the same results (Kong et al. 2010). It was also demonstrated that the intensity of phase-contrast images is directly proportional to the level of Ca-DPA inside the spore (Kong et al. 2010). This observation suggests that phase-contrast microscopy alone could be used to estimate levels of Ca-DPA in individual spores and to measure the kinetics of Ca-DPA release upon germination.

Expanding the research focus from widely studied laboratory strains to more industrially relevant species and strains has allowed for the identification and characterization of novel cellular parameters that contribute to (super)dormancy and spore germination heterogeneity (A.O. Krawczyk, E.M. Berendsen, A. de Jong, J. Boekhorst, M.H.J. Wells-Bennik, O.P. Kuipers, R.T. Eijlander, unpublished results). A combination of advanced genome sequencing and phenotypic characterization using single-cell techniques may provide more insight into one of the main remaining questions in spore germination research (Setlow 2014): What happens during the highly variable and often long lag period between the exposure of spores to nutrient germinants and the commitment of spores to germinate?

Flow Cytometry and Single-Cell Sorting

Flow cytometry (FCM) in combination with the use of fluorescent probes has been used extensively in microbiology. Applications include the detection and identification of microorganisms, assessment of the viability and activity of vegetative cells, and assessment of the germination and outgrowth of *Bacillus* spores (Cronin & Wilkinson 2010, den Besten et al. 2012, Mols & Abee 2011, Müller & Nebe-von-Caron 2010). Apart from heterogeneity in spore germination, considerable variability also occurs in lag periods between the different phases of the outgrowth of germinated spores (Stringer et al. 2005, Yi & Setlow 2010).

A pipeline was recently developed that allows for the selection of subpopulations of germinated and (super)dormant spores (upon single or successive exposure to nutrient and non-nutrient germinants such as Ca-DPA) and further assessment of their germination and outgrowth potential (**Figure 3**) (Y. Xiao, M. Tempelaars, T. Abee, unpublished data). For this purpose, single-cell sorting using FCM is applied to introduce single spores into individual wells of 96- or 384-well plates containing liquid (food) media and/or agar-based media at selected temperatures. Subsequent germination and outgrowth behavior in the wells can be assessed by optical density measurements of the multi-well plates. This way, the effects of a range of highly relevant parameters that may determine spore (super)dormancy and outgrowth can be determined. These include (*a*) the impact

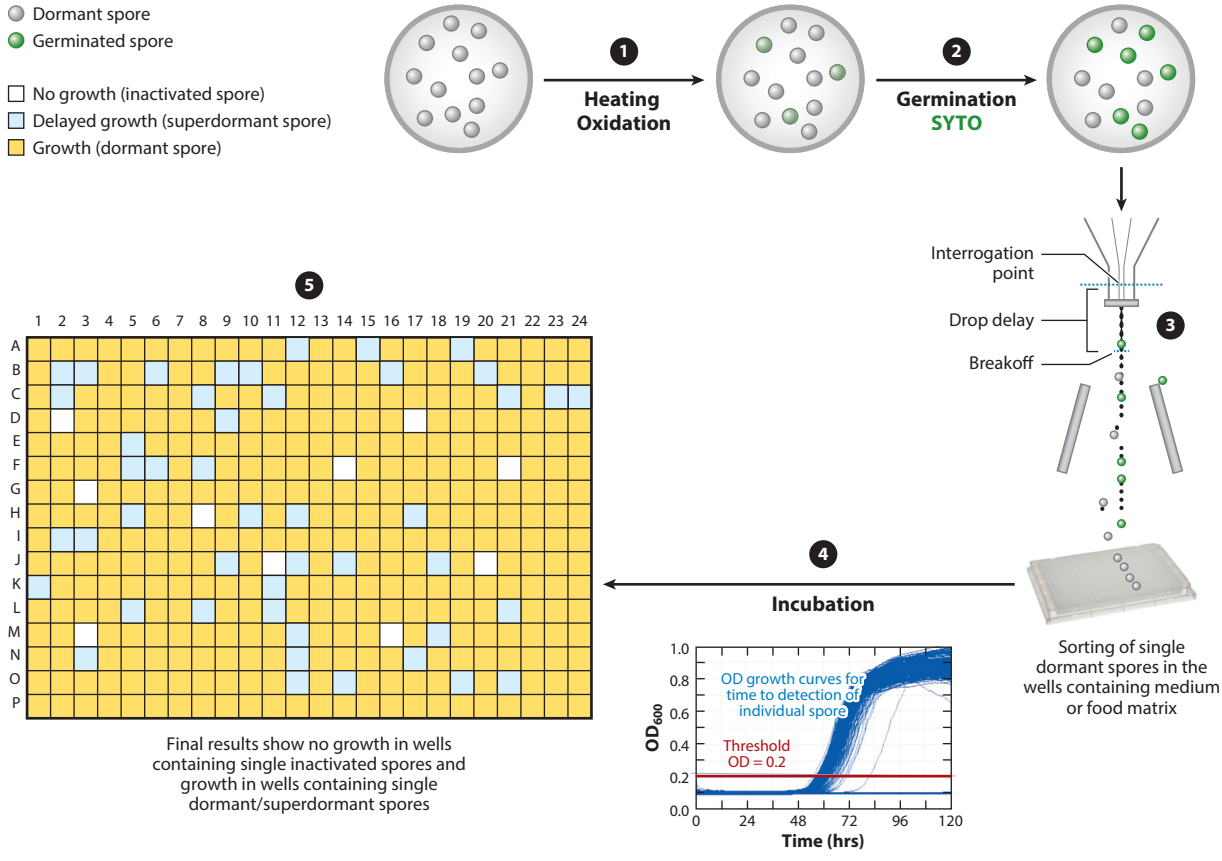

Figure 3

Sorting of spore populations using flow cytometry, followed by incubation of individual spores in a single well of a multi-well plate that contains the selected medium. ❶ Heating/oxidation: Spores are pretreated by heating or oxidation. ❷ Germination SYTO: Pretreated spores are exposed to a specific germinant, and germinated spores can be stained by fluorescence dye SYTO (*green*), which is specifically bound to nucleotides in the spore core. ❸ Sorting: Stained spores are discarded based on their significantly higher fluorescence intensity as detected by flow cytometry. Unstainable spores are individually sorted in the wells containing medium or food matrix. ❹ Incubation: Optical densities of the cultures in the multi-well plate are measured to assess germination and outgrowth of sorted spores in time. ❺ Final results show no growth in wells containing inactivated spores, growth in wells containing dormant spores, and delayed growth in wells containing superdormant spores. Abbreviations: OD, optical density; SYTO, SYTO(R)9 Green-Fluorescent Nucleic Acid Stain (Molecular probes, Carlsbad, California).

of diversity using spores derived from *Bacillus* spp. and *Clostridium* spp. isolates from different environments (e.g., soil, food, and clinical samples); (*b*) differences in sporulation history, including temperature and media effects; (*c*) the impact of processing, including heating, HHP, and exposure to disinfectants; (*d*) the germination and outgrowth in liquid media versus growth on agar surfaces; and (*e*) the effect of aerobic versus anaerobic conditions. Recent studies using a collection of twenty different *B. cereus* food isolates showed large differences in relative fractions of superdormant spores between different strains, and large heterogeneity in outgrowth performance of sorted single (super)dormant spores (Y. Xiao, M. Tempelaars, T. Abee, unpublished data). This approach will lead to further understanding of spore superdormancy and will allow for identification and quantification of abiotic factors and spore parameters that contribute to this phenomenon.

BOTTLENECKS IN PREDICTIONS: VARIABILITY IN SPORE INACTIVATION, GERMINATION, AND OUTGROWTH

Many models of various detail and complexity exist to describe survival and growth of spore-forming bacteria (Mafart et al. 2010, Zwietering & den Besten 2011). To make relevant predictions of the behavior of particular species in practice, two important sources of variability are (*a*) large differences between various strains belonging to the same species and (*b*) the heterogeneous behavior of individual cells within an isogenic population, both with respect to growth performance and inactivation robustness. These sources of variability are often much more important than the exact description of the kinetics of a single experiment.

Variability in Germination and Outgrowth

Our ability to predict the growth behavior of spore-forming bacteria requires quantitative information on the duration of the lag time (including germination time) and the growth rate of the organisms in the environment of interest. Growth rates are generally much better quantitatively described than the first parameter. Lag time is associated with the period between the end of dormancy of a spore and the time at which cell division starts. As discussed in the section Germination and Recovery, quantification of lag times is challenged by large variability in behavior of individual spores. This complicates prediction of lag times based on germination duration and poses challenges in modeling of germination and outgrowth processes (Augustin 2011). For instance, no clear correlation was observed between the times for germination, emergence, recovery, and doubling of individual spores of nonproteolytic *C. botulinum* (Stringer et al. 2005, 2009, 2011; Webb et al. 2007). This is consistent with observations on *B. cereus* spores in which germination efficiency of individual spores was not a good predictor for variability in final outgrowth (den Besten et al. 2012).

When spores are sublethally damaged and/or outgrowth conditions are suboptimal, then lag time and outgrowth behavior of the individual spores have been demonstrated to be very heterogeneous (discussed in the section Germination and Recovery, and in references therein). Nevertheless, assumptions are used in risk-assessment studies on lag times of spore-forming bacteria. Membré et al. (2008) developed a lag-time model based on the gamma concept (Zwietering et al. 1992); it took into account single-spore variability in lag time as a function of storage temperature, pH, water activity, the effect of heat treatment, and the effect of spore concentration (heat-treatment survivors). Membré et al. (2008) applied a model to predict the lag time of heat-treated *B. cereus* spores in chilled foods, and this serves as an example of the integration of lag-time variability in risk assessment.

The occurrence of variations in growth rates between strains or species of spore-forming bacteria is also well recognized. Growth ranges have been reported for groups of strains that share ecological similarities. *B. cereus* strains are often grouped on the basis of their psychrotrophic and mesophilic growth characteristics. Also, growth ranges have been reported for strains with different characteristics and origins (e.g., emetic, diarrheal, or food and environmental *B. cereus* isolates) (Carlin et al. 2006, Pielaat et al. 2005). Guinebretière et al. (2008) reported the growth ranges of the seven genetic groups of *B. cereus*. Afchain et al. (2008) proposed mean cardinal temperatures for six of these genetic groups of *B. cereus*, and yet another study described the variability in cardinal growth parameters for these groups (Carlin et al. 2013). Importantly, these cardinal growth parameters and corresponding variabilities can be used to estimate the growth performance of different genetic groups of *B. cereus*, which provides more precision in quantitative exposure assessment than does taking the *B. cereus* group as a whole.

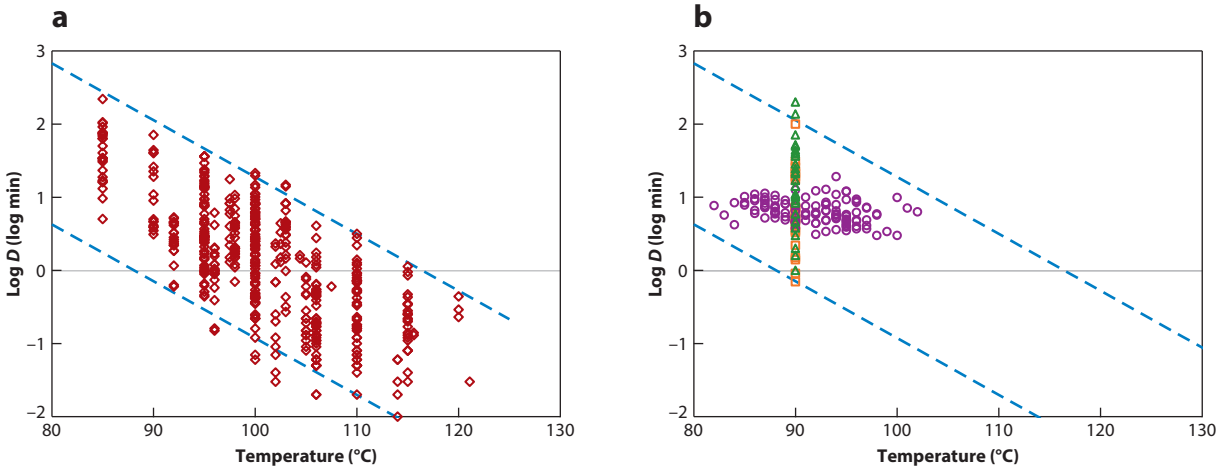

Figure 4

Heat resistance of *Bacillus cereus* spores as a function of temperature. (*a*) $\text{Log}_{10}D$ values versus temperature for *Bacillus cereus* spores (*red diamonds*) reported by van Asselt & Zwietering (2006), with the blue dashed lines representing the 95% prediction intervals based on all data. (*b*) The 95% prediction intervals reported by van Asselt & Zwietering (2006), the $\log_{10}D_{90}$ data of the psychrotrophic strains (*orange squares*) and of the mesophilic strains (*green triangles*) reported by Membré et al. (2006), and the $\log_{10}D$ data reported by Luu-Thi et al. (2014) (*purple circles*).

Variability in Spore Heat Inactivation

Commonly used parameters to describe heat inactivation are the *D*-value, which is the time needed to reduce the microorganisms by 90%; and the *z*-value, which is the temperature increase needed to reduce the *D*-value by 90%. Reported values of these parameters vary among studies. Integration of the findings of individual studies in a meta-analysis allows for global estimation of parameters and quantification of their variabilities. The meta-analysis performed by van Asselt & Zwietering (2006), for instance, compiled *D*-values from inactivation studies that were obtained over a long period of time in various laboratories. Using data on various strains and different applied methods in a range of products and media, the authors reported the $\log_{10}D_{\text{ref}}$-value and corresponding variability expressed in the standard deviation for different pathogenic sporeformers. The width of the 95% prediction interval of $\log_{10}D_{120}$ (the *D*-value at 120°C) for *B. cereus* spores was as large as 2.2 $\log_{10}$ (see **Figure 4*a***). Notably, Membré et al. (2006) reported D_{90}-values (the *D*-value at 90°C) for spores of numerous psychrotrophic and mesophilic *B. cereus* strains that coincided with the 95% prediction interval reported by van Asselt & Zwietering (2006) (**Figure 4*b***). Such quantitative information on variability in heat resistance is useful to improve quantitative exposure assessments and has been taken into account in some risk-assessment studies (Afchain et al. 2008, Daelman et al. 2013, Membré et al. 2006). As intrastrain diversity in heat resistance is well recognized, Luu-Thi et al. (2014) assessed the strain variability with respect to heat resistance of *B. cereus* spores that were produced under well-controlled standardized conditions. The meta-analysis data of van Asselt & Zwietering (2006) could be used to benchmark this strain variability in spore heat resistance with the overall variability in spore heat resistance (**Figure 4*b***). This highlighted that strain variability in spore heat resistance accounted for approximately two-thirds of the variability found in the literature (as retrieved by van Asselt & Zwietering 2006). The next step is a comparison of strain variability with other sources of variability, including that introduced by biologically independent experiments, to better understanding the different sources of variability. When major sources of

variability can be linked with genetic or physiological biomarkers, this will ultimately lead to a better understanding of underlying biological principles and allow for optimized control measures and interventions.

SUMMARY POINTS

1. Novel insights into which genes determine traits such as the heat resistance of spores and spore germination of *Bacillus* species present in the food chain were obtained by integrated approaches using whole-genome sequencing, transcriptomics, and bioinformatics, in combination with phenotypic characterization of spores.
2. Resistance properties of spores vary between species and strains in foods and in food ingredients and are largely determined by genetic (possibly mobile) elements. In addition, environmental conditions during spore formation and spore maturation also contribute to variation.
3. The impact of conditions during sporulation that determine germination properties and heterogeneity therein can be studied at the single-cell level using current microscopy and spectroscopy techniques. This will bring about novel insights regarding these complex events.
4. Spore germination and emergence of spores is not always understood in food isolates under conditions prevailing in the food chain but can now be studied using single-spore sorting (flow cytometry) followed by assessment of germination and outgrowth in time in (model) food matrices.
5. Poor germination efficacy of spores has been shown to hamper enumeration on standard laboratory media, leading to gross underestimation of highly heat-resistant spores in the absence of suitable germination triggers.
6. Quantification of the effect of various factors that determine inactivation of spores, germination, lag time, and growth are crucial for the prediction of the behavior of spore-forming bacteria in the food chain.
7. For predictive modeling purposes and ultimately improved control of spores in the food chain, it was established that variability in spore inactivation and outgrowth is in large part related to variations between strains and heterogeneous behavior of individual cells.

FUTURE ISSUES

1. Unraveling the complex processes of sporulation and germination, as well as the factors that influence spore properties such as heat resistance in diverse *Bacillus* spp. and *Clostridium* spp., will be supported by multidisciplinary integrative approaches that use techniques such as high-throughput phenotypic screening, genomics and transcriptomics, and advanced imaging.
2. Further insights into the mechanisms that determine biological phenomena such as heat resistance, germination, and outgrowth allow for improved detection of relevant traits in the food chain and improved enumeration of species. They also allow for potential novel intervention strategies that prevent germination and outgrowth.

3. Ultimate prediction (using models) of the behavior of sporeformers in the food chain requires knowledge on factors that determine spore survival and outgrowth, as well as information on how different factors contribute to variability in these characteristics.
4. When introducing specific spores into the environment in high numbers (as, for instance, animal probiotics and agricultural applications for insect control and improved crop yields), care must be taken to counterselect for undesirable traits (e.g., high spore heat resistance) that have potentially negative effects in the food chain.

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