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An integrative approach to identify *Bacillus weihenstephanensis* resistance biomarkers using gene expression quantification 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.

1. Introduction

The *Bacillus cereus* group comprises six closely related species: *Bacillus cereus* and *Bacillus anthracis*, known as human pathogens, *Bacillus thuringiensis* used as biopesticide, *Bacillus mycoides* and *Bacillus pseudomycoides* which are characterized by rhizoidal colonies on agar medium and *Bacillus 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 (Kotirantaa 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

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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., 2010a,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* ATCC 14579. 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; Rantsiou et al., 2011). The aim of this study was to characterize *Bacillus 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.

2. Materials and methods

2.1. 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 (100 rpm) 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.

2.2. 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 16–24 h at 30 °C. Acid inactivation kinetics were performed in triplicate from three independent standardized inocula (replicates A, B, and C).

2.3. 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).

2.4. 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 × *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 × *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,