



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 provide 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*, *lacI*, *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.

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1. 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, 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

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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 analyse 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* (Ceragioli et al., 2010; den Besten et al., 2009; Mols et al., 2010a, 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.

2. Materials and methods

2.1. 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.

2.2. Mild stress exposures

To quantify the stress adaptive behaviour of *B. weihenstephanensis* to conditions which may be encountered in the 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 ($\text{OD}_{600\text{ nm}}$ 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.

2.3. 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. The 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.

2.4. 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 follows:

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

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

(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 (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.

2.5. 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

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