Quantification of *Bacillus cereus* stress responses

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Quantification of *Bacillus cereus* stress responses

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Abstract

The microbial stability and safety of minimally processed foods is controlled by a deliberate combination of preservation hurdles. However, this preservation strategy is challenged by the ability of spoilage bacteria and food-borne pathogens to adapt to stressing environments providing cell robustness. *Bacillus cereus* is a toxin-producing, spore-forming bacterium, and is able to survive minimal processing conditions. A quantitative approach was followed to gain insight in *B. cereus*’ stress adaptive behavior at population, individual cell and molecular level. *B. cereus*’ ability to adapt to salt stress and gain robustness towards subsequent heat challenge-stress exposure was quantified in detail using primary kinetics models. The adaptive salt stress response was influenced by the adaptation-stress concentration, the growth phase of the cells, strain diversity and the culturing temperature during adaptation-stress treatment. The nonlinear nature of the heat inactivation kinetics suggested heterogeneity within the population with respect to stress adaptive behavior. The direct-imaging-based Anopore technology was used to quantitatively describe the population heterogeneity of *B. cereus* upon mild and severe salt stress treatments and during low temperature growth. Fluorescent labeling of cells provided insights in the origin of stress-induced population heterogeneity. Then, to elucidate adaptive salt stress responses at molecular level, the genome-wide transcriptome profiles of mildly and severely salt-stressed cells were compared. Various transcriptome responses could be correlated to phenotypic features of salt stress-adapted cells. Comparison of the transcriptome profiles of salt stress-adapted cells to those that were exposed to mild heat, acid and oxidative stress, directed to potential cellular biomarkers for stress adaptation. The selected candidate-biomarkers – the transcriptional regulator σB (activating general stress responses), catalases (removing reactive oxygen species), and chaperones and proteases (maintaining protein quality) – were measured upon adaptation-stress treatment at transcript, protein and/or activity level, and their induction was correlated to adaptation-stress induced robustness towards challenge-stress. Various candidate-biomarkers were suitable to predict the robustness level of adaptation-stress pretreated cells towards challenge-stress, and are therefore potential predictive cellular indicators for adaptation-stress induced robustness. The predictive potential of transcripts differed from that of proteins and activity level, underlining the significance to evaluate predictive potential of candidate-biomarkers at different functional cell levels. This quantitative understanding of *B. cereus*’ stress adaptive behavior provides mechanistic insights and opens up avenues to come to a mechanism-based approach for designing mild preservation strategies.
Chapter 1

Introduction and outline of the thesis
Abstract

The trend towards milder processing conditions in the food industry addresses the consumers’ demand for fresh and nutritious food products that require little preparation time. Mild processing conditions do not result in complete inactivation of microorganisms, and the spore-former Bacillus cereus is able to survive in minimally processed foods. B. cereus is a toxin-producing, food-borne pathogen that can evoke two types of gastrointestinal diseases, namely, emesis and diarrhea. Its ability to adapt to mild stressing environments and gain cell robustness necessitates a deliberate combination of mild preservation factors in minimally processed foods. In this research project, a mathematical modeling approach was used and combined with individual cell imaging analyses and molecular techniques for elucidating B. cereus’ behavior in response to mild stressing conditions. This chapter introduces B. cereus as food-borne pathogen and includes an introduction on the research topics and an outline of the thesis.
Bacillus cereus is a spore-forming bacterium. Bacillus cereus is a Gram-positive, facultative anaerobic bacterium belonging to the genus Bacillus within the family Bacillaceae. A common feature for bacilli is the production of endospores which are highly resistant to chemicals, heat, UV light, and desiccation (77). Spores are formed inside the mother cell upon starvation and released into the environment after lysis of the mother cell and can survive for years (72). The first isolated B. cereus strain is known as the type strain B. cereus ATCC (American Type Culture Collection) 14579, and was isolated from air in a cow-shed in 1887 (31). The genome sequence of B. cereus ATCC 14579 became publically available in 2003 (41), enabling us to better understand the genetic basis of many features of its behavior. Phase-contrast images of exponentially growing vegetative cells, sporulating cells and spores of B. cereus ATCC 14579 are shown in Figure 1.

![Figure 1. Vegetative cells and spores of Bacillus cereus ATCC 14579. Phase-contrast images of vegetative cells (a), sporulating cells (b, kindly provided by Marcel H. Tempelaars), and spores (c).](image)

The Bacillus cereus group. B. cereus belongs to a group of closely related bacilli, the B. cereus group, also known as B. cereus sensu lato. The B. cereus group comprises six species: B. mycoides, B. pseudomycoïdes, B. weihenstephanensis, B. anthracis, B. thuringiensis, and B. cereus (sensu stricto) (84). These species have been viewed as separate species, mainly by their phenotypic characteristics such as pathogenicity patterns and growth characteristics.

B. mycoides and B. pseudomycoïdes are the least-studied species within the B. cereus group. B. mycoides is phenotypically differentiated from the other B. cereus group species by its rhizoidal colony shape. In 1998, the species B. mycoides was separated into two groups, and the new species name B. pseudomycoïdes was proposed (68). B. weihenstephanensis is a psychrotolerant species that can grow below 7°C, but not at 43°C, and has specific signature sequences in the 16S rDNA and the cold shock protein cspA gene.
Psychrotolerant strains from the *B. cereus* group are not necessarily *B. weihenstephanensis* strains because also *B. cereus* isolates that were able to grow below 7°C have been identified (81). *B. thuringiensis* is characterized by its production of insecticidal crystal proteins (Cry and Cyt) and sprays containing these toxins have been widely used for the biological control of insects in crop protection (32). The genes encoding the toxins are located on large plasmids (75). Transgenic expression of Cry toxins in corn and cotton crops has been proven to control insect pests efficiently resulting in increased crop yields and reduction of the use of chemical insecticides (73). *B. thuringiensis* is usually considered harmless to humans, although human infections may occur (36). *B. anthracis* causes the fatal disease anthrax in animals and humans. This species carries two large plasmids encoding the main virulence factors: pXO1 encoding the anthrax toxin complex, and pXO2 containing an operon for the synthesis of a polyglutamate capsule, a structure that is important for the ability to evade the host immune system (64). In recent years, *B. anthracis* has become a notorious member of the *B. cereus* group due to the use of *B. anthracis* spores as biological weapon (43). *B. cereus sensu stricto* species comprises all strains of the *B. cereus* group that do not belong to any of the above-mentioned species due to the absence of distinctive traits (80). This species includes pathogenic strains that are associated with non-gastrointestinal and gastrointestinal illnesses. Non-gastrointestinal illnesses have been especially associated with immunologically compromised patients, neonates and drug addicts, and includes meningitis and pneumonia (23, 45). *B. cereus* is commonly known as food-borne pathogen causing gastrointestinal diseases (24, 76, 80).

**Bacillus cereus as pathogenic organism.** *B. cereus*’ ability to form endospores provides this organism an ultimate surviving and spreading advantage in a wide range of niches. *B. cereus* is ubiquitously present in nature and has been found in many types of soils, dust, cattle feed and dung, and in guts of invertebrates (42, 76). Not surprisingly, *B. cereus* has been isolated from a variety of food products (4, 16, 18, 93), including rice, pastry, vegetables and vegetable products, milk and milk products and ready-to-eat foods. *B. cereus* is able to evoke two types of food-borne gastrointestinal diseases, namely, emesis and diarrhea.

The emetic syndrome (an intoxication) is caused by the cereulide toxin, which is preformed in foods and elicits vomiting 1 h to 6 h after ingestion (76). The emetic type of disease is associated mostly with starchy food, like rice and pasta dishes (76). Because cereulide is heat-, acid-, and trypsin-stable, it will not be inactivated upon reheating of food or, after ingestion, in the gastrointestinal tract. The emetic symptoms are usually relatively
Introduction and outline of the thesis

Mild, but occasionally have been implicated in severe forms of food poisoning resulting in acute liver failure (20, 60). Cereulide is structurally related to the potassium ionophore valinomycin (3, 63), and is toxic to mitochondria causing inhibition of fatty acid oxidation (63, 80). The emetic toxin is synthesized enzymatically by a nonribosomal peptide synthetase, and the cereulide synthetase (ces) gene cluster is located on a plasmid (21). Cereulide is produced by specific subgroups of \textit{B. cereus} (25, 89) and a few \textit{B. weihenstephanensis} isolates (83). The synthesis of cereulide is influenced by temperature, and maximal production appeared to occur at lower temperatures (12°C to 15°C) (29).

The diarrheal syndrome (a toxico-infection) occurs after consumption of \textit{B. cereus} spores or vegetative cells and is caused by several enterotoxins, which are produced by vegetative cells multiplying in the small intestine. The diarrheal type of food-borne disease is accompanied by cramps and diarrhea occurring 8 h to 16 h after ingestion (76). A wide range of food products is associated with the diarrheal syndrome, including meat products, soups, vegetable, sauces and milk products. The most-intensively studied enterotoxins that have been associated with the diarrheal disease are haemolysin BL (Hbl), non-haemolytic enterotoxin (Nhe) and cytotoxin K (CytK). These enterotoxins belong to the family of pore-forming toxins and showed cytotoxic activity (58, 59). In contrast to Hbl and CytK, Nhe has long been considered to be non-haemolytic and to display cytotoxic properties only (10, 55, 58). However, a recent study showed that Nhe also displays haemolytic activity (28).

Both Hbl and Nhe are three-component toxin complexes. Hbl consists of the three proteins B, L1 and L2, and Nhe is composed of the three proteins NheA, NheB and NheC, and all three components are necessary for maximal cytotoxic activity (9, 55). Both Hbl and Nhe are encoded by three chromosomal genes co-transcribed as operons (\textit{hblCD4} [35, 74] and \textit{nheABC} [55], respectively). CytK is a single component protein toxin and is transcribed from the \textit{cytK} gene (58). Hbl, Nhe, and CytK are part of a large virulence regulon of \textit{B. cereus} that is controlled by the transcriptional regulator PtcR (33). The protein components of Hbl and Nhe and CytK are secreted from the \textit{Bacillus} cell by secretion machineries (80, 88). In addition to Hbl, Nhe and CytK, \textit{B. cereus} produces several less well-characterized proteins, including cereolysin O (48), haemolysin II (6) and haemolysin III (7).

The presence of virulence genes highly varies among \textit{B. cereus} isolates, and a Dutch survey showed that almost all \textit{B. cereus} isolates, originating from a wide range of food samples, harbored genes of at least one enterotoxin (93). The level of toxin expression is controlled in response to cell density, environmental conditions and the metabolic state of the cell (24, 80), and plays a role in the pathogenic potential of \textit{B. cereus}. The number of \textit{B. cereus} cells required to cause disease has not be determined, but in most foods incriminated
in disease cases the level was at least $10^5$ CFU g$^{-1}$ food (76, 80). *B. cereus* related food-borne diseases occur frequently, but estimation of the actual incidence is difficult. Because the symptoms are generally mild and recovery usually occurs within 24 h, most of the food poisoning cases are not diagnosed, resulting in an under representation of the number of *B. cereus* infections in the statistics of food-borne diseases. Nevertheless, the majority of the food-borne outbreaks in The Netherlands in 2008, with known causative agents, was caused by *B. cereus* (22). Most of the strains related to cases or outbreaks of *B. cereus* food-borne poisoning showed to be unable to grow below 10°C (2, 34), pointing to the importance of appropriate refrigeration of foods contaminated with *B. cereus* to control its growth and toxin production in foods. Non-adequate chilling was indeed reported in various *B. cereus* food-borne incidents (20, 27, 57, 60).

*Bacillus cereus* as spoilage organism. One of the industrial environments where *B. cereus* is causing spoilage problems, is the dairy industry. The omnipresence of *B. cereus* in the farm environment makes it impossible to avoid the presence of *B. cereus* in raw milk (92). The pasteurization process kills vegetative cells and growth competition in pasteurized milk from other vegetative bacteria is therefore eliminated. Spores are able to survive pasteurization heating regimes, and can germinate in pasteurized milk even more efficient than in raw milk (82). Because spores are hydrophobic, they easily attach to the surfaces of process lines and subsequently can form biofilms. These multi-cellular biofilm complexes, consisting of spores, vegetative cells and exopolymeric substances, are a continuous source of contamination of pasteurized milk (4). During storage, generally below 7°C, the psychrotolerant *B. cereus* strains are able to grow (51) causing ‘bitty cream’ and ‘sweet curdling’ defects (19, 62). Reported detrimental symptoms upon consumption of pasteurized milk kept at refrigeration temperatures are rare (50), indicating that *B. cereus* is mainly a spoilage organism in milk rather than a cause of food-borne diseases.

Adaptive stress responses in minimal processing. The last decades there has been an increasing trend in production and sales of mildly processed ready-to-use foods (16, 70). Spore-formers like *B. cereus* are able to survive mild processing and are therefore challenging the food processing industry to control their growth in minimally processed foods. The microbial safety and stability of minimally processed foods is based on simultaneous or successive application of mild preservation factors, known as hurdles (53). An appropriate combination of hurdles controls the growth of microorganisms and stabilizes the sensory and nutritive properties. However, application of mild preservation
stresses has a fundamental impact on the behavior of stress-exposed cells (1, 8). Upon stress sensing, bacteria are able to activate adaptive stress networks leading to transcription, translation and active stress-related cellular components. An activated adaptive stress response provides cell resistance to harsher conditions including stresses other than the one that induced the adaptive stress response (Figure 2).

Figure 2. Upon sensing mild adaptation-stress(es) bacteria activate adaptive stress response mechanisms resulting in transcription, translation and active stress-related cellular components providing cell protection to subsequent severe challenge-stress conditions.

These adaptive stress responses are of practical importance for minimally processed foods because activation of the adaptive stress response upon exposure to the first hurdle may render the organism more resistant to the subsequent hurdle(s). Therefore, adaptive stress responses may antagonize the benefits of the hurdle preservation strategy, and this underlines the need to better understand adaptive stress response mechanisms.

Previous studies showed that some mild stress conditions could indeed induce (cross-)protective effects towards otherwise lethal stress conditions for *B. cereus* (12, 13, 71, 86). The adaptive stress response of *B. cereus* upon both mild heat and acid stress treatment has been investigated in more detail using proteomic and transcriptomic approaches. Mild heat exposure resulted in increased synthesis of a set of conserved heat shock proteins, including the chaperones DnaJ, DnaK, GroEL, and the proteases ClpC and ClpP (71) that play a role in repair and maintenance of protein quality (65). In addition, the general stress response transcriptional regulator σB (86) and thioredoxin, a protein known to be involved in cellular defense against oxidative stress (67), were produced upon mild heat stress treatment. As expected, exposure of *B. cereus* cells to mild heat stress resulted in
increased transcription of the corresponding genes of these mild heat-induced proteins (87). Also mild acid stress treatment induced chaperones (71), $\sigma^B$ (86), and oxidative stress-related proteins (12), and these responses were also shown to be induced at transcriptome level upon exposure to inorganic and organic acid (66).

To date, only limited information is reported about the salt stress response of *B. cereus*. But interestingly, the available data showed that in addition to mild heat and mild acid stress treatment, also mild salt stress exposure induced some proteins that are involved in maintenance of protein quality (DnaK), defense mechanisms against oxidative stress (thioredoxin), and the transcriptional regulator $\sigma^B$ (71, 86), pointing to a possible role of these stress-related factors in general stress adaptation. Analyses of the transcriptome profiles of *B. cereus* cells in response to salt stress exposure will provide a better understanding of the underlying mechanisms of the adaptive salt stress response of *B. cereus*. A highly interesting frontier of knowledge is the linkage of molecular responses in the cell to phenotypic features. Upon stress exposure a wide spectrum of cellular responses are activated, and some of these key responses might potentially function as biomarker for phenotypic behavior. The search for biomarkers for adaptation-stress induced cell resistance should direct to cellular indicators of which the induction levels upon adaptation-stress treatment are correlated to adaptation-stress induced cell resistance, and therefore could be used to early detect and predict this stress adaptive behavior.

Most of the studies that investigated adaptive stress response mechanisms of pathogens, used optimal culturing temperature during adaptation-stress treatment (see e.g. references 12, 13, 47, 56, 71, 78, 86). However, during processing, distribution and storage the temperature of foods may be lower because chilling is commonly used in the minimal processing food chain. The limited available data describing the effect of low incubation temperature on the adaptive stress response showed that the adaptive acid stress response of *Listeria monocytogenes* was reduced when the adaptation-stressing temperature decreased (46). Therefore, investigation of the effect of low incubation temperature on adaptive stress responses of food-borne pathogens is of great relevance to provide realistic estimations of the impact of adaptive stress responses on microbial behavior in the chilled food chain.

**Heterogeneity in stress response.** Individual bacterial cells within a population can display variable degree in their stress responses, even in a homogeneous environment, and this heterogeneity is of particular interest when we wish to control bacterial growth or to inactivate bacteria. Heterogeneity in growth performance and stress resistance can be manifested in the occurrence of a subpopulation that show a different behavior compared to
the main fraction of the population. Heterogeneous stress resistance can be confirmed to be non-inheritable or inheritable (i.e. physiological or genotypic, respectively) (5, 11). A non-inheritable heterogeneous phenotype is only temporarily displayed, and rapidly reverses to the original phenotype. An inherited heterogeneous phenotype is displayed by genetic variation within the population and these genetic changes are relatively stable and can be detected as mutations using molecular techniques and through selection for the mutated phenotype. Selection for the mutated phenotypes occurs when only the mutated fraction is able to survive the applied selection regime. Noteworthy, inheritance of a phenotype from one generation to the next does not always depend on mutations in DNA sequences, and this so-called epigenetic inheritance (e.g. methylation of the DNA) (90) is challenging our search for underlying mechanisms of population heterogeneity. The presence of individual cells in a population that exhibit dissimilar phenotypes can increase the overall fitness of the population and this ‘bet hedging’ strategy of bacteria was described as a means by which a population ensures that a part of the offspring will be optimally suited for the future environment (90).

Heterogeneity within a population results in a non-uniform response, but this heterogeneity at the individual cell level is masked in conventional microbial culturing techniques that rely on data averaged across high numbers of cells. Therefore, indirect and direct methods have been developed to study heterogeneity of responses of individual cells. A frequently used indirect method to estimate the lag times of individual cells under stressing conditions is a turbidimetric approach (see e.g. references 30, 54, 79). The detection times or optical density growth curves are determined for single cell-originated cultures, and the variability of individual lag times can be estimated by extrapolation over time assuming that after the first division, cells are growing immediately at optimum specific growth rate. Direct methods make use of microscopic imaging of individual microorganisms (e.g. time-lapse microscopy) in order to determine their growth characteristics without extrapolation over time (26, 69, 85, 91).

Recently, a direct imaging method has been developed that allows monitoring of growth of individual cells until the microcolony stage (38-40). Strips made of porous aluminum oxide, sold as Anopore, are used as a microbial culture support. This material is highly porous (40% by volume) and when Anopore strips are placed upon an appropriate nutrient agar base, it retains microorganisms on the planar surface while allowing nutrients from the agar to pass through the pores. A thin layer of medium fluid on top of the strips, drawn up by the capillary action of the pores, enables dividing cells to slide along the surface to form an expanding microcolony during incubation of the strips. At regular time
intervals during growth, an Anopore strip is transferred to a microscope glass slide, covered with a thin film of agar supplemented with a fluorescent dye, to stain the microcolonies and to image the microcolonies at the specific time point. The stained microcolonies are imaged directly, without coverslip, using a fluorescence microscope, and the saved images are analyzed quantitatively using ImageJ software to calculate the area of each microcolony per imaging time point. Because a high number of microcolonies (preferably 100 to 150 microcolonies) are imaged for each imaging time point, the average and variability of microcolony size can be estimated. Figure 3 shows the experimental set up to monitor bacterial growth on Anopore strips.

Optimal growth conditions can be created using a nutrient-rich agar base, e.g. brain heart infusion agar, and by incubation of the strips at optimal culturing temperature. Growth monitoring during stressing conditions can be simulated by supplementing the agar with a stress factor (37), e.g. sodium chloride, and/or incubation of the strips at other non-optimal growth conditions.

Figure 3. Experimental set up to monitor microbial growth on Anopore strips. Bacteria are inoculated on top of Anopore strips which are placed upon an appropriate nutrient agar base. The strips are incubated until the cells have grown until microcolonies. For each imaging time point, an Anopore strip is transferred to a microscopic glass slide to stain the microcolonies with a fluorescent dye. The microcolonies are imaged directly using a fluorescence microscope. The saved images are analyzed quantitatively using ImageJ software to calculate the area of each microcolony.

**Modeling as tool for quantification.** Adaptive stress responses and population heterogeneity influence growth and inactivation kinetics of food-borne pathogens in minimally processed food. Modeling of these phenomena allows to translate qualitative,
descriptive microbiology to a quantitative one and relies on the development and use of mathematical models to describe these phenomena. Many models are currently available in the literature that can be used to quantitatively describe observations and to translate observations into kinetic parameters and useful overviews of the broad spectrum of models are given by McKellar and Lu (61) and Brul and others (14).

The premise of predictive modeling is that responses of microbial populations are reproducible and are affected by environmental conditions that can be quantitatively described. Consequently, quantitative knowledge about the impact of environmental factors on microbial responses can be used to predict responses by monitoring the environment. The use of mathematical models to concisely and quantitatively describe the impact of environmental factors on microbial responses is therefore the starting point to predict microbial behavior.

Mathematical modeling of the impact of adaptation-stress treatment on microbial responses starts with collecting data. To quantify the effect of adaptation-stress pretreatment on cell resistance towards a challenge-stress, in this first step of data collection, inactivation data are collected of unstressed cells, i.e. the reference condition, and inactivation data of adaptation-stress pretreated cells during exposure to a challenge-stress to determine their specific robustness (Figure 4). In order to test response differences, it is of importance to independently reproduce data because response differences should be significant with respect to our ability to reproduce effects (94). After data collection, a mathematical model is selected that is able to describe the inactivation curvatures in a statistically acceptable way for both unstressed and adaptation-stress pretreated cells. Preferably, a model is selected of which the model parameters have a biological meaning and can be easily recognized in the curvatures allowing a transparent comparison. When model parameters reflect specific curvature characteristics (e.g. shoulder phase), it allows exclusion of a model parameter for those specific conditions where the corresponding curvature characteristic is not significant. This principle of parsimony, meaning that the number of model parameters for adequate description of the data should be as low as possible, results in reduction of model complexity for those conditions where it is statistically acceptable. After model fitting, the model parameter estimates for each condition are used to quantify the effect of adaptation-stress pretreatment on cell resistance towards a challenge-stress.
Figure 4. Models as tool to quantitatively describe and compare observations. Inactivation data of unstressed (reference) cells (-s) and adaptation-stress pretreated cells (+s) are collected. To quantitatively describe the inactivation curvature characteristics, a mathematical model is selected that is able to describe the specific inactivation curvature features of unstressed and adaptation-stress pretreated cells. After fitting the selected model to the data, the model parameter estimates (e.g. the first decimal reduction time $\delta$) are used to quantify response differences.

Quantitative knowledge about the effects of adaptive stress responses on inactivation and growth dynamics can provide valuable information for quantitative microbiological risk assessment (QMRA) studies, in particular for the exposure assessment part of these studies. QMRA is widely used to characterize health risks associated to the potential presence of microbial hazards in foods. A QMRA consists of four stages: i) hazard identification, ii) exposure assessment, iii) hazard characterization, and iv) risk characterization (15, 49). The exposure assessment part of a QMRA focuses on the assessment of the dose (number of cells) of the hazard present in the food at the moment of consumption. Because *B. cereus* is commonly recognized as a microbial hazard in minimally processed foods (16), quantitative knowledge about its stress adaptive behavior in these foods will allow us to estimate the impact of these traits on the dose of *B. cereus* cells at the moment of consumption.
Outline of this thesis. The research described in this thesis involved the quantification of *B. cereus*’ adaptive stress responses at three levels, namely, population level, individual cell level and molecular level, respectively, and aimed to quantitatively correlate these responses. Quantification of *B. cereus*’ stress adaptive behavior at these three levels allowed us to obtain comprehensive, quantitative knowledge about its dynamics and population heterogeneity in response to stress exposure and directed to biomarkers inside the cell that were suitable to predict adaptive traits of this food-borne pathogen. This quantitative and mechanistic understanding of *B. cereus*’ adaptive stress responses opens avenues to integrate adaptive stress responses in quantitative exposure assessment studies. The approach followed to identify biomarkers for stress adaptive behavior can be the starting point to use biomarkers for early detection and prediction of stress adaptive behavior, and can help to optimize application of mild preservation factors in minimally processed foods without affecting necessary safety margins.

Previously, the effect of adaptation-stress pretreatment on resistance of *B. cereus* cells towards a challenge-stress was mostly investigated for just one condition per adaptation-stress factor, and the end-point method was used to evaluate the effect of adaptation-stress pretreatment on cell resistance. The main disadvantage of the end-point method is that it does not provide information about the inactivation kinetics that could provide valuable knowledge for quantitative exposure assessment studies focusing on this pathogen. Therefore, in Chapter 2, the effect of different levels of salt stress pretreatment on heat resistance was quantified in detail for two mesophilic *B. cereus* strains using primary kinetic models. The fitting performances of various models were statistically compared, and based on statistical indices and model characteristics the best model was selected to reliably estimate the effect of salt stress preexposure on heat resistance.

Chilling of foods is commonly used in the minimal processing food chain to control microbial growth and low temperature might affect the impact of adaptive stress responses. Therefore, in Chapter 3, the effect of culturing temperature on salt-induced heat resistance was described for three *Bacillus* strains, which represent three distinct lineages of the *B. cereus* group (44).

After quantification of adaptive stress responses for the population as a whole, *B. cereus*’ stress adaptive behavior was quantitatively assessed at individual cell level. In Chapter 4, the direct-imaging-based Anopore technology was applied to monitor the initial growth dynamics of individual *B. cereus* cells in response to mild and severe salt stress.
The heterogeneity in growth performance was quantified and the growth kinetics of cells cultured on Anopore strips were compared to those in liquid culture. Also the population heterogeneity of \textit{B. cereus} cells cultured at low culturing temperature was quantified and these results are described in \textbf{Chapter 5}. Fluorescent labeling of individual cells allowed to investigate the origin of the observed cold-induced population heterogeneity in more detail.

To further elucidate adaptive salt stress mechanisms at molecular level, genome-wide transcriptome profiles of mildly and severely salt-stressed \textit{B. cereus} cells were examined and these results are described in \textbf{Chapter 6}. The transcriptome profiles of mildly and severely salt-stressed cells were compared and pointed to salt stress specific transcription patterns. Several salt-induced transcriptome responses could be correlated to phenotypic characteristics of salt-stressed cells, and this linkage revealed a temporal shift between various transcriptome responses and corresponding phenotypic features of severely salt-stressed cells.

The whole genome transcriptome profiles of \textit{B. cereus} upon exposure to three other adaptation-stress conditions – mild heat, acid and oxidative stress – have recently been published (17, 66, 87), allowing to compare the transcriptome responses of salt-stressed \textit{B. cereus} cells to those induced upon mild heat, acid and oxidative stress treatment. This comparison, described in \textbf{Chapter 7}, revealed a remarkable confined overlap of differentially expressed genes upon treatment to those four adaptation-stress conditions and directed to several candidate-biomarkers for stress adaptive behavior. To evaluate whether the selected candidate-biomarkers could predict the adaptation-stress induced robustness level towards challenge-stress, the selected candidate-biomarkers were quantitatively measured upon adaptation-stress treatment, and their induction levels were correlated to adaptation-stress induced robustness towards challenge-stress. A framework was designed to systematically evaluate the predictive potential of each candidate-biomarker. Both short- and long-term biomarkers could be identified that were quantitatively correlated to adaptation-stress induced heat, acid and/or oxidative stress robustness, respectively, and are therefore potential predictive cellular indicators for adaptation-stress induced robustness towards these challenge-stresses. An overview of the research topics addressed in the thesis chapters is given in Figure 5.
Chapter 2
salt-induced heat resistance

Chapter 3
culturing temperature-dependent adaptive salt stress responses

Chapter 4
salt-induced population heterogeneity

Chapter 5
low temperature-induced population heterogeneity

Chapter 6
phenotype and transcriptome responses to salinity

Chapter 7
biomarkers for adaptation-stress induced robustness

Figure 5: Overview of the research themes – population, individual cell and molecular level – and research topics addressed in the thesis chapters.

Chapter 8 includes a summarizing discussion, concluding remarks and future perspectives.

References


Chapter 1


Quantification of the effects of salt stress and physiological state on thermotolerance of *Bacillus cereus* ATCC 10987 and ATCC 14579

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Abstract

The food-borne pathogen *Bacillus cereus* can acquire enhanced thermal resistance through multiple mechanisms. Two *B. cereus* strains, ATCC 10987 and ATCC 14579, were used to quantify the effects of salt stress and physiological state on thermotolerance. Cultures were exposed to increasing concentrations of sodium chloride for 30 min, after which their thermotolerance was assessed at 50°C. Linear and nonlinear microbial survival models, which cover a wide range of known inactivation curvatures for vegetative cells, were fitted to the inactivation data and evaluated. Based on statistical indices and model characteristics, biphasic models with a shoulder were selected and used for quantification. Each model parameter reflected a survival characteristic, and both models were flexible, allowing a reduction of parameters when certain phenomena were not present. Both strains showed enhanced thermotolerance after preexposure to (non)lethal salt stress conditions in the exponential-phase. The maximum adaptive stress response due to salt preexposure demonstrated for exponential-phase cells was comparable to the effect of physiological state on thermotolerance in both strains. However, the adaptive salt stress response was less pronounced for transition- and stationary-phase cells. The distinct tailing of strain ATCC 10987 was attributed to the presence of a subpopulation of spores. The existence of a stable heat-resistant subpopulation of vegetative cells could not be demonstrated for either of the strains. Quantification of the adaptive stress response might be instrumental in understanding adaptation mechanisms and will allow the food industry to develop more accurate and reliable stress-integrated predictive modeling to optimize minimal processing conditions.
Quantification of salt-induced thermotolerance

Introduction

*Bacillus cereus* is a spore-forming Gram-positive rod causing both food spoilage and food poisoning. The organism is ubiquitous in soil, in spices, on grass, in dairy cattle feed, and in dung and is an inevitable low-grade contaminant of pasteurized milk products (15). Toxins produced by vegetative cells of *B. cereus* can be the causative agents of two types of gastrointestinal disease, i.e., emesis and diarrhea. The diarrheal type is caused by heat-labile enterotoxins produced in the intestine, whereas emetic outbreaks are associated with the heat-stable cereulide toxin produced in the food product (8).

Consumer demand for mildly processed foods challenges the food industry to produce tasty, nutritious, and microbiologically safe minimally processed products (16). A minimally processed food product is microbiologically stable and safe because of the presence of a set of preservation hurdles that is specific for the particular food. An appropriate combination of preservation hurdles controls microbial spoilage and the growth of pathogenic microorganisms and stabilizes the sensory and nutritive properties of a food. During processing, microorganisms in the food undergo various kinds of stresses, and these stresses have a fundamental impact on the behavior of the stress-exposed cells. Bacteria have evolved adaptive networks to face the challenges of changing environments (1). Upon triggering of the adaptive networks, the so-called adaptive stress response, the bacteria gain increased resistance towards conditions which would be lethal for the cells if the stress response was not activated. This is of practical importance for minimally processed foods, as the adaptive stress response to the first hurdle may render the organism more resistant to the subsequent hurdle(s). Therefore, the adaptive stress response may counterbalance the benefits of the hurdle concept.

The effect of stress preexposure on thermotolerance of vegetative cells of *B. cereus* has been investigated previously (4, 22, 28). However, in most investigations, thermotolerance was assessed in exponentially growing cultures after preexposure to just one condition per stress-inducing factor, and the end-point method was used to evaluate the effect of stress preexposure on thermotolerance. The main disadvantage of the end-point method is that it does not provide information on the thermal death kinetics, which could provide valuable knowledge for quantitative risk assessment studies and might reflect the mechanisms by which stress preexposure influences thermotolerance. In the last decades, a number of mathematical primary models have been developed to quantitatively describe microbial inactivation. Primary kinetic models describe microbial survival as a function of time. An important aspect of modeling is the possibility of reliable estimation of the effects
of various stress conditions on the number of surviving microorganisms. Therefore, in this study we evaluated the fitting performance of different primary models, which cover a wide range of inactivation curvatures for vegetative cells. The most suitable models were selected in order to identify the survival curvature characteristics in more detail and to quantify the effects of the adaptive stress response and physiological state on the inactivation kinetics.

Materials and methods

**Bacterial strains and culture conditions.** *Bacillus cereus* ATCC 10987, isolated from a study on cheese spoilage in Canada in 1930 (24), and *Bacillus cereus* ATCC 14579, isolated from the air in a cow shed in the United Kingdom (9), were used in this study. The cultures were stored frozen (-80°C) in brain heart infusion (BHI) broth (Becton Dickinson, France) supplemented with 25% glycerol (Sigma). The bacteria were cultivated before each experiment in 10 ml BHI broth and incubated in a water bath at 30°C with aeration at 200 rpm (Julabo SW20; Julabo Labortechnik GmbH, Germany) for 16 h to 24 h.

**Thermal inactivation experiments with and without preexposure of cells to salt.** To evaluate the effects of preexposure to salt and of physiological state on thermostolerance, the following procedure was used. Erlenmeyer flasks (250 ml) with 50 ml of sterile BHI broth were inoculated with an overnight culture to reach an optical density (OD) of approximately 0.025 at 600 nm (Novaspec II spectrophotometer; Pharmacia Biotech, United Kingdom). The flasks were incubated in a water bath at 30°C with aeration at 200 rpm until a specific OD value was reached, depending on the desired physiological state (exponential-phase, OD = 0.5; transition-phase, OD = 5; stationary-phase, OD = 10 to 12). Cells were harvested by centrifugation of 20 ml of culture (3,660 × g, 5 min, 20°C) (Mistral 3000i; MSE, United Kingdom), and the supernatant was removed immediately after centrifugation. To preexpose the harvested cells to salt, the cells were resuspended in 20 ml BHI broth containing sodium chloride (VWR-International, France) at various concentrations (final concentrations of sodium chloride were as follows: for exponential-phase cells, 1%, 2.5%, and 5%; and for transition- and stationary-phase cells, 2.5% [wt/vol]) and incubated for 30 min in a water bath (30°C, 200 rpm). After preexposure to salt, the cells were spun down and resuspended in 2 ml BHI broth. When cells were not preexposed to sodium chloride, 20 ml of culture was centrifuged and concentrated in 2 ml BHI broth.
Quantification of salt-induced thermotolerance

To inactivate the (non)preexposed cells, six tubes (Greiner Bio-One, Germany) containing 20 ml of preheated BHI broth were placed in a water bath (GFL 1083; Gesellschaft Labortochnik GmbH, Germany) with aeration (150 rpm). The desired temperature (50°C) of the preheated BHI broth was checked with a digital thermometer (TFX 392 SK; Gullimex Instruments, Germany). From the concentrated culture, 200 µl was inoculated into each tube. At constant intervals, 1-ml samples were taken in duplicate, and serial dilutions were made in 9 ml peptone saline solution (1 g neutralized bacteriological peptone [Oxoid, United Kingdom] supplemented with 8.5 g sodium chloride per liter). From the appropriate dilution, 50 µl was spread in duplicate on BHI agar plates (BHI broth supplemented with 15 g agar [Oxoid, United Kingdom] per liter) by use of a spiral plater (Eddy Jet; IUL Instruments, Spain). Plates were incubated at 30°C for 24 h, and the results were expressed in log10 CFU ml⁻¹ (detection limit of the method, 1.3 log10 CFU ml⁻¹). For all experimental conditions, three experiments were performed on different days.

Microbial survival models. The following models were used to fit the inactivation data.

(i) The first-order model (31) was determined with the following equation:

$$\log_{10} N(t) = \log_{10} N(0) - \frac{t}{D}$$

where \(t\) is the inactivation treatment time (min), \(D\) is the decimal reduction time, which is the time needed for 1 log₁₀ reduction of the population (min); \(N(0)\) is the initial population (log₁₀ CFU ml⁻¹); and \(N(t)\) is the population at time \(t\) (log₁₀ CFU ml⁻¹).

(ii) The Weibull model (7) was determined with the following equation:

$$\log_{10} N(t) = \log_{10} N(0) - \left(\frac{t}{\delta}\right)^{\beta}$$

where \(\delta\) is the first decimal reduction time (min) and \(\beta\) is a fitting parameter which defines the shape of the curve. \(\beta\) values of < 1 correspond to concave upward survival curves, \(\beta\) values of > 1 correspond to concave downward curves, and a \(\beta\) value of 1 corresponds to a straight line (same as equation 1).

(iii) The biphasic linear model, or two-population model, was proposed by Cerf. The biphasic model assumes the existence of two populations, with one heat-sensitive and one heat-resistant population (6). The biphasic linear model can be formulated as follows:

$$\log_{10} N(t) = \log_{10} N(0) + \log_{10} \left[ (1 - f) \cdot 10^{-\frac{t}{D_{sens}}} + f \cdot 10^{-\frac{t}{D_{res}}} \right]$$
where \((1 - f)\) and \(f\) are the fractions of the heat-sensitive and heat-resistant populations, respectively, and \(D_{sens}\) and \(D_{res}\) are the decimal reduction times of the two populations, respectively (min).

(iv) The biphasic logistic model (30) assumes the existence of a primary heat-sensitive population and a secondary heat-resistant population and aims to take into account a shoulder for both populations. The biphasic logistic model is formulated as follows:

\[
\log_{10}\left(N(t) = \log_{10}\left(N(0) + \log_{10}\left(\frac{1 - f}{1 + \exp(-k_{sens} \cdot t_s)} + \frac{f}{1 + \exp(-k_{res} \cdot (t - t_s))}\right)\right)\right) (4)
\]

where \(t_s\) is the duration of the shoulder (min), \((1 - f)\) and \(f\) are the fractions of the heat-sensitive and heat-resistant populations, respectively, and \(k_{sens}\) and \(k_{res}\) are the maximum specific inactivation rates for the heat-sensitive and heat-resistant populations, respectively (min\(^{-1}\)). The respective decimal reduction times can be calculated from the inactivation rates by the equation \(D = \ln(10)/k\). When no shoulder or second population is present, then \(t_s = 0\) or \(f = 0\), respectively.

(v) The modified Gompertz model (27) was determined with the following equation:

\[
\log_{10}\left(N(t) = \log_{10}\left(N(0) + a \cdot \exp[-b \cdot \exp(c \cdot t)] - a \cdot \exp[-\exp(b)]\right)\right) (5)
\]

where \(a\), \(b\), and \(c\) are fitting parameters.

(vi) The reparameterized Gompertz model, as modified by Zwietering et al. (33), was determined with the following equation:

\[
\log_{10}\left(N(t) = \log_{10}\left(N(0) + A \cdot \exp\left[-\exp\left\{\frac{k \cdot e}{A} (t_s - t) + 1\right\}\right]\right) \right) (6)
\]

where \(t_s\) is the duration of the shoulder (min), \(k\) is the maximum specific inactivation rate (log\(_{10}\) min\(^{-1}\)), and \(A\) is the difference between the population at the end of the inactivation period and the initial population (log\(_{10}\) CFU ml\(^{-1}\)).

(vii) The Baranyi model was proposed by Baranyi et al. (2) and considers an inactivation curve as the mirror image of a growth curve. The Baranyi growth model can be written with the following equations (with \(m = 1\) and \(\nu = \mu\) (2):

\[
\log_{10}\left(N(t) = \log_{10}\left(N(0) + \frac{\mu}{\ln(10)} \cdot A(t) - \frac{1}{\ln(10)} \cdot \ln\left[1 + \frac{\exp[\mu \cdot A(t)] - 1}{10^{\log_{10}(N(0))}}\right]\right)\right) (7a)
\]
where \( \mu \) is the maximum specific growth rate (min\(^{-1}\)), \( N_{\text{final}} \) is the final population (log\(_{10}\) CFU ml\(^{-1}\)), and \( A(t) \) is as defined by Baranyi et al. (2).

\[
A(t) = t + \frac{1}{\mu} \ln \left[ \exp \left( \mu \cdot t \right) + \exp \left( -\mu \cdot t_{\text{lag}} \right) - \exp \left( -\mu \cdot t - \mu \cdot t_{\text{lag}} \right) \right]
\]  (7b)

where \( t_{\text{lag}} \) is the duration of the lag period of the growth curve (min).

(viii) The Geeraerd model assumes that the total population \( N \) equals the sum of two subpopulations, with one of them having more heat resistance (\( N = N_{\text{sens}} + N_{\text{res}} \)) (11). Inactivation of cells ensues when a critical component (\( C_c \)) is inactivated or destructed. The following equations define the model:

\[
\frac{dN_{\text{sens}}(t)}{dt} = -k_{\text{sens}} \cdot N_{\text{sens}}(t) \left[ \frac{1}{1 + C_c(t)} \right]  \]  (8a)

\[
\frac{dN_{\text{res}}(t)}{dt} = -k_{\text{res}} \cdot N_{\text{res}}(t) \left[ \frac{1}{1 + C_c(t)} \right]  \]  (8b)

\[
\frac{dC_c(t)}{dt} = -k_{\text{sens}} \cdot C_c(t)  \]  (8c)

where \( C_c(0) = \exp(k_{\text{sens}} \cdot t_s)-1 \).

The solution of this set of differential equations can be expressed as follows (11; A. H. Geeraerd, personal communication):

\[
\log_{10} N(t) = \log_{10} N(0) + \log_{10} \left\{ (1-f) \cdot \exp(-k_{\text{sens}} \cdot t) \cdot \frac{\exp(k_{\text{sens}} \cdot t_s)}{1 + [\exp(k_{\text{sens}} \cdot t_s)-1] \cdot \exp(-k_{\text{sens}} \cdot t)} + f \cdot \exp(-k_{\text{res}} \cdot t) \cdot \left[ \frac{\exp(k_{\text{sens}} \cdot t_s)}{1 + [\exp(k_{\text{sens}} \cdot t_s)-1] \cdot \exp(-k_{\text{sens}} \cdot t)} \right]^{k_{\text{res}}/k_{\text{sens}}} \right\}  \]  (9)

where \( t_s \) is the duration of the shoulder (min), \( (1-f) \) and \( f \) are the fractions of the heat-sensitive and heat-resistant populations, respectively, and \( k_{\text{sens}} \) and \( k_{\text{res}} \) are the maximum specific inactivation rates of the heat-sensitive and heat-resistant populations, respectively (min\(^{-1}\)).

**Model fitting and model selection.** The above models were fitted to the inactivation data per the experimental conditions in TableCurve 2D (Windows v. 2.03) and checked in Microsoft Excel by using the Excel Solver add-in. The Baranyi growth model was fitted to the mirror image of the inactivation data points in TableCurve following the procedure.
suggested by Baranyi et al. (2), and the freeware MicroFit 1.0 (Institute of Food Research, Norwich, United Kingdom) was used to check the TableCurve fitting results when the experimental data set was limited to 100 data points.

The criteria used to select the most adequate model to fit the experimental data were as follows: applicability for both strains ATCC 10987 and ATCC 14579 and the different experimental conditions, statistics (MSE_{model}, r^2, A_f, and F test), biological meaning of the parameters, and reflection of a proposed inactivation mechanism.

The statistical indices used to compare the models are discussed below (26).

(i) MSE_{model}. The mean square error of the model (MSE_{model}) is the residual sum of squares (sum of the squared differences between the observed values and the fitted values) divided by the degrees of freedom. The lower the MSE_{model} is, the better is the adequacy of the model to describe the data. It was calculated by the following equation:

$$\text{MSE}_{\text{model}} = \frac{\text{RSS}}{\text{DF}} = \frac{\sum_{i=1}^{n} \left( \log_{10} N'_{\text{observed}} - \log_{10} N'_{\text{fitted}} \right)^2}{n - s}$$

where RSS is the residual sum of squares, DF is the degrees of freedom, n is the number of data points, s is the number of parameters of the model, \(N'_{\text{observed}}\) is the observed population level (log_{10} CFU ml^{-1}), and \(N'_{\text{fitted}}\) is the fitted population level (log_{10} CFU ml^{-1}).

(ii) \(r^2\). The regression coefficient (\(r^2\)) is the proportion of the total variation of the data explained by the model. The value can range between 0 and 1, and the higher the value, the better the fit of the model. \(r^2\) was calculated by the following equation:

$$r^2 = 1 - \frac{\text{RSS}}{\text{TSS}}$$

where RSS is the residual sum of squares, and TSS is the total sum of squares, which is the total amount of variation present when the observed values are compared to the grand average of the observed values.

(iii) \(A_f\). The accuracy factor (\(A_f\)) shows the accuracy of the model and indicates how close the fitted values are, on average, to the observed values. The larger the value, the less accurate the model, and when \(A_f\) is equal to 1, there is perfect agreement between the fitted and the observed values. An \(A_f\) of 2 indicates that the fitted values are, on average, different by a factor of 2 on the log_{10} scale from the observed values (i.e., either half as large or twice as large). The \(A_f\) was calculated by the following equation:

$$A_f = 10^{\left[ \frac{\sum_{i=1}^{n} \left| \log_{10} \left( \text{log}_{10} N'_{\text{fitted}} / \log_{10} N'_{\text{observed}} \right) \right|}{n} \right]}$$

40
where $n$ is the number of data points.

(iv) $F$ test. The $F$ test was used to decide if the fitting performance of a model was statistically accepted. The $f$ value was calculated by the following equation:

$$f = \frac{\text{MSE}_{\text{model}}}{\text{MSE}_{\text{data}}}$$  \hspace{1cm} (13)

where $\text{MSE}_{\text{model}}$ is the mean square error of the model, and $\text{MSE}_{\text{data}}$ is the mean square error of the data for replicate values, which indicates the measuring error. $\text{MSE}_{\text{data}}$ was determined by the deviation of the observed values from the mean value at one time point, $i$, and was calculated as follows:

$$\text{MSE}_{\text{data}} = \frac{\text{RSS}}{\text{DF}} = \frac{\sum_{i=1}^{m} \sum_{j=1}^{k} (\text{average}_{\log_{10} N_{i}} - \log_{10} N_{ij})^2}{n - m}$$  \hspace{1cm} (14)

where $n$ is the number of data points, $m$ is the number of time points (sampling times), $k$ is the number of replicates at each time point, average $N_i$ is the mean value of the population at time point $i$ ($\log_{10}$ CFU ml$^{-1}$), and $N_{ij}$ is the population at time point $i$ for specific replicate $j$ ($\log_{10}$ CFU ml$^{-1}$).

The $f$ value was tested against an $F$ table value (95% confidence). If the $f$ value was smaller than the $F$ table value ($F_{\text{DF}_{\text{model}}, \text{DF}_{\text{data}}}$), then the $F$ test was accepted, and this indicated that the model described the observed data well.

Reduction of model parameters. After selection of the most adequate model(s) for fitting the data using the criteria mentioned above, a stepwise procedure was followed in order to reduce, where possible, the number of parameters of the selected model per experimental condition. The selected model was fitted to all the replicate data together per experimental condition, and the confidence interval (95%) of the parameter estimate was used to evaluate whether a parameter could be excluded from the model. If the confidence interval (95%) of the parameter estimate included zero, the parameter was regarded as nonsignificant. After the exclusion of a nonsignificant parameter, the model with the reduced number of parameters was refitted to the data to check for another nonsignificant parameter, which was subsequently excluded as well. The stepwise procedure was followed until only the significant parameters remained in the model. However, due to the reduction of parameters, there was a risk that the reduced model failed to describe the data acceptably. This can be the case if by reducing one parameter, a second parameter also becomes redundant. Therefore, the $F$ test was applied each time after the exclusion of one nonsignificant parameter to evaluate if the reduction of parameters was still statistically acceptable (33). If
the $F$ test was not accepted, the model reduction was not performed regardless of whether a nonsignificant parameter was included in the model. The $f$ value to test if a parameter could be excluded was calculated by the following equation:

$$f = \frac{(RSS_2 - RSS_1)(DF_2 - DF_1)}{RSS_1 / DF_1}$$

where $RSS_1$ is the residual sum of squares of the full model, $RSS_2$ is the residual sum of squares of the reduced model, and $DF_1$ and $DF_2$ are the degrees of freedom for the full and reduced models, respectively, calculated by the formula:

$$DF = \text{number of data points} - \text{number of parameters}.$$

The $f$ value was tested against an $F$ table value (95% confidence, $F_{DF_1,DF_2}$). If the $f$ value was smaller than the $F$ table value, the $F$ test was accepted and the nonsignificant parameter was excluded from the model.

**Statistical analysis of model parameters.** For all experimental conditions, three independent experiments were performed on different days, and the selected, reduced model was fitted to the reproductions individually. One-way analysis of variance and $t$ tests (one-sided) were performed in order to compare the average parameter estimates for the different conditions and to investigate if there were any significant effects of preexposure to salt and of physiological state on the inactivation kinetic parameters (SPSS, version 11.5.0).

**Results**

To select the sodium chloride concentrations for salt preexposure, the growth of *B. cereus* ATCC 10987 and ATCC 14579 was determined in BHI broth supplemented with 1%, 2.5%, 5%, and 10% sodium chloride (wt/vol) for 300 min. The two *B. cereus* strains were able to grow in the presence of salt in the growth medium (1% and 2.5%), although exposure to 2.5% sodium chloride resulted in an initial decline in the number of cells. Higher salt concentrations (5% and 10%) proved to be lethal for the bacterial cells (data not shown). The concentrations of 1%, 2.5%, and 5% sodium chloride were chosen in order to investigate the effects of preexposure to nonlethal and lethal salt conditions on thermotolerance.

**Effects of salt stress and physiological state on thermotolerance: strain diversity and population heterogeneity.** Figure 1 illustrates the influences of preexposure to salt and
physiological state on thermotolerance at 50°C for *B. cereus* ATCC 10987 (panel a) and ATCC 14579 (panel b). The thermotolerance of exponential-phase cells of both strains was highly increased by preexposure to 1%, 2.5%, and 5% sodium chloride, resulting in a lower inactivation rate for strain ATCC 10987 compared to the experimental condition without preexposure to salt and an additional shoulder period for strain ATCC 14579. Preexposure to 1% and 2.5% salt seemed to be most effective. The observed shoulder for strain ATCC 14579 after preexposure to salt could not be dismissed as an experimental artifact of cell clumping. Wet mounts of cell suspensions collected at the start of the inactivation showed no aggregation.

Figure 1. Thermal inactivation of *Bacillus cereus* ATCC 10987 (panel a) and ATCC 14579 (panel b) cells at 50°C with(out) preexposure to sodium chloride for 30 min (sodium chloride concentrations for exponential [E]-phase cells, 1%, 2.5%, 5%; sodium chloride concentration for transition [T]- and stationary [S]-phase cells, 2.5% [wt/vol]). Detection limit was 1.3 log₁₀ cfu ml⁻¹.
Both strains demonstrated a growth cycle-dependent effect on thermotolerance. Transition- and stationary-phase cells exhibited more resistance to heat than exponential-phase cells. The adaptive stress response was also influenced by physiological state; the effect of preexposure to salt on thermotolerance was less pronounced for transition- and stationary-phase cells than for exponential-phase cells for both strains.

Strain ATCC 10987 showed distinct tailing in the exponential-, transition-, and stationary-phases (Figure 1a), indicating the presence of a highly heat-resistant subpopulation. To investigate the potential presence of spores in very small numbers at the start of inactivation, suspensions from the three growth phases were heated for 15 min at 75°C to kill the vegetative cells. The observed tailing was indeed explained by minor fractions of spores present in the suspensions at the start of the inactivation, since the subpopulation detected after heating at 75°C corresponded to the tailing fraction. The presence of spores in the exponentially growing culture could be explained by the transfer of spores from the overnight culture during inoculation of the fresh medium. Germination of spores during incubation to exponential growth phase was apparently limited. The failure of these spores to germinate was not investigated any further. Strain ATCC 14579 was also able to form spores in the stationary-phase, but the fraction of spores was very small, such that no spores could be detected at the start of inactivation.

**Assessment of model adequacy and model selection.** The inactivation kinetics of the two *B. cereus* strains studied were further analyzed by fitting eight microbial survival models to the experimental data. The fitting performances of these models were assessed statistically and evaluated in order to select the most suitable model(s) to quantify in more detail specific curvature characteristics and to quantify the effects of preexposure to salt and of growth phase on thermotolerance.

To compare the fitting performances of the models, MSE$_{model}$, $r^2$, $A_F$, and the $f$ value were calculated. Comparison of the indices of the models showed that overall, the first-order model and the Weibull model did not describe the inactivation data for strains ATCC 10987 (Table 1) and ATCC 14579 (Table 2) acceptably, and neither did the biphasic linear model for the latter strain, which could be confirmed by the $F$ test. When the $F$ test was accepted (bold values), this indicated that the model described the observed inactivation data well. The rejection of the biphasic linear model for strain ATCC 14579 was caused by the inability of this model to describe the shoulder curvature.
Table 1. Statistical indices of microbial survival models used for fitting the inactivation data for *B. cereus* ATCC 10987

<table>
<thead>
<tr>
<th>Growth phase</th>
<th>Statistical index</th>
<th>Models (no.(^3))</th>
<th>Linear</th>
<th>Weibull</th>
<th>Biphasic linear</th>
<th>Biphasic logistic</th>
<th>Gompertz</th>
<th>Reparameterized Gompertz</th>
<th>BARANYI</th>
<th>GEERAERT</th>
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<tbody>
<tr>
<td>% NaCl</td>
<td></td>
<td>(2)</td>
<td>(3)</td>
<td>(4)</td>
<td>(5)</td>
<td>(4)</td>
<td>(4)</td>
<td>(5)</td>
<td>(4)</td>
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</tr>
<tr>
<td>Exponential</td>
<td>(r^2)</td>
<td>0.2284</td>
<td>0.9022</td>
<td>0.9798</td>
<td>0.9796</td>
<td>0.9756</td>
<td>0.9753</td>
<td>0.9751</td>
<td>0.9798</td>
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<tr>
<td>0%</td>
<td>MSE(_{modd})</td>
<td>2.5136</td>
<td>0.3746</td>
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<td>0.0712</td>
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<td>1.79</td>
<td>1.78</td>
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<td>1.78</td>
<td>1.79</td>
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<td>MSE(_{modd})</td>
<td>1.1110</td>
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<tr>
<td>2.5%</td>
<td>MSE(_{modd})</td>
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<td>0.4567</td>
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<td>1.41</td>
<td>1.41</td>
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<tr>
<td>5%</td>
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<td>0.9045</td>
<td>0.9820</td>
<td>0.9823</td>
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<td>0.9808</td>
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<td>1.38</td>
<td>1.38</td>
<td>1.38</td>
<td>1.38</td>
<td>1.38</td>
<td>1.38</td>
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<td>Stationary</td>
<td>(r^2)</td>
<td>0.7037</td>
<td>0.8355</td>
<td>0.9462</td>
<td>0.9590</td>
<td>0.9572</td>
<td>0.9572</td>
<td>0.9499</td>
<td>0.9592</td>
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<td>1.35</td>
<td>1.35</td>
<td>1.35</td>
<td>(r^2)</td>
</tr>
</tbody>
</table>

\(^{a}\)Number of parameters per model; \(^{b}\) Bold value indicates that the \(F\) test was accepted; \(^{c}\) Goodness of fit (0/8, 8/8); 0/8, model was accepted for none of the eight experimental conditions, 8/8, model was accepted for all experimental conditions.
### Table 2. Statistical indices of microbial survival models used for fitting the inactivation data for *B. cereus* ATCC 14579

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<thead>
<tr>
<th>Growth phase</th>
<th>Statistical index</th>
<th>Linear</th>
<th>Weibull</th>
<th>Biphasic linear</th>
<th>Biphasic logistic</th>
<th>Gompertz</th>
<th>Reparameterized Gompertz</th>
<th>Barany</th>
<th>Geeraerd</th>
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<tr>
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<td>1.45</td>
<td>1.45</td>
<td>1.46</td>
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</tr>
</tbody>
</table>

Goodness of fit<sup>a</sup><br>1/8 2/8 2/8 8/8 8/8 8/8 7/8 8/8

<sup>a</sup>Number of parameters per model; <sup>b</sup> Bold value indicates that the $F$ test was accepted; <sup>c</sup> Goodness of fit (1/8, 8/8): 1/8, model was accepted for one of the eight experimental conditions, 8/8, model was accepted for all experimental conditions.
The Baranyi model was statistically acceptable for most of the experimental conditions, except for transition-phase cells of strain ATCC 10987 with preexposure to 2.5% salt and exponential-phase cells of strain ATCC 14579 without preexposure to salt. One of the noticeable differences between the Baranyi model and other sigmoid curves, such as the modified Gompertz model, is that the mid-phase of the model curvature is very close to linear. This property of the Baranyi model was not applicable for exponential-phase cells of strain ATCC 14579 without preexposure to salt, as this condition showed a nonconstant inactivation rate in the mid-phase.

The statistical indices of the modified Gompertz model, the reparameterized Gompertz model, the biphasic logistic model, and the Geeraerd model were similar in being accepted statistically for all experimental conditions. To choose the most suitable of these remaining models, the following criteria were used: biological meaning of the parameters and reflection of a proposed inactivation mechanism. The modified Gompertz model contains mathematical parameters which have no biological interpretation. Reparameterization, resulting in the reparameterized Gompertz model, replaces the mathematical parameters with parameters that have biological meaning. As expected, the two Gompertz models gave similar statistical indices. The term \( a \cdot \exp[-\exp(b)] \) is disregarded in the reparameterized modified Gompertz model, and the effect of this model reduction on the statistical indices was negligible. Parameter \( A \) of the reparameterized Gompertz model represents the difference between the population at the end of the observation and the initial population and might reflect a highly resistant subpopulation present in the tail of the sigmoid curvature. The latter phenomenon was applicable for strain ATCC 10987 since the presence of spores was confirmed. The reparameterized Gompertz model did not reflect the population heterogeneity for strain ATCC 14579 suitably, as this strain did not show distinct tailing caused by a subpopulation of cells for which the inactivation rate was equal to zero. In addition, description of a curvature without a tail (linear inactivation or linear inactivation with shoulder) using the reparameterized Gompertz model resulted in a nonrealistic estimation of parameter \( A \). Therefore, the two sigmoid models were not regarded as adequate, although the number of parameters was less (four parameters) than that for the biphasic logistic model and the Geeraerd model (five parameters). The biphasic logistic model and the Geeraerd model assume the presence of a primary heat-sensitive and a secondary heat-resistant population, and both models include a shoulder period \( (t_s) \). The parameters of the biphasic logistic and Geeraerd model can be given biological meaning, have clear significance, and can be recognized easily in the inactivation curvature. Moreover, both models are flexible, allowing a decrease in the
number of parameters when certain phenomena are not present. The biphasic logistic model is derived from a logistic-based model. The Geeraerd model is based on the interesting features of a dynamic model and addresses the hypothesis of the presence of a pool of protective or critical components ($C_c$) around or in each cell (10, 11). Gradually, this pool is destroyed, undergoing first-order inactivation, and becomes approximately zero. Towards this time, the heat-sensitive and heat-resistant populations are inactivated following first-order kinetics. Taking into account these aspects, the Geeraerd model was considered the most adequate model. The dynamic properties of this model and the underlying hypothesis about the occurrence of a shoulder in an inactivation curve were preferred over the logistic-based properties of the biphasic logistic model. However, both the biphasic logistic and Geeraerd models were used to quantify the effects of salt stress and growth phase on thermotolerance, as both models were able to fit the inactivation characteristics occurring for strains ATCC 10987 and ATCC 14579 suitably.

Reduction of model parameters. The number of parameters of the selected biphasic logistic and Geeraerd models was reduced, where possible, to decrease the model complexity. A stepwise procedure was followed, and the $F$ test was used to determine whether the reduction of parameters was acceptable. Because the tailing fraction for the three physiological growth phases of strain ATCC 10987 corresponded to a minor population of spores, the inactivation rate of the heat-resistant subpopulation ($k_{res}$) was set at zero, resulting in a four-parameter model before the stepwise procedure was applied for this strain. It should be noted that the fixation of parameter $k_{res}$ at zero resulted in a nonaccepted $F$ test for one experimental condition (transition-phase with preexposure to 2.5% salt) for the biphasic logistic and Geeraerd models. The measuring error for this experimental condition was very small (MSE$_{data}$ = 0.05), as replicate experiments produced small variation. Visual inspection of the fitting performances of the biphasic logistic and Geeraerd models showed that the adequacies of both models were sufficient, although the $F$ test was not accepted due to the low MSE$_{data}$. Tables 3 and 4 show the parameter estimates obtained with the reduced biphasic logistic and reduced Geeraerd models. The numbers of parameters of the reduced biphasic logistic and reduced Geeraerd models were similar, except for one experimental condition for strain ATCC 10987 (transition-phase without preexposure to salt). For this condition, parameter $t_s$ was not significant for the biphasic logistic model ($P = 0.12$) but was significant for the Geeraerd model ($P = 0.01$).

Strain ATCC 10987 showed a significant fraction, $f$, of heat-resistant cells for the
Table 3. Parameter estimates of the biphasic logistic model

<table>
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<tr>
<th>Strain</th>
<th>Growth phase</th>
<th>%NaCl</th>
<th>Parameter valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>logN0 N(0) f kren kres f_0</td>
</tr>
<tr>
<td>ATCC 10987</td>
<td>Exponential</td>
<td>0%</td>
<td>6.85  5.94x10^-6  6.00 NS 0^a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1%</td>
<td>6.84  6.10x10^-6  0.74 NS 0^a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5%</td>
<td>6.48  1.54x10^-5  0.65 NS 0^a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5%</td>
<td>5.04  4.59x10^-4  1.18 NS 0^a</td>
</tr>
<tr>
<td></td>
<td>Transition</td>
<td>0%</td>
<td>7.74  9.42x10^-7  0.69 NS 0^a</td>
</tr>
<tr>
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<td></td>
<td>2.5%</td>
<td>7.61  2.48x10^-6  0.57 NS 0^a</td>
</tr>
<tr>
<td></td>
<td>Stationary</td>
<td>0%</td>
<td>7.56  3.94x10^-6  0.43 NS 0^a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5%</td>
<td>7.39  1.72x10^-5  0.61 5.63 0^a</td>
</tr>
<tr>
<td>ATCC 14579</td>
<td>Exponential</td>
<td>0%</td>
<td>6.96  1.65x10^-3  0.84 NS 0.22</td>
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<td>1%</td>
<td>6.92  1.19x10^-3b 0.91 8.86 0.27</td>
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<td>2.5%</td>
<td>6.70  2.89x10^-4b 0.57 9.05 0.14b</td>
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<td>5%</td>
<td>6.49  5.66x10^-4b 0.63 5.84 0.17</td>
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<td>Transition</td>
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<td>2.5%</td>
<td>7.95  NS 0.35 13.50 NS</td>
</tr>
<tr>
<td></td>
<td>Stationary</td>
<td>0%</td>
<td>8.04  3.63x10^-4b 0.38 NS 0.12</td>
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<td>2.5%</td>
<td>8.07  1.41x10^-3b 0.49 5.02 0.15</td>
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</tbody>
</table>

^aParameter was fixed at 0 because the inactivation rate kren was 0 for the heat-resistant population of spores at 50ºC; ^bParameter was not significant for this experimental condition but the parameter was not excluded from the model as the F test showed that the phenomenon of two populations was significant; ^cNS, nonsignificant parameter for this experimental condition.

Various experimental conditions, and this fraction could be attributed to the presence of spores at the start of inactivation. Further heterogeneity within the vegetative cell population of strain ATCC 10987 could not be statistically validated, as the biphasic logistic and Geeraerd models were sufficient to describe the data acceptably and further model complexity could not be substantiated. The presence of spores at the start of inactivation was not observed for strain ATCC 14579. The existence of a heat-sensitive and a heat-resistant vegetative population was statistically acceptable for strain ATCC 14579 under most of the experimental conditions, although the parameter f was not significant. Reduction of the nonsignificant parameter f also resulted in redundancy of the parameter kren, which was not statistically acceptable according to the F test. The presence of a mutated heat-resistant subpopulation of vegetative cells for strain ATCC 14579 was evaluated by collecting colonies at the time points corresponding to the end of the inactivation curvatures. Cell suspensions obtained from these colonies did not exhibit
### Table 4. Parameter estimates of the Geeraerd model

<table>
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<tr>
<th>Strain</th>
<th>Growth phase</th>
<th>%NaCl</th>
<th>log$_{10} N(0)$</th>
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<th>$k_{res}$</th>
<th>$t_s$</th>
<th>$k_{co}$</th>
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<tbody>
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<td>Exponential</td>
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<td>5.94x10$^{-6}$</td>
<td>5.65</td>
<td>NS</td>
<td>0$^a$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1%</td>
<td>6.99</td>
<td>4.30x10$^{-6}$</td>
<td>0.71</td>
<td>NS</td>
<td>0$^a$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5%</td>
<td>6.63</td>
<td>1.07x10$^{-5}$</td>
<td>0.62</td>
<td>NS</td>
<td>0$^a$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5%</td>
<td>5.11</td>
<td>3.76x10$^{-4}$</td>
<td>1.06</td>
<td>NS</td>
<td>0$^a$</td>
</tr>
<tr>
<td>Transition</td>
<td>0%</td>
<td>7.62</td>
<td>1.25x10$^{-6}$</td>
<td>0.71</td>
<td>1.73$^c$</td>
<td>NS</td>
<td>0$^a$</td>
</tr>
<tr>
<td></td>
<td>2.5%</td>
<td>7.77</td>
<td>1.65x10$^{-6}$</td>
<td>0.55</td>
<td>NS</td>
<td>0$^a$</td>
<td></td>
</tr>
<tr>
<td>Stationary</td>
<td>0%</td>
<td>7.73</td>
<td>2.60x10$^{-6}$</td>
<td>0.41</td>
<td>NS</td>
<td>0$^a$</td>
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</tr>
<tr>
<td></td>
<td>2.5%</td>
<td>7.39</td>
<td>1.72x10$^{-5}$</td>
<td>0.61</td>
<td>5.68</td>
<td>0$^a$</td>
<td></td>
</tr>
<tr>
<td>ATCC 14579</td>
<td>Exponential</td>
<td>0%</td>
<td>7.05</td>
<td>2.10x10$^{-3}$</td>
<td>0.77</td>
<td>NS</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>6.92</td>
<td>1.22x10$^{-3}$b</td>
<td>0.91</td>
<td>8.87</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.5%</td>
<td>6.70</td>
<td>3.30x10$^{-4}$b</td>
<td>0.57</td>
<td>9.07</td>
<td>0.14b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>6.49</td>
<td>6.89x10$^{-4}$b</td>
<td>0.63</td>
<td>5.88</td>
<td>0.17</td>
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</tr>
<tr>
<td>Transition</td>
<td>0%</td>
<td>7.86</td>
<td>8.40x10$^{-5}$b</td>
<td>0.47</td>
<td>6.76</td>
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</tr>
<tr>
<td></td>
<td>2.5%</td>
<td>7.95</td>
<td>NS</td>
<td>0.35</td>
<td>13.52</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Stationary</td>
<td>0%</td>
<td>8.13</td>
<td>1.27x10$^{-4}$b</td>
<td>0.35</td>
<td>NS</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.5%</td>
<td>8.07</td>
<td>1.94x10$^{-4}$b</td>
<td>0.49</td>
<td>5.20</td>
<td>0.15</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Parameter was fixed at 0 because the inactivation rate $k_{res}$ was 0 for the heat-resistant population of spores at 50°C; $^b$Parameter was not significant for this experimental condition but the parameter was not excluded from the model as the $F$ test showed that the phenomenon of two populations was significant; $^c$Parameter was significant ($P = 0.01$) when the model was fitted to all the replicate data together of this experimental condition. When replica curves were fitted individually, the average parameter estimate was statistically not significant ($P = 0.14$); $^d$NS, nonsignificant parameter for this experimental condition.

Enhanced thermotolerance compared to the original inactivation curves, indicating that the biphasic curvature was not caused by the presence of a genetically more resistant vegetative subpopulation. Furthermore, in addition to the presence of a fraction of spores, a stable heat-resistant subpopulation of vegetative cells could not be demonstrated experimentally for strain ATCC 10987 either.

**Quantification of effects of salt stress and physiological state on thermotolerance.**

After selection of the biphasic logistic model and the Geeraerd model as the most suitable models and reduction of the number of parameters for some experimental conditions, the parameter estimates of these models were used to compare the different experimental conditions. The replicate experiments for each condition were fitted individually with the reduced biphasic logistic and reduced Geeraerd models. The average parameter estimates

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50
were statistically compared using analysis of variance and \( t \) tests. The results of the \( t \) tests are shown in Tables 5 and 6. \( P \) values shown refer to statistical analyses using the Geeraerd model.

The preexposure of exponential-phase cells to salt resulted in an adaptive response rendering the exponential-phase cells more resistant to heat, and the largest increase in heat resistance of exponential-phase cells was achieved after preexposure to 2.5% salt (Tables 3 and 4). At this concentration, strain ATCC 10987 showed a nine times decreased inactivation rate compared to that for exponential-phase cells without salt preexposure. However, significant differences after preexposure to 1% and 5% salt could not be confirmed. Also, strain ATCC 14579 showed the lowest inactivation rate after preexposure to 2.5% salt and displayed the most extended shoulder period after this pretreatment. This inactivation rate was significantly lower than the inactivation rate after preexposure to 1% salt but not significantly different from the inactivation rate after preexposure to 5% salt. The shoulder period after preexposure to 2.5% salt was comparable to that after preexposure to 1% salt and was prolonged compared to that after preexposure to 5% salt. As a consequence, 2.5% salt was chosen to investigate the effects of salt preexposure on the heat resistance of transition- and stationary-phase cells.

The inactivation kinetics of transition- and stationary-phase cells revealed that the physiological state of the cells influenced the heat resistance of cells. Transition- and stationary-phase cells of strain ATCC 10987 exhibited enhanced resistance to heat compared to exponential-phase cells. Stationary-phase cells of strain ATCC 10987

<table>
<thead>
<tr>
<th>Growth phase % NaCl</th>
<th>P value for difference in ( k_{\text{trans}} ) values(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp 0%</td>
<td>Exp 1%   Exp 2.5% Exp 5% Trans 0% Trans 2.5% Stat 0% Stat 2.5%</td>
</tr>
<tr>
<td>Exp 0%</td>
<td>0.00</td>
</tr>
<tr>
<td>Exp 2.5%</td>
<td>0.00</td>
</tr>
<tr>
<td>Exp 5%</td>
<td>0.00</td>
</tr>
<tr>
<td>Trans 0%</td>
<td>0.00</td>
</tr>
<tr>
<td>Trans 2.5%</td>
<td>0.00</td>
</tr>
<tr>
<td>Stat 0%</td>
<td>0.00</td>
</tr>
<tr>
<td>Stat 2.5%</td>
<td>0.00</td>
</tr>
</tbody>
</table>

\(^a\)Exp, exponential; trans, transition; stat, stationary.
\(^b\)Bold value indicates that the two compared parameter estimates were significant different according to the \( t \) test (one-sided).
appeared to be the most resistant, as reflected in the lowest inactivation rate. The heat resistance of strain ATCC 14579 was maximal for transition-phase cells. Transition-phase cells showed a significant shoulder period \((P = 0.01)\) and a similar inactivation rate to that of stationary-phase cells.

The adaptive response to salt was significantly influenced by the physiological state of the cells. Strain ATCC 10987 did not obtain enhanced thermostolerance by preexposure to 2.5% salt in the transition-phase, but stationary-phase cells were a little more resistant after preexposure to salt, since a just significant shoulder period was observed \((P = 0.05)\). Strain ATCC 14579 showed an adaptive response in both the transition- and stationary-phases, as a significantly enhanced shoulder period was observed in both phases \((P = 0.00)\).

To compare the effects of the salt stress response and physiological state on thermostolerance, the thermostolerance of exponential-phase cells after preexposure to 2.5%

![Table 6. Comparison of parameter estimates of the Geeraerd model for B. cereus ATCC 14579, using P values](image)

<table>
<thead>
<tr>
<th>Growth phase</th>
<th>% NaCl and parameter(a)</th>
<th>Exp 0%</th>
<th>Exp 1%</th>
<th>Exp 2.5%</th>
<th>Exp 5%</th>
<th>Trans 0%</th>
<th>Trans 2.5%</th>
<th>Stat 0%</th>
<th>Stat 2.5%</th>
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<tr>
<td>(k_{\text{res}})</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Exp 0%</td>
<td></td>
<td>0.12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp 1%</td>
<td></td>
<td>0.11</td>
<td>0.01</td>
<td></td>
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<td>0.43</td>
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<td></td>
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<tr>
<td>Exp 5%</td>
<td></td>
<td>0.01</td>
<td>0.00</td>
<td>0.04</td>
<td>0.01</td>
<td>0.17</td>
<td>0.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trans 0%</td>
<td></td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.01</td>
<td></td>
<td>0.04</td>
<td>0.10</td>
</tr>
<tr>
<td>Trans 2.5%</td>
<td></td>
<td>0.01</td>
<td>0.00</td>
<td>0.02</td>
<td>0.01</td>
<td>0.17</td>
<td>0.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stat 0%</td>
<td></td>
<td>0.15</td>
<td>0.04</td>
<td>0.45</td>
<td>0.45</td>
<td>0.18</td>
<td>0.04</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>Stat 2.5%</td>
<td></td>
<td>0.15</td>
<td>0.04</td>
<td>0.45</td>
<td>0.45</td>
<td>0.18</td>
<td>0.04</td>
<td>0.10</td>
<td></td>
</tr>
</tbody>
</table>

\(\text{t}\) 
| Exp 0%       | NS          |        |        |          |        |          |            |        |          |
| Exp 1%       | NS          |        |        |          |        |          |            |        |          |
| Exp 2.5%     | NS          | 0.42   |        |          |        |          |            |        |          |
| Exp 5%       | NS          | 0.01   | 0.02   |          |        |          |            |        |          |
| Trans 0%     | NS          | 0.01   | 0.01   | 0.00     | 0.00   | 0.00     |            |        |          |
| Trans 2.5%   | NS          | 0.01   | 0.01   | 0.00     | 0.00   |          |            |        |          |
| Stat 0%      | NS          | NS     | NS     | NS       | NS     | NS       | NS         |        |          |
| Stat 2.5%    | NS          | 0.00   | 0.00   | 0.50     | 0.41   | 0.00     | NS         |        |          |

\(a\)Exp, exponential; trans, transition; stat, stationary.

\(b\)Bold value indicates that the two compared parameter estimates were significant different according to the \(t\) test (one-sided); NS, one of the two compared parameter estimates was a nonsignificant model parameter and was excluded from the model.
salt was compared to the thermotolerance of transition- and stationary-phase cells without preexposure to salt. The effect of growth phase on thermotolerance was comparable to the maximum adaptive salt stress response in exponential-phase cells for both strains. Transition-phase cells of strain ATCC 10987 showed a thermotolerance comparable to that of exponential-phase cells after preexposure to 2.5% salt. Stationary-phase cells of strain ATCC 10987 were significantly more heat resistant than exponential-phase cells after preexposure to 2.5% salt. Transition-phase cells of strain ATCC 14579 showed maximum heat resistance and were slightly more resistant than exponential-phase cells after preexposure to 2.5% salt.

The reported fractions in Tables 3 and 4 show the estimated spore fractions in the exponential-, transition-, and stationary-phases for strain ATCC 10987 at the start of inactivation. The observed levels of spores in the exponentially growing cultures of strain ATCC 10987 were similar for the four exponential growth phase conditions tested \( (P = 0.97) \). Spores were formed further at the end of the growth cycle, as the transition- and stationary-phase cultures exhibited significantly larger amounts of spores than the exponential-phase cultures \( (P = 0.00) \), with the highest level of spores found in the stationary-phase cultures.

Discussion

A deliberate combination of preservation hurdles can be used to design nutritious, tasty, and microbially safe minimally processed foods, and quantitative risk assessment studies can be used to optimize the balance between food quality and microbial safety. In many studies, it has been shown that adaptation to a certain type of stress may protect cells against other severe homologous or heterologous stresses (e.g., see references 3, 4, 17, 22, and 28). Since nonstressed microorganisms are often used in quantitative risk assessment studies, preservation measures based on these data might not be sufficient to ensure the safety of processed food. Therefore, in this study the effect of salt stress on thermotolerance was quantitatively assessed by using microbial survival models which were adequate for fitting the survival characteristics of strains ATCC 10987 and ATCC 14579.

**Experimental design and fitting performances of survival models.** Considering the biological variation mentioned by Browne and Dowds (4), inactivation experiments were reproduced on three different days, and sampling was performed in duplicate. The observed variation between days was obvious and was reflected in the measuring error. The
measuring error influenced the selection of a statistically accepted model and was represented in the \( f \) value. The higher the measuring error, the less complex a model has to be to describe the data acceptably. It is important to include this biological variation in the quantification, and therefore reproductions have to be performed to provide valuable information for quantitative risk assessment studies. In addition, the experimental procedure influences the inactivation curvatures, which necessitates an unambiguous description of the experimental process in order to compare the data to those in other studies and to evaluate the findings.

The models used in this study describe the various survival curvatures known for vegetative cells. Both the Baranyi model and the modified Gompertz model were used to describe the sigmoid curvature. The fitting performance of the modified Gompertz model was better than that of the Baranyi model and might be preferred when the experimental data do not show linear behavior in the mid-phase, as this results in a better description of the data.

The statistical analyses performed with the biphasic logistic model and the Geeraerd model resulted in similar conclusions. One striking difference was observed between the fitting performances of both models, namely, in the estimation of the parameter \( \log_{10} N(0) \). When the shoulder period \( t_s \) was not significant in both models, the parameter \( \log_{10} N(0) \) was estimated to be lower by the biphasic logistic model than by the Geeraerd model. Both models can fit a curvature without a shoulder period by selecting a \( t_s \) of 0. However, the shoulder curvature is not completely eliminated from the biphasic logistic model when \( t_s = 0 \), resulting in a lower estimation of \( \log_{10} N(0) \).

Parameter \( f \) of the biphasic logistic and Geeraerd models was not significant for most of the experimental conditions tested for strain ATCC 14579 but was not excluded from the models because the \( F \) test showed that the biphasic inactivation was significant (Tables 3 and 4). Also, the parameter \( k_{	ext{es}} \) was not significant for one experimental condition for strain ATCC 14579 (exponential-phase cells with preexposure to 2.5% salt) but was not excluded. The confidence intervals of both parameters were large when the parameters were not significant but not excluded (data not shown). Zwietering et al. mentioned that parameters which are strongly correlated are difficult to estimate and have large confidence intervals (32). The correlation matrices of the experimental conditions revealed that parameters \( f \) and \( k_{	ext{es}} \) were strongly correlated (\( > 0.99 \)) when both parameters were not significant and not excluded (exponential-phase cells with preexposure to 2.5% salt). The correlation between both parameters was lower (\( > 0.94 \)) when only parameter \( f \) was
nonsignificant and not excluded. Parameters were less correlated (< 0.90) when both parameters were significant (exponential-phase cells without preexposure to salt).

**Effect of salt preexposure on thermotolerance.** The sodium chloride concentrations used in this study were both nonlethal and lethal for strains ATCC 10987 and ATCC 14579 (Figure 1; Tables 3 and 4). Preexposure to 5% sodium chloride resulted in a significant decrease in cells at the start of inactivation \[
\log_{10} N(0)\]. When cells are exposed to lethal salt stress conditions, two phenomena can take place, namely, the inactivation of bacterial cells and the induction of an adaptive response in the surviving cells, and this agrees with observations in *Listeria monocytogenes* (19, 20). In our study, differences in the heat sensitivities of cells after preexposure to nonlethal and lethal salt stress conditions could not be confirmed, indicating that cells which are able to survive lethal stress conditions are still capable of demonstrating an adaptive stress response that might be comparable to the adaptive stress response of cells which are exposed to nonlethal stress conditions.

Increased thermotolerance by short-term preexposure of cells to salt was observed previously in *B. cereus* ATCC 14579 (22) and *B. cereus* NCIMB 11796 (4). Periago et al. showed an overlap in proteins induced by heat shock and salt stress exposure as well as induction of non-heat-shock-specific proteins during salt stress exposure (22). Several heat shock proteins induced during salt stress exposure belong to the group of chaperones and proteases, and these proteins act together to maintain quality control of cellular proteins (1). Increased production of heat shock proteins after salt stress exposure in *B. cereus* was also demonstrated by Browne and Dowds (4) and was observed in other bacilli as well (21, 23, 29). In addition to de novo protein synthesis during preexposure to salt, Periago et al. mentioned an increase in thermotolerance after preexposure to salt in the presence of chloramphenicol, indicating an alternative and complementary mechanism (22). It has been shown that compatible solutes such as glycine betaine can function as thermoprotectants in *Bacillus subtilis* (12).

Thermotolerance and adaptive stress response are affected by strain diversity, physiological state, and population heterogeneity. Strains ATCC 10987 and ATCC 14579 showed differences in thermotolerance. Consequently, the adaptive stress responses of both strains cannot be compared straightforwardly, as preexposure to salt resulted in a lower inactivation rate for strain ATCC 10987 and an additional shoulder period for strain ATCC 14579. Thus, it was not feasible to conclude which strain showed the maximum adaptive stress response.
Cells toward the end of the growth cycle appeared to be most resistant to thermal stress. Cells were highly sensitive to heat during the exponential growth phase and became more resistant to heat during the transition- and stationary-phases, as observed previously for *B. cereus* NCIMB 11796 (4) and for other bacteria (see, e.g., reference 18). Induction of a generalized stress response and additional physiological changes provide enhanced resistance for cells toward the end of the growth cycle (25). Moreover, the transient decline in pH of the culture during the growth cycle, reaching pH values of 7.2 and 6.5 in the exponential-phase for *B. cereus* ATCC 10987 and the transition-phase for *B. cereus* ATCC 14579, respectively (data not shown), may have contributed in the latter case to the enhanced stress resistance of these cells. As shown by Browne and Dowds (3), 40 min of exposure of exponential-phase cells of *B. cereus* NCIMB 11796 to pH 6.3 resulted in enhanced stress resistance. Our study has shown that the effect of physiological state on heat resistance was comparable to the maximum adaptive response to salt demonstrated in exponential-phase cells. This indicates that two different stresses may provide similar increased resistance levels.

A growth cycle-dependent effect of salt adaptation was observed for both *B. cereus* strains. These results are consistent with other studies, which examined differences in heat shock induced thermotolerance for exponential- and stationary-phase cells (14, 19).

In the current study, we quantified the adaptive stress response in three different growth phases in more detail and demonstrated that the significance of the adaptive stress response was strain and growth phase dependent.

The inactivation kinetics of both strains showed heterogeneous heat resistance within the population. The tailing of strain ATCC 10987 was explained by a minor fraction of spores present at the start of inactivation, and concealment of heat-resistant vegetative cells in the tail could not be confirmed. No spores could be detected at the start of inactivation for strain ATCC 14579. The biphasic nature of the inactivation curvature for strain ATCC 14579 suggested heterogeneity within the vegetative cell population. However, genotypic heterogeneity was not found by assessment of the thermotolerance of survivors from the end of the inactivation curvature. Other studies agree with our observation suggesting that tail survivors are not genotypically distinct (5, 13). The reported phenotypic biphasic inactivation might be of practical importance in processing because a subpopulation is able to display greater resistance than that of the majority of the population, which might influence the safety margin settings.

In conclusion, based on statistical indices and model characteristics, biphasic models with a shoulder period were selected. Both models could be used to quantify in
detail the effect of salt stress response on thermal inactivation kinetics for exponential-, transition-, and stationary-phase cells of strains ATCC 10987 and ATCC 14579. Each model parameter was used to characterize a survival characteristic, and both models were flexible, allowing a reduction of parameters when certain phenomena were not present. Strain diversity had the greatest impact on thermotolerance and survival curvatures. The maximal adaptive salt stress response in exponential-phase cells was comparable to the effect of physiological state on thermotolerance. The adaptive salt stress responses of transition- and stationary-phase cells were less pronounced than that of exponential-phase cells. Quantification of the adaptive stress response might be instrumental to understanding the adaptation mechanisms and might allow the food industry to develop more accurate and realistic quantitative risk assessments.

References


Quantification of the effect of culturing temperature on salt-induced heat resistance of *Bacillus* species

Heidy M. W. den Besten, Erik-Jan van der Mark, Lonneke Hensen, Tjakko Abee, Marcel H. Zwietering

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Abstract

Short- and long-term exposure to mild stress conditions can activate stress adaptation mechanisms in pathogens, resulting in a protective effect toward otherwise lethal stresses. The mesophilic strains *Bacillus cereus* ATCC 14579 and ATCC 10987 and the psychrotolerant strain *B. weihenstephanensis* KBAB4 were cultured at 12°C and 30°C until the exponential growth phase (i) in the absence of salt, (ii) in the presence of salt, and (iii) with salt shock after they reached the exponential growth phase and subsequently heat inactivated. Both the first-order model and the Weibull model were fitted to the inactivation kinetics, and statistical indices were calculated to select for each condition the most appropriate model to describe the inactivation data. The third-decimal reduction times (which reflected the times needed to reduce the initial number of microorganisms by three decimal powers) were determined for quantitative comparison. The heat resistance of both mesophilic strains increased when cells were salt cultured and salt shocked at 30°C, whereas these salt-induced effects were not significant for the psychrotolerant strain. In contrast, only the psychrotolerant strain showed salt-induced heat resistance when cells were cultured at 12°C. Therefore, culturing temperature and strain diversity are important aspects to address when adaptive stress responses are quantified. The activated adaptive stress response had an even larger impact on the number of surviving microorganisms when the stress factor (i.e., salt) was still present during inactivation. These factors should be considered when stress-integrated predictive models are developed that can be used in the food industry to balance and optimize processing conditions of minimally processed foods.
**Introduction**

*Bacillus cereus* is a widespread, spore-forming pathogen that can be isolated from a range of different food products (4, 27), including pastry, vegetables and vegetable products, milk and milk products, and ready-to-eat foods. This toxin-producing pathogen can cause diarrhea and emesis (13, 25). The diarrheal syndrome is caused by several enterotoxins which are produced by vegetative cells in the small intestine. The emetic toxin, cereulide, causes emesis and is produced in foods before ingestion. Adequate chilling of foods is important to control the growth and toxin production of enterotoxin-producing (17) and emetic toxin-producing (7, 18) *B. cereus* strains.

During processing and storage of mildly processed foods, bacteria are exposed to one or more preservation stresses, known as hurdles (16). While individual hurdles might not be effective in controlling microbial growth, the right combination of hurdles can be powerful in controlling microbial growth in minimally processed foods. However, the potential of *Bacillus* to become more resistant to stresses challenges the effectiveness of minimal processing. Several studies have demonstrated that exposure to mild stressing conditions can result in the increased resistance of both mesophilic and psychrotolerant members of the *B. cereus* group (2, 3, 5, 21, 22, Chapter 2). These studies used optimal culturing temperature during mild stress exposure to investigate adaptive stress responses. However, during processing, distribution, and storage, the temperature of foods may be lower because chilling is commonly used in the minimal processing food chain. Therefore, investigation of the effect of low incubation temperature on the adaptive stress response of food-borne bacteria is of great relevance and could provide valuable information for quantitative exposure assessment studies.

In the study described here, three representatives of the *B. cereus* group (12), namely, the mesophilic strains *B. cereus* ATCC 14579 and ATCC 10987 and the psychrotolerant strain *Bacillus weihenstephanensis* KBAB4, were cultured at 30°C in the absence and presence of mild salt stress, after which their heat resistance was assessed. Moreover, the culturing of cells was also performed at 12°C to determine the effect of a lowered culturing temperature on the adaptive salt stress response. The third-decimal reduction time estimates were determined to evaluate the effects of the various culturing variables on the heat resistance of the three strains.
Materials and methods

**Bacterial strain and culturing conditions.** The mesophilic strains *B. cereus* ATCC 14579 (8, 11) and *B. cereus* ATCC 10987 (23) and the psychrotolerant strain *B. weihenstephanensis* KBAB4 (15) were used throughout this study. The cultures were stored frozen in brain heart infusion (BHI) broth (Becton Dickinson, France) supplemented with 25% (vol/vol) glycerol (Sigma, The Netherlands) at -80°C. The bacteria were cultivated before each experiment in 10 ml BHI broth and incubated overnight at 30°C with shaking at 200 rpm (Innova 4335; New Brunswick Scientific, The Netherlands). Afterwards, these cultures were inoculated in Erlenmeyer flasks (250 ml) containing 50 ml fresh BHI broth and incubated at 30°C (Julabo SW22; Julabo Labortechnik, Germany) and at 12°C (Forma orbital shaker 481; Thermo Electron Corporation) with shaking at 200 rpm until the stationary growth phase. The latter temperature was chosen because various isolates of the *B. cereus* group were unable to grow at 10°C (1).

**Heat inactivation without or with preexposure to salt.** To investigate the effect of salt culturing and salt shock on subsequent heat resistance, the following procedure was followed. The stationary-phase cultures, which were precultured at 30°C, were inoculated in Erlenmeyer flasks containing 50 ml fresh BHI broth and BHI broth supplemented with 2.5% (wt/vol) sodium chloride (VWR, Belgium) and were further incubated at 30°C with shaking at 200 rpm, until the cells were exponentially growing (absorbance at 600 nm, 0.4 to 0.5; Novaspec II spectrophotometer; Pharmacia Biotech, United Kingdom). When they reached this optical density, both exponentially growing cultures, which were cultured in the absence of supplementary salt (untreated) or in the presence of supplementary salt (salt cultured), were further incubated for an extra period of 30 min at 30°C with shaking at 200 rpm. The concentration of 2.5% sodium chloride for salinity stressing was chosen because we previously demonstrated that short-term exposure of exponentially growing cells of *B. cereus* ATCC 14579 and ATCC 10987 to this salt concentration resulted in optimal salt-induced heat resistance for both strains (5, Chapter 2). To expose exponentially growing cells to a salinity upshift for a short time interval rather than being cultured for a long-term interval in the presence of salt, the cells were cultured in BHI broth until the exponential growth phase, after which the BHI broth was supplemented with 2.5% (wt/vol) sodium chloride. These salt-shocked cultures were also incubated for 30 min at 30°C with shaking at 200 rpm. To investigate the effect of a lowered culturing temperature on the adaptive salt stress response, a similar procedure was followed to produce untreated, salt-cultured, and
salt-shocked cultures at 12°C. The pH of the untreated, salt-cultured, and salt-shocked cultures of the three strains, grown at 30°C and 12°C, was measured and did not reach values below pH 7.0.

To heat inactivate the cells, the cultures were subsequently added (1:100, vol/vol) to 20 ml BHI broth and BHI broth supplemented with 2.5% (wt/vol) sodium chloride that were preheated to 50°C, 48°C, and 44.5°C for *B. cereus* ATCC 14579, *B. cereus* ATCC 10987, and *B. weihenstephanensis* KBAB4, respectively, and the cells were heat inactivated at the selected lethal temperatures with shaking at 200 rpm (Julabo SW22; Julabo Labortechnik). Before and after heat exposure, samples were taken and decimal dilutions were made in peptone saline solution (1 g neutralized bacteriological peptone [Oxoid, United Kingdom] supplemented with 8.5 g sodium chloride per liter). The appropriate dilutions were surface plated, in duplicate, on BHI agar plates (BHI broth supplemented with 15 g agar [Oxoid] per liter) using a spiral plater (Eddy Jet; IUL Instruments, Spain). The plates were incubated at 30°C for 16 h to 24 h, and the numbers of CFU were expressed in log_{10} CFU ml^-1. For all experimental conditions, three independent reproductions were performed.

**Microbial survival model fitting.** Two microbial survival models were fitted to the inactivation data of the three reproductions together for each experimental condition for the three strains using the program TableCurve 2D (Windows, version 2.03).

(i) The first-order model was as follows:

\[
\log_{10} N(t) = \log_{10} N(0) - \frac{t}{D}
\]

where log_{10} N(t) is the log 10 number of microorganisms at time t (min), log_{10} N(0) is the initial log_{10} number of microorganisms, and D is the time needed to reduce the number of microorganisms by one decimal (min).

(ii) The Weibull model was as follows:

\[
\log_{10} N(t) = \log_{10} N(0) - \left(\frac{t}{\delta}\right)^{\beta}
\]

where \( \delta \) is the first-decimal reduction time (min) and \( \beta \) is a fitting parameter that defines the shape of the curve.

To evaluate the model-fitting performances of the first-order model and the Weibull model, a procedure similar to that described previously was followed (5, Chapter 2). The following statistical indices were calculated.
(i) MSE model. The lower the mean square error of the model (MSE\text{model}) is, the better is the adequacy of the model to describe the data. It was calculated as follows:

\[
\text{MSE}\text{model} = \frac{\text{RSS}}{\text{DF}} = \frac{\sum_{i=1}^{n} \left( \log_{10} N_{\text{observed}}^{i} - \log_{10} N_{\text{fitted}}^{i} \right)^2}{n - s}
\]

where RSS is the residual sum of squares, DF is the degrees of freedom, \(n\) is the number of data points, \(s\) is the number of parameters of the model, \(\log_{10} N_{\text{observed}}^{i}\) is the observed \(\log_{10}\) number of microorganisms for data point \(i\), and \(\log_{10} N_{\text{fitted}}^{i}\) is the fitted \(\log_{10}\) number of microorganisms for data point \(i\).

(ii) MSE data. The lower the mean square error of the data (MSE\text{data}), which indicates the measuring error, is, the less variation was observed between the reproductions per experimental condition. It was calculated as follows:

\[
\text{MSE}\text{data} = \frac{\text{RSS}}{\text{DF}} = \frac{\sum_{i=1}^{n} \sum_{j=1}^{k} (\text{average} \log_{10} N^{i} - \log_{10} N_{j}^{i})^2}{n - m}
\]

where \(n\) is the number of data points, \(m\) is the number of sampling time points, \(k\) is the number of reproductions at each sampling time point, average \(\log_{10} N^{i}\) is the mean value of the \(\log_{10}\) number of microorganisms at sampling time point \(i\), and \(\log_{10} N_{j}^{i}\) is the \(\log_{10}\) number of microorganisms at sampling time point \(i\) for specific reproduction \(j\).

(iii) \(F\) test. The \(F\) test was used to determine if the fitting performance of the model was statistically acceptable. The \(f\) value was calculated by the equation:

\[
f = \frac{\text{MSE}\text{model}}{\text{MSE}\text{data}}
\]

The \(f\) value was tested against an \(F\) table value (95% confidence). If the \(f\) value was smaller than the \(F\) table value (\(F_{DF\text{model}}^{DF\text{data}}\)), the \(F\) test was accepted and this indicated that the model fitting was statistically acceptable.

For some experimental conditions, the fitting performances of both the first-order model and the Weibull model were statistically acceptable, according to the \(F\) test (equation 5). To evaluate whether the extra model parameter \(\beta\) of the Weibull model significantly improved the model description of these inactivation data sets, the 95% confidence interval of model parameter \(\beta\) was calculated and it was checked whether this confidence interval included the value of 1. Furthermore, an additional \(F\) test was performed (28):

\[
f = \frac{(\text{RSS}_2 - \text{RSS}_1)/(\text{DF}_2 - \text{DF}_1)}{\text{MSE}\text{data}}
\]
where RSS₁ is the residual sum of squares of the Weibull model; RSS₂ is the residual sum of squares of the first-order model; and DF₁ and DF₂ are the degrees of freedom for the Weibull model and the first-order model, respectively. Note that DF₂ − DF₁ equals 1, because the difference between the number of model parameters of the Weibull model and the first-order model is 1. The f value was tested against an F table value (95% confidence, (F_{DF_{num}}^{1})). If the f value was smaller than the F table value, the F test was accepted and this indicated that model parameter β did not significantly improve the description of the inactivation data.

**Comparison of model parameters.** In order to compare the various conditions, the third-decimal reduction time, which reflected the time needed to reduce the initial number of microorganism by three decimal powers, was estimated for each condition. The third-decimal reduction time could be estimated without extrapolation outside the experimental ranges. For each condition, the selected model (first-order model or Weibull model) was fitted to the three independent reproductions individually, and the average third-decimal reduction time estimate was calculated. Independent t tests (two-sided) were performed to compare the average third-decimal reduction time estimates of the different conditions and to investigate if there were significant effects of short- and long-term salt stress exposure at 30°C and 12°C on subsequent heat resistance (SPSS, version 15.0.1, for Windows).

**Results**

**Effect of salt culturing and salt shock on subsequent heat resistance.** The mesophilic strains *B. cereus* ATCC 14579 and ATCC 10987 and the psychrotolerant strain *B. weihenstephanensis* KBAB4 were cultured until the exponential growth phase (i) in the absence of salt (untreated), (ii) in the presence of salt (salt cultured), and (iii) with salt shock for 30 min after they reached the exponential growth phase (salt shocked). Subsequently, the heat resistance of the untreated, salt-cultured, and salt-shocked cells was assessed. Figure 1 shows the inactivation kinetics of the cells that were cultured at 30°C. The temperatures selected to inactivate the three strains were not similar, namely, 50°C, 48°C, and 44.5°C for *B. cereus* ATCC 14579, *B. cereus* ATCC 10987, and *B. weihenstephanensis* KBAB4, respectively. These inactivation temperatures were chosen to obtain comparable inactivation kinetics for the untreated cultures of the three strains (Figure 1a to c). The heat resistance of both mesophilic strains, *B. cereus* ATCC 14579 and ATCC 10987, increased when cells were salt cultured at 30°C (Figure 1d and e) or pre-
exposed to salt for 30 min at 30°C (Figure 1g and h) compared to the heat resistance of the untreated cultures of both strains, whereas the effects of salt culturing or salt shock on heat resistance were less pronounced for the psychrotolerant strain, *B. weihenstephanensis KBAB4* (Figure 1f and i).

The addition of salt in the inactivation medium did not increase the heat resistance of cells that were not preexposed to salt (Figure 1a to c) at 30°C. However, the presence of salt in the inactivation medium had a notable increasing protective effect for the salt-cultured and salt-shocked cells of all the three strains (Figure 1d to i).

**Figure 1.** Heat inactivation kinetics of *Bacillus cereus* ATCC 14579 (a, d, and g), *Bacillus cereus* ATCC 10987 (b, e, and h), and *Bacillus weihenstephanensis* KBAB4 (c, f, and i). Cells were cultured at 30°C until the exponential growth phase in the absence of salt (a, b, and c), in the presence of salt (d, e, and f), or with salt shock for 30 min after they reached the exponential growth phase (g, h, and i) and were subsequently exposed to heat in the absence of salt (◇) and in the presence of salt (△). Continuous curves, fitting of the selected microbial survival model; black lines, fitting of the Weibull model; gray lines, fitting of the first-order model.
Effect of culturing temperature on salt-induced heat resistance. To study the effect of a lowered culturing temperature on the adaptive responses to salt stress, cells were also cultured at 12°C in the absence of salt (untreated) and in the presence of salt (salt cultured) or were salt shocked at 12°C for 30 min after they reached the exponential growth phase at 12°C (salt shocked) (Figure 2). In contrast to culturing at 30°C, neither of the mesophilic strains showed increased heat resistance when the strains were cultured in the presence of salt at 12°C (Figure 2d and e) or were salt shocked after they reached the exponential growth phase at 12°C (Figure 2g and h) compared to the heat resistance of cells that were not preexposed to salt stress before heat inactivation (Figure 2a and b). However, salt culturing (Figure 2f) and, to a more limited extent, salt shock at 12°C (Figure 2i) affected

![Figure 2](image_url)

Figure 2. Heat inactivation kinetics of *Bacillus cereus* ATCC 14579 (a, d, and g), *Bacillus cereus* ATCC 10987 (b, e, and h), and *Bacillus weihenstephanensis* KBAB4 (c, f, and i). Cells were cultured at 12°C until the exponential growth phase in the absence of salt (a, b, and c), in the presence of salt (d, e, and f), or with salt shock for 30 min after they reached the exponential growth phase (g, h, and i) and were subsequently exposed to heat in the absence of salt (○) and in the presence of salt (△). Continuous curves, fitting of the selected microbial survival model; black lines, fitting of the Weibull model; gray lines, fitting of the first-order model.
the heat resistance of the psychrotolerant strain, resulting in increased heat resistance of this strain compared to that after culturing at 12°C without salt stress (Figure 2c). The presence of salt in the inactivation medium increased the heat resistance of the untreated, salt-cultured, and salt-shocked cells of *B. weihenstephanensis* KBAB4 (Figure 2c, f, and i).

Because species of the *B. cereus* group are spore-formers, the potential presence of spores was determined in the untreated, salt-cultured, and salt-shocked cultures of the three strains grown at 30°C and 12°C. For that, the cultures were heated for 15 min at 75°C to kill the vegetative cells and to determine the number of spores. The number of spores in all the cultures was below the detection limit of 5 spores per ml, and therefore, spores could not have contributed to the observed inactivation curvature characteristics shown in Figure 1 and 2.

**Quantification of the effect of culturing temperature on salt-induced heat resistance.**
In order to select the most appropriate model to quantitatively describe the heat inactivation kinetics of the three strains, the first-order model and the Weibull model were fitted to the replicate inactivation data together for each experimental condition. According to the $F$ test, which statistically evaluated the mean square error of the model fitting compared to the measuring error (mean square error of the data) (equation 5), the fitting performance of the Weibull model was statistically acceptable for all the conditions (12 inactivation data sets per strain). For some experimental conditions, the performances of both the first-order model and the Weibull model were statistically acceptable, according to this $F$ test. For these conditions, an additional $F$ test (equation 6) was performed to evaluate whether the model parameter $\beta$ of the Weibull model significantly improved the description of the inactivation data compared to that achieved with the first-order model. When the exclusion of model parameter $\beta$ was statistically acceptable, the model parameter $\beta$ was fixed equal to 1, resulting in the use of the first-order model to describe these inactivation data sets (10 out of 36 inactivation data sets). Under most of the conditions for which the exclusion of model parameter $\beta$ was statistically acceptable, the confidence interval of model parameter $\beta$ of the Weibull model also included 1, confirming an acceptable reduction of model complexity. For one experimental condition, the exclusion of model parameter $\beta$ of the Weibull model, although it was statistically different from 1, was statistically acceptable according to the $F$ test, indicating that in some boundary cases contradictory results can be obtained. The continuous curves in Figure 1 and Figure 2 show the fitting of the selected survival model (first-order model or Weibull model) for each experimental condition.
To quantify and evaluate the overall effects of the various culturing variables on heat resistance, the time to reach a 3-log_{10} reduction of the initial population was estimated for each experimental condition. For each experimental condition, the selected survival model was fitted to the individual reproductions, and the average estimate of the third-decimal reduction time was calculated. Figures 3 to 5 show the third-decimal reduction time estimates of the untreated, salt-cultured, and salt-shocked cells cultured at 12°C and 30°C of *B. cereus* ATCC 14579 (Figure 3), *B. cereus* ATCC 10987 (Figure 4), and *B. weihenstephanensis* KBAB4 (Figure 5). The 95% confidence intervals of the third-decimal reduction time estimates clearly visualize the statistical comparison. Cells of the mesophilic strains *B. cereus* ATCC 14579 and ATCC 10987 that were salt cultured and salt shocked at 30°C were significantly more heat resistant than cells that were cultured at 30°C without salt stress (Figure 3 and 4) (*P* < 0.05), and the third-decimal reduction time estimates of these salt-cultured and salt-shocked cells were two to five times higher than those of untreated cells. The statistical analyses confirmed that culturing temperature had a significant effect on the adaptive salt stress responses because salt culturing and salt shock did not significantly increase the level of heat resistance when both strains were cultured at 12°C (*P* > 0.05). The culturing temperature also significantly affected the adaptive salt...
Chapter 3

Figure 4. Third-decimal reduction time estimates of *Bacillus cereus* ATCC 10987 inactivated in the absence of salt (open bars) and in the presence of salt (gray bars). Before heat inactivation, the cells were cultured at 12°C and 30°C until the exponential growth phase in the absence of salt (untreated), in the presence of salt (salt cultured), or with salt shock for 30 min after they reached the exponential growth phase (salt shocked). Error bars indicate 95% confidence intervals.

stress response of the psychrotolerant strain *B. weihenstephanensis* KBAB4 (Figure 5), as salt culturing and salt shock increased the third-decimal reduction time estimates two to five times only when cells of *B. weihenstephanensis* KBAB4 were cultured at 12°C.

Figure 5. Third-decimal reduction time estimates of *Bacillus weihenstephanensis* KBAB4 inactivated in the absence of salt (open bars) and in the presence of salt (gray bars). Before heat inactivation, the cells were cultured at 12°C and 30°C until the exponential growth phase in the absence of salt (untreated), in the presence of salt (salt cultured), or with salt shock for 30 min after they reached the exponential growth phase (salt shocked). Error bars indicate 95% confidence intervals.
The parameter estimates presented in Figure 3 to 5 can also be used to evaluate the impacts of the various culturing variables on heat resistance and to distinguish main variables from side variables. Figures 3 to 5 unambiguously show that for the cultures in which the salt adaptive stress response was significantly activated, the heat resistance significantly increased when salt was also present during the inactivation treatment, resulting in very heat resistant cells for all the three strains. Moreover, although salt culturing and salt shock at 30°C did not significantly increase the heat resistance for *B. weihenstephanensis* KBAB4, the presence of salt during inactivation of these salt-preexposed cells also resulted in significantly more heat-resistant cells. The third-decimal reduction time estimates of these very heat-resistant cells of *B. cereus* ATCC 14579, *B. cereus* ATCC 10987, and *B. weihenstephanensis* KBAB4 were 5 to 15 times higher than those cells that were not exposed to salt during culturing and subsequent heat inactivation.

**Discussion**

In minimally processed foods, various hurdles are combined to control food quality and microbial safety. Quantification of food production and distribution processes contributes to the balance and optimization of the quality and safety of these foods. Previous studies demonstrated that the adaptive stress responses of food-borne pathogens can have a large impact on their resistance (e.g., see references 2, 3, 5, 14, 22, 24, Chapter 2) and should therefore be addressed in quantitative assessments of the inactivation kinetics of pathogens in the food chain.

Heating is a widely used method for microbial inactivation, and a thorough literature survey showed that classical first-order inactivation kinetics are the exception rather than the rule (26). Also in the present study, for most of the conditions tested, the nonlinear Weibull model described the inactivation data significantly more adequately than the first-order model. However, when a reduction of model complexity was statistically acceptable, the first-order model was preferred to describe the inactivation data. This principle of parsimony, meaning that the model with the smallest number of parameters that adequately represents the data is preferred above a more complex model, is a strong model selection criterion. The biological variation influenced the most appropriate model selection because the higher the measuring error is, the simpler a model can be to adequately describe the data, and therefore, reproductions of experiments should be performed to evaluate if deviations from first-order kinetics are statistically relevant. In this study, the third-decimal reduction times were estimated to evaluate the effects of the various culturing...
variables on subsequent heat resistance. The third-decimal reduction time is an interpretable model parameter that could be easily recognized in the inactivation curvatures and reflected the time needed to produce a substantial inactivation of the pathogen without extrapolation outside the experimental ranges, and therefore, these estimates were used to evaluate the overall effects of culturing variables and to separate the main culturing variables from side culturing variables. Noteworthy are that the conclusions based on the third-decimal time estimates were rather comparable to those based on the first-decimal reduction time estimates (data not shown), confirming the observed trends.

The adaptive salt stress response depended on the culturing temperature, the stress exposure time (salt cultured versus salt shocked), and differences between the strains. Both salt culturing and salt shock at 30°C increased the subsequent heat resistance of mesophilic strains *B. cereus* ATCC 14579 and ATCC 10987, whereas these salt-induced effects were not significant for psychrotolerant strain *B. weihenstephanensis* KBAB4. Increased heat resistance after short-term preexposure to salt was previously observed for *B. cereus* ATCC 14579 (5, 22, Chapter 2), *B. cereus* ATCC 10987 (5, Chapter 2), *B. cereus* NCIMB 11796 (3), and *B. weihenstephanensis* DSM11827 (21). It was demonstrated that some proteins induced upon heat shock were also induced after salinity upshift, and this could have contributed to the salt-induced cross-protective effects toward heat. Moreover, complementary adaptation mechanisms can be involved, as genes encoding various osmoprotectant transporters were highly upregulated in *B. cereus* ATCC 14579 in response to increased osmolarity (6, Chapter 6), and it was shown that osmoprotectants (e.g., glycine betaine) can serve as thermoprotectants (10). The extended use of chilling in the minimally processed food chain underlines the need to also investigate the adaptive stress responses of bacteria at nonoptimal incubation temperatures. As indicated by the limited available data describing the effect of low incubation temperature, the adaptive acid stress response of *Listeria monocytogenes* was reduced when the stressing temperature decreased (14). In the present study, we demonstrated that salt-induced thermostolerance was strongly influenced by the incubation temperature. Long- and short-term exposure to salt stress at 12°C did not significantly increase the heat resistance of either of the mesophilic strains. In contrast, psychrotolerant strain *B. weihenstephanensis* KBAB4 showed substantially increased heat resistance when cells were exposed to salt stress at 12°C, pointing to the importance of including differences between strains when adaptive stress responses are to be quantified.

The impact of an activated salt stress response on heat resistance can be larger when the stress factor is also present in the inactivation medium. The protective effect of salt in the inactivation medium was previously demonstrated for other pathogens, such as
Salmonella enterica serovar Typhimurium (19), and tended to protect spores of B. cereus against heat (20). This may be explained by the increased stability of proteins (9). Our study showed that heat inactivation of salt-adapted cells in the presence of salt resulted in highly heat resistant cells of B. cereus ATCC 14579, B. cereus ATCC 10987, and B. weihenstephanensis KBAB4, and this can be of relevance for food-processing conditions when mild stress applications are subsequently followed by a heating processing step.

This study showed that culturing temperature and strain diversity are important aspects to address when adaptive stress responses are quantified and need to be considered in evaluations of adaptive stress responses. An activated adaptive response can have an even larger impact on the number of surviving organisms when the stress factor is still present during heat inactivation. These factors should be considered when stress-integrated predictive models are developed in order for the latter to be able to provide reliable predictions of the inactivation kinetics of microorganisms in minimally processed foods.

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References


Quantitative analysis of population heterogeneity of the adaptive salt stress response and growth capacity of *Bacillus cereus* ATCC 14579

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Abstract

Bacterial populations can display heterogeneity with respect to both the adaptive stress response and growth capacity of individual cells. The growth dynamics of *Bacillus cereus* ATCC 14579 during mild and severe salt stress exposure were investigated for the population as a whole in liquid culture. To quantitatively assess the population heterogeneity of the stress response and growth capacity at a single cell level, a direct imaging method was applied to monitor cells from the initial inoculum to the microcolony stage. Highly porous Anopore strips were used as a support for the culturing and imaging of microcolonies at different time points. The growth kinetics of cells grown in liquid culture were comparable to those of microcolonies grown upon Anopore strips, even in the presence of mild and severe salt stress. Exposure to mild salt stress resulted in growth that was characterized by a remarkably low variability of microcolony sizes, and the distributions of the log_{10}-transformed microcolony areas could be fitted by the normal distribution. Under severe salt stress conditions, the microcolony sizes were highly heterogeneous, and this was apparently caused by the presence of both a nongrowing and growing population. After discriminating these two subpopulations, it was shown that the variability of microcolony sizes of the growing population was comparable to that of non-salt-stressed and mildly salt-stressed populations. Quantification of population heterogeneity during stress exposure may contribute to an optimized application of preservation factors for controlling growth of spoilage and pathogenic bacteria to ensure the quality and safety of minimally processed foods.
Introduction

*Bacillus cereus* is a common contaminant of various foods (2, 9), causing both spoilage and food poisoning. Vegetative cells of *B. cereus* can produce two types of toxins that can harm the consumer (11). The heat-stable emetic toxin is formed in the food itself, while the diarrheal toxins, called enterotoxins, are produced during intestinal growth of *B. cereus*.

In the last decades, there has been a substantial increase in production and sales of mildly processed ready-to-use foods (9). The microbial stability and safety of minimally processed foods are based on the use of various mild preservation factors. However, successive application of mild preservation factors can influence the ability of the organism to survive and grow in the food. *B. cereus* gains increased resistance during exposure to mild stresses by triggering of the so-called adaptive stress response (7, 8, 10, 29, Chapter 2). Furthermore, it is becoming apparent that there is heterogeneity within a population in the way cells deal with stress, even in a homogeneous environment. A fraction of a microbial population may be more stress resistant, resulting in a biphasic nature of the stress response, and such heterogeneous resistance can be confirmed to be genotypic (1, 20, 27) or nongenotypic (5, 10, 16, Chapter 2). The adaptive stress response and population heterogeneity might affect safety margin settings for processing conditions and, therefore, should be addressed to optimally apply preservation factors in minimally processed foods.

Indirect and direct methods have been developed to study the variability of responses of individual cells. A method that is frequently used is an indirect approach based on turbidity measurements (for example, references 13, 14, 22, and 25). Detection times or optical density growth curves of single cell-generated cultures are used to estimate the variability of the lag times of individual cells, assuming that the specific growth rate is constant after the first division for each population engendered by a single cell. Direct methods make use of imaging of individual cells (12, 26, 32, 35) or spores (31) and can provide growth kinetics of individual cells and spores without extrapolation over time.

In this study, we used a direct method to observe the growth of individual vegetative cells from the initial inoculum stage to the microcolony stage on a planar, ceramic material, sold under the trade name of Anopore. Anopore is a rigid aluminum oxide that is extremely porous (pore density is up to 50%) compared to other membranes (19). Recently, strips of Anopore were shown to be an effective support for culturing and imaging of microorganisms (17, 18). Microbial growth is possible on Anopore strips when these are placed upon agar, which supplies the nutrients to the cells from beneath, through the pores. A thin layer of water on top of the Anopore strips, drawn up by capillary action...
of the pores, enables the cells to slide along a surface rather than being layered in one location. This might influence the specific growth rate of the cells, as it was shown that the growth rate of cells growing as surface colonies on a gelatin layer was smaller than the growth rate of cells in broth (6). The estimated doubling times of *Escherichia coli* 2613 and *Enterobacter aerogenes* grown on Anopore strips were, however, found to be comparable to the estimated doubling times in broth under nonstressing conditions (17, 18).

To date, quantitative, direct observations of the first generations of single cells have been focused on the growth of cells after stress exposure. Further knowledge about the behavior of individual cells during the early phase of stress exposure is necessary when the population heterogeneity of the stress response has to be quantified. In this study, the growth parameters of the bacterial population as a whole during mild and severe salt stress exposure were investigated for *B. cereus* ATCC 14579 and compared to the growth kinetics of individual microcolonies. Moreover, the heterogeneity of the adaptive stress response and growth capacity of individual microcolonies were quantitatively assessed.

**Materials and methods**

**Bacterial strains and culturing conditions.** *Bacillus cereus* ATCC 14579 was stored frozen (-80°C) in brain heart infusion (BHI) broth (Becton Dickinson, France) supplemented with 25% (vol/vol) glycerol (Sigma, The Netherlands). The concentration of sodium chloride in BHI broth without extra supplementation of sodium chloride was 0.5%. The bacteria were cultivated before each experiment in 10 ml BHI broth and incubated at 30°C with shaking at 200 rpm (SW20; Julabo Labortechnik GmbH, Germany) for 12 h to 18 h. To produce exponentially growing cells, this culture was diluted 1:200 (vol/vol) in an Erlenmeyer flask (250 ml) containing 50 ml fresh BHI broth. The flask was incubated at 30°C with shaking at 200 rpm until the optical density was 0.4 to 0.5 at 600 nm (Novaspec II spectrophotometer; Pharmacia Biotech, United Kingdom).

**Growth in broth.** To determine the growth kinetics of the population as a whole in liquid culture, an exponential-phase inoculum was added to 50 ml BHI broth, 1:100 (vol/vol), in duplicate, after which the initial sample was taken (t = 0). To expose the cells to non-salt, mild salt, and severe salt stress conditions, BHI broth without and with supplementation of sodium chloride (VWR-International, France) was added (the supplementary concentrations of sodium chloride were 2.5% and 5% [wt/vol], respectively), after which the suspensions were incubated at 30°C with shaking at 200 rpm. At constant intervals, 1-ml aliquots were
Growth dynamics of salt-stressed *Bacillus cereus*

taken and serial dilutions were made in 9 ml of peptone saline solution (1 g neutralized bacteriological peptone [Oxoid, United Kingdom] supplemented with 8.5 g sodium chloride per liter). Fifty-µl aliquots of the appropriate dilutions were surface plated on BHI agar plates (BHI broth supplemented with 12 g agar [Oxoid, United Kingdom] per liter) by using a spiral plater (Eddy Jet; IUL Instruments, Spain). Appropriately diluted aliquots of the mildly and severely salt-stressed cells were also plated on BHI agar plates supplemented with 2.5% and 5% (wt/vol) sodium chloride. The plates were incubated at 30°C for 16 h to 48 h, depending on the sodium chloride concentration of the BHI agar plates. The results are expressed in log_{10} CFU ml^{-1}.

**Growth on Anopore strips.** Anopore strips (8 by 36 mm; 60 µm thick; 3 × 10^9 pores per cm²; pore density up to 50%; a gift from PamGene International, ‘s-Hertogenbosch, The Netherlands) were manufactured, washed, and sterilized as previously described (17). The strips were placed upon BHI agar plates and prewarmed for a minimum of 30 min at 30°C in a moist chamber. To inoculate the Anopore strips with cells, exponentially growing cells were diluted 1:10, 1:100, and 1:1,000 (vol/vol) in prewarmed BHI broth at 30°C. The dilutions were inoculated in 2-µl aliquots on the upper surface of each Anopore strip. The plates were closed with Parafilm and then incubated at 30°C in a moist chamber. To expose the cells to mild salt stress and severe salt stress conditions, exponentially growing cells were inoculated on Anopore strips which were placed upon BHI agar plates supplemented with 2.5% and 5% (wt/vol) sodium chloride, respectively.

For each imaging time point, one Anopore strip was transferred right side up to a microscope slide (50 by 76 by 1 mm) covered with a 1-mm-thick film of 1% (wt/vol) solidified low-melting-point agarose (Invitrogen, The Netherlands). The agarose was dissolved in peptone saline solution, and 5 µM SYTO-9 dye (Invitrogen, The Netherlands) was added to stain the microcolonies on the Anopore strip. Staining was for 20 min at room temperature in the dark. For the sake of simplicity, the term microcolony is used for colonies that consisted of a single cell or a few cells at the start of the incubation as well as for microcolonies later on in time after expansion.

**Microscopy and image analysis.** Strips were imaged directly (without the use of a coverslip or immersion oil) using an Olympus BX-41 fluorescence microscope equipped with 10× and 50× UmPlanFl1 objective lenses and U-MWIBA and U-M41007 filters (17). A Kappa charge-coupled device camera controlled by Kappa Image Base software was used to capture the images. The saved images were analyzed quantitatively using ImageJ.
software to implement background correction, conversion to a binary image, and calculation of microcolony area. The total cell area of each microcolony was measured two-dimensionally in pixels, excluding the intercell area. It was assumed that the ratio between cell volume and cell area was constant over time, supposing both that the increase of cell number corresponded with the measured increase of cellular surface and that the bacterial cell shape was constant during the experimental time.

The intervals of imaging were 15, 20, and 30 min for the reference condition (non-salt), mild salt (2.5%), and severe salt (5%) stress conditions, respectively. Images of the microcolonies were obtained until layers of cells within the microcolonies were observed. An average number of 85 (between 47 and 199) microcolony images per imaging time point were processed.

**Quantification of heterogeneity.** Microsoft Excel was used to calculate the distribution of microcolony areas per imaging time point for each experimental condition, and the observed frequency distributions were presented in histograms. To optimally envisage the increase of microcolony area over time, the binning of the histograms was based on the number of cells per microcolony. In order to estimate the number of cells per microcolony, the areas of the individual microcolonies were divided by the area of an average single cell measured at the first imaging time point of (non-)stress exposure.

The extreme value, gamma, lognormal, and Weibull distributions were fitted to the observed frequency distributions of microcolony areas using @RISK 4.5.5 (professional edition; Palisade Europe, Middlesex, United Kingdom), which is an add-in program for Microsoft Excel. The goodness of fit of the four distributions was evaluated using the chi-square ($\chi^2$) test, the Anderson-Darling (A-D) test, and the Kolmogorov-Smirnov (K-S) test. After that, the individual microcolony areas were log$_{10}$-transformed, and the variance and the mean of the log$_{10}$-transformed microcolony areas were calculated for each imaging time point. The observed frequency distributions of the log$_{10}$-transformed microcolony areas were tested for normality using the $\chi^2$ test, the A-D test, and the K-S test, and SPPS software (version 12.0.1) was used to verify the K-S test.

**Determination and comparison of growth kinetics in broth and on Anopore strips.** The specific growth rate in broth was estimated by linear regression using the average viable counts per time point obtained with BHI agar plates (in log$_{10}$ CFU ml$^{-1}$). A straight line was fitted to the mean values that appeared to represent exponential growth. The data points of an initial lag phase or decline phase preceding the exponential growth phase were excluded.
from the linear regression analysis. To improve the objectivity of the exclusion of points from the linear regression analysis, the lag-exponential growth model \( (33) \) was fitted to the data points, and the upper 90% of the data points of the exponential growth phase were included in the analysis. The lag-exponential growth model was extended with a stationary phase when the data points showed three phases. Consequently, 80% of the data points of the exponential growth phase were included in the linear regression analysis. Slopes were derived from the linear regression analysis, and the specific growth rate \( \mu (\log_{10} \text{h}^{-1}) \) was determined from the formula \( \mu = \text{slope} \). The specific growth rate of the microcolonies on Anopore strips was estimated by using a procedure similar to the one used to estimate the specific growth rate in broth but using the mean microcolony area per time point after log\(_{10}\)-transformation of each microcolony area. Comparison of the estimated specific growth rate in broth and on Anopore strips was made with Student’s \( t \) test (two-sided).

Results

**Population dynamics in broth.** The growth response of *B. cereus* ATCC 14579 was investigated after abrupt shifts to non-salt stress (reference), mild salt stress (2.5%), and severe salt stress (5%) conditions. The growth dynamics of the three experimental conditions are shown in Figure 1. Compared to the reference condition (Figure 1a), exposure to 2.5% salt (Figure 1b) resulted in an initial period of reduced growth before exponential growth started. An initial decline in viable counts was observed after the abrupt shift to 5% salt, and exponential growth was resumed after a lag period (Figure 1c). The viable counts obtained with BHI agar plates were used to estimate the specific growth rate for the three experimental conditions. As expected, the specific growth rate in broth decreased with increasing salt concentration (Table 1).

<table>
<thead>
<tr>
<th>% NaCl (wt/vol)</th>
<th>Specific growth rate (log(_{10}) h(^{-1})) (95% CI(^a))</th>
<th>On Anopore</th>
<th>In Broth</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.74 (0.64 – 0.84)</td>
<td>0.78 (0.75 – 0.81)</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>0.58 (0.52 – 0.65)</td>
<td>0.62 (0.57 – 0.67)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.39 (0.31 – 0.48)</td>
<td>0.36 (0.33 – 0.38)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) CI, confidence interval.
To further assess the population growth response, cells were plated both on nonselective plates (BHI agar plates) and on selective plates (BHI agar plates supplemented with 2.5% and 5% salt). Figure 1b shows that the viable counts on nonselective and selective media were comparable after the abrupt exposure to 2.5% salt. However, the osmotic shift to 5% salt resulted in a remarkable initial difference in viable counts on nonselective and on selective plates (Figure 1c). After this initial stage, the viable counts on both nonselective plates and plates supplemented with 2.5% salt became comparable but were still higher than the viable counts on plates supplemented with 5% salt. A viable count

Figure 1. Growth of *Bacillus cereus* ATCC 14579 in BHI broth without addition of salt (a) or with addition of 2.5% salt (b) or 5% salt (c) at 30°C. Viable cells were plated on BHI agar plates (□), BHI agar plates with the addition of 2.5% salt (△), and BHI agar plates with the addition of 5% salt (★), and growth of individual microcolonies on Anopore strips placed upon BHI agar plates was determined without addition of salt (d) or with the addition of 2.5% salt (e) or 5% salt (f) at 30°C. The areas of individual microcolonies per imaging time point were measured two-dimensionally in pixels and log_{10}-transformed. Data points represent the average microcolony size per imaging time point (○). For the 5% salt stress condition, the data points represent the average microcolony sizes of the growing population only. The specific growth rates were estimated by linear regression using the time points that represented exponential growth (continuous line).
Growth dynamics of salt-stressed *Bacillus cereus*

Stasis was reached on nonselective and both selective media before exponential growth resumed.

Viable count data describe the culturability of the population as a whole. To examine the effect of an abrupt severe osmotic shift on the viability of the individual cells, the membrane integrity of the individual cells was tested during exposure to 5% salt, using both SYTO-9 and propidium iodide (PI) nucleic acid dyes. The permeant nucleic acid dye SYTO-9 can generally enter all bacteria in a population, while the impermeant nucleic acid dye PI penetrates only bacteria with damaged membranes (LIVE/DEAD BacLight bacterial viability kit) (15). The loss of culturability of a fraction of the population after the severe osmotic shift was contributory to the loss of membrane integrity, as the percent reduction in viable counts was found to correlate closely with the percentage of PI-labeled cells at the time that exponential growth resumed (data not shown).

**Population dynamics on Anopore strips.** To determine the growth dynamics of individual cells after osmotic shifts in more detail, growth of individual microcolonies was monitored using Anopore strips, which were placed upon BHI agar plates without the addition of salt (Figure 2), with addition of 2.5% salt (Figure 3), and with addition of 5% salt (Figure 4). The 100-fold dilution of the inoculated culture resulted in a suitable density of microcolonies on the Anopore strips and, therefore, this dilution factor was used to determine the increase of microcolony area over time for each of the three experimental conditions. The observed frequency distributions of the number of cells per microcolony and example images of microcolonies of *B. cereus* ATCC 14579 are shown in Figure 2, 3, and 4, respectively, for the non-salt, 2.5% salt, and 5% salt stress conditions. The frequency distributions and the images illustrate that the size of the microcolonies expanded over time. The monitoring times were 2 h, 3.7 h, and 7.5 h for the three different growth conditions. At the initial imaging time point, the maximum bin number of the histograms was up to 8 cells per microcolony. Microscopic observations of exponentially growing cells in suspension confirmed this range of cells per microcolony. The first imaging time point of stress exposure showed that the average single cell area was comparable for the non-salt stress and 2.5% salt and 5% salt stress conditions. Moreover, single cells that could be individually discriminated later on during the experimental time did not show a difference in measured cellular surfaces for the three experimental conditions. As the microcolony size expanded over time, the number of cells per microcolony increased, and the histograms shifted to the right (Figure 2, 3, and 4). A maximum of five generation times was monitored.
Figure 2. Example images of *Bacillus cereus* ATCC 14579 cultured on Anopore strips, which were placed upon BHI agar plates without addition of salt at 30°C. Observed and fitted frequency distributions of the number of cells per microcolony are shown for imaging time points (t) at 0, 0.5, 1, 1.5, and 2 h. The histograms show observed frequencies of numbers of cells per microcolony. Continuous curves show fitted normal distributions of the log10-transformed microcolony areas.

until the cells formed a second layer within the microcolony, resulting in a maximum bin number of 256 cells per microcolony at the final imaging time point. Exposure to 2.5% salt and 5% salt showed a period of reduced or no growth. Furthermore, the number of bins per histogram notably increased over time when the microcolonies were exposed to 5% salt.
Figure 3. Example images of *Bacillus cereus* ATCC 14579 cultured on Anopore strips, which were placed upon BHI agar plates with the addition of 2.5% salt (wt/vol) at 30°C. Observed and fitted frequency distributions of the number of cells per microcolony are shown for imaging time points ($t$) at 0, 1, 1.7, 2.3, 3, and 3.7 h. Histograms show observed frequencies of numbers of cells per microcolony. Continuous curves show fitted normal distributions of the log$_{10}$-transformed microcolony areas.
Figure 4. Example images of Bacillus cereus ATCC 14579 cultured on Anopore strips, which were placed upon BHI agar plates with the addition of 5% salt (wt/vol) at 30°C. Observed and fitted frequency distributions of the number of cells per microcolony are shown for imaging time points (t) at 0, 2.5, 4.5, 5.5, 6.5, and 7.5 h. Histograms show observed frequencies of numbers of cells per microcolony. Continuous curves show fitted normal distribution of the log10-transformed microcolony areas; solid lines represent the distribution of all microcolonies, and dashed lines represent the distribution of the growing microcolonies only.
Quantification of population heterogeneity. To quantitatively describe the frequency distribution of the microcolony areas for each imaging time point, four commonly used statistical distributions were fitted per data set: the extreme value, gamma, lognormal, and Weibull distributions. The numbers of imaging time points for the three experimental conditions were 9, 12, and 16, respectively, for the non-salt stress condition and 2.5% and 5% salt stress conditions. Based on the $\chi^2$, the A-D, and the K-S test statistics, the four distributions were ranked for each data set in order to choose the best overall distribution. The A-D and the K-S statistics gave quite similar results and ranked the lognormal distribution as the best distribution. Based on the $\chi^2$ test, both the extreme value and the lognormal distribution were selected as appropriate distributions, but the distribution ranking was influenced by the number and location of the bins of the distribution. Neither the A-D test nor the K-S test requires binning, and they may therefore be less arbitrary (36).

The advantage of the lognormal distribution is that the distribution parameters themselves have a specific meaning, parameter $a$ being the mean and parameter $b$ describing the spread of the data (23). Moreover, when a variable is lognormally distributed, the logarithm of the variable is normally distributed (34).

To examine the distributions of the microcolony areas in more detail, the normal distribution was fitted to the log10-transformed microcolony areas for each imaging time point, and the $\chi^2$, the A-D, and the K-S tests were applied to test the normality of the data sets. The continuous curves in Figure 2, 3, and 4 show the frequencies generated by the normal distribution. For the reference condition (Figure 2) and the 2.5% salt stress condition (Figure 3) the log10-transformed microcolony areas were normally distributed according to both the $\chi^2$ test and the K-S test ($P > 0.05$), but the A-D test was not accepted for all the data sets (5 out of 21). The A-D statistic highlights differences between the two tails of the fitted distribution and the input data (3), and the data sets which were not accepted according to the A-D test were indeed less symmetric at the two tails. In addition, the normal distribution could not be applied to all data sets for the 5% salt stress condition (Figure 4). After 5 h, the distributions were significantly different from the normal distribution ($P < 0.05$) according to both the A-D test and the K-S test, but the $\chi^2$ test did not confirm this finding. The histograms in Figure 4 supported the findings of the A-D and the K-S tests, as the symmetry of the observed frequency distributions decreased over time, resulting in a negatively skewed distribution.

The spread of each data set was illustrated by the number of bins and the width of the frequency distribution and could be expressed in the variance. The variances of the observed frequency distributions for the non-salt stress condition and the 2.5% salt stress
condition were similar and rather stable over time (Figure 5). This indicated that exposure to mild salt stress resulted in growth that was characterized by a noticeably low variability of the individual microcolony sizes over time, and this variability was comparable to that during non-salt stress exposure. By contrast, the variances of the distributions during exposure to 5% salt clearly increased for the last imaging time points. The final imaging time point for the 5% salt stress condition showed that most of the microcolonies were able to resume growth during 5% salt stress exposure (85% of the microcolonies), while a subpopulation of microcolonies failed to do so during the time scale of the experiment. To investigate this finding further, the distribution of the growing subpopulation at the final imaging time point was tested for normality using the three statistical tests, and this was confirmed ($P > 0.05$). Subsequently, the nongrowing population as observed at the final imaging time point was also marked for the other imaging time points. The frequency distribution of the nongrowing population was separated from the total population for each imaging time point, showing two subpopulations, a growing and nongrowing population per time point. Figure 4 shows the fitted normal distribution of the growing population for each data set. The distributions of the growing population were tested for normality, and this was confirmed for all imaging time points using both the $\chi^2$ and the K-S tests ($P > 0.05$). The A-D test was not accepted for these data sets (3 out of 16), of which the two tails of the observed frequency distribution were less symmetric. Figure 5 illustrates that the

Figure 5. Variances of the observed frequency distributions of the log10-transformed microcolony areas versus the imaging time points per salt stress condition. The intervals of imaging were 15, 20, and 30 min for conditions of non-salt stress (0%; □), mild salt stress (2.5%; △), and severe salt stress (5%; ◇ distribution of all microcolonies, and ■, distribution of the growing microcolonies only).
discrimination of two subpopulations resulted in a remarkable decrease of the variance for the last imaging time points. The variances of the observed frequency distributions of the growing population were comparable to the variances of the distributions for the non-salt and 2.5% salt stress conditions.

Finally, the average microcolony size was calculated for each imaging time point and used to estimate the specific growth rate of the microcolonies on Anopore strips. The calculation of the average per time point for the 5% salt stress condition was restricted to the growing subpopulation. Figure 1d, e, and f show the growth of *B. cereus* ATCC 14579 on Anopore strips placed upon BHI agar plates without addition of salt (Figure 1d) and with the addition of 2.5% salt (Figure 1e) and 5% salt (Figure 1f). The non-salt stress condition showed no initial lag phase. The onset of the exponential growth phase for the 2.5% and 5% salt stress conditions was, respectively, after 1.7 h and 5 h, preceding a phase of reduced growth or a lag phase. The estimated specific growth rates of the microcolonies on Anopore strips decreased with increasing hypertonic conditions (Table 1).

**Comparison of growth kinetics in broth and on Anopore strips.** It has been shown in other studies that lag time and initiation of growth under stressful conditions can be affected by the size of the inoculum (28, 30) and, therefore, a similar inoculum size was used in both the broth and the Anopore experimental setups. Comparison of the growth kinetics obtained in broth and on Anopore strips suggested that the onsets of the exponential phases were comparable (Figure 1a versus d and b versus e), except for the 5% salt stress condition. In the latter situation, the exponential growth phase started after 6 h and 5 h, respectively, in broth (Figure 1c) and on Anopore strips (Figure 1f). The specific growth rates in broth and on Anopore strips for each experimental condition were statistically compared, and confidence intervals are presented in Table 1. The specific growth rates in broth were not significantly different from the specific growth rates on Anopore strips for the three tested experimental conditions (*P* > 0.05). Moreover, the linear regression lines of both the viable count data and the microcolony data were found to be parallel for the non-salt, 2.5%, and 5% salt stress conditions (Figure 1a versus d, b versus e, and c versus f), as the differences between the viable counts and the mean values of the log_{10}-transformed microcolony areas remained constant over time for each experimental condition.
Discussion

The plate count method was used to investigate the effects of mild and severe salt stress exposure on the population dynamics of *B. cereus* ATCC 14579 as a whole. It is becoming increasingly known that single cells that have originated from the same population exhibit marked variability in stress resistance (1, 5, 10, 16, 20, 27, Chapter 2). Therefore, in this study the effects of abrupt shifts to hypertonic environments were further examined at the single cell level. Direct imaging methods are well suited to reveal the heterogeneity within a microbial population, and in the present study Anopore strips were used to quantitatively assess the heterogeneity of the stress exposure response and growth capacity of individual *B. cereus* ATCC 14579 microcolonies over time.

Growth of a bacterial population is determined by the proliferation of individual cells and results in an increase in cell number and total cell volume over time. Using direct imaging, the growth of microcolony area was determined by measuring the individual microcolony areas two-dimensionally in pixels at regular intervals. In order to estimate the proliferation of cells using these data, it was assumed that the ratio between cell volume (three dimensions) and cell area (two dimensions) was constant during the experimental time scale. Consequently, it was assumed both that the increase in cell number corresponded with the measured increase of cellular surface and that the bacterial cell shape did not change over time. Our study showed that the specific growth rate of cells in liquid culture was similar to that of cells grown in microcolonies, both in the absence and the presence of salt stress. Moreover, the onset of the exponential growth phase was comparable in both experimental setups. These findings illustrated the remarkable similarity of growth kinetics of cells grown in broth and on Anopore strips and supported the acceptability of the assumption.

Previous studies have shown that several statistical distributions can be used to adequately describe the variability of the lag times and turbidity detection times of unstressed and stressed single cell-generated bacterial populations (for example, references 13, 14, and 22) and the variability of interdivision times of cells after stress exposure (21). Our study confirmed the findings of others (36), that the statistic used to test the acceptance of distribution fitting influences the conclusions of acceptance. McKellar and Hawke proposed to choose a distribution of which the parameters are interpretable (23). In this study, the log10-transformed microcolony areas were normally distributed during non-salt stress and mild salt stress exposure, and the normal distribution could also be applied to the distributions of the growing subpopulation under severe salt stress conditions. The range of
the variances of the distributions was rather small, and the use of exponentially growing cells might have affected this remarkably low variability of individual microcolony sizes over time.

Both the viable count growth curves and the direct imaging approach showed that after an abrupt osmotic shift to 5% salt, a fraction of cells within the initial population was able to resume exponential growth, indicating heterogeneity in the population’s response to severe salt stress. It should be noted that the initial reduction of viable counts after the severe osmotic shift differed from the fraction of the nongrowing population on Anopore strips, but this phenomenon was not investigated any further. Labeling of cells which were grown in liquid culture using the fluorogenic nucleic acid stain propidium iodide demonstrated that loss of membrane integrity coincided with the loss of culturability. Loss of culturability of a subpopulation of cells by abrupt severe osmotic shifts within the growth-permissive range was previously observed for Salmonella enterica serovar Typhimurium (24) and Listeria monocytogenes (28). It can be hypothesized that the resumption of growth after an initial lag period was due either to the recovery of a fraction of the population or was caused by a very small highly resistant subpopulation which was able to grow exponentially, directly after the osmotic shift without or with having an initial period of recovery and adaptation. Moreover, it may be the case that the resumption of growth was the net result of different responses by subpopulations, as hypothesized by Mellefont et al. (24). A loss of viability as well as recovery, stress adaptation, and exponential growth might occur simultaneously in different subpopulations. Considering the microcolony data as well as the viable count growth curves, the most likely scenario seems that the resumption of growth was the result of the recovery and adaptation of a fraction of the population. The microcolony data suggested that a large fraction of the population was able to initiate growth after an initial lag period. In addition, the growth experiment in broth demonstrated that a viable count stasis was reached on nonselective and selective media before exponential growth was resumed. This may indicate that this fraction of the population had adapted to the stressful environment and could resuscitate and proliferate even under severe stress conditions. However, the possible presence of a very small highly resistant subpopulation, which was able to initiate exponential growth immediately after the osmotic shift without having a recovery and adaptation period, could not be dismissed either, as this fraction of the population can be rather small and may not be differentiated from the recovering cells.

Anopore is a highly inert and resistant material and is unlikely to be damaged by most stress applications, including heat. The rigidity of the Anopore strips facilitates the
transfer of strips from one condition to another in order to successively expose cells to various stresses. Therefore, for further research, the Anopore approach may provide possibilities to assess the first responses of cells to a succession of environmental changes.

The recent interest in the underlying sources of population heterogeneity emphasizes that it is a well-recognized problem within the field of quality and safety of minimally processed foods (4, 5). This study showed that Anopore strips were well suited to monitor individual microcolonies at the early stage of stress exposure. Furthermore, this direct imaging approach could be used to quantitatively describe the population heterogeneity of the adaptive stress response and growth capacity of individual microcolonies. Quantification of population heterogeneity during stress exposure might contribute to the optimal assessment of safety margin settings for food processing conditions in order to guarantee the quality and safety of minimally processed foods.

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References


Chapter 5

Direct-imaging-based quantification of *Bacillus cereus* ATCC 14579 population heterogeneity at a low incubation temperature

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Abstract

*Bacillus cereus* ATCC 14579 was cultured in microcolonies on Anopore strips near its minimum growth temperature to directly image and quantify its population heterogeneity at an abusive refrigeration temperature. Eleven percent of the microcolonies failed to grow during low temperature incubation, and this cold-induced population heterogeneity could be partly attributed to the loss of membrane integrity of individual cells.
Bacillus cereus is a food poisoning- and food spoilage-causing organism that can be found in a large variety of foods (4, 23). There are two illnesses associated with B. cereus, namely, emetic and diarrheal intoxication (17, 24). Most of the strains related to cases or outbreaks of B. cereus food-borne poisoning were shown to be unable to grow at 7°C (1, 12). The average temperatures of domestic refrigerators have been investigated in various surveys around the world and often ranged from 5°C to 7°C, but extreme values exceeded 10°C to 12°C (5, 16). Inadequate chilling was indeed reported in various incidents of B. cereus food-borne illness (7, 8, 18, 19), pointing to the importance of appropriate refrigeration of foods contaminated with B. cereus to control its growth and toxin production in foods (9).

Several studies have demonstrated that microorganisms can show diversity in their population stress response, even in an apparently homogeneous stress environment (6, 11, 21, 22, Chapter 4). However, only very limited data describing the heterogeneity in growth performance of individual cells from food-borne pathogens cultured at low temperatures are available (10). Because inadequate chilling of food is one of the factors that contribute to the number of incidents of B. cereus food-borne illness, there is a need for better understanding of its growth performance at lowered incubation temperatures. In this study, we used the direct-imaging-based Anopore technology (6, 13–15, Chapter 4) to quantitatively describe the population heterogeneity of B. cereus ATCC 14579 cells at 12°C. The minimum temperature for the growth of B. cereus ATCC 14579 in brain heart infusion (BHI) broth is 7.5°C (personal communication from F. Carlin), but various food-borne-associated B. cereus isolates were shown to be unable to grow at 10°C (1). Therefore, in this study, a culturing temperature of 12°C was chosen, to mimic temperature abuse of refrigerated foods. In addition, the membrane integrity of cells was assessed using both membrane permeant and impermeant nucleic acid dyes in order to get more insight into cellular characteristics that may contribute to heterogeneity in growth response.

Growth performance in broth at a low incubation temperature. Previous studies have shown that the temperature history of the inoculum culture and its growth conditions have pronounced effects on the growth performance of cells (see, e.g., references 2, 10, 20, and 25). Therefore, an exponentially growing working culture that was already precultured in BHI broth at 12°C, with shaking at 200 rpm, was used in this study to assess the effect of a low incubation temperature rather than the effect of temperature down-shock on the growth performance of B. cereus ATCC 14579. The cold-adapted, exponentially growing working culture (optical density at 600 nm of 0.4 to 0.5) was inoculated in fresh, precooled (12°C)
BHI broth in duplicate and further incubated at 12°C with shaking at 200 rpm. At regular time intervals, appropriately diluted aliquots were plated on BHI agar plates and BHI agar plates supplemented with 2.5% and 5% (wt/vol) sodium chloride to test cell injury. At the initial sampling point \( t = 0 \) h, the viable counts on the BHI plates supplemented with 5% salt were lower than the counts on the BHI plates and the BHI plates supplemented with 2.5% salt (Figure 1a). After this initial time point, the plate counts were similar on the three different plating media. The growth kinetics showed an initial lag period, after which

![Graph](image)

**Figure 1.** (a) Growth of *Bacillus cereus* ATCC 14579 in BHI broth at 12°C. Cells were plated on BHI agar plates (○), BHI agar plates plus 2.5% salt (□), and BHI agar plates plus 5% salt (△). (b) Growth of microcolonies on Anopore strips placed on BHI agar plates at 12°C. The area of each microcolony per imaging time point was measured in pixels and log_{10}-transformed. Data points represent the average microcolony size of the grown population of microcolonies per imaging time point (○). The specific growth rates in broth and on Anopore strips were estimated by linear regression using the time points that represented exponential growth (continuous lines).
Cold-induced population heterogeneity

exponential growth resumed at 6 h (Figure 1a) with a specific growth rate of 0.051 log\(_{10}\) h\(^{-1}\) (95% confidence interval, 0.045 to 0.056). To check possible effects of medium conditioning on the observed lag phase, the exponentially growing working culture was also inoculated into culture supernatant, which was prepared by filtering a cold-adapted exponentially growing culture through a membrane filter, and further incubated at 12°C with shaking at 200 rpm. However, the use of culture supernatant did not reduce the observed lag phase (data not shown). Both the lag phase and the lower viable counts on the BHI plates supplemented with 5% salt after inoculation of the inoculum pointed to the susceptibility of the exponentially growing culture at this low incubation temperature.

Quantitative analysis of population heterogeneity on Anopore strips. Cells were cultured in microcolonies on porous Anopore strips to directly image and quantify the heterogeneous growth response. Anopore strips (96% ethanol sterilized) were placed on precooled (12°C) BHI agar plates, and the cold-adapted exponentially growing working culture was diluted in precooled (12°C) BHI broth and inoculated in 2-μl aliquots onto the upper surface of each Anopore strip (8 by 36 mm by 60 μm, 0.2-μm-diameter pore size, pore density of up to 50%; Whatman International, Maidstone, United Kingdom). The plates were sealed with Parafilm to prevent dehydration of the agar surface. A thin layer of medium fluid on top of the Anopore strip, drawn up by the capillary action of the pores, allows daughter cells to slide along the planar surface of the Anopore strip to form an expanding one-cell-layer-thick microcolony during growth. The plates containing the Anopore strips were incubated at 12°C. At regular time intervals, an Anopore strip was transferred to a microscope slide (50 by 76 by 1 mm) covered with a 1-mm-thick film of 1% (wt/vol) solidified low-melting-point agarose. The agarose was dissolved in peptone saline solution containing 1.7 μM SYTO-9 dye to stain the microcolonies on the Anopore strip. The term microcolony was used for colonies that consisted of a single cell or a few cells at the start of the incubation, as well as for the expanded microcolonies later on in time after incubation. The Anopore strips were imaged using a Zeiss Axiosplan 2 Imaging microscope equipped with an LD Plan-Neofluar 63×/0.75 Corr Ph2 objective lens and a single-band fluorescein isothiocyanate (FITC) filter. A charge-coupled-device (CCD) camera (Quantix; Photometrics, United States) controlled by Imaging Pro Plus 5.0 software was used to capture images of the microcolonies. The saved images of the microcolonies were used to measure the area of each microcolony in pixels, excluding the intercell area, using ImageJ software (version 1.34s). An average number of 103 microcolony images per imaging time point were analyzed to determine the distribution of microcolony size for
each time point, following the procedure described previously (6, Chapter 4). Figure 2 shows examples of images of *B. cereus* ATCC 14579 cells grown on Anopore strips. The

Figure 2. Examples of images of *Bacillus cereus* ATCC 14579 cultured on Anopore strips which were placed upon BHI agar plates at 12°C. Observed and fitted frequency distributions of the number of cells per microcolony for the imaging time points (t) 0, 6, 12, 18, and 24 h are shown. Histogram shows observed frequencies of number of cells per microcolony. Continuous curves show fitted normal distributions of the log_{10}-transformed microcolony areas, solid lines represent the distribution of all microcolonies, and dashed lines represent the distribution of grown microcolonies only.
distribution at the first imaging time point shows that the sizes of the microcolonies ranged from 1 cell per microcolony up to 16 cells per microcolony. Phase-contrast microscopic imaging of the cold-adapted inoculum confirmed this size range. The growth kinetics of the microcolonies also showed an initial lag phase before exponential growth resumed at 6 h (Figure 2). The growth of the microcolonies was monitored up to 24 h, as a second layer of cells within the microcolonies was observed after this imaging time point. The resumption of microbial growth resulted in expansion of microcolony size because the number of cells forming the microcolony increased over time, and the frequency distributions of microcolony size shifted to the right (Figure 2). The reproducibility of the frequency distribution at the final imaging time point ($t = 24$ h) was confirmed in an independent reproduction of cell culturing on Anopore strips. The data in Figure 3 show that the variability of microcolony sizes increased after the resumption of growth. To quantitatively assess the observed heterogeneity of microcolony sizes, the frequency distributions per imaging time point were statistically tested for normality using the Anderson-Darling test and the Kolmogorov-Smirnov test as described earlier (6, Chapter 4). The continuous curves in Figure 2 show the fitted normal distributions. The observed frequency distributions were normally distributed until 6 h according to the two statistical tests ($P > 0.05$). After this imaging time point, the normal distribution could not be acceptably fitted.

Figure 3. The variances of the observed frequency distributions of the log$_{10}$-transformed microcolony areas versus the imaging time points at 12°C and 30°C. The intervals of imaging were 3 h at 12°C and 15 min at 30°C. ◇, Distribution at 12°C of all microcolonies; ◈, distribution at 12°C of grown microcolonies only; □, distribution of microcolonies at 30°C (see reference 6, Chapter 4).
according to both tests \( (P < 0.05) \). The deviation from normality was maximal at the final imaging time point \( (t = 24 \text{ h}) \). Both the images and the observed frequency distribution at this time point indicated that some microcolonies, represented by the first three bins of the frequency distribution, failed to continue growing at 12°C. In order to quantitatively describe the population heterogeneity, this nongrown population, 11% of the microcolonies, was separated from the grown population of microcolonies for each imaging time point. This mathematical procedure resulted in both a nongrown population and a grown population of microcolonies per imaging time point. The frequency distribution of the grown population for each imaging time point was tested for normality, and this was confirmed by both the A-D test and the K-S test \( (P > 0.05) \). The fitted normal distributions of the grown population are also shown in Figure 2. The discrimination of the two populations per time point resulted in a rather stable variance of microcolony sizes of the grown population over time (Figure 3). In our previous study, the Anopore technology was used for quantification of the population heterogeneity of \( B. \) \( cereus \) ATCC 14579 grown at 30°C in the absence and presence of osmotic stress (6, Chapter 4). Culturing of cells at 30°C resulted in a homogeneous growth response expressed in a stable variance of microcolony sizes over time (Figure 3) (see reference 6, Chapter 4). Exposure to osmotic stress \( (5\% \ [\text{wt/vol}] \text{ sodium chloride}) \) at 30°C (6, Chapter 4) induced a heterogeneous growth response similar to that observed in the present study at 12°C.

In order to estimate the specific growth rate of cells grown on Anopore strips, the average microcolony sizes of the grown population of microcolonies from imaging time point 6 h until 24 h were used for linear regression (Figure 1b). The estimated specific growth rate, \( 0.045 \log_{10} \text{h}^{-1} \) (95% confidence interval, 0.035 to 0.055), was not significantly different from the estimated specific growth rate of cells cultured in broth \( (P > 0.05) \). This correspondence of the growth kinetics of cells grown on Anopore strips and in broth was previously demonstrated for \( B. \) \( cereus \) ATCC 14579 at 30°C and during exposure to additional osmotic stress conditions \( (2.5\% \text{ and } 5\% \ [\text{wt/vol}] \text{ sodium chloride}) \) at 30°C (6, Chapter 4). The Anopore growth system’s characteristics might have contributed to this remarkable similarity of the initial growth kinetics of cells grown on Anopore strips and in broth compared to those of other surface growth systems (3).

**Membrane integrity of individual cells grown in microcolonies.** An advantage of the Anopore technology is that growth kinetics, as well as characteristics of individual cells, can be studied directly using different fluorescent dyes. Therefore, extra Anopore strips were used at each imaging time point to examine the membrane integrity of the individual...
cells within the microcolonies by double staining these microcolonies using 1.7 μM SYTO-9 dye and 10 μM propidium iodide (PI) dye. When SYTO-9 dye and PI are used in combination, intact cells are labeled green and cells with damaged membranes are labeled red. The double-stained microcolonies were imaged using a dual-band FITC/TxRed filter, and a CCD camera (Axiocam MRc; Zeiss, The Netherlands) controlled by Axiovision 4.2 software was used to capture images of the double-stained microcolonies. The results of double staining of the exponentially growing inoculum at the initial imaging time point \((t = 0\, \text{h})\) demonstrated that about 3 to 5% of the microcolonies consisted of cells that had lost their membrane integrity during the low temperature preincubation. This percentage is higher than the 0.5 to 1% found for exponentially growing cultures of \(B.\, cereus\) ATCC 14579 cultured at 30°C (data not shown).

Representative images of the nongrown population and the grown population of microcolonies at the final imaging time point \((t = 24\, \text{h})\) are shown in Figure 4a and b, respectively. The nongrown population showed green-labeled microcolonies, red-labeled microcolonies, and microcolonies of which the individual cells were either green- or red-

![Figure 4. Labeling of Bacillus cereus ATCC 14579 microcolonies with SYTO-9 and propidium iodide after 24 h of culturing on Anopore strips which were placed upon BHI agar plates at 12°C. At this imaging time point, two populations of microcolonies were observed, a nongrown population (a) and a grown population (b).](image-url)
labeled (Figure 4a). The observation of green-labeled, nongrown microcolonies suggested that the loss of culturability of the nongrown microcolonies was not only due to loss of membrane integrity. This is in agreement with the finding that the percentage of nongrown microcolonies (11%) was higher than the percentage of red-labeled microcolonies at the initial imaging time point. Most of the grown microcolonies were either fully green-labeled or mainly green-labeled (Figure 4b). Interestingly, though, some grown microcolonies consisted of a large number of red-labeled cells, indicating that these cells’ membranes were compromised at some point during growth at 12°C (Figure 4b). This finding pointed also to the heterogeneous growth response of *B. cereus* ATCC 14579 at a low incubation temperature.

**Concluding remarks.** The results of this study demonstrate that one of the most commonly used methods to control bacterial growth, chilling, results in a heterogeneous growth performance of *B. cereus* ATCC 14579. The direct-imaging-based Anopore technology was shown to be well suited to quantify in detail the cold-induced population heterogeneity for this strain. This study provides prospects for quantifying the population heterogeneity at lower incubation temperatures for other spore-forming and non-spore-forming food-borne pathogens, including strains which are able to produce toxins at abusive temperatures, such as emetic *B. cereus* (9). Our study showed that 11% of the microcolonies of *B. cereus* ATCC 14579 failed to continue growing at 12°C. Translating this to foods, in the case of initial low levels of contaminants, i.e., 1 to 5 cells per product unit, this observed population heterogeneity has an impact on the variability in microbial outgrowth. However, at higher contamination levels in foods, such a fraction of nongrowing cells will be overgrown by the larger fraction of growing cells. This indicates that the observed chill-induced population heterogeneity is too limited to have a major impact on the total exposure assessment when foods are contaminated with high concentrations of *B. cereus* and stored at an abusive refrigeration temperature.

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Phenotypic and transcriptomic analyses of mildly and severely salt-stressed *Bacillus cereus* ATCC 14579 cells

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Abstract

Bacteria are able to cope with the challenges of a sudden increase in salinity by activating adaptation mechanisms. In this study, exponentially growing cells of the pathogen *Bacillus cereus* ATCC 14579 were exposed to both mild (2.5% [wt/vol] NaCl) and severe (5% [wt/vol] NaCl) salt stress conditions. *B. cereus* continued to grow at a slightly reduced growth rate when it was shifted to mild salt stress conditions. Exposure to severe salt stress resulted in a lag period, and after 60 min growth had resumed, with cells displaying a filamentous morphology. Whole-genome expression analyses of cells exposed to 2.5% salt stress revealed that the expression of these cells overlapped with the expression of cells exposed to 5% salt stress, suggesting that the corresponding genes were involved in a general salt stress response. Upregulation of osmoprotectant, Na⁺/H⁺, and di- and tripeptide transporters and activation of an oxidative stress response were noticeable aspects of the general salt stress transcriptome response. Activation of this response may confer cross-protection against other stresses, and indeed, increased resistance to heat and hydrogen peroxide could be demonstrated after preexposure to salt. A temporal shift between the transcriptome response and several phenotypic responses of severely salt-stressed cells was observed. After resumption of growth, these cells showed cellular filamentation, reduced chemotaxis, increased catalase activity, and optimal oxidative stress resistance, which corresponded to the transcriptome response displayed in the initial lag period. The linkage of transcriptomes and phenotypic characteristics can contribute to a better understanding of cellular stress adaptation strategies and possible cross-protection mechanisms.
Introduction

*Bacillus cereus* is a spore-forming Gram-positive bacterium that is frequently isolated from foods (6, 43). It is able to cause two types of gastrointestinal diseases, emesis and diarrhea. The emetic syndrome (an intoxication) is caused by a heat-, acid-, and trypsin-stable toxin, cereulide, and the symptoms may occur after ingestion of foods contaminated with the toxin (9, 34). The diarrheal syndrome (a toxico-infection) is caused by one or more enterotoxins produced by vegetative cells of *B. cereus* in the small intestine. The number of *B. cereus* cells required to cause disease is relatively high, and in most cases at least $10^5$ CFU g$^{-1}$ food has been found in foods implicated in disease (34).

Salt is widely used as a food additive in the food industry to control bacterial growth (1). Bacteria employ several strategies to adapt to adverse conditions, and upon activation of the so-called adaptive stress response bacteria can become more robust. Exposure to sodium chloride has been shown to induce a protective response in *B. cereus*, which enables this organism to survive under otherwise lethal conditions (5, 8, 26, Chapter 2). The use of various mild preservation methods in foods is becoming more prevalent, underlining the significance of a better understanding of the stress responses of pathogens to ensure the microbial safety of foods.

When bacteria are challenged by increased salinity in their environment, water efflux occurs, and this results in a reduction in the cellular turgor. To restore and maintain cellular turgor, bacteria initiate a two-step adaptation response. Initially, K$^+$ is taken up by the bacterial cell, and subsequently osmoprotectants, like glycine betaine, are imported by transport systems (21, 24, 30). For *Bacillus subtilis* five osmoprotectant uptake transport systems (OpuA to OpuE) have been described (21, 24, 30). OpuA, OpuB, and OpuC are multicomponent ABC transporters, whereas the OpuD and OpuE symporters each consist of a single component. In the genome sequence of *B. cereus* ATCC 14579 (18), several open reading frames putatively encoding osmoprotectant transporters have been identified, indicating the importance of these transporters in *B. cereus* ATCC 14579. To date, limited information is available about the underlying mechanisms of the salt stress response of *B. cereus*. A proteome study performed by Browne and Dowds (5) showed that enzymes involved in the central metabolism were induced in *B. cereus* NCIMB 11796 during exposure to salt stress. Furthermore, salt stress is known to induce the alternative transcription factor σ$^B$ protein in *B. cereus* ATCC 14579 (35), suggesting a role for this regulator in the salt stress response of this strain.
In this study, we performed genome-wide comparative transcriptional analyses of B. cereus ATCC 14579 in response to 2.5% and 5% sodium chloride and combined these analyses with phenotypic analyses. The transcriptome profiles for the two salt stress conditions were compared in order to investigate the overlap in the responses, the so-called general salt stress response, and to identify specific responses of mildly and severely salt-stressed cells. Moreover, we linked observed transcriptome expression patterns to several responses of the salt-stressed cells.

Materials and methods

**Bacterial strain and culture conditions.** Mesophilic strain B. cereus ATCC 14579 was used throughout this study. The culture used was stored frozen in brain heart infusion (BHI) broth (Becton Dickinson, France) supplemented with 25% (vol/vol) glycerol (Sigma, The Netherlands) at -80°C. The bacteria were cultivated before each experiment in 10 ml BHI broth and incubated at 30°C with shaking at 200 rpm (Innova 4335; New Brunswick Scientific, The Netherlands) for 12 h to 18 h. To produce two independent biological replicates of exponentially growing cultures, two stationary-phase cultures were diluted 1:100 (vol/vol) in two Erlenmeyer flasks (250 ml) containing 50 ml fresh BHI broth. The flasks were incubated at 30°C with shaking at 200 rpm until the cells were growing exponentially (absorbance at 600 nm, 0.4 to 0.5; Novaspec II spectrophotometer; Pharmacia Biotech, United Kingdom). When an optical density at 600 nm (OD600 nm) of 0.4 to 0.5 was reached, decimal dilutions were prepared using 9 ml of a peptone saline solution (1 g neutralized bacteriological peptone [Oxoid, United Kingdom] supplemented with 8.5 g NaCl per liter) to determine the viable counts at time zero. Fifty-microliter aliquots of the appropriate dilutions were surface plated on BHI agar plates (BHI broth supplemented with 15 g agar [Oxoid] per liter) using a spiral plater (Eddy Jet; IUL Instruments, Spain). To study the effects of exposure to mild and severe salt stress on the growth of B. cereus ATCC 14579, BHI broth supplemented with 7.5% and 15% (wt/vol) NaCl, respectively, was added (the final supplementary concentrations of NaCl were 2.5%, and 5% [wt/vol], respectively), after which the cultures were incubated further at 30°C with shaking at 200 rpm. At constant intervals, appropriately diluted aliquots of the mildly and severely salt-stressed cells were plated on BHI agar plates. The plates were subsequently incubated for 16 h to 24 h at 30°C, and the results were expressed in log_{10} CFU ml^{-1}. 
Phenotype and transcriptome response to salinity

RNA isolation. RNA was isolated from both unstressed and salt-stressed cultures. To do this, four 50 ml-cultures were incubated in 250 ml-Erlenmeyer flasks at 30°C with shaking at 200 rpm. When the absorbance at 600 nm reached 0.4 to 0.5, one culture was used for extraction of RNA for the reference unstressed sample (time zero). Twenty milliliters of this unstressed culture was transferred into a 50-ml Falcon tube (Greiner Bio-One, Germany) and processed as described below. The remaining three cultures were exposed to 2.5% NaCl by adding 25 ml of BHI broth supplemented with 7.5% NaCl and incubated further at 30°C with shaking at 200 rpm. At three time points, after 10, 30, and 60 min of exposure to salt, 30 ml of the mildly salt-stressed cells was transferred into 50-ml Falcon tubes. A similar procedure was used to expose the cells to 5% NaCl for 10, 30, and 60 min. Subsequently, the unstressed and salt-stressed cultures were pelleted by centrifugation at 15,000 × g at 4°C for 30 s (Eppendorf 5804 R centrifuge; Eppendorf, Germany). After the supernatant was decanted, 1 ml of phenol-based RNA extraction buffer (TRI reagent; Ambion, United Kingdom) was added to the cell pellets. Subsequently, the cell pellets were resuspended, snap-frozen in liquid nitrogen, and stored at -80°C. The experiments used to obtain RNA samples were performed in duplicate. After the cell pellets were defrosted, the cells were disrupted by bead beating (Mini Beadbeater-8; Biospec Products, United States) with zirconium beads (diameter, 0.1 mm; Biospec Products), and the RNA was isolated as previously described (35).

cDNA synthesis, Cy dye labeling, and microarray hybridization. cDNA synthesis and Cy3 and Cy5 dye labeling of the cDNA samples were performed as previously described (37). Subsequently, the Cy5-labeled cDNA samples prepared from the mildly and severely salt-stressed cells were hybridized (concentration ratio, 1:1; 300 to 400 ng Cy dye-labeled cDNA per sample) with the Cy3-labeled reference sample (time zero). Independent biological duplicates of the reference sample and the mildly and severely salt-stressed samples were hybridized with the dyes swapped. After hybridization in a hybridization oven (Agilent), the microarrays were washed and blown dry with nitrogen gas.

In this study, custom-made B. cereus ATCC 14579 microarrays were used, which were printed by Agilent.

Microarray scanning and data analysis. The microarrays were scanned using an Agilent microarray scanner (G2565BA). The data were extracted from the microarrays using Agilent’s Feature Extraction software (version 8.1.1.1). The data extraction procedure included LOWESS normalization for the raw data. The web-based VAMPIRE microarray
suite (17) was used for further statistical analysis of the normalized data. The data for duplicate experiments were merged, and significantly differentially expressed microarray spots were identified using a threshold for the false discovery rate of less than 0.05. Subsequently, the expression ratios of the spots representing the same open reading frame were averaged. An open reading frame was considered to be differentially expressed when all spots representing the open reading frame were significantly differentially expressed based on the VAMPIRE output. The transcriptional expression data for a total of 5,058 open reading frames for 2.5% and 5% salt-stressed cells were compared.

**Catalase activity absorbance assay.** The catalase activity absorbance assay used was based on a previously described experimental procedure (7, 38). Untreated exponentially growing cells and cells which were salt stressed (2.5% or 5% NaCl) for 10 to 60 min were washed in phosphate-buffered saline (PBS) (138 mM NaCl, 2.7 mM KCl, 140 mM Na2HPO4, 1.8 mM KH2PO4; pH adjusted to 7.4 with HCl) and subsequently diluted (1:10, vol/vol) in PBS supplemented with H2O2 (final concentration, 40 mM). The decrease in absorbance at 240 nm was measured over time at 30°C with a spectrophotometer (Spectramax Plus 384; Molecular Devices, United States). One unit of catalase activity was defined as a decrease in the absorbance at 240 nm of 1 unit per minute. The rate of decrease for each sample was corrected for the amount of untreated or salt-stressed cells added to the assay buffer (absorbance at 600 nm and the assay dilution factor, as cell suspensions were diluted 1:10 when they were exposed to H2O2). For all experimental conditions, three reproductions were performed.

**Fluorescence microscopy.** Exponentially growing cultures were exposed to 5% NaCl for 150 min and subsequently double stained; 30 µM of the red fluorescent membrane dye FM4-64 (Invitrogen, The Netherlands) and 2 µM of the green fluorescent nucleic acid dye SYTO-9 (Invitrogen) were added to 1-ml aliquots of a culture and incubated for 15 min at room temperature in the dark. Cells were imaged using a Zeiss Axioskop fluorescence microscope with a fluorescein isothiocyanate filter set (magnification, ×1,000; Carl Zeiss, Germany). Images were obtained with a Canon Powershot G3 digital camera.

**Chemotaxis assays.** Exponentially growing cultures and cultures which were exposed to 5% NaCl for 10, 60, and 150 min were pelleted by centrifugation at 15,000 × g at 4°C for 1 min (Eppendorf centrifuge 5804 R; Eppendorf). The sample volume of the salt-stressed cells was adjusted based on the equivalent cellular density of unstressed cells (OD600 nm) in
50 ml. The pellets of unstressed and salt-stressed cells were resuspended in 30 ml of PBS agar (PBS supplemented with 0.3% [wt/vol] agar) at 42°C, resulting in a cellular density in the agar of approximately 8 log_{10} CFU ml^{-1} agar. Each mixture was equally divided and poured in four 9-cm-diameter petri dishes. Sterile filter disks (6-mm blank paper disks; Becton Dickinson) were placed in the middle of the petri dishes on the solidified cell-agar mixture, and subsequently, 5-µl aliquots of stock solutions of trehalose and alanine (0.4 M) were pipetted on top of the disks. The plates were incubated at 30°C, and after 1, 2, 3, and 4 h of incubation the plates were examined for chemotaxis.

**Determination of heat resistance and H_{2}O_{2} resistance of salt-stressed cells.** Exponentially growing cultures and cultures which were exposed to 2.5% and 5% NaCl for 10 to 150 min were added to 20 ml preheated BHI broth (1:100, vol/vol) at 50°C and then inactivated at 50°C with shaking at 200 rpm (Julabo SW22; Julabo Labortechnik, Germany). Before exposure and after 1, 2, 4, 6, and 8 min of 50°C-treatment, samples were taken, and appropriate dilutions were surface plated in duplicate on BHI agar plates. The plates were incubated for 16 h to 24 h at 30°C. A similar procedure was used to determine the H_{2}O_{2} resistance of exponentially growing cultures and salt-stressed cultures exposed to 1 mM H_{2}O_{2}. Both the heat and H_{2}O_{2} inactivation experiments were performed in duplicate.

**Microarray data accession number.** The microarray data have been deposited in the GEO database under accession number GSE13713.

**Results**

**Effect of 2.5% and 5% NaCl stress on growth kinetics.** To investigate the effect of exposure to mild and severe salt stress on the growth kinetics of *B. cereus* ATCC 14579, exponentially growing cultures (OD_{600 nm} ~ 0.4 to 0.5) were challenged with 2.5% and 5% NaCl. Exposure to 2.5% salt resulted in a slightly reduced growth rate (Figure 1). Cultures challenged with 5% salt showed an initial lag phase. After this lag phase, cellular growth resumed during exposure to 5% salt and resulted in an increase in absorbance (Figure 1a) at 60 min. However, this resumption of growth did not result in an increase in the viable counts (Figure 1b). Microscopic observations of the cells revealed that filamentous cells were formed during exposure to 5% salt for more than 60 min. This explains the discrepancy between the observed absorbance values and the viable counts after the resumption of growth during exposure to severe salt stress.
**General transcriptome response to NaCl stress.** Microarray analyses were performed to obtain insight into the molecular response of *B. cereus* ATCC 14579 to both mild and severe salt stress. Exponentially growing cells were exposed to 2.5% and 5% salt, and samples were taken after 10, 30, and 60 min of exposure and compared to an untreated reference sample. The number of differentially expressed genes for each condition (2.5% salt for 10 min, 2.5% salt for 30 min, 2.5% salt for 60 min, 5% salt for 10 min, 5% salt for 30 min, and 5% salt for 60 min) is shown in Figure 2. This figure shows that the number of differentially expressed genes in 2.5% salt-stressed cells was lower than the number of differentially expressed genes in 5% salt-stressed cells. Exposure of cells to 2.5% salt resulted in 153 differentially expressed genes at 10 min. After this first sampling time point, the number of differentially expressed genes decreased for the 30- and 60-min time points, at which there were 45 and 63 differentially expressed genes, respectively.
Figure 2. Number of differentially expressed genes in *B. cereus* ATCC 14579 after exposure to 2.5% and 5% salt for 10, 30 and 60 min. The gray bars indicate the genes that were differentially up- or downregulated after exposure to 2.5% salt for 10 min, as well as at the time points indicated.

Approximately 95% of the differentially expressed genes (*n* = 147) in 2.5% salt-stressed cells exposed for 10 min were also differentially expressed at one or more time points during exposure to 5% salt. The overlapping transcriptome responses during mild and severe salt stresses included upregulation of genes encoding transporters involved in osmoregulation, genes encoding transcriptional regulators, and oxidative stress response-related genes and downregulation of genes involved in nucleotide and amino acid transport and metabolism. To provide more insight into the overlap of the transcriptome responses during exposure to mild and severe salt stresses, a selection of representative genes is shown in Figure 3 (for a complete list of genes differentially expressed in cells exposed to 2.5% salt for 10 min and in cells exposed to 5% salt stress at one or more time points, see Table S1 in the supplemental material). To optimally visualize the overlap in gene expression, Figure 3 shows the expression ratio after exposure to 2.5% salt for 10 min and the average ratio after exposure to 5% salt for 10, 30, and 60 min for each gene included. In the genome of *B. cereus* ATCC 14579 genes encoding various osmoprotectant transporters were identified (18). The osmoprotectant symporter genes *opuD* (BC0555) and *opuE* (BC3644), a gene encoding a proline/betaine transporter (BC3000) belonging to the major
facilitator transporter family, and a gene encoding a proton-dependent di- and tripeptide transporter (BC0684) were among the most highly expressed genes upon exposure to salt stress. In contrast, the genes encoding the multicomponent osmoprotectant ABC transporters OpuA (represented by BC2791) and OpuB/OpuC (represented by BC2232) were not differentially expressed during exposure to 2.5% and 5% salt stress. Furthermore, the upregulation of Na+/H+ antiporters (represented by BC1612) and other cation transporters (represented by a zinc-transporting ATPase gene, BC0596) was upregulated during both 2.5% and 5% salt stress. The transport of cations may contribute to the cation
homeostasis inside the cell upon exposure to NaCl stress. These findings emphasized the importance of osmoprotectant, sodium, and other cation transporters in the salt stress response of *B. cereus* ATCC 14579.

The expression of the gene encoding the alternative transcription factor σ^B (sigB, BC1004), which is involved in the global adaptation response to stress, was upregulated during exposure to both mild and severe salt stresses. Transcription of sigB in cells exposed to 5% salt increased over time, and the expression ratio at 60 min was comparable to that of cells exposed to 2.5% salt for 10 min. Likewise, the activator of σ^B, rsbY (BC1006), and many other members of the σ^B regulon (37) were also upregulated. This finding supported the putative involvement of σ^B in the salt stress response of *B. cereus* ATCC 14579. However, no differences in the growth kinetics were observed when exponentially growing cells of the sigB deletion mutant FM1400 (35), the rsbY deletion mutant FM1401 (36), and parental strain ATCC 14579 were exposed to 2.5% and 5% salt (data not shown). In addition to upregulation of the transcriptional regulator gene sigB, two putative CarD-like transcriptional regulators (25) were highly expressed (represented by BC4714).

Exposure to both mild and severe salt stresses induced genes that have been described to be involved in the oxidative stress response of bacterial cells. The main vegetative catalase gene katA (BC1155) and the σ^B-dependent catalase gene katE (BC0863) (37) were upregulated. In addition, an alkyl hydroperoxide reductase gene, ahpC (BC0377), that has been demonstrated to be involved in scavenging endogenous hydrogen peroxide in *Escherichia coli* (28), and a gene encoding an antioxidant protein (BC5044) were found to be upregulated. In cultures of *B. subtilis* grown under high-salinity conditions, various iron limitation-related genes were upregulated (13, 33). In this study we also observed upregulation of genes involved in iron homeostasis, including a gene encoding a ferrochelatase (BC1154), which is neighbor of katA.

The overlap in the responses during exposure to 2.5% and 5% salt not only encompassed upregulated genes but also included repression of 61 genes (see Table S1 in the supplemental material). Several genes involved in nucleotide transport and metabolism (represented by a uracil phosphoribosyltransferase gene, BC3891) and in amino acid transport and metabolism (represented by an L-serine dehydratase gene, BC4136) were repressed upon exposure to salt stress. It is likely that a reduced growth rate allowed reductions in amino acid and pyrimidine metabolism, and this is consistent with previous findings for *B. subtilis* (33) and *E. coli* (40).
Transcriptome response to 5% NaCl stress. Exposure of *B. cereus* ATCC 14579 to 5% salt stress resulted in a greater transcriptome response than exposure to 2.5% salt stress (Figure 2). The observed differences between the growth kinetics during exposure to severe salt stress and the growth kinetics during exposure to mild salt stress may have contributed to the 5% salt stress-specific transcriptome profile. Genes putatively involved in various biological processes were found to be up- or downregulated in cells exposed to severe salt stress. Figure 4 shows the expression ratios for genes which were representative of the 5% salt stress-specific transcriptome response. The expression ratios for the representative genes after exposure to 5% salt for 10 min, 30 min, and 60 min are shown in this figure.

Exposure to 5% salt stress resulted in an initial lag phase before growth had resumed after 60 min of exposure. The transcriptome analysis revealed that genes involved in protein synthesis were upregulated during the lag phase at 30 min with exposure to 5% salt. Genes encoding an RNA polymerase (represented by *rpoB* [BC0122]), protein

Figure 4. Representative genes which were differentially expressed in *B. cereus* ATCC 14579 during exposure to 5% salt. The black bars, gray bars, and open bars indicate the expression ratios after exposure to 5% salt for 10, 30, and 60 min, respectively.
Phenotype and transcriptome response to salinity

translation elongation factors (represented by \textit{tufA} [BC0129]), and large- and small-subunit ribosomal proteins (represented by \textit{rpsJ} [BC0130]) were upregulated. After 60 min of exposure upregulation of tricarboxylic acid cycle genes (represented by the 2-oxoglutarate dehydrogenase gene, \textit{odhA} [BC1252]) was observed to correspond to the resumption of growth at this time point. Increased expression of tricarboxylic acid cycle proteins after resumption of growth was demonstrated previously for salt-challenged \textit{B. subtilis} cells (15). Our transcriptome data showed that components of the electron transport chain were also upregulated at 60 min (represented by the cytochrome \textit{d} ubiquinol oxidase subunit I gene, \textit{cydA} [BC1938]).

Resumption of growth resulted in formation of filamentous cells. Autolysins are involved in hydrolyzing peptidoglycan, which allows the daughter cell to separate (31), and indeed, in our study the putative autolysin gene \textit{yocH} (BC0679) and genes encoding a murein hydrolase exporter (represented by \textit{ywbH} [BC3669]) were downregulated during 5% salt stress. Moreover, the putative cell division inhibitor \textit{yfhF} (BC0497) gene was upregulated.

Exposure to severe salt stress affected the expression of genes involved in cell motility. Genes encoding proteins of the flagellar apparatus (represented by \textit{flgK} [BC1636] and \textit{flgB} [BC1641]) were repressed. Furthermore, genes described to be involved in chemotaxis were downregulated. For example, genes encoding methyl-accepting chemotaxis proteins, also known as chemoreceptors (represented by BC0422), and genes involved in the regulation cascade of chemotaxis (represented by \textit{cheY} [BC1627]) were repressed.

\textbf{Oxidative response is activated in NaCl-adapted cells.} Catalase activity is an important defense mechanism against hydrogen peroxide stress. Exposure to both 2.5% and 5% salt stresses resulted in upregulation of genes encoding catalases, represented by \textit{katA} and \textit{katE} in Figure 3. Therefore, catalase activity in exponentially growing cells and in cells exposed to 2.5% and 5% salt stresses was assessed (Figure 5). The catalase activity of 2.5% salt-adapted cells was significantly \((P < 0.05)\) higher than that of unstressed cells. Five percent salt stress induced a lag phase, and cells in this adaptation phase (5% salt for 30 min) did not show increased catalase activity despite upregulation of \textit{kat} genes. When cellular growth had resumed at 60 min, the catalase activity in the cells adapted to severe salt stress was significantly higher \((P < 0.05)\) than that in unstressed cells. The increased catalase activity in cells adapted to both mild and severe salt stresses was confirmed by visualization of the catalase activity on a native polyacrylamide gel (data not shown).
Figure 5. Catalase activities in exponentially growing cells and in cells exposed to 2.5% and 5% salt for 10 to 60 min. The error bars indicate standard errors.

**Adaptation to 5% NaCl results in filamentation and reduced chemotaxis.** Exposure to 5% salt resulted in filamentation of cells, and the morphology of the filamentous cells was studied in more detail by using fluorescence microscopy. Cells exposed to 5% salt were stained using the green fluorescent nucleic acid stain SYTO-9 and FM 4-64, a red lipophilic dye for staining membranes. Figure 6a shows a phase-contrast image of filamentous cells exposed to 5% NaCl for 150 min, and Figure 6b and c show cells labeled with SYTO-9 and FM 4-64. SYTO-9 stains the nucleoid inside the cell, and Figure 6b shows that regularly

Figure 6. *B. cereus* ATCC 14579 exposed to 5% salt for 150 min: phase-contrast micrograph (a) and micrograph of cells labeled with SYTO-9 (a green fluorescent nucleic acid stain) (b), and FM 4-64 (a red fluorescent membrane stain) (c).
spaced nucleoids were present in filamentous cells. Staining with the membrane stain FM 4-64 resulted in visible areas with dense red fluorescence, indicating that cell membrane septa were formed in the filamentous cells (Figure 6c).

Several cell motility and chemotaxis genes were found to be downregulated upon exposure to 5% salt, and because cell motility and an active chemotactic machinery is required for chemotaxis (2), we tested the chemotactic behavior of cells exposed to 5% salt with the attractants trehalose and alanine. The chemotactic behavior of exponentially growing cells and that of cells exposed to 5% salt for 10 min and 60 min were comparable with both attractants (data not shown). However, prolonged exposure to 5% salt resulted in reduced chemotactic behavior with trehalose and alanine. The halo of the unstressed exponentially growing cells was denser than that of 5% salt-stressed cells, indicating the reduced chemotactic behavior of the latter type of cells (see Figure S1 in the supplemental material).

**NaCl stress induces cross-protection against heat and H₂O₂.** The transcriptome analyses of salt-stressed *B. cereus* ATCC 14579 cells revealed several features that could result in increased cellular resistance to other stress conditions. Therefore, the heat resistance and H₂O₂ resistance were determined for unstressed cells and for cells which were preexposed to 2.5% or 5% salt for 10 to 150 min. Cells preexposed to 2.5% salt (2.5% salt for 10 min, 2.5% salt for 30 min, and 2.5% salt for 60 min) were found to be more heat resistant than exponentially growing cells (0%) (Figure 7a). Cells exposed to 5% salt for 10 to 30 min were still in a lag phase, and these cells were very heat sensitive (5% salt for 10 min and 5% salt for 30 min). When 5% salt-stressed cells had resumed growth (5% NaCl for 60 min), the heat sensitivity decreased and was comparable to that of unstressed cells. Prolonged preexposure to 5% salt (5% salt for 90 min and 5% salt for 150 min) increased the heat resistance.

Preexposure to both mild and severe salt stress induced cross-protection against H₂O₂ stress (Figure 7b). Cells exposed to 5% salt stress which had resumed growth (5% salt for 60 min, 5% salt for 90 min, and 5% salt for 150 min) were found to be the most H₂O₂-resistant cells.
Figure 7. Inactivation of viable cells after heat treatment at 50°C for 2 min (a) or after exposure to 1 mM H₂O₂ for 1 min (b) for exponentially growing *B. cereus* ATCC 14579 cells and for cells which were preexposed to 2.5% and 5% salt for 10 to 150 min. Inactivation was determined by subtracting the log₁₀-number of surviving cells after lethal treatment from the log₁₀-number of cells at the start of the inactivation. The error bars indicate standard errors.

Discussion

Salt is a commonly used food additive, and bacteria can activate various cellular adaptation mechanisms to cope with salinity. In this study we described the phenotype and transcriptome responses of the pathogen *B. cereus* ATCC 14579 to salt stress. Two concentrations of added sodium chloride were used, 2.5% and 5%, in order to obtain insight into the overlap between the salt stress responses and concentration-dependent parameters. Moreover, the use of various time frames for exposure to salt stress allowed adequate linkage of observed transcriptome responses to the phenotypic behavior of cells.

The transcriptome data confirmed that induction of osmoprotectant transporters is important in the mild and severe salt stress responses of *B. cereus* cells, which is in agreement with findings for other bacteria, such as *B. subtilis*, *E. coli*, and *Listeria monocytogenes* (21, 30). It has been demonstrated for *B. subtilis* that the substrate affinity of the glycine betaine ABC transporters OpuA and OpuC is in the same micromolar range as that of the glycine betaine symporter OpuD (20). However, the uptake via the ion-dependent symporters encoded by *opuD* and *opuE* seemed to be more favorable in nutrient-rich BHI medium than via ABC transporters, because the osmoprotectant ABC transporters
were not differentially expressed in *B. cereus* ATCC 14579 upon salt stress. The gene encoding a putative di- and tripeptide transporter (BC0684) was also highly upregulated in *B. cereus* ATCC 14579 during exposure to both mild and severe salt stress. Previously, it has been demonstrated that a homologous gene (34%) in *L. monocytogenes* EGD-e (*dptT*, LMO0555), encoding a di- and tripeptide transport system that supplies the cells with proline-containing peptides, is involved in salt stress protection (44). A similar function in the osmoregulation of *B. cereus* ATCC 14579 is conceivable as well. A transcriptome study of *B. subtilis* cells grown under high-salinity conditions showed that genes involved in the synthesis of the osmoprotectant proline were highly upregulated in this bacterium (33). However, induction of these genes was not observed in our study of the *B. cereus* ATCC 14579 salt stress response. This is consistent with previous investigations of de novo synthesis of compatible solutes in various *Bacillus* species (23), which demonstrated that proline was endogenously synthesized in osmotically stressed *B. subtilis* cells but not in *B. cereus* DSM31. In addition to proline, *B. subtilis* is also able to synthesize glycine betaine when the precursor choline is provided. A genome-wide search for orthologues of the *gbsAB* genes, which are known to be involved in glycine betaine synthesis in *B. subtilis* (4), revealed that these genes are not present in the genome of *B. cereus* ATCC 14579. These findings suggest that the osmoadaptation of *B. cereus* ATCC 14579 in BHI medium involves mainly the import of osmoprotectants rather than the synthesis of these compounds.

The upregulation of *sigB* and σ^B^-dependent genes during exposure to both mild and severe salt stresses and the salt stress-induced translation of σ^B^ in *B. cereus* ATCC 14579 (35) suggested a role for σ^B^ during exposure to salt stress in this organism. For *B. subtilis* it has been shown that σ^B^ and σ^B^-dependent proteins were strongly induced in response to salt stress at both the transcriptome (27) and proteome (15) levels. A *sigB* deletion mutant of *B. subtilis* was strongly impaired in osmotic stress adaptation (16, 39), as were a number of deletion mutants with mutations in σ^B^-dependent genes (16). The osmoprotectant transporter OpuE was found to be σ^A^- and σ^B^-dependent in *B. subtilis* (32), indicating a physiological function in osmoregulation of a σ^B^-dependent gene. In the current study, we demonstrated that the growth kinetics of exponentially growing cells of the *B. cereus* ATCC 14579 *sigB* deletion mutant FM1400 (35) and the *rsbY* deletion mutant FM1401 (36) after addition of salt were comparable to those of parental strain ATCC 14579. Previously, our laboratory showed that exposure to mild heat highly induced transcription of the σ^A^-regulon (37), and σ^B^ was found to be involved in the adaptive heat shock response of *B. cereus* ATCC 14579 (35). The set of σ^B^-dependent genes in *B. cereus*
ATCC 14579 appeared to be much smaller than that in other Gram-positive species, such as
*B. subtilis* and *L. monocytogenes* (37), and the $\sigma^B$-regulon of this strain does not include
genes encoding osmoprotectant transporters. Additionally, a large proportion of genes in
the *B. cereus* ATCC 14579 $\sigma^B$-regulon have unknown functions. Apparently, the role of $\sigma^B$
and its regulon in the salt shock response of *B. cereus* ATCC 14579 is limited, although
transcription and translation of $\sigma^B$ are induced by salt stress.

Two putative CarD-like transcriptional regulators, first described in *Myxococcus xanthus* (25), were highly expressed (BC3648 and BC4714) in *B. cereus* ATCC 14579
upon exposure to mild and severe salt stresses. To date, the information on CarD-like
transcriptional regulators is limited. Recently, it was demonstrated that in *Borrelia burgdorferi*, a spirochete causing Lyme disease, a homologous gene for a CarD-like
transcriptional regulator was expressed mainly during cultivation at a lower temperature
(45). It is known that osmotic and chill stresses can trigger similar features in bacterial
cells, indicating the overlap in the two responses (29, 41, 42). The role of the putative
CarD-like transcriptional regulators in the stress response of *B. cereus* ATCC 14579
remains to be elucidated.

Exposure of exponentially growing cells to 5% salt resulted in an initial lag phase
immediately after the salinity upshift, and filamentous cells were formed upon resumption
of growth. Various bacteria have been shown to form filamentous cells under severe stress
conditions (12, 19). The putative cell division inhibitor gene *yfhF* was found to be
upregulated in *B. subtilis* upon exposure to salt stress (27), indicating that cell division
might be affected. In this study, we showed that cell separation rather than septum
formation was impaired in the filamentous *B. cereus* ATCC 14579 cells. Our transcriptome
data revealed that genes encoding the FtsZ protein and the MinCD complex, which are
known to be involved in ring formation at the midcell and correct septum placement (11),
were not differentially expressed during exposure to severe salt stress in *B. cereus* ATCC
14579. On the other hand, genes involved in cell separation were downregulated, and these
findings correspond to the inhibition of cell separation observed later when growth
resumed.

Cell motility is required for chemotaxis toward attractants (2). The downregulation
of genes involved in flagellum assembly and the chemotactic machinery during exposure to
severe salt stress in this study suggested that the chemotactic behavior might be affected in
the cells. Repression of genes and proteins involved in chemotaxis and motility was
observed previously for *B. subtilis* exposed to high salinity (13, 15, 33) and also for *B.
cereus* ATCC 14579 in response to bile salts (22). To test the chemotactic behavior of
unstressed and salt-stressed cells, we used an assay in which we exposed high numbers of unstressed and salt-stressed cells to attractants in phosphate-buffered agar. This procedure allowed us to visualize the chemotactic behavior of *B. cereus* ATCC 14579 cells after brief incubation of the chemotactic plates, which resulted in increased cell density in the middle of the plate in the case of positive taxis. This chemotactic assay is growth rate independent because nutrients were not available in the agar and differences between unstressed and stressed cells could be observed in a short experimental time frame. We used this assay to demonstrate differences in the chemotactic behaviors of unstressed and salt-stressed cells because growth rate differences between unstressed and stressed cells may affect results obtained in cell motility assays which use nutritious media and longer incubation times to visualize chemotactic performance. The motility and chemotaxis genes were down-regulated in cells exposed to 5% salt for 10 to 60 min, but the chemotactic behavior of these cells was not affected. This indicated that the cells were still able to sense and move toward attractants, despite the observed growth arrest in the severely salt-stressed cells. Therefore, it is conceivable that other cellular parameters contributed to the temporal growth inhibition. Prolonged exposure to 5% salt (150 min) resulted in reduced chemotaxis compared to that of unstressed cells, indicating that there was a temporal shift between the observed transcriptome responses of severely salt-stressed cells during the lag period and the phenotypic responses of these cells when growth resumed.

Our transcriptome data revealed that oxidative stress-related genes were up-regulated during exposure to salt stress. Salt-induced expression of genes and proteins involved in the oxidative stress response was previously observed for *B. subtilis* (15, 27, 33). In our study, we demonstrated that upregulation of *kat* genes indeed resulted in increased catalase activity in salt-adapted cells compared to unstressed *B. cereus* ATCC 14579 cells. Cells adapting to severe salt stress, which were still in the lag phase, showed upregulation of *kat* genes, but the highest catalase activity and greatest resistance to H$_2$O$_2$ stress were observed in cells that had resumed growth during exposure to severe salt stress. Also, these findings demonstrate that there is a temporal shift between the transcriptome response of cells and development of the corresponding phenotype. In addition to salt-induced cross-protection against H$_2$O$_2$ stress, preexposure to salt induced cross-protection against heat. Aerobic heat stress has been demonstrated to impose an oxidative burden of reactive oxygen species in *E. coli* (3), and a recent study of *E. coli* showed that continuous osmotic and/or heat stresses induced upregulation of genes involved in the defense against oxidative stress damage (10). Therefore, it is conceivable that an activated oxidative stress response might confer cross-protection against other stresses. Furthermore, salt stress
induced the transcription of osmoprotectant transporters, and it has been demonstrated that osmoprotectants (e.g., glycine betaine) can also provide protection against other stresses, such as heat (14) and chilling (41).

The whole-genome expression analyses of mildly and severely salt-stressed B. cereus cells revealed an overlap in the transcriptome responses. The so-called general salt stress transcriptome response involved activation of genes encoding osmoprotectant, Na+/H+, and di- and tripeptide transporters and an oxidative stress response. Activation of this general salt stress response may have contributed to the salt-induced cross-protection against heat and H₂O₂. Analysis of the transcriptome and phenotype responses in severely salt-stressed cells revealed a temporal shift between these responses for cellular filamentation, chemotactic performance, catalase activity, and optimal oxidative stress resistance. Therefore, comparison of mild and severe stress transcriptome profiles in combination with assessment of phenotypic characteristics can contribute to a better understanding of cellular stress adaptation strategies and possible cross-protection mechanisms.

Acknowledgments

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References


Supplementary material
Table S1. Expression ratios of *B. cereus* ATCC 14579 genes which were differentially expressed after exposure to 2.5% salt for 10 min and the expression ratios of these genes at 30 and 60 min of 2.5% salt exposure and at 10, 30 and 60 min of 5% salt exposure

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Figure S1. Chemotaxis of *B. cereus* ATCC 14579 cells to the attractant trehalose when cells were not exposed to salt (a), and after exposure to 5% salt for 150 min (b). Images of the chemotaxis plates were taken after 3 h of incubation.
Short- and long-term biomarkers for bacterial robustness: A framework for quantifying correlations between cellular indicators and stress adaptive behavior

Heidy M. W. den Besten, Aarathi Arvind, Heidi M. S. Gaballo, Roy Moezelaar, Marcel H. Zwietering, Tjakko Abee

Submitted for publication
Abstract

The ability of microorganisms to respond and to adapt to changing environments challenges the prediction of their history-dependent behavior. Cellular biomarkers that are quantitatively correlated to stress adaptive behavior will facilitate our ability to predict the impact of these adaptive traits.

Here, we present a framework for identifying cellular biomarkers for adaptation-stress induced microbial robustness towards challenge-stresses. Several candidate-biomarkers were selected by comparing the genome-wide transcriptome profiles of our model-organism *Bacillus cereus* upon exposure to four adaptation-stress conditions (mild heat, acid, salt and oxidative stress). These candidate-biomarkers—a transcriptional regulator (activating general stress responses), enzymes (removing reactive oxygen species), and chaperones and proteases (maintaining protein quality)—were quantitatively determined at transcript, protein and/or activity level upon exposure to mild heat, acid, salt and oxidative stress for various time intervals. Both unstressed and adaptation-stress treated cells were also exposed to challenge-stress conditions (severe heat, acid and oxidative stress) to quantify the robustness advantage provided by adaptation-stress pretreatment. To evaluate whether the candidate-biomarkers could predict the challenge-stress robustness enhancement elicited by adaptation-stress pretreatment, the biomarker responses upon adaptation-stress treatment were correlated to adaptation-stress induced robustness towards heat, acid and oxidative challenge-stress. Both short- and long-term biomarkers could be identified of which their induction levels were quantitatively correlated to adaptation-stress induced enhanced robustness towards heat, acid and/or oxidative challenge-stress, respectively, and are therefore predictive cellular indicators for adaptation-stress induced enhanced robustness.

The identified biomarkers are among the most consistently induced cellular components in stress responses and ubiquitous in biology, supporting extrapolation to other microorganisms than *B. cereus*. Our quantitative, systematic approach provides a framework to search for these biomarkers and to evaluate their predictive quality at different functional cell levels in order to select promising biomarkers that can serve to early detect and predict adaptive traits. This mechanistic basis for stress adaptive behavior is a starting point towards mechanism-based predictive modeling of microbial fitness and robustness.
Introduction

Bacteria are constantly faced with changing environmental conditions and have evolved sophisticated mechanisms to adapt to changing environments. Environmental changes trigger a cascade of cellular events, and regulatory networks of microorganisms serve to prime cells to be prepared for later challenges even before they arise (23, 38). The adaptive stress response is a crucial survival strategy for a wide spectrum of microorganisms, including food spoilage bacteria, pathogens and organisms used in functional food applications, and prediction of this stress adaptive behavior will allow to control and/or exploit these adaptive traits.

Bacteria are not only exposed to changing environments in their natural habitats but also in industrial processing, and activation of stress adaptation mechanisms can provide cell robustness to harsher stress conditions including stresses other than the one that induced the adaptive stress response. This so-called cross-protection phenomenon challenges the hurdle preservation strategy that targets to guarantee the microbial safety and stability as well as the sensory and nutritional quality of minimally processed foods by simultaneous or successive application of multiple mild preservation factors (22). While individual hurdles may not be effective in controlling growth of food spoilage bacteria and pathogens, the right combination of hurdles allows to control growth of microorganisms and to minimize organoleptic changes in foods. However, the ability of spoilage and pathogenic microorganisms to adapt to stressing environments could antagonize the benefits of the hurdle preservation strategy, because long- and short-term exposure to mild stress conditions showed to induce (cross-)protection towards otherwise lethal challenge-stress conditions (1, 18, 30) and might even affect the virulence of pathogens (7, 14). The ability of microorganisms to gain cellular robustness upon activation of adaptive stress responses is also beneficial for various industrial applications (29), including the development of reliable starter cultures (31) and selection of robust probiotic strains (8).

Previous studies demonstrated that our model-organism Bacillus cereus gained increased resistance upon preexposure to several mild food preservation stresses (3, 4, 10, 25, Chapter 2), underlining the significance of a better understanding of its stress adaptive behavior. The availability of complete genome sequences of microbes and the development of functional genomics technologies have provided a wealth of data and opportunities to better understand stress adaptation mechanisms. Comparison of genome-wide transcriptome profiles of microorganisms in response to diverse environmental conditions can reveal general stress response features (5, 15, 16, 26), and could lead to identification of
cellular indicators for stress adaptive behavior. Recently, we investigated the genome-wide transcriptome response of *B. cereus* to several mild adaptation-stresses (6, 11, 24, 36, Chapter 6), and these transcriptome analyses gave insight into general and stress-specific adaptive responses, and opened avenues towards identification of potential cellular biomarkers that can predict stress adaptive behavior. Here, we present a framework for the identification of such cellular biomarkers for adaptation-stress induced enhanced robustness towards challenge-stresses. We identified both short- and long-term biomarkers of which their induction levels were quantitatively correlated to the adaptation-stress induced robustness level of the cell. The identification of predictive cellular indicators for stress adaptive behavior will enable the prediction of the impact of adaptive responses during mild stress (processing) conditions on subsequent microbial robustness, and can be exploited to control these stress adaptive traits.

Materials and methods

**Bacterial strain and preculturing conditions.** *Bacillus cereus* ATCC 14579 was used as model-organism throughout the study. Stock cultures grown in brain heart infusion (BHI, Becton Dickinson, France) broth were stored at −80°C in 25% (vol/vol) glycerol. To prepare precultures, 10 ml BHI broth was inoculated with a droplet of the stock culture and incubated over night at 30°C with shaking at 200 rpm.

**Adaptation-stress treatment.** The precultures were inoculated in Erlenmeyer flasks containing 50 ml fresh BHI broth and incubated at 30°C with shaking at 200 rpm until the cells were exponentially growing (absorbance value at 600 nm of 0.4 to 0.5). Upon reaching this optical density, the exponentially growing cells were exposed to four mild, adaptation-stress conditions for 2, 5, 10, 15, 20, 30 and 60 min with shaking at 200 rpm. The following adaptation-stress conditions were applied: heat stress (43°C); acid-shock (pH 5.5, adjusted with 37% hydrochloric acid, at 30°C); osmotic-upshift (1.5% [wt/vol] sodium chloride, at 30°C); and oxidative stress (0.1 mM H<sub>2</sub>O<sub>2</sub>, at 30°C). Preliminary experiments had demonstrated that exposure to those selected conditions for 15 min resulted in optimal heat resistance (Figure S1 in the supplementary material).

**Determination of challenge-stress robustness following adaptation-stress pretreatment.** Both unstressed and adaptation-stress treated cells were subsequently exposed to three severe challenge-stress conditions to determine their specific robustness
using the inactivation procedure described previously (11, Chapter 6). The following
inactivation conditions were chosen: heat stress (50°C); low pH (pH 3.3, adjusted with 37%
hydrochloric acid, at 30°C); and oxidative stress (0.2 mM H$_2$O$_2$, at 30°C). Before and after
inactivation treatment, samples were taken and decimal dilutions were made in peptone
saline solution (1 g neutralized bacteriological peptone [Oxoid, United Kingdom]
supplemented with 8.5 g sodium chloride per liter). After acid-inactivation treatment,
samples were decimally diluted in BHI broth to ensure no further acid-inactivation during
diluting. The appropriate dilutions were surface plated, in duplicate, on BHI agar plates
(BHI broth supplemented with 15 g agar [Oxoid, United Kingdom] per liter) using a spiral
plater (Eddy Jet; IUL Instruments, Spain) and the plates were incubated at 30°C for 16 h to
24 h. The experiments to inactivate unstressed and adaptation-stress pretreated cells were
reproduced 2 to 3 times on different days. The robustness of both unstressed and
adaptation-stress pretreated cells was determined as the number of microorganisms
surviving the inactivation treatment, $N(t)$, compared to the initial number of
microorganisms before the inactivation treatment at $t = 0$, $N(0)$. t-Tests were performed to
compare the log$_{10}$-robustness, $\log_{10}\frac{N(t)}{N(0)}$, of adaptation-stress pretreated cells to that of
unstressed cells (with $P < 0.05$ as significance threshold).

**Determination of candidate-biomarker induction upon adaptation-stress treatment.**
Ten candidate-biomarkers were quantitatively measured before and after adaptation-stress
treatment for 2 to 60 min, namely, catalase activity, the proteins SigB, ClpC and ClpP, and
the transcripts $sigB$, $catA$, $catE$, $clpB$, $clpC$, $clpP$. The experimental procedures followed to
measure and to quantify the responses of these candidate-biomarkers upon adaptation-stress
treatment are described below.

**Catalase activity assay.** A previously described procedure was used to determine
the catalase activity of unstressed cells and adaptation-stress treated cells (11, 37, Chapter
6). Briefly, cells were washed in phosphate-buffered saline and subsequently exposed to
hydrogen peroxide, and the decrease in absorbance at 240 nm was measured over time at
30°C with a spectrophotometer (Spectramax Plus 384; Molecular Devices, USA). One unit
of catalase activity was defined as a decrease in absorbance at 240 nm of 1 unit per minute.
The rate of decrease for each sample was corrected for the amount of cells used in the assay
and standardized to absorbance value of 0.5 at 600 nm. For all adaptation-stress treatment
intervals, three biologically independent catalase activity experiments were performed.

The catalase activity was also visualized by catalase activity staining on a native
polyacrylamide gel. For that, total proteins were extracted from unstressed and adaptation-
stress treated cultures following a similar procedure as previously described (25, 37). Subsequently, fifty micrograms of protein extracts were separated on a native 10% Tris-HCl polyacrylamide gel (Criterion; Bio-Rad Laboratories, USA). Catalase activity was visualized as described previously (39), which results in yellow catalase bands against a dark-green background.

**Western blotting.** Total cellular protein was extracted from four biologically independent cultures of unstressed and adaptation-stress treated cells. Forty micrograms of protein extracts were separated by using 15% Tris-HCl gels for SigB and ClpP, and 7.5% Tris-HCl gels for ClpC. Immunoblotting was performed as described previously (34) with anti-SigB antibodies raised against the SigB protein of *B. cereus* (34), and anti-ClpC and anti-ClpP antibodies raised against these proteins of *B. subtilis* (25). Immunocomplexes were visualized using Chemiluminescent Peroxidase Substrate-3 (Sigma-Aldrich, Germany) and scanned with a Chemiluminescence scanner with ChemiDoc XRS software (Bio-Rad Laboratories, USA). The band intensity was quantified using Quantity One software (version 4.6.1; Bio-Rad Laboratories, USA) with background subtraction.

**RNA isolation and RT-PCR.** RNA was isolated from two biologically independent cultures of unstressed and adaptation-stress treated cells. For that, 10 ml of culture was transferred into a 50-ml Falcon tube, and spun down at 13,000 × g for 30 s at 4°C. After the supernatant was decanted, the cell pellets were immediately resuspended in 1 ml of TRI-reagent (Ambion, UK) and snap-frozen in liquid nitrogen. The RNA was further extracted as described previously (34). Synthesis of cDNA and real time(RT)-PCR was carried out as described previously (35), with 16S-rRNA as reference gene, and *sigB*, *catA*, *catE*, *clpB*, *clpC* and *clpP* as target genes. The primers of the reference and target genes are listed in Table S1 in the supplementary material. The relative expression ratios of the target genes were calculated as previously described (27).

**Evaluation of candidate-biomarker induction.** The relative induction levels of the candidate-biomarkers in adaptation-stress treated cells compared to unstressed cells were log10-transformed and averaged for the biologically independent reproductions for each adaptation-treatment time point. *t*-Tests were performed to evaluate whether the induction of the candidate-biomarker in adaptation-stress treated cells was statistically significant (with *P* < 0.05 as significance threshold).

**Correlation between adaptation-stress induced robustness and candidate-biomarkers responses.** The robustness of adaptation-stress treated cells (s) was also relatively expressed to that of unstressed cultures (uns) and subsequently log10-transformed,
Biomarkers for adaptation-stress induced robustness

log₁₀\left[ \frac{N_{t0}/N_{0k}}{N_{t0\text{trans}}/N_{0\text{trans}}} \right] \), and averaged for the biological reproductions. Then, for each candidate-biomarker, the adaptation-stress induced candidate-biomarker responses were quantitatively correlated to adaptation-stress induced robustness towards challenge-stress per adaptation-stress and challenge-stress pair. The Pearson correlation coefficient r was calculated to test the significance of each correlation using PASW software.

Results

Experimental strategy for identifying biomarkers for adaptation-stress induced robustness. In search of potential biomarkers for adaptation-stress induced robustness, the genome-wide transcriptome profiles of our model-organism B. cereus ATCC 14579 grown until the mid-exponential growth phase and then exposed to various mild adaptation-stress conditions (6, 11, 24, 36, Chapter 6) were compared. This comparison revealed a remarkable limited number of genes (see Table S2 in the supplementary material) that were differentially expressed upon treatment to all those adaptation-stress conditions. This overlap of transcriptome responses included various defense mechanisms and regulatory and metabolic pathways, including cellular defense mechanisms against oxidative stress, factors involved in repair and maintenance of cellular protein quality and energy production, and transcriptional regulators. In order to select the most promising functional categories that could point to candidate-biomarkers, the expression ratios of the differentially expressed genes were compared. Genes were marked of which the expression ratios were at least five upon exposure to one adaptation-stress condition and at least two upon exposure to two other adaptation-stress conditions. These genes represented three main functional categories: (1) members of the general stress response regulon controlled by transcriptional regulator σB; (2) cellular defense mechanisms against oxidative stress; and (3) repair and maintenance of cellular protein quality. This transcription signature of stress adaptation seemed to be stress-independent, supporting its suitability as a source for identification of potential biomarkers for stress adaptation. Based on these three categories, candidate-biomarker transcripts for stress adaptive behavior were assigned, namely that of sigB, catA, catE, clpB, clpC and clpP. Conceivably, not only transcripts but also proteins and enzymes could function as biomarker, and therefore, also the proteins SigB, ClpC and ClpP, and catalase activity were included as candidate-biomarkers, resulting in ten candidate-biomarkers (Figure 1a).
To quantify the stress adaptive behavior of *B. cereus* to a wide spectrum of stress conditions imposed on bacteria, four adaptation-stress conditions were used to mildly stress the cells for various time intervals, namely, heat stress (43°C), acid-shock (pH 5.5), osmotic-upshift (1.5% NaCl), and oxidative stress (0.1 mM H₂O₂) (see Figure S1 in the...
supplementary material for more details about the procedure to select these adaptation-stress conditions for adaptation experiments. Adaptation-stress treated cells were subsequently exposed to three more severe, normally lethal, challenge-stress conditions – heat stress (50°C), low pH shock (pH 3.3) and high oxidative stress (0.2 mM H₂O₂) – to quantify adaptation-stress induced enhanced robustness towards these challenge-stresses. Severe osmotic-upshift (up to 30% sodium chloride [wt/vol]) did not result in inactivation kinetics comparable to those observed with the other three challenge-stress conditions (data not shown) and was therefore not used as challenge-stress. To evaluate whether one or more of the selected candidate-biomarkers could quantitatively predict the robustness enhancement following adaptation-stress pretreatment, the induction of the candidate-biomarkers was determined upon adaptation-stress treatment and correlated to adaptation-stress induced robustness towards heat, acid and oxidative challenge-stress (Figure 1b). After correlating the adaptation-stress induced biomarker and robustness responses, the Pearson correlation coefficient was calculated to evaluate the significance of the correlations in order to select potential biomarkers for adaptation-stress induced enhanced robustness (Figure 1b).

**Induction of candidate-biomarkers and challenge-stress robustness in response to adaptation-stress treatment.** Mild heat treatment resulted in induction of almost all candidate-biomarkers, but their induction patterns differed (Figure 2a). The relative transcription levels of *sigB* and *catE*, which is a regulon member of σ^B^ (36), displayed transient increased expression up to 15 min of heat treatment and decreased afterwards, whereas the transcription patterns of *clpB*, *clpC* and *clpP* were rather constant over time. Upon 60 min of mild heat treatment, the relative transcription levels of *sigB* and *catE* increased again. The gene encoding the main vegetative catalase, *catA*, was just significantly transcribed at one mild heat treatment time point, and also, no increased catalase activity was observed in mild heat-treated cells compared to unstressed cells. This confirmed that the role of catalase CatE is less significant in total cellular catalase activity compared to the main vegetative catalase CatA (37). Mild heat treatment resulted in increased production of the proteins SigB, ClpC and ClpP compared to that of unstressed cells and the production patterns of both Clp proteins were comparable.

Mild heat pretreatment provided a robustness advantage towards severe heat treatment, and also conferred cross-protection towards acid challenge-stress, whereas no increased resistance towards oxidative challenge-stress was observed upon mild heat pretreatment (Figure 2b).
Figure 2. Induction of candidate-biomarkers and robustness in response to heat adaptation-stress treatment. 

a) Candidate-biomarkers – the transcripts of *sigB*, *catA*, *catE*, *clpB*, *clpC* and *clpP*, the proteins SigB, ClpC and ClpP, and catalase activity – were quantitatively measured in unstressed (uns) cultures and upon 43°C stress (s) treatment for 2, 5, 10, 15, 20, 30 and 60 min. The columns mark the log10-fold induction after adaptation-stress treatment compared to unstressed cells. 

b) Unstressed and 43°C-treated cells were inactivated by exposure to 50°C for 5 min, pH 3.3 at 30°C for 3.5 min, and 0.2 mM H2O2 at 30°C for 2 min, to determine their heat, acid and oxidative challenge-stress robustness, respectively. The columns mark the number of microorganisms surviving the challenge-stress treatment compared to the initial number of microorganisms (%). Error bars represent standard errors of the reproductions.
Besides mild heat treatment, cells were also short- and long-term exposed to mild acid, salt and oxidative stress and the induction patterns of the candidate-biomarkers upon these adaptation-stress treatments are shown in Figure 3a, 4a and 5a, respectively. The induction patterns of the candidate-biomarkers were highly affected by the type of adaptation-stress condition applied. In addition to mild heat treatment, also mild acid and salt treatment significantly increased transcription of \textit{sigB} and \textit{catE} and led to increased SigB protein levels compared to unstressed cells, whereas these responses were not significant in mild oxidative stress-treated cells. Mild acid treatment for up to 30 min elicited just significantly increased transcription of \textit{clpB}, \textit{clpC}, and \textit{clpP} compared to unstressed cells, but increased production of the ClpC and ClpP proteins could not be demonstrated. Both mild salt and oxidative stress did not significantly increase the transcription of \textit{clpB}, \textit{clpC}, and \textit{clpP} nor did these mild stress conditions lead to higher levels of the ClpC and ClpP proteins. In contrast to mild heat treatment, exposure to mild acid, salt and oxidative stress resulted in significantly increased transcription of \textit{catA} and cellular catalase activity.

Mild acid pretreatment resulted in enhanced resistance towards severe acid stress and provided also cross-protection towards oxidative challenge-stress, whereas no significantly increased resistance towards heat challenge-stress was observed (Figure 3b). On the other hand, mild salt pretreatment provided heat and oxidative challenge-stress cross-protection, but did not confer cross-protection towards acid challenge-stress (Figure 4b). Mild oxidative stress-pretreatment provided cells enhanced robustness towards heat, acid, and also oxidative challenge-stress, and therefore, only this adaptation-stress condition conferred enhanced robustness to all three challenge-stresses tested (Figure 5b). In Table S3 in the supplementary material, the effect of the different adaptation-stress pretreatments on heat, acid and oxidative challenge-stress resistance of \textit{B. cereus} is summarized.

\textbf{Framework for identifying biomarkers for adaptation-stress induced robustness.} To evaluate whether one or more of the candidate-biomarkers could predict the adaptation-stress induced robustness level of the cell, the individual candidate-biomarker responses were correlated to adaptation-stress induced robustness towards heat, acid and oxidative challenge-stress for each adaptation-stress condition.
Figure 3. Induction of candidate-biomarkers and robustness in response to acid adaptation-stress treatment.  
a) Candidate-biomarkers – the transcripts of sigB, catA, catE, clpB, clpC and clpP, the proteins SigB, ClpC and ClpP, and catalase activity – were quantitatively measured in unstressed (uns) cultures and upon pH 5.5 stress (s) treatment for 2, 5, 10, 15, 20, 30 and 60 min. The columns mark the log_{10}-fold induction after adaptation-stress treatment compared to unstressed cells. b) Unstressed and pH 5.5-treated cells were inactivated by exposure to 50°C for 5 min, pH 3.3 at 30°C for 3.5 min, and 0.2 mM H_2O_2 at 30°C for 2 min, to determine their heat, acid and oxidative challenge-stress robustness, respectively. The columns mark the number of microorganisms surviving the challenge-stress treatment compared to the initial number of microorganisms (%). Error bars represent standard errors of the reproductions.
Figure 4. Induction of candidate-biomarkers and robustness in response to salt adaptation-stress treatment. a) Candidate-biomarkers – the transcripts of $\text{sigB}$, $\text{catA}$, $\text{catE}$, $\text{clpB}$, $\text{clpC}$ and $\text{clpP}$, the proteins SigB, ClpC and ClpP, and catalase activity – were quantitatively measured in unstressed (uns) cultures and upon 1.5% NaCl stress (s) treatment for 2, 5, 10, 15, 20, 30 and 60 min. The columns mark the log$_{10}$-fold induction after adaptation-stress treatment compared to unstressed cells. b) Unstressed and 1.5% NaCl-treated cells were inactivated by exposure to 50°C for 5 min, pH 3.3 at 30°C for 3.5 min, and 0.2 mM H$_2$O$_2$ at 30°C for 2 min, to determine their heat, acid and oxidative challenge-stress robustness, respectively. The columns mark the number of microorganisms surviving the challenge-stress treatment compared to the initial number of microorganisms (%). Error bars represent standard errors of the reproductions.
Figure 5. Induction of candidate-biomarkers and robustness in response to oxidative adaptation-stress treatment. a) Candidate-biomarkers – the transcripts of *sigB*, *catA*, *catE*, *clpB*, *clpC* and *clpP*, the proteins SigB, ClpC and ClpP, and catalase activity – were quantitatively measured in unstressed (uns) cultures and upon 0.1 mM H$_2$O$_2$ stress (s) treatment for 2, 5, 10, 15, 20, 30 and 60 min. The columns mark the log$_{10}$-fold induction after adaptation-stress treatment compared to unstressed cells. b) Unstressed and 0.1 mM H$_2$O$_2$-treated cells were inactivated by exposure to 50°C for 5 min, pH 3.3 at 30°C for 3.5 min, and 0.2 mM H$_2$O$_2$ at 30°C for 2 min, to determine their heat, acid and oxidative challenge-stress robustness, respectively. The columns mark the number of microorganisms surviving the challenge-stress treatment compared to the initial number of microorganisms (%). Error bars represent standard errors of the reproductions.
A decision flow chart was designed to evaluate whether the candidate-biomarker that was induced upon adaptation-stress treatment could indeed predict the robustness level of adaptation-stress pretreated cells (Figure 6a). Three types of biomarkers were formulated, namely, long-term biomarkers, short-term biomarkers, and no-response biomarkers, and these three types of biomarkers will be discussed below. A stepwise procedure was followed to select those conditions where the candidate-biomarkers functioned as long-term biomarkers or as short-term biomarkers. First, the adaptation-stress induced biomarker and robustness responses upon adaptation-stress treatment for 2, 5, 10, 15, 20, 30 to 60 min (7 treatment time points) compared to that of unstressed cells were correlated for each adaptation-stress and challenge-stress pair, and the Pearson correlation coefficient was tested for significance ($P < 0.05$). When the correlation was significant, then the treatment time point of 60 min was excluded from the analysis, after which the correlation of the remaining treatment time points was tested for significance. The stepwise procedure was followed until the exclusion of treatment time point of 10 min. When the adaptation-stress induced candidate-biomarker and robustness responses were significantly correlated for all adaptation time intervals, then the candidate-biomarker was qualified as long-term biomarker for that adaptation-stress and challenge-stress pair. The candidate-biomarker was qualified as short-term biomarker when the adaptation-stress induced biomarker and robustness responses were only significantly correlated for short-term adaptation time intervals. When adaptation-stress treatment for up to 60 min did not significantly induce the candidate-biomarker and also no significant induction of robustness was observed upon adaptation-stress pretreatment, then the candidate-biomarker was qualified as no-response biomarker.

Several biomarkers were suitable to predict the robustness enhancement following adaptation-stress pretreatment for more than one adaptation-stress and challenge-stress pair. To evaluate whether the biomarker could predict the robustness enhancement towards one specific challenge-stress originated from multiple adaptation-stress pretreatments, the adaptation-stress induced biomarker and robustness responses were combined and the correlation was tested for significance. When this correlation remained significant ($P < 0.05$), then the biomarker was defined as convergent-application biomarker (Figure 6b). The biomarker was defined as divergent-application biomarker when it could predict the robustness enhancement towards multiple challenge-stresses upon pretreatment to a specific adaptation-stress (Figure 6c).
a

induction of biomarker

yes

induction of robustness

yes

biomarker and robustness correlated upon inclusion of all adaptation intervals

yes

biomarker and robustness also correlated for short-term adaptation intervals

yes

long-term biomarker

no

biomarker and robustness only correlated for short-term adaptation intervals

yes

short-term biomarker

no

induction of robustness

no

b

long-term biomarker

short-term biomarker

no-response biomarker

correlation coefficients all positive or all negative

yes

biomarker is correlated to a specific challenge-stress robustness for multiple adaptation-stresses, combined

yes

convergent-application biomarker

multiple adaptation-stresses, challenge-stress specific

no

long-term biomarker

short-term biomarker

no-response biomarker

correlation coefficients all positive or all negative

yes

biomarker is adaptation-stress specific and correlated to multiple challenge-stress robustness, combined

yes

divergent-application biomarker

adaptation-stress specific, multiple challenge-stresses
Identification of biomarkers for adaptation-stress induced robustness. The step-wise procedure was followed to select those conditions for which the candidate-biomarker could predict the robustness level of the cell following adaptation-stress pretreatment. The SigB protein functioned as a long-term biomarker for mild heat- and salt-induced heat challenge-stress robustness (Figure 7). SigB was also significantly correlated to heat challenge-stress robustness upon mild acid pretreatment, but because mild acid pretreatment did not significantly induce heat challenge-stress robustness (Figure 3), SigB was not identified as a long-term biomarker for this condition. Since SigB could predict the heat challenge-stress robustness status of both mild heat- and salt-pretreated cells, and remained significantly correlate to heat challenge-stress robustness when these mild heat- and salt-induced responses were combined, SigB was qualified as convergent-application biomarker for heat challenge-stress robustness. In addition to SigB protein, also the proteins ClpC and ClpP were identified as convergent-application biomarkers for heat challenge-stress robustness, but their potential to predict heat challenge-stress robustness were related to mild heat- and acid-pretreated cells (see Figure S2 and S3 in the supplementary material). Noteworthy, both ClpC and ClpP proteins could predict the enhanced heat challenge-stress robustness levels provided by mild heat pretreatment only for short adaptation intervals (2 to 30 min). Additionally, SigB was suitable to predict the robustness advantage towards both acid and oxidative challenge-stress originating from preexposure to mild acid stress. Since SigB could still predict the mild acid-induced robustness towards acid and oxidative challenge-stress when these responses were combined, it also functioned as divergent-application biomarker.
Figure 7. Protein SigB as potential biomarker for adaptation-stress induced robustness. Induction of protein SigB upon adaptation-stress treatment (43°C, △; pH 5.5, ○; 1.5% NaCl, △; 0.1 mM H₂O₂, □) was correlated to adaptation-stress induced robustness towards challenge-stress (50°C, open symbols; pH 3.3, light gray filled symbols; 0.2 mM H₂O₂, dark gray filled symbols). Each adaptation-stress was applied for 2, 5, 10, 15, 20, 30 and 60 min and induction of SigB and robustness for adaptation-stress pretreated cells (s) was relatively expressed to that of unstressed cells (uns) (log s/uns). Robustness of adaptation-stress pretreated cells and unstressed cells was determined as the number of bacteria surviving the challenge-stress treatment compared to the initial number of bacteria. The graph boxes show the conditions for which SigB was qualified as biomarker for adaptation-stress induced robustness: black box represents a long-term biomarker; double black box represents a convergent-application biomarker; double gray box represents a divergent-application biomarker.
Catalase activity could predict the robustness level towards oxidative challenge-stress upon pretreatment to more than two adaptation-stress conditions (Figure 8). Mild heat stress did not induce catalase activity and this corresponded to no induction of oxidative challenge-stress robustness, and therefore, catalase activity acted as a no-response biomarker for this condition. Additionally, catalase activity functioned as long-term biomarker for oxidative challenge-stress robustness originated from mild acid and oxidative stress pretreatment. Because, catalase activity could still predict the oxidative challenge-stress robustness status when these three adaptation-stress induced responses were combined, it pointed to the potential of catalase activity to act as a vigorous convergent-application biomarker for oxidative challenge-stress robustness. Additionally, catalase activity significantly correlated to robustness towards multiple challenge-stresses induced upon pretreatment to mild acid or mild oxidative stress, and functioned therefore also as divergent-application biomarker.

The transcripts of both clpC and clpP were qualified as convergent-application biomarkers for acid challenge-stress robustness upon mild acid and salt pretreatment (Figure S4 and S5 in the supplementary material). In addition to protein SigB and catalase activity, clpC and clpP were also suitable to predict the robustness advantage towards multiple challenge-stresses, namely acid and oxidative challenge-stress, following mild acid pretreatment. Furthermore, also the transcript catE (Figure S6 in the supplementary material) showed similar divergent predictive potential for those conditions. Detailed supplementary information about the predictive potential of the proteins ClpC and ClpP, and the transcripts sigB, catA, catE, clpB, clpC and clpP is shown in Figures S2 to S9 in the supplementary material, and summarized for all the candidate-biomarkers in Table S4. Noteworthy, the transcript candidate-biomarkers were for various conditions identified as short-term biomarkers rather than long-term biomarkers.

A concluding overview of the predictive potential of the candidate-biomarkers is presented in Table 1, and highlights that some biomarkers have more potential than others to predict stress adaptive behavior. The SigB protein was suitable to predict the robustness enhancement towards heat challenge-stress upon mild heat and salt adaptation-stress pretreatment and was therefore qualified as convergent-application biomarker for heat challenge-stress robustness (Table 1a, see also Figure 7). Next to SigB, also the proteins ClpC and ClpP were qualified as convergent-application biomarkers for heat challenge-stress robustness, but their potential to predict this stress adaptive behavior was related to mild heat- and acid-pretreated cells. The predictive potential of the transcripts clpC and clpP were comparable and both were qualified as convergent-application biomarkers for
Figure 8. Catalase activity as potential biomarker for adaptation-stress induced robustness. Induction of catalase activity upon adaptation-stress treatment (43°C, ◇; pH 5.5, ◦; 1.5% NaCl, △; 0.1 mM H$_2$O$_2$, □) was correlated to adaptation-stress induced robustness towards challenge-stress (50°C, open symbols; pH 3.3, light gray filled symbols; 0.2 mM H$_2$O$_2$, dark gray filled symbols). Each adaptation-stress was applied for 2, 5, 10, 15, 20, 30 and 60 min and induction of catalase activity and robustness for adaptation-stress pretreated cells (s) was relatively expressed to that of unstressed cells (uns) (log s/uns). Robustness of adaptation-stress pretreated cells and unstressed cells was determined as the number of bacteria surviving the challenge-stress treatment compared to the initial number of bacteria. The graph boxes show the conditions for which catalase activity was qualified as biomarker for adaptation-stress induced robustness: black box represents a long-term biomarker; dashed gray box represents a no-response biomarker; double black box represents a convergent-application biomarker; double gray box represents a divergent-application biomarker.
Table 1a. Convergent-application biomarkers for adaptation-stress induced robustness

<table>
<thead>
<tr>
<th>Convergent-application biomarkera</th>
<th>Adaptation-stressb</th>
<th>Challenge-stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>multiple adaptation-stresses, challenge-stress specific</td>
<td>heat, salt</td>
<td>heat</td>
</tr>
<tr>
<td>SigB</td>
<td>heat</td>
<td>heat</td>
</tr>
<tr>
<td>sigB</td>
<td>heat</td>
<td>heat</td>
</tr>
<tr>
<td>catalase activity</td>
<td>heat, acid, H₂O₂</td>
<td>H₂O₂</td>
</tr>
<tr>
<td>catA</td>
<td>heat</td>
<td>heat</td>
</tr>
<tr>
<td>catE</td>
<td>heat</td>
<td>heat</td>
</tr>
<tr>
<td>ClpC</td>
<td>acid</td>
<td>acid</td>
</tr>
<tr>
<td>ClpP</td>
<td>acid, salt</td>
<td>acid</td>
</tr>
<tr>
<td>clpB</td>
<td>acid</td>
<td>acid</td>
</tr>
<tr>
<td>clpC</td>
<td>acid, salt</td>
<td>acid</td>
</tr>
<tr>
<td>clpP</td>
<td>acid</td>
<td>acid</td>
</tr>
</tbody>
</table>

1gray text indicates that the cellular indicator was not identified as a convergent-application biomarker for adaptation-stress induced robustness.

1italic text indicates that adaptation-stress treatment did not significantly induce the biomarker and also did not provide a robustness enhancement upon pretreatment (no-response condition).

acid challenge-stress robustness following mild acid and salt stress preexposure. Catalase activity emerged as a convergent-application biomarker for oxidative challenge-stress robustness, which could be elicited by multiple adaptation-stress pretreatments (e.g. mild heat, acid and oxidative stress) (see also Figure 8). Several candidate-biomarkers were correlated to robustness towards multiple challenge-stresses upon pretreatment to a specific adaptation-stress (Table 1b). In addition to SigB, four other biomarkers – catalase activity, catE, clpC and clpP – showed similar predictive potential by emerging as divergent-application biomarker for acid and oxidative challenge-stress robustness following mild acid challenge-stress robustness following mild acid and salt stress preexposure. Catalase activity emerged as a convergent-application biomarker for oxidative challenge-stress robustness, which could be elicited by multiple adaptation-stress pretreatments (e.g. mild heat, acid and oxidative stress) (see also Figure 8). Several candidate-biomarkers were correlated to robustness towards multiple challenge-stresses upon pretreatment to a specific adaptation-stress (Table 1b). In addition to SigB, four other biomarkers – catalase activity, catE, clpC and clpP – showed similar predictive potential by emerging as divergent-application biomarker for acid and oxidative challenge-stress robustness following mild

Table 1b. Divergent-application biomarkers for adaptation-stress induced robustness

<table>
<thead>
<tr>
<th>Divergent-application biomarkera</th>
<th>Adaptation-stress</th>
<th>Challenge-stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>adaptation-stress specific, multiple challenge-stresses</td>
<td>acid</td>
<td>acid, H₂O₂</td>
</tr>
<tr>
<td>SigB</td>
<td>acid</td>
<td>acid, H₂O₂</td>
</tr>
<tr>
<td>sigB</td>
<td>H₂O₂</td>
<td>heat, H₂O₂</td>
</tr>
<tr>
<td>catalase activity</td>
<td>acid</td>
<td>acid, H₂O₂</td>
</tr>
<tr>
<td>catA</td>
<td>acid</td>
<td>acid, H₂O₂</td>
</tr>
<tr>
<td>catE</td>
<td>acid</td>
<td>acid, H₂O₂</td>
</tr>
<tr>
<td>ClpC</td>
<td>acid</td>
<td>acid, H₂O₂</td>
</tr>
<tr>
<td>ClpP</td>
<td>acid</td>
<td>acid, H₂O₂</td>
</tr>
<tr>
<td>clpB</td>
<td>acid</td>
<td>acid, H₂O₂</td>
</tr>
<tr>
<td>clpC</td>
<td>acid</td>
<td>acid, H₂O₂</td>
</tr>
<tr>
<td>clpP</td>
<td>acid</td>
<td>acid, H₂O₂</td>
</tr>
</tbody>
</table>

1gray text indicates that the cellular indicator was not identified as a divergent-application biomarker for adaptation-stress induced robustness.
acid pretreatment. Therefore, this study also showed that various biomarkers can have similar predictive potential.

Discussion

The adaptive stress response of bacteria is a crucial mode of cellular protection and allows bacteria to survive in changing environments. Cellular biomarkers that are quantitatively correlated to stress adaptive behavior will allow to predict the impact of changing environments on bacterial fitness, robustness and survival. The availability of complete genome sequences of a wide variety of bacteria has been instrumental in the development of functional genomics technologies that furthered our understanding of bacterial stress adaptation mechanisms. These technologies, that make use of holistic and unbiased approaches, can direct our rational search for critical cellular components that may function as biomarkers for stress adaptive behavior and subsequent enhanced robustness under challenging conditions. In this study, we identified various transcript biomarkers and also biomarkers at protein and activity level, all with predictive quality. We proposed a framework for selecting those cellular biomarkers for adaptation-stress induced robustness and we systematically evaluated the predictive potential of several candidate-biomarkers for our model-organism *B. cereus*. Based on a genome-wide comparison of transcriptome profiles of *B. cereus* cells exposed to four mild adaptation-stress conditions, several potential biomarkers were selected. These were the general stress response transcriptional regulator $\sigma^B$, catalases involved in $\text{H}_2\text{O}_2$-scavenging, and chaperones and ATP-dependent Clp proteases involved in protein repair and maintenance. Since several adaptation-stress conditions induced the transcription of genes involved in these stress responses, these findings support their significance in *B. cereus* general stress response. Activation of cellular mechanisms involved in stress response-signaling and -regulation upon exposure to stress conditions as well as activation of systems involved in oxidative stress defense and maintenance of protein quality might be an essential and universal mode of microbial stress adaptation and enhancement of bacterial robustness. Therefore, the canonical induction of these stress responses extends far beyond the species *B. cereus*, and has been demonstrated for other bacteria including *Bacillus subtilis*, *Listeria monocytogenes* and *Escherichia coli* upon exposure to stress conditions (2, 26, 32), but also in yeast by a wide variety of environmental changes (5, 15). In the present study, the induction of $\sigma^B$, catalases, chaperones and ATP-dependent Clp proteases were quantitatively determined at transcript, protein and/or activity level upon adaptation-stress treatment and these responses were
correlated to adaptation-stress induced challenge-stress robustness, aiming to evaluate their predictive biomarker potential at different functional cell levels. Our study shows that the predictive potential of cellular indicators is highly influenced by the functional cell level at which the indicator is measured. The SigB protein and catalase activity were identified as long-term biomarkers for various adaptive stress responses. Moreover, both biomarkers could be employed as convergent- and divergent-application biomarkers as their predictive ability was not restricted to a single adaptation-stress and challenge-stress pair. These findings underlined the high predictive quality of these biomarkers and pointed to a promising role in prediction of stress adaptive behavior. In contrast, the catA transcript of the main vegetative catalase was not suitable to predict the challenge-stress robustness level of adaptation-stress pretreated cells for any of the tested conditions, underpinning the significance to evaluate the predictive potential of cellular indicators at different functional cell levels. In addition, our results demonstrated that the predictive potential of biomarkers could manifest across multiple time scales because some biomarkers, such as the ClpC and ClpP proteins, as well as the clpC, clpP, sigB and catE transcripts, acted as short-term biomarker and predicted the adaptation-stress induced robustness under challenge-stress conditions for rather short adaptation intervals.

The adaptive traits of bacteria can antagonize food processing strategies that rely on combining mild preservation treatments to control bacterial growth of spoilage and pathogenic bacteria, but are also crucial for various industrial functional food applications. The identification of potential biomarkers for adaptation-stress induced robustness towards challenge-stresses contributes to a better understanding of bacterial stress adaptation mechanisms and can guide our search to control and/or exploit these adaptive traits. Promising candidate-biomarkers might be species- and genus-specific because the functional conservation of stress-related cellular factors differs among species and genera, and the predictive value of biomarkers might even be strain-specific. The role and regulation of key regulators of general stress responses as well as cellular mechanisms that are crucial for controlling protein quality and defending against oxidative stresses have been shown to differ between closely-related genera. The transcriptional regulator $\sigma^B$ functions as central regulator of general stress responses in Gram-positive bacteria with a low GC content including the genera Bacillus, Staphylococcus and Listeria with variations in regulon-members and -size (33, 36). This sigma factor is absent in various lactic acid bacteria (19, 31, 33) suggesting that lactic acid bacteria have developed different stress regulatory networks. Clp proteases and chaperones and the main vegetative catalase, predominantly controlled by the CtsR and/or HrcA repressors (13) and by PerR (17, 20, 28)
in low GC Gram-positive bacteria, respectively, are widely conserved in microorganisms (12, 13, 21) and play indispensable roles in cellular repair and defense strategies. Despite apparent variations in their mechanisms of expression control between species and genera, these canonical stress-related components are among the most consistently induced components in microbial stress responses and ubiquitous in biology. We showed that these stress indicators can serve as predictors of stress adaptive behavior in B. cereus and it is conceivable that they may also be employed in other microorganisms. Variations in functional conservations and regulatory circuits necessitates a profound validation of biomarker quality, and our study provides a quantitative approach to systematically search for these biomarkers and evaluate their predictive potential.

In conclusion, we presented a framework for identifying cellular biomarkers for stress adaptive behavior and to statistically evaluate their predictive potential at different functional cell levels. This quantitative approach opens avenues towards prediction of microbial performance using cellular biomarkers. Moreover, this study presented a mechanistic basis for stress adaptive behavior and is thus a starting point towards mechanism-based predictive modeling of bacterial fitness and robustness.

Acknowledgment

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References


Supplementary material
Figure S1. Selection of adaptation-stress condition for adaptation experiments. Inactivation kinetics of *Bacillus cereus* ATCC 14579 cells at 50°C following adaptation-stress pretreatment for 15 min. Four mild adaptation-stress conditions were used for pretreatment, namely, heat stress (a), acid-shock (b), osmotic-upshift (c) and oxidative stress (d), respectively. Pretreatment with 43°C (a), pH 5.5 (b), 1.5% NaCl (c) and 0.1 mM H$_2$O$_2$ (d) resulted in subsequent optimal heat resistance.
Figure S2. Protein ClpC as potential biomarker for adaptation-stress induced robustness. Induction of protein ClpC upon adaptation-stress treatment (43°C, △; pH 5.5, □; 1.5% NaCl, △; 0.1 mM H₂O₂, □) was correlated to adaptation-stress induced robustness towards challenge-stress (50°C, open symbols; pH 3.3, light gray filled symbols; 0.2 mM H₂O₂, dark gray filled symbols). Each adaptation-stress was applied for 2, 5, 10, 15, 20, 30 and 60 min and induction of ClpC and robustness for adaptation-stress pretreated cells (s) was relatively expressed to that of unstressed cells (uns) (log \(s/uns\)). Robustness of adaptation-stress pretreated cells and unstressed cells was determined as the number of bacteria surviving the challenge-stress treatment compared to the initial number of bacteria. The graph boxes show the conditions for which ClpC was qualified as biomarker for adaptation-stress induced robustness: gray box represents a short-term biomarker; dashed gray box represents a no-response biomarker; double black box represents a convergent-application biomarker. Short-term biomarkers are only correlated to robustness for short-term adaptation intervals, excluding long-term intervals (black filled symbol).
Figure S3. Protein ClpP as potential biomarker for adaptation-stress induced robustness. Induction of protein ClpP upon adaptation-stress treatment (43°C, pH 5.5, O2; 1.5% NaCl, Δ; 0.1 mM H2O2, □) was correlated to adaptation-stress induced robustness towards challenge-stress (50°C, open symbols; pH 3.3, light gray filled symbols; 0.2 mM H2O2, dark gray filled symbols). Each adaptation-stress was applied for 2, 5, 10, 15, 20, 30, and 60 min and induction of ClpP and robustness for adaptation-stress pretreated cells (s) was relatively expressed to that of unstressed cells (uns) (log s/uns). Robustness of adaptation-stress pretreated cells and unstressed cells was determined as the number of bacteria surviving the challenge-stress treatment compared to the initial number of bacteria. The graph boxes show the conditions for which ClpP was qualified as biomarker for adaptation-stress induced robustness: gray box represents a short-term biomarker; dashed gray box represents a no-response biomarker; double black box represents a convergent-application biomarker. Short-term biomarkers are only correlated to robustness for short-term adaptation intervals, excluding long-term intervals (black filled symbol).
Figure S4. Transcript clpC as potential biomarker for adaptation-stress induced robustness. Induction of transcript clpC upon adaptation-stress treatment (43°C, ○; pH 5.5, △; 1.5% NaCl, □; 0.1 mM H\textsubscript{2}O\textsubscript{2}, ▽) was correlated to adaptation-stress induced robustness towards challenge-stress (50°C, open symbols; pH 3.3, light gray filled symbols; 0.2 mM H\textsubscript{2}O\textsubscript{2}, dark gray filled symbols). Each adaptation-stress was applied for 2, 5, 10, 15, 20, 30 and 60 min and induction of clpC and robustness for adaptation-stress pretreated cells (s) was relatively expressed to that of unstressed cells (uns) (log s/uns). Robustness of adaptation-stress pretreated cells and unstressed cells was determined as the number of bacteria surviving the challenge-stress treatment compared to the initial number of bacteria. The graph boxes show the conditions for which clpC was qualified as biomarker for adaptation-stress induced robustness: black box represents a long-term biomarker; gray box represents a short-term biomarker; dashed gray box represents a no-response biomarker; double black box represents a convergent-application biomarker; double gray box represents a divergent-application biomarker. Short-term biomarkers are only correlated to robustness for short-term adaptation intervals, excluding long-term intervals (black filled symbol).
Figure S5. Transcript clpP as potential biomarker for adaptation-stress induced robustness. Induction of transcript clpP upon adaptation-stress treatment (43°C, ▽; pH 5.5, ▽; 1.5% NaCl, △; 0.1 mM H$_2$O$_2$, □) was correlated to adaptation-stress induced robustness towards challenge-stress (50°C, open symbols; pH 3.3, light gray filled symbols; 0.2 mM H$_2$O$_2$, dark gray filled symbols). Each adaptation-stress was applied for 2, 5, 10, 15, 20, 30 and 60 min and induction of clpP and robustness for adaptation-stress pretreated cells (s) was relatively expressed to that of unstressed cells (uns) (log s/uns). Robustness of adaptation-stress pretreated cells and unstressed cells was determined as the number of bacteria surviving the challenge-stress treatment compared to the initial number of bacteria. The graph boxes show the conditions for which clpP was qualified as biomarker for adaptation-stress induced robustness: black box represents a long-term biomarker; gray box represents a short-term biomarker; dashed gray box represents a no-response biomarker; double black box represents a convergent-application biomarker; double gray box represents a divergent-application biomarker. Short-term biomarkers are only correlated to robustness for short-term adaptation intervals, excluding long-term intervals (black filled symbol).
Figure S6. Transcript *catE* as potential biomarker for adaptation-stress induced robustness. Induction of transcript *catE* upon adaptation-stress treatment (43°C, ○; pH 5.5, ○; 1.5% NaCl, △; 0.1 mM H_2 O_2, □) was correlated to adaptation-stress induced robustness towards challenge-stress (50°C, open symbols; pH 3.3, light gray filled symbols; 0.2 mM H_2 O_2, dark gray filled symbols). Each adaptation-stress was applied for 2, 5, 10, 15, 20, 30 and 60 min and induction of *catE* and robustness for adaptation-stress pretreated cells (s) was relatively expressed to that of unstressed cells (uns) (log s/uns). Robustness of adaptation-stress pretreated cells and unstressed cells was determined as the number of bacteria surviving the challenge-stress treatment compared to the initial number of bacteria. The graph boxes show the conditions for which *catE* was qualified as biomarker for adaptation-stress induced robustness: black box represents a long-term biomarker; gray box represents a short-term biomarker; double gray box represents a divergent-application biomarker. Short-term biomarkers are only correlated to robustness for short-term adaptation intervals, excluding long-term intervals (black filled symbols).
Figure S7. Transcript $\text{sigB}$ as potential biomarker for adaptation-stress induced robustness. Induction of transcript $\text{sigB}$ upon adaptation-stress treatment ($43^\circ\text{C}$, $\triangle$; pH 5.5, $\bigcirc$; 1.5% NaCl, $\triangle$; 0.1 mM $\text{H}_2\text{O}_2$, $\square$) was correlated to adaptation-stress induced robustness towards challenge-stress ($50^\circ\text{C}$, open symbols; pH 3.3, light gray filled symbols; 0.2 mM $\text{H}_2\text{O}_2$, dark gray filled symbols). Each adaptation-stress was applied for 2, 5, 10, 15, 20, 30 and 60 min and induction of $\text{sigB}$ and robustness for adaptation-stress pretreated cells (s) was relatively expressed to that of unstressed cells (uns) (log $s/uns$). Robustness of adaptation-stress pretreated cells and unstressed cells was determined as the number of bacteria surviving the challenge-stress treatment compared to the initial number of bacteria. The graph boxes show the conditions for which $\text{sigB}$ was qualified as biomarker for adaptation-stress induced robustness: black box represents a long-term biomarker; gray box represents a short-term biomarker. Short-term biomarkers are only correlated to robustness for short-term adaptation intervals, excluding long-term intervals (black filled symbols).
Figure S8. Transcript *catA* as potential biomarker for adaptation-stress induced robustness. Induction of transcript *catA* upon adaptation-stress treatment (43°C, ◆; pH 5.5, ○; 1.5% NaCl, △; 0.1 mM H₂O₂, □) was correlated to adaptation-stress induced robustness towards challenge-stress (50°C, open symbols; pH 3.3, light gray filled symbols; 0.2 mM H₂O₂, dark gray filled symbols). Each adaptation-stress was applied for 2, 5, 10, 15, 20, 30 and 60 min and induction of *catA* and robustness for adaptation-stress pretreated cells (s) was relatively expressed to that of unstressed cells (uns) (log s/uns). Robustness of adaptation-stress pretreated cells and unstressed cells was determined as the number of bacteria surviving the challenge-stress treatment compared to the initial number of bacteria. For non of the adaptation-stress and challenge-stress pairs, *catA* was qualified as biomarker.
Figure S9. Transcript clpB as potential biomarker for adaptation-stress induced robustness. Induction of transcript clpB upon adaptation-stress treatment (43°C, △; pH 5.5, ○; 1.5% NaCl, △; 0.1 mM H₂O₂, □) was correlated to adaptation-stress induced robustness towards challenge-stress (50°C, open symbols; pH 3.3, light gray filled symbols; 0.2 mM H₂O₂, dark gray filled symbols). Each adaptation-stress was applied for 2, 5, 10, 15, 20, 30 and 60 min and induction of clpB and robustness for adaptation-stress pretreated cells (s) was relatively expressed to that of unstressed cells (uns) (log s/uns). Robustness of adaptation-stress pretreated cells and unstressed cells was determined as the number of bacteria surviving the challenge-stress treatment compared to the initial number of bacteria. The graph boxes show the conditions for which clpB was qualified as biomarker for adaptation-stress induced robustness: black box represents a long-term biomarker; dashed gray box represents a no-response biomarker.
Table S1. RT-PCR primers used in this study

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<th>Primername</th>
<th>Gene-no</th>
<th>Sequence (5′-3′)</th>
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<td>AATTCCGAGCAACGCGAAGAC</td>
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<td>16SrRNA_Rev</td>
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<td>BC1004</td>
<td>CAAATCTGATGAAAGCGGCGAG</td>
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<tr>
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<td>CGGTCCGCTTTGGAATAGCG</td>
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<td>catA_Forw</td>
<td>BC1155</td>
<td>CTGGAACCCCAACGCAAGGTG</td>
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<td>BC1155</td>
<td>CGAACAGGTACACGAGTAGCA</td>
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<td>clpP_Forw</td>
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Table S2. Overlap of transcriptome responses upon adaptation-stress treatment

<table>
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<th>Gene-no a, c, d</th>
<th>Annotation</th>
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<td>Heat</td>
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<tr>
<td>BC0099 d</td>
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<td>BC0100 d</td>
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<td>BC0101 c</td>
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<tr>
<td>BC0102 c</td>
<td>ClpC</td>
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<td>FruE protein</td>
<td>2.04</td>
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<td>BC0377 c</td>
<td>Alkyl hydroperoxide reductase</td>
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</tr>
<tr>
<td>BC0387</td>
<td>hypothetical protein</td>
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<td>BC0503</td>
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<td>BC1003 b</td>
<td>Anti-sigmaB factor</td>
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<td>BC1005 b</td>
<td>Bacterioferritin</td>
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<td>BC1006 b</td>
<td>PP2C phosphatase</td>
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<td>BC1154 c</td>
<td>Ferrochelatase</td>
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<td>BC1155 b, c</td>
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<td>BC3402</td>
<td>Arsenate reductase family protein</td>
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<td>BC3647</td>
<td>N-ethylmaleimide reductase</td>
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<td>BC5401</td>
<td>Lipase/acylhydrolase</td>
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aThe comparison of the genome-wide transcriptome profiles of B. cereus ATCC 14579 upon treatment to four mild adaptation-stress conditions – heat stress (36), acid-shock (24), osmotic-upshift (11, Chapter 6, M. Mols and A. Streng, unpublished data) and oxidative stress (6) – for 10 min, revealed a limited number of genes that were differentially expressed upon treatment to all four adaptation-stress conditions. The values denote the transcription ratios of these genes for adaptation-stress treated cells compared to unstressed cells (mid-exponential growth phase with OD 600 nm 0.4 to 0.5). The microarray data of the mild heat, acid, salt and oxidative stress adaptation experiments have been deposited in the GEO database with accession numbers GSE6005, GSE15140, GSE13713 and GSE18807, respectively. The genes of which the expression ratios were at least five upon treatment to one adaptation-stress condition and at least two upon treatment to two other adaptation-stress conditions, represented three functional categories: members of the general stress regulon controlled by the transcriptional regulator σB (9, 36); cellular defense mechanisms against oxidative stress; repair and maintenance of cellular protein quality.

bGene is part of the σB-regulon.
cGene is associated with cellular defense mechanisms against oxidative stress.
dGene is associated with repair and maintenance of cellular protein quality.
Table S3. Adaptation-stress induced (cross-)protection

<table>
<thead>
<tr>
<th>Adaptation-stress</th>
<th>Challenge-stress</th>
<th>Heat</th>
<th>Acid</th>
<th>H$_2$O$_2$</th>
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<tbody>
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<td>Heat</td>
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<td>+</td>
<td></td>
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<tr>
<td>Acid</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
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<td>Salt</td>
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<td>H$_2$O$_2$</td>
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aPlus symbol (+) represents enhanced robustness towards challenge-stress (heat, 50°C; acid, pH 3.3; oxidative stress, 0.2 mM H$_2$O$_2$) upon adaptation-stress pretreatment (heat, 43°C; acid, pH 5.5; salt, 1.5% NaCl; oxidative stress, 0.1 mM H$_2$O$_2$).
<table>
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<th>Biomarker</th>
<th>Challenge-stress</th>
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<th>Salt</th>
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<td>L</td>
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<tr>
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<sup>a</sup>Gray fills represent those conditions for which adaptation-stress pretreatment resulted in enhanced robustness towards challenge-stress. Long-term biomarker, L; Short-term biomarker, S; No-response biomarker, N.
Chapter 8

Summarizing discussion, conclusions and future perspectives
The increasing trend in production and sales of mildly preserved ready-to-use foods (11, 46) challenges the food industry to optimize mild preservation strategies. The application of various mild preservation factors in minimally processed foods, known as hurdles, assures the microbial safety of minimally processed foods and also stabilizes the sensory and nutritive characteristics. However, the ability of pathogenic microorganisms to adapt to stressing environments could antagonize the benefits of the hurdle preservation strategy, because exposure to mild stress conditions can provide enhanced cell resistance towards otherwise lethal stress conditions (1, 28, 50). *Bacillus cereus* is frequently isolated from ready-to-use foods (11, 17, 59) and most of the strains related to cases or outbreaks of *B. cereus* food-borne poisoning were shown to be unable to grow at 7°C (2, 23), underlining the significance of appropriate refrigeration of foods contaminated with *B. cereus* to control its growth and toxin production. In this thesis, a quantitative approach was followed to gain insight in *B. cereus*’ stress adaptation mechanisms. Its stress adaptive behavior was quantified at population level, individual cell level, and at molecular level, with the aim to correlate molecular adaptive stress responses and individual cell performance to the population dynamics of *B. cereus* as a whole (Figure 1).

Figure 1. Overview of the research themes – population, individual cell and molecular level – and the research topics addressed in the thesis chapters. *B. cereus*’ stress adaptive behavior was quantified at different levels aiming to quantitatively link molecular adaptive stress responses and individual cell performance to the population dynamics as a whole.
Quantification of the adaptive stress response at population level. At the time this research project started, only limited information was available about \textit{B. cereus}' adaptive stress responses. The studies that had investigated its stress adaptive behavior had shown that short-term exposure to various mild stress conditions enhanced the resistance of \textit{B. cereus} towards subsequent heat challenge-stress exposure (7, 8, 47). The greatest cross-protection was provided by preexposure to salt stress, and this prompted us to quantify this adaptive salt stress response in more detail. Two mesophilic strains, \textit{B. cereus} ATCC 10987 and ATCC 14579, were short-term exposed to mild and severe salt stress conditions after which their resistance towards heat challenge-stress was determined. Linear and nonlinear primary survival models, which cover a wide range of known inactivation curvatures for vegetative cells, were fitted to the heat inactivation kinetics of both unstressed and salt stress preexposed cells to quantitatively describe the inactivation curvature characteristics. The results are described in Chapter 2. Various statistical indices ($r^2$, $A_p$, mean square error of the model) and the biological variation, i.e. the day-to-day variation, were calculated to statistically evaluate the fitting performances of the models. The biological variation between reproductions, reflected in the mean square error of the data, influenced the model selection because the higher the measuring error, the less complex a model had to be to describe the data statistically acceptably. A thorough literature survey performed by Van Boekel (52) confirmed that first-order heat inactivation kinetics are the exception rather than the rule. However, when specific curvature characteristics are statistically significant, taken into account the biological variation, then a more complex model is needed to fit the data adequately. In Chapter 2, the rather complex biphasic models with a shoulder were selected for quantification because these models could describe the heat inactivation data of unstressed and salt stress preexposed cells of both strains statistically acceptably. Moreover, the model parameters of these models reflected specific inactivation phenomena that could be recognized in the inactivation curvature (e.g. shoulder) allowing to exclude a model parameter for those specific conditions where the corresponding curvature characteristic was not statistically significant.

The quantitative comparison of the impact of both mild and severe salt stress preexposure on heat resistance demonstrated that especially mild salt stress conditions provided \textit{B. cereus} cells optimal protection towards subsequent heat challenge-stress exposure. This trend was also observed when \textit{B. cereus} cells were short-term exposed to acid stress prior to heat inactivation (Figure 2, unpublished data). Exposure of exponentially growing \textit{B. cereus} ATCC 14579 cells to an abrupt acid shock of pH 5.5 using
Figure 2. Growth of *B. cereus* ATCC 14579 in BHI broth at 30°C (a) without addition of stress (◇), and after acid shock to pH 5.5 using hydrochloric acid (HCl, □) and lactic acid (HL, △) as acidulants. Heat inactivation kinetics of *B. cereus* ATCC 14579 in BHI broth at 50°C (b) after reaching the mid-exponential growth phase (◇), and after preexposure to pH 5.5 for 30 min using HCl (□) and HL (△) as acidulants.

Hydrochloric acid (HCl) as acidulant reduced the growth rate compared to unstressed cells (Figure 2a), whereas using lactic acid (HL, 2 mM undissociated acid) as acidulant resulted in an initial growth arrest (Figure 2a). Heat inactivation following acid pretreatment for 30 min, revealed that the heat resistance of HCl-preexposed cells was higher than that of untreated cells, while preexposure to HL did not provide cross-protection towards heat challenge-stress (Figure 2b).

Further investigations along this line using stress conditions other than salt and acid stress for preexposure (Chapter 7) confirmed that especially mild stress conditions, that significantly reduced the growth rate but did not introduce a bacteriostatic effect or reduction in viable counts, provided optimal protection towards subsequent heat challenge-stress exposure. However, also bacteriostatic conditions and even conditions that resulted in reduction of viable counts were able to provide enhanced cell resistance, indicating that in the latter situation, the culturable fraction of *B. cereus* cells was still capable of demonstrating an adaptive stress response (Chapter 2). This phenomenon was previously also demonstrated for the food-borne pathogen *Listeria monocytogenes* (42) and highlights that activation of adaptive stress responses and loss of culturability can occur.
simultaneously and points to population heterogeneity with respect to stress adaptive behavior.

The heat resistance of transition- and stationary-phase *B. cereus* cells was higher than that of exponential-phase cells. Towards the end of the growth cycle, general stress responses are activated providing resistance towards challenge-stress (49). A further increase of heat resistance of transition- and stationary-phase cells in response to adaptation-stress pretreatment (e.g. salt) may therefore be less pronounced for transition- and stationary-phase cells compared to exponentially growing cells as demonstrated for *B. cereus*. These results are consistent with findings in other Gram-positive and Gram-negative bacteria (36, 42), and as a result, the physiological state of cells used for stress adaptation experiments will affect the impact of stress adaptive behavior on cell robustness.

Among the many ways to control bacterial growth in minimally processed foods, lowering of the temperature is one of the most widely used. Therefore, the effect of culturing temperature on the adaptive salt stress response was quantitatively assessed for three *Bacillus* strains, namely, the mesophilic strains *B. cereus* ATCC 14579 and ATCC 10987 and the psychrotolerant strain *Bacillus weihenstephanensis* KBAB4, which represent three distinct lineages of the *B. cereus* group (39). These results are described in Chapter 3. The three *Bacillus* strains were cultured until the exponential growth phase at 12°C and 30°C in the absence and in the presence of salt, and were also short-term salt shocked after reaching the exponential growth phase prior to heat inactivation. This allowed us to quantify the effects of culturing temperature and mild stress exposure time (salt culturing versus salt shock) on salt-induced heat resistance. The fitting performance of the Weibull model was statistically adequate to describe the inactivation data of all three strains, indicating that the more complex models as described in Chapter 2 were not needed for statistically better description of the inactivation data. The experimental procedure followed for the salt stress adaptation experiments in Chapter 3 slightly differed from that following in Chapter 2 because cultures were not concentrated before heat inactivation, resulting in a lower initial cell concentration, \( \log_{10} N(0) \), at the start of the inactivation. The maximum reduction, \( \log_{10} N(0) - \log_{10} N(\text{detection limit}) \), was therefore lower in the procedure following in Chapter 3 compared to Chapter 2 resulting in inactivation curvatures that could be adequately described with a less complex inactivation model.

The third decimal reduction times (that reflected the times needed to reduce the initial number of microorganisms with three decimal powers) were used to quantify the effects of culturing temperature and mild stress exposure time on salt-induced heat
resistance. Culturing temperature during mild stress exposure had a significant impact for the three tested strains. The heat resistance of both mesophilic strains increased when cells were salt cultured or salt shocked at 30°C, whereas these effects were not significant for the psychrotolerant strain. In contrast, only the psychrotolerant strain showed salt-induced increased heat resistance when the cells were short- or long-term preexposed to salt at 12°C. The effect of mild stress exposure time (salt culturing versus salt shock) on the magnitude of the adaptive salt stress response was rather limited and of minor importance compared to the effect of culturing temperature. For cultures in which the adaptive salt stress response was activated, the cross-protective effect towards heat challenge-stress was even larger when salt was also present during the heat inactivation treatment. This may be explained by the increased stability of proteins (26). This latter is of relevance for food processing conditions where mild stress applications are subsequently followed by a (re)heating step.

Quantification of the adaptive stress response at individual cell level. Within a bacterial population, even between genetically identical cells there is phenotypic variation resulting in heterogeneous behavior of the population as a whole. This heterogeneity in performance within a population can be manifested in various ways (4, 5). We demonstrated in Chapter 2 and 3 that the heat inactivation kinetics of both unstressed cells and salt stress-adapted cells deviated from first-order kinetics, pointing to heterogeneity in their behavior. We further elaborated on this and quantitatively described the growth performance of B. cereus ATCC 14579 in response to mild and severe salt stress exposure at individual cell level. These results are described in Chapter 4. Cells were cultured from the single cell stage until microcolonies on porous Anopore strips (32-34) to directly image and quantify the growth dynamics of individual cells. Exposure of B. cereus cells to mild salt stress resulted in a noticeably low variability of microcolony sizes over time, and this variability was comparable to that of cells that continued growing in the absence of salt. This indicated that mild salt stress treatment did not introduce heterogeneity in growth performance. In contrast, exposure to severe salt stress induced a lag phase and a subpopulation of cells that did not continue growing after the initial lag phase. However, this subpopulation of non-growing microcolonies was rather small (15% of the total number of microcolonies), and such a limited fraction of nongrowing cells will be quickly overgrown by the growing population of cells. This indicates that the observed salt-induced population heterogeneity is too limited to have a major impact on the final number of cells when foods are initially contaminated with higher levels of B. cereus cells.
The growth kinetics of cells cultured on Anopore strips were compared to those cultured in liquid culture and were similar both in the absence and the presence of salt stress. An advantage of the direct-imaging-based Anopore technology is that growth kinetics as well as physiological characteristics of individual cells of both the growing and nongrowing populations of microcolonies can be studied using different fluorescent dyes. Along this line, the population heterogeneity of *B. cereus* ATCC 14579 during low temperature incubation was quantified in Chapter 5 and the membrane integrity of cells was assessed in order to get more insight into physiological characteristics of cells that may have contributed to the heterogeneity in growth response. The growth of *B. cereus* was monitored at 12°C to mimic temperature abuse of refrigerated foods. Culturing of cells at this low incubation temperature revealed a subpopulation of nongrowing microcolonies, and this fraction of nongrowing microcolonies was rather comparable to that in response to severe salt stress exposure. The growth kinetics at low temperature of the growing population of microcolonies was again similar to that of cells cultured in broth, underpinning the remarkable similarities in growth kinetics of cells cultured on Anopore strips and those in broth under various growth conditions compared to other surface growth systems (6). Double staining of the nongrowing and growing microcolonies using the fluorescent dyes SYTO-9 and propidium iodide revealed that the cold-induced population heterogeneity could partly be attributed to the loss of membrane integrity of cells.

**Salt stress adaptation mechanisms.** To further elucidate adaptive salt stress mechanisms at molecular level, the genome-wide transcriptome profiles of mildly and severely salt-stressed *B. cereus* ATCC 14579 cells were examined using DNA microarrays. The comparison of the transcriptome profiles of mildly and severely salt-stressed cells provided insight into general salt stress responses and concentration-dependent responses. These results are described in Chapter 6. The transcriptome response of mildly salt-stressed cells displayed a large overlap with that of severely salt-stressed cells and this general salt stress transcriptome response included upregulation of osmoprotectant transporters, Na⁺/H⁺ transporters and di- and tripeptide transporters, the general stress response regulator σ^B and members of its regulon, and activation of oxidative stress-related responses. The importance of osmoprotectants in maintaining cellular turgor in response to osmotic upshift has also been demonstrated for other Gram-positive and Gram-negative bacteria (24, 38, 51), and our transcriptome data suggested that *B. cereus* mainly depends on importing these compounds via transporters rather than synthesizing those in nutrient-rich, high-osmotic medium.
The upregulation of sigB and $\sigma^B$-dependent genes during both mild and severe salt stress exposure and the salt stress-induced translation of $\sigma^B$ in *B. cereus* ATCC 14579 (55, Chapter 7), pointed to a possible role for $\sigma^B$ during salt stress exposure. However, the growth kinetics of exponentially growing cells of the *B. cereus* ATCC 14579 sigB deletion mutant (55) and the rsbY deletion mutant (56) after addition of salt stress were comparable to the parental strain. The $\sigma^B$-regulon size of *B. cereus* ATCC 14579 appeared to be much smaller than that of other Gram-positive species, such as *Bacillus subtilis* and *L. monocytogenes* (57), and does not include genes encoding osmoprotectant transporters (18, 57). Additionally, numerous genes in the *B. cereus* ATCC 14579 $\sigma^B$-regulon have unknown functions (18, 57). Therefore, the functional role of $\sigma^B$ in salt stress adaptation of *B. cereus* remains to be elucidated.

Activation of oxidative stress-related responses upon mild and severe salt stress exposure included upregulation of genes encoding catalases and resulted in increased catalase activity in salt-adapted cells. Likely, this played a role in the observed enhanced resistance towards H$_2$O$_2$-challenge stress for salt-adapted cells. Activation of oxidative stress-related responses upon salt stress exposure also may have contributed to the salt-induced protective effects towards other severe challenge-stresses, such as heat (Chapter 2, 3, 6 and 7), based on the observation that aerobic heat stress imposed an oxidative stress burden in *B. cereus* cells (43). Moreover, complementary adaptation mechanisms can be involved in salt-induced cross-protection towards heat challenge-stress. It was demonstrated that osmoprotectants (e.g. glycine betaine) can serve as thermoprotectants (30), and that some proteins induced upon heat shock were also induced after salinity up-shift (47).

Exposure to severe salt stress resulted in transcription of an additional set of genes that were not differentially transcribed during mild salt stress exposure. A temporal shift was observed between the transcriptome response upon severe salt stress treatment and several corresponding phenotypes. After resumption of growth during severe salt stress exposure, the cells showed cellular filamentation, reduced chemotaxis performance and increased catalase activity, which corresponded to the transcriptome response already displayed during the initial lag period. This underpinned that adaptation to severe stress conditions is not an instantaneous process and development of corresponding phenotypes takes (adaptation) time. The use of varying time frames for adaptive stress exposure, allowed for an adequate linkage of observed transcriptome responses to phenotypic behavior of cells, and therefore contributed to a better understanding of stress adaptation mechanisms.


**Biomarkers for adaptation-stress induced robustness.** The ability of *B. cereus* to activate stress adaptation mechanisms providing cell robustness to harsher stress conditions including stresses other than the one that induced the adaptive stress response (7, 8, 47, and Chapter 2, 3, 6 and 7) challenges the prediction of its stress adaptive behavior. The availability of genome-wide transcriptome profiles of *B. cereus* in response to various adaptation-stress conditions – mild heat, acid, salt and oxidative stress (14, 44, 57, Chapter 6) – opened avenues to unbiasedly search for general adaptation-stress indicators that could function as biomarkers for stress adaptive behavior. The results of our systematic search for these biomarkers are described in Chapter 7. The comparison of the adaptation-stress induced transcriptome profiles revealed a limited number of genes that were differentially transcribed upon exposure to all those adaptation-stresses. This transcription signature of stress adaptation seemed to be adaptation-stress independent and directed to potential biomarkers for stress adaptation. Several candidate-biomarkers were selected which have known canonical roles in stress responses, namely the transcriptional regulator $\sigma^B$ (activating general stress responses), catalases (removing reactive oxygen species), and chaperones and proteases (maintaining protein quality). These candidate-biomarkers were quantitatively measured at transcript, protein and/or activity level upon exposure to mild heat, acid, salt and oxidative stress for various adaptation time intervals. Adaptation stress-treated cells were also exposed to challenge-stress conditions (severe heat, acid and oxidative stress) to quantify their robustness level following adaptation-stress pretreatment.

To assess whether the selected candidate-biomarkers – the proteins SigB, ClpC and ClpP, the transcripts *sigB*, *clpB*, *clpC*, *clpP*, *catA* and *catE*, and catalase enzyme activity – could indeed predict the robustness level of adaptation-stress pretreated cells and therefore could function as biomarkers, they were correlated to adaptation-stress induced robustness towards heat, acid and oxidative challenge-stress. A decision flow chart was designed to systematically evaluate the predictive potential of the candidate-biomarkers for each adaptation-stress and challenge-stress pair. Most of the selected candidate-biomarkers were suitable to predict the robustness level of adaptation-stress pretreated cells, but their predictive potential was determined by the type of adaptation-stress and challenge-stress. The SigB protein was identified as biomarker for heat challenge-stress robustness because it was suitable to predict the heat challenge-stress robustness level of mild heat and salt stress pretreated cells. Also the ClpC and ClpP proteins functioned as biomarker for heat challenge-stress robustness but their predictive potential was related to mild heat and acid stress pretreated cells. The predictive potential of the transcripts *clpC* and *clpP* was comparable and both emerged as biomarkers for acid challenge-stress robustness following
mild acid and salt stress pretreatment. Catalase activity was suitable to predict the robustness level towards oxidative challenge-stress upon pretreatment to mild heat, acid and oxidative stress, and it was the only biomarker that could predict the robustness level of cells towards a challenge-stress upon pretreatment with three types of adaptation-stresses, underlining its significant predictive potential for oxidative challenge-stress robustness.

General stress response regulators and cellular mechanisms involved in controlling protein quality and defending against oxidative stresses, have known crucial roles in adaptive stress responses in other bacterial species than *B. cereus*, including *B. subtilis*, *L. monocytogenes* and *Escherichia coli* (3, 48, 54), and also in yeast in response to a wide variety of environmental changes (13, 21). The canonical roles of these cellular components in adaptive stress responses suggest that they may contribute to (cross-)protective phenomena. Moreover, these cellular components may also have predictive potential in other microorganisms, but differences in functional conservation of these stress-related factors between species and genera will challenge our search for promising biomarkers. Clp proteases, chaperones and cellular systems involved in oxidative stress defense are widely conserved with variation in regulation mechanisms of expression control (20, 31), and are promising candidate-biomarkers in other organisms than *B. cereus*. The role and regulation of $\sigma^B$ in *B. cereus* differs from that of closely related food-borne pathogens such as *L. monocytogenes* (27). Various $\sigma^B$-dependent genes identified so far in *L. monocytogenes* have known functions in acid stress responses (glutamate decarboxylase system [25, 37, 58]), osmotic stress responses (di/tripeptide- and osmoprotectant-transporters [25, 37]), and protein quality maintenance (proteases [15, 58]), and this may support a promising role for $\sigma^B$ in prediction of stress adaptive behavior in this food-borne pathogen. In contrast, the low GC Gram-positive lactic acid bacteria lack $\sigma^B$ (29, 53), and regulation of general stress responses involves other regulatory systems and possibly interesting biomarker candidates. CovRS- and Rgg-like regulators has been shown to influence transcription of general stress response proteins including chaperones, proteases and oxidoreductases in streptococci species (16, 22), and CtsR and/or HrcA have known regulatory functions in expression control of chaperones and proteins in various lactic acid bacteria (20) and also in bifidobacteria (60). These regulatory systems function mainly as repressors and have autoregulatory properties, which makes it intricate to hypothesize their potential predictive roles in stress adaptation. RpoS ($\sigma^s$) is the master regulator of the general stress response in Gram-negative enteric bacteria including *E. coli* and *Salmonella* (1) and is involved in stress adaptation (19), advocating biomarker potential for this general stress response regulator. We demonstrated the necessity to measure promising candidate-biomarkers at
different functional cell levels (transcript, protein and activity level) because the predictive potential of transcripts differed from that of proteins and activity level. Our study provides a systematic, quantitative approach to search for biomarkers for stress adaptive behavior and to statistically evaluate the predictive potential of candidate-biomarkers at different functional cell levels in order to select promising biomarkers that can serve to early detect and predict adaptive traits.

Conclusions and future perspectives. The results described in this thesis provide quantitative insights in the adaptive stress responses of *B. cereus* at population, individual cell and molecular level. It shows the potential of quantitatively correlating findings at molecular and individual cell level to phenotypic behavior of the population as a whole for elucidating microbial adaptive traits. We quantitatively evaluated the impact of adaptive stress responses on growth and inactivation kinetics, and provided quantitative information for exposure assessment studies that focus on this food-borne pathogen. Quantitative microbiological risk assessment (QMRA) is increasingly used as a tool to evaluate food-borne related health risk (10, 41), and the exposure assessment part of a QMRA focuses on the estimation of the number of cells of the hazard present in the food at the moment of consumption. *B. cereus* is commonly recognized as a hazard in mildly processed products, such as ready-to-use foods (11, 59), and these products often rely on refrigeration to control bacterial growth. The final contamination level of these foods is affected by the initial contamination level of the product, the ability of the pathogen to grow, and the reduction of the pathogen before consumption during for example reheating of the food. Outgrowth of psychrotolerant *B. cereus* strains will not be prevented by refrigeration of foods and some psychrotolerant strains were associated with *B. cereus* food-borne poisoning cases (23). The prevalence of mesophilic strains in ready-to-eat foods is higher than that of psychrotolerant strains (59) and their growth potential is influenced by the reliability of the chilled chain conditions and the domestic refrigerator temperature (12, 35, 45). Most outbreaks of food-borne *B. cereus* poisoning have been caused by mesophilic strains that can grow at temperatures as low as 10°C (2, 23), pointing to the importance of appropriate refrigeration of foods contaminated with *B. cereus* to control its growth and toxin production.

The heterogeneity in growth performance of *B. cereus* during exposure to a single mild stress condition was demonstrated to be rather limited (Chapter 4 and 5). A Dutch survey showed that the contamination level of ready-to-use products varied from 1-2 log_{10} CFU g\(^{-1}\) to 5-6 log_{10} CFU g\(^{-1}\) (59). When foods are contaminated with these *B. cereus* cell levels, then the observed population heterogeneity will not largely influence the variability
of outgrowth. However, the hurdle technology makes use of multiple mild stress factors, and this can result in more variability in outgrowth. The direct-imaging-based Anopore technology provides prospects for quantifying population heterogeneity when various mild stress conditions are applied successively or simultaneously and it opens up the way to get more insight in the impact of combined stress conditions on physiological characteristics of individual cells. Moreover, this technology also provides openings to investigate in vivo gene expression profiles of specific genes of interest in individual cells during stress exposure in reporter gene fusions studies, allowing to indirectly measure promoter activity during stress exposure. These individual cell-based approaches will contribute to a better understanding of stress adaptation mechanisms and will provide insights in the origins of population heterogeneity.

The results described in Chapter 2, 3, 6 and 7 demonstrated that adaptive stress responses have a significant impact on inactivation kinetics, and these phenomena are influenced by the adaptation-stress concentration, growth phase, strain diversity, culturing temperature and population heterogeneity. Storage of foods at abusive refrigeration temperatures allows both psychrotolerant and mesophilic cells to continue growing, but we demonstrated that the salt-induced heat resistance enhancement was not significant for both mesophilic strains tested when they were cultured at abusive refrigeration temperature, in contrast to the psychrotolerant strain. Our study provides a quantitative estimation of the impact of adaptive salt stress responses on heat resistance because (third-)decimal reductions times of unstressed cells were compared to those of adaptation-stress pretreated cells. These kinetic values should not be considered as exact values, but can give directions for the order of magnitude of the impact of stress adaptive behavior on cell resistance. Further investigation along this line for a wider spectrum of mesophilic and psychrotolerant strains would substantiate the observed trend of differences between mesophilic and psychrotolerant strains. This will provide valuable information for microbiological exposure assessment studies that describe the changes of microbiological hazards along the processing steps in the food chain and that are focusing on specific processes where these quantified aspects are of relevance.

The drive to use more mechanism-based approaches for designing and applying preservation hurdles in minimally processed foods, urged the search for biomarkers to early detect and predict stress adaptive behavior. Prediction of phenotypic behavior using cellular indicators is a key area of research (9, 40, 53), and quantitatively correlating microbial responses at molecular and phenotypic level can provide mechanistic understanding of stress adaptive behavior and leads for identifying cellular indicators for bacterial
performance. In this thesis, we quantitatively correlated induction profiles of cellular components to phenotypic data for elucidating stress adaptation mechanisms and we were able to identify biomarkers for stress adaptive behavior that resulted in enhanced robustness. We demonstrated that some cellular components, such as the SigB protein and catalase activity, were suitable to predict the robustness level towards challenge-stress of adaptation-stress pretreated cells. If a correlation is significant for a single combination of adaptation-stress and challenge-stress, this is no proof of a mechanistic relation, but if this correlation holds for various conditions, the evidence becomes stronger, and this could indeed be demonstrated for these cellular indicators. Both SigB and catalase activity could predict the robustness enhancement towards a specific challenge-stress originating from multiple adaptation-stress pretreatments, and therefore emerged as convergent-application biomarkers. In addition, both biomarkers were also suitable to predict the robustness enhancement towards multiple challenge-stresses upon pretreatment to a specific adaptation-stress and could therefore also be employed as divergent-application biomarkers. Further investigation along these lines by varying adaptation-stress concentrations will reveal how vigorous these quantified correlations are for \textit{B. cereus}, and is required to understand the limits of reliability of the predictive potential of biomarkers. Moreover, it will strengthen our insights whether extrapolation of results obtained with one set of environmental conditions can be extrapolated to another set of environmental conditions and can support convergent and divergent predictive potential of biomarkers. Robust biomarkers will be of indisputable significance for developing tools to screen for resistant or sensitive cells and will complement an empirical approach of optimally applying successively or simultaneously various mild preservation hurdles.

Adaptive stress responses are crucial defense strategies in living cells, and the identified biomarkers are among the most consistently induced components in microbial stress responses and ubiquitous in biology. Therefore, extrapolation to other eukaryotic and prokaryotic cell types including pathogenic and non-pathogenic microorganisms is plausible and merits further studies. Our study provides a framework to systematically search and evaluate predictive potential of candidate-biomarkers at different functional cell levels. When (certain levels of) extrapolation to other microorganisms than \textit{B. cereus} is substantiated, then the development of mechanism-based predictive models that are able to predict microbial kinetics by monitoring cellular indicators may be within our reach. These mechanism-based predictive models will contribute to design effective, intelligent mild preservation strategies to come to a proper, optimized balance between food safety and food quality.
References


Samenvatting


In het eerste deel van het onderzoek is de hitteresistentie van twee mesofiele *B. cereus* stammen na blootstelling aan zout onderzocht. Blootstelling aan lage en hogere concentraties zout resulteerde in verhoogde hitteresistentie van exponentieel groeiende cellen, en dit adaptatiedrag is in detail gekwantificeerd met behulp van kinetische modellen in hoofdstuk 2. Verschillende inactivatiemodellen zijn getoetst om zo het meest geschikte inactivatiemodel te kunnen selecteren om de hitte-inactivatie kinetiek van niet gestresseerde bacteriën en zout-geadapteerde bacteriën kwantitatief te beschrijven. De verkregen hitte-inactivatie kinetiekinparameters zijn vervolgens gebruikt om het effect van zoutblootstelling op hitteresistentie te kwantificeren.

Omdat mild geconserveerde producten vaak in de koelkast bewaard moeten worden, is de invloed van lage temperatuur op zoutadaptatie gekwantificeerd en beschreven in hoofdstuk 3. De temperatuur tijdens blootstelling aan zout bleek de invloed van grote invloed te zijn op de mate van zoutadaptatie en daaropvolgende hitteresistentie. Wanneer de temperatuur tijdens zoutblootstelling verlaagd werd, veranderde de hitteresistentie van beide mesofiele stammen niet, ondanks de voorafgaande blootstelling aan zout. Dit in tegenstelling tot de psychrotolerante stam waarbij juist de combinatie van lage temperatuur en blootstelling aan zout resulteerde in verhoogde hitteresistentie.

Uit de resultaten die beschreven zijn in de hoofdstukken 2 en 3 bleek ook dat het adaptatiedrag van individuele bacteriën binnen een grote populatie niet voor alle individuele bacteriën hetzelfde behoeft te zijn. Deze heterogeniteit in adaptatie is daarom
nader onderzocht in hoofdstuk 4 en 5. De groei van een groot aantal individuele cellen werd gevolgd tot aan de microkolonie-fase waardoor de heterogeniteit binnen een populatie kon worden gekwantificeerd. Naast blootstelling aan een hoge zoutconcentratie (hoofdstuk 4), bleek ook blootstelling aan lage temperatuur te resulteren in een subpopulatie van cellen die niet uitgroeide tot een microkolonie (hoofdstuk 5). Het kleuren van cellen met de fluorescerende verbinding propidium-iodide wees uit dat de populatieheterogeniteit bij lage temperatuur deels werd veroorzaakt doordat het celmembraan beschadigd was.


blootstelling. Vervolgens is de mate van verhoogde resistentie tijdens letale stress-blootstelling door voorafgaande milde stress-blootstelling bepaald, en vergeleken met de mate van inductie van de kandidaat-biomarkers tijdens milde stress-blootstelling. Hieruit bleek dat de gemeten cellulaire niveaus van een aantal kandidaat-biomarkers zeer goed overeen kwamen met het resistentieniveau van milde stress-geadapteerde cellen. Deze biomarkers zouden daarom gebruikt kunnen worden om het adaptatiegedrag, dat leidt tot verhoogde resistentie, in een vroeg stadium te kunnen aantonen en te voorspellen. Daarnaast bleek dat het belangrijk is om potentiële biomarkers op verschillende functionele niveaus in de cel te meten, omdat de voorspellende waarde van RNA transcripten verschilde met die van eiwitten en het activiteitsniveau van enzymen.

Stress-adaptatie is van wezenlijk belang voor levende cellen, omdat het vitaliteit waarborgt in een veranderende omgeving. De geïdentificeerde biomarkers staan bekend om hun cruciale rol hierin en het is aannemelijk dat soortgelijke biomarkers in andere micro-organismen ook een functie zouden kunnen hebben in het voorspellen van stress-adaptatiegedrag. De aanpak die in dit proefschrift beschreven is, biedt mogelijkheden om op zoek te gaan naar kandidaat-biomarkers in andere pathogenen dan *B. cereus*, en daarnaast, om de voorspellende waarde van deze kandidaat-biomarkers op verschillende functionele niveaus in de cel te evalueren. Stress-adaptatiegedrag dat leidt tot verhoogde resistentie kan voor fermentatie-organismen en probiotica juist gewenst zijn en biomarkers die het resistentieniveau voorspellen kunnen helpen om stammen te selecteren en condities te kiezen die het gewenste resistentieniveau verhogen. Omdat in dit proefschrift het stress-adaptatiegedrag op moleculair niveau kwantitatief gecorreleerd is aan fenotypisch gedrag, is het een eerste én duidelijk stap op weg naar het voorspellen van bacterieel gedrag middels het meten van moleculaire indicatoren en zou kunnen leiden tot het ontwikkelen van modellen die dit gedrag voorspellen. Voorspelling modellen die gebaseerd zijn op mechanistische kennis van de bacteriële cel dragen bij om milde conserveringsmethoden verder te ontwikkelen en zo de balans tussen veiligheid en kwaliteit van voedingsproducten te optimaliseren.
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Heidy
List of publications


Zwietering, M. H., H. M. W. den Besten (2010). Modelling: One word for many activities and uses. Accepted for publication in Food Microbiol.


VLAG graduate school activities

**Discipline specific activities**

*Courses*
- Reaction kinetics in food science, VLAG, Wageningen (2004)
- Food fermentation, VLAG, Wageningen (2008)

*Meetings*
- Seminar Safe Consortium, Brussels, Belgium (2006, oral presentation)
- FoodMicro 2006, Bologna, Italy (2006, poster presentation)
- NVvM meetings, Papendal (2006, poster presentation; 2007 and 2009, oral presentation)
- 5th ICPMF, Athens, Greece (2007, two oral presentations)
- FoodMicro 2008, Aberdeen, United Kingdom (2008, two oral presentations)
- 6th ICPMF, Washington, USA (2009, oral presentation)

**General courses**
- PhD week, VLAG, Bilthoven (2004)
- Scientific writing, WGS, Wageningen (2006)
- Writing grant proposals, WGS, Wageningen (2008)

**Other activities**
- Preparation of PhD research proposal (2004)
- WCFS / TIFN C009 Project meetings
- Program 3 WE-days (2004-2008; 2006, organization social program)
- Work discussion-meetings, Laboratory of Food Microbiology (2004-2009)
- VLAG PhD study trip Laboratory of Food Microbiology, South Africa (2005, organization)
- VLAG PhD study trip Laboratory of Food Microbiology, Canada (2008)
- Lecturer and supervisor of practical courses of the MSc and BSc Food Microbiology programs of Wageningen University (2004-2009)
- Member VLAG PhD council (2004-2008)