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Identification of mRNA as biomarker of *Bacillus weihenstephanensis* acid resistance: toward the integration of Omic data into predictive microbiology

Noémie Desriac

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présentée par

Noémie DESRIAC

Préparée à ADRIA Développement et au
Laboratoire Universitaire de Biodiversité et
d'Ecologie Microbienne

Identification d'ARNm
en tant que biomarqueurs
de résistance acide
chez *Bacillus
weihenstephanensis* :
vers une intégration de
données transcriptomiques
dans la prévision du
comportement bactérien

Thèse soutenue le 04 Juillet 2013

devant le jury composé de :

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A ma fille, tendrement

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Chapter 1

General introduction and thesis outline

Predictive microbiology

The microbiological safety of food is of fundamental importance to all companies involved in the production, processing, distribution, retail and regulation of foods and drinks. Indeed, incidences of bacterial contamination of food have huge consequences resulting in illnesses, deaths, and loss of production, public confidence in the food industry (Harris, 1997). Furthermore, the costs of food borne illness include the burden of medical treatment and industry losses (Harris, 1997).

Good Hygienic Practice (GHP) and the implementation of Hazard Analysis Critical Control Point (HACCP) system are the main measures used to ensure food safety. This preventive approach is a base of the European food law ensuring the protection of the public health. Supporting the GHP and the HACCP system, the regulation EC n°2073/2005 of the European parliament lays down the microbiological criteria for foodstuffs. Although these tools play key roles, a range of other objective control measures and risk assessment procedures are steadily being adapted to varying degrees by both governmental and industry (Basset *et al.*, 2012). Indeed, approach to estimate risk caused by pathogenic microorganisms in the food chain sits within the framework of Microbiological Risk Assessment (MRA) as described by the Codex Alimentarius Commission (Codex, 1999).

MRA is a scientific process in which the hazards and risk factors are identified, and the risk profile is determined. By condensing the scientific data to an assessment of the human health risk related to the specific food borne hazard, this process is now in use to establish national and international food safety objectives. The MRA is usually seen as particularly appropriate to governmental food safety organizations and the related decision-making tools. But this could be as well of relevance to numerous industrial applications, such as shelf-life determination, heat treatment optimisation, raw material selection, assessment of non thermal inactivation processes and new formulation development (European regulation, EC2073, 2005). MRA has thus emerged as a comprehensive and systematic approach for addressing the risk for the consumer health, but could be as well adapted to microbial spoilage events in food products and for the optimisation of processes to limit alteration risks (Rigaux, 2013).

Predictive microbiology is increasingly used as a tool for Microbiological Risk Assessment (MRA). Over the last decades, this research has gained more and more interest as shown by the increasing number of articles published with the keyword “predictive microbiology” since 1960 (Figure 1). Predictive microbiology is a research area in which microbial and mathematical knowledge is combined for the development of mathematical models that describe microbial evolution in foods. The basic idea underlying predictive microbiology is that the behaviour of microorganisms is

deterministic and able to be predicted from knowledge of the microorganism itself, and the immediate environment of the microorganism. Thus a detailed knowledge of microbial response to environmental conditions enables objectives evaluation of the effect of processing, distribution and storage operations on the microbiological safety and quality of foods by distilling the microbial behaviour knowledge into mathematical models.

Early modeling studies mostly concerned thermal inactivation of pathogenic bacteria (Esty and Meyer, 1922), but latter modeling studies dealt also with descriptions of the effect of constraints on microbial growth and most often describing the effect of temperature as a sole or the main controlling factor. Today, predictive models have been developed for both spoilage and pathogenic organisms and many models are currently available in the literature and can be used to quantitatively describe observations and translate observations into kinetic parameters (McKellar and Lu, 2004; Brul *et al.*, 2007). Nevertheless these food predictive microbiology models have been mainly developed using culture based methods and worst case *scenari* to ensure food safety. However, it is now commonly recognized that this yields to over estimations in microbial quantitative risk assessment, and could be improved by integrating the bacterial adaptive responses implemented upon a variety of stresses through the integration of the associated physiological mechanisms.

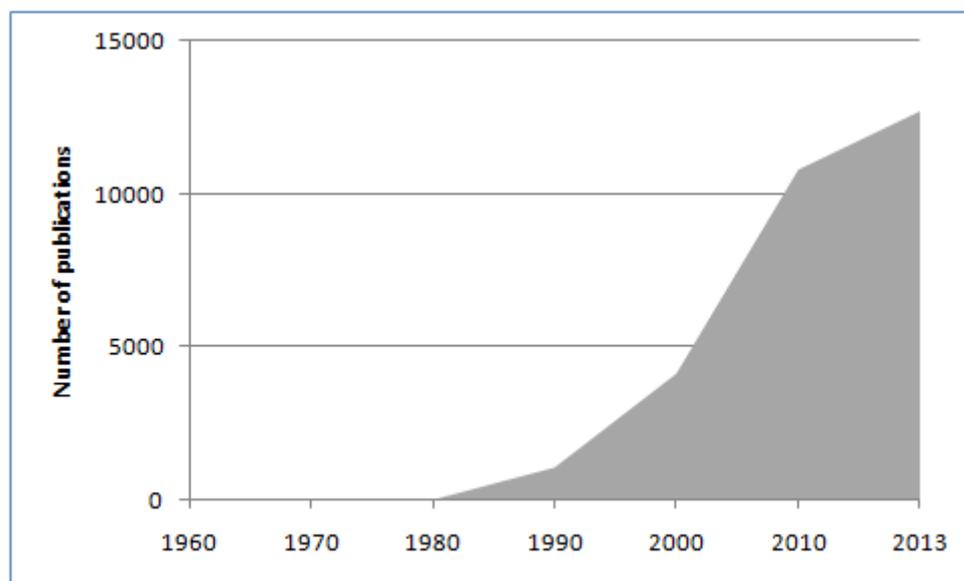


Figure 1 : Growth over the past fifty years in the number of publications citing “predictive microbiology” as keywords. Source: PubMed March 2013.

The adaptive stress response includes mechanisms developed by the bacteria to adapt and survive to dynamic environmental conditions. Indeed when exposed to changing environments microorganisms have developed stress adaptation strategies that trigger cascade of cellular events and regulatory networks yielding the induction of cell resistance to harsher stress conditions (Mitchell *et al.*, 2009;

Wolf *et al.* 2008). Such adaptive stress response and associated cross-protection toward otherwise lethal stresses, is a crucial survival strategy for a wide spectrum of microorganisms, including food spoilage bacteria, pathogens and technological interest microorganisms. Thus, this phenomenon appears of practical importance for food industry particularly with the increasing trend in the production and sales of mildly processed foods (Carlin *et al.*, 2000; Peck *et al.*, 2008). Indeed, the microbial safety and stability of these food products are based on simultaneous or successive application of mild preservation factors, known as hurdles (Leistner and Gorris, 1995). While individual hurdle 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 qualitative properties loss of minimally processed food. Among more than 60 preservative hurdles, the most used in food preservation are temperature, water activity, acidity, redox potential, preservative such as nitrite or sorbate, and competitive microorganisms (Leistner, 2000).

'Omic' technologies

In the last decade, omics approaches have influenced tremendously the way of studying the biology of microorganisms. Omics technologies comprise high-throughput techniques directed to understand the cell metabolism as one integrated system, rather than merely a collection of independent parts, by using information about the relationships between different molecules (Zhang *et al.*, 2010). These include genome-wide sequencing tool, genome-wide transcript and protein analysis and assessment of the metabolic profile of microorganisms and generate a deluge of data. The most comprehensive analyses are those at the genome sequence level (Brul *et al.*, 2012) but, while genomes are fixed except for mutations encountered during DNA replication and sequence deletion/insertion events, transcriptomes, proteomes and metabolomes vary in response to environmental conditions. Jointly with the development of bioinformatics, omics technologies are expected to play an important role in understanding how bacteria can respond and survive to stressing conditions, yet the translation of 'omic' data to bacterial behaviour cells remains a significant challenge (Brul *et al.*, 2012). Indeed, one of the major challenges will be to increment omics generated data in decision making tools developed to support food safety and quality issues (Brul *et al.*, 2012; Havelaar *et al.*, 2010, McMeekin *et al.*, 2008; Ranstiou *et al.* 2011). Up to know two main different approaches are known with the identification and quantification of (i) signalling and metabolic pathway with flux balance analysis (Kauffman *et al.*, 2003; Metris *et al.*, 2011), or (ii) biomarkers (den Besten *et al.*, 2010; Kort *et al.*, 2008).

Any anatomic or physiologic finding such as proteins, genes, metabolites or minerals, could be considered as biomarker provided there is a validated link between the biomarker and a relevant physiologic, toxicologic, pharmacologic, or clinical outcome. The official US National Institute of Health definition of a biomarker is “a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (Atkinson *et al.*; 2001). This definition commonly recognized could be adapted for food processes and safety issues as: a characteristic that is objectively measured and evaluated as an indicator of bacterial responses to food processes and stress conditions. Advances in the knowledge of model organisms paved the way for the identification of potential cellular biomarkers involved in bacterial survival, virulence or stress resistance. This is for instance the case for well-known food-borne pathogens and spoilers, among which *B. cereus* group is one representative. In 2010, den Besten *et al.* described a strategy to identify biomarkers for cell robustness. Shortly, both unstressed and mild stress treated cells were exposed to lethal stress conditions (severe heat, acid and oxidative stress) to quantify the robustness advantage provided by mild stress pre-treatment. Robustness was defined by the ratio of $\log_{10}N(t)/\log_{10}N_0$, with $N(t)$ being the survivors quantified on agar plate at a defined time and N_0 the inoculum (unstressed cells) used for inactivation treatment. Based on available Omic data, this framework enabled the identification of candidate biomarkers, *i.e.* enzymatic activity, protein levels and genes expression RT-qPCR quantifications. Linear correlations between induced biomarker and induced robustness (Figure 2) upon exposure to mild stress, revealed three kinds of biomarkers defined as “no-response biomarker”, “short-term biomarker” and “long-term biomarkers”.

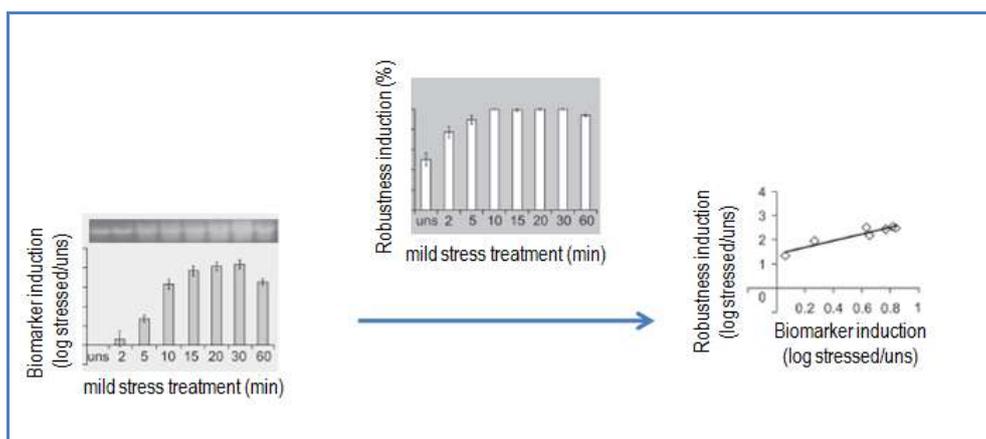


Figure 2: Conceptual scheme for measuring and correlating mild stress induced biomarker and robustness responses (adapted from Abee *et al.*, 2011; den Besten *et al.*, 2010). To evaluate whether the candidate biomarker could predict the robustness level of mild stress pre-treated cells, the mild stress induced biomarker and robustness responses were correlated to evaluate the significance of their linear correlation, which was done using a Pearson test. Given stressed conditions was relative to the associated unstressed sample (uns).

In the opposite to the other omics data, mRNA gene expressions could be easily quantified and key mRNAs variation with time and exposure to distinct stresses could be routinely analyzed. Indeed, even if transcripts do not reflect the whole story of the cell fitness, reverse transcription quantitative PCR (RT-qPCR) is considered as the gold standard for accurate, sensitive and fast measurement of gene expression patterns (Nolan *et al.*, 2006). Over the last ten years, many qPCR-based methods have been developed to detect, identify and/or quantify pathogens in food (Postollec *et al.*, 2011). Application of RT-qPCR to study population dynamics and activities through quantification of gene expression are already available and appear as a new trend in bacterial behaviour descriptions and associated metabolic activities (Carey *et al.*, 2009; Falentin *et al.*, 2010; Hierro *et al.*, 2006; Juste *et al.*, 2013; Senouci-Rezkallah *et al.*, 2010; Torriani *et al.*, 2008; Ulve *et al.*, 2008). However it is important to bear in mind that while RT-qPCR offers many practicability and sensitivity advantages, numerous quality controls shall be checked all among the entire workflow, in order to confirm the robustness of the quantitative results (Bustin *et al.*, 2009; Bustin *et al.*, 2010; Derveaux *et al.*, 2010).

Bacillus cereus group

The *Bacillus cereus sensu lato* complex, also known as the *B. cereus* group, is a subdivision of the *Bacillus* genus which includes rod-shaped, aerobic or facultative anaerobic organisms that can form endospores. The genus *Bacillus*, belonging to the phylum Firmicutes, comprises a diverse group of Gram-positive and Gram-variable bacteria. The *B. cereus* group comprises six species: *B. cereus sensu stricto* and *B. anthracis*, known as human pathogen, *B. thuringiensis* used as biopesticide, *B. mycoides* and *B. pseudomycoides* which are characterized by rhizoidal colonies on agar medium and *B. weihenstephanensis* psychrotolerant strains (Lechner *et al.*, 1998; Nakamura and Jackson, 1995; Nakamura, 1998). Phase-contrast image of exponentially growing vegetative cells, sporulating cells and spores of *B. weihenstephanensis* KBAB4 are shown in Figure 3. The species in the *B. cereus* group are genetically closely related (Ash *et al.*, 1991) and shared specific genetic elements not present in other *Bacillus* species which do not show any pattern, either in their sequences or genomic locations, which allows to differentiate between the member species of the group (Tourasse *et al.*, 2006). Despite their close relatedness, the different species within the *B. cereus* group have very different phenotypic characteristics and medical relevancies (Jensen *et al.*, 2003; Ehling-Schulz *et al.*, 2006; Andrup *et al.*, 2008). Based on both genotypic and phenotypic aspects, Guinebretière *et al.* (2008) proposed to classify *B. cereus* group species into seven groups and provided evidence for a multiemergence of psychrotolerance in this group. Furthermore this classification appeared are more suitable than species affiliation for determining potential virulence (Guinebretière *et al.*, 2010).

Spores and vegetative cells of *B. cereus* are widely encountered in environment such as in soil (von Stetten *et al.*, 1999), considered as its natural habitats, rhizosphere (Berg *et al.*, 2005), animal such as insect (Luxananil *et al.*, 2001) or mammals (Stenfors-Arnesen *et al.*, 2008). Thus *B. cereus* can contaminate raw foods such as milk (Larsen and Jorgensen, 1997; Bartoszewicz *et al.*, 2008), rice (Ankolekar *et al.*, 2009), meat (Smith *et al.*, 2004) but also in processed foods, such as dried milk (Reyes *et al.*, 2007), cheese (Pirttijarvi *et al.*, 1998) or rice and pasta (Mortimer and McCann, 1974; Dierick *et al.*, 2005; Delbrassine *et al.*, 2011), and ready-to-eat food (Wijnands *et al.*, 2005). Furthermore it has been demonstrated that upon ingestion of contaminated foods, *B. cereus* can enter the human gastro-intestinal tract (Turnbull and Kramer, 1985) and may cause food-borne gastro-intestinal diseases, namely, emesis and diarrhea. Nevertheless, the prevalence of *B. cereus* induced food-borne is difficult to determine, because the symptoms associated are generally mild, and thus support that many *B. cereus* infections are not reported and that the prevalence is largely underestimated (Cadel-six *et al.*, 2011; Stenfors-Arnesen *et al.*, 2007).

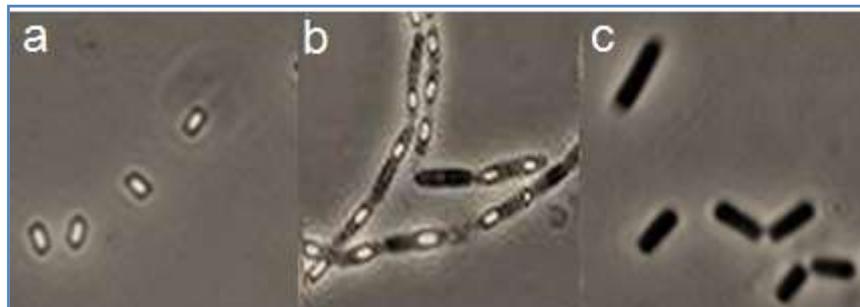


Figure 3: Vegetative cells and spores of *B. weihenstephanensis* KBAB4. Phase-contrast images of vegetative cells (a), sporulating cells (b), and spores (c, kindly provided by Eugénie Baril, LUBEM Quimper, 2011).

In France between 2006 and 2010, *B. cereus* represents, depending of the year the fourth or sixth causative agent of food poisoning outbreaks (Cadel-six *et al.*, 2011). The emetic symptoms are due to the production of cereulide, an emetic toxin produced in food or in the intestine, whereas the diarrhetic symptoms are due to the production of enterotoxins (hemolysin BL, HBL), non hemolytic enterotoxin (NHE) and CytK in the intestine, (Kotiranta *et al.*, 2000; Lund and Granum, 1997; Lund *et al.*, 2000). Up to now, *B. weihenstephanensis* has never been correlated with food-borne diseases, but some strains have been shown to be emetic (Hoton *et al.*, 2009; Thorsen *et al.*, 2006) and some others, such as the KBAB4 strain, possess genes involved in enterotoxin production (Lapidus *et al.*, 2008; Réjasse *et al.*, 2012; Stenfors *et al.*, 2002; Stenfors Arnesen *et al.*, 2011). In addition, conjugation and transfer of toxin encoding plasmids were shown to occur among strains of the *B. cereus* group (Hoffmaster *et al.*, 2004; Hoffmaster *et al.*, 2006; Van der Auwera *et al.*, 2007). Furthermore, the increasing demand for ready-to-eat foods could raise the issue associated with psychrotolerant bacteria, since they represent the most probable risk in refrigerated food poisoning (Valero *et al.*, 2007).

Besides being an important food-borne pathogen, *B. cereus* is a notorious food spoilage organism. Food spoilage is caused by the growth and associated metabolites of unwanted bacteria in food and causes enormous expenses for food industry (Gram *et al.*, 2002). *B. cereus* causes mainly spoilage of milk and dairy products (Anderson *et al.*, 1995; de Jonghe *et al.*, 2010; Meer *et al.*, 1991), thereby shortening the shelf-life of these products. Spoilage of pasteurized milk by *Bacilli* may cause defects such as off flavours and structural defects mainly due to the production of hydrolytic extracellular enzymes such as proteolytic, lipolytic and/or phospholipolytic enzymes (Meer *et al.*, 1991). The off-flavours can be bitter, putrid, rancid, fruity, yeasty or sour. Bitter flavour is caused by protease activity on the milk proteins, while rancid and fruity flavours are caused by lipases (Heyndrickx and Scheldeman, 2002). For instance, *B. cereus* produces a chymosin-like protease enzyme which is reportedly responsible for degradation of milk casein, resulting in coagulation (sweet curdling) and finally a bitter-tasting product (Frank, 1997). The production of a phospholipase C which degrades fat globule membranes, resulting in fat aggregation in cream has been also reported in *B. cereus* (Frank, 1997).

Furthermore, spores and vegetative cells can attach to processing equipment and food contacting surface yielding to biofilm formation and continuous source of (re)contamination (Wijman *et al.*, 2007). It could be also noted the importance of psychrotolerant strain of *B. cereus* which may grow at low temperature and subsequently induce food spoilage when storage temperature is abused. The spoilage effects associated with psychrotolerant *Bacilli* are quite similar to those observed for other bacteria namely off-flavours and structural defects (Heyndrickx and Scheldeman, 2002) which render food products unacceptable to the consumer from a sensory point of view (Gram *et al.*, 2002). Thus, the economic impact of *B. cereus* in food and food processing lines is substantial.

Thesis outline

The research described in this thesis was initiated to establish a transcriptomic based approach allowing the identification of *B. weihenstephanensis* resistance biomarkers. Quantification of *B. weihenstephanensis* acid resistance, by combining both predictive microbiology tool and Omic technologies, opens avenues to integrate bacterial physiology in quantitative microbial risk assessment. Indeed, the identification of biomarkers may lead to early detect and predict the acid stress resistance of *B. weihenstephanensis*, and may serve as starting point to offer new decision making tools which may help in designing and/ or optimizing safer and more environmental friendly mildly preserved foods. An overview of the research topics addressed in the thesis chapter is given in the Figure 4.

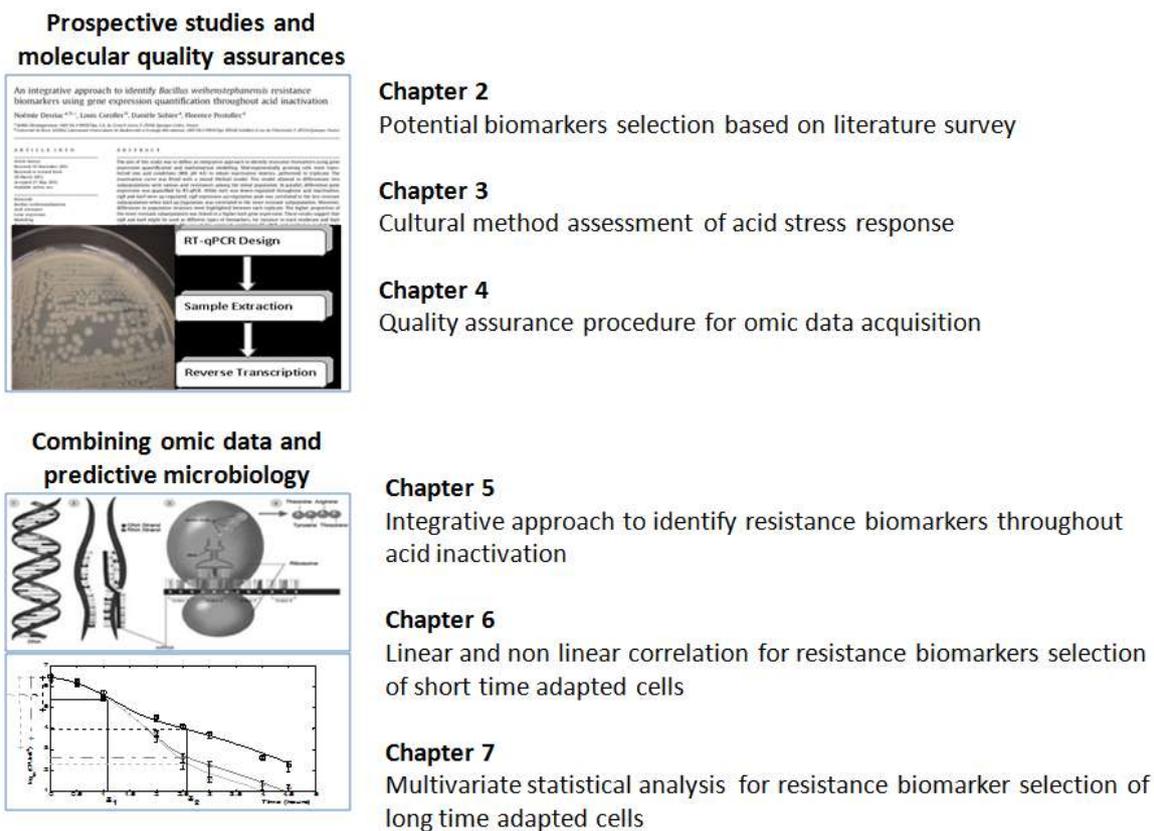


Figure 4: Overview of the research chapter addressed in this thesis.

First, a literature survey was done on the currently knowledge of *B. cereus* acid adaptive stress response (**Chapter 2**) to have a more comprehensive insight in the capabilities of *B. weihenstephanensis* to overcome acid conditions. This will allow the selection of genes which may serve as potential biomarkers. One of the most affecting factors influencing bacterial cells resistances is the microorganism physiological state.

Thus in **chapter 3**, the impact of the physiological state of vegetative cells of *B. weihenstephanensis* on the subsequent acid resistance is investigated. The impact of the stress intensity on the bacterial resistance is also investigated through the determination of the acid sensitivity of *B. weihenstephanensis*. Investigation of acid sensitivity may be of interest for the calculation of process efficiency and highlights the expected key role of biomarkers in tailor-made process optimization tools.

Thenceforth, a reverse transcription quantitative PCR (RT-qPCR) method and the necessary associated quality assurance procedure to ensure accurate and reliable analyses of gene expressions which may be further used as potential biomarkers are implemented and described in **chapter 4**.

In the **chapter 5**, an integrative approach is implied in order to identify *B. weihenstephanensis* resistance biomarkers using gene expression quantification throughout acid resistance. During food processing, bacteria may be exposed to multiple and successive hurdles, thus considering cell history

or physiological state in the search of molecular biomarkers of bacterial behaviour is crucial. To adapt the cells to new environments, either short or long time exposures are reported in the literature.

In the **chapter 6**, the bacterial behaviour under lethal acid exposure is investigated after preliminary exposure to short mild stressed conditions. Gene expressions of adapted cells are then linearly or non-linearly correlated to the subsequent acid resistance in order to find relevant acid resistance biomarkers of *B. weihenstephanensis*.

In the **chapter 7**, a multivariate statistical analysis approach is used to obtain an integrated view of the biological impact of the survival ability of *B. weihenstephanensis* cells under lethal acid conditions which are preliminary adapted by long time exposures in mild stressed conditions. This last study allows a mathematical selection of acid resistance biomarkers and shows results in how used omics data and more precisely gene expression patterns in the bacterial behaviour prediction.

Finally, **chapter 8** presents a summarizing discussion, concluding remarks and perspectives of the research described in this thesis.

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Chapter 2

***Bacillus cereus* cell response upon exposure to acid environment: towards the identification of potential biomarkers**

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Abstract

Microorganisms are able to adapt to different environments and evolve rapidly, allowing them to cope with their new environments. Such adaptive response and associated cross-protections towards other lethal stresses, is a crucial survival strategy for a wide spectrum of microorganisms, including food spoilage bacteria, pathogens and technological microflora and probiotic microflora used in functional food applications. The growing demand for minimal processed food yields to an increasing use of combination of hurdles or mild preservation factors in the food industry. A commonly used hurdle is low pH which allows the decrease in bacterial growth rate but also the inactivation of pathogens or spoilage microorganisms. *Bacillus cereus* is a well-known food-borne pathogen leading to economical and safety issues in food industry. Because survival mechanisms implemented will allow bacteria to cope with environmental changes, it is important to provide understanding of *B. cereus* stress response. Thus this review deals with the adaptive traits of *B. cereus* cells facing to acid stress conditions. The acid stress response of *B. cereus* could be divided into four groups (i) general stress response (ii) pH homeostasis, (iii) metabolic rearrangements and (iv) secondary oxidative stress response. This current knowledge may be useful to understand how *B. cereus* cells may cope to acid environment such as encountered in food products and thus to find some molecular biomarkers of the bacterial behaviour. These biomarkers could be furthermore used to develop new microbial behaviour prediction tools which can provide insights into underlying molecular physiological state that govern the behaviour of microorganisms and thus opening the avenue toward the detection of stress adaptive behaviour at an early stage and the control of stress-induced resistance throughout the food chain.

Introduction

Bacillus cereus is a Gram positive, facultative anaerobic bacterium belonging to the genus *Bacillus* which may produce endospores. The *B. cereus* group comprises seven recognized species: *B. cereus* and *B. anthracis*, known as human pathogens, *B. thuringiensis* used as biopesticide, *B. mycoides*, *B. pseudomycoides* characterized by rhizoidal formations, *B. weihenstephanensis* including psychrotolerant strains and *Bacillus cytotoxicus* which is the last identified specie (Guinebretière *et al.*, 2013). Furthermore, in 2008, Guinebretière *et al.* proposed a division of the *Bacillus cereus sensu lato* into seven major groups (I-VII) using both genetic and phenotypic criteria. Each group corresponds to different virulence potential and to specific thermotypes, showing clear differences in their ability to grow at low or high temperatures (Auger *et al.*, 2008; Guinebretière *et al.*, 2008; Lapidus *et al.*, 2008). Spores and vegetative cells of *B. cereus* are widely encountered in environment such as in soil, considered as its natural habitat, in rhizosphere (Berg *et al.*, 2005), in insects (Luxananil *et al.*, 2001) or mammals (Stenfors-Arnesen *et al.*, 2008).

B. cereus is a known food-borne human pathogen which frequently causes illnesses. It can cause two types of food poisoning (i) the diarrheic syndrome due to the production in the intestine of enterotoxins such as hemolysin BL (HBL), non hemolytic enterotoxin (NHE) and CytK, and (ii) emetic syndrome due to the production of the cereulide, an emetic toxin produced in food (Kotiranta *et al.*, 2000; Lund and Granum, 1997; Lund *et al.*, 2000). Within the seven groups of *Bacillus sensu lato* the involvement of groups II, III, IV, V and VII in food outbreaks have been reported (Cadel six *et al.*, 2012). *Bacillus cereus* is associated to a large number of food products such as rice, pasta and milk or mayonnaise-based ready-to-eat (RTE) food salad (Delbrassine *et al.*, 2011; Larsen and Jorgensen, 1999; Mortimer and McCann, 1974; Parry and Gilbert, 1980; Reyes *et al.*, 2007; Valero *et al.*, 2007). Nevertheless, the prevalence of *B. cereus* inducing food-borne diseases is difficult to determine and may be underestimated, because the symptoms associated with *B. cereus* infections or intoxications are generally mild and not always reported (Cadel-six *et al.*, 2011; Stenfors-Arnesen *et al.*, 2008). However, more severe cases which may lead to fatal cases have also been reported, demonstrating the possible severity of the emetic syndrome (Dierick *et al.*, 2005; Naranjo *et al.*, 2011; Shiota *et al.*, 2010).

B. cereus is also known for its ability to cause food spoilage which may lead to enormous expenses for food industry (Gram *et al.*, 2002). *B. cereus* causes mainly spoilage of milk and dairy products (Andersson *et al.*, 1995; de Jonghe *et al.*, 2010; Meer *et al.*, 1991;), thereby shortening the shelf-life of these products. Spoilage of pasteurized milk by *Bacilli* may cause defects such as off flavours and structural defects mainly due to the production of hydrolytic extracellular enzymes such as

proteolytic, lipolytic and/or phospholipolytic enzymes (Meer et al., 1991). The off-flavours can be bitter, putrid, rancid, fruity, yeasty or sour. Bitter flavour is caused by protease activity on the milk proteins, while rancid and fruity flavours are caused by lipases (Heyndrickx and Scheldeman, 2002). For instance, *B. cereus* produces a chymosin-like protease enzyme which is reportedly responsible for degradation of milk casein, resulting in coagulation (sweet curdling) and finally a bitter-tasting product (Frank, 1997). The production of a phospholipase C which degrades fat globule membranes, resulting in fat aggregation in cream has been also reported in *B. cereus* (Frank, 1997). To prevent growth of microorganisms, food is often processed and preserved. Usually food products were sterilized by heat, but nowadays in response to consumers demand the food industry use milder conditions for preservation (Abee and Wouters, 1999). Combinations and applications of different preservation steps, named hurdles, are frequently applied in modern food industries to ensure the microbial safety and stability and preserve the sensory and nutritional quality (Leistner and Gorris, 1995; Leistner, 2000). Mild heat treatment, low pH and cold storage are examples of hurdles used to preserve food. In order to cope with these environmental dynamics, microorganisms have developed stress adaptation strategies that lead to the possibility that bacteria overcome harsher conditions for a variety of stresses. The heat and salt stress responses of *B. cereus* have been studied (see for e.g. Browne and Dowds, 2001; den Besten *et al.*, 2007, 2009; Periago *et al.*, 2002; van Schaick *et al.*, 2004a) since a few years and more recently, the acid stress response of *B. cereus* have been investigated too (Mols *et al.*, 2010 a and b). In 2011, a minireview describing the acid stress response of both germinating spores and vegetative cells of *B. cereus* was published (Mols and Abee, 2011b). However, in food both spores and vegetative cells of *B. cereus* could be found and in order to inactivate spores in food, drastic treatments which will affect the organoleptic aspect of the food are necessary. Thus, another way to limit the prevalence of *B. cereus* in food, could be to focus on vegetative cells. Indeed, in minimally processed food, initial contaminant spores may germinate and thus, inactivating or limiting the growth of the subsequent vegetative cells with mild preservation processes will allow to minimize the prevalence of *B. cereus* in such kind of food products.

Because *B. subtilis*, used as model microorganism, and *B. cereus* possess many differences such as the genome size or the regulation and the regulon of the general stress response (Anderson *et al.*, 2005; de Been *et al.*, 2011), this review will detail the vegetative cells acid stress response of *B. cereus*. Data on the response of other Gram-positive bacteria upon exposure to low pH are available and have been reviewed by Cotter and Hill in 2003.

Here, mechanisms of acid resistance in *B. cereus* are reviewed as (i) the involvement of the general stress response in acid stress response, (ii) the pH homeostasy maintaining, (iii) metabolic

rearrangements and alkali production and (iv) the secondary oxidative stress response observed upon exposure to low pH.

General stress response

The ability of bacteria to respond rapidly to changing environmental conditions is a prerequisite for survival in their habitats. This bacterial stress response is triggered by a change in the microorganism growth conditions. Such a change triggers a cascade of events that will lead to increase stress resistance of the bacterial cell, most often against not only the stress to which it was exposed but also other stresses, thereby ensuring its survival under a variety of conditions. A common strategy that bacteria use to counter stressful conditions is to activate a specific alternative sigma factor, which leads to the transcription of a set of genes (a so-called regulon), the products of which protect the cell against adverse conditions (Kazmierka *et al.*, 2005). In several Gram-positive bacteria, the alternative sigma factor σ^B is the key sigma factor controlling the general stress response (Kazmierka *et al.*, 2005; van Schaick *et al.*, 2004b). This factor is a secondary subunit of RNA polymerase that is known to play an important role in regulating gene expression when there are major changes in the environment. Upon binding of σ^B to core of RNA polymerase, genes located downstream a promoter that can be recognized by the σ^B -RNA polymerase complex are then transcribed. The role of σ^B and its regulation has been extensively studied in the Gram-positive model-organism *Bacillus subtilis* (for a comprehensive review see Hecker *et al.*, 2007). In *B. cereus*, σ^B is activated in several stress conditions such as ethanol, NaCl exposure or H₂O₂ and acid shock. However the largest up-regulation of σ^B is observed in response to heat shock (van Schaick *et al.*, 2004a).

σ^B regulation. In all Gram-positive bacteria the activation of σ^B confers protection to cell against adverse conditions. In *B. subtilis*, σ^B activity is controlled by RsbVW partner-switching, a mechanism which is highly conserved in species that contain σ^B , including *B. cereus* strains (de Been *et al.*, 2010 and 2011). In non-stressed cells, σ^B is present in an inactive form by complexation with the anti-sigma factor RsbW. In this form, σ^B is unable to bind to RNA polymerase and thus cannot initiate the transcription of stress response genes. Under stress, an anti-sigma factor antagonist, RsbV, can bind to RsbW, thereby forming an RsbV-RsbW complex. This leads to the release of σ^B , which can then bind to RNA polymerase, leading to the transcription of σ^B -dependent genes. In addition, RsbW acts as a kinase of RsbV, thereby providing a negative feedback on σ^B activation. Under stress conditions, RsbV is dephosphorylated by one or more specific PP2C-type phosphatases, resulting in the sequestration of RsbW and the activation of σ^B . This mechanism is conserved in *Bacilli* (van Schaick *et*

al., 2005), *Staphylococcus aureus* (Palma and Cheung, 2001; Pané-Farré *et al.*, 2006; Senn *et al.*, 2005;) and *Listeria monocytogenes* (Wiedmann *et al.*, 1998; Ferreira *et al.*, 2004). However, there are considerable differences in the upstream part of the σ^B activation pathway (Ferreira *et al.*, 2004; van Schaick *et al.*, 2004a), reflecting differences in the mechanisms of stress sensing and signaling in various bacteria.

In the human pathogen *B. cereus*, the mechanism of σ^B activation has been studied only more recently (van Schaick *et al.*, 2004a and b, 2005, 2007; de Been *et al.*, 2010, 2011). It has been shown that σ^B activation is governed by a single PP2C-type phosphatase, RsbY, which carries an N-terminal REC domain (van Schaick *et al.*, 2005). The *rsbY* gene is unique to *B. cereus* and to closest relatives and is transcribed both from σ^A -dependent promoter (constitutive sigma factor) and σ^B -dependent promoter. Because, the RsbY carries an N-terminal REC domain it could be suggested that RsbY is activated through a mechanism which involves phosphorylation of a conserved aspartate residue in the REC domain by a kinase. In 2010, de Been and co-workers identified that this PP2C-type phosphatase RsbY receives its input from the multi-sensor hybrid kinase RsbK (Figure 1).

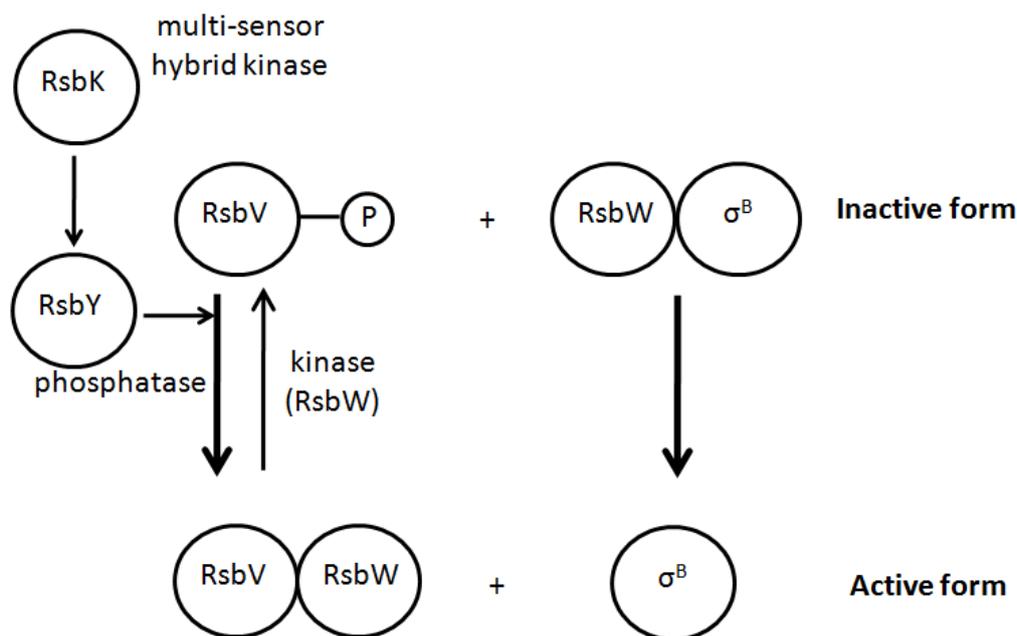


Figure 1: Schematical representation of the activation of *B. cereus* σ^B . Upon different stressing conditions, RsbK auto-phosphorylates a conserved histidine residue. The phosphoryl group is then transferred to RsbY. RsbV is then dephosphorylated by RsbY, resulting in the sequestration of RsbW and the activation of σ^B . The anti-sigma factor RsbW also acts as a kinase of RsbV, thereby providing a negative feedback on σ^B activation.

RsbK contains both sensor histidine kinase and response regulator domains, and *rsbK* gene is located close to *sigB* on the genome of *B. cereus* ATCC 14579 (Figure 2). They also realized a genome survey (de Been *et al.*, 2011) indicating that RsbK and RsbY should constitute one functional module for the control of σ^B activity in members of the *B. cereus* group, including the pathogens *B. thuringiensis*, *B. anthracis* and the psychrotolerant *B. weihenstephanensis*.

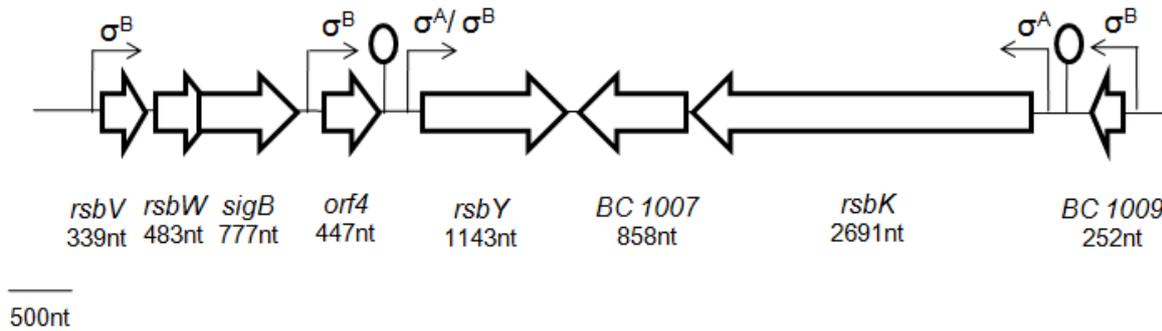


Figure 2: Overview of the genetic organization of *sigB* cluster of *Bacillus cereus* ATCC 14579. Hairpin structure of transcription termination sites is indicated as well as the location of σ^B and σ^A . *sigB* codes for the alternative sigma factor σ^B and *rsbV*, *rsbW* and *rsbY* code for its regulatory proteins. *orf4* encodes a bacterioferritin. *rsbK* encodes a hybrid kinase which may constitute with RsbY one functional module for the control of σ^B activity in members of the *B. cereus* group (de Been *et al.*, 2011).

One exception concerns the *Bacillus cytotoxicus* genome which lacks the entire SigB operon, including *sigB* gene and the primary regulatory loci that control the SigB activity, *rsbV* and *rsbW* (Lapidus *et al.*, 2008; Scott and Dyer, 2012). Orthologous RsbKY signalling modules were found in four other *bacilli* outside the *B. cereus* group. However, the RsbKY modules in these other *bacilli* strains were not connected to *sigB* in terms of genomic context (de Been *et al.*, 2010). Analysis of the transcriptional organization of the σ^B operon revealed that this operon is transcribed as a 2.1-kb transcript encompassing *rsbV*, *rsbW*, *sigB* and *orf4* (Figure 2). *orf4*, encoding a bacterioferritin (Wang *et al.*, 2009), is also under the control of an additional σ^B -dependent promoter and is a member of the σ^B regulon in *B. cereus*. The *rsbY* gene was found directly downstream of the σ^B operon in *B. cereus* group and is transcribed both from σ^B and σ^A -RNA polymerase (van Schaick *et al.*, 2004a; Wang *et al.*, 2009). Once stress condition is sensed and signalled through the regulatory cascade, the activation of σ^B as well as the transcription of the set of σ^B -regulated genes occurred allowing bacterial resistance. In 2004, van Schaik and colleagues demonstrated that upon stress conditions, the level of σ^B raised rapidly. Thereby they showed that an addition of 4% ethanol, 2.5% NaCl as well as heat (42°C) or acid shock (pH 5.2) have an impact on σ^B level. They also highlighted a limited effect of ATP depletion on σ^B level, showing that, unlike in *B. subtilis*, the σ^B response could occur solely in response to changes of environmental conditions.

σ^B regulated genes and acid stress. As mentioned, the activation of the σ^B response will allow the transcription of the set of genes encoding for proteins with specific functions, which will protect the cell against stress. The identification of the complete σ^B regulon in *B. subtilis* (Helmann *et al.*, 2001; Petershon *et al.*, 2001; Price *et al.*, 2001), *L. monocytogenes* (Kazmierczak *et al.*, 2003), and *S. aureus* (Bischoff *et al.*, 2004; Pané-Farré *et al.*, 2006) by DNA microarray technology revealed that a low number of genes have an obvious role in the stress response of these organisms and may contribute

to stress resistance. For instance, *L. monocytogenes* σ^B regulates the *gadB* gene (Kazmierczak *et al.*, 2003) that is involved in acid stress resistance (discussed in the section 3.2) and into several metabolic pathways like the amino-acids pathway. Furthermore, in both *L. monocytogenes* and *S. aureus*, some pathogenic traits seem to be governed by σ^B . While in *B. cereus* ATCC 14579 and in a *sigB* null mutant, the production of virulence factors is the same (van Schaick *et al.*, 2004a), a *B. anthracis* $\Delta sigB$ strain was affected in its virulence (Fouet *et al.*, 2000). Indeed, the injection of spores of *B. anthracis* 7702 strain or its $\Delta sigB$ derivative in 10 mice showed that the number of deaths with given doses of the $\Delta sigB$ strain were similar to those obtained with the 1 log unit lower doses of the parental strain suggesting a 1 log unit difference in the 50% lethal dose (Fouet *et al.*, 2000). These findings suggest that σ^B has evolved in response to specific niches (Ferreira *et al.*, 2004). In addition, the *B. cereus* σ^B regulon differs from *B. subtilis*, *L. monocytogenes* and *S. aureus* ones. Actually, it has been highlighted that the transcription of approximately 30 genes are σ^B -dependent in *B. cereus* ATCC 14579 (van Schaick *et al.*, 2004a and b, 2007; de Been *et al.*, 2010) among which the majority are organized in operons (Table 1) whereas around 100 genes are σ^B -dependent in *B. subtilis* and *S. aureus* (Bischoff *et al.*, 2004; Helmann *et al.*, 2001; Pané-Farré *et al.*, 2006; Petershon *et al.*, 2001). In *L. monocytogenes*, 54 genes were up-regulated in a σ^B -dependent fashion upon stress exposure. However, the total number of σ^B -dependent genes in *L. monocytogenes* may be higher, as the microarray used in that study had only limited genome coverage (Kazmierczak *et al.*, 2003).

The comparison of *B. cereus*, *B. subtilis*, *L. monocytogenes* and *S. aureus* σ^B regulons showed that genes are generally conserved among the four Gram-positive bacteria to same extent as all the other genes of the genome. However, only three genes (*rsbV*, *rsbW*, and *sigB*) are conserved in their σ^B dependency in all four bacteria, suggesting that the σ^B regulon of the different gram positive bacteria has evolved to perform niche specific functions (van Schaick *et al.*, 2007). Containing of 14 unknown proteins, the role of the σ^B regulon of *B. cereus* to counteract the acid stress has not been studied in detail. Microarray analyses were performed on *B. cereus* ATCC 14579 cells exposed 10 min to a lethal (pH 4.5) or not (pH 5.4) acid stress (Mols *et al.*, 2010b). The variation of the σ^B -dependent gene expression was reported except for BC0999 and BC1012 genes encoding for two hypothetical proteins. Eleven genes belonging to the σ^B regulon showed an over-expression both in lethal and non lethal acid stress. Among them, the σ^B operon containing *rsbV*, *rsbW* and *sigB*, *orf4* and *rsbY*, as well as the two genes *BC1000* and *BC1009* are up-regulated (≥ 1.5 fold in both conditions). In the same way, *BC0862* and *BC0998* genes are over expressed. The first one encoding the YfiT protein is known to be heat-shock inducible both in *B. cereus* and *B. subtilis* but its role in stress response remains unclear, whereas the second gene encodes the YfkM protease that may acts to degrade incorrectly folded proteins (van Schaick *et al.*, 2004b; Helmann *et al.*, 2001).

Gene accession	Alias	Annotation
BC0861		Hypothetical protein
BC0862	<i>yfkM</i>	Protease I
BC0863	<i>katE</i>	Catalase
BC0995		Hypothetical protein
BC0996		Hypothetical protein
BC0998	<i>yflT</i>	General stress protein
BC0999	<i>csbD</i>	Hypothetical protein
BC1000		Hypothetical protein
BC1001		Hypothetical protein
BC1002	<i>rsbV</i>	Anti- σ^B factor antagonist
BC1003	<i>rsbW</i>	Anti- σ^B factor
BC1004	<i>sigB</i>	RNA polymerase sigma factor σ^B
BC1005	<i>orf4</i>	Putative bacterioferritin
BC1006	<i>rsbY</i>	PP2C-type RR; regulates σ^B activity
BC1007	<i>cheR</i>	Chemotaxis protein methyltransferase
BC1008	<i>rsbK</i>	Multi-sensor hybrid kinase
BC1009		Hypothetical protein
BC1010		Hypothetical protein
BC1011		Hypothetical protein
BC1012	<i>ybjQ</i>	Hypothetical protein
BC1154	<i>hemH-2</i>	Ferrochelatase
BC1155	<i>katA</i>	Catalase
BC2108	<i>sigZ</i>	ECF sigma factor
BC2638		Spore germination protein LC
BC3129	<i>corA</i>	Mg ²⁺ and Co ²⁺ transporter
BC3130		Hypothetical protein
BC3131		Hypothetical protein
BC3132	<i>yflT</i>	General stress protein
BC4640	<i>ytfJ</i>	Hypothetical protein
BC4641	<i>ytfI</i>	Hypothetical protein
BC5390	<i>cwlJ</i>	Cell wall hydrolase
BC5391	<i>gerQ</i>	Spore coat protein

Table 1: *B. cereus* ATCC 14579 σ^B regulon members adapted from de Been *et al.*, 2010.

The genes *BC1154* encoding a ferrochelatase and *BC1155* encoding the main vegetative catalase *KatA* were up-regulated both in lethal and nonlethal conditions but more upon mild acid exposure (from 7.8 fold to 44 fold) than upon lethal acid stress (from 1.5 to 3.7 fold). Eight other σ^B -dependent genes confirms that the response differ with the intensity of acid stress. For example, *BC0863* and *BC3129* genes, encoding respectively the catalase *KatE* and a magnesium cobalt transporter *CorA*, were up-regulated upon non lethal acid stress whereas they were down regulated in lethal acid stress.

Furthermore, *clp*, *groES* and *dnaK* genes encoding repair and chaperone proteins and heat stress regulators *ctsR* and *hrcA* (van de Guchte *et al.*, 2002) were also shown to be up-regulated upon exposure to acid conditions in *B. cereus* (Mols *et al.*, 2010b; Mols and Abee, 2011b).

pH homeostasis

The external pH as well as the presence of weak acid determines the intracellular pH (pH_{int}). Weak acids under their unprotonated form can diffuse into the cell and dissociate, releasing a proton and leading to acidification of the cytoplasm. Strong acids are not able to permeate through the cell membrane but by lowering the external pH and increasing the pH gradient, they increase the proton permeability and thereby lead to the reduction of pH_{int} (Abee and Wouters, 1999; Beales, 2004; Cotter *et al.*, 2000; Krebs *et al.*, 1983). Bacteria can survive thanks to their ability to regulate their pH_{int} , a process primarily driven by controlled movement of cations across the membrane (Beales, 2004). However, this ability to maintain pH_{int} (pH homeostasis) can be overtaken at low extracellular pH value, leading to the cell death (Booth and Kroll, 1989; Beales, 2004). For instance, *B. cereus* ATCC 14579 cells grown at pH 7.00 or 5.50 showed pH_{int} values of 7.10 and 6.22, respectively, whereas a 40 min exposure of cells at pH 4.00 decreased the pH_{int} of 2.02 units and was combined to a population reduction of 2.35 log (Senouci-Rezkallah *et al.*, 2011).

Therefore to survive in acidic conditions, microorganisms activate enzymes contributing to maintain their pH homeostasis, such as proton pumps and consuming proton reactions like glutamate decarboxylation.

F_1F_0 -ATPase and Proton Transporter. In aerobic organisms, the active transport of H^+ is coupled with electron transport in respiratory chains whereas anaerobic bacteria carry out H^+ transport via H^+ -ATPase molecules using energy from ATP hydrolysis (Gandhi and Chikindas, 2007; Shabala *et al.*, 2002). In *L. monocytogenes*, a facultative anaerobic bacterium, the use of both processes has been demonstrated (Shabala *et al.*, 2002). The multi-subunit enzyme F_1F_0 -ATPase, that is highly conserved, serves as a channel for proton translocation coupled with ATP synthesis or hydrolysis (Fillingame and Divall, 1999; Yoshida *et al.*, 2001). ATP synthesis is generated by the expense of proton motive force (PMF) whereas ATP hydrolysis generates a PMF that can facilitate the extrusion of proton from the cytoplasm (Cotter and Hill, 2003).

Since *B. cereus* is a facultative anaerobic bacterium, it could be supposed that this bacterium may use both ATP hydrolysis and synthesis to maintain its pH homeostasis like as shown in *L. monocytogenes*. In fact, F_0F_1 -ATPase encoding genes were down-regulated in *B. cereus* ATCC 14579 and *B. cereus* ATCC10987 exposed to non-lethal acid conditions, and were not repressed upon exposure to lethal acid stresses, indicating that *B. cereus* does not use F_0F_1 -ATPase to extrude proton (Mols *et al.*, 2010a and b). Down regulation of F_0F_1 -ATPase genes could be explained by the cells trying to prevent excessive inward flux of proton via this ATPase upon exposure to acid conditions (Mols *et al.*, 2010b). This down-regulation has also been demonstrated in *S. aureus* where the expression of F_0F_1 -ATPase

encoding-genes was clearly reduced to about 50 % (Bore *et al.*, 2007). But in these both cases, the ATPase could still play an important role by pumping H⁺ out of the cells, since Arikado and others (1999) suggest that the enzyme regulation occurs mainly at the post-transcriptional level.

Other proton transporters may also play key role in pH homeostasis. Indeed, in early 1970s, it has been shown that an activity in biological membranes couples the fluxes of Na⁺ and H⁺ (Mitchell, 1961; Mitchell and Moyle, 1967; West and Mitchell, 1974). They suggested that Na⁺/H⁺ antiporters are involved in the homeostasis of both Na⁺ and H⁺ in cells. Since then, Na⁺/H⁺ as well as K⁺/H⁺ antiporter activity has been found in cytoplasmic membranes of many cells. These monovalent cation/proton antiporters are especially known to be involved in bacterial pH homeostasis under alkaline challenge (Krulwich *et al.*, 1999). Interestingly, Mols and colleagues (2010b) showed that *napA* encoding a Na⁺/H⁺ antiporter was highly up-regulated upon exposure to lethal pH whereas it was down regulated upon non lethal acid exposure (Mols *et al.*, 2010b).

Thus, F₀F₁-ATPase and antiporter gene regulations under lethal and non lethal conditions indicated fine balance between ATP synthesis on one hand and proton pumps regulating pH_{int} at the expense of ATP on the other hand (Mols and Abee, 2011b).

Amino-acids decarboxylases systems. Amino acid decarboxylases function to control the pH of the bacterial environment by consuming hydrogen ions as part of the decarboxylation reaction (Gale and Epps, 1942). Examples of this are the lysine, arginine, and glutamate decarboxylases, which operate by combining an internalized amino acid with a proton and exchanging the resultant product for another amino acid substrate. Furthermore, the products of amino acid decarboxylases consist of basic amines (pKa value around 10) which may be responsible of a slight increase of extracellular pH (Olson, 1993). Lysine decarboxylase is an enzyme that converts lysine to cadaverine and was found to be an important system involved in acid resistance and in pH_{int} homeostasis in several Gram-negative bacteria including *E. coli*, *Vibrio vulnificus*, *Vibrio parahaemolyticus* and *Salmonella typhimurium* (Bearson *et al.*, 1997; Eun Rhee *et al.*, 2002; Moreau, 2007; Tanaka *et al.*, 2008). Arginine decarboxylase enzyme has one substrate (L-arginine) and two products, CO₂ and agmatine, which has been showed to be a competitive inhibitor in *E. coli* (Blethen *et al.*, 1968). Glutamate decarboxylase (GAD) converts glutamate in γ -aminobutyrate (GABA) and is well described in, for instance, *L. monocytogenes* (Gandhi and Chikindas, 2007). In *L. monocytogenes*, the increase of acid tolerance due to glutamate decarboxylase system is only provided when glutamate decarboxylase acts in concert with glutamate/GABA antiporter (Cotter *et al.*, 2000).

Today, only few pieces of information are available on these systems in *B. cereus*. Mols and co-workers (2010b) showed that the glutamate decarboxylase gene (*gad*) is not present in the genome of *B. cereus* ATCC 14579 whereas it is present in *B. cereus* ATCC 10987 and many other strains.

However, they did not see any difference in growth under acidic conditions between these strains. As previously described for *L. monocytogenes*, it may be possible that the glutamate decarboxylase does not contribute to the growth of *B. cereus* ATCC 10987 in acidic environments because the gene encoding the glutamate/GABA antiporter is lacking. In 2011, Senouci-Rezkallah *et al*, studied the impact of amino-acid presence on the acid tolerance response of *B. cereus* ATCC 14579 strain. They observed that survival to an acid shock at pH 4.0 of *B. cereus* cells grown at pH 7.0 was enhanced in the presence of glutamate and strongly enhanced in the presence of arginine or lysine. However, the presence of these amino-acids had no impact on the acid tolerance of acid pre-adapted cells when submitted to acid shock. They also observed that the presence of glutamate, arginine or lysine increased the pH_{int} of *B. cereus* cells grown at pH 7.0 during exposure at pH 4.0 whereas it had no significant influence on pH_{int} cells grown at pH 5.5 during acid shock, suggesting an induction of these systems under these growth conditions (Figure 3).

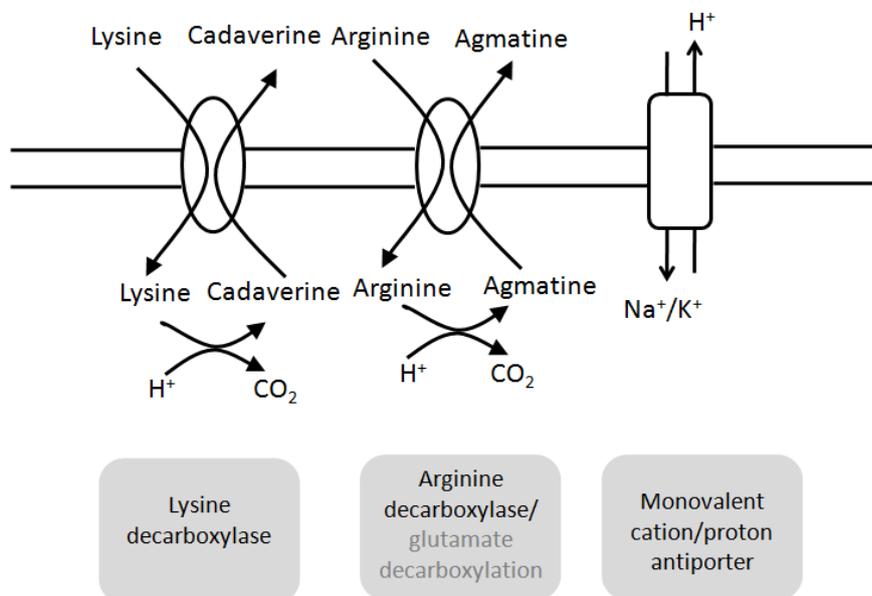


Figure 3: Graphical representation of the biological systems involved in pH homeostasis of *Bacillus cereus* cells. Lysine decarboxylase converts lysine to cadaverine, arginine decarboxylase converts arginine to agmatine, consuming a proton and producing a CO₂. Glutamate decarboxylation converts glutamate in γ -aminobutyrate, Note that this enzyme is not present in all *B. cereus* strain as shown for *B. cereus* ATCC 14579 and in this strain the glutamate may be decarboxylated by the arginine decarboxylase. Monovalent cation/proton antiporters may also play key role, by proton extrusion, in pH homeostasis of *B. cereus* particularly upon exposure to lethal pH.

As *B. cereus* ATCC 14579 does not possess the gene encoding for the glutamate decarboxylase, the glutamate may be decarboxylated by the arginine decarboxylase, as described in *Lathyrus sativus* (Ramakrishna and Adiga, 1975).

Metabolism modification and alkali production

Depending on their environment, bacteria will establish specific pathways allowing their survival or growth. Several metabolic pathways have been associated with bacterial growth at low pHs.

Upon acid stress, alkali production is often observed and well established in oral bacteria for example (Burne and Marquis, 2000). Urea and arginine are two major substrates for alkali generation by oral biofilms colonizing the teeth. Urea is in all salivary gland secretions and is rapidly hydrolyzed to ammonia and CO₂ by bacterial ureases while arginine is primarily catabolised to ornithine, ammonia and CO₂. The increased tolerance of cells results from the production of NH₃, which combines with protons in the cytoplasm to produce NH₄⁺ raising the internal pH. Nevertheless, it could bear in mind that these two systems are not present in all oral bacteria (Lemos and Burne, 2008) and other mechanisms play important role in their acid tolerance.

Ammonia producing mechanism. Arginine deiminase or the ADI pathway (for review see Lu, 2006) has been identified in a variety of Gram-positive bacteria, including *Bacillus* spp, *L. monocytogenes*, and several lactic acid bacteria (LAB) (Cunin *et al.*, 1986; Ryan *et al.*, 2009). The ADI pathway converts arginine to citrulline and ammonia. Subsequently, citrulline is metabolised into ornithine generating carbon dioxide, ammonia and ATP. Arginine and ornithine are exchanged via an antiporter importing arginine and exporting ornithine. The resulting NH₃ rapidly reacts with H⁺ and helps to alkalize the environment consuming proton and forming ammonium. Furthermore, the generated ATP can enable extrusion of cytoplasmic protons by the F₀F₁-ATPase as shown in *L. monocytogenes* or in LAB (van de Guchte *et al.*, 2002). This system has three main enzymes, ADI, ornithine transcarbamylase, and carbamate kinase, encoded by *arcA*, *arcB* and *arcC*, respectively. They appear to be inherently acid-tolerant, displaying activity at pH 3.1 and even lower in some species (Casiano-Colon and Marquis, 1988; Curran *et al.*, 1995). The role of the ADI pathway in acid stress resistance has been established in bacteria such as streptococci and *L. monocytogenes*. *Streptococcus sanguis* showed arginine deiminase activity upon exposure to pH 3.5 leading to an increase resistance (Curran *et al.*, 1995) and ADI negative mutants of *L. monocytogenes* were more sensitive to low pHs (Ryan *et al.*, 2009). In *B. cereus*, arginine deiminase gene *arcA* showed significant up-regulation in *B. cereus* ATCC 14579 and ATCC 10987 upon exposure to non lethal acid shock at pH 5.4, whereas exposure to a lethal acid shock pH 4.5 revealed no significant induction (Mols *et al.*, 2010b). Senouci-Rezkallah and colleagues also demonstrated a 6 fold up-regulation of the *arcA* gene in acid adapted cells grown at pH5.5 compared to non preadapted cells grown at pH 7. These data

suggest that the ADI pathway may be of great importance for *B. cereus* survival in low pH environments. Nevertheless, this role seems to be restricted to non lethal acid stress (Figure 4).

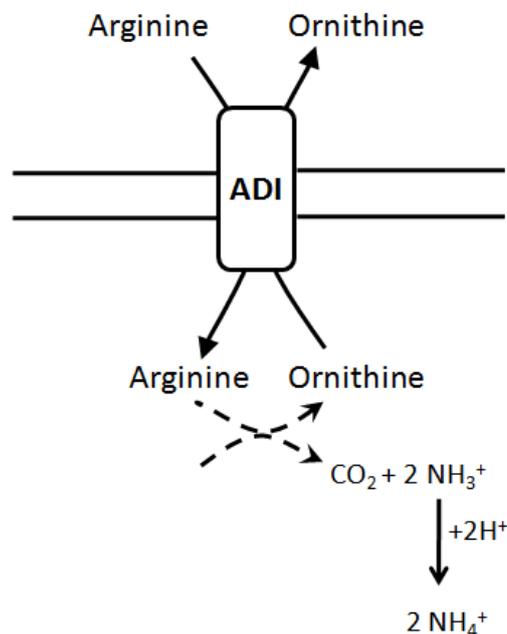


Figure 4: Graphical representation of the arginine deiminase (ADI) pathway implied in the acid stress response of *Bacillus cereus*. The ADI pathway converts arginine to citrulline and ammonia. Citrulline is then metabolised into ornithine generating carbon dioxide and ammonia. Arginine and ornithine are exchanged via an antiporter importing arginine and exporting ornithine. Ammonia reacts with proton forming ammonium.

Another ammonia producing mechanism associated with low pH resistance is urease. The urease enzyme catalyses the hydrolysis of urea, generating two molecules of ammonia and one molecule of carbon dioxide. Urease is well studied in the human pathogen *Helicobacter pylori* in which urease plays an important role in the ability of stomach colonization and in virulence (Eaton et al., 1991). Urea is present in various environments in which *B. cereus* can be found, including soil, food, and the human body, where urea is present in all fluids and is finally excreted in the urine as a detoxification product (Burne and Chen, 2000; Mobley and Hausinger, 1989). In 2004, Rasko and colleagues identified an urease cluster composed of nine genes (from *BCE3657* to *BCE3666*) in the genome of *B. cereus* strain ATCC 10987 that is not present in all sequenced strains belonging to *B. cereus* (Mols and Abee, 2008). This cluster harbours three genes *ureA*, *ureB* and *ureC*, encoding structural enzymes, four genes (*ureEFGD*) encoding accessory proteins, and two additional genes *ureI* and *nikT* respectively encoding a putative urea (acetamine) transporter and a nickel transporter. In 2008, Mols and Abee showed that among 49 *B. cereus* strains, isolated from various sources and including clinical, environmental and food isolates, together with the type strain ATCC 14579 and other sequenced strains such as ATCC 10987 and PAL25, 10 strains are *ureABC* PCR-positive and 9 also possess urease activity. They also showed that urease activity did not contribute to *B. cereus* survival capacity under acid shock conditions.

Fermentative pathways. The metabolic pathways involved in fermentative growth, such as lactate, alcohol and butanediol pathways are involved in *B. subtilis* cell acid stress response as shown by their up-regulation upon low pH of growth (Cruz Ramos *et al.*, 2000; Wilks *et al.*, 2009). AlsS condenses two molecules of pyruvate to form acetolactate, converted to acetoin by spontaneous decarboxylation at low pH and by the action of the acetolactate decarboxylase (AlsD) (Renna *et al.*, 1993). Acetoin can then be excreted by the cells. Alcohol dehydrogenase (Adh) is an NAD(P)-dependent dehydrogenase that may remove acidity and transfer electrons to the electron transfer chain (Reid and Fewson, 1994). Lactate dehydrogenase, encoded by *ldh*, is a cytoplasmic NADH-linked enzyme that converts pyruvate to lactate to remove acidic compounds, restoring the NAD⁺/NADH balance. During fermentation, Ldh is the key enzyme involved in reoxidation of the NADH formed by glycolysis (Cruz-Ramos *et al.*, 2000). In *Streptococcus oralis* and *Streptococcus mutans*, it was shown that many enzymes involved in glycolysis are up-regulated during growth at low pH (Wilkins *et al.*, 2001, 2002). It has been suggested that the increase in the amount of these proteins may result in an increase in ATP production and consequently increased proton extrusion via the F₁F₀-ATPase. In *B. subtilis*, acid conditions also up-regulate a large number of NAD(P)-dependent dehydrogenases such as alanine dehydrogenase (*ald*), succinate-semialdehyde dehydrogenase (*gabD*), and several putative formate dehydrogenases (*fdhD*, *yjgC*, *yrhE*, and *yrhG*). These enzymes are able to remove acidity through NAD(P)H which transfers electrons to the electron transport system and pumps protons out of the cell (Wilks *et al.*, 2009).

In 2010, Mols and colleagues showed that a set of 25 genes of *B. cereus* ATCC 14579 are differentially expressed in lethal or non-lethal organic or inorganic acid conditions and a set of 146 genes for all non-lethal acid conditions. Up-regulation concerned mainly genes involved in energy metabolism, oxidative and general stress response. Pyruvate metabolism, the tricarboxylic acid cycle (TCA) and other fermentation pathways were induced to maintain intracellular ATP levels and/or the redox balance. In lethal acid conditions, increase of lactate dehydrogenase (*ldh*) and cytochrome bd oxidase (*cydAB*) gene expression are shown. In *B. subtilis*, these genes are co-ordinately expressed together with the lactate permease gene *lctP* and the formate-nitrite transporter gene *ywcJ* and under control of the negative regulator YdiH (Rex) (Larsson *et al.*, 2005). Lactate dehydrogenase in concert with the cytochrome bd oxidase has been proposed to function as an alternative electron transport chain (Chai *et al.*, 2009). The *alsSD* is also up-regulated upon exposure to lethal or non lethal acid stresses, but this up-regulation is less pronounced upon lethal conditions than under non lethal acid stress. Together with the *alsSD* genes, *cydAB*, *ldh* and *lctP* form a distinct regulon, which is part of the Fnr regulon (Reents *et al.*, 2006). By analogy with *B. subtilis*, the induction of these genes may be

associated to a changing in NADH/NAD^+ ratio. It could be noted that under lethal concentration of acetic acid *B. cereus* showed some resemblance with the response of *S. aureus* cells deficit of *murF* which exhibit a reduced peptidoglycan synthesis. Indeed, these cells down-regulated iron uptake associated genes and, induced *ldh*, lactate permease, and formate/nitrite transporter protein genes (Sobral *et al.*, 2007).

Mols and co-workers also investigated in 2010, the impact of lethal hydrochloric acid stress on *B. cereus* ATCC 14579 cells. Genes encoding alcohol dehydrogenases and lactate dehydrogenases appeared to be also induced upon exposure to lethal acid stresses (Figure 5).

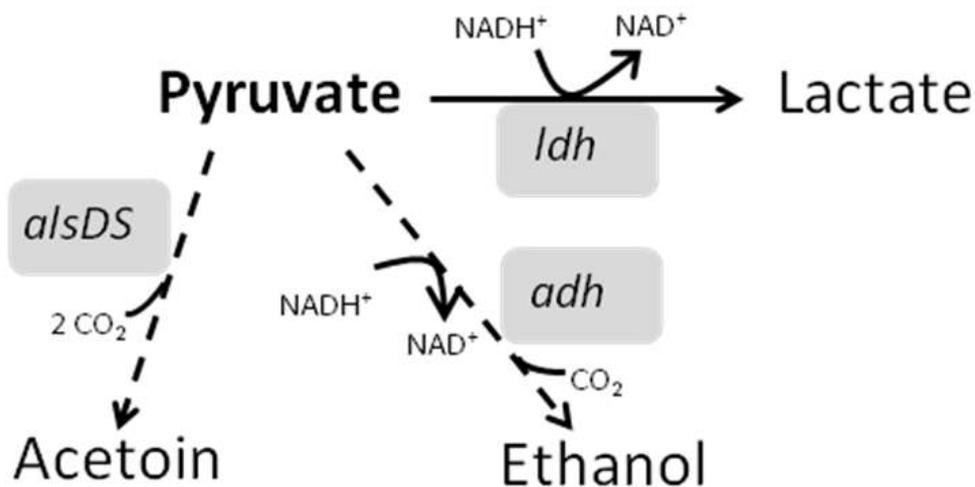


Figure 5: Graphical representation of the fermentative pathways, such as acetoin production (*alsDS*), alcohol (*adh*) and lactate dehydrogenases (*ldh*), which represent the most notable metabolic rearrangements showed upon exposure to acid stress conditions.

Therefore, the conversion of pyruvate to ethanol or lactate, generating CO_2 and dissipating H^+ , may be an ultimate futile response of *B. cereus* to deal with low intracellular pH_{int} or restoration of NAD^+/NADH balance. Furthermore some metabolism rearrangements were also found specially correlated with lactic acid or acetic acid stress. For instance, several genes involved in glycolysis are moderately up-regulated upon exposure to lactic acid stress (2mM undissociated acid) (Mols *et al.*, 2010a). Nevertheless, their functions remain to be elucidated.

Secondary oxidative stress

Microorganisms have a limited tolerance for oxygen. This property is most evident in the case of obligate anaerobes and microaerophiles, which cannot grow in air saturated media, but it applies as well to aerobes. Aerobic organisms use molecular oxygen (O_2) for respiration or oxidation of nutrients to obtain energy. Reactive oxygen species (ROS), such as superoxide (O_2^-), hydrogen

peroxide (H_2O_2), and the highly reactive hydroxyl radical (OH^\cdot), are generated continuously in cells growing aerobically. Therefore aerobic microorganisms could survive only because they contain antioxidant defences. The biological targets for the ROS are DNA, RNA, proteins and lipids. In some bacteria, such as *Borrelia burgdorferi*, the membrane could be the primary target of ROS, (Boylan *et al.*, 2008). However, it has been shown that the most damaging effect of ROS in bacteria results from the interactions of H_2O_2 with Fe^{2+} , generating reactive OH by the Fenton reaction. Because the Fe^{2+} is localized along the phosphodiester backbone of nucleic acid, DNA is a major target of OH (reviewed by Imlay, 2003). The effect of reactive oxygen species on proteins is the oxidation of thiols, resulting in disulfide bond formation (Leichert *et al.*, 2003). Living organisms have developed various defences to protect themselves against ROS damage, some are enzymatic (catalases, superoxide dismutases (SOD), thioredoxins, and peroxidases) and others are non-enzymatic (glutathione, vitamin A, C,...). In bacteria, oxidative stress is sensed and lead to the activation of specific transcriptional regulators which will induce defence mechanisms when ROS concentration exceeds a critical level (Dempfle, 1991; Farr and Kogoma, 1991; Storz and Imlay, 1999). Furthermore, it is known that some other stresses could generate a bacterial response similar to the oxidative stress response. Indeed, this secondary oxidative stress response has been described upon exposure to salt, heat or cold temperature, bile, or after starvation and more recently upon lethal antibiotic concentrations for *Bacillus* (Ceriagioli *et al.*, 2010; den Besten *et al.*, 2009; Hecker and Volker, 2004; Hecker *et al.*, 2007; Mols and Abee, 2011a).

Mols and co-workers showed that acid-stressed *B. cereus* cells revealed a major oxidative response, as other Bacilli (Mols *et al.*, 2010b and 2011a and b). In sum, *B. cereus* cells exposed to lethal or non lethal organic or inorganic acid stresses induce a set of genes such as those coding for superoxide dismutase, catalases and thioredoxins, which are known to be involved in bacterial oxidative stress response. Using fluorescent probes HFP (3'-p-hydroxyphenyl fluorescein), authors correlated the induction of these genes upon lethal conditions to the formation of ROS. Because the formation of ROS is apparent in *Bacillus spp.* upon lethal stresses (Mols *et al.*, 2009; Mols and Abee, 2011a; Hecker and Volker, 2004), ROS may be part of a common mechanism of cellular death in respiring *Bacillus* species. Based on phenotypic and transcriptomic results, a model for environmental stresses was proposed with induction of radical formation, including OH^\cdot and ONOO^- in *Bacillus spp* (Mols and Abee, 2011a). Rapidly, environmental stresses induce perturbation of the electron transfer chain (ETC), where free electrons prematurely leak to oxygen resulting in the formation of superoxide (O_2^-). The ETC perturbation is confirmed by the expression of alternatives, such as cytochrome d ubiquinol oxidase (CydAB) and nitrate/nitrite reductase (Nar/Nas). Upon the action of superoxide dismutase, the O_2^- is converted into hydrogen peroxide H_2O_2 leading to a primary oxidative stress response (for a

critical review of *B. subtilis* oxidative stress response see Zuber, 2009). By Fenton reaction and iron-sulfur cluster damaging, the H_2O_2 could be transformed into the highly toxic OH^\cdot radicals. Furthermore, O_2^- can rapidly react with nitric oxide (NO) to form another highly toxic compound, ONOO^- , which may have a damaging effect that could lead to cell death (for more details see Mols and Abee, 2011a).

From physiological knowledge to the identification of potential biomarkers

The knowledge regarding the adaptive stress response of bacteria is of interest for the food industry, particularly with the increasing trend in the production of minimally processed foods where various stresses are combined to control microbial growth. Indeed the implementation of the bacterial adaptive traits in predictive microbiology concept will lead to a more accurate prediction of the bacterial behaviour under specific conditions. However, one of the major challenges will be to increment these data in decision making tools developed to support food safety and quality issues (Brul *et al.*, 2012; Havelaar *et al.*, 2010, McMeekin *et al.*, 2008; Ranstiou *et al.*, 2011). Up to now two main different approaches to integrate bacterial behaviour into predictive modelling are known with the identification and quantification of (i) signalling and metabolic pathway with flux balance analysis (Kauffman *et al.*, 2003; Metris *et al.*, 2011), or (ii) biomarkers (den Besten *et al.*, 2010; den Besten *et al.*, 2013; Desriac *et al.*, 2012; Desriac *et al.*, 2013). The official US National Institute of Health definition of a biomarker is “a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (Atkinson *et al.*; 2001). When applied to industrial process conditions, we propose to adapt this commonly medical oriented definition as: a characteristic that is objectively measured and evaluated as an indicator of bacterial responses to food processes and stress conditions. In 2010, den Besten *et al.* described a strategy to identify biomarkers for cell robustness of *B. cereus*. Shortly, both unstressed and mild stress treated cells were exposed to lethal stress conditions (severe heat, acid and oxidative stress) to quantify the robustness advantage provided by mild stress pre-treatment. Robustness was defined by the ratio of the survivors quantified on agar plate at a defined time and the inoculum (unstressed cells) used for inactivation treatment. This framework enabled the identification of candidate biomarkers, *i.e.* enzymatic activity, protein level and gene expression obtained by Reverse Transcription followed by quantitative PCR (RT-qPCR). Linear correlations between induced biomarker and induced robustness upon exposure to mild stress, revealed three kinds of biomarkers defined as “no-response biomarker”, “short-term biomarker” and “long-term biomarker”. For instance, protein SigB, *clpC* and *clpP* transcripts were

suitable to predict the robustness advantage towards lethal acid and oxidative stresses following mild acid stress pretreatment. If this first approach proposed linear correlation, in 2012 we proposed an integrative approach encompassing both gene expression quantification throughout bacterial inactivation and mathematical modeling of the bacterial behaviour to identify different molecular biomarkers to further predict the acid resistance of *B. weihenstephanensis*. The *sigB* gene was proposed as biomarker to track moderate acid resistance whereas *katA* was identified as biomarker to track high acid resistance. Indeed, surviving bacterial kinetics of *B. weihenstephanensis* under lethal acid conditions (pH 4.6), have a biphasic patterns meaning that the bacterial population could be divided into two subpopulations with different acid resistances. By correlating the proportion of the two subpopulations with the gene expression, it could be shown that the highest expression of *sigB* was observed when the sensitive subpopulation represented the majority of the bacterial population. At the opposite the *katA* up regulation was correlated to the more resistant subpopulation. In 2013, we also proposed both linear and non linear correlations between gene expression and acid survival ability of short adapted cells were. Two kinds of biomarkers were defined with (i) direct biomarker genes for which the expression patterns upon mild stress treatment were linearly correlated to induced acid resistance; and (ii) long-acting biomarker genes which were up regulated for mild stress adaptation times of 30 minutes at maximum and linked to increased resistance over studied time (60 min).

However if the selection of potential biomarkers offers new perspectives for the prediction of bacterial behaviour and physiology, one of the key challenges will be to increment these data into mathematical model to predict growth or inactivation, during industrial processes to offer decision making tools for food safety and quality management (Brul *et al.*, 2012; Desriac *et al.*, 2012; Desriac *et al.*, 2013). The development of such models will contribute to support the food business sector competitiveness by optimizing inactivation process monitoring, eco efficient processing and accurate shelf life products. Thus, the integration of bacterial physiological state into predictive microbiology behaviour will offer new tailor made decision making tools which could be used for getting the proper balance between food safety and food quality.

Conclusion

Nowadays, food industries use mild preservation and processing techniques, in response to consumers demand for fresher, healthier and better foods and because mild preservation techniques save energy and are more environmental friendly. These mild preservation techniques, such as hurdles, may lead to the survival of spoilage and/or pathogenic microorganisms. Therefore the

adaptive stress response and the physiology of bacteria is an important subject. One of the organisms that can spoil food and cause food-borne illnesses is *B. cereus*. This review describes the current knowledge on *B. cereus* acid stress response. *B. cereus* can encounter acid conditions in foods and upon ingestion *B. cereus* has to overcome the acid barrier of the human stomach (Clavel *et al.*, 2004; Wijnands *et al.*, 2009). For the control of *B. cereus* in food, it is especially relevant to understand how *B. cereus* cells grow and survive in acidic conditions. This knowledge may then be applied to optimize food production processes and storage conditions, with the aim to minimizing the threat of *B. cereus* induced food-borne illnesses. The acid stress response of *B. cereus* could be divided into four groups (i) general stress response (ii) pH homeostasis, (iii) metabolic rearrangements and (iv) secondary oxidative stress response (Figure 6).

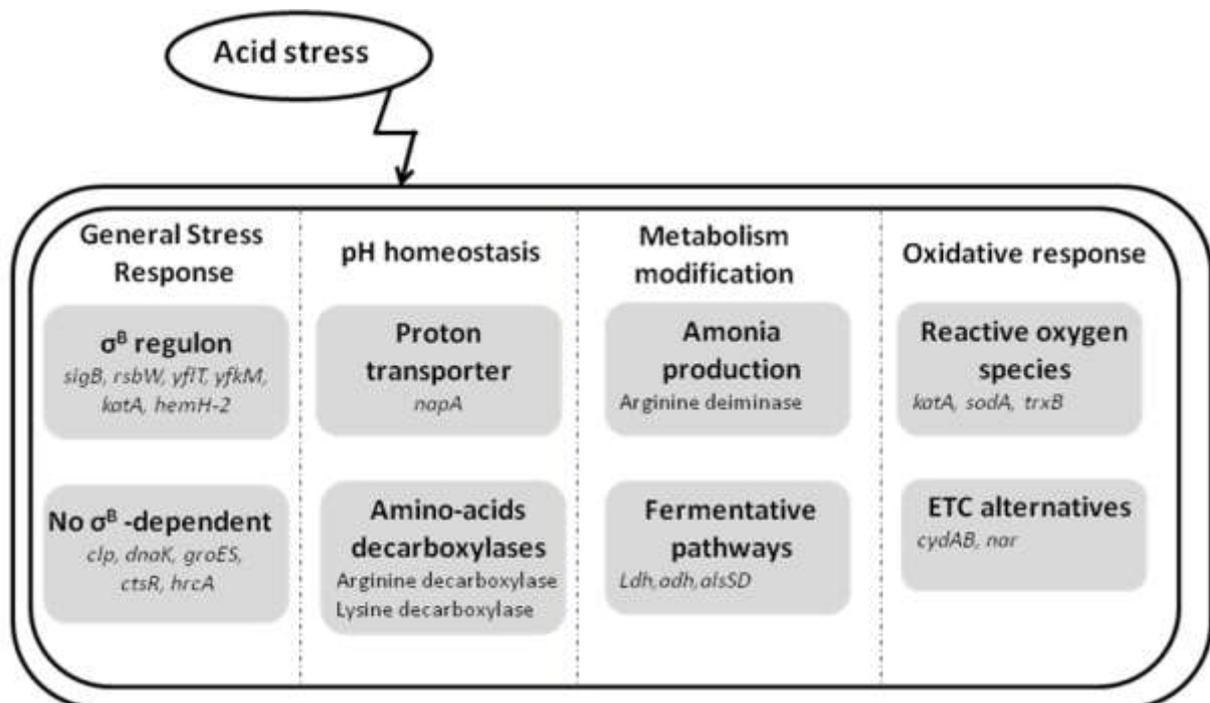


Figure 6: Global representation of acid stress-associated mechanisms in *Bacillus cereus* which could be divided into four different groups: (i) general stress response, (ii) pH homeostasis, (iii) metabolic rearrangements and (iv) oxidative response. The general stress response is governed by the σ^B factor (encoded by *sigB*) which regulated the expression of approximately 30 genes among which *rsbW* (anti σ^B factor), *yfjT* (general stress protein), *yfkM* (protease), *katA* (catalase), *hemH-2* (ferrochelataase). However this group also contains genes which are putatively not only induced by low pH, but may be involved in a more general response to stresses and which are not under the control of σ^B such as *clp* (protease), *dnaK*, *groES* (chaperone proteins), *ctsR* and *hrcA* (transcriptional regulators). pH homeostasis involves monovalent cation/proton antiporter (*napA*) and amino-acids decarboxylases. The most notable metabolic rearrangements were fermentative pathways, such as acetoin production (*alsDS*), alcohol (*adh*) and lactate dehydrogenases (*ldh*) and the production of ammonia throughout the arginine deiminase system. At last, due to the perturbation of the electron transfer chain upon acid stress exposure, an oxidative response involving catalase (*katA*), dismutase (*sodA*), thioredoxins such as *trxB* and alternative for the electron transfer chain (ETC) such cytochrome *d* ubiquinol oxidase (*cydAB*) and nitrate/nitrite reductase (*nar/nas*).

The general stress response involves genes that are putatively not only induced under low pH exposure, but may be involved in a more general response to stresses. In *B. cereus*, the induction of

transcriptional regulator such as σ^B factor, *ctsR* and *hrcA* occurs when cells are exposed to an acid stress. Due to a positive feedback of σ^B on its regulon, the σ^B operon is also up-regulated upon both lethal and non lethal acid stresses. Furthermore, some genes of the σ^B -regulon are also up-regulated in both acid stress intensity (*BC0998*, *katA*, *BC1145*, *orf4*, *rsbY*, *BC1000* and *BC1009*) whereas others showed an up-regulation only upon exposure to non lethal acid stress such as *BC1011*, *BC3131*, *katE* and *corA*. Other genes that are known to be involved in various stresses, including protein repair chaperones *groEL*, *dnaK* and *clp* are up-regulated upon exposure to acid condition in *B. cereus*. The pH homeostasis is maintained using decarboxylation of amino acids like arginine, lysine and glutamate, however the decarboxylation systems used remains unclear, and antiporter like Na^+/H^+ antiporter encoding by the *napA* gene which is highly up-regulated upon exposure to lethal pH. The most notable metabolic rearrangements show upon exposure to mainly organic acid stress are fermentative pathways, such as acetoin production (AlsDS), alcohol (*AdhA*), lactate dehydrogenases (*Ldh*) and rerouting of pyruvate metabolism. Another key mechanism in acid response of *B. cereus* cells is the arginine deiminase (ADI) pathway that will induce proton consumption. At last, the secondary oxidative stress response is correlated to the electron transfer chain perturbations generating superoxide and therefore other reactive oxygen species inducing oxidative stress mechanisms.

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Chapter 3

Sensitivity of *Bacillus weihenstephanensis* to acidic changes of the medium is not dependant on physiological state

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Abstract

This study aims to quantify the effect of salt and acid preliminary exposure on acid resistance of vegetative cells of *Bacillus weihenstephanensis*. The psychrotolerant strain KBAB4 was cultured until the mid-exponentially phase (i) in BHI, (ii) in BHI supplemented with 2.5% salt or (iii) in BHI acidified at pH 5.5 with HCl. The growing cells were subsequently inactivated in lethal acid conditions ranging from 4.45 to 4.70. Based on statistical criteria, a primary mixed-Weibull model was used to fit the acid inactivation kinetics. The acid resistance was enhanced for acid-adapted cells and decreased for salt-adapted cells. The secondary modeling of the bacterial resistance allowed the quantification of the change in pH leading to a ten fold variation of the bacterial resistance, *i.e.* cells sensitivity (z_{pH}). This sensitivity was not significantly affected whatever the preliminary mild exposure and the presence of sub-populations with different acid resistances. These results highlighted that pre-incubation conditions influence bacterial acid resistance without affecting the sensitivity to acidic modifications, with a 10 fold reduction of *Bacillus* acid resistance observed for a reduction of 0.37 pH unit. Quantification of such adaptive stress response might be instrumental in quantitative risk assessment more particularly in food formulation, particularly for low-acid minimally processed foods.

Introduction

Spore forming bacteria cause major problems in the food industry since they may be involved in both food poisoning and food spoilage, raising major safety and economical issues. The *Bacillus cereus* group consists of six closely related species: *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis* and *B. cereus (sensu stricto)*. *B. cereus* and *B. anthracis* are known as human pathogens, *B. thuringiensis* is used as biopesticide, *B. mycoides* and *B. pseudomycoides* are characterised by rhizoidal formations when grown on agar medium. The *B. weihenstephanensis* specie includes strains which can grow under chilled temperature (Lechner *et al.*, 1998) leading major problem in ready-to-eat or ready-to-cook foods stored at chilled temperatures, also known as refrigerated processed foods of extended durability (REFPEDs).

The heat resistance of *Bacillus* spore was extensively studied and is used for FSO establishment (Membre *et al.*, 2006). The influence of sporulation environment of *Bacillus* (Baril *et al.*, 2011a, and 2011b; Baril *et al.*, 2012; Garcia *et al.*, 2010; Gonzalez *et al.*, 1999; Planchon *et al.*, 2011) is known to have a great impact on spore resistance properties and spore formation abilities. Recently, Baril *et al.* (2012) reported that sporulation boundaries of *B. weihenstephanensis* were included within the range of temperatures, pH and water activities supporting growth. A decrease in spore heat resistance and sporulation rate was observed for spores of *B. weihenstephanensis* produced for non optimal growth conditions. For instance, the time to get one spore per ml was tenfold longer when sporulation occurred at 10°C than at 30°C (Baril *et al.*, 2011a). Since a low sporulation rate and low resistance of spores was observed under sublethal conditions, *Bacillus* vegetative cells resistance appears as key issue for food industry particularly for minimally processed food through the food process and storage.

To ensure the microbial food safety and stability, the food industry uses combination of hurdles, mild preservation factors (Leistner and Gorris, 1995). A commonly used hurdle is a low pH which allows the decrease in growth rate but also the inactivation of pathogens or spoilage microorganisms (Coroller *et al.*, 2006; Greenacre *et al.*, 2003; Ita and Hutkins, 1991). For low-acid minimally processed foods, the main microbiological hazard is the presence of psychrotrophic, sporulated bacteria (Valero *et al.*, 2000) among which *B. weihenstephanensis* is representative. Although *B. weihenstephanensis* spoilage ability which limit shelf life product and generate economic losses (Abee and Wouters, 1999; Andersen-Borge *et al.*, 2001; Priest, 1993) is well established, its pathogenicity remains uncertain. Indeed some *B. weihenstephanensis* strains, among which the

KBAB4 strain, may be cytotoxic (Lapidus *et al.*, 2008; Hoton *et al.*, 2009; Réjasse *et al.*, 2012; Stenfors *et al.*, 2002). Thus, in this study the *B. weihenstephanensis* KBAB4 strain was used as bacterial model. The aim was to investigate the acid resistance of vegetative cells with (adapted cells) and without (non adapted cells) preliminary exposure to mild stress conditions. Since physiological state may greatly influence the bacterial resistance of vegetative cells, adaptation to mild saline and acid stress conditions were tested. To further apprehend the impact of lethal pH conditions on the acid resistance, the sensitivity was also determined.

Materials and Methods

Bacterial strain pre-incubation and adaptation conditions. The psychrotolerant *B. weihenstephanensis* KBAB4 strain, kindly provided by the Institut National de la Recherche Agronomique (INRA, Avignon, France), was used throughout this study. The strain was stored at -80°C in brain heart infusion (BHI, Biokar Diagnostic, Beauvais, France) supplemented with 50% (v/v) glycerol. Firstly, bacteria (1ml of glycerol stock) were grown in 100 ml of BHI broth incubated at 30°C for 8 h under shaking conditions (100 rpm) and an aliquot (1 ml) was transferred into a second flask of 100 ml BHI broth incubated for 15 h in the same conditions. For non adapted cells (control), a portion (0.1 ml) of the pre-culture was transferred in a flask containing 100 ml of BHI broth and incubated at 30°C with shaking at 100 rpm, until the cells reached the mid-exponentially phase ($OD_{600\text{ nm}}$ values of 0.20 ± 0.02 meaning approximately 10^7 CFU.ml⁻¹). In order to test different physiological state on the bacterial resistance behaviour, preliminary mild stress exposure was used. For mild stress adapted cells, the transfer was done in BHI supplemented with HCl (pH 5.5) or NaCl (2.5% w/v) and incubated at 30°C under shaking conditions (100 rpm) until a $OD_{600\text{ nm}}$ values of 0.15 ± 0.03 corresponding to around 10^7 CFU.ml⁻¹. Bacterial enumeration was done on Nutrient Agar (Biokar Diagnostic) using a SPIRAL plater (AES Chemunex, Combours, France) and serial dilutions using Tryptone Salt (Oxoid, Dardilly, France). Plates were subsequently incubated for 16 to 24 h at 30°C.

Acid inactivation. Adapted and non-adapted mid exponentially cells were subsequently submitted to 6 acid lethal conditions with a pH ranging from 4.40 to 4.7. At appropriate time from 5 min to 6 h, samples were taken to acquire inactivation kinetics. The survivor enumeration was done according the ISO 7218 (2007). Survivors were quantified on Nutrient Agar (Biokar Diagnostic) using a SPIRAL plater (AES Chemunex) and serial dilutions in Tryptone Salt (Oxoid). More than 20 CFU per Petri dishes were counted leading to a detection limit equal to 400 CFU.ml⁻¹. Plates were subsequently incubated for 16 to 24 h at 30°C. Acid inactivation kinetics were performed in three biological

replicates from independent frozen cells batches. To ensure the absence of spores in both pre-incubation and inactivation media, aliquots of 2 ml were submitted to heat treatment at 70°C for 5 min (Baril *et al.*, 2011b) and potential germinating spores were then quantified on nutrient agar (Biokar Diagnostic) medium at 30°C after 24 h incubation.

Estimation of the bacterial resistance. Three primary mathematical models were used to fit the inactivation kinetics.

(i) The first-order model was as follow

$$\log_{10} N(t) = \log_{10} N_0 - \frac{t}{D} \quad (1)$$

Where $N(t)$ is the concentration of survivors expressed in \log_{10} CFU.ml⁻¹ at time 't'; N_0 is the inoculum concentration in \log_{10} CFU.ml⁻¹; t is the time of exposure to stress (in hours) and D is the time necessary to obtain the logarithm decrease of the bacterial population.

(ii) The Weibull model was as follows:

$$\log_{10} N(t) = \log_{10} N_0 - \left(\frac{t}{\delta} \right)^p \quad (2)$$

Where δ is the first-decimal reduction time (hours) and p is a shape parameter

(iii) The biphasic mixed-Weibull model, which assumed the existence of two sub-populations, one more sensitive and one more resistant sub-population (Coroller *et al.*, 2006), was determined with the following equation:

$$N(t) = \frac{N_0}{1 + 10^\alpha} \left[10^{-\left(\frac{t}{\delta_1} \right)^p + \alpha} + 10^{-\left(\frac{t}{\delta_2} \right)^p} \right] \quad (3)$$

Similarly, p and δ parameters represent the shape parameter and the first decimal reduction time, respectively. Index 1 refers to the more sensitive sub-population while index 2 refers to the more resistant sub-population. α corresponds to $\log_{10}(N_{01}/N_{02})$ and is function of the proportion of each sub-population in the inoculum N_0 . For instance, when $\alpha = 2$, this means that 1 inoculated cell belongs to sub-population 2, while 100 cells belongs to the sub-population 1; and when $\alpha = 3$, this means that 1 inoculated cell belongs to the sub-population 2, while 1,000 belongs to the sub-population 1.

Primary model fitting performances. The three models were used to fit the inactivation kinetics obtained for three replicates, for each experimental condition, using the minimization of the sum of

square error. The minimum values were computed with a non linear fitting module (LSQCURVEFIT, MATLAB 6.5.1, Optimization Toll box, The Mathworks, Massachusetts, USA). To evaluate the model-fitting performances and to select the most adequate model the r^2 , the MSE , the AIC_c and the F test were calculated as indicated below. The regression coefficient r^2 is the proportion of the total variation of the data explained by the model. The value shall range between 0 and 1. The higher the value is, the better the fitting of the model is (den Besten *et al.*, 2006). Regarding the mean square error of the model value (MSE_{model}), the lower the value is, the better the model is to describe the experimental data (den Besten *et al.*, 2006). The corrected Akaike Information Criterion (AIC_c) (Hurvich and Tsai, 1995), allows comparison of models by taking both the goodness of fit and the parsimony into account. The lower the AIC_c value is, the best the model is. The F test was used to decide whether the fitting performance of a model was statistically accepted. The f value was compared to F table value (0.05 significance level). If the calculated f value is smaller than the F value from *the* table, the F test is accepted and indicates that the model fitting is statistically acceptable.

Primary model simplification. After the selection of the most adequate model used to fit experimental kinetics (using the criteria mentioned above), the model was simplified to enable easy comparison of bacterial population behaviour. The simplification was validated only if the AIC_c value obtained with the simplified model was lowest than the AIC_c obtained with the initial model. In this study, simplifications concerned the equivalence of the inactivation kinetic shape parameter (ρ) to directly compare calculated acid resistance as well as the proportion of the two sub-populations (α) to fix one α per physiological state studied (control, salt- and acid-adapted cells).

The parameters of the simplified model were then estimated with a non linear fitting module (LSQCURVEFIT, MATLAB 6.5.1, Optimization Toll box, The Mathworks, Massachusetts, USA) and their confidence intervals calculated using the well known re-sampling method called the bootstrap method, with 10,000 iterations (Huet *et al.*, 2004).

Secondary model fitting. This modelling step enabled to quantify the impact of lethal pH conditions on the acid-resistance. For both adapted and control cells, the influence of environmental factor on the inactivation rate was evaluated using a Bigelow-like model described by the following equation:

$$\log_{10}(\delta) = \log_{10}(\delta^*) - \frac{pH^* - pH}{z_{pH}} \quad (8)$$

where δ is the first reduction time, pH^* is the reference pH treatment fixed, and z_{pH} is a distance of pH from pH^* , which leads to a 10-fold reduction of the δ value. The reference pH used was arbitrary

fixed at 4.6 since this lethal treatment was applied to every bacterial physiological state tested. The parameters were estimated with a fitting module (NLINFIT, MATLAB 6.5.1, Optimization Toll box, The Mathworks, Massachusetts, USA). Then independent t tests (two-tailed) were performed to compare the different estimated z_{pH} *i.e.* the different estimated cell sensitivities, and thereby the influence of environmental pH on adapted and non-adapted cells.

Results

The psychrotolerant strain *B. weihenstephanensis* KBAB4 was cultured until the exponential growth phase (i) at low pH (acid-adapted cells), (ii) in the presence of salt (salt-adapted cells), and (iii) in media without supplementation (control cells). Subsequently, the acid resistance of control cells (pH 4.40 to pH 4.70), salt-adapted (pH 4.50 to pH 4.70) and acid-adapted (pH 4.45 to pH 4.70) cells were assessed following an exposure to low pH ranging from 4.40 to 4.70 (Figure 1). At pH 4.40, 4 log population was inactivated after less than 30 minutes of acid exposure (data not shown). This fast decrease was followed by a few bacterial enumerations which did not allow survival curve fitting. At pH 4.80 a bacterial growth was observed (data not shown), that is consistent with previous works (Baril *et al.*, 2012). These observations emphasize the limited range of pH that may be used in order to study the acid resistance of *B. weihenstephanensis* KBAB4.

Assessment of primary model adequacy and model selection. To assess the bacterial acid resistance, the inactivation kinetics of *B. weihenstephanensis* were fitted using three microbial survival mathematical models. The fitting performance of these models was evaluated in order to select the most adequate model to quantify acid bacterial resistance and the effect of preliminary saline and acid mild stresses on it.

To compare the fitting performances of the three models, MSE_{model} , r^2 , AIC_c and the f value were calculated for 12 tested conditions performed in three replicates (Table 1). Using the F test, the linear model appeared statistically acceptable for the two third of inactivation experiments and the Weibull model appeared acceptable for most of the experimental conditions, except for 2 conditions *i.e.* salt-adapted cells submitted to pH 4.60 and for acid-adapted cells exposed to pH 4.70. Unlike the first models, the mixed-Weibull model was acceptable for all experimental conditions despite that in some cases no biphasic pattern was observed on the survival kinetics (at pH 4.70 and 4.55) and in these cases only one δ value was estimated ($\delta_1 = \delta_2$). This observation was reflected by the AIC_c and

the MSE_{model} criteria which highlight that the mixed Weibull model is over parameterized and could be simplified.

Primary model simplification. Despite that the mixed-Weibull model was the most suitable to describe the bacterial kinetics, the number of model parameters could be reduced to lower the complexity of the secondary model analysis. The following stepwise procedure was done and validated using the AIC_C criterion. Indeed, the AIC_C value was calculated at 56.55 for the mixed-Weibull model while it was equal to 8.36 for the simplified mixed-Weibull model. In studied conditions, all inactivation kinetics showed a similar concave shape, underlying close bacterial resistance distributions within the population, whatever the pre-incubation conditions or stress intensity were. Thus a single p value was determined for all inactivation kinetics of *B. weihenstephanensis* KBAB4. As recommended by Couvert *et al.* (2005), the improvement of the fitting quality was performed using a global p value that was estimated with the whole set of data. This further means that each kinetic point has the same weight in the value estimation and thus associated δ values could be directly compared. Furthermore, since the α parameter varies according to the cell pre-treatment exposures (Coroller *et al.*, 2006) only one α was estimated for salt-adapted, acid-adapted and control cells corresponding to three physiological states. As for the estimation of p , α parameters were estimated using the whole set of targeted data.

Effect of stress intensity and physiological state on acid resistance. After selection of the most adequate model, the estimated parameters were used to compare the different experimental conditions. Only one p value was estimated at 2.33 ± 0.06 for the whole set of data. The α parameters were estimated at 2.51 ± 0.21 , 2.04 ± 0.08 and 2.17 ± 0.28 for control, salt-adapted and acid-adapted cells, respectively. Thus, no significant variation ($p > 0.05$) was observed in the proportion of the two sub-populations whatever the physiological state. Therefore the variation of bacterial resistances as function of the physiological state (control salt-adapted and acid-adapted cells), or the stress intensity (lethal pH ranging from 4.40 to 4.70) was represented by the variation of δ_1 and δ_2 values (Table 2). Whatever pre-incubation conditions, and the sub-populations, it was not surprising to observe that the bacterial resistance decreased when the stress intensity increased. Furthermore, salt-adapted cells were less resistant than control cells; and the latter less resistant than acid-adapted cells ($p < 0.05$).

Pre-incubation conditions	Acid lethal conditions		Linear	Weibull	Mixed-Weibull	Simplified Mixed-Weibull
BHI, 30°C	pH 4.70	r^2	0.914	0.891	0.861	0.865
		MSE_{model}	0.143	0.131	0.130	0.150
		AIC_C	33.635	31.827	34.735	34.243
		f	0.914	0.836	0.833	0.956
BHI, 30°C	pH 4.65	r^2	0.907	0.915	0.916	0.922
		MSE_{model}	0.181	0.122	0.094	0.105
		AIC_C	36.199	25.795	21.278	18.898
		f	1.608	1.085	0.837	0.931
BHI, 30°C	pH 4.60	r^2	0.937	0.921	0.926	0.924
		MSE_{model}	0.144	0.132	0.099	0.121
		AIC_C	25.886	25.065	21.131	20.503
		f	1.148	1.052	0.789	0.966
BHI, 30°C	pH 4.55	r^2	0.940	0.947	0.927	0.920
		MSE_{model}	0.081	0.053	0.056	0.072
		AIC_C	16.100	-0.856	4.179	10.009
		f	1.335	0.865	0.912	1.181
BHI, 30°C	pH 4.50	r^2	0.791	0.724	0.672	0.718
		MSE_{model}	0.422	0.410	0.383	0.392
		AIC_C	59.712	61.608	60.278	56.636
		f	0.890	0.865	0.808	0.827
BHI, 30°C	pH 4.45	r^2	0.894	0.931	0.920	0.918
		MSE_{model}	0.187	0.089	0.080	0.098
		AIC_C	40.721	17.583	17.496	18.352
		f	1.930	0.919	0.821	1.008
Salt 2.5% BHI, 30°C	pH 4.70	r^2	0.956	0.938	0.926	0.922
		MSE_{model}	0.071	0.073	0.067	0.084
		AIC_C	9.047	11.344	10.409	14.432
		f	0.894	0.918	0.845	1.065
Salt 2.5% BHI, 30°C	pH 4.60	r^2	0.883	0.851	0.934	0.903
		MSE_{model}	0.214	0.199	0.069	0.119
		AIC_C	43.856	42.838	16.323	24.263
		f	3.616	3.357	1.160	2.018
Salt 2.5% BHI, 30°C	pH 4.50	r^2	0.978	0.969	0.983	0.977
		MSE_{model}	0.082	0.085	0.041	0.065
		AIC_C	8.427	10.978	3.401	3.757
		f	2.104	2.197	1.049	1.676
pH 5.5 BHI, 30°C	pH 4.70	r^2	0.942	0.986	0.989	0.988
		MSE_{model}	0.086	0.015	0.009	0.012
		AIC_C	17.483	-49.231	-64.554	-60.005
		f	11.817	2.067	1.293	1.660
pH 5.5 BHI, 30°C	pH 4.60	r^2	0.945	0.947	0.961	0.962
		MSE_{model}	0.098	0.068	0.039	0.045
		AIC_C	19.207	8.774	-6.876	-7.139
		f	2.162	1.513	0.857	1.001
pH 5.5 BHI, 30°C	pH 4.45	r^2	0.914	0.981	0.985	0.983
		MSE_{model}	0.247	0.040	0.026	0.036
		AIC_C	32.811	-3.470	-8.699	-8.378
		f	8.671	1.411	0.915	1.271

Table 1: Statistical indices calculated to select bacterial survival mathematical model used to fit *B. weihenstephanensis* inactivation kinetics. Bold value indicates that the F test was acceptable.

Pre-incubation conditions	Acid lethal conditions	Log δ_1 (h)	Log δ_2 (h)
BHI, 30°C	pH 4.70	0.58 ± 0.08	-
BHI, 30°C	pH 4.65	0.37 ± 0.03	0.66 ± 0.07
BHI, 30°C	pH 4.60	0.22 ± 0.07	0.59 ± 0.10
BHI, 30°C	pH 4.55	0.29 ± 0.01	-
BHI, 30°C	pH 4.50	-0.16 ± 0.10	0.18 ± 0.20
BHI, 30°C	pH 4.45	-0.29 ± 0.06	-0.16 ± 0.18
Salt 2.5% BHI, 30°C	pH 4.70	0.03 ± 0.07	0.44 ± 0.03
Salt 2.5% BHI, 30°C	pH 4.60	-0.18 ± 0.02	0.09 ± 0.03
Salt 2.5% BHI, 30°C	pH 4.50	-0.35 ± 0.04	0.02 ± 0.07
pH 5.5 BHI, 30°C	pH 4.70	0.54 ± 0.01	0.84 ± 0.03
pH 5.5 BHI, 30°C	pH 4.60	0.36 ± 0.01	0.69 ± 0.03
pH 5.5 BHI, 30°C	pH 4.45	-0.02 ± 0.02	0.15 ± 0.01

Table 2: The log of the first decimal decrease in hour (\pm standard deviation) for the more sensitive sub-population (δ_1) and the more resistant sub-population (δ_2) estimated using a mixed-Weibull model with a single p value at 2.33 ± 0.06 and an α parameter of 2.22 ± 0.21 for control cells (BHI, 30°C), 2.04 ± 0.08 for salt-adapted cells (Salt 2.5%, BHI, 30°C) and equal to 2.17 ± 0.28 for acid adapted cells (pH 5.5, BHI, 30°C).

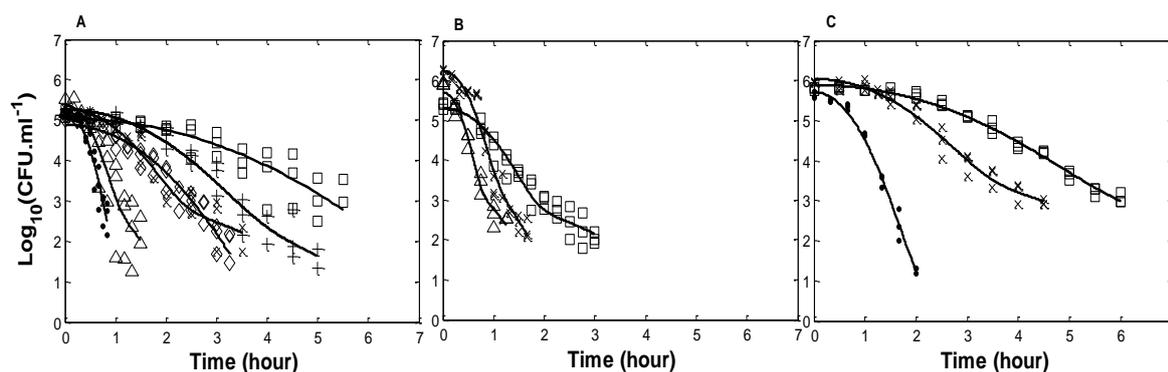


Figure 1: Acid inactivation kinetics of *B. weihenstephanensis* KBAB4 cells cultured in BHI *i.e.* control cells (A), in BHI supplemented with 2.5% NaCl, *i.e.* salt-adapted cells (B) and, in BHI supplemented with HCl *i.e.* acid-adapted cells (C) during exposure to an acidic lethal conditions at pH 4.70 (\square), 4.65 ($+$), 4.60 (\times), 4.55 (\circ), 4.50 (Δ) and 4.45 (\bullet). Continuous curves correspond to the fitting of experimental data using a simplified mixed-Weibull model.

To quantify the influence of environmental factor on the bacterial resistance for both adapted and control cells a secondary Bigelow-like model was used. The sensitivity of cells to the pH variation or the influence of the acidic environment on the bacterial resistance was estimated by the z_{pH} value. z_{pH} value corresponds to the slope of the log of δ as a function of pH, which therefore represents the pH change leading to a 10 fold reduction of the δ value. Using the Bigelow-like model the log of the bacterial resistances at reference pH (4.60) were estimated for both sensitive and resistant sub-populations of control salt-adapted and acid-adapted cells of *B. weihenstephanensis* (Table 3).

Pre-incubation conditions	Sub-population	Bacterial resistance pH 4.6 ($\text{Log}_{10} \delta$)	z_{pH} (pH unit)
BHI, 30°C	Sensitive	0.24 ± 0.05	0.30 ± 0.03
BHI, 30°C	Resistant	0.41 ± 0.08	0.32 ± 0.05
Salt 2.5% BHI, 30°C	Sensitive	-0.17 ± 0.04	0.52 ± 0.06
Salt 2.5% BHI, 30°C	Resistant	0.18 ± 0.07	0.48 ± 0.10
pH 5.5 BHI, 30°C	Sensitive	0.32 ± 0.02	0.45 ± 0.02
pH 5.5 BHI, 30°C	Resistant	0.60 ± 0.06	0.36 ± 0.03

Table 3: Estimated parameters using the Bigelow-like model for each subpopulation of adapted and non-adapted cells of *B. weihenstephanensis*.

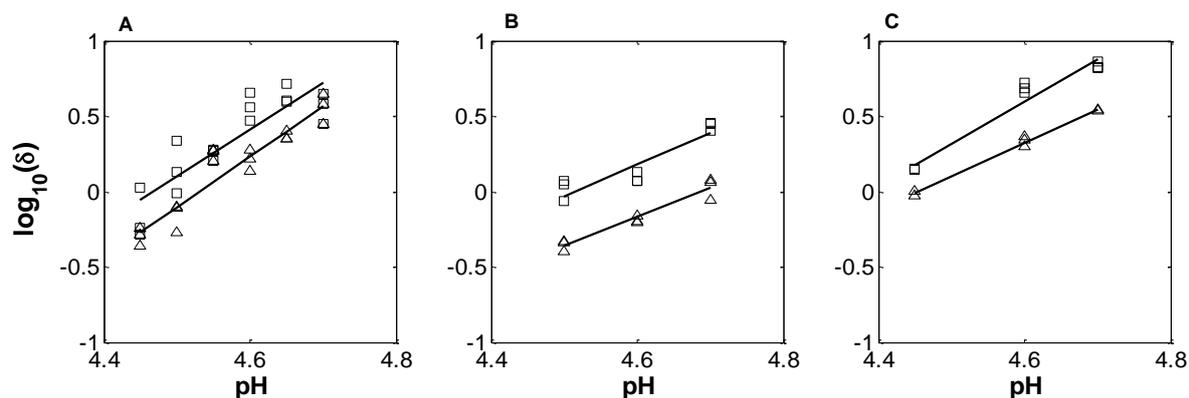


Figure 2: First decimal decrease of the most sensitive sub-population, δ_1 (Δ) and the more resistant sub-population, δ_2 (\square) estimated using a mixed-Weibull model as a function of the pH intensity. Secondary model is represented for control cells pre-cultured in BHI(A), salt-adapted cells pre-cultured in BHI with 2.5%NaCl(B) and; (C) acid-adapted cells pre-cultured in BHI supplemented with HCl. Continuous lines correspond to estimated values of the secondary Bigelow-like model determined for each sub-population.

Naturally, the resistant sub-population showed enhanced resistance at reference pH as compared to the sensitive sub-population. In the mean time for tested conditions, the bacterial sensitivity of each sub-population was estimated. Since these z_{pH} values were not significantly different ($p < 0.05$), the whole set of δ values was used to estimate only one z_{pH} (0.37 ± 0.04) whatever the sub-population and the conditions of adaptation (validated by a F test). Therefore an estimated variation of 0.37 pH unit will yield to a 10 fold variation of the bacterial resistances, whatever the pre-incubation condition or the sub-population studied. The determination of the sensitivity (z_{pH}) underlines that the bacterial resistance followed the same pattern when facing acidic changes of the environment.

Discussion

In minimally processed foods, various hurdles are combined to control food microbial quality and safety. Previous studies demonstrated that the adaptive stress responses of microorganisms strongly impact on their resistance (e.g., see Browne and Dowds, 2001 and 2002; Hill *et al.*, 2002;

Koutsoumanis *et al.*, 2003; Skandamis *et al.*, 2008) and should therefore be addressed in quantitative microbial risk assessments when referring to pathogen or spoilage organism inactivation throughout the food chain. In this study the acid resistance and sensitivity of control, salt-and, acid-adapted cells of *B. weihenstephanensis* were quantified using linear and non linear models.

The fitting performance of the mixed-Weibull model was considered as the best to quantify the acid bacterial resistance. This primary model assumes that the population is divided into two sub-populations with different acid resistance characteristics and thus allows to highlight a more resistant sub-population. On the one hand, acid-adapted cells showed an enhanced acid resistance as compared to control cells. This enhanced resistance to severe acid environment after preliminary exposure to mild acid environment was already reported in various species such as *Salmonella*, *Listeria*, *Vibrio*, *Bacillus* and *Escherichia* (Browne and Dowds, 2002, Cheng *et al.*, 2003; Chen *et al.*, 2009; Foster and Hall, 1990; Kroll and Patchett, 1992; Leyer *et al.*, 1995, Senouci-Rezkallah *et al.*, 2011). On the other hand, salt-adapted cells appeared less resistant to subsequent lethal pH exposure than control cells. This is also in agreement with previous finding on *B. cereus* ATCC 14975 (den Besten *et al.*, 2010). Therefore, prior exposure of *B. weihenstephanensis* to mild stress environments result in enhanced (acid-adapted cells) or reduced acid resistance (salt-adapted cells). Since during food processing, bacteria may be exposed to multiple and successive hurdles (Leistner, 2000), considering cell history or physiological state is crucial and may strongly impact on the resistance to further stresses. Knowing that *B. weihenstephanensis* cells will be less acid resistant after prior exposure to salt conditions is of practical importance to refine food processes. Furthermore, to evaluate the impact of pH variation on the bacterial resistance the sensitivities were quantified for the three different physiological states. Whatever the pre-incubation conditions (adapted and non-adapted cells) or the sub-populations studied (acid resistant and acid-sensitive), the acidic change will affect the variation of the bacterial resistance in the same way. This information may be a key issue for industrial process optimisation since whatever the contaminant physiological state (cell history) a decrease of 0.37 pH unit will lead a 10 fold reduction of acid resistance.

These findings were also reported for *B. weihenstephanensis* and *B. licheniformis* spores for which the heat sensitivity was not modified whatever the sporulation environmental conditions tested (Baril *et al.*, 2011a). Therefore it may be supposed that the bacterial sensitivity of a given strain (z value) is mainly dependent of physico-chemical environment, whereas the bacterial resistance (δ value) is affected by both cell history and the stress intensity. In the case this assumption is generalised, the food environment variation would have similar impact on the bacterial resistance whatever cell history. This simplification may be of interest for the calculation of process efficiency.

However the bacterial physiological state of the initial food contaminant needs to be known and correlate to the bacterial resistance. That underlines the necessity to find molecular biomarkers that could reflect the bacterial fitness and which could be easily quantified. Indeed, finding molecular biomarkers to characterise the physiological state under specific conditions is today considered as a key issue for the area of food safety and quality (den Besten *et al.*, 2010; den Besten *et al.*, 2012; Desriac *et al.*, 2012; Kort *et al.*, 2008). Thus, the combination of molecular tools and predictive microbiology concepts appears to be an interesting challenge for food formulation and preservation optimization (Brul *et al.*, 2012, Desriac *et al.* 2012, Havelaar *et al.*, 2010, Mc Meekin *et al.*, 2008, Rantsiou *et al.*, 2011).

Conclusion

This study highlights that the pre-incubation conditions, and therefore the bacterial physiological state, strongly impacts bacterial acid resistance without affecting the sensitivity to acidic modifications. Quantification of such adaptive stress response might be instrumental to evaluate the survival potential of a cell for successive hurdles and thus to evaluate the bacterial exposure of consumers. Knowing bacterial sensitivity yield the possibility to offer tailor-made approach for food industry process optimization. Nevertheless, one of the key challenges will be to find bacterial behavior biomarkers in order to quantify the bacterial physiology throughout food process and thus offer decision making tools for food safety and quality management.

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Chapter 4

The step by step RT-qPCR quality assurance procedure set up to identify biomarkers of *Bacillus weihenstephanensis*

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Abstract

Omics data bases have exploded, opening the avenue to take the bacterial physiological state into consideration in Microbiological Risk Assessment (MRA) through the integration of molecular biomarkers. Gene expression levels are recognized as relevant biomarker to describe bacterial behaviour and reverse transcription quantitative PCR (RT-qPCR) is considered as the gold standard for accurate, sensitive and fast measurement of gene expression. However numerous critical points may arise throughout the entire workflow of RT-qPCR data acquisition influencing accuracy of the results and reliability of the conclusions. That is why in 2009, the minimum information for publication of quantitative real-time PCR experiment, named MIQE, was published. However publications still lack of transparency regarding the key assay parameter validations for accurate design and reporting of qPCR experiments in publication, in particular dealing with *Bacilli*. Herein, the step-by step RT-qPCR quality controls established for the selection of *Bacillus weihenstephanensis* resistance biomarkers was described. Throughout this example, appropriate quality procedures and quality controls that shall be set up and carefully assessed from the sample preparation to the data analyses to ensure reliable interpretations in RT-qPCR were depicted. The results led to the identification of *B. weihenstephanensis* resistance biomarkers to lethal acid stress (pH 4.6).

Introduction

Predictive microbiology is one of the Microbiological Risk Assessment (MRA) components as described in the European Regulation EC2073 (2005). The MRA and related modelling approaches are usually seen as particularly appropriated to governmental food safety organizations and the related decision-making tasks and tools. But this is also of relevance to numerous industrial applications, such as shelf-life determination, heat treatment optimisation, raw material selection, assessment of non thermal inactivation processes and new formulation development. Food predictive microbiology models were for many years mainly developed using culture based methods and worst case *scenari* to ensure food safety. It is now commonly recognized that this yields to over or under estimations in microbial quantitative risk assessment, and could be improved by integrating the bacterial and related cellular adaptive response to various stresses. In the mean time, transcriptomics, proteomics, and more generally Omics databases have exploded, offering wonderful opportunities to develop new methodologies to qualify cellular physiology and improve risk assessment (Brul *et al.*, 2012; Havelaar *et al.*, 2010; Mcmeekin *et al.*, 2008; Rantsiou *et al.*, 2011). Indeed, this will allow the identification of genes that encode key proteins involved in stress survival, cell recovery and growth, but as well information related to bacterial resistance or sensitivity and descriptive parameters that will further be incremented in mechanistic models (Brul *et al.*, 2012; den Besten *et al.*, 2010; Desriac *et al.*, 2012).

The application of Omics technologies is expected to play an important role in understanding how bacteria can adapt to stress conditions. One of the major challenges will be the use of data generated by these new techniques with the required quality assurance procedures in decision making related food safety and quality issues. This work focuses on quantification of transcriptomic biomarkers of *B. weihenstephanensis*, *i.e.* a characteristic that is objectively measured and evaluated as a biologic processes indicator (Atkinson *et al.*, 2001), in order to improve predictive microbiology accuracy. Applications of reverse transcription combined to quantitative PCR (RT-qPCR) to study population dynamics and activities through quantification of gene expression are already available and appear as a new trend in bacterial behaviour descriptions (Carey *et al.*, 2009; Falentin *et al.*, 2010; Senouci-Rezkallah *et al.*, 2011; Torriani *et al.*, 2008; Ulve *et al.*, 2008). While RT-qPCR offers many practicability and sensitivity advantages, numerous quality controls shall be checked all among the entire workflow, in order to confirm the robustness of the quantitative results (Bustin *et al.*, 2009; Bustin *et al.*, 2010).

Indeed, in 2009, Bustin and coworkers published the MIQE Guidelines due to the lack of consensus existing on how to perform and interpret quantitative real time (qPCR) experiments. In 2010, they proposed an abridged set of guidelines for covering the key parameters of every qPCR assay (Bustin *et al.*, 2010) essential for allowing reviewers, editors and readers to evaluate the technical merits of scientific publications. However this study reported the RT-qPCR quality controls mentioned in 25 reviewed publications (Borgmeier *et al.*, 2011; Brillard *et al.*, 2010; Broussolle *et al.*, 2010; Cadot *et al.*, 2010; Ceuppens *et al.*, 2013; Dommel *et al.*, 2011; den Besten *et al.*, 2010; Desriac *et al.*, 2012; Frenzel *et al.*, 2010; Frenzel *et al.*, 2012; Garcia *et al.*, 2010; Guillemet *et al.*, 2013; Huillet *et al.*, 2012; Kovac *et al.*, 2013; Lindbäck *et al.*, 2012; Martinez-Blanch *et al.*, 2011; Melegard *et al.*, 2011; Pandiani *et al.*, 2010; Park *et al.*, 2012; Reiter *et al.*, 2011; Senouci *et al.*, 2011; Sklyarova *et al.*, 2012; Velho *et al.*, 2011, Zhu *et al.*, 2011) relying on *Bacilli* since 2010 (Table 1) and still shows a lack of transparency regarding validation of the key assay parameters. Indeed, only 1 to 2 publications mentioned the major criteria such as the absence of RT-qPCR inhibitors, the use of inter-run calibrators (IRCs), or the limit of detection of the method. Interestingly, even though the other criteria listed are more often mentioned, none is systematically reported.

% of publications detailing information	
RT-qPCR design	
Accession number	56%
Number of replicate	80%
Inter-run calibration IRCs	8%
Assay optimisation and validation	
Primer and probe sequences	84%
<i>In silico</i> /empirical validation	32%
Amplicon size	24%
PCR efficiency	40%
PCR specificity	48%
Limit of detection	8%
RNA extraction/RT-PCR	
RNA quantity	84%
RNA quality	32%
DNA free ^a	68%
Inhibition free	4%
Priming conditions for RT	72%
Reagent/protocols	72%
NTC	24%
Data analysis	
Efficiency correction	48%
Reference genes normalization ^b	92%
Stability reference genes	28%
Statistical analysis	36%

Table 1: Key criteria mentioned in RT-qPCR experiments articles dealing with *Bacillus* since 2010. ^a Article which mentioned DNase treatment was assigned as DNA free even though control of the DNA treatment efficiency was not

mentioned.^b Note that 92% of articles mentioned a normalization using reference gene nevertheless only 16% used multiple reference genes normalization with at least 3 reference genes as advised by Vandesompele *et al.*, 2002.

Herein, the RT-qPCR quality assurance procedure (Figure 1) applied for the search of resistance biomarkers of *B. weihenstephanensis* to acidic stress is detailed in order to allow accurate and reliable analyses of gene expressions. Note that, if some criteria could be done only when validated the assay procedure, some other shall be done for each RT-qPCR sample and are indicated in bold in the following figure.

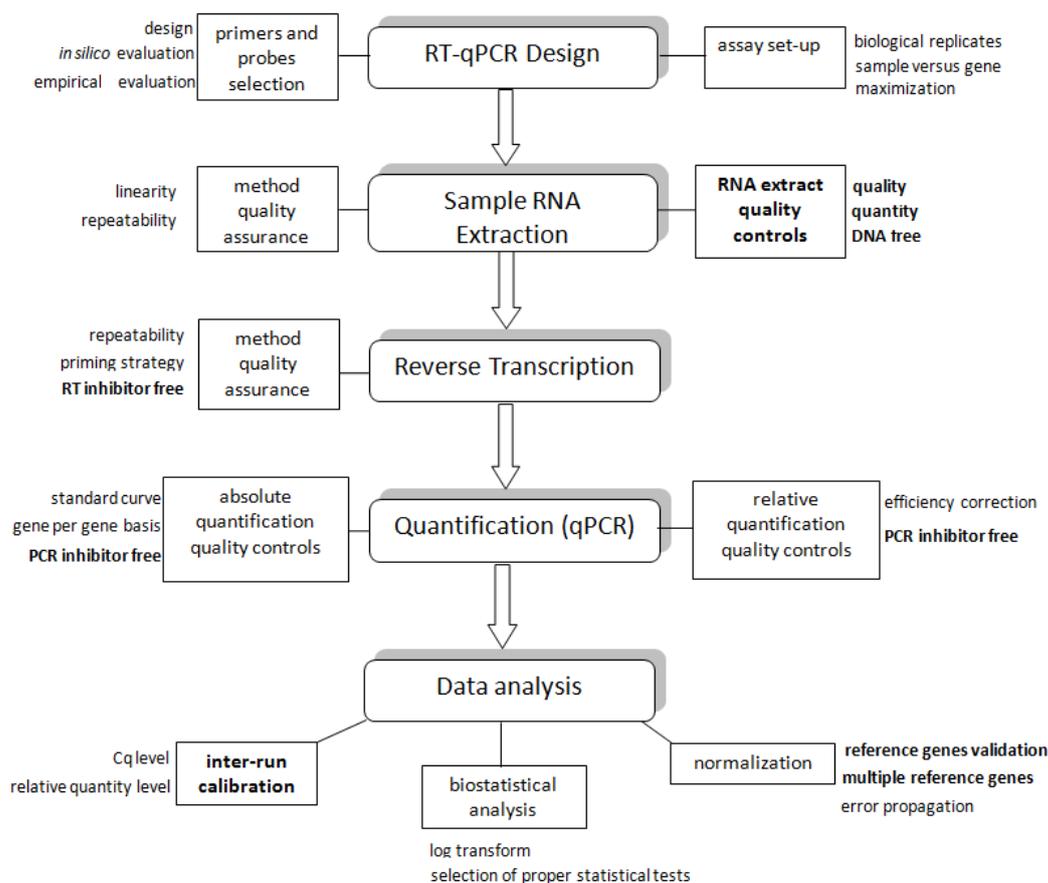


Figure 1: Key criteria required for accurate and reliable biomarker quantification using RT-qPCR mRNA quantifications. This workflow represents the different working steps and associated quality controls performed in this study. Procedure validation implies indicated key criteria which shall be done at least one time when setting up the assay. While in bold quality, controls which shall be done for each RT-qPCR samples.

RT-qPCR design and calibration

Proper set-up of the experiment saves time, decreases reagent cost and increases the precision and accuracy of the results. Nevertheless, the experimental design is one of the most neglected points in setting up an RT-qPCR methodology procedure. In this study, each biological sample was done in three independent replicates, from the bacterial sampling to the RT-qPCR quantitative data till

independently reproduced data which shall be produced to test response differences (Derveaux *et al.*, 2010).

For PCR quantification, a specific rotative PCR device based on the Pall GeneDisc Technology was developed to facilitate data acquisition and decrease data dispersion errors. Briefly, GeneDisc® plate disposable device (Pall GeneDisc Technologies, Bruz, France) is composed of 36 PCR wells preloaded with specific primers and probes for the detection of 36 different cDNAs within a single PCR run (Table 2). Primers and TaqMan® probe sets were designed in order to be used with the same PCR cycling program. This tailor made GeneDisc® plate disposable device offers multiplexing capacity as it simultaneously runs 36 real-time PCR tests for 1 sample load.

To correct run to run variation, so-called inter-run calibrators (IRCs) were used. These IRCs consist in three known genomic DNA concentrations and were performed on a gene per gene basis for each qPCR device batch. The calibration was done by the quantification cycle (Cq) measurement as described by Vermeulen *et al.*, 2009. Briefly, the Cq average difference value resulting from three DNA quantities for a given gene from two different device batches were used as the gene specific Cq IRC factor to obtain calibrated Cq values. The estimated Cq IRC factor obtained for each gene varied in absolute values from 0.00 to 3.27 Cq, underlying the necessity to correct run to run or batch to batch variations on a gene per gene basis.

Assay optimisation and validation

Primers and probes were at first manually designed then *in silico* and empirically evaluations were used in the validation step of the selected oligonucleotides. Oligonucleotide sequences were tested for specificity using NCBI's BLAST software and were compared to the whole *B. weihenstephanensis* KBAB4 genome. Oligonucleotides with BLAST score values for the targeted gene greater than 30 were retained whereas primers and probes pair with BLAST score values greater than 20 for unspecific genes were excluded. The Gibbs free energy (ΔG) of hairpin, dimer and cross-dimer was calculated using the Beacon Designer free edition tool for assessing the primer secondary structure stability. Primers and/or probes showing stable secondary structures were avoided according to ΔG values advised in the technical note of the software.

Gene name	Gene number	Annotations	Efficacy	r ²	Intercept	Amplicon size (bp)	Forward	Reverse	Probe
<i>16s*</i>	BcerKBAB4_0001	16S ribosomal RNA	97.2	0.988	42.23	112	AATGGACGGTACAAGAGC	TACTAGCGATTCCAGCTTCA	TCCGAACCTGAGAACGGTTTTATGAGA
<i>23s*</i>	BcerKBAB4_0004	23S ribosomal RNA	96.9	0.980	42.81	126	GCTGATAAGTAGGCAAAATCC	GGTACAGGCCACTTTTATCT	CCATCACAGCTCAGCCCTCAC
<i>atpA</i>	BcerKBAB4_5105	F0F1 ATP synthase	102.3	0.981	42.46	141	CTTAGAGAAAACAACGTAGG	CCTAATGGGTTTACAACACG	CTTACCGGATTTCTGTGAAGGCTCT
BcerKBAB4_0325	BcerKBAB4_0325	peroxiredoxin	98.6	0.995	44.52	94	GTATCTACAGACACTCACTTCA	GAGTTGGGTCCACCACATCA	CCGATAGTTCTTGAGCTATCATGGCCAT
BcerKBAB4_0904	BcerKBAB4_0904	Bacterioferritin	99.1	0.994	44.25	126	AGATTTAGCAGAGAAATACTCG	GCATGTCCCTGTTCAATCACTA	TACGAGAAACCGTAGCTGCATATGG
BcerKBAB4_1716	BcerKBAB4_1716	Major facilitator transporter	103.8	0.997	44.16	140	TGGCAGAGATTAGCAGT	AAGCTGAGGTTACAAATGCT	TGCAGAAAACAACGCTCAACTAAACGG
BcerKBAB4_2108	BcerKBAB4_2108	Aldehyde dehydrogenase	101.2	0.993	44.07	157	CCACTTTCTTCGTATGCATTG	CTACTTTGGGACTTCTGTAA	CTACCTACAACAGATCAAGGTCCA
BcerKBAB4_4399	BcerKBAB4_4399	EmrB/OacA family drug resistance transporter	101.7	0.987	43.14	99	TTTCTCACGGTACAGCAAC	GAGCAGTAGTTTGTGGCT	TTGAACCAAGCTACTTGTCTTGACGTA
<i>bkdR</i>	BcerKBAB4_4008	Transcriptional regulator	96.5	0.995	43.09	149	AGGAGATTTCAGAGAGAC	GTCTCTTACATTAGGACCAT	CTGGTATATCTCTTACCGTGAGGA
<i>clpC</i>	BcerKBAB4_0076	ATP-dependent Clp protease	101.9	0.990	43.74	142	CGAAGCTCGTAAGCTAGG	GGAGTTGTAACACTTGTGTG	TGCTGTACACTTCCACTTCCAG
<i>codY</i>	BcerKBAB4_3651	Transcriptional repressor	100.1	0.987	44.06	123	GAGAGATGCTGACACAATGT	GCATGTGCTTCATAGTTC	CGACGACTTACAACGAAACAGT
<i>ctsR</i>	BcerKBAB4_0073	Transcriptional regulator	95.7	0.991	44.08	136	CGTTAGAAAGAGGATTCGTAGT	CCTTGTGCAAGGCTATGAT	TAATGGGAATGTAACCTCTCTCCACT
<i>cydA</i>	BcerKBAB4_4629	Cytochrome bd-1 oxidase subunit I	97.9	0.985	43.62	146	GTGCTTCTGTATGTGGATG	TGTAACAAGAGGCCAGAAAT	CGCTTACTGTAACCTCACTCAATGTATGTTCT
<i>dps</i>	BcerKBAB4_1875	DNA-protecting protein	98.8	0.996	45.08	129	TCATTTGGTATGTGACAGGAC	GCTAACGGTTTTCTCTTAAC	TGCTCCAGATGCAACGCTGTAC
<i>fabF</i>	BcerKBAB4_1083	3-oxoacyl-(acyl carrier protein) synthase II	100.6	0.998	42.49	112	CAGAGTCAAGTGTCAAT	CGCTGGATTACTTTAAAGCG	TCACCTGCTTACTCTTAAGCAA
<i>groES</i>	BcerKBAB4_0245	Chaperone protein	96.1	0.995	44.82	114	CGAGTCTTGAATAATGGTG	AATCAAGTAGTCTGTACCTTCG	CCAATTGAACGCACATCTTCTCTATTG
<i>gyrA*</i>	BcerKBAB4_0006	DNA gyrase subunit A	104.0	0.992	43.05	147	TTGGGACAAAGTAACGGTATG	GAACATTTACATCTCTTCGCAC	CGCTTGTCTACTTCCGCAAAAGAG
<i>katA</i>	BcerKBAB4_1058	Catalase	96.0	0.998	44.79	136	ATCCACTAGATCCGACGAA	ACAGTTGCACTTGGAGTAA	CGCTTGTCTACTTCCGCAAAAGAG
<i>lacI</i>	BcerKBAB4_4066	LacI transcriptional regulator	100.1	0.993	43.20	149	CAATGTTATACGATGCAAGAC	TCTTCAAGTATGCGAATGC	TAAACCAAGCACTACTTGTATGCTGTCG
<i>lexA</i>	BcerKBAB4_3377	Transcriptional repressor	104.4	0.998	44.65	143	CCAATCATCGGAAAGTTACTG	GCTTCAATCATGCTATCTCA	CTACACTTCCGACTGCTGTAATCGGT
<i>mntH</i>	BcerKBAB4_1735	Manganese transport protein	104.9	0.990	43.95	138	GCTTTGGTAAATACTCGCTT	CATAAACCGATGGAACCTGG	TCAGGAAGATTTCTCCCAAGTAGCGAT
<i>napA</i>	BcerKBAB4_1526	Potassium efflux system protein	101.9	0.995	44.06	142	GCAGGACTTGAAACAGACT	AGACAGCATTCCCTTGTTC	CAACGTAACCACTACGAAATGGAAGA
<i>narL</i>	BcerKBAB4_1974	Respiratory nitrate reductase	103.9	0.991	43.13	142	GTGGTTCGAAAGTCTTTT	GGCACTAAATAACATGGACAAG	CCATACTGCGAAATAGACCCATACTGT
<i>nos</i>	BcerKBAB4_5238	Nitric-oxide synthase	103.9	0.982	43.12	112	ATTGATTCGGTATGCAAGAT	ATTCGTACTTCTCTTTCG	CGCAGTGGAAATGAGAGTCAACCAATC
<i>perR</i>	BcerKBAB4_0453	Peroxide regulator	96.4	0.992	45.63	110	TCCTTATGGAGGATGGAAAC	CCTTATGACACTTGGACAA	CGAAGCCTGCTGTTTTTTCAGCTT
<i>relA</i>	BcerKBAB4_4254	Pyrophosphokinase	98.7	0.997	42.41	105	ACTAGCTGATCGTCTTCAAC	TGTGCTAAAGGTGCAAAAGAT	TGCTTCTCTTGAGGCAAAATGTTTCA
<i>rsbW</i>	BcerKBAB4_0902	Anti- σ^H factor	102.8	0.994	42.84	128	TCGTTTAAACAATGGCTGCT	CTCCAACGCTCTTTTATATGC	TCTTCCATATCGTATAAGCAAAGCC
<i>sigA*</i>	BcerKBAB4_4144	RNA polymerase sigma factor	98.0	0.992	43.04	135	TTAGATGATGAGCAACTCG	ATCCTTAAGACGCTTACTACG	TTGAGGAATACGTTCTCTGTTACGC
<i>sigB</i>	BcerKBAB4_0903	General Stress Sigma Factor	100.5	0.995	44.32	94	AACTGGCGATAGAGAGCTA	AGAACTTCTCTTCGGATAGT	CGCAATCTATAAATCTTCCGTTGAAGC
<i>sigG</i>	BcerKBAB4_3730	Sporulation sigma factor	97.6	0.989	45.15	104	ACGATGGAGGAGATCCCTATC	ACGCTTACTTCTCTTTC	TTCTCAACCCCACTGCTCGCTCT
<i>sigH</i>	BcerKBAB4_0088	RNA polymerase sigma F	104.2	0.984	41.12	140	ACAAGCTGCTTCGTTCAA	TTCTCATCGTAAATGGCT	TTTTCGAGGTAGACACAGTTTCAGCA
<i>sigM</i>	BcerKBAB4_3069	Stress Sigma Factor	101.4	0.981	43.67	139	CAGAGAGATGATGATCGCAA	TCTAATACAGTAGCACCCCTCT	TAGCTTGCCTCTTACCACCTGG
<i>sodA</i>	BcerKBAB4_5239	Superoxide dismutase	91.7	0.980	44.60	107	ATCAACTTCCAAAGCAGCT	GGTGTATCTTGATGGGTTG	CAAGCCATCCATAGCCACTTCCAA
<i>spxA</i>	BcerKBAB4_1097	Transcriptional regulator	92.6	0.986	44.30	141	AGATGATTCGTGACTATCCAG	GCTGTCTTCCAGTAATTTG	CATCTTGGTTTAACTACTTCTGAAGG
<i>trxB</i>	BcerKBAB4_4951	Thioredoxin reductase	97.0	0.982	43.43	103	TGCAATATTCGTTTTCAAGCG	ACCATCAATACTGACCAAT	CCAAGTCTTCTTCCACTGGCACAC
<i>tuf*</i>	BcerKBAB4_0103	Elongation factor	95.9	0.992	44.93	114	CAACATCGGTCCTACTT	ACGAAAAACCTCAGCTTTGAA	CTTGTCCACCTGGATGCTCTCAC

Table 2: Targeted genes and potential reference genes optimized in this study. * potential reference genes

In order to avoid hinder primers and probes annealing due to the secondary structure of both targeted cDNA and PCR products, their folding structures were analyzed using the mFold Web Server with default settings, as well as 50 nM and 3 nM concentrations for Na^+ of Mg^{2+} , respectively, for an annealing temperature set at 64°C. The Figure 2 describes the positions of primers and probes selected for the 16S gene PCR product according to previously described assurance procedures. After *in silico* assay evaluations, primers specificity was assessed using a no template control qPCR (NTC) which may give no Cq and by scruting the melt curves resulting of qPCR runs with SYBR Green chemistry. The standard curves were further performed with hydrolysis probes in order to estimate the PCR efficiency. A dilution series of genomic DNAs were used as input material yielding to six concentrations from 2 log to 7 log of equivalent copy per genome (ECG) per PCR well. Note that the standard curve extremities (2 and 7 log of ECG), as well as the point 5 log of ECG were done in three replicates. All primer/probe sets showed satisfying amplification efficiency values comprised between 92 and 104%, a r^2 of at least 0.980, and closed intercepts 43.72 ± 0.98 (Table 2). Note that the standard curve could be based on known concentration of genomic DNA, cloned targeted sequence in linear or circular plasmid, RT-qPCR products or commercially synthesised oligonucleotides. However comparison study⁴¹ showed that targeted sequences were noticeably more stable than PCR products and thus standard quality and stability should be routinely tested for assays using absolute quantification by qPCR.

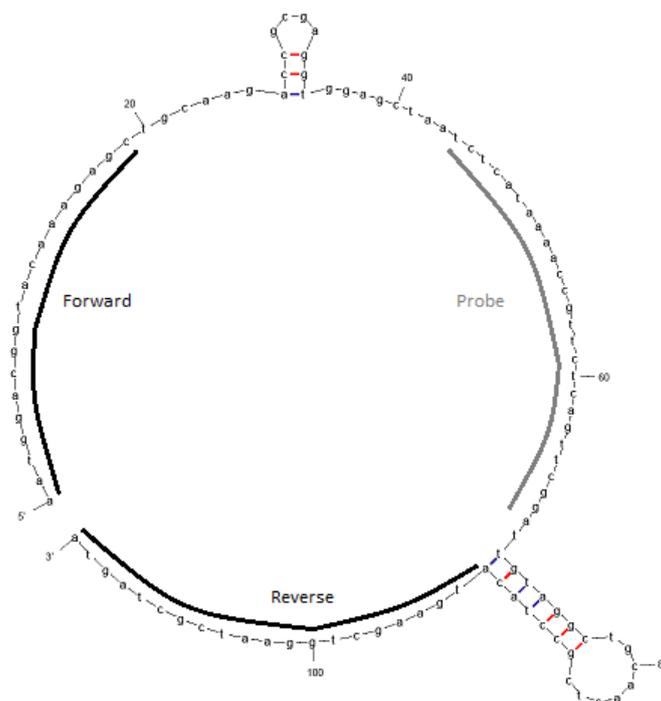


Figure 2: 16S cDNA mfold using performed by mFold Web Server using default settings for concentration of Na^+ of 50mM Na^+ , 3mM Mg^{2+} and a for temperature of 64°C. This mfold shows that no cDNA secondary structure could hamper the annealing of primers and probe.

RNA extraction

The accuracy of gene expression analyses is recognized to be influenced by both RNA quantity and quality. In this study, RNA extraction was performed using the RNeasy® Mini kit (Qiagen, Courtaboeuf, France), after sample stabilisation using RNA protect reagent (Qiagen) as recommended by the manufacturer. The two bacterial lysis technical procedures proposed were compared, and bacterial disruption was performed using lysozyme and proteinase K digestion. Indeed, the combination of enzymatic lysis using lysozyme and proteinase K digestion provided higher RNA extract concentration ($340 \text{ ng} \cdot \mu\text{l}^{-1} \pm 33.31$ for $10^8 \text{ CFU} \cdot \text{ml}^{-1}$) rather than a combination of lysozyme and mechanical disruption with bead beating ($125.33 \text{ ng} \cdot \mu\text{l}^{-1} \pm 22.51$ for $10^8 \text{ CFU} \cdot \text{ml}^{-1}$).

The linearity and the reproducibility of the RNA extraction step were evaluated from $10^3 \text{ UFC} \cdot \text{ml}^{-1}$ to $10^8 \text{ UFC} \cdot \text{ml}^{-1}$ using RT-qPCR with SYBR Green chemistry and *tuf* primers with standard curve which theoretically corresponds to $-3.382 \text{ Cq} + 41.310 \log_{10} \text{ ECG/PCR well}$. The linear correlation coefficient between the bacterial enumeration and the PCR quantifications was equal to 0.999, with a slope of 0.98 ± 0.05 and an intercept equals to 0.29 ± 0.26 (Figure 3A). These data suggested that the RNA extraction was reproducible (standard error < 10%) and linear between 10^3 to $10^8 \text{ UFC} \cdot \text{ml}^{-1}$.

The RNA quality was assessed using microfluidic analysis (RQI, Experion, Bio-Rad, Marnes la Coquette, France). RNA extracted from bacterial cells upon sub-lethal conditions (BHI, 30°C, $\pm \text{HCl pH } 5.5$, $\pm \text{NaCl } 2.5\%$, $\pm \text{H}_2\text{O}_2 \text{ } 0.1 \text{ mM}$) gave RQI value greater than 5 as advised by Fleige and Pfaffl⁴² (Figure 3B). For cells submitted to lethal conditions (BHI, 30°C, HCl pH 4.6), repeatable RQI of 2.4 ± 0.5 were estimated among 39 samples. Nevertheless, the RT-qPCR products are of 157 pb at maximum (Table 2) and Fleige and Pfaffl⁴² pointed out that short RT-qPCR products ranging from 75pb to 250pb are independent of the RNA quality. The variation of gene expression was studied using a normalisation step with reference genes in order to remove sampling differences, such as RNA quality, and identify real specific gene variation (Fleige and Pfaffl, 2006; Vandesompele *et al.*, 2002). Under these experimental conditions, the normalized expression differences measured with RT-qPCR using poor RNA quality as input material are expected to be similar to those obtained from high quality samples.

Contaminating genomic DNAs were removed from each RNA samples using the DNA-free™ Kit (Ambion, Cambridge, UK) according to manufacturer's recommendations. No template reverse transcription qPCR controls were then performed for each sample and indicated that contaminating genomic DNAs were successfully removed from RNA samples. Efficient removal of contaminating genomic DNAs was estimated over 100 bacterial samples.

Reverse transcription and quality controls

As basic statements, the total RNAs amounts, priming strategy and reaction conditions (Stahlber *et al.*, 2004 a and b) were fixed to avoid RT-qPCR variations due to the reverse transcription step and to compare gene expression measurements. An adjusted volume of RNA template corresponding to 200 ng of the total RNA extract was used to reverse-transcribed into cDNA with a blend of random hexamers using an iScript cDNA synthesis kit (Bio-Rad, Marnes la Coquette, France). The reaction was followed by a reverse transcriptase denaturation step (5 min at 80°C). The main advantage of this priming strategy was to reverse transcribed all mRNA at once and thus opens the avenue toward the study of any new targeted mRNA at will during the ongoing study.

The last RT-qPCR quality control assessment concerned the presence of potential inhibitors. For that purpose, dilutions of RNA samples were performed and quantified in three replicates by RT-qPCR. No significant variation in the estimated copy numbers ($p < 0.05$) was found, confirming the absence of both reverse transcription and qPCR inhibitors. Moreover, repeatability of the reverse transcription step was demonstrated.

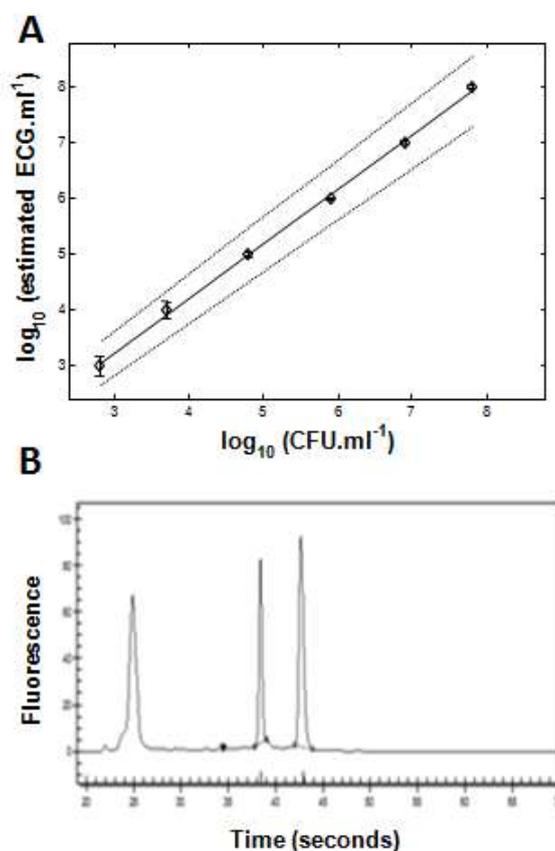


Figure 3: RNA extraction quality controls A- The linearity of the RNA extraction method with (○) the mean of three independent replicates. The error bars represent the standard error of the mean. The solid line represents the linear regression whereas the dashed lines represent the 95% confidence bound of the linear regression. B- Microfluidic

analysis of RNA extract quality with an RQI value estimated at 9.8 using the Experion Technology (Bio-Rad, Marnes la Coquette, France).

Data analysis

Two quantification strategies can be used in RT-qPCR assays. The levels of expressed genes may be measured by an absolute or a relative quantification. The absolute quantification approach relies on the PCR signal to input copy number using a calibration curve (Bustin, 2000; Pfaffl and Hageleit, 2001). While the relative quantification measures the relative change in mRNA levels across multiple samples as a function of the level of another RNA sample. The relative quantification strategy was indeed selected to facilitate gene expression comparison within a set of samples and to identify key biomarkers in bacterial metabolic response to environmental conditions. The determined C_q values were transformed to RNA quantities according to the delta C_q transformation. The calculation took into account the efficiency correction of each qPCR assay after getting the mean of the gene expression measurements for a given sample.

Several strategies, as well as their advantages and limits, have been described by Hugett *et al.* (2005) to ensure the reliability of the determined gene expression patterns, *i.e.* to remove experimental variation from the true biological variation. In 2005, the reference gene concept has been selected as the preferred way of normalizing RT-qPCR data (3rd London qPCR symposium, April 2005). The reference gene concept is particularly attractive since the reference genes are internal controls that are affected as the sample by all sources of variation during the RT-qPCR workflow. However, while reference genes have the intrinsic capacity to capture all non-biological variations, the literature describes substantial evidences that most of the commonly used reference genes are regulated under some circumstances (Dheda *et al.*, 2005; Wong and Medrano, 2005). Therefore it is strongly advised to check the expression stability of the selected reference genes at each sample level. In this study, five candidate genes (*16S*, *23S*, *tuf*, *sigA*, *gyrA*) were selected as potential reference genes for *B. weihenstephanensis*. In this case, the reference genes were expressed in the cell, and their mRNAs were submitted to same sampling, nucleic acid extraction, DNase treatment, storage, reverse transcription and real time PCR as targeted mRNAs. The stability of the gene reference mRNAs was evaluated as recommended by Vandesompele *et al.* (2002). The gene expression stability measure (*M*) was calculated as the average pair-wise variation for the considered mRNA in comparison to the other tested gene expressions. This measure relies on the principle that the expression ratio of two ideal reference genes is identical in all samples, regardless of the experimental conditions. Furthermore Vandesompele *et al.* (2002) turned out that three stables genes are necessary and sufficient for homogeneous samples. The calculated *M* values of *16S*, *23S* and *tuf* genes were of 0.67,

0.70 and 0.85, respectively, and thus these genes could be used as reference. The normalization factor was calculated as the geometric mean of reference genes quantities (Vandesompele *et al.*, 2002) and was used to underline the variation in gene expression throughout experimental samples. The error propagation rules were calculated as described by the authors of GeNorm using the standard error (SE) values since the SE adds confidence to the calculated mean. To identify relevant differences in gene expression between samples, the normalized relative gene expression was log transform making the data distribution more symmetrical (Derveaux *et al.*, 2010; Bengtsson *et al.*, 2005).

Accurate results for biomarkers identification

Using this method, the gene expression of *B. weihenstephanensis* mid-exponentially phase cells submitted to a lethal acid stress (pH4.6) was quantified throughout acid inactivation kinetics in order to identify some bacterial resistance biomarkers. The significance of the up-regulation of the log normalized results was analyzed using t-Tests. For instance, *spxA* and *katA* encoding respectively for a transcriptional regulator and a catalase were significantly up-regulated from 2h to 4h of acid inactivation time ($p < 0.05$). The highest up-regulation was observed at 2h of acid inactivation for *spxA* with $11.1 \text{ fold} \pm 2.6$ and at 4h for *katA* with 12.9 ± 0.1 fold more in acid stressed cells than in unstressed cells. These two genes are known to be involved in oxidative stress response and their up-regulation in *B. weihenstephanensis* during acid stress concurs with previous studies on the acid stress response of *B. cereus* (Mols *et al.*, 2010a and b). Other patterns were also observed with for instance *sigB*, encoding the general stress sigma factor, which was 19.5 ± 5.9 fold more expressed after 2h of acid inactivation whereas no significant variation was found after 3h and 4h of acid treatment. *narL* which encodes a nitrate reductase was significantly down-regulated throughout the acid inactivation kinetics. Therefore *spxA*, *katA* and *sigB* may be used as biomarkers to track acid resistance of *B. weihenstephanensis* as suggested by Desriac *et al.* (2012) whereas *narL* may serve as viability biomarker as suggested by Kort *et al.* (2008).

Conclusion

RT-qPCR is a fast, accurate, sensitive and cost-effective method for gene expression analyses, and is recognized as the gold standard to identify and select gene expression that may serve as molecular biomarkers of stress response. The relative quantification appears as an efficient strategy to identify

and select relevant biomarkers, while the absolute quantification plays an important role in the construction of mathematical models to further predict the bacterial behaviour in food industry process. However to ensure reliability of the observed RT-qPCR results, appropriate quality procedures shall be set up and the quality controls shall be carefully assessed from the sample preparation to the data analyses. This study underlines the lack of transparency regarding the RT-qPCR quality controls already advised by the MIQE and MIQE précis 3 years ago. Thus we proposed a workflow representing the different working steps and necessary associated quality controls, *i.e.* needed for both procedure validation and each RT-qPCR samples. This step by step schematical assay validation allows to ensure the reliability of RT-qPCR data acquisition and result interpretation. Bearing in mind that these quality procedures and controls are essential for using mRNA gene expression as biomarkers, they remain essential for all RT-qPCR experiments.

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Chapter 5

An integrative approach to identify *Bacillus weihenstephanensis* resistance biomarkers using gene expression throughout acid inactivation

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Abstract

The aim of this study was to define an integrative approach to identify resistance biomarkers using gene expression quantification and mathematical modelling. Mid-exponentially growing cells were transferred into acid conditions (BHI, pH 4.6) to obtain inactivation kinetics, performed in triplicate. The inactivation curve was fitted with a mixed Weibull model. This model allowed to differentiate two subpopulations with various acid resistances among the initial population. In parallel, differential gene expression was quantified by RT-qPCR. While *narL* was down-regulated throughout acid inactivation, *sigB* and *katA* were up-regulated. *sigB* expression up-regulation peak was correlated to the less resistant subpopulation when *katA* up-regulation, was correlated to the more resistant subpopulation. Moreover, differences in population structure were highlighted between each replicate. The higher proportion of the more resistant subpopulation was linked to a higher *katA* gene expression. These results suggest that *sigB* and *katA* might be used as different types of biomarkers, for instance to track moderate and high acid-resistance, respectively. The use of this approach combining RT-qPCR and predictive modelling to track cellular biomarker variations appears as an interesting tool to take into account physiological cell responses into mathematical modelling, allowing an accurate prediction of microbial behaviour.

Introduction

The *Bacillus cereus* group comprises six closely related species: *B. cereus* and *B. anthracis*, known as human pathogens, *B. thuringiensis* used as biopesticide, *B. mycoides* and *B. pseudomycoides* which are characterized by rhizoidal colonies on agar medium and *B. weihenstephanensis* psychrotolerant strains. This group is known for its ability to produce enzymes during vegetative growth that have been associated with food spoilage which causes high economic losses for the food industry (Gram *et al.*, 2002). Moreover, *B. cereus* is a well known food-borne human pathogen that causes two types of food poisoning. Diarrheic symptoms are due to the production of enterotoxins (hemolysin BL, HBL), non hemolytic enterotoxin (NHE) and cytotoxin CytK in the intestine after food consumption. Emetic symptoms are caused by the production of cereulide, an emetic toxin produced in food (Koritanta *et al.*, 2000; Lund and Granum, 1997; Lund *et al.*, 2000). To our knowledge, the *B. weihenstephanensis* species have never been correlated with food-borne diseases, but two strains have been shown to be emetic (Thorsen *et al.*, 2006) and some others, such as the KBAB4 strain, possess genes involved in enterotoxin production. In addition, *B. weihenstephanensis* may be of concern for food safety due to its potentiality to exchange toxin genes (Lapidus *et al.*, 2008). To preserve food quality and safety, combinations of hurdles are widely used in food production (Leistner, 2000) such as acidity, temperature (high or low), water activity, preservatives, redox potential and competitive microorganisms. Furthermore, the increasing demand for ready-to-eat foods raises the question of whether psychrotolerant bacteria present a hazard in these food products because of their ability to grow at refrigerated temperatures. *B. cereus* sensu lato can persist in acidic environments (Neumann and Martinoia, 2002) and upon ingestion of contaminated food, the microorganism has to survive gastric passage in order to reach the intestine (Clavel *et al.*, 2004, Wijnands *et al.*, 2009).

Food process optimisation and quantitative microbial risk assessment may be evaluated using mathematical models to predict microbial growth, survival and/or inactivation based on food physico-chemical features (Sanaa *et al.* 2004). Nowadays, genome sequencing and related 'omics' technologies offer a wealth of information to explore complex biological pathways in a quantitative and integrative manner. Transcriptomic analysis of the stress response of *B. cereus* paved the way for the identification of potential cellular biomarkers involved in bacterial survival, virulence or stress resistance (Ceragioli *et al.*, 2010; den Besten *et al.*, 2009; Mols *et al.*, 2010 a and b). Den Besten *et al.* (2010) reported the identification of biomarkers after correlating enzyme activity, protein content or gene expression with induced thermal resistance in *B. cereus* ATCC14579. Complementary to global omic technologies, real-time PCR (qPCR) and reverse-transcription qPCR (RT-qPCR) are considered as methods of choice for quantitative analyses of specific genes and their expression (Nolan *et al.*, 2006). Over the last ten years, many qPCR-based methods have been developed to detect, identify

and/or quantify pathogens in food (Postollec *et al.*, 2011). More recently, RT-qPCR was described to study microbial growth dynamics and associated metabolic activities in food, yielding the possibility to evaluate microbial risk assessment (Carey *et al.*, 2009, Falentin *et al.*, 2010, Hierro *et al.*, 2006, Senouci-Rezkallah *et al.*, 2011, Torriani *et al.*, 2008, Ulve *et al.*, 2008).

Combining molecular tools and predictive microbiology concepts to incorporate bacterial physiology into prediction of growth and inactivation appears to be an interesting challenge for food formulation and preservation optimisation (Havelaar *et al.*, 2010, McMeekin *et al.*, 2008, Ranstiou *et al.*, 2011). The aim of this study was to characterize *B. weihenstephanensis* acid resistance, *i.e.* under bactericidal conditions and to develop an integrative approach to identifying and quantifying potential biomarkers, *i.e.* expression of specific genes that could further predict the impact of acid stress on bacterial survival. For this purpose, *B. weihenstephanensis* KBAB4 acid inactivation kinetics were fitted using a mixed Weibull mathematical model while gene expression was recorded by RT-qPCR throughout four hours of acid exposure. To further extend this approach to different bacterial physiological states, we chose three genes known to be involved in various *B. cereus* stress responses: *sigB*, involved in the general stress response, *kata*, encoding a major catalase, and *narL*, encoding a respiratory nitrate reductase.

Materials and Methods

Bacterial strain and culture conditions. *B. weihenstephanensis* KBAB4, kindly provided by the Institut National de la Recherche Agronomique (INRA, Avignon, France), was used in this study. Its genome sequence is already available (Lapidus *et al.*, 2008). The strain was stored at -80°C in brain heart infusion (BHI, Biokar Diagnostic, Beauvais, France) supplemented with 50% (v/v) glycerol.

Standardized inocula were obtained using three successive cultures. Bacteria were first grown in 100 ml of BHI broth incubated at 30°C for 8 h under shaking conditions (100rpm) and an aliquot (1%) was transferred into a second flask of 100 ml BHI broth incubated for 15 h in the same conditions. Then a portion (0.1%) was transferred into a third flask containing 100 ml of BHI broth incubated in the same conditions.

In the latter condition, bacterial growth began at an average of 10^5 CFU.ml⁻¹ and reached 10^7 CFU.ml⁻¹ after 4 h with an OD_{600 nm} values of 0.20 ± 0.02 , corresponding to mid-exponential cells. To ensure the absence of spores in both growth and inactivation media, aliquots of 2 ml were submitted to heat treatment at 70°C for 5 min (Baril *et al.*, 2011) and potential germinating spores were then quantified on nutrient agar (Biokar Diagnostic) medium at 30°C after 24 h incubation.

Acid inactivation kinetics. A volume of 15 ml of 10^7 CFU.ml⁻¹ of mid-exponential cells was transferred into 285 ml of BHI broth supplemented with HCl to reach a final pH of 4.6 allowing

bacterial decrease at a rate compatible with sampling for RNA extraction. In order to exclude possible additional bacteriostatic or bactericidal effects associated with the use of certain organic acids, HCl was chosen as acidifier to evaluate the sole impact of proton acidification. Bacterial suspensions were further incubated at 30°C under shaking conditions (100 rpm) to quantify survivors throughout the inactivation kinetics. The inoculum was also used as a no-stress control and was analyzed similarly to the acid-treated samples.

Aliquots were sampled after 5 min, 1 h, 2 h, 3 h and 4 h of exposure at pH 4.6. For each sampling time, an aliquot was dedicated to molecular analysis while a second one was used for bacterial counts. Survivors were quantified on Nutrient Agar (Biokar Diagnostic) after appropriate dilutions in Tryptone Salt (Oxoid, Dardilly, France) using a SPIRAL plater (AES Chemunex, Combourg, France) according to ISO 7218. Plates were subsequently incubated for 16 to 24 h at 30°C. Acid inactivation kinetics were performed in triplicate from three independent standardized inocula (replicates A, B, and C).

Fitting of bacterial survival kinetics. A mixed Weibull model was used to fit experimental data to determine the bacterial stress resistance as already reported by Coroller *et al.* (2006). This primary model assumes that the population is divided into two subpopulations with different acid resistance characteristics as follows:

$$N(t) = \frac{N_0}{1 + 10^\alpha} \left[10^{-\left(\frac{t}{\delta_1}\right)^p + \alpha} + 10^{-\left(\frac{t}{\delta_2}\right)^p} \right] \quad (1)$$

where $N(t)$ is the concentration of survivors expressed in \log_{10} CFU.ml⁻¹ at time 't'; N_0 is the inoculum concentration in \log_{10} CFU.ml⁻¹; t is the sampling time; p is a shape parameter; δ_1 and δ_2 are treatment times, in hours, necessary to obtain the first logarithm decrease for subpopulations 1 and 2, respectively. The second subpopulation is assumed to be more resistant to acid stress than subpopulation 1 ($\delta_1 < \delta_2$). α corresponds to $\log_{10}(N_{01}/N_{02})$ and represents the proportion of each subpopulation in the inoculum N_0 . For instance, when $\alpha = 2$, this means that 1 inoculated cell belongs to subpopulation 2, while 100 cells belongs to the subpopulation 1. The minimization of the sum of square error (SSE) was used to fit acid-inactivation kinetics. The minimum values were computed with a non linear fitting module (NLINFIT, MATLAB 6.5.1, Optimization Toll box, The Mathworks, Massachusetts, USA).

RNA extraction and cDNA synthesis. For both the inoculum (no-stress control) and acid-treated samples, 10 ml of cell suspensions were used for RNA extraction. After centrifugation ($10\,000 \times g$, 4°C , 10 min), the pellets were resuspended in 1 ml RNA Protect reagent (Qiagen, Courtaboeuf, France) and then incubated for 5 min at room temperature. After further centrifugation ($10\,000 \times g$, 4°C , 10 min), the cell pellets were frozen and stored at -80°C until RNA extraction (within 30 days maximum). Thawed cell pellets were disrupted by lysozyme and proteinase K digestion as recommended by the manufacturer (Qiagen). RNA purification was performed according to RNeasy[®] Mini kit (Qiagen) instructions. RNA quantity and quality were assessed using NanoDrop 2000 (NanoDrop Technologies, Wilmington, USA) and microfluidic analysis (RQI, Experion, Bio-Rad, Mity Mory, France). As advised by Bustin *et al.* (2009) to compare different samples, an adjusted volume of RNA template corresponding to 100 ng of the total RNA extract was reverse-transcribed into cDNA with a blend of random hexamers using an iScript cDNA synthesis kit (Bio-Rad). The reaction was followed by a reverse transcriptase denaturation step (5 min at 80°C).

Primer design and quantitative PCR. cDNA amounts were quantified by qPCR. Based on gene sequences obtained from NCBI databases (Table 1), primers were manually designed and were evaluated for primer dimers and hairpin formation using Beacon Designer software (Premier Biosoft International, Palo Alto, CA, USA). Amplification was performed in a final volume of 25 μl containing iQ[™] SYBR[®] Green Supermix (Bio-Rad), 500nM of each primer and 2.5 μl of cDNA. The absence of genomic DNA in RNA samples was checked using RNA as a template for qPCR instead of cDNA. Moreover, to verify the absence of primer dimers or hairpin formation, a blank sample (containing water instead of the cDNA template) was included in each assay. All quantifications were carried out in a CFX 96[™] thermal cycler (Bio-Rad) with an initial denaturation step at 95°C for 3 min, followed by 40 cycles of 10 s at 95°C , 10 s at 62°C and 30 s at 72°C . To minimize inter-run variations, a calibrator sample consisting in three DNA samples of known quantity was used to adjust all quantitative cycle Cq values (Bio-Rad CFX manager software). Based on a literature, three genes involved in *B. cereus* stress responses (Mols *et al.*, 2010a, van Schaik *et al.*, 2004) were chosen, *i.e.* *sigB*, encoding a general stress transcription factor, *narL*, encoding a respiratory nitrate reductase and *katA*, encoding a catalase. Furthermore, the expression of three reference genes was evaluated in order to represent relative quantification after gene stability standardization (Vandesompele *et al.*, 2002).

Gene expression quantification. Gene expression quantification was performed at different times of acid inactivation for each replicate. RT-qPCR results were expressed using both absolute and

relative quantification. Gene expression variation throughout acid stress was compared to gene expression in the control.

For absolute quantification, standard curves were generated by plotting Cq values as a function of the logarithm of known genomic DNA. Genomic DNA was expressed in genome copy number as previously described (Falentin *et al.*, 2010). qPCR efficiency coefficients (E) were calculated using the formula $E=10^{-1/\text{slope}}$ (Table 1).

Gene	Accession number	Molecular function	Primer sequence	Amplicon length (bp)	Efficiency (%)
Targeted genes					
<i>sigB</i>	BcerKBAB4_0903	General stress sigma factor	F:AACTTGCGATAGAGGAG TA R:AGAACTTCTCTTCGGATAGT	94	91.8
<i>narL</i>	BcerKBAB4_1974	Respiratory nitrate reductase, gamma subunit	F:GTGGTTCCGAAGTCTCTTT R:GGCACTAAATACATGGACAAG	142	102.7
<i>katA</i>	BcerKBAB4_1058	Catalase	F:ACCAAACTCTCGTACAGC R:TTACGAATACACCGTGAGC	147	94.4
Reference genes					
<i>tuf</i>	BcerKBAB4_0103	Elongation factor EF-Tu	F:CAACATCGGTGCTCTACTT R:ACGAAAACCTCAGCTTTGAA	114	97.5
<i>gyrA</i>	BcerKBAB4_0006	DNA gyrase subunit A	F:TTGGGACAAGTAACGGTATG R:GAACATTTACATCCTCTTCGAC	147	91.7
<i>sigA</i>	BcerKBAB4_4144	RNA polymerase sigma factor RpoD	F:TTAGATGATGGACGAACTCG R:ATCCTTAAGACGCTTACTACG	135	96.6

Table 1: Selected genes for RT-qPCR quantifications. F: Forward, R: Reverse

It was assumed that all RT-qPCR gene expression measurements were comparable because the same priming strategy and reaction conditions were used for reverse transcription and the samples contained the same total amount of RNA (Stahlberg *et al.*, 2004). Mean values were calculated in order to analyze the triplicate as a whole. To visualize the gene expression dynamics throughout acid inactivation, the percentage of each targeted mRNA expression was calculated as the genome copy number per ml divided by the sum of genome copy number per ml of the three targeted genes. For instance, at a given sampling time %*katA* expression = (*katA* gene expression in copy genome per ml)*100/ (gene expression in copy genome per ml of *katA*+*sigB*+*narL*).

For relative quantification, the mean of Cq values of the triplicate (replicates A, B, and C) was converted into fold-changes (as compared with the control) after normalization using the expression of three reference genes (*tuf*, *gyrA*, *sigA*) as recommended by Vandesompele *et al.* (2002). The normalization factor is equal to the geometric mean of relative quantities of reference gene expressions and varies throughout acid inactivation. Gene expression stability of the three reference genes (*M*) was also evaluated using the Bio-Rad CFX Manager software which calculates the average pair-wise variation for reference genes. The purpose of such normalization was to remove the sampling differences (such as RNA quantity or quality) in order to identify real specific gene variation

(Vandesompele *et al.*, 2002). The difference between gene expressions observed in each subpopulation was tested using one-way ANOVA (MATLAB 6.5.1, The Mathworks).

Results and Discussion

The acid inactivation of vegetative cells of *B. weihenstephanensis* KBAB4 was investigated using complementary approaches. Bacterial resistance was quantified after fitting a mathematical model to survivor counts. In parallel, RT-qPCR were performed on targeted genes involved in bacterial response to stress conditions. The integration of acid inactivation modelling and gene expression quantification was carried out in order to further identify *B. weihenstephanensis* KBAB4 relevant resistance biomarkers expressed throughout acid inactivation.

Determination of acid stress resistance. Bacterial growth curves showed good repeatability with, for instance, mid exponential cells concentration at $7.0 \pm 0.1 \log_{10}$ CFU.ml⁻¹ after 4 h of culture. Inactivation kinetics determined by colony enumerations showed slight variation. Nevertheless, for each inactivation point the standard deviation of the bacterial counts never exceeded 0.2 \log_{10} CFU.ml⁻¹. The three independent replicates A, B and C showed a biphasic shape and were treated as a whole (Figure 1). The fitting with a mixed Weibull model was performed to estimate the acid resistance of mid-exponential cells. The parameters used to describe the triplicate inactivation were the following: (i) one shape parameter p was estimated for the concave shape of the survival curve ($p = 1.52 \pm 0.46$), (ii) one initial population size ($N_0 = 6.44 \pm 0.20 \log_{10}$ CFU.ml⁻¹), (iii) values for the resistance of subpopulation 1 ($\delta_1 = 1.08 \pm 0.23$ h) and subpopulation 2 ($\delta_2 = 2.24 \pm 0.69$ h), and (iv) one α parameter estimated at 1.68 ± 0.50 .

Fitting experimental survival counts with a mixed Weibull model assumes the presence of subpopulations with different resistance to acid stress. In this study, the most sensitive subpopulation had a δ_1 value of 1.08 h while the first decimal decrease of subpopulation 2 was observed after 2.24 h of acid exposure. Therefore, twice the duration of exposure to stress was required to yield a first decimal reduction of the second subpopulation. It should be noted that the absence of spores at the start and at the end of acid inactivation was systematically checked to ensure that the bacterial population was only made by vegetative cells. The biphasic pattern of the inactivation kinetics was already reported in the case of non-thermal inactivation of vegetative cells such as *Salmonella*, *Listeria monocytogenes*, *Staphylococcus aureus* (Buchanan *et al.*, 1994; Coroller *et al.*, 2006; Whiting *et al.*, 1996). Classically, such heterogeneity within the inoculum is not caused by the presence of a genetically more resistant vegetative subpopulation (Buchanan *et al.*, 1994; den

Besten *et al.*, 2006; Humpheson *et al.*, 1998) but varies with cellular physiological state or with stress intensity (Coroller *et al.*, 2006; Greenacre *et al.*, 2003, Virto *et al.*, 2005).

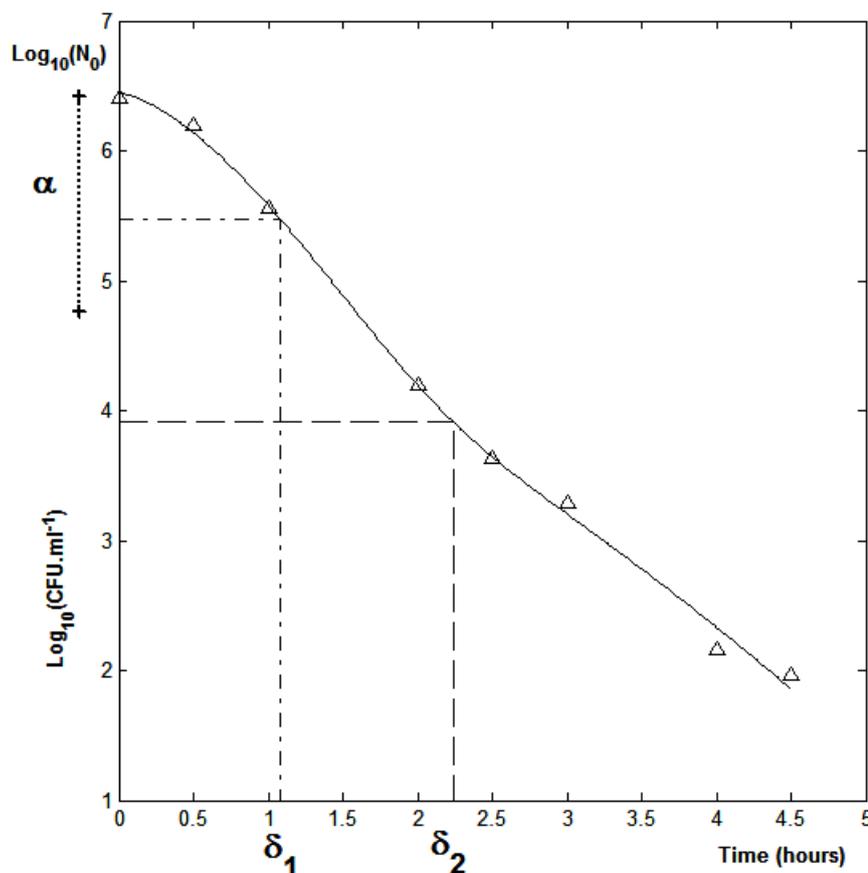


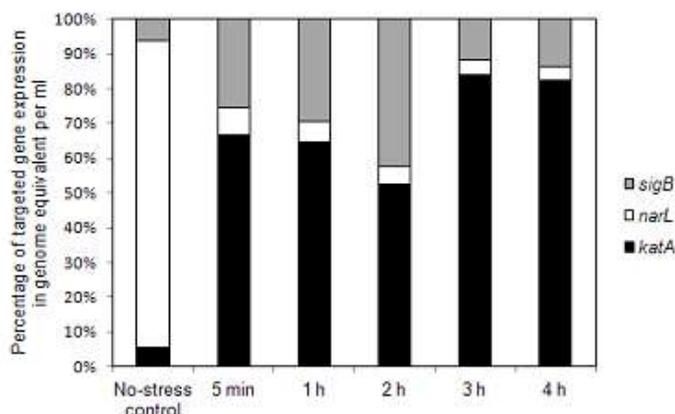
Figure 1 : *B. weihenstephanensis* KBAB4 survival kinetic of mid-exponentially grown cells exposed to acid conditions during 4 h (BHI, pH 4.6). Mean value of experimental counts performed for independent replicate are indicated as circle and fitted with a mixed Weibull model indicated as a dark grey line. Fitting of experimental survival counts enabled the determination of various parameters, in particular the acid resistance of subpopulation 1 ($\delta_1 = 1.08$ h) and subpopulation 2 ($\delta_2 = 2.24$ h) as well as the proportion of each subpopulation. The d parameter corresponds to the treatment time required to obtain a 90% decrease of each subpopulation.

Gene expression quantification upon acid inactivation. In parallel to survival kinetics, the expression of some genes was tracked throughout the acid inactivation using RT-qPCR. Quantifications reported in this study were performed with a set of quality controls already detailed in previous work (Postollec *et al.*, 2011). RNA extraction of three independent replicates of mid-exponential cells gave reproducible RNA concentration of 16.3 ± 3.7 ng. μl^{-1} with satisfactory quality with a RQI value greater than 5 as advised by Fleige and Pfaffl, 2006. For all acid-treated samples, RNA quality was reproducible with a RQI greater than 2 and did not impact on RT-qPCR efficiency (efficiency greater than 90%). Reverse transcription and qPCR were also evaluated for their reproducibility and showed satisfactory results for each targeted gene. For example, the gene

expression quantification of *tuf* gave a Cq of 17.01 ± 0.1 for the control. qPCR efficiencies were comprised between 91.7% and 102.7% (Table 1). For acid-treated samples, the difference in Cq values between triplicate RT-qPCR assays was less than 10 % of the mean value (Langmann *et al.*, 2003), except for one quantification corresponding to *katA* after 1 hour of inactivation (12 %).

Based on the absolute gene expression quantification expressed in \log_{10} of genome copy number per ml, the percentages of expression of *sigB*, *katA*, and *narL* were calculated (Figure 2A).

2A



2B

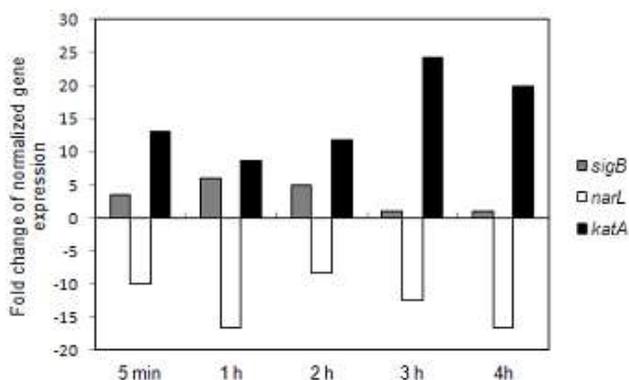


Figure 2: *sigB*, *narL* and *katA* gene expression of *B. weihenstephanensis* KBAB4 throughout acid inactivation expressed as (A) percentages of targeted mRNA expression, calculated based on genome copy number per ml, for samples from the inactivation kinetics study and for the inoculum indicated as no-stress control. (B) Fold change of the normalized expression of *sigB*, *narL* and *katA* using three reference genes (*tuf*, *gyrA* and *sigA*) and compared to the no-stress control throughout acid inactivation.

Even though the quantitative information is lost, this representation allows easy comparison of targeted gene expressions. In control cells, *narL* represented the vast majority of targeted gene expression (approximately 90%) while during acid exposure it represented less than 10% of targeted gene expression. On the contrary, *sigB* and *katA* represented approximately 10% expression of the three targeted genes in control cells and their expression increased up to 90% after acid exposure. Even though further investigations are needed to estimate the mRNA stability throughout acid

inactivation, the observed gene expression dynamic and peak suggest that RT-qPCR pattern reflect bacterial survival physiology.

In parallel, gene expression changes were quantified using the Cq values after reference gene normalization. The three reference genes used to calculate the normalization factor were *tuf*, *gyrA*, and *sigA* for which gene expression stability M , was below 1.5 in agreement of the requirements reported by Vandesompele *et al.* (2002). After normalization, expression of the targeted genes was presented as fold-changes as compared to control (Figure 2B). Throughout acid inactivation, up-regulation was observed for *sigB* and *katA*, while down-regulation was observed for *narL*. Note that variable expression peaks were observed for both up-regulated and down-regulated genes throughout acid exposure. The expression of *sigB* was up-regulated during the first part of the kinetics with a peak at 2 h (4.9 fold \pm 1.3). *katA* was up-regulated throughout acid inactivation with a maximal value at 3 h (24.3 fold \pm 2.6). RT-qPCR provides an interesting tool to quantify physiological responses for a more comprehensive view of cellular activities (Falentin *et al.*, 2010, Torriani *et al.*, 2008). The genes used in this study are known to be involved in *B. cereus* response to various stresses (den Besten *et al.*, 2009, Mols and Abee 2011 b, Mols *et al.* 2010 a, b, van Schaik *et al.*, 2004). Up-regulation of *sigB*, involved in general stress response, during the first 2 h of acid exposure seemed to be associated with an early bacterial response and moderate acid stress resistance. Similarly, the expression of *katA*, encoding a major catalase, seemed to be involved both in early stress response and in a more specific response associated with greater resistance after longer acid exposure. *katA* is known to play a key role during oxidative stress and to be σ^B -dependent (de Been *et al.*, 2010, Mols and Abee, 2011 a). Up-regulation of *sigB* and *katA* in *B. weihenstephanensis* KBAB4 during acid stress concur with other studies on the response of acid stressed *B. cereus* cells (Mols *et al.*, 2010 a, b). By contrast, the observed down-regulation of *narL* in *B. weihenstephanensis* KBAB4 differed from the response reported for *B. cereus* ATCC 14579. Indeed, Mols *et al.* (2010 a) reported over-expression of the nitrate and nitrite reductases involved in nitrogen metabolism. Furthermore correlation of the differential expression of several genes, such as *katA* and *narL* induction, with a secondary oxidative stress response in *B. cereus* was reported upon acid stress (Mols *et al.*, 2010 a). As in *B. cereus*, up-regulation of the catalase gene in *B. weihenstephanensis* could be due to the implementation of a secondary oxidative response upon acid stress. Nevertheless, the differences in the expression of genes such as *narL* between these two species remain to be elucidated and may result from differences in cell physiology.

Integrative analysis for the identification of acid resistance biomarkers. Fitting surviving bacterial counts with a mathematical model throughout acid inactivation enabled the identification

of two subpopulations with different acid resistance, *i.e.* a more sensitive subpopulation 1 (first log decrease obtained after $1.08 \text{ h} \pm 0.23$ acid exposure) and a more resistant subpopulation 2 (δ_2 of $2.24 \text{ h} \pm 0.69$). Such biphasic pattern was already reported for acid inactivation on vegetative cells and was associated to the presence of different cellular physiological state or stress intensity (Coroller *et al.*, 2006; Greenacre *et al.*, 2003, Virto *et al.*, 2005). Because *kata* and *sigB* gene expressions were up-regulated during acid inactivation, a one-way ANOVA analysis was performed to determine whether given gene expression could be associated with a given subpopulation. Significant differences ($p < 0.05$) were observed for both *sigB* and *kata*, which yields to a higher *sigB* induction in the subpopulation1 and higher *kata* induction in subpopulation 2. No significant variation ($p > 0.05$) was observed for *narL* gene expression throughout inactivation.

As already mentioned by Zwietering and den Besten (2011), the deviations resulting from independent biological reproductions are more important than deviations resulting from experimental errors. Indeed, the inactivation kinetic observed in this study for the replicate C was slightly different from the other two, yielding a difference of only 1% in the initial population. In order to highlight differences in the population structure, mathematical fitting was performed for each replicate using the same estimated N_0 , ρ , δ_1 and δ_2 , and only the proportion of each subpopulation, α , was different for each replicate. The proportion of each subpopulation throughout acid inactivation is represented in Figure 3 for each replicate. After 2 hours acid exposure, subpopulation 2 represented 86% of the population in replicate C as compared to 10 to 20% in the other replicates. While after 4 hours acid exposure, all subpopulation 1 was inactivated and only subpopulation 2 remained.

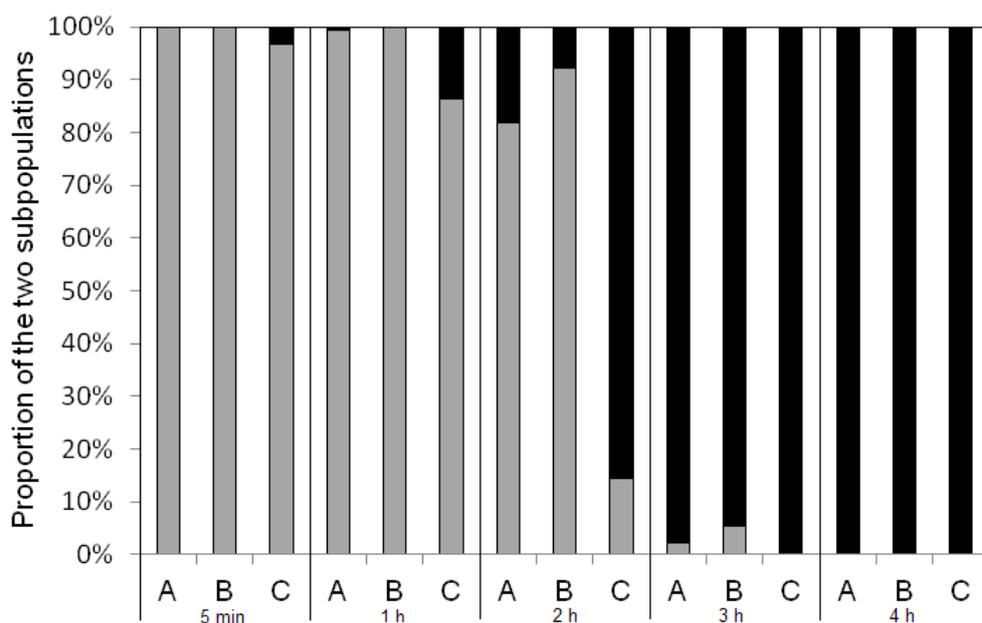


Figure 3: Proportion of the two subpopulations observed for *B. weihenstephanensis* KBAB4 throughout acid inactivation. These proportions were calculated using the mixed Weibull model and are indicated for each replicates (A, B, and C). The

grey bars represent the percentage of subpopulation 1 and the black bars represent the percentage of subpopulation 2, for which the first decimal decrease is observed at 2.24 h of acid exposure in studied conditions.

Based on RT-qPCR results expressed in genome copy number per ml, the percentage of gene expression attributed to each replicate was calculated. While after 1 and 2 h of acid exposure, *katA* gene expression was mainly provided by replicate C, with respectively 90% and 70% (Figure 4). In contrast, after 3 and 4 h of acid exposure when mainly subpopulation 2 remained, *katA* expression was equivalent whatever the replicate (approximately 1/3 for each replicate). Following these results, the *katA* up-regulation could be correlated to the more resistant subpopulation or to the most resistant replicate. The expression of given genes of interest may be defined as biomarkers, *i.e.* a characteristic that is objectively measured (with satisfactory accuracy and reproducibility), and evaluated as an indicator of normal or pathogenic biologic processes (Atkinson *et al.*, 2001). This study shows that combination of survival mathematical fitting and RT-qPCR quantification could lead to the identification of candidate biomarkers to track moderate resistance (*sigB*) or higher resistance (*katA*). Further investigations will be necessary to extend the use of these RT-qPCR biomarkers to other stresses. This study shows promising results to track bacterial resistances in food. Similarly, *narL* down regulation displays several interesting features, and may be considered as cell viability biomarker as suggested by Kort *et al.* (2008) because this gene is widely conserved within the bacterial kingdom and up regulated in unstressed cells as compared to acid-exposed bacteria. To confirm this hypothesis it is necessary to correlate *narL* expression with a loss of viability, and further investigations will be necessary.

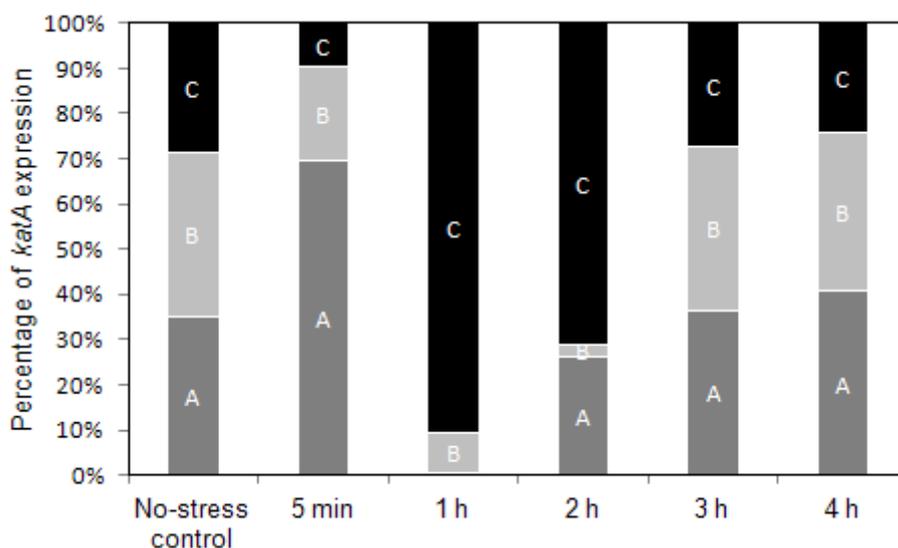


Figure 4: Percentages of *katA* expression calculated based on absolute quantification, and represented for each replicate (A, B, and C) throughout acid inactivation.

Conclusion

Based on an integrative approach, the response of *B. weihenstephanensis* KBAB4 to acid stress was investigated. The reported phenotypic biphasic inactivation and the presence of two subpopulations with different resistance were identified using modelling of survivors counts. These observations could be of importance for the food industry to refine safety procedures, as the presence of subpopulations with different behaviours may greatly impact the resistance of the whole population. By monitoring gene expression during acid inactivation, potential resistance biomarkers were proposed to track different acid resistant subpopulations. Further investigations involving a larger number of targeted genes or other bacterial physiological states are needed to accurately predict the acid resistance of *B. weihenstephanensis*. Nevertheless, this approach suggests that the most relevant genes reflecting specific bacterial resistance could be selected and combined to further extend to a predictive tool.

Clearly, such integrative approach offers new perspectives for the prediction of microbial behaviour and physiology using cellular biomarkers. One of the key challenges will be to increment these data into a mathematical model to predict bacterial behaviour, *i.e.* growth and inactivation, during industrial processes to offer decision-making tools for food safety and quality management.

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Chapter 6

Prediction of *Bacillus weihenstephanensis* acid resistance: The use of gene expression patterns to select potential biomarkers

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Abstract

Exposure to mild stress conditions can activate stress adaptation mechanisms and provides cross-resistance towards otherwise lethal stresses. In this study, an approach was followed to select molecular biomarkers (quantitative gene expressions) to predict induced acid resistance after exposure to various mild stresses, *i.e.* exposure to sublethal concentrations of salt, acid and hydrogen peroxide during 5 min to 60 min. Gene expression patterns of unstressed and mildly stressed cells of *Bacillus weihenstephanensis* were correlated to their acid resistance (3D value) which was estimated after exposure to lethal acid conditions. Among the twenty-nine candidate biomarkers, 12 genes showed expression patterns that were correlated either linearly or non-linearly to acid resistance, while for the 17 other genes the correlation remains to be determined. The selected genes represented two types of biomarkers, (i) four direct biomarker genes (*lexA*, *spxA*, *narL*, *bkdR*) for which expression patterns upon mild stress treatment were linearly correlated to induced acid resistance; and (ii) nine long-acting biomarker genes (*spxA*, BcerKBAB4_0325, *katA*, *trxB*, *codY*, *lacl*, BcerKBAB4_1716, BcerKBAB4_2108, *relA*) which were transiently up-regulated during mild stress exposure and correlated to increased acid resistance over time. Our results highlight that mild stress induced transcripts can be linearly or non-linearly correlated to induced acid resistance and both approaches can be used to find relevant biomarkers. This quantitative and systematic approach opens avenues to select cellular biomarkers that could be incremented in mathematical models to predict microbial behaviour.

Introduction

Bacteria are exposed to changing environments both in their natural habitats and at industrial sites. In order to cope with these environmental dynamics, microorganisms have developed stress adaptation strategies that provide cell induced resistance to harsher conditions for a variety of stresses. This well-known cross protection phenomenon appears of practical importance for food industry, particularly with the increasing trend of minimally processed foods, where mild preservation factors, called hurdles, are combined to ensure the microbial safety and stability of processed foods (Leistner and Gorris, 1995). However, the ability of microorganisms to adapt to stressing environments may antagonize the benefits of the hurdle strategy as adaptive responses may be activated upon exposure to stresses yielding induced resistance. Therefore the prediction of mild stress inducing resistance is important to control bacterial adaptive behaviour throughout the food chain.

Bacillus weihenstephanensis is a psychrotolerant bacterium belonging to the *Bacillus cereus* group which is challenging the food industry. Indeed this group is composed of closely related strains, which might produce toxins or enzymes involved in food safety or spoilage. To our knowledge, *B. weihenstephanensis* has never been correlated with food-borne diseases, but some strains have been shown to be emetic (Hoton *et al.*, 2009; Thorsen *et al.*, 2006) and some others, such as the KBAB4 strain, possess genes involved in enterotoxin production (Lapidus *et al.*, 2008; Réjasse *et al.*, 2012; Stenfors *et al.*, 2002; Stenfors Arnesen *et al.*, 2011). In addition, conjugation and transfer of toxin encoding plasmids were shown to occur at significant levels among strains of the *B. cereus* group (Hoffmaster *et al.*, 2004; Hoffmaster *et al.*, 2006; Van der Auwera *et al.*, 2007), and particularly *B. weihenstephanensis*, which may be of concern for food safety (Lapidus *et al.*, 2008). Furthermore, the increasing demand for ready-to-eat foods could raise the issue associated with psychrotolerant bacteria, since they represent the most probable risk in refrigerated food poisoning (Lapidus *et al.*, 2008).

Nowadays, the finding of biomarkers, *i.e.* cellular components that are objectively measured (with satisfactory accuracy and reproducibility), and evaluated as indicators of specific biologic processes (Atkinson *et al.*, 2001), appears as a new trend for microbiological food safety. Actually, genome sequencing and related 'omics' technologies offer a wealth of information to explore complex biological pathways in a quantitative and integrative manner. Therefore quantification both at population and molecular level of the microorganism responses to food preservation treatments become feasible and such insights might further be used in microbial risk assessment (Brul *et al.*, 2012). Over the last ten years, many qPCR-based methods have been developed to detect, identify

and/or quantify pathogens in food (Postollec *et al.*, 2011). Real-time PCR (qPCR) and reverse-transcription qPCR (RT-qPCR) are considered as methods of choice to analyze specific genes and their expression patterns (Nolan *et al.*, 2006). RT-qPCR was used to study microbial growth dynamics and associated metabolic activities in food (Carey *et al.*, 2009; Falentin *et al.*, 2010; Hierro *et al.*, 2006; Torriani *et al.*, 2008; Ulve *et al.*, 2008). Some studies focused on adaptive traits of *B. cereus* (Ceriagioli *et al.*, 2010; den Besten *et al.*, 2009; Mols *et al.*, 2010a; Mols *et al.*, 2010b; van Melis *et al.*, 2011; van Schaik *et al.*, 2007) allowing a better understanding of stress response mechanisms and identification of potential biomarkers to predict stress adaptive behaviour. In 2010, a framework was proposed for quantifying linear correlations between cellular indicators and mild stress induced robustness to evaluate predictive potential of candidate biomarkers (den Besten *et al.*, 2010a). In our study, we used this approach to investigate linear correlations between gene expression patterns and survival under lethal acid conditions. Moreover, an additional approach was proposed to correlate transient gene expression to induced acid resistance. Both correlations between gene expression data and survival ability were used to select relevant biomarkers for mild stress induced resistance. In this study the acid resistance was used as model, nevertheless, targeted genes are known to be involved in various stresses allowing to further explore their predictive potential for other stresses. The identification of such quantitative biomarkers would then allow prediction of the impact of adaptive responses, due to exposure to mild stress conditions, on resistance and could further be used as indicator to refine process conditions to limit and control these adaptive traits in food environment.

Materials and Methods

Bacterial strain and preculture conditions. The psychrotolerant *B. weihenstephanensis* KBAB4 strain, kindly provided by the Institut National de la Recherche Agronomique (INRA, Avignon, France), was used throughout this study. The strain was stored in Brain Heart Infusion broth (BHI broth, Biokar Diagnostic, Beauvais, France) supplemented with 50% (v/v) glycerol at -80°C . Bacteria (1 ml of glycerol stock) were first grown in 100 ml of BHI broth and incubated at 30°C for 8 h under shaking conditions (100 rpm) and an aliquot (1%) was transferred into a second flask of 100 ml BHI broth and incubated for 15 h in the same condition.

Mild stress exposures. To quantify the stress adaptive behaviour of *B. weihenstephanensis* to conditions which may be encountered in food industry, three conditions were used to mildly stress cells, namely, mild salt, acid and oxidative stresses. Mild stress pretreated cells were subsequently submitted to lethal acid conditions to quantify induced acid resistance after mild stress exposure. For

that purpose, a portion (0.1%) of the preculture was transferred into a flask containing 100 ml of BHI broth incubated at 30°C with shaking at 100 rpm, until the cells reached the mid-exponential phase ($OD_{600\text{ nm}}$ values of 0.20 ± 0.02). This culture was used, without (unstressed samples) and with mild stress exposure (mildly stressed samples), as inoculum for the acid inactivation which enabled the determination of acid resistance (3D values). The following mild stress conditions were applied: osmotic-upshift (2.5% w/v NaCl), acid-shock (pH 5.5 adjusted with 37% HCl) and oxidative stress (0.1 mM H_2O_2) for 5, 15, 30 and 60 min.

Determination of acid stress resistance. Both unstressed and mildly stressed cells were subsequently exposed to lethal acid condition to determine the acid resistance (3D values). For that, 1 ml of culture was transferred into 100 ml of BHI broth supplemented with HCl to reach pH 4.6. Bacterial suspensions were further incubated at 30°C for 4 h under shaking conditions (100 rpm) and the number of survivors was quantified at selected sampling time throughout the acid inactivation. Survivors were quantified on Nutrient Agar (Biokar Diagnostic) using a SPIRAL plater (AES Chemunex, Combourg, France), after appropriately diluting in Tryptone Salt broth (Oxoid, Dardilly, France). Plates were subsequently incubated for 16 to 24 h at 30°C. Acid inactivation experiments were performed in triplicate from three independent precultures.

Bacterial resistance fitting. Two models were used to estimate the acid resistance expressed as the 3D value meaning the time of acid exposure necessary to reduce the initial population by three log, *i.e.* 99.9% reduction of the initial population using a non linear fitting module in MATLAB (NLINFIT, MATLAB 6.5.1, Statistics Toolbox, The Mathworks, Massachusetts, USA).

(i) The first order model was as follow:

$$\log_{10} N(t) = \log_{10} N(0) - \frac{t}{D} \quad (1)$$

Where $N(t)$ is the concentration of survivors expressed in \log_{10} CFU ml^{-1} at sampling time ' t ' (h); $N(0)$ is the inoculum concentration in \log_{10} CFU ml^{-1} ; and D is the decimal reduction time, in h.

(ii) The Weibull model was as follow:

$$\log_{10} N(t) = \log_{10} N(0) - \left(\frac{t}{\delta} \right)^p \quad (2)$$

where δ is the first-decimal reduction time (h) and p is a shape parameter.

To evaluate the model fitting performances and to select the most appropriate model to describe the data, a procedure similar to that previously described by den Besten *et al.* (2010b) was followed. Briefly, F tests were performed to (i) determine the statistical acceptance of the fitting performance,

and (ii) test whether the extra parameter p of the Weibull model significantly improved the fitting of the inactivation data. Then for each condition, the selected model was fitted to each replicate and the average third decimal reduction time (3D value), *i.e.* the exposure time necessary to reduce the initial population by three log, was estimated.

RNA extraction and RT-qPCR. The expression of 29 genes selected as potential biomarkers and 3 reference genes, namely *16S*, *23S* and *tuf*, was quantitatively measured before and after 5, 15, 30 and 60 min of mild stress treatment (see table S1 in the supplementary data). For the three replicates of unstressed and pretreated cells, RNA extractions were realized as previously described by Desriac *et al.* (2012). Briefly, 10 ml of suspension was centrifuged and cell pellets were resuspended in 1 ml of RNA Protect reagent (Qiagen, Courtaboeuf, France) and incubated for 5 min at room temperature. After further centrifugation the cell pellets were frozen and stored at -80°C until cells disruption as recommended by the manufacturer (Qiagen). RNA purification was performed following RNeasy[®] Mini kit (Qiagen) instructions.

As advised by Stahlberg *et al.* (2004) an adjusted volume of total RNA corresponding to 100 ng was reverse-transcribed into cDNA using iScript cDNA synthesis kit (Bio-Rad, Mitry Mory, France). cDNA amounts were quantified by qPCR in a final volume of 25 μl containing iQ[™] SYBR[®] Green Supermix (Bio-Rad), 500nM of each primer and 2.5 μl of cDNA. The specificity and efficiency of each primer was determined by melt curve analysis and a dilution series of genomic DNA, respectively. Blank samples, containing water instead of the cDNA, as well as samples containing RNA as template were added to exclude genomic DNA contamination and to validate RT-qPCR results. All quantifications were carried out in a CFX 96[™] thermal cycler (Bio-Rad) as previously described (Desriac *et al.*, 2012). To minimize inter-run variations, a calibrator sample consisting of six known quantities of genomic DNA was used to adjust all quantitative cycle Cq values (Bio-Rad CFX manager software).

The gene stability of the three reference genes (M) was evaluated using the Bio-Rad CFX Manager software calculating the average pair-wise variation of reference genes (Vandesompele *et al.*, 2002). RT-qPCR results were expressed using relative quantification after normalization with *16S*, *23S* and *tuf* as reference genes. The mean Cq obtained for each targeted genes was converted into fold change, meaning the ratio of gene expression for stressed over unstressed samples.

Statistical analysis. Student's t -tests were performed to compare the estimated 3D values of mild stress adapted cells to that of unstressed cells ($p < 0.05$). Also the significance of gene induction or repression as compared to gene expression of the unstressed cells was evaluated by student's t -tests using the \log_{10} transformed expression values. Then, to investigate the correlation between acid

resistance and gene expression, the average 3D value of unstressed and mild stress adapted samples were correlated to the \log_{10} gene expression of these samples. A non linear correlation corresponded to a significant up-regulation ($p < 0.05$) of targeted genes for 5 and 15 min of mild stress exposure and no significant variation as compared to unstressed cells after 30 and/or 60 min, which could be linked to an increased resistance over time. The significance of a linear correlation between acid resistance and gene expression was evaluated by calculating the Pearson correlation coefficient r using PASW software ($p \leq 0.05$).

Results

Selection of potential biomarkers. In search of potential biomarkers to predict the impact of mild stress exposure on induced acid resistance of *B. weihenstephanensis*, a literature review was performed, and allowed the selection of 29 genes, *i.e.* potential biomarkers, that may play a key role in bacterial acid stress resistance (Ceriagioli *et al.*, 2010; Daniel *et al.*, 1997; den Besten *et al.*, 2009; ; Mols *et al.*, 2010a; Mols *et al.*, 2010b; Ratnayake-Lecamwasam *et al.*, 2001; Ter Beek *et al.*, 2008; van Melis *et al.*, 2011; van Schaik *et al.*, 2007; Wilks *et al.*, 2009; Zhang *et al.*, 2003; Zuber, 2009). Since cross-protection is a well-known phenomenon, selected genes (see table S1 in the supplementary data) were sorted into three main categories: (i) members of the general stress response regulon controlled by the transcriptional regulator σ^B and genes involved in repair and maintenance of cellular proteins (6 genes); (ii) genes involved in cellular defence mechanisms against oxidative stress (9 genes); (iii) genes encoding for regulators, transporters or enzymes involved in cell maintenance (14 genes). In order to find relevant biomarkers to predict resistance, gene expression quantifications were correlated to acid resistance expressed by 3D values (Figure 1).

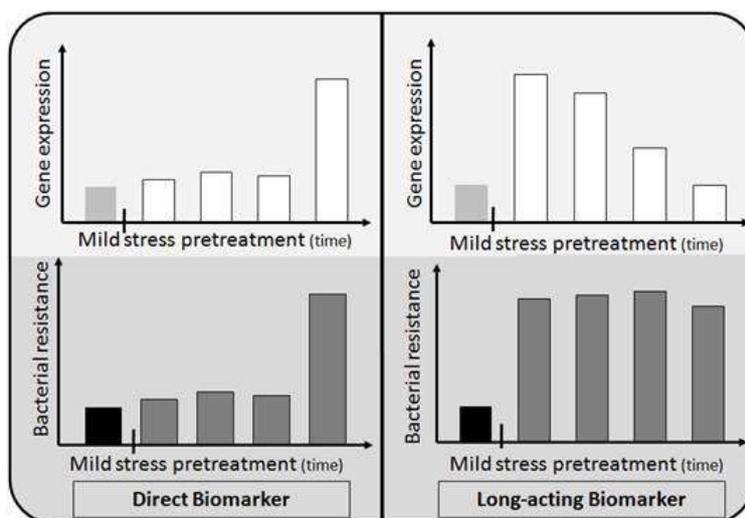


Figure 1 : Conceptual scheme used in this study to find relevant biomarkers for mild stress induced acid resistance. From the 29 targeted genes, linear and non linear correlations were observed for 12 genes showing two types of biomarkers defined as (i) direct biomarkers which expression patterns were linearly correlated to bacterial resistance, and (ii) long-acting biomarkers which were up-regulated for short mild stress adaptation times and linked to increased resistance over time.

Acid resistance and gene expression pattern in response to mild stress. Among the 29 candidate biomarkers, 17 genes showed non determined correlation with acid resistance while 12 genes showed expression patterns that could be correlated to acid resistance. Direct biomarker genes showed an expression pattern linearly correlated to bacterial resistance. While long-acting biomarkers showed gene expression which was transiently up-regulated and linked to a stable increase of acid resistance over mild exposure time. The impact of mild stress treatments on subsequent acid resistance (estimated by the 3D values) varied with the exposure time and mild stress conditions.

Both mild salt and acid stress treatments up to 30 min did not significantly change the 3D value compared to unstressed cells with a 3D value estimated at $2.5 \text{ h} \pm 0.5$, whereas 60 min significantly increased the acid resistance with 3D values of $6.2 \text{ h} \pm 0.2$ and $8.6 \text{ h} \pm 1.7$, respectively, for mild salt and acid stress treatments (Figure 2 and 3).

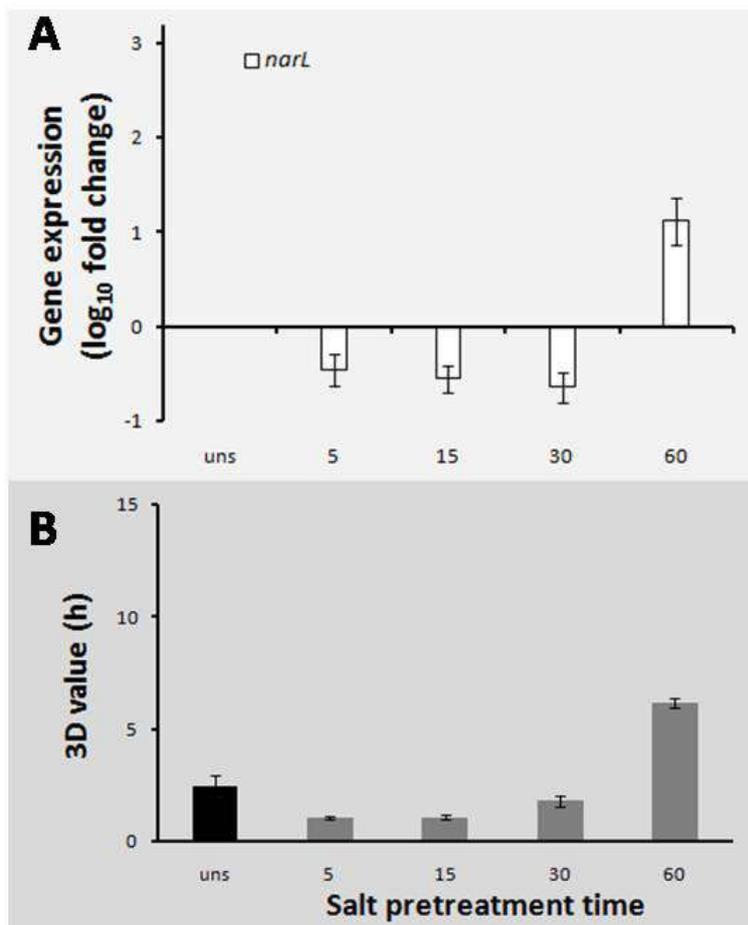


Figure 2: Selected biomarker for mild salt stress induced acid resistance. A- Gene expression as a function of salt pretreatment time. B- Quantified acid resistance of unstressed and stressed cells as a function of mild salt pretreatment time. *narL* (BcerKBAB4_1974) may serve as direct biomarker for acid resistance of mild salt stressed cells.

The expression pattern of *narL*, encoding a nitrate reductase involved in nitrogen metabolism (Mols and Abee, 2011), was linearly correlated to the bacterial resistance after mild salt stress exposure (Figure 2) with a significant Pearson correlation ($p < 0.05$) with a down regulation until 30 min and followed by an up-regulation after 60 min exposure. Upon mild acid exposure (Figure 3), a significant increase of acid resistance was observed after 30 and 60 min exposure. A linear correlation was observed with induced resistance and *bkdR*, *lexA* and *spxA* gene expressions. The *bkdR* gene encodes a σ^{54} -dependent transcriptional activator (Ter Beek *et al.*, 2008), and *lexA* and *spxA*, encode for regulators with key roles in oxidative stress response of *Bacillus* (Butalaa *et al.*, 2009; Zuber, 2009). These results, in particular the up-regulation of genes involved in the oxidative stress response upon mild acid stress exposure of *B. weihenstephanensis* KBAB4, are in agreement with findings already reported for *B. cereus* ATCC 14579 and ATCC 10987 (Mols *et al.*, 2010a).

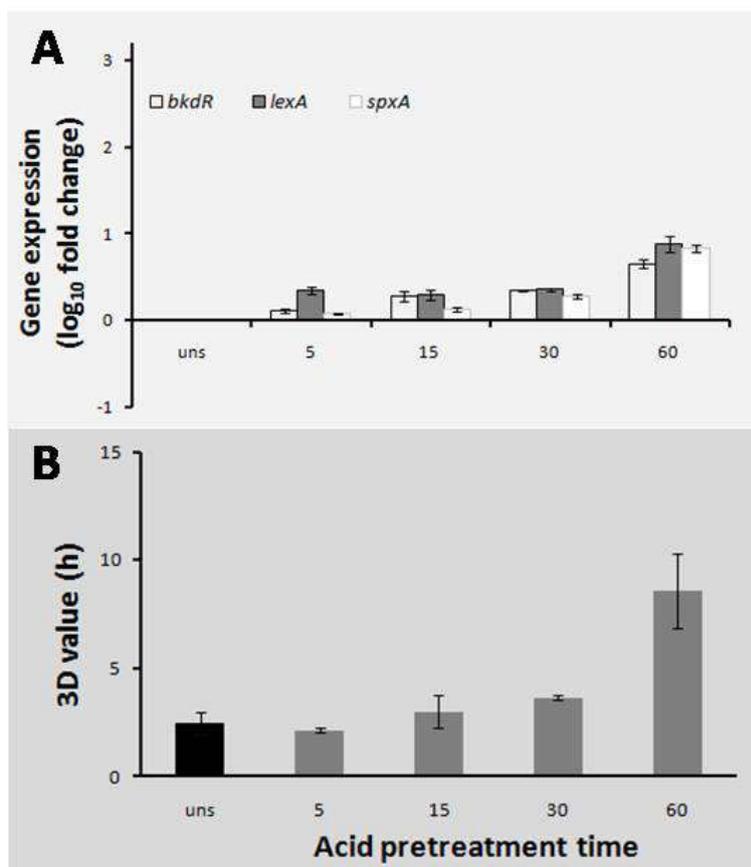


Figure 3: Selected biomarker for mild acid stress induced acid resistance. A- Gene expression as a function of acid pretreatment time. B- Quantified acid resistance of unstressed and stressed cells as a function of mild acid pretreatment time. *bkdR* (BcerKBAB4_4008), *lexA* (BcerKBAB4_3377) and *spxA* (BcerKBAB4_1097) genes may serve as direct biomarkers for acid resistance of mild acid stresses cells.

Mild oxidative stress exposure to 0.1 mM H₂O₂ for 5 to 60 min resulted in enhanced acid resistance of *B. weihenstephanensis* for all adaptation times (Figure 4). Note that no significant variation ($p > 0.05$) was observed, whatever the exposure times, with an average 3D value close to 5 h as compared to 2.5 h \pm 0.5 for unstressed cells. Two genes, *narL* and *lexA*, showed significant up-regulation in oxidative stressed cells as compared to unstressed cells (Figure 4). This up-regulation was linearly correlated to enhanced acid resistance and thus *narL* and *lexA* were defined as direct biomarkers upon mild oxidative stress conditions. Two other genes *sigB* and *rsbW* (members of the general stress response regulon) showed a linear correlation between their expression patterns and acid resistance (data not shown), but their up-regulation never exceeded two folds of variation. On the other hand, nine other genes showed interesting patterns with an up-regulation only for short exposure times (5 and 15 min) and no significant variation as compared to unstressed cells after 30 and/or 60 min of mild oxidative exposure. Four of these genes are known to be involved in oxidative stress defence, namely, BcerKBAB4_0325, encoding a peroxiredoxin, *trxB*, encoding a thioredoxin, *katA*, encoding the main vegetative catalase (de Been *et al.*, 2010), and *spxA*, encoding a

transcriptional regulator (Zuber, 2009). The other five genes have known functions in cell maintenance of *Bacillus* with genes encoding for regulators (*codY* and *laci*) (Daniel *et al.*, 1997; den Besten *et al.*, 2010a; Frenzel *et al.*, 2012; Lindbäck *et al.*, 2012; Ratnayake-Lecamwasam *et al.*, 2001), transporter (BcerKBAB4_1716) (Ceragioli *et al.*, 2010; den Besten *et al.*, 2010a) and enzymes (BcerKBAB4_2108 and *relA*) (den Besten *et al.*, 2010a; Wilks *et al.*, 2009; Zhang *et al.*, 2003).

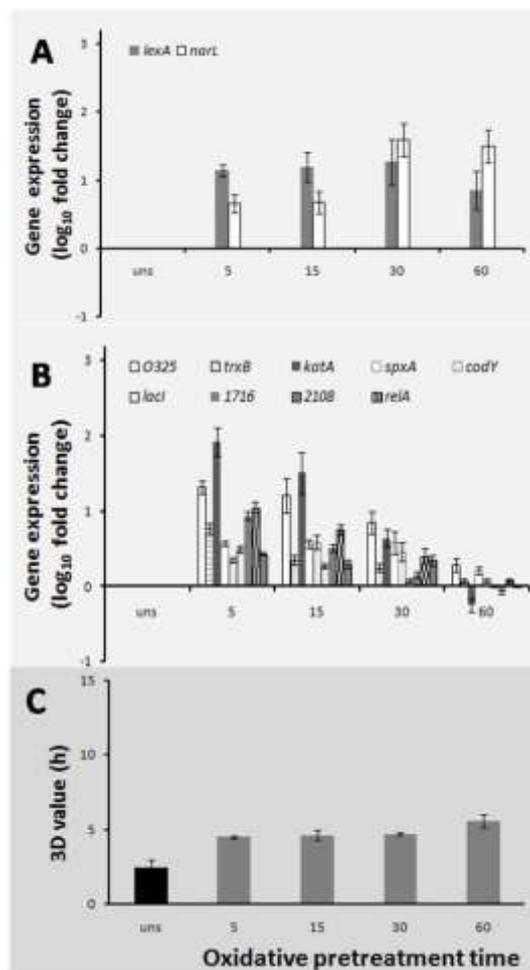


Figure 4: Selected biomarkers for mild oxidative stress induced acid resistance. A and B- Gene expression as a function of oxidative pretreatment time. C- Quantified acid resistance of unstressed and stressed cells as a function of mild oxidative pretreatment time. *lexA* (BcerKBAB4_3377) and *narL* (BcerKBAB4_1974) may serve as direct biomarkers for acid resistance of oxidative stress pretreated cells. BcerKBAB4_0325, *trxB* (BcerKBAB4_4951), *katA* (BcerKBAB4_1058), *spxA* (BcerKBAB4_1097), *codY* (BcerKBAB4_3651), *laci* (BcerKBAB4_3651), BcerKBAB4_2108, BcerKBAB4_1716 and *relA* (BcerKBAB4_4254) genes may serve as long-acting biomarkers for acid resistance of oxidative stress pretreated cells.

Potential biomarkers to predict acid resistance. Among the candidate biomarkers, 12 genes were correlated with acid resistance (Table 1). Four genes were found as direct biomarker genes, *i.e.* three, upon mild acid exposure (*lexA*, *spxA* and *bkdR*), two (*lexA* and *narL*) upon mild oxidative stress and one (*narL*) upon mild salt stress exposure. Both *lexA* and *narL* were up regulated and correlated to increase acid resistance after multiple sublethal stress exposures, highlighting that some biomarkers may have a broader predictive potential than others (den Besten *et al.*, 2010a).

Nevertheless it should be noted that no targeted gene could be defined as direct biomarker gene for all three tested mild stress pretreatments. Among the nine genes selected as long-acting biomarker, four genes have key roles in the oxidative stress response of *B. cereus* and five genes are known to be involved in cell maintenance (Table 1).

	Gene	Biomarker type	
		Direct	Long-acting
Oxidative response			
Regulator	<i>lexA</i>	Acid / Oxidative	
	<i>spxA</i>	Acid	Oxidative
Removing ROS	BcerKBAB4_0325		Oxidative
	<i>katA</i>		Oxidative
	<i>narL</i>	Salt / Oxidative /	
	<i>trxB</i>		Oxidative
Cell maintenance			
Regulator	<i>bkdR</i>	Acid	
	<i>codY</i>		Oxidative
	<i>laci</i>		Oxidative
Transporter	BcerKBAB4_1716		Oxidative
Enzymes	BcerKBAB4_2108		Oxidative
	<i>relA</i>		Oxidative

Table 1: Genes selected as direct and long-acting biomarkers for acid resistance induced by mild acid, salt and oxidative stress exposures. ROS: reactive oxygen species.

Discussion

The adaptive stress response of bacteria is a crucial mode of cellular protection and allows bacteria to survive in fluctuating environments. It is well known that the adaptive stress response may provide cross protection against other stresses. This phenomenon appears of practical importance for food industry particularly with the increasing trend in the production of minimally processed foods where various stresses are combined to control microbial growth. The availability of complete genome sequence has been instrumental to better understand bacterial behaviour under specific conditions. Therefore, the combination of molecular tools and predictive microbiology concepts appears to be an interesting challenge for food formulation and preservation optimisation in order to incorporate bacterial physiology into bacterial growth and survival predictions (Brul *et al.*, 2012; Havelaar *et al.*, 2010; McMeekin *et al.*, 2008; Rantsiou *et al.*, 2011). In order to survive, bacteria are continuously sensing their environment to respond with appropriate changes in gene expression and protein activity (Boor, 2006). Thus gene expression is the first and often transient bacterial response to changing environments while functional proteins and enzymes might appear more stable to fulfil

their key roles in bacterial stress responses (den Besten *et al.*, 2013; Michalik *et al.*, 2012). Knowing that, it can be proposed that gene expression may be correlated to bacterial stress resistance not only in a linear way but also by more complex features. In this study, we presented an approach that encompasses both linear and non linear correlations between gene expression patterns and bacterial induced resistance in order to identify and select relevant quantitative biomarkers that could be included in predictive models to further refine bacterial behaviour predictions. Based on *B. cereus* and *B. subtilis* stress adaptive behaviour upon exposure to various stresses, 29 genes were selected as potential biomarkers. While the correlation of bacterial resistance and the expression of 17 genes remain to be determined, 12 genes showed promising expression patterns to predict bacterial acid resistance. Among which, half of them are known for their involvement in the oxidative stress response thus highlighting the importance of cross-protection in bacterial stress behaviour. Four genes could be selected as direct biomarkers as their expression was linearly correlated to an increased acid bacterial resistance. Eight additional genes, defined as long-acting biomarker genes, showed transient up-regulation for short mild stress treatment exposures that was correlated with stable increased of acid resistance during longer time intervals. These results underline the importance to consider non-linear correlations, particularly when focussing at transcriptional level in order to find relevant biomarkers. This is based on the notion that mRNAs can have short half-lives (Kushner, 1996), whereas the encoded proteins or enzymes may display prolonged stability and functionality (den Besten *et al.*, 2013; Michalik *et al.*, 2012) and contributing to enhanced resistance. Indeed transient gene expression patterns have been functionally linked to stable enzyme activity levels (den Besten *et al.*, 2010a), as shown for *kata* gene expression and corresponding catalase activity level in mild oxidative stressed cells of *B. cereus* ATCC 14579. In a recent study (den Besten *et al.*, 2013), the same transient gene expression patterns has been demonstrated for *kata* gene expression of *B. weihenstephanensis* KBAB4 upon mild oxidative stress treatment and was linked to stable enzyme activity levels and enhanced resistance. Furthermore, it should be noted that expression of *spxA* may serve both as direct and long-acting biomarker as expression of this gene could serve as direct biomarker for acid stress resistance induced by mild acid pre-exposure and as long-acting biomarker for acid stress resistance induced by mild oxidative pre-exposure. This may be explained by variation in stability of the encoded protein SpxA as function of the environmental conditions (Ghaemmaghani and Oas, 2001; Michalik *et al.*, 2009). Nevertheless most observed gene patterns remain uncorrelated to bacterial resistance and need further investigations.

The identification of biomarkers contributes to a better understanding of bacterial stress response and may be helpful in early detection of adaptive traits. Since functional conservation of stress-related cellular factors differs among species and genera, promising biomarkers might be species or

genus specific. Nevertheless, when biomarkers have predictive potential in several species, it will strengthen their predictive potential. In this study, we focussed on the psychrotolerant *B. weihenstephanensis* KBAB4 and five genes selected as potential biomarkers (BcerKBAB4_0325, *katA*, *lacI*, BcerKBAB4_1716 and BcerKBAB4_2108) have been demonstrated to be also up-regulated by multiple mild stresses in *B. cereus* ATCC 14579 (den Besten *et al.*, 2010a). Mild salt and acid stressed *B. weihenstephanensis* cells showed an up-regulation of genes belonging to the SigB regulon (data not shown) (*sigB*, *rsbW* and BcerKBAB4_0904) as already demonstrated for *B. cereus* ATCC 14579 cells (den Besten *et al.*, 2009; Mols *et al.*, 2010a; Mols *et al.*, 2010b), but the potential of these transcripts to function as biomarkers remains to be elucidated. In acid-adapted cells of *B. weihenstephanensis* KBAB4, four genes known for their involvement in the oxidative stress response of *B. cereus* showed a significant up-regulation. The up-regulation of such genes has been reported in *B. cereus* ATCC 14579 acid stressed cells (Mols *et al.*, 2010a) and was associated with a secondary oxidative stress response (for a review see Mols and Abee, 2011), and may be a common component of multiple stress stimuli (Reder *et al.*, 2012). The enhanced acid resistance of mild oxidative stressed *B. weihenstephanensis* cells confirmed the significant role of the oxidative stress response in cross-protection, as was already observed in *B. cereus* (den Besten *et al.*, 2010a; Mols *et al.*, 2010a). Therefore genes involved in various bacterial stress responses appear of practical importance as they might function as biomarker for multiple bacterial stress resistances. Furthermore the overlap in up-regulation of genes and adaptive behaviour in both *B. weihenstephanensis* and *B. cereus* revealed similar adaptive traits between two species of the *B. cereus* group, and point to common and important roles in stress adaptation.

In conclusion, we demonstrated how gene expression profiles can be used to identify cellular biomarkers that can serve to early detect and predict bacterial behaviour. This quantitative approach encompasses both linear and non-linear correlations of gene expression and bacterial resistance and leads to the identification of biomarkers to track bacterial resistance. Moreover this study supports the extrapolation of such biomarkers to others related species and bacterial resistances and underlines the possibility to predict the microbial behaviour using molecular biomarkers.

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Supplemental data

Gene	Gene number	Annotations	Biomarkers type	Pearson coefficient (stress exposure)
			Reference	
<i>16S*</i>	BcerKBAB4_r0001	16S ribosomal RNA	gene	
			Reference	
<i>23S*</i>	BcerKBAB4_R0004	23S ribosomal RNA	gene	
<i>atpA</i> ^(c)	BcerKBAB4_5105	FOF1 ATP synthase subunit alpha	ND	
BcerKBAB4_0325 ^(b)	BcerKBAB4_0325	Peroxiredoxin	Long-acting	
BcerKBAB4_0904 ^(a)	BcerKBAB4_0904	Bacterioferritin	ND	
BcerKBAB4_1716 ^(c)	BcerKBAB4_1716	Major facilitator transporter	Long-acting	
BcerKBAB4_2108 ^(c)	BcerKBAB4_2108	Aldehyde dehydrogenase	Long-acting	
BcerKBAB4_4399 ^(c)	BcerKBAB4_4399	EmrB/QacA family drug resistance transporter	ND	
<i>bkdR</i> ^(c)	BcerKBAB4_4008	PAS modulated σ^{54} specific transcriptional regulator	Direct	0.917 (acid)
<i>clpC</i> ^(a)	BcerKBAB4_0076	ATP-dependent Clp protease ATP-binding subunit ClpC	ND	
<i>codY</i> ^(c)	BcerKBAB4_3651	Transcriptional repressor	Long-acting	
<i>ctsR</i> ^(a)	BcerKBAB4_0073	Transcriptional regulator	ND	
<i>cydA</i> ^(c)	BcerKBAB4_4629	Cytochrome bd-I oxidase subunit I	ND	
<i>dps</i> ^(c)	BcerKBAB4-1875	DNA-protecting protein	ND	
<i>fabF</i> ^(c)	BcerKBAB4_1083	3-oxoacyl-(acyl carrier protein) synthase II	ND	
<i>groES</i> ^(a)	BcerKBAB4_0245	Chaperone protein	ND	
<i>katA</i> ^(b)	BcerKBAB4_1058	Catalase	Long-acting	
<i>lacI</i> ^(c)	BcerKBAB4_4066	LacI transcriptional regulator	Long-acting	
			Direct	0.907 (acid)/0.888 (oxidative)
<i>lexA</i> ^(b)	BcerKBAB4_3377	SOS response transcriptional repressor	Direct	
<i>mntH</i> ^(c)	BcerKBAB4_1735	Manganese transport protein MntH	ND	
<i>napA</i> ^(c)	BcerKBAB4_1526	Potassium efflux system protein	ND	
			Direct	0.972 (salt)/0.886(oxidative)
<i>narL</i> ^(b)	BcerKBAB4_1974	Respiratory nitrate reductase, gamma subunit	Direct	
<i>nos</i> ^(b)	BcerKBAB4_5238	Nitric-oxide synthase	ND	
<i>perR</i> ^(b)	BcerKBAB4_0453	Peroxide stress response regulator	ND	
<i>relA</i> ^(c)	BcerKBAB4_4254	GTP pyrophosphokinase	Long-acting	
<i>rsbW</i> ^(a)	BcerKBAB4_0902	Anti-sigmaB factor	ND	
<i>sigB</i> ^(a)	BcerKBAB4_0903	General Stress Sigma Factor	ND	
<i>sigM</i> ^(c)	BcerKBAB4_3069	Stress Sigma Factor	ND	
<i>sodA</i> ^(b)	BcerKBAB4_5239	Superoxide dismutase	ND	
			Direct/Long-acting	0.987(acid)
<i>spxA</i> ^(b)	BcerKBAB4_1097	Transcriptional regulator Spx	acting	
<i>trxB</i> ^(b)	BcerKBAB4_4951	Thioredoxin reductase	Long-acting	
			Reference	
<i>tuf*</i>	BcerKBAB4_0103	Elongation factor EF-Tu	gene	

Table S1: Targeted and reference genes (*) used in this study with ^(a) members of the general stress response regulon controlled by the transcriptional regulator σ^B and genes involved in repair and maintenance of cellular proteins; ^(b) genes involved in cellular defence mechanisms against oxidative stress; ^(c) genes encoding for regulators, transporters or enzymes involved in cell maintaining. Direct biomarker genes showed an expression pattern linearly correlated to bacterial resistance; while long-acting biomarkers showed gene expression which was transiently up-regulated and linked to a stable increase of acid resistance over mild exposure time. Seventeen genes showed non determined (ND) correlation with acid resistance.

Chapter 7

mRNAs biomarkers selection based on Partial Least Square algorithm in order to further predict *Bacillus weihenstephanensis* acid resistance

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Abstract

In order to integrate Omics data to quantitative microbiological risk assessment in foods, gene expressions may serve as bacterial behaviour biomarkers. In this study an integrative approach encompassing predictive modelling and mRNAs quantifications, was followed to select molecular biomarkers to further predict the acid resistance of *Bacillus weihenstephanensis*. A multivariate analysis was performed to correlate the acid bacterial resistance and the gene expression of vegetative cells with or without exposure to stressing conditions. This mathematical method provides the advantage to take gene expressions and their interactions into account. The use of the Partial Least Squares algorithm allowed the selection of nine genes as acid resistance biomarkers among the 30 targeted genes. According to their involvement in the general acid stress response of *Bacillus*, these genes were assigned to three different biological modules namely, metabolic rearrangements, general stress response and oxidative stress response. The oxidative stress response appeared as the major activated biological module in *B. weihenstephanensis* cells submitted to acid stress conditions. Furthermore, as a firstly described model, the developed concept showed promising results to further be used to predict bacterial resistance using gene expression. Thus, this study underlines the possibility to integrate the bacterial physiology state, using Omics biomarkers, into bacterial behaviour modelling and thereby further improve microbial risk assessment accuracy. This will certainly enable new decision making approaches in the food safety and quality framework, by avoiding over risk estimation and thus over-processing.

Introduction

Approach to estimate risks caused by pathogenic microorganisms in the food chain sits within the framework of Microbiological Risk Assessment (MRA) as described by the Codex Alimentarius Commission, the international agency for setting food standards (Codex, 1999). The MRA is usually seen as particularly appropriated to governmental food safety and quality organizations with related decision-making tasks and tools. But this has also relevance to numerous industrial applications, such as shelf-life determination, heat treatment optimisation, raw material selection, assessment of non thermal inactivation processes and new formulation development. MRA is as well adapted to microbial spoilers and spoilage events, in food products and processes. Within this framework, the level of undesirable microorganisms in the food chain is normally assessed using classical tools and approaches for microbial identification, enumeration, and prediction of behaviour. It is now commonly recognized that such decision making tools often over-estimate the risks, and could be improved by integrating the bacterial and related cellular adaptive response to various stresses. In the meantime, Omics data bases have exploded, offering wonderful opportunities to develop new methodologies and improve available approaches in risk analyses (Brul *et al.*, 2012; Havelaar *et al.*, 2010; McMeekin *et al.*, 2008; Rantsiou *et al.*, 2011). Over the last ten years, many qPCR-based methods have been developed to detect, identify and/or quantify pathogens in food (Postollec *et al.*, 2011). Real-time PCR (qPCR) and reverse-transcription qPCR (RT-qPCR) are considered as methods of choice to analyze specific genes and their expression patterns (Nolan *et al.*, 2006). RT-qPCR was used to study microbial growth dynamics and associated metabolic activities in food (Carey *et al.*, 2009; Falentin *et al.*, 2010; Hierro *et al.*, 2006; Torriani *et al.*, 2008; Ulve *et al.*, 2008).

Bacillus weihenstephanensis is a psychrotolerant bacterium belonging to the *Bacillus cereus* group which is of major concerns for the food industry. Indeed this group is composed of closely related strains, which might produce toxins or enzymes involved in food spoilage. To our knowledge, *B. weihenstephanensis* has never been correlated with food-borne diseases, but some strains have been shown to be emetic (Hoton *et al.*, 2009; Thorsen *et al.*, 2006) and some others, such as the KBAB4 strain, possess genes involved in enterotoxin production (Lapidus *et al.*, 2008; Réjasse *et al.*, 2012; Stenfors *et al.*, 2002; Stenfors Arnesen *et al.*, 2011). In addition, conjugation and transfer of toxin encoding plasmids were shown to occur at significant levels among strains of the *B. cereus* group (Hoffmaster *et al.*, 2004; Hoffmaster *et al.*, 2006; Lapidus *et al.*, 2008; Van der Auwera *et al.*, 2007), and particularly *B. weihenstephanensis*, which may synthesize toxin depending on the environmental conditions, and particularly at low temperature (Stenfors-Arnesen *et al.*, 2007 and 2011; Thorsen *et al.*, 2006). Furthermore, the increasing demand for ready-to-eat foods could raise

the issue associated with psychrotolerant bacteria, since they represent the most probable risk in refrigerated food poisoning (Lapidus *et al.*, 2008).

To ensure the microbial food safety and stability, the food industry uses combination of hurdles, mild preservation factors (Leistner, 1995). A commonly used hurdle is a low pH which allows the decrease in growth rate but also the inactivation of pathogens or spoilage microorganisms (Coroller *et al.*, 2006; Greenacre *et al.*, 2003; Ita et Hutkin, 1991). Thus, the finding of acid resistance biomarkers for *Bacillus*, *i.e.* cellular components that are objectively measured (with satisfactory accuracy and reproducibility), and evaluated as indicators of specific biologic processes (Atkinson *et al.*, 2001), appears as practical importance for food industry.

Some approaches to select molecular biomarkers of *Bacillus* have been already presented (den Besten *et al.*, 2010; den Besten *et al.*, 2013; Desriac *et al.*, 2012; Desriac *et al.*, 2013). These studies showed linear or non linear correlations between gene expression and bacterial resistance. Nevertheless, they are based on correlation of a gene expression with bacterial resistance without considering gene expression interactions. In this study, a multivariate statistical approach was used to obtain an integrated view of the biological impact of the survival ability of *Bacillus weihenstephanensis* under lethal acid conditions. Partial Least Square regressions (PLS) approach allows overcoming problem of inter-correlated predictor variable and data matrices where the number of variables exceeds the number of samples (Kettaneh-Wold, 1992; Kvalheim and Karstang, 1989).

The objective was to find among the targeted genes those which are mostly correlated to the acid resistance of vegetative cells of *Bacillus weihenstephanensis* with (adapted cells) or without (unstressed cells) preliminary exposure to sub lethal stress conditions (acid, temperature, oxidative adaptation conditions). Since targeted genes are known to be involved not only in bacterial acid stress response but also in a variety of stresses such as salt and oxidative stresses, it should be feasible to further explore their predictive potential for other lethal stresses.

Materials and Methods

Bacterial strain and pre-culture conditions. The psychrotolerant *Bacillus weihenstephanensis* KBAB4 strain, kindly provided by the Institut National de la Recherche Agronomique (INRA, Avignon, France), was used in this study. Its genome is already available (Lapidus *et al.*, 2008). The strain was stored at -80°C in Brain Heart Infusion broth (BHI broth, Biokar Diagnostic, Beauvais, France) supplemented with 50% (v/v) glycerol. Bacteria (1ml of glycerol stock) were first grown in 100 ml BHI

broth and incubated at 30°C for 8 h under shaking conditions (100rpm) and an aliquot (1ml) was transferred into a second flask of 100 ml BHI broth and incubated for 15 h in the same conditions.

Mild stress exposure adaptation. In order to integrate the cellular adaptive stress response of *B. weihenstephanensis* to conditions which may be encountered in food industry and thus different physiological states, three conditions were used to mildly stress cells, namely, cold, acid and salt stresses. In these conditions, bacterial growth began at an average of 10^5 CFU.ml⁻¹ and reached around 10^7 CFU.ml⁻¹ after 4 to 70 h of incubation time, corresponding to mid-exponentially phase cells which were used as inocula for acid resistance determination. For non adapted cells or unstressed cells, a portion (0.1 ml) of the pre-culture incubated 15h was transferred in a flask containing 100 ml of BHI broth and incubated at 30°C with shaking at 100 rpm, until 10^7 CFU.ml⁻¹ ($OD_{600nm} = 0.20 \pm 0.02$). For mild cold-adapted cells, the portion was transferred in a flask containing 100 ml of BHI broth and incubated at 8°C with shaking at 100 rpm, until reached 10^7 CFU.ml⁻¹ ($OD_{600nm} = 0.33 \pm 0.03$). At last, for mild acid- and salt-adapted cells, the transfer was done in BHI supplemented with HCl (pH 5.5) and NaCl (2.5% w/v) respectively, and incubated at 30°C under shaking conditions (100 rpm) ($OD_{600nm} = 0.15 \pm 0.03$).

Acid inactivation kinetics. Mild stress grown cells and unstressed cells were subsequently submitted to lethal acid conditions to quantify the resulting acid bacterial resistance. A volume of 15 ml of 10^7 CFU.ml⁻¹ of non adapted, cold-, acid- and salt- adapted mid-exponentially cells was transferred into 285 ml of BHI broth supplemented with HCl to reach a final pH of 4.6. In order to exclude possible additional bacteriostatic or bactericidal effects associated with the use of certain organic acids, HCl was chosen as acidifier to evaluate the mere impact of proton acidification. Bacterial suspensions were further incubated at 30°C under shaking conditions (100 rpm). Aliquots were sampled after appropriated incubation time of exposure at pH 4.6. For each sampling time, an aliquot was dedicated to molecular analysis while a second was used to determine the number of survivors. Note that sampling of inocula *i.e.* mid exponential non adapted, cold-, acid- and, salt-adapted cells of *B. weihenstephanensis* were also performed for both molecular analysis and bacterial enumeration. Bacterial counts were performed on Nutrient Agar (Biokar Diagnostic) using a SPIRAL plater (AES, Chemunex, Combourg, France), after appropriately diluting in Tryptone Salt broth (Oxoid, Dardilly, France). Plates were subsequently incubated for 16 to 24 h at 30°C. Acid inactivation experiments were performed in three biological replicates using three different aliquots of frozen cells. To ensure the absence of spores in both growth and inactivation media, aliquots of 2 ml were

submitted to heat treatment at 70°C for 5 min (Baril *et al.*, 2011) and potential germinating spores were then quantified on nutrient agar (Biokar Diagnostic) medium at 30°C after 24h of incubation.

Bacterial resistance fitting and estimation. To determine the acid bacterial resistance, a Weibull model was used (Marfart *et al.*, 2002).

$$\log_{10}\left(\frac{N}{N_0}\right) = -\left(\frac{t}{\delta}\right)^p \quad (1)$$

where p is a shape parameter, δ is the first decimal reduction time (h) and t the sampling time.

In this study, the acid resistance of the bacterial population is needed for any time of sampling in order to be able to correlate the acid resistance to the gene expression at each sampling time. We choose to qualify the bacterial resistance by the time of exposure to stress, needed for the next decimal reduction. Using the equation (1), the bacterial reduction could be computed as follows:

$$dn = p\delta^{-p}t^{p-1}dt \quad (2)$$

where dn corresponds to the bacterial reduction for dt the variation of time (Mafart and Coroller, 2012). Knowing the equation (2), the decimal reduction n between time t and time $t+i$ could be written as:

$$n = \int_t^{t+i} p\delta^{-p}t^{p-1}dt \quad (3)$$

Choosing to qualify the bacterial resistance population at time ' t ' of sampling by using the time needed to reduce the population of 1 log or 90%; the previous equation could be written as follows:

$$\int_t^{t+\delta_t} p\delta^{-p}t^{p-1}dt = 1 \quad (3)$$

where δ_t is the time necessary to reduce 90% of the survival bacterial population sampled at the time ' t '. Thus the bacterial resistance at time ' t ' or instant δ could be defined as $t+\delta_t$ which represents the total exposure time to stress conditions necessary to reduce 90% of the bacterial population at time ' t '. This mathematical equation was used to determine the acid resistance (instant δ) of each sampling time using the estimated parameters of the Weibull model. This model was fitted on the inactivation curves using non linear fitting module in MATLAB (NLINFIT, MATLAB 7.0.1, Statistic Toolbox, The Mathworks, Massachusetts, USA).

RNA extraction and cDNA synthesis. For both inocula and acid-treated samples, 10 ml of cells suspensions were used for RNA extraction as previously described by Desriac *et al.* (2012 and 2013). Briefly, after centrifugation (10 000×g, 4°C, 10 min) cell pellets were resuspended in 1 ml of RNA protect (Qiagen, Courteboeuf, France) and incubated for 5 min at room temperature. After further centrifugation, the cell pellets were frozen and stored at -80°C until cell disruption, performed as recommended by the manufacturer (Qiagen). RNA purification was performed following RNeasy® Mini Kit (Qiagen) instruction. RNA quantity and quality was assessed using NanoDrop 2000 (NanoDrop Technologies, Wilmington, USA) and microfluidic analysis (RQI, Experion, Bio-Rad, Mity Mory, France). As advised by Stahlberg *et al.* (2004), an adjusted volume of total RNA corresponding to 200 ng was reverse-transcribed into cDNA using iScript cDNA synthesis kit (Bio-rad). The reaction was followed by a reverse transcriptase denaturation step (5 min 80°C). Contaminating genomic DNAs were removed from each sample using the DNA-free™ Kit (Ambion, Cambridge, UK) according to manufacturer's recommendations. The absence of genomic DNAs in RNA sample was checked using RNA as template for qPCR.

Primer design and quantitative PCR. cDNA amounts were quantified by qPCR using hydrolysis probes technology. Based on sequences obtained from NCBI databases, primers and probes of 33 studied genes were manually designed and were evaluated for primer dimers and hairpins formation using Beacon Designer software (Premier Biosoft International, Palo Alto, CA, USA). Amplification was performed using a specific rotative PCR device based on the Pall GeneDisc Technology developed to facilitate data acquisition and decrease data dispersion errors. Tailor made and ready to use GeneDisc® plate allowed the quantification of the 33 different cDNAs, among which 3 reference genes, within a single PCR run. To correct run to run variation, so-called inter-run calibrators were used. The latter consisted in three known genomic DNA concentrations and were performed on a gene per gene basis for each device batches. The calibration was done on the quantification cycle (Cq) level as described by Vermeulen *et al.* (2009). A dilution series of genomic DNAs was used as input material to estimate the PCR efficiency. All primer pairs and probes showed satisfying efficiency values comprised between 92 and 104%, an R^2 of at least 0.980, and close intercepts of 43.72 ± 0.98 .

Gene expression quantification. Gene expression quantification was performed at different sampling times of lethal acid treatment of adapted and unstressed cells as well as for the inocula, in three replicates. RT-qPCR results were expressed in relative quantity scaled to the lowest expression level using the delta Cq transformation with efficiency correction, after getting the mean of the gene expression measurements for the three replicates of a given sample. Then normalization was

performed using the expression of three reference genes as recommended by Vandesompele *et al.* (2002). The normalization factor is equal to the geometric mean of relative quantities of reference expressions and varied from one sample to others. Gene expression stability of the three reference genes was assessed by calculating the average pair-wise variation, known as *M* value, which was equal to 0.76, 0.79 and 0.98 for *16S*, *23S* and *tuf*, respectively, underlying satisfying stability (Vandesompele *et al.*, 2002).

Multivariate analysis. A multivariate statistical approach was used to obtain an integrated view of the impact of the acid stress exposure on *Bacillus weihenstephanensis* gene expression. The objective was to find among the targeted genes those which are mostly correlated to the acid resistance of vegetative cells of *B. weihenstephanensis*. Due to the greater number of gene expressions as compared to the number of samples, Partial Least Square regressions were used to identify gene expressions that were significantly correlated to the acid bacterial resistance. As advised by Derveaux *et al.* (2010), the normalized relative quantities of gene expression was log transformed making the data distribution more symmetrical (Bengtsson *et al.*, 2005). Furthermore, based on literature, targeted genes could be assigned in three main biological modules *i.e.* general stress response, oxidative response and metabolic rearrangements.

PLS was used to select among the 30 targeted genes those that were correlated to bacterial acid resistance according to their variable importance in projection value (VIP). The variables with VIP values over 0.8 were retained (Thabius *et al.*, 2011). These were reassigned to their respective biological module and orthogonal PLS (OPLS) on each biological module was done. OPLS was used to remove irrelevant variation in the gene expression data *i.e.* the non-correlated systematic variation in gene expression, making interpretation of the resulting PLS model easier (Trygg and Wold, 2002). If OPLS model was non-significant (according to cross-validation ANOVA analysis), a new gene selection was performed as function of their VIP values and the significance of their PLS regression coefficients until getting significant OPLS model.

Then OPLS scores from each biological module were used to construct hierarchical OPLS in order to evaluate the module which mostly impacts on the acid bacterial resistance of *B. weihenstephanensis*. All calculations were performed using Simcap+ version 13.0 software (Umetrics, Umea, Sweden) with unit of variance scaling of the data. Model validation was performed using R^2 value (total variance explained) over 0.5 and Q^2 value (variance predicted after cross validation) at least equal to 2/3 of R^2 (Thabius *et al.*, 2011). Additional model robustness validation was performed using permutation tests (100 permutations) for PLS and analysis of variance testing of cross-validated predictive residuals (CV-ANOVA) for PLS and OPLS (Erikson *et al.*, 2008). The permutation testing method first estimates a

PLS model and its R^2 and Q^2 ; then, with fixed X, the order of the elements in the Y-vector is randomly permuted a number of times, here 100 times. Each time a new PLS model is fitted using X and the permuted Y, providing a reference distribution of R^2 and Q^2 for random data. These distributions are then used to appraise the statistical significance R^2 and Q^2 parameters of the original PLS model. R^2 and mostly Q^2 of the permuted model must decrease, especially, Q^2 must have negative values.

Results

Potential Biomarkers. In search of potential biomarkers to predict the acid resistance of *B. weihenstephanensis*, a survey was performed in proteomics and transcriptomics available databases, and allowed the selection of 30 genes (supplemental data), *i.e.* potential mRNA biomarkers, that may play a key role in bacterial acid stress resistance (Ceriagioli *et al.*, 2010; Daniel *et al.*, 1997; den Besten *et al.*, 2009; Mols *et al.*, 2010a; Mols *et al.*, 2010b; Mols and Abee, 2011a; Mols and Abee, 2011b; Ratnayake-Lecamwasam *et al.*, 2001; Ter Beek *et al.*, 2008; van Melis *et al.*, 2011; van Schaik *et al.*, 2007; Wilks *et al.*, 2009; Zhang *et al.*, 2003; Zuber *et al.*, 2009). Mols and Abbe (2011) reported four different groups in the acid stress associated mechanisms of *B. cereus* cells with (i) general stress response, (ii) metabolic rearrangement, (iii) pH homeostasis and (iv) oxidative response. In this study, metabolic rearrangement and pH homeostasis were considered as one biological module since the metabolic rearrangements are involved in internal pH maintaining. Consequently, selected genes were assigned in three different biological modules (see Figure 1) namely, general stress response (9 genes), oxidative stress response (9 genes) and metabolic rearrangements (12 genes).

Multivariate analysis for the identification of acid resistance biomarkers. To identify among the candidate biomarkers those which may serve as acid resistance biomarkers of *B. weihenstephanensis*, the gene expression of the 30 potential biomarkers were correlated to the acid resistance. Since the gene expressions were quantified for a given sampling time, the corresponding bacterial resistance was calculated as an instant δ (hour) meaning the time of exposure to stress needed to reduce of 90% the sampling bacterial population. Furthermore, to avoid the correlation of non-biological variation of gene expression with acid resistance, the Cq values were transformed as relative quantities and normalized using 3 stable references genes (*16S*, *23S*, and *tuf*) and further log transformed before correlation (Bengtsson *et al.*, 2005). Then gene expressions were correlated to the instant δ using PLS regression (Figure 1). The stepwise procedure allowed the selection of 20 genes for which the VIP values were greater than 0.8 and the construction of a significant PLS model

($p = 0.01$ after cross-validation ANOVA). The PLS model could explained 90.1% of the acid resistance variation ($R^2 = 0.901$ and $Q^2 = 0.778$) and was further validated through the response permutation testing with a $R^2 = 0.537$ and $Q^2 = -0.348$.

Among the 20 selected genes, 4 genes were assigned in the oxidative module (*cydA*, *katA*, *nos* and *perR*); 7 genes were assigned to the general stress response module (*clpC*, *ctsR*, *dps*, *groES*, *napA*, *rsbW* and *sigB*) and; 9 genes were assigned to the metabolic rearrangements module (*atpA*, BcerKBAB4_1716, BcerKBAB4_4399, *bkdR*, *codY*, *fabF*, *narL*, *relA* and *sigH*). Then for each biological module OPLS model was constructed. OPLS model was then tested for significance using cross-validation ANOVA, and a new selection of gene was performed based on the VIP and the coefficient values until getting significant OPLS model ($p < 0.05$). This new selection decreased the number of potential biomarkers from 20 to 15 genes (Figure 1) with 4 genes in the oxidative module (*cydA*, *katA*, *nos* and *perR*); 5 genes in the general stress response (*clpC*, *ctsR*, *dps*, *napA* and *rsbW*); and 6 genes for the metabolic rearrangements module (*atpA*, *codY*, *fabF*, *narL*, *relA* and *sigH*). Then to evaluate the influence of each biological module on the acid resistance, a hierarchical OPLS (H-OPLS) was constructed. The H-OPLS was based on the score obtained from the OPLS of each biological module and was validated using cross validation ANOVA ($p = 0.0006$). The VIP obtained for each biological module underlined that each biological module has equivalent influence on the acid resistance of *B. weihenstephanensis*.

At last, to reduce once more the number of potential biomarkers, a new selection based on the VIP value of selected genes was done. Since no biological module appeared as more important than another, the 3 genes with the greater VIP value in each biological module were selected as biomarkers of the acid resistance of *B. weihenstephanensis*. Thus using this multivariate analysis, 9 genes were selected as the more relevant biomarkers of acid resistance of *B. weihenstephanensis* namely, *codY*, *cydA*, *dps*, *fabF*, *katA*, *napA*, *nos*, *rsbW* and *sigH*.

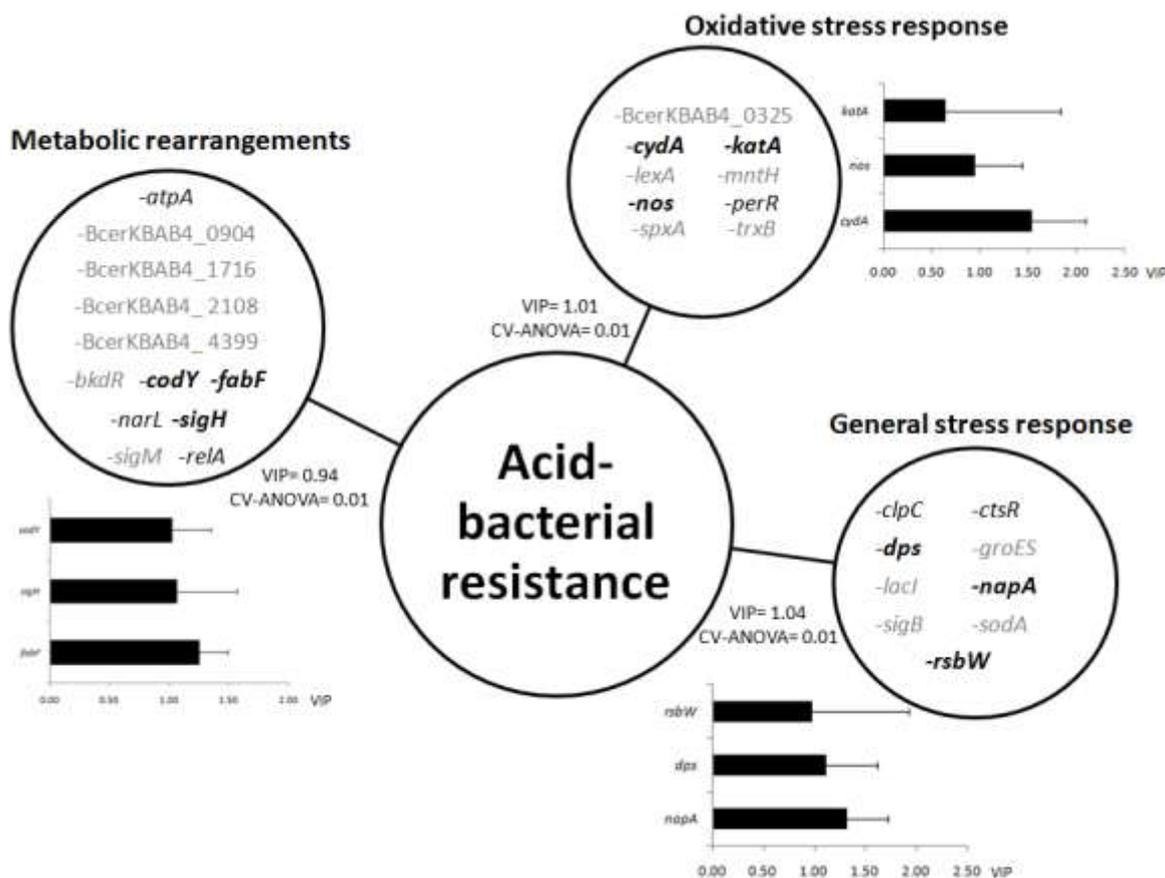


Figure 1: Overview of mRNA biomarkers selection based on PLS regressions. General stress response, oxidative stress response and metabolic rearrangements correspond to three biological modules involved in the acid stress response of *B. cereus*. In grey, genes which were deleted during PLS regressions analysis and in black, genes which were used to performed OPLS and H-OPLS models. Bold genes correspond to selected genes and showed the highest variable importance VIP. Bar diagrams correspond to the VIP values of genes selected as biomarkers in this study. VIP and CV-ANOVA correspond respectively, to the VIP of each biological module obtained using the H-OPLS and to the cross-validation ANOVA obtained for OPLS models of the different biological modules (models were considered as significant when CV-ANOVA value was lower than 0.05).

Acid resistance and selected biomarkers patterns. Although no biological module appeared most important in the construction of the PLS model, one of this may appear most important in the physiological response of *B. weihenstephanensis* upon lethal acid exposure. To investigate the influence of each biological module on the bacterial response, the percentage of expression of the sum of the three selected biomarkers was calculated for each module based on the RT-qPCR results expressed in relative quantities (Figure 2A). It could be noted that whatever the growth conditions encountered for the inoculum, *i.e.* unstressed, acid-, salt- or cold-adapted cells, the percentage of gene expression associated to the oxidative module increased when the cells were exposed to the lethal acid stress. As for instance, in unstressed cells of *B. weihenstephanensis*, the percentage of gene expression associated to the oxidative response increased from 30% to more than 60 % after 2h of lethal acid exposure. Furthermore, this increase in expression of genes associated with oxidative stress response was linked to an increase in the bacterial resistance (Figure 2B). Regarding the acid

resistance of the different inocula, the same pattern could be observed. Indeed, as already reported, the most acid resistant cells were cells grown under sublethal acid conditions (acid adaptation with pH5.5) and the percentage of gene expression associated to oxidative stress was close to 40 % whereas the two other modules represented approximately 30 %. In cells which were the less resistant to the lethal acid stress exposure *i.e.* cold- and salt-adapted cells, the percentage of gene expression associated to oxidative stress response was the less represented module.

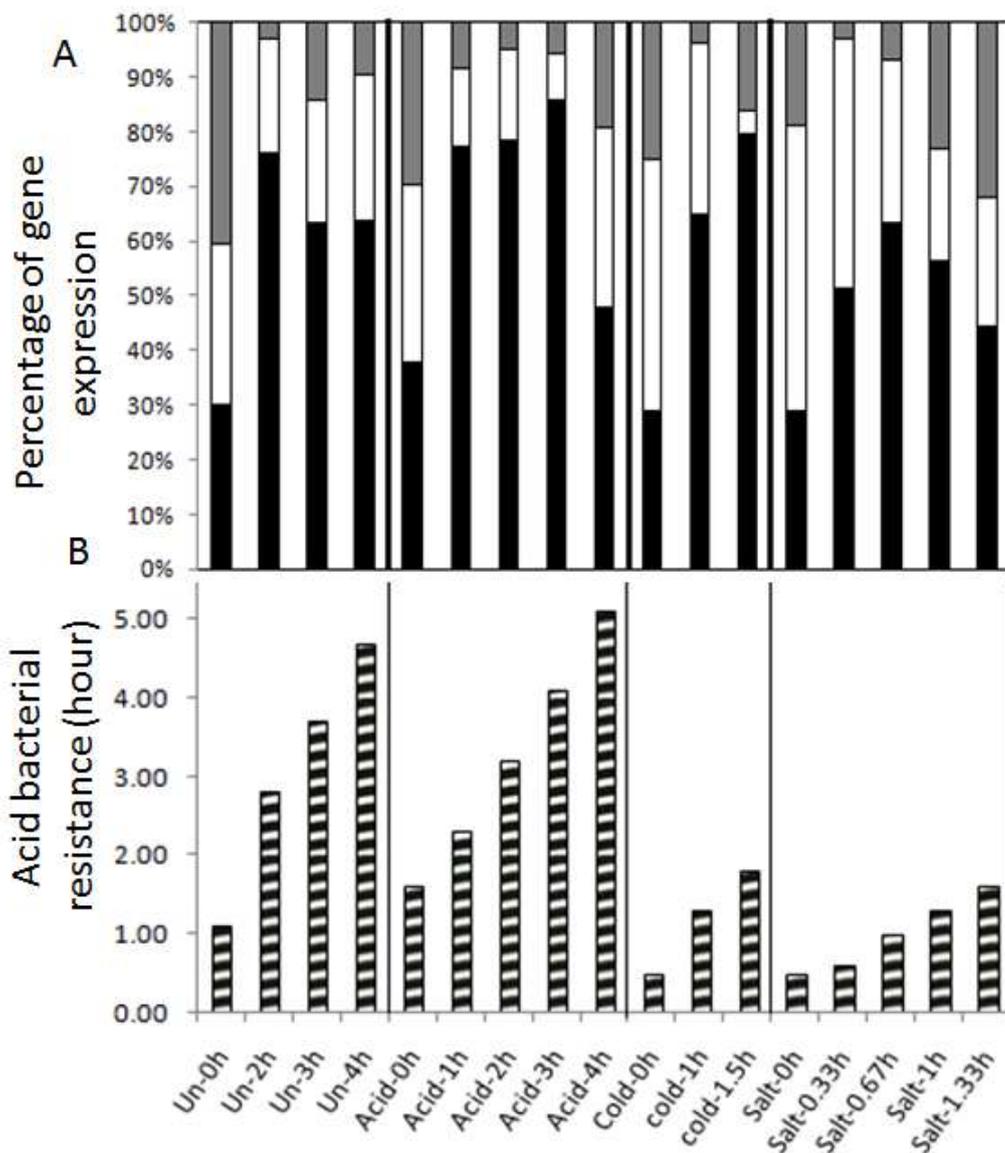


Figure 2: Gene expressions and associated bacterial behaviour, *i.e.* acid resistance. A- The percentage of gene expression associated to the oxidative stress response module (black bars), the general stress response (white bars) and, to the metabolic rearrangements module (grey bars). Hatched bars represent the instant δ computed from the estimated parameters of the Weibull model. Samples were named first as function of their preliminary growth conditions (Un- for unstressed and Acid-, Cold- and Salt- for adapted cells) followed by the exposure time (h) of the acid inactivation treatment pH4.6.

Discussion

The combination of molecular tools and predictive microbiology concepts appears to be an interesting challenge for food formulation and preservation optimisation in order to incorporate bacterial physiology into bacterial growth and survival predictions (Brul *et al.*, 2012; Desriac *et al.*, 2012 and 2013; Havelaar *et al.*, 2010; McMeekin *et al.*, 2008; Rantsiou *et al.*, 2011). Thus the finding of molecular biomarkers to characterize the physiological state under specific conditions remains a key issue (den Besten *et al.*, 2010; and 2013; Desriac *et al.*, 2012 and 2013; Kort *et al.*, 2008). In order to survive, bacteria are continuously sensing their environment to respond with appropriate changes in gene expression and protein activity (Boor, 2006). Thus gene expression is the first and often transient bacterial response to changing environment. Some recent studies focussed on the finding of molecular biomarkers using gene expression to characterize the bacterial behaviour of *Bacillus* (den Besten *et al.*, 2010; and 2013; Desriac *et al.*, 2012 and 2013; Kort *et al.*, 2008). These studies highlighted the potential of using gene expression patterns as biomarkers using linear correlation between one gene pattern and given bacterial behaviour. Knowing that gene expression belongs to complex biological systems for which part of regulatory networks remains unknown, it is clearly conceivable that focusing at only one gene may not be sufficient to further describe bacterial behaviour under a variety of stress. That is why, in this study, a multivariate analysis based on bacterial acid resistance modelling and mRNA quantification was used in order to investigate and further predict the response of *B. weihenstephanensis* KBAB4 to lethal acid stress. Indeed PLS was used for the regression modeling because it overcomes the problems of inter-correlated predictor variables and data matrices where the number of variables exceeds the number of samples (Kettaneh-Wold 1992; Kvalheim 1989). In other word, the PLS regression allowed to take into account interactions between gene expressions as encountered during gene regulations. Based on *B. cereus* and *B. subtilis* stress adaptive behaviour upon exposure to various stresses, 33 genes were selected as potential biomarkers. Then using PLS based approach, 9 genes were selected namely, *codY* which encodes for transcriptional repressor, *cydA* which encodes for the cytochrome bd-I oxidase subunit I, *dps* which encodes for a DNA-protecting protein, *fabF* encoding for 3-oxoacyl synthase II, *katA* encoding for a catalase, *napA* encoding for a potassium efflux system protein, *nos* encoding for nitric-oxide synthase, *rsbW* encoding for anti-sigma B factor and *sigH* encoding for a RNA polymerase factor. These 9 genes were assigned to different biological module to investigate the impact of each biological module on the acid bacterial resistance of *B. weihenstephanensis*. *cydA*, *katA* and *nos* were assigned to the oxidative stress response module. Indeed, cytochrome bd oxidase (*cydA*) genes may act as an alternative complex IV of the electron transfer chain (Mols *et al.*, 2010)

and is part of the larger anaerobic responsive Fnr regulon (Reents *et al.*, 2006); *kata* encodes for a catalase showing clear association with the oxidative stress response and ; *nos* which forms nitric oxide from arginine, which putatively protects cells from H₂O₂-induced DNA damage by inhibition of the Fenton reaction and activation of catalase (Gusarov and Nudler, 2005; Shatalin *et al.*, 2008).

dps, *napA* and *rsbW* genes were assigned to the general stress response since *dps* and *rsbW* encode respectively for DNA-protecting protein and the anti-sigma B factor and since in *B. subtilis* the corresponding *napA* gene was regulated by the σ^B factor (Helmann *et al.*, 2001; wilks *et al.*, 2008). At last *codY*, *fabF* and *sigH* genes were assigned to the metabolic rearrangements. CodY is a conserved global transcriptional regulator that mediates beneficial changes in response to fluctuations of nutrient availability in low G+C Gram-positive bacteria (Sonenshein, 2005; Frenzel *et al.*, 2012). As instance, CodY is a transcriptional regulator involved in the control the *ureABC* expression in *B. subtilis* (Wraig *et al.*, 1997). *sigH* which is particularly known for its involvement in sporulation was also involved in adaptation to nutrient deficiency (Britton *et al.*, 2002). Indeed, in *sigH B. subtilis* mutant cells, the majority of altered genes are involved in metabolism, transport nutrients, and antibiotic production and is, for instance, involved in the regulation of the *ureABC* expression in *B. subtilis* (Wraig *et al.*, 1997). As of *fabF*, it is involved in the fatty acid biosynthesis. Regarding the VIP associated to each biological module in the H-OPLS, these biological modules have equivalent influence on the acid resistance of *B. weihenstephanensis* (Figure 1). Nevertheless investigating the percentage of expression of each module using the RT-qPCR results, the oxidative stress module was showed to be the main response upon acid lethal stress conditions whatever the prior bacterial growth conditions (Figure 2). This finding is consistent with previous studies (Mols *et al.*, 2010 a and b) where *B. cereus* cells revealed a major oxidative stress response under exposure to acid conditions. This secondary stress response would be due to perturbation of the electron transfer chain, where free electrons prematurely leak to oxygen result in the formation of superoxide (for a comprehensive review see Mols and Abee 2011a). The overlap in adaptive behaviour in both *B. weihenstephanensis* and *B. cereus* points to common and important role of a given gene in stress responses. This supports the possibility of extrapolation of such biomarkers to other related species. If the selection of potential biomarkers offers new perspectives for the prediction of bacterial behaviour and physiology, one of the key challenges will be to increment these data into mathematical model to predict growth or inactivation, during industrial processes to offer decision making tools for food safety and quality management (Desriac *et al.* 2012, 2013). PLS model offers the advantage of rapid selection of biomarkers but could also be used as mathematical model to predict the bacterial behaviour. Even though a complete set of data is needed to validate the developed model, the prediction feasibility was analyzed using conditions with sufficient available

inputs. Thus, the absolute quantifications of the nine selected mRNA biomarkers were used for unstressed, acid and salt adapted samples. Using this PLS model the acid resistance of the cold-samples were further predicted. The acid resistance were predicted to 0.74 h, 0.97 h and 2.08 h respectively for the cold adapted inoculum, after 1h and 2h for which the acid resistances were observed at 0.5 h, 1.3 h and 1.8 h. Further investigations are currently in progress in order to validate the selection of potential biomarkers for a larger variety of stresses and thus a larger number of bacterial physiological states and to validate the model in industrial conditions. Nevertheless these results highlight the possibility to integrate bacterial physiological state using mRNAs quantification into microbial behaviour prediction. This promising work opens the avenue toward the implementation of new MRA tailor made tools for food industry.

Conclusion

Based on both predictive microbiology and mRNAs quantifications, the response of *B. weihenstephanensis* KBAB4 to acid stress was investigated. The acid bacterial resistance was determined after mathematical modelling of experimental acid inactivation kinetics through the determination of the instant δ values for each sampling time. RT-qPCR quantifications were performed to quantify the gene expression of 33 potential biomarkers at each sampling time. Then a multivariate analysis was used to correlate the gene expression to the acid resistance. PLS based analysis allowed the selection of nine genes as biomarker of the acid resistance of *B. weihenstephanensis* taking the correlation between the different biomarkers into account. Furthermore, this approach showed some promising results in how integrate Omics data in bacterial behaviour modelling through the prediction of bacterial behaviour using mRNAs quantification. Moreover an overlap of *B. weihenstephanensis* and *B. cereus* adaptive behaviour was observed supporting thereby the extrapolation of such generic biomarkers and concept to other related species.

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Chapter 8

Summarizing discussion, concluding remarks and perspectives

Introduction

Preservation of food requires the control of microorganisms throughout the food chain, and proper monitoring to ensure the safety and stability of the product during its shelf life. Approaches to estimate risks posed by pathogenic microorganisms in the food chain are captured in the framework of Microbiological Risk Assessment (MRA). Within the MRA framework, the fate of undesirable microorganisms in the food chain is assessed using classical tools such as cultural based method or PCR methods. If these methods allow microbial identification, enumeration and/or prediction of microbial behaviour (Brul *et al.*, 2012), they do not assess the bacterial physiological state. It is now recognized that this yields over or under estimations in microbial quantitative risk assessment. Such tools can be more specific, sensitive and relevant by providing insights into the underlying molecular physiological mechanisms that govern the behaviour of the microorganisms. In order to incorporate the microorganisms physiological state into MRA, two main different approaches are known with the identification and quantification of (i) metabolic pathways with flux balance analysis (Kauffman *et al.*, 2003; Metris *et al.*, 2011), or (ii) biomarkers (den Besten *et al.*, 2010; Kort *et al.*, 2008).

Minimally processed food products with high organoleptic and nutritious quality is gaining importance in food markets through the world and leads to possible threat: the survival of spoilage and/or pathogenic microorganisms (Abee and Wouters, 1999; Carlin *et al.*, 2000; Valero *et al.*, 2000). These products rely on the application of various mild preservation factors, known as hurdles (Leistner, 2000; Leistner and Gorris, 1995). They offer a high quality to the consumers, retaining the typical characteristics of fresh foods and assuring the microbial safety and stability. However the ability of microorganisms to adapt to stressing environments could antagonize the benefits of the hurdle preservation strategy by providing enhanced cell resistance towards lethal stress conditions (Abee and Wouters, 1999; Hill *et al.*, 2002; Skandamis *et al.*, 2008). For low-acid minimally processed foods, the main microbial hazard is the presence of psychrotrophic, sporulated bacteria that are able to survive mild heat treatment (that could even stimulate spore germination) and grow at chilled temperature (Valero *et al.*, 2000). *Bacillus weihenstephanensis* is a psychrotolerant bacterium belonging to the *Bacillus cereus* group which is composed of spore forming bacteria that may be involved in food safety or spoilage (**Chapter 1**).

This thesis described transcriptomic based approaches to identify molecular biomarker that may be further used to improve effective controlling of psychrotrophic *B. cereus* strains resistance. *B. cereus* can encounter acid conditions in foods which often have a low pH with or without the addition of organic acids, and upon ingestion to overcome the acid barrier of the human stomach. This thesis

focussed on the bacterial acid resistance which appears of great importance for the control of *B. cereus*. However this approach may be applied to other hurdles and may further be used to optimize food production processes and storage conditions, with the aim of getting the proper balance between food safety and food quality.

Bacillus cereus acid stress response and potential biomarkers selection

The availability of omics data of *B. cereus* response to acid conditions opened avenues to search for acid stress indicators that could function as biomarkers of resistance behaviour. The official US National Institute of Health definition of a biomarker is “a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (Atkinson *et al.*; 2001). This definition commonly recognized could be adapted to food processes and safety issues as: a characteristic that is objectively measured and evaluated as an indicator of bacterial responses to food processes and stress conditions.

The current knowledge on the *B. cereus* acid response is described in the **chapter 2** and allowed the selection of acid stress indicators that could function as biomarkers of resistance behaviour. The acid stress response of *B. cereus* could be divided into four groups (i) general stress response (ii) pH homeostasis, (iii) metabolic rearrangements and (iv) secondary oxidative stress response. The general stress response involves genes that are putatively not only induced under low pH exposure, but may be involved in a more general response to stresses. In *B. cereus*, the induction of transcriptional regulator such as σ^B factor, *ctsR* and *hrcA* occurs when cells are exposed to an acid stress. Due to a positive feedback of σ^B on its regulon, the σ^B operon is also up-regulated upon both lethal and non lethal acid stresses. Other genes that are known to be involved in various stresses, including protein repair chaperones *groEL*, *dnaK* and *clp* are up-regulated upon exposure to acid condition in *B. cereus*. The pH homeostasis is maintained using decarboxylation of amino acids like arginine, lysine and glutamate, however the decarboxylation systems used remains unclear and antiporter like Na^+/H^+ antiporter encoding for the *napA* gene is highly up-regulated upon exposure to lethal pH. The most notable metabolic rearrangements shown upon exposure to mainly organic acid stress are fermentative pathways, such as acetoin production (AlsDS), alcohol (AdhA), lactate dehydrogenases (Ldh), rerouting of pyruvate metabolism and the arginine deiminase (ADI) pathway that will induce proton consumption. The secondary oxidative stress response is correlated to the electron transfer chain perturbations generating superoxide and other reactive oxygen species inducing oxidative stress mechanisms.

Using this current knowledge, 31 genes were selected as potential biomarkers of acid resistance of *B. weihenstephanensis*. Five genes were selected for their potential use as reference genes. It could be noted that if the selected genes play key role in acid stress response of *B. cereus*, they are also known to be involved in various stress responses such as salt, oxidative, or heat bacterial stress responses. Likewise, these potential biomarkers have known crucial roles in adaptive stress responses of other bacterial species, including *Bacillus subtilis*, *Escherichia coli* and *Listeria monocytogenes* (Allen *et al.*, 2008; Petershon *et al.*, 2001; Van der Veen *et al.*, 2007). This is of importance to further extend our biomarkers to a variety of stresses which may be encountered in food processes and/or to other bacteria. Indeed, the canonical roles of these cellular components in adaptive stress responses suggest that they may contribute to (cross-) protective phenomena and that they may also have predictive potential in other bacteria. However, differences in functional conservation of these stress related factors between species and genera will challenge our search for successful biomarkers.

Impact of physiological state on the acid resistance and development of molecular method to track this feature

At the time this study started, limited information was available about the acid resistance of vegetative cells of *B. weihenstephanensis*. Spores are highly resistant to acid stress, but sporulation boundaries were similar to or included within the growth boundaries and thus no sporulation rate was observed under lethal acid conditions (Baril *et al.*, 2012). Therefore, another way to limit the prevalence of *B. cereus* in food, could be to focus on their less resistant form *i.e.* on vegetative cells. This may be of practical importance for food industry and particularly for minimally processed food. It is commonly recognized that the previously bacterial environment, or in other word the bacterial physiological state, will greatly impact the subsequent bacterial resistance.

In order to quantify the effect of preliminary exposure to stressing conditions encountered in food environment on the acid resistance of *B. weihenstephanensis* vegetative cells, the KBAB4 strain was cultured until the mid-exponentially phase in optimal growth conditions, mild salt and acid stressed conditions (**Chapter 3**). The growing cells were subsequently inactivated in lethal acid conditions ranging from 4.45 to 4.70. Linear and non linear primary survival models, which cover a wide range of known inactivation curvatures for vegetative cells, were fitted to the acid inactivation data in order to evaluate which model is the most adequate to describe the data obtained. To further apprehend the impact of lethal pH conditions on the acid resistance, the sensitivity, *i.e.* the change in pH leading to a ten folds variation of the bacterial resistance was also determined. The quantitative comparison

of the impact of the physiological state on the acid bacterial resistance showed that the acid resistance was enhanced for acid-adapted cells and decreased for salt-adapted cells. This latter is of relevance for food processing conditions where mild stress applications are successive since *B. weihenstephanensis* cells will be less acid resistant after prior exposure to salt conditions. The secondary modeling of the bacterial resistance allowed the quantification of the change in pH leading to a ten fold variation of the bacterial resistance, *i.e.* cells sensitivity (z_{pH}). This sensitivity was not significantly affected whatever the preliminary mild exposure and the presence of sub-populations with different acid resistances. These results highlighted that growth conditions influence bacterial acid resistance without affecting the sensitivity to acidic modifications, with a 10 fold reduction of *Bacillus* acid resistance observed for a reduction of 0.37 pH unit. These findings were also reported for *B. weihenstephanensis* and *B. licheniformis* spores for which the heat sensitivity was not modified whatever the sporulation environmental conditions tested (Baril *et al.*, 2011).

Therefore it may be supposed that the bacterial sensitivity of a given strain is mainly dependent of abiotic factors, whereas the bacterial resistance is affected by both biotic and abiotic factors. In the case this assumption is generalised, the food environment variation would have similar impact on the bacterial resistance whatever cell history. This simplification may be of interest for the calculation of process efficiency. However the bacterial physiological state of the food contaminant needs to be known and correlate to the bacterial resistance. That underlines the necessity to find molecular biomarkers that could reflect the bacterial fitness and which could be easily quantified. Thus a molecular method was developed (**Chapter 4**) to obtain insights into the underlying molecular physiological mechanisms of the acid bacterial responses. These quantitative data were then correlated to the bacterial behaviour observed using cultural based methods. Since gene expression levels are recognized as relevant biomarker to describe bacterial behaviour and since reverse transcription quantitative PCR (RT-qPCR) is considered as the gold standard for accurate, sensitive and fast measurement of gene expression, the present work has focused on the use of gene expression as biomarker. To ensure the quality of the gene expression data a step by step RT-qPCR quality controls was established. From the sample preparation to the data analysis, every quality controls and assurance procedures are in agreement with the current recommendations performed to ensure reliable RT-qPCR results.

Combining Omics data and predictive microbiology

To improve the quantitative microbiological risk assessment (MRA), currently recognized to yield to over or under estimations of the risk, one promising way is to take into account the bacterial adaptive traits observed throughout the food process. For that purpose, the identification of biomarkers with predictive potential appeared as a new trend, and was paved by the availability of bacterial insights into the molecular physiological state that govern the behaviour of the microorganisms. The methods used in this study to search for these biomarkers could be summarized as described in the Figure 1.

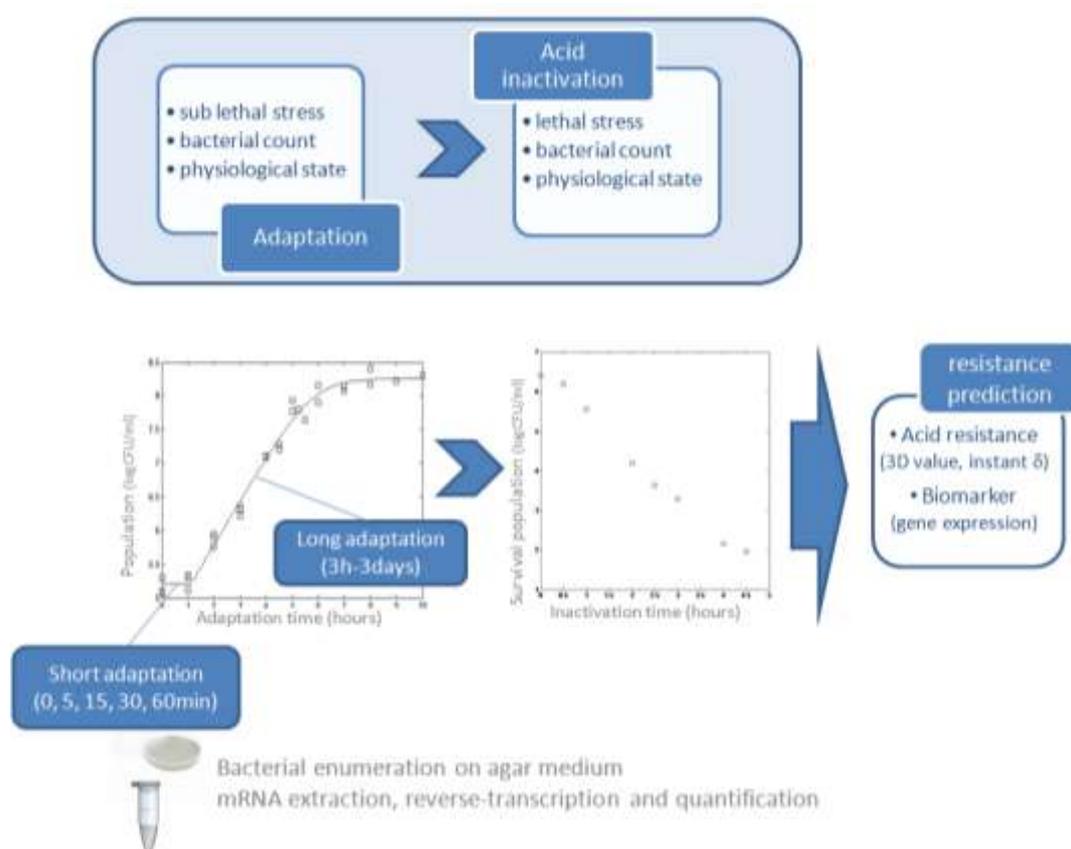


Figure 1: Schematic view of experimental design proposed in this study in which adapted cells are transferred to lethal acid conditions to determine inactivation kinetics. For each sampling time (throughout adaptation and inactivation), *B. weihenstephanensis* acid biomarkers are quantified by RT-qPCR and acid resistance is determined by classical mathematical models to constitute the database for further identify acid resistance biomarkers using various correlation kinds.

Based on an integrative approach encompassing both gene expression quantification throughout bacterial inactivation and mathematical modeling of the bacterial behaviour, the identification of different molecular biomarkers was done (**Chapter 5**). The *sigB* gene was proposed as biomarker to track moderate acid resistance of *B. weihenstephanensis* whereas *katA* was identified as biomarker to track high acid resistance. Indeed, fitting surviving bacterial counts of *B. weihenstephanensis*

under lethal acid conditions (pH 4.6), allowed the identification of a biphasic patterns meaning that the bacterial population could be divided into two subpopulations with different acid resistances. By correlating the proportion of the two subpopulations with the gene expression, it could be shown that the highest expression of *sigB* was observed when the sensitive subpopulation represented the majority of the bacterial population. At the opposite the *katA* up regulation was correlated to the more resistant subpopulation. *sigB* encodes the general stress sigma factor and its activation will allow the transcription of a set of genes encoding for proteins with specific functions, which will protect the cell against stress exposure. In *B. cereus*, σ^B is activated in several stress conditions such as ethanol, NaCl exposure or H₂O₂ and acid shock. However, the largest activating effect is observed with heat shock (van Schaik *et al.*, 2004a). Moreover, this key sigma factor controlling the general stress response is present in several gram-positive bacteria such as *Listeria* and *Staphylococcus* (Kazmierckza *et al.*, 2005; van Schaik *et al.*, 2004b) supporting the promising biomarker role of *sigB* in prediction of stress adaptive behaviour in foodborne microorganisms. *katA* encodes a catalase which convert hydrogen peroxide into water (Imlay, 2003) and its up-regulation in acid stress response was linked to the formation of reactive oxygen species due to perturbation of the aerobic electron transfer chain (Mols and Abee, 2011; Mols *et al.*, 2010b). Likewise for *sigB*, scavengers of reactive oxygen species such as catalases, are ubiquitous in biology and have known crucial roles in stress adaptation and survival in other species than *B. cereus*, including *Bacillus subtilis* (Hoper *et al.*, 2005; Pethersohn *et al.*, 2001) and also yeasts (Causton *et al.*, 2001). Recently, den Besten *et al.* (2013) have shown that the catalase activity may be linearly correlated to the induction of resistance of mild stressed cells of *B. weihenstephanensis* towards severe oxidative and heat stresses and thus may be used as potential biomarker of cell robustness. All these informations point out the predictive potential of *katA*.

In 2010, a framework was proposed for quantifying linear correlations between cellular indicators and mild stress induced robustness to evaluate the predictive potential of candidate biomarkers (den Besten *et al.*, 2010). Based on this approach, we proposed to investigate the linear correlations between the thirty targeted gene expression patterns and the survival ability of *B. weihenstephanensis* KBAB4 under lethal acid conditions after short exposure to sublethal stresses, *i.e.* mild salt, acid and hydrogen peroxide stress exposures during 5 to 60 minutes. In order to survive, bacteria are continuously sensing their environment to respond with appropriate changes in gene expression and protein activity (Boor, 2006). Thus gene expression is the first and often transient bacterial response to changing environments while functional proteins and enzymes might appear more stable to fulfil their key roles in bacterial stress responses (den besten *et al.*, 2013;

Michalik *et al.*, 2012). Knowing that, it is conceivable that gene expression may be correlated to bacterial resistance not only in a linear way but also by more complex feature.

Thus an additional approach was proposed to correlate transient gene expression patterns to induced acid resistance. Both linear and non linear correlation between gene expression and acid survival ability of short adapted cells were described in the **chapter 6**. Two kinds of biomarkers were defined with (i) direct biomarker genes for which the expression patterns upon mild stress treatment were linearly correlated to induced acid resistance; and (ii) long-acting biomarker genes which were up regulated for mild stress adaptation times of 30 minutes at maximum and linked to increased resistance over studied time (60 min). *narL* gene which encode a nitrate reductase involved in nitrogen metabolism (Mols and Abee, 2011) may serve as direct biomarker for acid resistance of mild salt stressed cells. Three genes named *bkdR*, *lexA* and *spxA* may serve as direct biomarkers for acid resistance of mild acid stressed cells. The *bkdR* gene encodes a σ^{54} -dependent transcriptional activator (Ter Beek *et al.*, 2008), and *lexA* and *spxA*, code for regulators with key roles in oxidative stress response of *Bacillus* (Butalaa *et al.*, 2009; Zuber, 2009). At last two genes, *lexA* and *narL* were found as direct biomarkers for acid resistance of mild oxidative stressed cells. If no targeted genes could be defined as direct biomarkers for the three tested preliminary mild stress exposure, it could be note that both *lexA* and *narL* were up-regulated and correlated to increase acid resistance after two sublethal stress preexposures. These finding highlight that some biomarkers may have a broader predictive potential than other (den Besten *et al.*, 2010). Regarding the non linear correlation, nine genes may serve as long acting biomarkers for acid resistance of mild oxidative stressed cells. Four of these genes are known to be involved in oxidative stress defence, namely, BcerKBAB4_0325, encoding a peroxiredoxin, *trxB*, encoding a thioredoxin, *kata*, encoding the main vegetative catalase (de Been *et al.*, 2010), and *spxA*, encoding a transcriptional regulator (Zuber, 2009). The other five genes have known functions in cell maintenance of *Bacillus* with genes encoding for regulators (*codY* and *laci*) (Daniel *et al.*, 1997; den Besten *et al.*, 2010; Frenzel *et al.*, 2012; Lindbäck *et al.*, 2012; Ratnayake-Lecamwasam *et al.*, 2001), transporter (BcerKBAB4_1716) (Ceragioli *et al.*, 2010; den Besten *et al.*, 2010) and enzymes (BcerKBAB4_2108 and *relA*) (den Besten *et al.*, 2010; Wilks *et al.*, 2009; Zhang *et al.*, 2003). The comparison of the number of gene which may serve as direct biomarker and the number of gene which may serve as long-acting biomarker showed the importance to consider non linear correlations in the search of potential biomarkers, particularly when focusing at transcriptional level. This is based on the notion that mRNAs can have short half-lives (Kushner, 1996), whereas the encoded proteins or enzymes may display prolonged stability and functionality (den Besten *et al.*, 2013; Michalik *et al.*, 2012), contributing to enhanced resistance. It

could be also noted that among the selected potential biomarkers of this chapter, five genes have been demonstrated to be also up-regulated by multiple mild stresses in *B. cereus* ATCC 14579 (den Besten *et al.*, 2010). Similar adaptive traits such as the significant role of the oxidative stress response in cross-protection (den Besten *et al.*, 2010; Mols *et al.*, 2010b; Chapter 5, 6,7), were revealed between two *B. cereus* ATCC 14579 and *B. weihenstephanensis* KBAB4. These findings point out to common and important roles in stress adaptation and support the extrapolation of such biomarkers to other related species and bacterial resistances.

However, using both linear and non linear correlation the majority of gene expression patterns (17) were not correlated to the bacterial resistance underlying the necessity to find other approaches which may explain a greater part of the variability of gene expression observed. Up to now the correlation between gene expression and bacterial resistance in the search of molecular biomarker for food associated microorganisms, are based on gene per gene correlation, another approach may be to take gene correlation into account. In the Chapter 6, the identification of acid resistance biomarkers was based on the gene expressions after short mild stress exposures (*i.e.* until 60 min). However to adapt the cells to new environment, either short (den Besten *et al.*, 2010; den Besten *et al.*, 2013; Kort *et al.*, 2008) or long time (Dubois-Brissonet *et al.*, 2000; Coroller *et al.*, 2006) exposure to mild stress are reported. The major difference between these two protocols relies in the fact that (i) short exposures do not imply growth while (ii) long adaptation time refers to exposure of few hours to several days allowing the multiplication of vegetative cells.

In the **Chapter 7**, we used a multivariate statistical approach based on Partial Least Square (PLS) algorithm to obtain an integrated view of the biological impact of the survival ability of *B. weihenstephanensis* under lethal acid conditions. The identification of acid resistance biomarkers was made using gene expressions of long adapted cells and gene expressions throughout acid inactivation kinetics. This statistical approach allowed to find among the targeted genes those which are mostly correlated to the acid resistance. The step-wise procedure for PLS, described in the Figure 2, allowed the selection of 9 genes as the more relevant biomarkers for acid resistance of *B. weihenstephanensis*.

The *cydA* gene encodes a cytochrome bd oxidase which may act as alternative complex IV of the electron transfer chain (Mols *et al.*, 2010b) and is part of the larger anaerobic responsive Fnr regulon (Reents *et al.*, 2006). *kata* and *nos* which encodes a nitric oxide synthase, which putatively protects cells from H₂O₂-induced damage by inhibition of the Fenton reaction and activation of catalase (Gusarov and Nudler, 2005; Shatalin *et al.*, 2008). *dps* and *rsbW* encode respectively for a DNA-protecting protein and the anti- σ^B factor and, *napA* encode a antiporter which is regulated by the σ^B

factor in *Bacillus subtilis* (Helmann *et al.*, 2001; Wilks *et al.*, 2008). CodY is a conserved global transcriptional regulator that mediates beneficial changes in response to fluctuation of nutrient availability in low G+C Gram-positive bacteria (Sonenshein, 2005; Frenzel *et al.*, 2012). *sigH* is particularly known for its involvement in sporulation, however this sigma factor play also key role in adaptation to nutrient deficiency (Britton *et al.*, 2002; Wray *et al.*, 1997) and *fabF* is involved in the fatty acid biosynthesis (Ter Beek *et al.*, 2008).

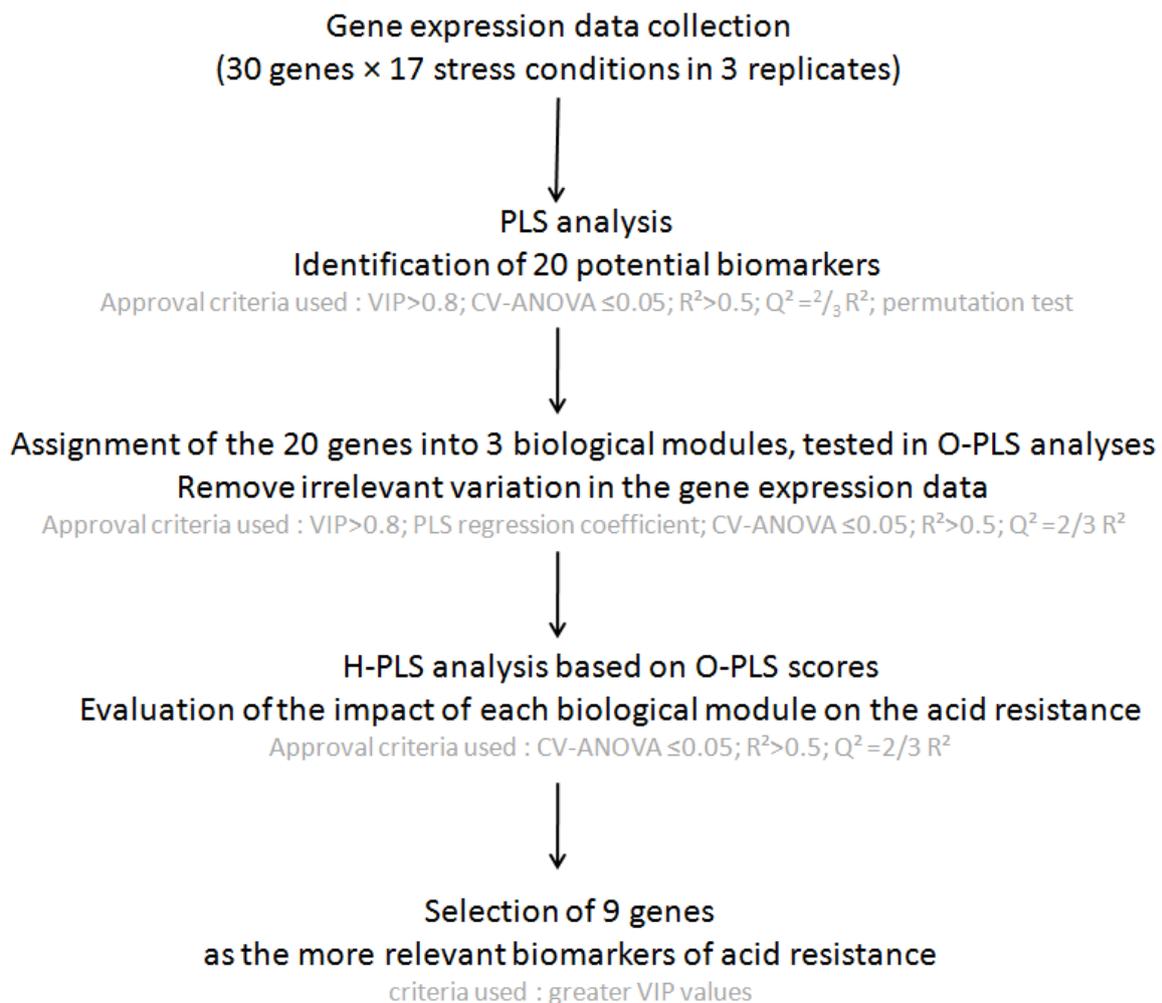


Figure 2 : Stepwise procedure used for gene selection as biomarker of *B. weihenstephanensis* acid resistance using a Partial Least Square approach

cydA, *katA* and *nos* belong to the general stress response biological module, *dps*, *napA* and *rsbW* to the oxidative stress response module and at last *codY*, *fabF* and *sigH* to the metabolic rearrangements. The VIP values obtained by H-PLS for each biological module underlined that each module has equivalent influence on the acid resistance of *B. weihenstephanensis*. Note that to reduce the number of resistance biomarkers only 3 genes of each biological module was chosen. However, 5 other genes (*atpA*, *narL*, *relA*, *clpC* and *ctsR*) could be also selected as resistance biomarkers since they were not statistically rejected throughout the entire PLS workflow.

Interestingly, regarding the percentage of expression associated to each module, a variation in the proportion of each biological module may be observed depending of the environmental conditions. Indeed, the oxidative biological module appeared as the most activated module when cells are submitted to a lethal acid conditions, as demonstrated for *B. cereus* (Mols *et al.*, 2010 a and b). The overlap in the adaptive behaviour of *B. cereus* and *B. weihenstephanensis* supports the feasibility of extrapolate such biomarkers to other related species.

At last, if the selection of potential biomarkers offers new perspectives for the prediction of bacterial behaviour and physiology, one of the key challenges will be to increment these data into mathematical model to predict growth or inactivation, during industrial processes to offer decision making tools for food safety and quality management (Brul *et al.*, 2012; Chapter 5, 6). PLS model offers the advantage that it could be used as mathematical model to predict the bacterial behaviour. First experiments performed to test the feasibility of the PLS model to predict the bacterial behaviour showed promising results. The acid resistances of three independent samples were predicted to 0.74 h, 0.97 h and 2.08 h whereas the acid resistances observed was equal to 0.5 h, 1.3 h and 1.8 h, respectively. Nevertheless it is important to bear in mind that further investigations are necessary to validate the PLS model.

Conclusion and future perspectives

Adaptive stress responses are crucial defense strategies in living cells and allow bacteria to survive in changing environment challenging the prediction of their history-dependent behaviour. Thus the finding of molecular biomarkers that are quantitatively correlated to stress adaptive behaviour appears as a promising area. The aim of this thesis was to identify gene expressions which may serve as molecular biomarkers of the acid resistance of *B. weihenstephanensis*. The psychrotolerant *B. weihenstephanensis* is a commonly recognized spoilage agent in foods and particularly in mildly processed foods (Antolinos *et al.*, 2012) since the preservation of these products is based on the combination of hurdles technologies (Leistner and Goris, 1995; Leistner, 2000).

The approaches used in this study which combine bacterial predictive modelling and gene expression quantification allowed the identification of molecular biomarkers (Figure 3). In order to mimic food processes and formulation preservatives compounds, various stress exposures were used, named salt, cold acid and oxidative stresses with short or long time exposures. Despite different approaches to select the biomarkers and different experimental conditions, some genes appeared to be identified as molecular biomarkers in all studied conditions underlying their predictive potential. Indeed *katA* which was selected as acid resistance biomarker whatever the selection method used.

Alike, the *narL* gene was also retained using the three approaches developed in this study. However the pattern allowing the correlation between gene expression and bacterial behaviour may vary as function of the studied conditions. As instance, using a PLS approach (Chapter 7) the *katA* was showed to be linearly correlated to the acid resistance whereas its expression was not linearly correlated when a linear approach on a gene per gene basis was done (Chapter 6). At the opposite, this gene was also selected as long acting biomarker meaning that its expression was non-linearly correlated to the acid resistance of short oxidative adapted cells. This variation could be explain by the fact that using a PLS approach the correlation between various biomarkers was taken into account.

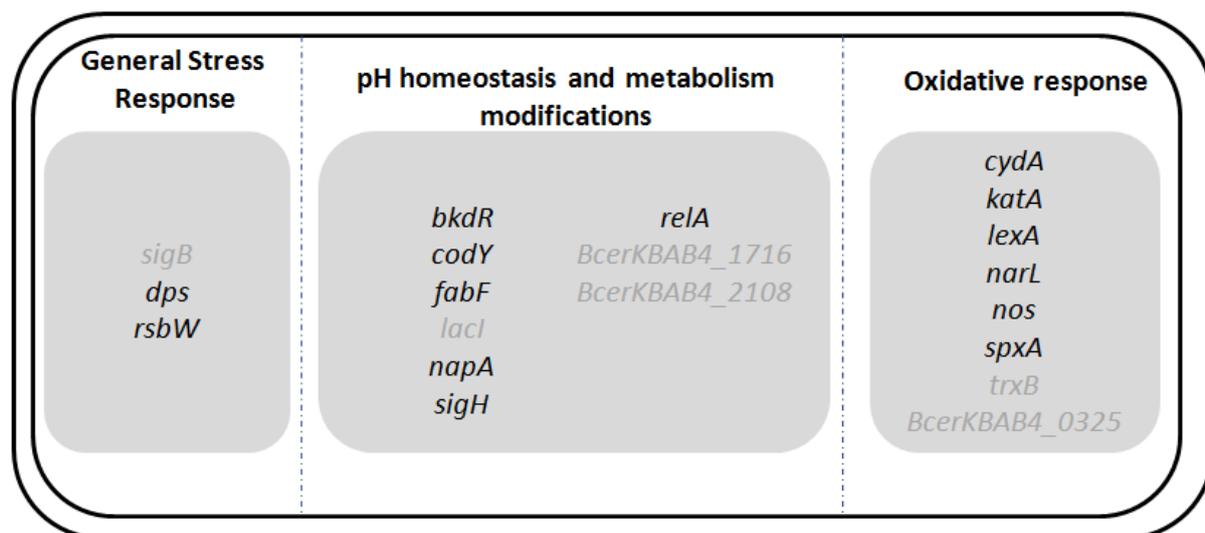


Figure 3: Genes selected, in this study, as acid resistance biomarkers of *B. weihenstephanensis*. Note that the classification of genes in the general stress response, pH homeostasis and metabolism rearrangement or oxidative response may vary since one gene may be involved in various biological modules. In grey the genes for which the mathematical way to correlate the gene expression pattern and the bacterial resistance was not determined.

Furthermore, among the 20 genes selected in this study as acid resistance biomarkers, 7 genes possessed correlation with the bacterial resistance which remains unclear. Indeed, as shown by the long-acting biomarkers, the mathematical correlation which will allow to further predict the bacterial resistance was not determined yet. Some other genes such as *bkdR*, *spxA*, *narL* and *lexA* showed linear correlation with the bacterial resistance however this correlation is not demonstrated for all studied conditions. Thus the predictive potential of these biomarkers appears limited to a set of conditions which will be not sufficient to evaluate all conditions that may be encountered in the food industry.

Thus further investigations using a largest number of environmental conditions which may be subsequent or simultaneous are necessary. This will reveal how vigorous these biomarkers are for predict the bacterial resistance of *B. weihenstephanensis*. These investigations are also required to understand the limits of the reliability of the predictive potential biomarkers. It could be also note

that through the obtained results some similar patterns in the adaptive response of the collection strains of *B. cereus* and *B. weihenstephanensis* were underlined, and thus support the plausible extrapolation to other related species and merit further investigations.

The working plan of the further investigations could be:

- (i) the acquisition of different physiological states after exposure to various environmental conditions
- (ii) the qualification of the physiological states by specific biomarkers quantification at the population and single cell level
- (iii) the bacterial resistance prediction by integrating cell histories,
- (iv) the implementation of mechanism-based predictive models based on PLS algorithm which will be able to predict the microbial ability to growth or to survive by monitoring gene expression.

Indeed, this thesis also underlines the predictive potential of PLS model with the first described bacterial resistance prediction by using gene expression data. Furthermore, it is important to bear in mind that the description of the full biological pathway is not required for acid resistance prediction but only key biomarkers quantification. The development of such models will contribute to support the food business sector competitiveness by optimizing inactivation process monitoring, eco efficient processing and accurate shelf life products. Thus, through the integration of bacterial physiological state into predictive microbiology behaviour, we will be able to offer new tailor made decision making tools which could be used for getting the proper balance between food safety and food quality.

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Compendium

Introduction

La sécurité microbiologique des aliments est d'une importance fondamentale les entreprises impliquées dans la production, la distribution et la vente de denrées alimentaires. En effet, la présence de microorganismes dans les produits alimentaires peut engendrer des conséquences économiques telles qu'une diminution de la production (Harris, 1997), ou encore sanitaires avec des maladies voire des décès; ces dernières engendrant elles-mêmes un coût en matière de traitement médical et donc des pertes dans le secteur de santé publique (Harris, 1997). Pour assurer la sécurité des aliments, un système qui identifie, évalue et maîtrise les dangers a été mis en place sous le nom de HACCP (Hazard Critical Control Point). Bien que ce système et les bonnes pratiques d'hygiène jouent un rôle clé, d'autres mesures sont aujourd'hui impliquées dans l'évaluation des risques microbiologiques (Basset *et al.*, 2012). Par exemple, un des outils couramment employé est la microbiologie prévisionnelle. La microbiologie prévisionnelle permet de prévoir si, et à quelle vitesse, les microorganismes vont se multiplier, survivre ou mourir en fonction du procédé, de la formulation de l'aliment et des conditions de stockage. Elle apparaît donc comme un outil d'aide à la décision qui permet de prévoir le comportement bactérien par le biais de modèles mathématiques. Aujourd'hui de nombreux modèles sont disponibles (McKellar et Lu, 2004 ; Brul *et al.*, 2007) et permettent de décrire le comportement bactérien en se basant sur des paramètres biologiques, physiques ou empiriques. Néanmoins, ces modèles de microbiologie prévisionnelle ont été principalement développés par le biais de méthodes culturelles et sont majoritairement basés sur le cas le plus défavorable. Il est aujourd'hui reconnu que cette approche engendre des sur-estimations du risque d'origine microbienne.

Une voie prometteuse pour améliorer la prévision du comportement bactérien est de prendre en compte les capacités adaptatives des microorganismes. En effet, la capacité de la cellule bactérienne à survivre ou croître dans des conditions environnementales données va dépendre de son histoire cellulaire ou de son état physiologique. Si ces conditions sont compatibles avec la vie, un équilibre s'installe entre le microorganisme et le système dans lequel il évolue. Dans la plupart des cas, cet équilibre est précaire et perturbé par les variations physiques, chimiques et biologiques de l'environnement. La survie des microorganismes est alors liée à leur capacité à s'adapter à ces variations et à maintenir leurs conditions cytoplasmiques à un niveau compatible avec la vie. En microbiologie, tout facteur ou condition environnementale va nuire à la croissance ou à la survie des

microorganismes est appelé stress (Youssef et Juneja, 2003). L'adaptation des microorganismes à ces conditions de stress va conditionner leurs comportements futurs face à de nouvelles conditions.

L'intégration en microbiologie prévisionnelle de ces réponses d'adaptation au stress est un enjeu devenu plus accessible par le développement des biotechnologies. En effet, ces dernières décennies ont vu le développement des technologies dites 'Omics': génomique, transcriptomique, protéomique, métabonomique et/ou métabolomique. L'accessibilité de ces techniques a largement influencé la façon d'étudier la réponse biologique des microorganismes face à des conditions environnementales variables (Brul *et al.*, 2012). Néanmoins, l'intégration des données Omics en microbiologie prévisionnelle, en vue de décrire le comportement bactérien, reste un défi. Cette modélisation du comportement des microorganismes permettrait d'offrir de nouveaux outils d'aide à la décision, d'affiner les procédés alimentaires tout en garantissant la sécurité des aliments. Ces objectifs sont d'autant plus importants qu'une demande croissante des consommateurs pour des aliments à hautes qualités organoleptiques et nutritionnelles, ainsi que pour des aliments prêts à l'emploi a engendré l'augmentation de la vente de produits utilisant des traitements assainissant limités. Parmi plus d'une soixantaine moyens de préservation disponibles, la température, l'activité de l'eau, l'ajout d'acides et de conservateurs tels que les nitrites ou sorbates apparaissent comme les facteurs « leviers » majoritairement utilisés (Leistner, 2000). Le principe de cette préservation des aliments repose sur le fait qu'un seul facteur limitant peut ne pas être suffisant pour inhiber totalement le développement microbien mais que leur association permet de contrôler le comportement bactérien tout en minimisant leur impact sur les qualités organoleptiques de l'aliment. Pour ces aliments peu transformés, le risque microbiologique majeur concerne la présence de bactéries psychrotrophes sporulées qui possèdent la capacité de résister à une grande variété de stress sous forme spores, et pouvant croître aux températures de réfrigération.

Aujourd'hui dans le but d'intégrer des données Omics et donc la physiologie cellulaire en microbiologie prévisionnelle, deux approches sont principalement utilisées. Elles consistent en: l'identification et la quantification de flux métabolomiques (Kauffman *et al.*, 2003 ; Métris *et al.*, 2011) ou de biomarqueurs (den Besten *et al.*, 2010 ; Kort *et al.*, 2008). Ces derniers peuvent être définie comme toute entité biologique (gènes, protéines, métabolites, minéraux) pouvant être reliée à un comportement biologique, pathologique ou pharmacologique et pouvant être quantifiée avec une précision et une reproductibilité satisfaisantes peut ainsi servir de biomarqueur (Atinkson *et al.*, 2001).

Bacillus cereus est une bactérie sporulée, aéro-anaérobie facultative et thermorésistante. Ces caractéristiques lui confèrent également une résistance particulière à l'action de composés bactéricides, ou de désinfectants, aux radiations, ou à la dessiccation. Le groupe *Bacillus cereus* est

composé de 7 espèces apparentées (Guinebretière *et al.*, 2008 ; Guinebretière *et al.*, 2010), dont trois espèces *B. cereus*, *B. thuringiensis* et *B. weihenstephanensis* ne peuvent être distinguées par les méthodes de référence permettant l'identification, la recherche et le dénombrement au sein des aliments de *B. cereus* (ISO 7932 et ISO 21871). *Bacillus cereus* peut produire deux types de toxines; une toxine émétique préformée dans les aliments et résistante à une large gamme de températures et de pH, et des toxines diarrhéiques produites pendant la croissance dans l'intestin grêle après ingestion de cellules végétatives ou de spores en quantité suffisante. Le syndrome émétique est caractérisé par des nausées, des vomissements et des crampes abdominales se produisant 1 à 5 h après l'ingestion des aliments contaminés, avec rétablissement dans les 6 à 24 h. Les symptômes associés sont semblables à ceux générés par les entérotoxines de *Staphylococcus aureus* préformés dans les aliments. Ce type d'intoxication alimentaire est relié à la production d'un dodécadepsipeptide cyclique, le céréulide, et nécessite l'ingestion de ce dernier. Le syndrome diarrhéique est attribué à des entérotoxines : l'hémolysine BL (Hbl) complexe à trois composantes (Hbl B, Hbl L1 et Hbl L2), l'hémolysine Nhe elle aussi composée de trois sous-unités (NheA, NheB et Nhe C) et la cytotoxine K (CytK) (Koritanta *et al.*, 2000 ; Lund and Granum, 1997 ; Lund *et al.*, 2000). Ces toxines sont produites dans le tube digestif de l'hôte et, pour être actives, les deux premières doivent être composées de leurs différentes sous-unités. Les temps d'incubation après ingestion des plats contaminés varient de 6 à 15 h avec rétablissement dans les 24 à 48 h et peut facilement être confondu avec celle provoquée par *Clostridium perfringens*. En 2010, *B. cereus* est à l'origine de 210 cas de toxi-infections collectives confirmés et serait l'agent suspecté pour 490 cas (Données épidémiologiques, Institut National de Veille Sanitaire).

En plus de leur caractère pathogène, les bactéries appartenant au groupe *B. cereus* sont aussi reconnues pour leurs capacités d'altération des aliments. La détérioration des aliments est causée par la croissance de bactéries indésirables dans ces derniers et peut entraîner d'importantes pertes pour l'industrie alimentaire (Gram *et al.*, 2002). Les altérations d'aliments causées par *Bacillus* sont principalement des défauts organoleptiques tels que des saveurs désagréables avec des notes rances, fruitées, levurées ou amères (Meer *et al.*, 1991) ou encore des défauts de texture rendant l'aliment impropre à la consommation. Les concentrations bactériennes associées aux aliments altérés sont proches de 10^6 UFC.g⁻¹ d'aliments (Meer *et al.*, 1991).

L'espèce *Bacillus weihenstephanensis* est une espèce du groupe *B. cereus* pouvant croître aux températures de réfrigération habituelles des denrées alimentaires. La présence de cette bactérie a été rapportée dans des produits laitiers (Bartoszewicz *et al.*, 2008 ; Christiansson *et al.*, 1999 ; Heyndrickx and Scheldeman, 2002 ; Svensson *et al.*, 2004), des ovo-produits (Baron *et al.*, 2007 ; Jan *et al.*, 2011), et dans une très grande variété de produits végétaux (Valero *et al.*, 2002). Les capacités

d'altération de ces bactéries psychrotolérantes sont très proches de celles des autres *Bacilli* avec des saveurs désagréables et des défauts de texture (Heyndrickx et Scheldeman, 2002). Ainsi, si les capacités d'altération de l'espèce *B. weihenstephanensis* sont définies, son potentiel de pathogénicité reste conditionnel puisque certaines souches de *B. weihenstephanensis* peuvent produire des toxines émétiques ou diarrhéiques (Lapidus *et al.*, 2008 ; Réjasse *et al.*, 2012 ; Stenfors *et al.*, 2002 ; Stenfors Arnesen *et al.*, 2011) mais qu'à ce jour aucune souche appartenant à cette espèce n'a été associée à une toxi-infection alimentaire.

Aujourd'hui, les outils de quantification de l'expression génique, en particulier la rétro-transcription suivie d'une quantification en temps réel via une réaction en chaîne par polymérase (RT-qPCR), permettent une quantification précise des variations de l'expression des gènes dans le temps. L'expression génique est l'une des premières réponses du microorganisme face à un environnement stressant. En effet, après avoir perçu les variations environnementales, l'expression des gènes est modulée permettant ainsi la production de protéines adaptées à la survie du microorganisme dans les nouvelles conditions. Ce travail de thèse a donc eu pour objectif d'identifier des ARNm en tant que biomarqueurs de résistance acide de *B. weihenstephanensis*. Il repose sur la corrélation de l'expression génique, quantifiée par RT-qPCR, à la résistance bactérienne au stress acide quantifiée par le biais de modèles mathématiques (Figure 1). Le travail réalisé est présenté au travers de 8 chapitres.

Après une introduction générale, le **chapitre 2** est consacré à un état des lieux sur les connaissances actuelles de la réponse physiologique de *B. cereus* face à un stress acide. Le **chapitre 3** correspond à l'étude de l'impact de l'état physiologique sur la résistance acide de *B. weihenstephanensis*. Dans ce cadre, l'état physiologique est caractérisé par des conditions de culture. Après avoir souligné le rôle clé que peuvent jouer les ARNm en tant que biomarqueurs de résistance, le **chapitre 4** décrit la méthode de RT-qPCR mise en place pour quantifier l'expression de gènes. Les chapitres suivants sont consacrés à l'identification de biomarqueurs de résistance acide de *B. weihenstephanensis*. Ainsi, le **chapitre 5** met en évidence une corrélation entre l'expression de certains gènes d'intérêt et la résistance des cellules soumises à un stress acide. Le **chapitre 6** présente une approche comprenant à la fois des corrélations linéaires et non linéaires entre l'expression génique et le comportement bactérien. Le **chapitre 7**, quant à lui, décrit une approche statistique multivariée qui permet d'étudier l'expression des gènes dans leur ensemble. Le dernier chapitre (**chapitre 8**) est consacré à une synthèse de l'ensemble des travaux présentés.

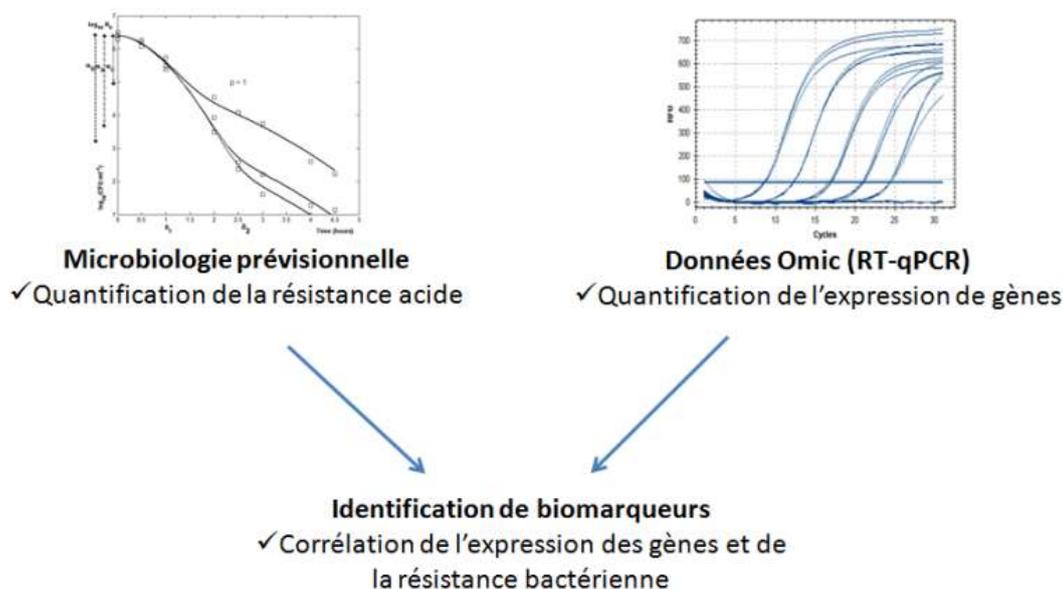


Figure 1 : Représentation schématique des outils utilisés dans cette étude afin de déterminer des biomarqueurs de résistance acide de *B. weihenstephanensis*.

Réponse physiologique de *B. cereus* face à un stress acide (Chapitre 2)

Cette étude d'identification de biomarqueur de la résistance acide de *B. weihenstephanensis* débute par un état de l'art sur la réponse physiologique de cellules végétatives de *B. cereus* face à un stress acide et donne lieu à une sélection de gènes pouvant servir de biomarqueurs. La **réponse au stress acide de *B. cereus* est divisée en 4 groupes majoritaires** avec (i) l'implication de la **réponse générale au stress** connue sous le nom de GSR (General Stress Response); (ii) le **maintien de l'homéostasie** cellulaire; (iii) les **réarrangements métaboliques** et; (iv) la réponse à un stress oxydatif, dite **réponse oxydative secondaire** (Figure 2).

Une stratégie communément employée par les bactéries pour faire face à des conditions de stress, est l'activation d'un facteur sigma alternatif spécifique qui va permettre la transcription d'un ensemble de gènes ou de régulons dont les produits vont protéger la cellule face aux conditions hostiles (Kazmierckza *et al.*, 2005). Il s'agit de la réponse générale au stress. Dans un grand nombre de bactéries Gram positif, le facteur sigma alternatif σ^B est le facteur clé contrôlant la GSR (Kazmierckza *et al.*, 2005 ; van Schaik *et al.*, 2004b). Il correspond à une sous unité de l'ARN polymérase. Chez *B. cereus*, cette sous unité est activée face à des conditions de stress diverses parmi lesquelles l'exposition à de l'éthanol, du sel ou encore face à un stress oxydatif ou acide.

Un second point clé dans la réponse au stress acide des bactéries est le maintien de l'homéostasie cellulaire, c'est-à-dire la régulation du pH interne de la cellule à un pH permettant la vie, via l'induction d'enzymes telles que des pompes à protons ou encore des enzymes responsables de

réactions consommatrices de protons. Comme le montre la sur-expression de *napA*, codant pour un transporteur Na^+/H^+ , au sein de cellules de *B. cereus* soumises à un stress acide létal, d'autres transporteurs sont impliqués dans le maintien de l'homéostasie de *B. cereus*.

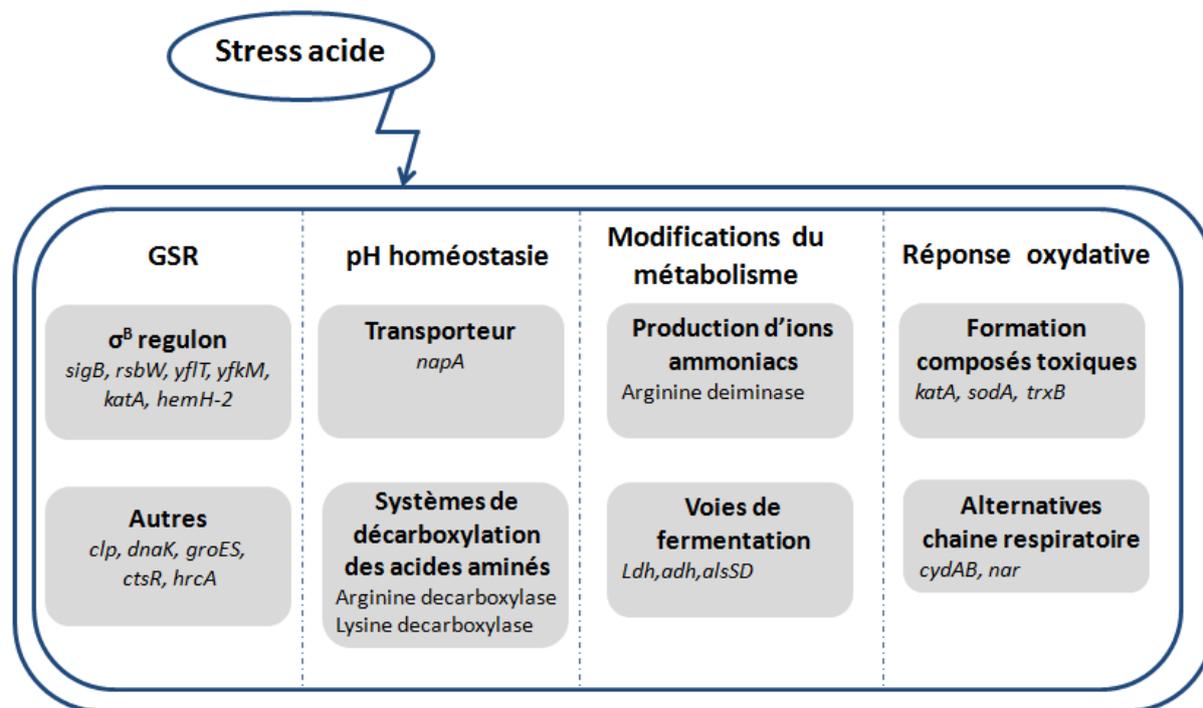


Figure 2 : Représentation des 4 réponses physiologiques mises en place par des cellules de *B. cereus* soumises à un stress acide. La réponse générale au stress ou GSR est principalement gouvernée par le facteur σ^B (*sigB*) qui régule l'expression d'une trentaine de gènes dont *rsbW* (anti-facteur σ^B), *yfjT* (protéine de stress), *yfkM* (protéase), *katA* (catalase), *hemH-2* (ferrochelatase). Ce groupe contient également des gènes non contrôlés par σ^B et pouvant être induits face à différents stress : *clp* (protease), *dnaK* et *groES* (protéines chaperonnes), *ctsR* et *hrcA* (régulateurs transcriptionnels). Le maintien de l'homéostasie cellulaire implique des transporteurs de protons (*napA*) ou des systèmes de décarboxylation des acides aminés. Les réarrangements métaboliques les plus notables sont les voies fermentatives telles que la production d'acétoïne (*alsSD*), d'alcool (*adh*), de lactate (*ldh*) déshydrogénases et la production d'ions ammoniacs à travers le système arginine déiminase. Enfin, la quatrième réponse correspond à l'induction d'une réponse secondaire oxydative due à des perturbations de la chaîne de transfert des électrons. Elle implique l'induction de gènes codant pour des catalases (*katA*), des dismutases (*sodA*) ou encore des thioredoxines (*trxB*) et des gènes codant pour la mise en place de solutions alternatives à la chaîne de transfert des électrons (*cydAB, nar/nas*).

L'implication des systèmes de décarboxylation des acides aminés dans la réponse physiologique de *B. cereus* face à un stress acide a été démontrée. En effet, des cellules de *B. cereus* préalablement cultivées en présence de lysine, arginine ou glutamate sont plus résistantes à un choc acide (Senouci-Rezkallah *et al.*, 2011) que des cellules préalablement cultivées sans ces acides aminés.

Afin de prévenir l'acidification de leur pH interne, les microorganismes peuvent aussi produire des composés alcalins ou modifier leurs métabolismes afin de produire des composés neutres. Ces mécanismes ont été largement étudiés chez les bactéries de la sphère bucale et chez les bactéries lactiques. Les mécanismes communément rencontrés sont les systèmes produisant des ions ammoniacs tels que la voie de l'arginine déiminase (ADI) ou de l'hydrolyse de l'urée. En effet l'ion

ammoniaque généré réagit rapidement avec un proton permettant ainsi l'alcalinisation de l'environnement en consommant un proton pour former un ion ammonium. Il a été démontré que la voie ADI pouvait jouer un rôle clé dans la résistance acide de *B. cereus* exposé à des conditions non létales de stress alors que cette voie ne semble pas impliquée lors d'exposition à des conditions létales (Mols *et al.*, 2010b; Senouci-Rezkallah *et al.*, 2011). L'activité uréase, quant à elle, a été mise en évidence dans 10 isolats de *B. cereus* sur 49, néanmoins elle ne contribue pas à augmenter leur survie face à un stress acide (Mols *et al.*, 2008). Concernant les voies de fermentation comme l'alcool déshydrogénase ou lactate déshydrogénase, elles sont induites face à un stress acide léthal et seraient une futile et ultime réponse des cellules pour restaurer la balance NAD^+/NADH (Mols *et al.*, 2010b). Enfin la dernière réponse connue de *B. cereus* face à un stress acide est la réponse secondaire oxydative. Elle serait corrélée à une perturbation de la chaîne de transfert des électrons et correspondrait à la réponse majoritaire mise en place par les cellules de *B. cereus* (Mols *et al.*, 2010b). En effet, si la tolérance limitée pour l'oxygène des microorganismes anaérobies est évidente, elle est également existante chez les microorganismes aérobies. Les microorganismes capables de se développer en condition d'aérobie, utilisent l'oxygène pour obtenir de l'énergie et produisent des composés toxiques pour la cellule tels que O_2^- , H_2O_2 ou OH^\cdot lors de la respiration. Les cellules bactériennes ne peuvent dès lors survivre que si elles possèdent des systèmes de défense aux anti-oxydants. Chez *B. cereus* les systèmes anti-oxydants sont induits face à des conditions acides avec par exemple, la sur-expression d'un ensemble de gènes codant pour des superoxydes dismutases ou encore des catalases ou thiorédoxines (Mols *et al.*, 2010b). De plus, l'induction de certains gènes tels que *cydAB* codant pour un cytochrome *d* ubiquinol ou *nar* codant pour une nitrate réductase corrobore l'induction d'une réponse au stress oxydatif lors d'une soumission à un stress acide. Ces protéines peuvent effectivement servir d'alternative à la chaîne respiratoire. L'ensemble de toutes ces connaissances synthétisé dans le chapitre 2 a permis la sélection de 31 gènes jouant un rôle majeur dans la réponse au stress acide chez *B. weihenstephanensis* et pouvant potentiellement servir de biomarqueur de résistance (Tableau 1).

Gènes	N° NCBI	rôles
<i>16S*</i>	BcerKBAB4_r0001	16S ribosomal RNA
<i>23S*</i>	BcerKBAB4_R0004	23S ribosomal RNA
<i>atpA</i>	BcerKBAB4_5105	F0F1 ATP synthase subunit alpha
BcerKBAB4_0325	BcerKBAB4_0325	Peroxiredoxin
BcerKBAB4_0904	BcerKBAB4_0904	Bacterioferritine
BcerKBAB4_1716	BcerKBAB4_1716	Major facilitator transporter
BcerKBAB4_2108	BcerKBAB4_2108	Aldehyde dehydrogenase
BcerKBAB4_4399	BcerKBAB4_4399	EmrB/QacA family drug resistance transporter
<i>bkdR</i>	BcerKBAB4_4008	PAS modulated σ^{54} specific transcriptional regulator
<i>clpC</i>	BcerKBAB4_0076	ATP-dependent Clp protease ATP-binding subunit ClpC
<i>codY</i>	BcerKBAB4_3651	Transcriptional repressor
<i>ctsR</i>	BcerKBAB4_0073	Transcriptional regulator
<i>cydA</i>	BcerKBAB4_4629	Cytochrome bd-I oxidase subunit I
<i>dps</i>	BcerKBAB4_1875	DNA-protecting protein
<i>fabF</i>	BcerKBAB4_1083	3-oxoacyl-(acyl carrier protein) synthase II
<i>groES</i>	BcerKBAB4_0245	Chaperone protein
<i>gyrA*</i>	BcerKBAB4_0006	DNA gyrase subunit A
<i>katA</i>	BcerKBAB4_1058	Catalase
<i>lacI</i>	BcerKBAB4_4066	LacI transcriptional regulator
<i>lexA</i>	BcerKBAB4_3377	SOS response transcriptional repressor
<i>mntH</i>	BcerKBAB4_1735	Manganese transport protein MntH
<i>napA</i>	BcerKBAB4_1526	Potassium efflux system protein
<i>narL</i>	BcerKBAB4_1974	Respiratory nitrate reductase, gamma subunit
<i>nos</i>	BcerKBAB4_5238	Nitric-oxide synthase
<i>perR</i>	BcerKBAB4_0453	Peroxide stress response regulator
<i>relA</i>	BcerKBAB4_4254	GTP pyrophosphokinase
<i>rsbW</i>	BcerKBAB4_0902	Anti-sigmaB factor
<i>sigA*</i>	BcerKBAB4_4144	RNA polymerase sigma factor RpoD
<i>sigB</i>	BcerKBAB4_0903	General Stress Sigma Factor
<i>sigG</i>	BcerKBAB4_3730	Sporulation sigma factor
<i>sigH</i>	BcerKBAB4_0088	RNA polymerase sigma factor
<i>sigM</i>	BcerKBAB4_3069	Stress Sigma Factor
<i>sodA</i>	BcerKBAB4_5239	Superoxide dismutase
<i>spxA</i>	BcerKBAB4_1097	Transcriptional regulator Spx
<i>trxB</i>	BcerKBAB4_4951	Thioredoxin reductase
<i>tuf*</i>	BcerKBAB4_0103	Elongation factor EF-Tu

Tableau 1: Gènes sélectionnés dont l'expression pourrait servir de biomarqueur de résistance acide de *B. weihenstephanensis*. * correspond aux gènes de référence c'est-à-dire dont l'expression permettra de normaliser les résultats obtenus en RT-qPCR tout au long de cette étude.

Impact de l'état physiologique des cellules sur la résistance acide (Chapitre 3)

Face à un stress acide, les cellules de *B. cereus* peuvent induire plusieurs réponses physiologiques (Figure 2) qui vont lui permettre de résister à la pression engendrée par des conditions environnementales défavorables. Il est aujourd'hui reconnu que les réponses adaptatives sont dépendantes de l'histoire cellulaire et vont influencer le futur comportement cellulaire. Par exemple, des cellules de *B. cereus* pré-adaptées à un stress acide non létal seront plus résistantes à un stress acide létal (Jobin *et al.*, 2002; Senouci-Rezkallah *et al.*, 2011). Les capacités de survie de *B. cereus* face à un stress donné sont donc relatives aux conditions préalablement rencontrées par la cellule et donc

à l'état physiologique de cette dernière à l'instant t . Afin d'évaluer l'impact de l'état physiologique des cellules de *B. weihenstephanensis* sur leur résistance acide, des cellules préalablement adaptées à un stress non létal salin ou acide sont ensuite soumises à des stress létaux acides (pH 4.45 à 4.70). Les cinétiques d'inactivation acide ont été modélisées en utilisant des modèles linéaires et non linéaires afin de pouvoir quantifier la résistance bactérienne. Sur la base de critères statistiques liés à la qualité d'ajustement et au nombre de paramètres (moyenne des carrés des écarts du modèle, R^2 , AICc, test F), le modèle le plus adéquat pour décrire l'ensemble des données, parmi les 3 modèles testés, est le **modèle double Weibull** (Coroller *et al.*, 2006). Ce modèle repose sur l'hypothèse de la **présence de 2 sous-populations** au sein de la population bactérienne étudiée. La première sous-population est moins résistante au stress que la seconde. L'impact de l'état physiologique des cellules sur la résistance acide bactérienne a été évalué via l'utilisation de cellules préalablement soumises à des conditions optimales de croissance, préalablement soumises à un stress non létal salin, ou à un stress acide non létal. **La résistance acide la plus faible est observée pour des cellules préalablement soumises à un stress salin non létal, suivie par celle des cellules préalablement soumises à des conditions optimales de croissance et enfin celle des cellules préalablement adaptées à un stress non létal acide.** Ce résultat peut être d'importance pour l'ajustement des procédés industriels, notamment pour définir l'ordre des facteurs de préservation permettant de garantir la stabilité et la sécurité des aliments.

Afin d'appréhender l'impact de l'intensité du stress sur la résistance bactérienne, la sensibilité des cellules est décrite et quantifiée par l'utilisation d'un modèle secondaire de type Bigelow. La sensibilité des cellules (z_{pH}) est définie par la variation de pH nécessaire pour induire un changement de résistance bactérienne (δ) d'un facteur 10. Quelles que soient les conditions préalablement rencontrées par les cellules, **la sensibilité de *B. weihenstephanensis* face à un stress acide létal ne varie pas.** Ainsi une **réduction de pH de 0,37 unité va engendrer une diminution de la résistance acide de *B. weihenstephanensis* d'un facteur 10**, et ceci peu importe les conditions environnementales préalablement rencontrées par les cellules. Des résultats similaires ont déjà été mis en évidence pour des spores de *Bacillus licheniformis* et *B. weihenstephanensis* pour lesquelles la sensibilité au stress thermique ne varie pas en fonction des conditions de sporulation (Baril *et al.*, 2011). Dès lors, il peut être supposé que la sensibilité d'une souche bactérienne donnée est uniquement dépendante des propriétés abiotiques alors que la résistance bactérienne est elle influencée à la fois par les propriétés biotiques et abiotiques. Les variations de l'environnement liées à l'aliment ou au procédés de fabrication auraient alors le même impact sur la résistance bactérienne et ceci indépendamment des conditions préalablement rencontrées par les cellules. Cette observation présente un intérêt pour prévoir et calculer l'efficacité des procédés industriels

puisqu'en connaissant l'état physiologique des cellules à l'instant t , il serait alors facile d'estimer l'impact du procédé sur la résistance bactérienne. Cependant, l'état physiologique des cellules bactériennes ne peut être facilement et rapidement quantifié à ce jour. Ceci souligne donc l'importance de trouver des biomarqueurs rapidement quantifiables et corrélés à un état physiologique donné. La suite de cette étude se focalise donc sur l'identification de gènes comme biomarqueur de résistance en appliquant deux outils, la microbiologie prévisionnelle et l'utilisation des techniques de RT-qPCR.

Méthode de quantification par RT-qPCR (Chapitre 4)

L'étape suivante de ce travail a donc consisté à la mise en place d'une méthode de RT-qPCR permettant de quantifier l'expression des gènes sélectionnés comme potentiels biomarqueurs suite à l'analyse des données bibliographique (Tableau 1). Une exception concerne 5 gènes parmi les 36 sélectionnés. Ces 5 gènes sont des gènes de référence potentiels, c'est-à-dire des gènes dont l'expression ne varie pas en fonction des conditions environnementales. Afin de valider leur utilisation en tant que normalisateur d'expression génique, leur stabilité est évaluée. Elle repose sur le principe que le ratio entre l'expression de 2 gènes de référence est identique pour tous les échantillons et ceci quelles que soient les conditions environnementales (Vandesompele *et al.*, 2002). L'expression des autres gènes est quant à elle corrélée à la résistance acide des cellules et va permettre d'identifier les biomarqueurs d'intérêts. Néanmoins, si la RT-qPCR est aujourd'hui considérée comme la méthode de choix pour une quantification précise et sensible de l'expression géniques, de nombreux points critiques peuvent survenir tout au long de l'acquisition des données. Deux articles nommés 'MIQE' et 'MIQE précis' pour 'Minimal Information for publication of Quantitative real-time PCR experiments' présentent les contrôles nécessaires pour garantir la qualité des résultats de qPCR (Bustin *et al.*, 2009 ; 2010). **La méthode de RT-qPCR mise en place dans ces travaux de thèse afin d'obtenir des données fiables, précises et répétables est décrite dans ce chapitre.**

Un outil de quantification génique basé sur la technologie Pall GeneDisc a été développé afin de faciliter l'acquisition des données et de limiter la dispersion des erreurs sur les résultats. Cet outil est composé de 36 puits de PCR pré-remplis avec les sondes et amorces spécifiques à la détection de 36 ADN complémentaires (Tableau 1) et permet la quantification de l'ensemble des gènes en une seule expérience. Les variations de quantification liées aux différents lots de GeneDisc® Plate employés sont corrigées par le biais de calibrations (IRCs). Ces calibrations sont composés de 3 concentrations connues d'ADN génomique (ADNg).

La qualité de la conception des amorces et sondes TaqMan® a été évaluée *in silico* et de manière empirique. Les couples amorces/sonde utilisés montre une efficacité de PCR comprise entre 92 et 104% et un coefficient de corrélation R^2 d'au moins 0.980 avec des produits de PCR de 94 à 157 paires de bases (Chapitre 4). La répétabilité et la linéarité de l'étape d'extraction d'ARN ont été validées de 10^3 UFC.ml⁻¹ à 10^8 UFC.ml⁻¹.

La qualité des ARN extraits a été quantifiée par le biais d'analyses micro-fluidiques. Les ARN extraits de cellules de *B. weihenstephanensis* soumises à des conditions optimales de croissance ou des conditions non létales de croissance donnent un RQI supérieur à 5 comme recommandé par Fleige and Pfaffl (2006). Les ARN extraits des 39 échantillons soumis à des conditions létales de stress ont, eux un RQI égal à 2.4 ± 0.5 . Cette diminution de la qualité des ARN pourrait être due à l'état physiologique des cellules et n'influence pas la quantification de l'expression génique puisque Fleige et Pfaffl ont démontré (2006) que la quantification de produits de RT-qPCR inférieurs à 250 paires de bases était indépendante de la qualité des ARN.

L'absence de contaminants d'ADNg a également été validée par le biais de quantification par qPCR d'échantillons d'ARN non rétro-transcrits. Parmi plus de 100 échantillons testés aucune contamination à l'ADNg n'a été démontrée soulignant l'efficacité de la méthode employée pour éliminer l'ADNg. En ce qui concerne la rétro-transcription, la même stratégie d'amorçage et la même quantité d'ARN ont été utilisées pour pouvoir comparer les différents échantillons et limiter les variations de quantification due à l'étape de rétro-transcription (Stahlberg *et al.*, 2004a ; 2004b). La méthode choisie repose sur l'utilisation d'amorces aléatoires permettant ainsi la rétro-transcription de l'ensemble des ARN cibles en une seule expérience. Il peut également être noté que l'absence d'inhibiteur de RT-qPCR a été validée pour l'ensemble des échantillons. **La validation de la stabilité des gènes de référence par le biais de comparaison 2 à 2** (Vandesompele *et al.*, 2002), a permis de sélectionner 3 gènes (*tuf*, *23S*, *16S*). Leur expression a été utilisée afin de supprimer les variations d'expression génique non biologiques comme par exemple les variations dues à la qualité des ARN, ou la concentration bactérienne initiale.

Les chapitres suivant de cette étude se consacrent à l'identification de biomarqueurs de la résistance acide de *B. weihenstephanensis*. Plusieurs temps d'adaptation et plusieurs stress ont été utilisés pour faire varier l'état physiologique des cellules. L'expression génique est utilisée afin de quantifier l'état physiologique alors que la modélisation des cinétiques de survie est utilisée afin de quantifier la résistance acide de *B. weihenstephanensis*. Les données Omic et les données de microbiologie prévisionnelle sont ensuite confrontées pour identifier des biomarqueurs (Figure 3). D'après la définition de l'agence national de la santé des Etats-Unis (Atkinson *et al.*, 2001), un biomarqueur est

une caractéristique pouvant être objectivement quantifiée et évaluée comme indicateur d'un mécanisme biologique normal, pathogénique ou d'une réponse pharmacologique. Ici, l'expression génique est donc la caractéristique pouvant être objectivement quantifiée et évaluée quant à la résistance bactérienne, elle correspond au mécanisme biologique.

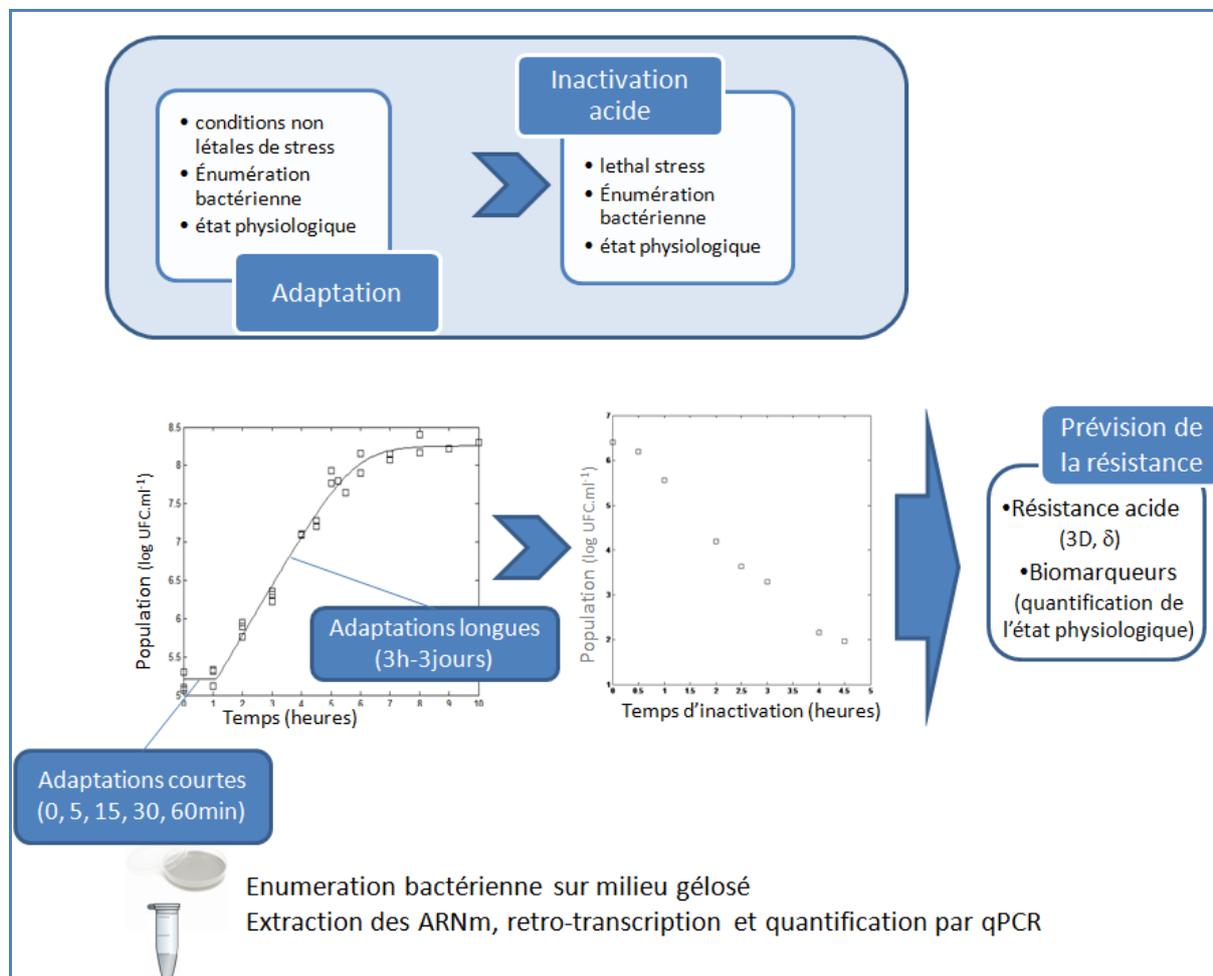


Figure 3 : Représentation schématique de l'obtention des différents états physiologiques de *B. weihenstephanensis*. L'expression génique peut être quantifiée à la fois lors d'adaptation courtes ou longues à des stress non létaux et/ou lors d'un stress acide léthal. La résistance bactérienne associée est quantifiée par le biais de paramètres mathématiques. La corrélation entre les expressions géniques et la résistance bactérienne associée est ensuite testée.

Comme indiqué précédemment la résistance bactérienne à un instant t dépend de l'état physiologique et donc des conditions environnementales préalablement rencontrées. Pour faire varier l'état physiologique des cellules, deux méthodologies ont été employées avec d'une part des adaptations courtes et d'autre part des adaptations longues dans des conditions environnementales non létales. Dans le premier cas aucune croissance ni inactivation n'est observée, alors que, dans le second cas, la croissance du microorganisme est notée. Comme décrit dans la Figure 3, l'expression génique est réalisée soit à la fin des adaptations soit au cours du stress acide léthal.

RT-qPCR pendant l'inactivation (Chapitre 5)

Basée sur une approche utilisant à la fois des outils de microbiologie prévisionnelle et de quantification de l'expression génique tout au long de l'inactivation des cellules bactériennes, une première sélection de biomarqueurs de résistance acide a été réalisée. Ainsi, **le gène *sigB* a été proposé comme biomarqueur de résistance acide modérée de *B. weihenstephanensis*, et *katA* a été identifié comme biomarqueur de résistance acide élevée.** En effet, la modélisation du comportement des cellules de *B. weihenstephanensis* dans des conditions acides létales a montré la présence de 2 sous-populations au sein de la population bactérienne. La première sous-population étant plus sensible au stress acide que la seconde. En comparant la proportion des deux sous-populations avec l'expression du gène, il a pu être montré que l'expression la plus haute de *sigB* était corrélée à la présence majeure de la sous-population sensible. A l'opposé, l'expression de *katA* a été corrélée à la présence majeure de la sous population la plus résistante.

Le gène *sigB* code pour le facteur sigma de la réponse au stress général et son activation permet la transcription d'un ensemble de gènes codant pour des protéines aux fonctions spécifiques permettant de protéger la cellule face à un stress. De plus, σ^B est induit dans un large nombre de stress bactériens et de bactéries distinctes, supportant dès lors la possibilité d'extrapoler son rôle de biomarqueur de résistance à d'autres stress que le stress acide et à d'autres souches bactériennes.

katA code pour une catalase qui convertit le peroxyde d'hydrogène en eau (Imlay, 2003) et sa sur-expression dans des conditions acides serait liée à la formation d'espèces réactives de l'oxygène formées suite à la perturbation de la chaîne de transfert d'électrons (Mols et Abee, 2011; Mols *et al.*, 2010b). De même que pour *sigB*, les catalases, sont omniprésentes chez les microorganismes et elles possèdent un rôle crucial dans l'adaptation au stress et la survie des espèces autres que *B. cereus* (Causton *et al.*, 2001; Hoper *et al.*, 2005; Petherson *et al.*, 2001). De plus récemment, den Besten *et al.* (2013) ont montré que l'activité de la catalase peut être corrélée linéairement à l'induction de la résistance de cellules de *B. weihenstephanensis*. Toutes ces informations soulignent le rôle de *katA* comme biomarqueur.

Adaptations courtes - corrélation linéaire ou non linéaire (Chapitre 6)

En 2010, une approche a été proposée pour identifier des biomarqueurs (den Besten *et al.*, 2010). Cette approche est basée sur des corrélations linéaires entre les biomarqueurs et la résistance bactérienne. Cependant, pour survivre dans des environnements évolutifs, les bactéries s'adaptent par des changements appropriés d'expression de leurs gènes et de l'activité de leurs protéines (Boor,

2006). L'expression des gènes est donc la première réponse bactérienne et elle est le plus souvent transitoire. L'activité enzymatique, au contraire, peut apparaître dans une condition donnée et rester stable (den Besten *et al.*, 2013; Michalick *et al.*, 2012). Connaissant ceci, l'existence d'une corrélation non linéaire entre l'expression génique et la résistance bactérienne apparaît clairement concevable. Cette hypothèse repose sur le fait que les ARN messagers ont des temps de demi-vie courts (Kushner, 1996) alors que les protéines ou enzymes résultantes peuvent être nettement plus stables suivant les conditions environnementales (den Besten *et al.*, 2013 ; Michalik *et al.*, 2012). C'est pourquoi, une étude corrélant de manière linéaire et non linéaire, l'expression de gènes et la résistance acide de *B. weihenstephanensis* KBAB4 est proposée dans ce chapitre.

Pour faire varier l'état physiologique, les cellules bactériennes ont été soumises pendant 5 à 60 minutes à des stress salin, acide ou oxydatif non létal et la résistance acide des cellules adaptées a ensuite été quantifiée. Le concept de corrélation employé est décrit dans la Figure 4. **Ces corrélations ont permis la définition de deux types de biomarqueurs (i) les 'direct biomarkers' qui correspondent aux gènes dont l'expression peut être linéairement corrélée à la résistance acide de *B. weihenstephanensis*; et (ii) les 'long-acting biomarkers' qui correspondent aux gènes dont l'expression augmente pour des temps d'adaptation allant de 5 à 15 ou 30 minutes et est corrélée à une induction de la résistance pour tous les temps d'adaptation.**

Suivant les conditions testées, 4 gènes peuvent servir de 'direct biomarkers' alors que 9 peuvent servir de 'long-acting biomarkers'. Les gènes pouvant servir de 'direct biomarkers', sont *lexA*, *spxA*, *narL* et *bkdR*. Ces gènes codent pour des régulateurs impliqués dans la réponse oxydative (*lexA*, *spxA*), une nitrate réductase pouvant servir d'alternative à la chaîne de transport des électrons (*narL*) et enfin pour un régulateur impliqué dans le maintien des cellules (*bkdR*). Les 9 gènes définis comme 'long-acting biomarker' sont impliqués dans la réponse oxydative (*spxA*, BcerKBAB4-0325, *katA*, *trxB*) ou dans la maintenance des cellules (*codY*, *lacI*, BcerKBAB4_1716, BcerKBAB4_2108, *relA*). Toutefois, l'expression de ces derniers gènes en tant que biomarqueur de la résistance acide n'a été à ce jour mis en évidence que pour des cellules préalablement adaptées à un stress oxydatif.

Concernant les gènes impliqués dans la GSR, aucune corrélation n'a pu être mise en évidence bien que leurs expressions varient avec l'exposition aux différentes conditions. Par exemple, une surexpression de *sigB* dans toutes les conditions acides et salines testées a été montrée alors qu'aucune variation significative n'était observée dans des cellules adaptées à un stress oxydatif.

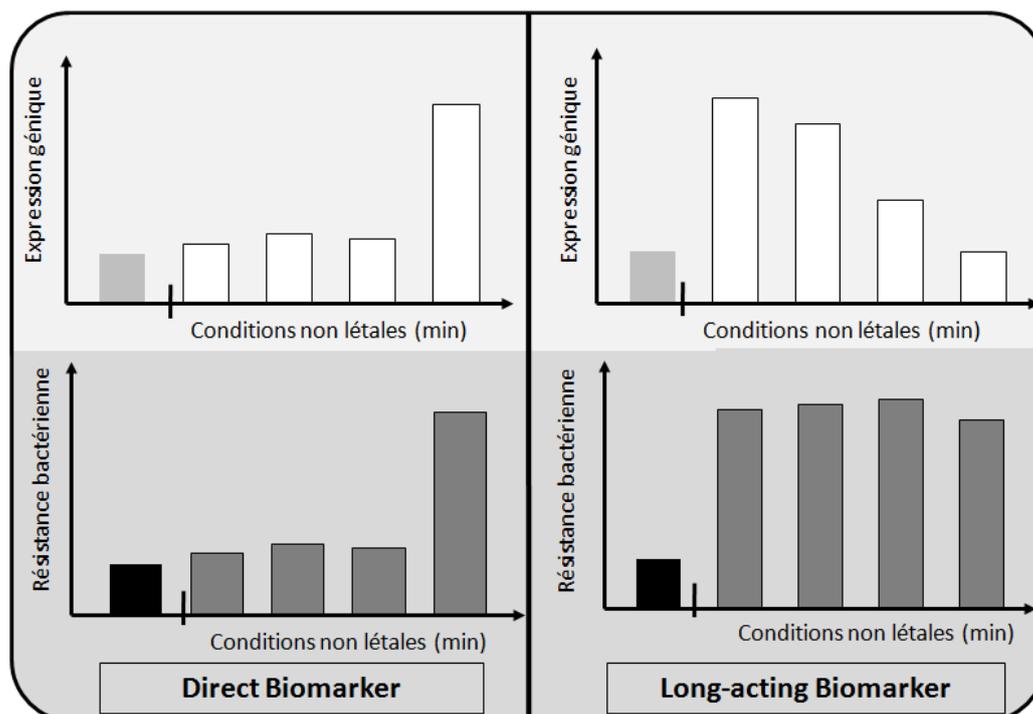


Figure 4 : Représentation du concept de corrélation employé dans cette étude pour l'identification de biomarqueurs de résistance acide. La corrélation linéaire ou non linéaire entre l'expression génique et la résistance acide associée a permis l'identification de 12 gènes définis comme (i) « direct biomarker » c'est-à-dire dont l'expression est linéairement corrélée à la résistance acide et (ii) les « long-acting biomarker » dont l'induction de l'expression est observée pour des temps d'adaptation compris entre 5 et 30 min au maximum et est corrélée à une augmentation de la résistance jusqu'à 60 min.

Il est de plus intéressant de noter que, parmi la trentaine de gènes ciblée, seuls 13 gènes ont pu servir de biomarqueur de résistance. Ainsi, pour plus de moitié des gènes ciblés, aucune corrélation entre l'expression génique et la résistance acide n'a été trouvée. Ceci souligne une faiblesse dans l'approche cherchant à corréler l'expression génique et la résistance bactérienne. Sachant que la régulation des gènes est dépendante de systèmes biologiques complexes dont la globalité des réseaux de régulation n'est pas encore connue, il est concevable que l'étude de l'expression des gènes sans prendre en compte leur corrélation soit insuffisante pour décrire le comportement bactérien. C'est pourquoi, toujours dans la recherche de biomarqueurs de résistance de *B. weihenstephanensis*, une quatrième approche basée sur une analyse statistique multivariée a été proposée (Chapitre 7).

Adaptation longues - analyse statistique multivariée (Chapitre 7)

L'analyse statistique multivariée employée permet d'obtenir une vision globale de l'impact du stress létal de nature acide sur l'expression des gènes de *B. weihenstephanensis*. **L'analyse utilisée ici est la PLS pour Partial Least Square. Cette méthode a pour avantage principal de prendre en compte les corrélations entre les variables explicatives** et apparaît donc comme adaptée dans l'étude de

l'expression génique afin de prendre en compte les corrélations entre les différents gènes. Ce type d'analyse a été réalisé en 2011 par Thabius *et al.* pour identifier la cible principale de l'oleoythanolamide pour réduire l'adiposité de souris possédant une masse grasse importante mais n'a jamais été utilisé à ce jour dans la recherche de biomarqueurs de comportement bactérien. Dans ce dernier chapitre de thèse, plusieurs analyses PLS et dérivées sont employées afin de sélectionner les biomarqueurs (Figure 5). Ces analyses sont également employées afin de mettre en évidence quelle réponse physiologique employée par la cellule (GSR, pH homéostasie et réarrangement métaboliques ou réponse oxydative) joue un rôle majoritaire dans la résistance acide de *B. weihenstephanensis*.

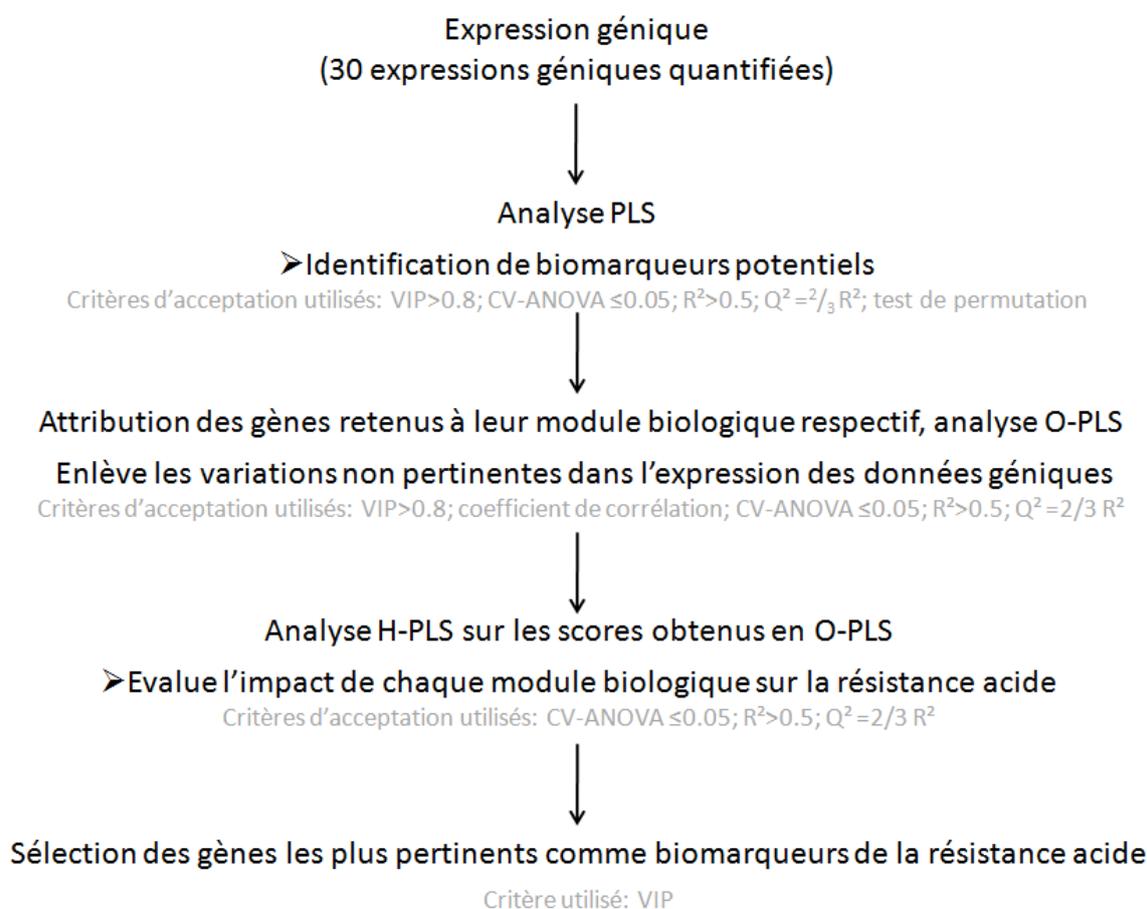


Figure 5 : Procédure d'analyses PLS utilisée pour sélectionner des biomarqueurs de résistance acide de *B. weihenstephanensis*.

Les différentes étapes de PLS ont permis la sélection de 9 biomarqueurs de résistance acide de *B. weihenstephanensis* (Figure 6). Cette analyse a également révélé une importance similaire des différents modules biologiques étudiés. De ce fait, 3 gènes sélectionnés appartiennent au module physiologique GSR (*dps*, *napA* et *rsbW*), 3 au module réarrangements métaboliques ou pH homéostasie (*codY*, *fabF*, *sigH*) et enfin, 3 au module réponse secondaire oxydative (*cydA*, *katA*, *nos*).

Toutefois, il peut être noté que si les modules utilisés ont des importances similaires en PLS, une analyse des données brutes d'expression génique a révélé une réponse majoritaire du module oxydatif. Ce résultat est en concordance avec des résultats obtenus précédemment concernant l'étude du comportement de *Bacillus cereus* ATCC 14579 sous des conditions acides stressantes (Mols *et al.*, 2010b).

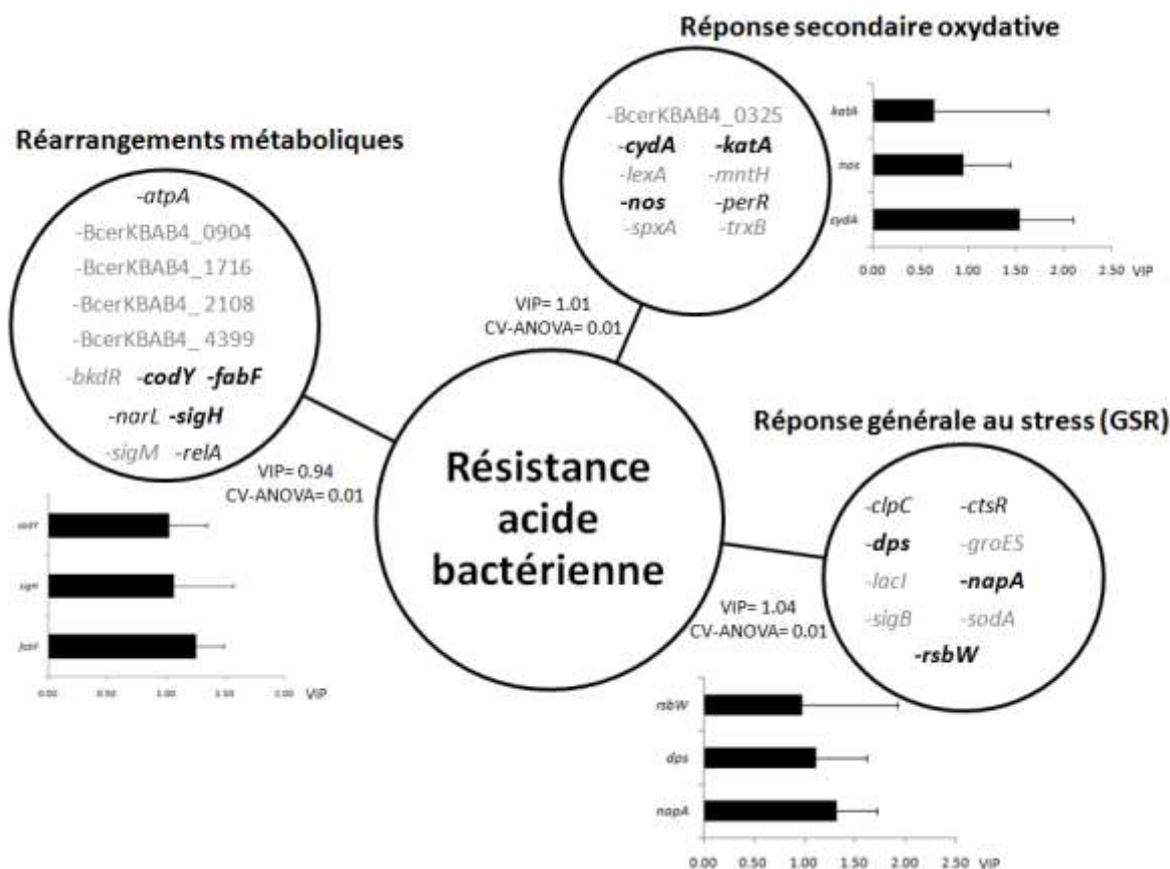


Figure 6 : Représentation globale de la sélection par analyses PLS des gènes pouvant servir de biomarqueurs de la résistance acide de *B. weihenstephanensis*. Réponse secondaire oxydative, GSR et réarrangements métaboliques représentent les différents modules biologiques auxquels appartiennent les différents gènes étudiés. En gris, les gènes éliminés par la première étape de PLS, en noir, les gènes utilisés lors de l'analyse O-PLS et H-PLS. Enfin en gras, les gènes sélectionnés (d'après leur VIP) comme biomarqueurs de résistance acide. Les histogrammes représentent les valeurs de VIP obtenues des gènes sélectionnés comme biomarqueurs. Les VIP et CV-ANOVA correspondent, respectivement, pour au VIP de chaque module biologique et à la validation croisée ANOVA obtenues pour chacune des O-PLS associées au module (le modèle O-PLS est validé si la valeur CV-ANOVA est inférieure à 0.05).

Fort de cette sélection de biomarqueurs, il reste à la mettre en œuvre via son **intégration dans des modèles mathématiques permettant la prévision du comportement bactérien**. La méthode PLS en plus de permettre une identification de biomarqueurs en prenant en compte les corrélations entre gènes, offre un second avantage. Elle peut être utilisée comme modèle mathématique pour prévoir le comportement bactérien. Et **les premiers résultats sont prometteurs avec une estimation de la résistance de 3 échantillons à 0,74 h, 0,97 h et 2,08 h, alors que les résistances observées sont respectivement de 0,5 h, 1,3 h et 1,8 h**. Cependant, des travaux supplémentaires sont nécessaires

pour valider le modèle PLS puis l'étendre à un plus grand nombre de stress bactériens pour pouvoir appréhender le large spectre d'états physiologiques pouvant être rencontrés chez les bactéries.

En conclusion, l'ensemble des travaux décrits dans ce manuscrit souligne l'intérêt d'utiliser des biomarqueurs pour caractériser le comportement bactérien. Cependant, si l'identification de ces biomarqueurs a fait l'objet de plusieurs études, **l'implémentation de ces derniers dans des outils d'aide à la décision reste un véritable challenge** (Brul *et al.*, 2012). La prévision du comportement bactérien par le biais de biomarqueurs permettrait d'augmenter la compétitivité du secteur alimentaire par une meilleure optimisation des procédés industriels, une économie de l'énergie, ou encore une meilleure précision dans les durées de conservation des denrées. Par ailleurs, l'extension de ces outils à des flores d'intérêt industriel serait un avantage pour l'industrie agroalimentaire. Par exemple, dans le Gouda et le Cheddar, des bactéries telles que *Lactococcus lactis* subsp *lactis* ou *L. lactis* subsp *cremoris*, sont d'une importance principale pour le développement de la saveur. En effet, en plus de leur rôle essentiel dans l'acidification du lait, ces microorganismes contribuent considérablement à la formation de l'arôme de fromage par la production de peptides et d'acides aminés, qui contribuent au goût de base de fromage, et la conversion des acides aminés en composés aromatiques (Kieronczyk *et al.*, 2003). Ainsi, l'habileté de ces microorganismes à croître joue un rôle essentiel dans la fabrication fromagère qui peut dès lors être améliorée en maîtrisant et en évaluant l'impact des différentes étapes de fabrication sur l'état physiologique bactérien. Ainsi si l'impact du procédé industriel recherché sur la physiologie des bactéries diffèreraient des flores d'intérêt sanitaire aux flores d'intérêt technologique, le principe resterait le même : **de l'état physiologique à l'instant présent dépendra le comportement futur des microorganismes** (Figure 7).

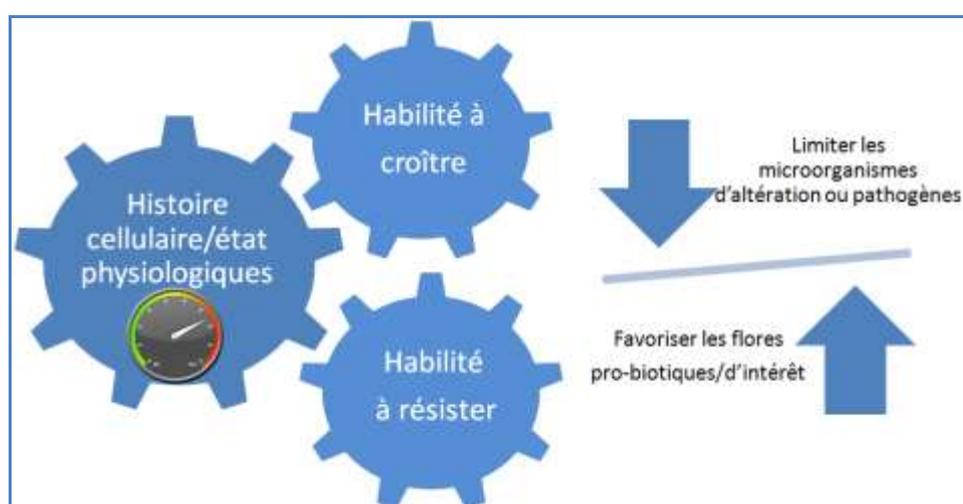


Figure 7 : Vue schématique de l'impact de l'état physiologique sur les problématiques de l'industrie agroalimentaire. La quantification de l'état physiologique via des biomarqueurs pourrait être appliquée pour une meilleure prévision du comportement bactérien et donc un affinement des procédés industriels.

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Valorization- Valorisation

List of publications-Publications avec comité de lecture

- Desriac N.**, Coroller L., Sohier D., Postollec F. 2012. An Integrative approach to identify *Bacillus weihenstephanensis* resistance biomarkers using gene expression quantification throughout acid inactivation. *Food Microbiology* 32, 172-178.
- Desriac N.**, Postollec F., Coroller L., Sohier D., Abee T., den Besten H.W.M. 2013. Prediction of *Bacillus weihenstephanensis* acid resistance: the use of gene expression patterns to select potential biomarkers. *International Journal of Food Microbiology* In press doi: 10.1016/j.ijfoodmicro.2013.03.014.
- Desriac N.**, Postollec F., Durand D., Leguerinel I., Sohier D., Coroller L. Sensitivity of *Bacillus weihenstephanensis* to acidic changes of the medium is not dependant on physiological state. Resubmitted in *Food Microbiology*
- Desriac N.**, Postollec F., Coroller L., Combrisson J., Hallier-Soulier S., Sohier D. The step by step RT-qPCR quality assurance procedure set up to identify biomarkers of *Bacillus weihenstephanensis*. Submitted in *Food Analytical Methods*.
- Desriac N.**, Coroller L., Jannic F., Postollec F., Sohier D. mRNAs biomarkers selection based on Partial Least Square algorithm in order to further predict *Bacillus weihenstephanensis* acid resistance. Submitted in *Plos One*
- Desriac N.**, Broussolle V., Postollec F., Mathot A.G., Sohier D., Coroller L., Leguerinel I. *Bacillus cereus* vegetative cells responses to acidic environments. In revision for submission in *Frontiers in Microbiology*

Meetings-Congrès

- Desriac N.**, Postollec F., Coroller L., Sohier D. 2011. Determination of acid stress bacterial resistance and viability biomarkers. **Medicen**, 19 April, Paris, France (oral presentation).
- Desriac N.**, Postollec F., Coroller L., Sohier D. 2011. The use of Omics biomarker to track the survival of *Bacillus weihenstephanensis* KBAB4 throughout acid inactivation. **IAFP European symposium**, 18-20 May, Ede, Netherlands 2011 (oral presentation).
- Desriac N.**, Postollec F., Coroller L., Sohier D. 2011. The use of Omics biomarker to track the survival of *Bacillus weihenstephanensis* KBAB4 throughout acid inactivation. **Journée des doctorants**

et post-doctorants en biologie santé en Bretagne, 20 June, Brest, France (oral presentation)

- Desriac N.**, Postollec F., Sohier D., Coroller L. 2011. Determination of acid stress bacterial resistance biomarker of *Bacillus weihenstephanensis*. **7thICPMF**, 12-15 September, Dublin, Ireland (oral presentation, best student award)
- Desriac, N.**, Postollec, F., Leguerinel, I., Sohier, D., Coroller, L. 2012. Sensitivity of *Bacillus weihenstephanensis* to acidic changes of the medium is not dependant on physiological state. **IAFP European Symposium**, 21-23 may, Varsaw, Poland (oral presentation).
- Postollec F., **Desriac N.**, Coroller L., Sohier, D. 2012. Quantification of acid-resistance biomarkers using RT-qPCR gene expression. **IAFP European Symposium**, 21-23 may, Varsaw, Poland.
- Desriac N.**, Postollec F., Den Besten H., Abee T., Coroller L., Sohier D. 2012. Prediction of *Bacillus weihenstephanensis* acid resistance: The use of gene expression patterns to find potential biomarkers. **Colloque UMT ACTIA**, 15 June Paris, France (poster presentation)
- Desriac N.**, Postollec F., Coroller L., Sohier D., Abee T., den Besten H. 2012. Prediction of *Bacillus weihenstephanensis* acid resistance: the use of gene expression patterns to find potential biomarkers. **23rd International ICFMH Symposium, Food Micro2012**, 3 -7 September, Istanbul, Turquie (oral presentation).
- Desriac N.**, Postollec F., Leguerinel I., Coroller L., Sohier D. 2013. Prediction of *Bacillus weihenstephanensis* acid resistance using gene expression quantification. **DREAM Project's International Conference**, 24-26 June, Nantes, France (accepted poster presentation).
- Desriac N.**, Postollec F., Leguerinel I., Coroller L., Sohier D. 2013. Reproducibility assessment of species-specific PCR methodology in order to monitor viable target microflora. **DREAM Project's International Conference**, 24-26 June, Nantes, France (accepted poster presentation).
- Desriac N.**, Coroller L., Postollec F., Combrisson J., Hallier-soulier S., Sohier D. 2013. RT-qPCR data acquisition workflow to use gene expression as biomarkers. **Microbial Spoilers in Food**, 1-3 July, Quimper, France (accepted poster presentation).
- Desriac N.**, Coroller L., Sohier D., Postollec F. 2013. mRNAs as biomarkers of acid resistance of *Bacillus weihenstephanensis*: How to select them? **Microbial Spoilers in Food**, 1-3 July, Quimper, France (accepted oral presentation).
- Desriac N.**, Postollec F., Coroller L., Sohier D. 2013. The step by step RT-quality assurance procedure for reverse transcription quantitative PCR: illustration with the *Bacillus weihenstephanensis* acid resistance biomarkers. **IAFP Annual meeting**, 29-31 July, Charlotte North Carolina, USA (accepted poster presentation).
- Desriac N.**, Coroller L., Sohier D., Postollec F. 2013. Prediction of *Bacillus weihenstephanensis* acid resistance using gene expression quantification as biomarker. **IAFP Annual meeting**, 29-31 July, Charlotte North Carolina, USA (accepted oral presentation).

Teaching-Enseignement

Practical courses of biochemistry-Travaux pratiques de Biochimie (72h), DUT, IUT QUIMPER, 2010-2011.

Microbiology-Microbiologie (16h and 2h of practical and theoretical courses, respectively), DUT, IUT QUIMPER, 2010-2011.

Molecular biology-Biologie moléculaire (48h and 8h of practical and theoretical courses, respectively), DUT, IUT QUIMPER, 2011-2012/2012-2013.



The use of Omic biomarkers to track the survival of *Bacillus weihenstephanensis* KBAB4 throughout acid inactivation

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Bacillus cereus is ubiquitously found in soil and may either cause emetic or diarrheal types of food-borne illnesses. To prevent bacterial development, food industries use preservation techniques like low pH and cold temperature storage. In this study, the acid resistance of *B. weihenstephanensis* KBAB4 psychrotrophic bacteria is investigated using microbiological and transcriptomic methods for various physiological states and various adaptations (cold, salt, acid...).

Standardized protocols were used to determine bacterial inactivation during an acid stress (pH 4.6). Population evolution was followed by CFU enumeration and kinetics were fitted using a mixed Weibull model. In parallel, the expression of fourteen genes, selected as potential biomarkers, was performed using RT-qPCR. Quantification of each gene transcription was related to two housekeeping reporter genes (*gyrA* and *sigA*) which showed the most stable expression (geNorm M value of 1.158).

In optimal conditions, exponentially cells submitted to an acid shock could be divided in two populations, *i.e.* one sensitive (first decimal decrease after 1h00 of inactivation) and one resistant (first decimal decrease after 3h00). In these conditions, gene expression showed up-regulation (9 genes) or down-regulation for 2 genes (*narL* and *napA*). Furthermore gene variation profile may vary during inactivation kinetic. For instance, *sigB* encoding general stress sigma factor, is up-regulated throughout inactivation with a peak after 2h00 (7.4 fold \pm 2.3) whereas *katB* encoding major catalase is up-regulated at the beginning of the kinetic and stays constant from 2h00 to 4h00 of inactivation (around 31 fold).

Acquisition of gene expressions along inactivation kinetics is in progress for various physiological states. These results will allow to develop a predictive tool that will correlate the genes expression to the bacterial resistance. The prediction of bacterial history-dependent behavior using gene expression will offer a decision making tool adapted to industrial processes.



Determination of acid-stress bacterial resistance and viability biomarkers of *Bacillus weihenstephanensis* KBAB4

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Bacillus cereus is a Gram-positive spore-forming bacterium that is ubiquitously found in soil and may either cause emetic or diarrheal types of food-borne illnesses. *B. cereus* is also an important spoilage bacterium, for instance, in dairy products yielding sweet curdling. To prevent bacterial development, food industries use preservation techniques like low pH and cold temperature storage. Furthermore, it is well known that the bacterial resistance depends on transcription of specific set of genes. In this study, the acid resistance of *B. weihenstephanensis* KBAB4 psychrotrophic bacteria is investigated for various physiological states and various adaptations (cold, salt, acid...) using classical microbiological method and the RT-qPCR genes expression quantifications.

A protocol was implemented in order to obtain homogeneous culture and reproducible physiological states without spore formation. Bacterial cells were submitted to an acid shock (pH 4.6) and the population evolution was followed by CFU enumeration on Nutrient Agar. Kinetics inactivation were fitted using a mixed Weibull model. Results show that in optimal conditions of growth the exponentially cells could be divided in two populations, *i.e.* one sensitive (first decimal decrease after 1h00 of inactivation) and one resistant (first decimal decrease after 3h00). Nevertheless the more resistant population represents only 1/1000 of the starting population. Because bacterial resistance varies depending on cell histories, whether cells are grown under salt or cold conditions, a selection of 36 genes was performed. These potential biomarkers were targeted using an optimized RT-qPCR protocol yielding gene expression quantification during acid inactivation kinetics.

In order to allow the simulation of bacterial stress response throughout industrial processes, genes expression could further be correlated to the bacterial acid stress resistance. More precisely, genes transcription and bacterial resistance will be included into mathematical models in order to offer a decision making tool adapted to industrial process and ensuring food safety.



Sensitivity of *Bacillus weihenstephanensis* to acidic changes of the medium is not dependant on physiological state

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Exposure to mild stress conditions can activate stress adaptation mechanisms in microorganisms. They might result in a protective effect toward otherwise lethal stresses.

This study aims to quantify the effect of salt and acid preexposure on acid-tolerance of vegetative cells of *Bacillus weihenstephanensis*.

The psychrotolerant strain KBAB4 was cultured until the exponential growth phase (i) in BHI, (ii) in BHI supplemented with 2.5% salt (iii) or BHI acidified at pH 5.5 with HCl. The growing cells were subsequently inactivated in an acidic suspension at a pH from 4.4 to 4.7. Linear and nonlinear microbial survivals models were fitted to the inactivation data. Based on statistical criterion, a Weibullian model was selected and used to describe the acid inactivation and the adaptation of the cells to stress.

The acid-tolerance was enhanced after growth in non lethal acid stress conditions whereas a decrease of the acid-tolerance was observed for cells grown in salt conditions. Both environmental growth and inactivation did not present influence on the shape of the inactivation kinetics. Only the scale parameter, which quantifies the bacterial resistance, depended on acid intensity and growth conditions. The secondary modeling of the bacterial resistance allowed to quantify sensitivity of the cells to acidic change of the medium. This sensitivity was not significantly affected whatever the growth conditions.

These results highlight that the growth conditions may influence the bacterial acid resistance without affecting the sensitivity to acidic modification. Quantification of such adaptive stress response might be instrumental in understanding adaptation mechanism. And naturally, a tool to link the variation of food composition to its repercussion on the bacterial exposure of consumers.



Prediction of *Bacillus weihenstephanensis* acid resistance: The use of gene expression patterns to find potential biomarkers

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Exposure to mild stress conditions can activate stress adaptation mechanisms yielding protection to lethal stresses. The aim of this study was to find molecular biomarkers that can predict stress resistance of the psychrotrophic strain *Bacillus weihenstephanensis* KBAB4. Selected mild stress conditions included exposure of mid-exponential phase cells in broth to NaCl (2.5% w/v), pH 5.5 (set with HCl), or 0.1 mM H₂O₂. Cells were imposed for 5, 15, 30 and 60 min, and during mild stress exposure the expression of 31 targeted genes was assessed using RT-qPCR. Expression data were represented in fold change using three reference genes and compared to the unstressed control. In parallel, non-stressed (control) and mild-stressed mid-exponential phase cells were submitted to a lethal acid treatment at pH 4.6 and the bacterial inactivation was followed by CFU enumeration. The bacterial resistance was deduced from the third decimal reduction times. Non-linear and linear correlations between gene expression data and survival capacity were analyzed in order to find relevant biomarkers.

Unstressed cells had a 3D value estimated at 2.5 h ± 0.5. The oxidative stress adaptation increased the bacterial resistance whatever the adaptation time with an average 3D value estimated around 5h. The impact of acid and salt stresses on the bacterial resistance varied depending on the adaptation times. Induced robustness was only observed after 60 min of adaptation resulting in a 3D value of 6.2 h ± 0.2 and 8.6 h ± 1.7 for salt- and acid-adapted cells, respectively. The correlation of bacterial resistance and gene expression allowed to define (i) direct biomarker genes of which their expression patterns were linearly correlated to the bacterial resistance; and (ii) long-acting biomarker genes which were up-regulated for short adaptation times, linked to increased resistance over time. This latter observation could be explained by the stability of the encoded functional proteins and enzymes. Our results highlight a new approach that encompasses linear and non linear correlations in order to find relevant biomarkers. Furthermore, this quantitative approach opens avenues towards prediction of microbial behavior using a variety of cellular biomarkers.



mRNA used as biomarkers for acid resistance of *Bacillus weihenstephanensis*: How to select them?

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It is now recognized that available tools for microbial quantitative risk assessment may yield to over/under estimations of process or shelf-life. In order to adjust bacterial behaviour simulations, the integration of bacterial physiology and related cellular adaptive response to various stresses still remains a challenge. Psychrotolerant *Bacillus weihenstephanensis* belongs to the *Bacillus cereus* group which is thus of industrial concern for minimally processed foods and particularly for low-acid minimally processed foods. In this study, both bacterial behaviour modelling and gene expression quantification were used in order to select relevant biomarkers to track acid stress resistance of *B. weihenstephanensis*

The strain KBAB4 was cultured during short (up to 60 min) or long adaptation times (from 4h to 72h) in various sublethal conditions (NaCl 2.5%, HCl pH 5.5, H₂O₂ 0.1mM, 8°C). The cells were further submitted to a lethal acid stress pH 4.6 to determine the bacterial acid resistance. The gene expression of about 30 targeted genes was quantified (i) throughout adaptation time and/or (ii) during inactivation treatment (acid lethal exposure) and correlated to the acid resistance. Both gene per gene correlation and multivariate analysis (Partial Least Square PLS) were used to select acid resistance biomarkers.

First, a gene per gene correlation between gene expression and bacterial resistance allowed the selection of two types of biomarkers i.e. (i) 4 direct biomarkers for which gene expression was linearly correlated to the subsequent bacterial and (ii) 9 long-acting biomarkers for which a transient mRNA up-regulation was correlated to increased acid resistance over time. However the correlation between bacterial resistance and the expression of 17 genes remains to be determined. These results highlighted the importance of more complex features to correlate the gene expression to the bacterial resistance. Thus, a multivariate analysis was performed to correlate the acid resistance and the gene expression and their correlation of 30 potential biomarkers. A step-wise procedure using Partial Least Square (PLS) algorithm was implemented and allowed the selection of 9 genes as the most relevant biomarkers. Furthermore, the prediction of feasibility on 3 independent samples was analyzed. For that purpose, a PLS model based on the absolute expression of the 9 selected mRNAs, was built to simulate the acid resistance. The acid resistance was estimated at 0.74h, 0.97h and 2.08h as compared to observed resistances of 0.5h, 1.3h and 1.8h, respectively. Even though a complete dataset is needed to validate the developed model, these results are promising.

Based on both predictive microbiology and mRNA quantifications, key molecular biomarkers were selected for acid resistance of *B. weihenstephanensis*. However, remaining key challenge are now to increment the selected biomarkers into mathematical modelling to offer new generation decision making tools. Herein, promising results using the PLS algorithm underlined the possibility to predict the bacterial behaviour using mRNA quantification. Even though a "proof of concept" validation is needed, these results are promising to integrate cell history and related physiology in predictive modelling to further improve MRA.



Prediction of *Bacillus weihenstephanensis* acid resistance using gene expression quantification as biomarker

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In order to integrate Omics data to quantitative microbiological risk assessment in foods, gene expressions may serve as bacterial behaviour biomarkers.

In this study an integrative approach was followed to select molecular biomarkers to further predict the acid resistance of *Bacillus weihenstephanensis*.

An integrative approach combining gene expression and survival capacity was used to determinate potential biomarkers of acid resistance of *B. weihenstephanensis*. The gene expression of 31 genes was quantified (i) throughout bacterial adaptation and correlated to a subsequent acid-resistance (3D values *i.e.* the time necessary to loss 99.9% of the bacterial population) and (ii) throughout bacterial inactivation and correlated to an instant first bacterial decrease, *i.e.* the time necessary to loss 90% of the bacterial population at time *t*.

With the quantification throughout bacterial adaptation 4 genes could be selected as direct biomarkers meaning that there was linear correlation between their expression and subsequent bacterial resistance. 9 other genes, named long-acting biomarker, showed an up-regulation during short adaptation time and were correlated to an increased acid-resistance over time highlighting the importance of non-linear correlation when focussing at transcriptional level. With quantification throughout bacterial inactivation 8 genes were selected using a Partial Least Square (PLS) regression. The main advantage of this mathematical selection is that it takes gene expression correlations into account by building a mathematical model. Further investigations are needed but the first analyse of this model showed that it may be further used to predict bacterial resistance using gene expression. Indeed, the bacterial resistance of two other samples was estimated at 2.1 h and 3.3 h whereas the resistances were estimated by fitting inactivation curves at 2.8 h and 3.7 h, respectively.

This study underlines the possibility to integrate the bacterial physiology state, using Omics biomarkers, into bacterial behaviour modelling and thereby further improve microbial risk assessment.

Prediction of *Bacillus weihenstephanensis* acid resistance: The use of gene expression patterns to find potential biomarkers

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Introduction & Objective

Bacteria are exposed to changing environments both in their natural habitats and in industrial processing. Exposure to mild stress conditions can activate stress adaptation mechanisms and provide resistance towards otherwise lethal stresses. Combining molecular tools and predictive microbiology concepts to incorporate bacterial physiology into prediction of growth and survival appears as an interesting challenge for food formulation and preservation optimisation. In this study, two approaches were followed to select molecular biomarkers to predict the acid resistance of *B. weihenstephanensis*.

Methods

- ▶ Mid exponentially cells of *B. weihenstephanensis* - optimal conditions (BH), 30°C, 100 rpm) and used as no-stress control - 5, 15, 30 and 60 min to sublethal conditions (NaCl (2.5%), HCl (pH 5.5); H₂O₂ 0.1mM).
- ▶ Determination of the acid stress resistance (pH 4.6) - Mixed Weibull model (δ value) - linear or Weibull (3D value).
- ▶ Quantification of targeted gene expression - Design primers/probes of 31 targeted genes (i.e. Potential biomarker) - RNA extraction and RT-qPCR¹ - Relative/absolute quantification
- ▶ Integrative approach combining gene expression and survival capacity

Determination of potential biomarkers using gene quantification throughout bacterial adaptation

- Both mild salt and acid treatment, up to 30 min, did not significantly change the 3D value as compared to the unadapted cells, whereas 60 min of treatment significantly increased the acid resistance resulting in 3D values of 6.2 h \pm 0.2 and 8.6 h \pm 1.7, respectively.
- Mild oxidative stress treatment resulted in enhanced acid resistance of *B. weihenstephanensis* over time. Depending on the adaptation time, no significant variation ($p > 0.05$) was observed with an average 3D value of approximately 5 h.

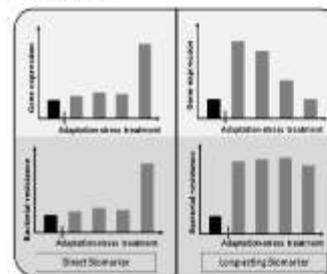


Figure 2: Conceptual scheme used to find relevant biomarkers for adaptation stress yielding an increased acid resistance.

Determination of potential biomarkers using gene quantification throughout bacterial inactivation

- The inactivation curve was fitted with a mixed Weibull model. This model allowed to differentiate two subpopulations with various acid resistances among the initial population.
- The most sensitive subpopulation had a δ_1 value (time necessary to obtain the first decimal decrease for subpopulation 1) of 1.08 h while the first decimal decrease of subpopulation 2 was observed after 2.24 h of acid exposure.

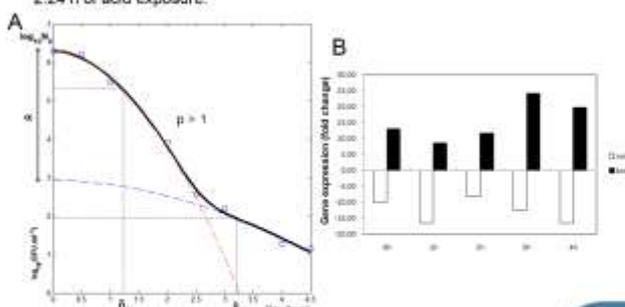


Figure 3: A- *B. weihenstephanensis* KBAB4 survival kinetic of mid-exponentially grown cells exposed to acid conditions (BH, pH4.6). B- Fold change of the normalized expression of *narL* and *katA* using three reference genes and compared to the no-stress control.

- Throughout acid inactivation, up-regulation was observed for *katA*, while down-regulation was observed for *narL*.
- Significant differences ($p < 0.05$) were observed for *katA* gene expression in the two subpopulations, which yields to a higher *katA* induction in subpopulation 2.

¹ Desriac et al., 2013. An integrative approach to identify *Bacillus weihenstephanensis* potential biomarkers using gene expression quantification throughout bacterial acid resistance in vitro. IF 191676, 2013, 02, 200.
² Mills et al., 2016. Isolation of acid-tolerant *Bacillus* strains results in higher acid tolerance and multiple acid-resistance mechanisms. International Journal of Food Microbiology 212 (2016) 1-10.

- Regarding the gene expression pattern of the 31 candidate biomarkers:
 - ✓ 4 genes could be selected as direct biomarker, i.e. Linear correlation between their expression and bacterial resistance.
 - ✓ 9 genes, named long-acting biomarker, showing an up-regulation during short adaptation time and increased resistance over time ... highlighting the importance of non-linear correlation particularly when focussing at transcriptional level.
 - ✓ Half of selected genes reveal a similar adaptive trait with *B. cereus* ATCC 14579² and underline the possibility to extrapolate such biomarkers to other related species.

Conclusions

- ▶ Promising results were obtained for this integrative approach which encompasses:
 - Gene expression quantification during inactivation and adaptation (biomarkers).
 - Bacterial resistances determined using mathematical models (bacterial behavior).
- ▶ Potential resistance biomarkers were proposed to:
 - track subpopulations with different acid resistance.
 - predict the increased of bacterial resistance.
- ▶ This study underlines the possibility to integrate the bacterial physiological state, using 'Omics' biomarkers, into bacterial behavior modeling and thereby further improve microbial risk assessment.
- ▶ One of the key challenge will be to increment these data into mechanism-based predictive modeling of microbial behavior.

Résumé

Outil d'évaluation des risques microbiologiques d'origine alimentaire, et d'optimisation des procédés de transformation, la microbiologie prévisionnelle ne considère pas l'influence de l'état physiologique des microorganismes sur leur comportement. L'objectif de ce travail est d'identifier des marqueurs permettant de rendre compte de l'impact de l'adaptation cellulaire face à un stress acide. Dans ce cadre, la résistance acide de *Bacillus weihenstephanensis* a été étudiée et des ARNm ont été identifiés en tant que biomarqueurs de résistance.

(i) La résistance acide a été quantifiée via des méthodes culturales pour différents passés cellulaires. L'adaptation à un stress salin est défavorable à la résistance acide en comparaison à des conditions optimales de croissance ou à une adaptation spécifique au stress acide. Néanmoins, la résistance acide de *B. weihenstephanensis* est modulée de la même façon par l'acidité de l'environnement, indépendamment des conditions de culture préalablement rencontrées.

(ii) Un outil basé sur la technologie Pall GeneDisc® a été développé pour permettre la quantification de l'expression génique par RT-qPCR.

(iii) La corrélation des données Omic et de résistance acide a permis la sélection de biomarqueurs permettant de différencier les cellules les plus résistantes des cellules les plus sensibles présentes au sein d'une population bactérienne.

(iv) Des corrélations linéaires et non linéaires ont également permis de définir des 'direct biomarker' qui correspondent aux gènes dont l'expression peut être linéairement corrélée à la résistance; et des 'long-acting biomarker' qui correspondent aux gènes dont l'expression transitoire est corrélée à une résistance acide stable.

(v) Une analyse multivariée a été utilisée afin de prendre en compte les interactions entre l'expression des différents gènes impliqués dans la résistance acide et a permis la sélection de 9 gènes comme biomarqueurs de résistance acide.

Enfin, des premières données prometteuses ont été obtenues. Il serait ainsi possible d'utiliser l'expression génique d'une population bactérienne à un instant donné pour prévoir sa survie dans un environnement léthal.

Mots clés : *Bacillus weihenstephanensis*, résistance acide, biomarqueurs, expression génique, microbiologie prévisionnelle.



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Abstract

Predictive microbiology is a tool to assess food microbiological risks and optimise food processes. However predictive microbiology which predicts the bacterial behaviour does not take bacterial physiological state into consideration. The aim of this work is to identify molecular biomarkers to assess the impact of bacterial adaptation on the subsequent acid resistance. In this study, the acid resistance of *Bacillus weihenstephanensis* was investigated and mRNAs were identified as acid resistance biomarkers.

(i) The bacterial acid resistance of different mild stress adapted cells was quantified using cultural methods. Mild salt-adapted cells were less resistant than cells grown in optimal conditions; and the latter less resistant than acid-adapted cells. However, the bacterial resistance of *B. weihenstephanensis* followed the same pattern when facing acidic changes of the environment and that, whatever the environmental condition previously encountered.

(ii) For RT-qPCR gene expression quantifications a specific rotative PCR device based on the Pall GeneDisc® Technology was developed.

(iii) Omic data and bacterial acid resistances correlation allows the selection of biomarkers to track the more resistant and the more sensitive cells present within the bacterial population.

(iv) Both linear and non linear correlations allowed to define two types of biomarkers: 'Direct biomarker' for which expression patterns upon mild stress treatment were linearly correlated to the subsequent acid resistance and 'long-acting biomarkers' which were transiently up-regulated during mild stress exposure and correlated to increased acid resistance over time.

(v) A multivariate analysis was performed to correlate the acid bacterial resistance and the gene expression of vegetative cells. This mathematical method provides the advantage to take gene expressions and their interactions into account and allowed the selection of 9 genes as acid resistance biomarkers of *B. weihenstephanensis*.

Finally, some promising results were also obtained. Thereby, it would be feasible to use gene expression at a given time to predict the bacterial survival behaviour in lethal acid conditions.

Keywords: *Bacillus weihenstephanensis*, acid resistance, biomarkers, gene expression, predictive microbiology.



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