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Title

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Running title

Acid-induced radical formation in *B. cereus*

Authors

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Summary

Acid stress resistance of the food-borne human pathogen *Bacillus cereus* may contribute to its survival in acidic environments, such as encountered in soil, food, and the human gastrointestinal tract. The acid stress responses of *B. cereus* strains ATCC 14579 and ATCC 10987 were analysed in aerobically grown cultures acidified to pH values ranging from pH 5.4 to pH 4.4 with HCl. Comparative phenotype and transcriptome analyses revealed three acid stress-induced responses in this pH range: growth rate reduction, growth arrest and loss of viability. These physiological responses showed to be associated with metabolic shifts and the induction of general stress response mechanisms with a major oxidative component, including up-regulation of catalases and superoxide dismutases. Flow cytometry analysis in combination with the hydroxyl ($OH\cdot$) and peroxynitrite ($ONOO^-$) -specific fluorescent probe 3’-(p-hydroxyphenyl) fluorescein (HPF), showed excessive radicals to be formed in both *B. cereus* strains in bactericidal conditions only. Our study shows that radicals can indicate acid-induced malfunctioning of cellular processes that lead to cell death.

Introduction

Bacillus cereus is a Gram-positive, spore-forming, facultative anaerobic, rod-shaped food-borne human pathogen that appears to be well-equipped to survive in various adverse conditions. The spores and vegetative cells of *B. cereus* can be found in a range of environments, such as soil (Von Stetten *et al.*, 1999; Vilain *et al.*, 2006), plant rhizosphere (Berg *et al.*, 2005), and various foods (Choma *et al.*, 2000; Rosenquist *et al.*, 2005). Besides being notorious for causing spoilage of dairy products, *B. cereus* is a food-borne pathogen that can cause two distinct types of disease, i.e., emesis and diarrhoea (Kotiranta *et al.*, 2000). The emetic syndrome occurs upon ingestion of the heat-stable toxin cereulide, which is produced in food by emetic *B. cereus* strains (Agata *et al.*, 2002). The diarrheal syndrome is associated with the action of enterotoxins, such as non-haemolytic enterotoxin (NHE) and cytotoxin K (CytK) (Granum and Lund, 1997), that are produced by vegetative cells inside the human small intestine (Stenfors Arnesen *et al.*, 2008). Before entering the small intestine and subsequent production of enterotoxins, *B. cereus* cells have to survive the low pH of the human stomach. Therefore, acid resistance is a key parameter in the pathogenic potential of enterotoxic *B. cereus* strains. Obviously, the highly resistant dormant spores of *B. cereus* can pass the stomach unaffected and germination in the acid environment of the small intestine is an important aspect of their pathogenic potential (Wijnands *et al.*, 2007; Hornstra *et al.*, 2009). Outside the human host, *B. cereus* may also be frequently exposed to acidic conditions including a range of low pH foods, where in specific cases organic acids have been added as preservatives (Brul and Coote, 1999). In conclusion, coping with low pH stress is an important feature in the performance of *B. cereus* in a variety of environments as described above, but also in other ecological niches such as soil and plant rhizosphere (Neumann and Martinoia, 2002).

Acid stress responses have mainly been studied in Gram-negative organisms, such as *Escherichia coli* and *Salmonella* Typhimurium (Richard and Foster, 2003), and in a select number of Gram-positive bacteria, such as lactic acid bacteria and *Listeria monocytogenes* (van de Guchte *et al.*, 2002; Cotter and Hill, 2003; Ryan *et al.*, 2008). These reviews highlight the importance of proton pumps, i.e., F_1F_0-ATPase, transcriptional regulators, such as RpoS (Gram-negatives) and σ^B (Gram-positives), proteins involved in protection of macromolecules, such as DnaK and GroESL, and enzymes that produce alkaline compounds, such as the ammonium forming enzymes urease and arginine deiminase. In contrast, the acid stress response of *B. cereus* has not been studied extensively. Available information is limited to alternative sigma factor σ^B expression upon exposure to a low pH (van Schaik *et al.*, 2004), and the acid tolerance response, which includes modulation of intracellular pH and protein synthesis (Browne and Dowds, 2002; Jobin *et al.*, 2002; Thomassin *et al.*, 2006). Additionally, the role of urease in acid resistance of a large number of *B. cereus* strains has been studied (Mols and Abee, 2008), and revealed that its role in acid resistance of *B. cereus* was limited.

Therefore, we set out to investigate the molecular mechanisms involved in acid stress response of *B. cereus* and to identify possible acid-induced inactivation mechanisms, by comparing responses of cells exposed to selected pHs leading to mild, bacteriostatic and bactericidal acid stress. To determine both general and phenotype-associated transcriptional responses, two model strains ATCC 14579, isolated from air (Ivanova *et al.*, 2003) and ATCC 10987, a food-isolate (Rasko *et al.*, 2004) were investigated. Recently, Kohanski and colleagues (2007) reported that the formation of reactive oxygen species (ROS), such as hydroxyl radicals (OH·), plays a role in antibiotic-induced inactivation of aerobically grown *E. coli* and *Staphylococcus aureus* cells. These ROS were suggested to originate from antibiotic-induced perturbation of the electron transfer chain resulting in the production of superoxide (O_2^-). O_2^- can damage iron-sulphur clusters and subsequently react with the released iron, resulting in OH· formation via the Fenton reaction. Therefore, flow cytometry analysis, in combination with the OH· and peroxynitrite ($ONOO^-$) -specific fluorescent probe 3’-(p-hydroxyphenyl) fluorescein (HPF) (Setsukinai *et al.*, 2003), was included in our study to detect ROS in (sub)lethally acid-stressed *B. cereus* cells. Our study provides evidence that radicals can indicate acid-induced malfunctioning of cellular processes and the stress-induced formation of reactive oxygen species as a common theme in bacterial stress response and cellular death is discussed.

Results

Physiological response to acid stress

The physiological response to acid stress was studied using *B. cereus* strains ATCC 14579 and ATCC 10987 by acidifying aerobically grown cultures to pH values ranging from pH 5.4 to pH 4.4 by addition of HCl (Fig. 1). Upon exposure to the different acid shocks, the growth of exponentially growing *B. cereus* cells was instantly affected. The two strains used showed different phenotypic responses to different levels of acidity. *B. cereus* strain ATCC 14579 showed to continue growth as reflected in an increase of colony forming units upon exposure to pH shocks as low as pH 5.0. This response is hereafter referred to as growth phenotype. Strain ATCC 14579 was inactivated at pH 4.6 and lower as shown by the inability to form colonies on BHI plates incubated at 30°C for 16 hours. This response is hereafter referred to as inactivation phenotype and the condition as bactericidal. Upon exposure to pHs between pH 5.0 and 4.7, ATCC 14579 showed a stable number of viable counts within the first hour of exposure. This response is hereafter referred to as survival phenotype and the condition as bacteriostatic. However, prolonged exposure (overnight) resulted in a decrease of viable counts (Fig. 2). The growth boundary of the other strain tested, *B. cereus* ATCC 10987, was determined at pH 5.0. Upon exposure to pHs higher than pH 5.0, ATCC 10987 was able to grow and at pHs lower than pH 5.0 this strain was inactivated. There was no apparent survival phenotype in the ATCC 10987 acid shock response using 0.1 pH unit intervals, as observed for ATCC 14579 between pH 5.0 and pH 4.7. The display of an intermediate physiological survival response by strain ATCC 14579 over the pH range 4.7 to 5.0, and the different inactivation boundaries were the main differences between the two strains tested.

Microarray analysis using hierarchical clustering

To investigate the impact of mild, bacteriostatic and bactericidal acid shocks on the gene expression of the two *B. cereus* strains, four pHs were selected based on the different phenotypic responses displayed by the two strains (Fig. 2A and 2B). At 0, 10, 30 and 60 minutes after the exposure to the different pH shocks, i.e., pH 5.4, pH 5.0, pH 4.8 and pH 4.5, RNA samples were collected and subsequent microarray analyses were performed. To compare the transcriptomes of both strains, data obtained of orthologous genes that are present in the genomes of both ATCC 14579 and ATCC 10987 (Ivanova *et al.*, 2003; Rasko *et al.*, 2004; Mols *et al.*, 2007) were collected and subjected to hierarchical clustering. The transcriptome profiles clustered in two different groups, with one cluster including samples obtained of the growth phenotype and the other cluster encompassing samples of the survival (strain ATCC 14579 only) and inactivation phenotypes (Fig. 3). The transcriptome profiles of cultures that showed growth after acid shock exposure clustered together independent of the exposure time and strain. Within these two major groups, the different branches of the hierarchical clustering were separated mostly depending on strain and exposure pH rather than exposure time. In conclusion, the exposure of *B. cereus* ATCC 14579 and ATCC 10987 to mild, bacteriostatic and bactericidal acid stress led to phenotype specific transcriptome profiles independent of exposure time.

The pH- and phenotype-specific responses were investigated by analyzing the transcriptome data obtained for genes showing significant differential expression in one or more conditions per strain. Groups of genes with similar expression profiles were identified using hierarchical clustering (*Supplementary material*). The results obtained for a selection of genes putatively involved in low pH or oxidative responses are presented and discussed below.

Acid shock response of low pH associated genes

A selection of genes, based on their putative role in acid stress response of other Gram-positive organisms (Cotter and Hill, 2003; Ter Beek *et al.*, 2008), was monitored profoundly. The selection includes transcription regulators, proton pumps, glutamate decarboxylase, production of alkaline compounds, protection of macromolecules, membrane synthesis, and multidrug transporters. The ratios of these genes obtained from cells exposed to pH 5.4 and pH 4.5 for 10, 30 and 60 minutes were averaged and plotted per gene (Fig. 4). In general, the average ratios, showing the up- or down-regulation, were less pronounced in inactivated cells. The genes encoding sigma factors σ^B and σ^H, involved in the global adaptive response to stress, were slightly up-regulated in growing cells of both strains. On the other hand, *codY*, which is a key regulator in the nutrient starvation response of Gram-positive organisms, showed no significant up-regulation. The major oxidative stress response regulator *perR* was one of the most up-regulated transcription regulators in both growing and inactivated cells, indicating an oxidative response upon low pH exposure. Previously, proton pumps, i.e., F_1F_0-ATPase, were shown to contribute to pH homeostasis in fermenting Gram-positives exposed to mild acid conditions (Cotter and Hill, 2003). In this study, genes encoding subunits of the F_1F_0-ATPase (represented by *atpA* in Fig. 4) were highly down-regulated in aerobically grown and exposed *B. cereus* cells upon exposure to sub-lethal pHs. Upon exposure to lethal acid shocks, genes encoding sodium-proton antiporters *napA* and *nhaC* were not down-regulated and *napA* even showed to be up-regulated. In *Listeria monocytogenes* (Cotter *et al.*, 2001) and *Lactococcus lactis* (Sanders *et al.*, 1998) acid-induced glutamate decarboxylase (*gad*), which catalyzes the decarboxylation of glutamate with concomitant consumption of protons, was found to play an important role in low pH survival. In *B. cereus* ATCC 10987, however, the *gad* gene, that is not present in the genome of ATCC 14579, showed not to be up-regulated upon low pH exposure. This is in line with the notion that *B. cereus* ATCC 10987 lacks a glutamate/GABA exchanger (Mols *et al.*, 2007), that is required to supply glutamate decarboxylase with its substrate (Cotter and Hill, 2003). Alkaline compound forming mechanisms, such as the arginine deiminase (ADI) pathway and the urease enzyme, are involved in acid tolerance of Gram-positive organisms (Cotter and Hill, 2003). Arginine deiminase (*arcA*), which is involved in acid resistance of streptococci (Curran *et al.*, 1995) and *L. monocytogenes* (Ryan *et al.*, 2009), showed significant up-regulation in both *B. cereus* strains upon exposure to sub-lethal acid shocks, whereas exposure to bactericidal acid shocks revealed no significant induction. Urease encoding genes, specific for ATCC 10987 (Mols *et al.*, 2007), were induced upon exposure to pH 5.4 (represented by *ureA* in Fig. 4A and 4B), but not in bactericidal conditions. Macromolecules are easily damaged during stress exposure, and their protection and repair is crucial for bacterial survival. DnaK and GroES are chaperones, preventing misfolding of proteins, and in *Streptococcus mutans* deletion of these chaperones resulted in less resistant cells (Lemos *et al.*, 2001). Notably, chaperone encoding genes *dnaK* and *groES* and protease encoding gene *clpC* were up-regulated upon exposure to sub-lethal acid shocks, whereas exposure to lethal pHs did not induce these genes in *B. cereus*.

Mild sorbic acid stress induces the expression of the fatty acid biosynthesis genes (*fab*), *bkdR* and a multidrug transport gene in *B. subtilis* (Ter Beek *et al.*, 2008). Two homologous multidrug systems (*mdr1* and *mdr2*) showed to be also up-regulated in sub-lethal inorganic acid stress in *B. cereus*. In contrast to sorbic acid stressed *B. subtilis*, fatty acid biosynthesis (represented by *fabF* in Fig. 4A and 4B) was down-regulated at pH 5.4 and no significant induction was found for branched-chain fatty acid biosynthesis (*bkdR*) in *B. cereus* upon exposure to (sub)lethal inorganic acid stress.

Oxidative response and rearrangements in energy metabolism

The response of several genes involved in oxidative stress and energy production were investigated in more detail (Fig. 5). Two distinct types of cytochrome oxidases showed different expression patterns. Cytochrome C oxidase, which acts as complex IV in aerobic conditions, was repressed in sub-lethal conditions in both strains. Cytochrome D ubiquinol oxidase, which can act as an alternative complex IV, was also down-regulated upon exposure to pH 5.4. However, it was highly induced in bacteriostatic and bactericidal conditions. Genes involved in oxidative stress, such as *sodA*, *katB* (Fig. 5) and *perR* (Fig. 4) were highly up-regulated in all acid shock conditions tested. The induction of these genes indicates that a low pH may induce the formation of oxidative compounds, such as H_2O_2. Nitric oxide (NO), formed from arginine by nitric oxide synthase (*nos*), putatively protects cells from H_2O_2-induced DNA damage by inhibition of the Fenton reaction and activation of catalase (Gusarov and Nudler, 2005; Shatalin *et al.*, 2008). Although *nos* was only slightly up-regulated in bactericidal conditions, the formation of nitric oxide may be inferred from the induction of nitric oxide dioxygenase (*hmp*) and a nitric oxide dependant transcriptional regulator (*dnrN*, *Supplementary material*). Nitric oxide dioxygenase facilitates the reaction of nitric oxide with oxygen to form nitrate. Nitrate reductase (*nar*) and nitrite reductase (*nas*) are involved in nitrogen metabolism and may serve as an alternative for aerobic respiration. Nitrate reductase and nitrite reductase genes are unique for strain ATCC 14579 and cluster together with nitrite extrusion protein *narK* (Mols *et al.*, 2007). The cluster, including *nar*, *nas* and *narK*, was highly up-regulated upon exposure to all acid shocks tested.

Besides genes involved in oxidative responses or energy metabolism, other genes were also up-regulated upon exposure to all the different acid shocks (see *Supplementary material*). Both strains showed to induce the expression of iron transporting and iron binding proteins, such as *feoB* (BC0709, BCE0783) and *dps* (BC2011, BC5044, BCE2092, BC5191). Furthermore, *mntH* (BC1803, BCE1960), encoding for manganese transport protein, also belonged to the group of up-regulated genes. Manganese and iron ions may play a role in oxidative stress response, conceivably acting as co-factors for superoxide dismutase proteins, and via other redox balancing mechanisms.

Inactivation associated radical formation

The induction of oxidative stress associated genes and a recent publication that showed that hydroxyl radicals (OH·) were formed upon exposure to bactericidal antibiotics in *Escherichia coli* and *Staphylococcus aureus* (Kohanski *et al.*, 2007), prompted us to investigate the formation of radicals upon low pH exposure of *B. cereus*. The formation of OH· and/or peroxynitrite ($ONOO^-$) in ATCC 14579 and ATCC 10987 cells was tested upon exposure to selected pHs (pH 5.4, pH 5.0, pH 4.8, and pH 4.5) at different intervals using the fluorescent probe 3’-(p-hydroxyphenyl) fluorescein (Fig. 6 and Fig. 7, respectively). Upon exposure to pH 4.5, ATCC 14579 was inactivated and this pH induced an increase of fluorescence indicating the formation of OH· and/or $ONOO^-$. The exposure to the other pHs tested, i.e., pH 5.4, 5.0, and 4.8, did not result in inactivation of the cells and also did not induce excess radical formation. Strain ATCC 10987 showed excess radical formation corresponding to the inactivation observed at pH 5.0, pH 4.8 and pH 4.5. At pH 5.4, where this strain was able to resume growth, no excess radical formation was measured.

The formation of the oxygen derived radicals OH· and ONOO$^-$ should be prevented when oxygen is not available during acid exposure. Indeed, no OH· and ONOO$^-$ formation was observed in both strains anaerobically exposed to similar low pH values. Correspondingly, increased acid resistance was observed in both strains when exposed anaerobically (data now shown). In addition, the generation of superoxide, a key precursor in OH· and ONOO$^-$ formation, was monitored upon low pH exposure using a superoxide-specific fluorescent probe. Detectable levels of superoxide were only found in aerobically exposed cells, and not in anaerobically exposed cells (Mols *et al.*, unpublished results). Taken together, these data provide evidence that the formation of hydroxyl radicals and/or peroxynitrite is associated with inactivation of *B. cereus* strains ATCC 14579 and ATCC 10987 exposed to low pH environments in the presence of oxygen.

Discussion

In this study, we describe the physiological and transcriptional responses of *Bacillus cereus* strains ATCC 14579 and ATCC 10987 to sub-lethal and lethal acid shocks. The two model strains were subjected to a range of pHs demonstrating that ATCC 14579 was more acid-resistant than ATCC 10987. ATCC 14579 survived acid conditions between pH 5.0 and pH 4.7 without growth or inactivation in the first hour of exposure. However, a prolonged exposure of ATCC 14579 to pH 4.8 resulted in a decrease of viable cells. In contrast, ATCC 10987 did not display this survival phenotype and was inactivated within the first hour of exposure to pHs lower than pH 5.0.

The exposure of *B. cereus* to sub-lethal and lethal acid stress resulted in distinct transcriptome profiles related to the physiological response displayed by the cultures. The concurrent analysis of two strains thus enables for distinguishing between phenotype-specific, stress level-specific and strain-specific transcriptome responses. Furthermore, the approach used showed not only the well-studied responses to mild pHs, including the induction of several general stress response genes, but also the response to lethal levels of acidity, an issue that has up to now mostly been neglected, as exemplified in recent studies on mild acid stress response of *Bacillus subtilis* (Wilks *et al.*, 2009). Cotter and Hill (2003) have reviewed the response of Gram-positive organisms to mild levels of acidity and mechanisms of acid resistance were described for fermentative lactic acid bacteria and *L. monocytogenes*, including roles of proton pumps, regulators, altered metabolism, protein and DNA repair, cell envelope alterations and alkali production. Using two model strains of *B. cereus*, we have demonstrated that protein and DNA repair, stress related transcriptional regulators, altered metabolism and alkali production were indeed induced at low pH. In contrast to fermentative lactic acid bacteria, F_1F_0-ATPase was not up-regulated in these respiring *B. cereus* strains upon exposure to acid, indicating that *B. cereus* does not use F_1F_0-ATPase to extrude protons under the conditions tested. Down-regulation of F_1F_0-ATPase is best explained by the cells trying to prevent excessive inward flux of protons via this ATPase upon exposure to acid conditions. Furthermore, no indications were found in the transcriptome analyses for low pH-induced membrane damage or rearrangement of membrane composition. For example, our experiments did not show an induction of fatty acid biosynthesis (*fab* genes), as was shown for *B. subtilis* exposed to mild sorbic acid stress (Ter Beek *et al.*, 2008). Furthermore, Ter Beek and colleagues (2008) reported that a putative multidrug resistance (mdr) transporter was induced in *B. subtilis* exposed to mild sorbic acid stress and they proposed this transporter to export sorbate anions from the cell. Two homologous genes in *B. cereus* were up-regulated upon exposure to inorganic acid stress at pH 5.4 that were not induced in response to lethal pH exposures. Since there is no apparent connection with sorbic acid stress and the induction of these putative mdr systems in *B. cereus*, their role in acid resistance of *B. cereus*, if any, remains to be elucidated.

The transcriptome analyses of the phenotypic responses to various levels of acidity revealed a major oxidative response. In bactericidal conditions, the oxidative response could be linked to the formation of OH· and/or $ONOO^-$ using flow cytometry in combination with the fluorescent probe HPF that specifically targets these reactive oxygen species. The observed oxidative burst in *B. cereus* may originate in a similar way as described for the formation of OH· radicals in *Escherichia coli* and *Staphylococcus aureus* upon exposure to bactericidal antibiotics in aerobic conditions (Kohanski *et al.*, 2007). The formation of OH· and $ONOO^-$ was not observed in anaerobically acid-stressed *B. cereus* cells, and correspondingly, increased acid resistance was observed under these conditions for both strains (data not shown). Based on phenotype and transcriptome analyses we propose a model for acid-induced radical formation, including OH· and $ONOO^-$, in *B. cereus* (Fig. 8). Acid stress may cause perturbation of the aerobic electron transfer chain (ETC) in *B. cereus* indicated by the differential expression of several genes potentially involved in ETC activity. This disturbance may cause premature leakage of electrons to oxygen leading to the formation of superoxide (O_2^-). Indeed, elevated levels of O_2^- could be detected in *B. cereus* cells upon exposure to lethal levels of acidity as indicated by staining of these cells with a superoxide-specific fluorescent probe (Mols *et al.*, unpublished results). Furthermore, the formation of O_2^- can be inferred from the induction of superoxide dismutase and catalase genes. Iron-sulphur clusters may subsequently be damaged by O_2^- releasing iron in the cytoplasm (Imlay, 2006). Free iron can react with hydrogen peroxide, originating from the dismutation of O_2^-, forming the highly toxic OH· radicals in the Fenton reaction (Imlay *et al.*, 1988). Furthermore, O_2^- can rapidly react with nitric oxide (NO) to form another highly toxic oxidative compound, $ONOO^-$ (Beckman and Koppenol, 1996). NO is formed by a reaction catalyzed by nitric oxide synthase (bNOS). Indirect indications for the formation of NO upon low pH exposure can be inferred from the up-regulation of nitric oxide dioxygenase and nitric oxide dependant regulator *dnrN*. The induction of bNOS activity, which is possibly regulated at protein level (Shatalin *et al.*, 2008), may initially have a positive effect on surviving oxidative stress. bNOS-derived NO may inhibit thiol reduction leading to the inhibition of the OH· forming Fenton reaction (Gusarov and Nudler, 2005; Sudhamsu and Crane, 2009). Furthermore, NO induces catalase activity in *B. anthracis* (Shatalin *et al.*, 2008) and inhibits the aerobic ETC (Husain *et al.*, 2008). On the other hand, NO facilitates the formation of $ONOO^-$, which may have a damaging effect that could lead to cell death. Nitric oxide dioxygenase and nitrite reductase are described to be possible NO dissipation routes (Payne *et al.*, 1997; Gardner, 2005). The genome of ATCC 14579 encodes both mechanisms and this strain showed to be more acid resistant than the nitrite/nitrate reductase deficient ATCC 10987 strain.

The phenomenon that exposure to stresses such as salt, heat, acid, and bile, results in secondary oxidative stress, has been described earlier for *B. cereus* and numerous other bacteria (Aldsworth *et al.*, 1999; Clements *et al.*, 1999; Hecker and Volker, 2001; Airo *et al.*, 2004; Banjerdkij *et al.*, 2005; Latifi *et al.*, 2005; Dodd *et al.*, 2007; Kim *et al.*, 2008), but up to now, this secondary oxidative stress response has not been linked to radical-associated cell death. Moreover, our findings are supported by earlier observations in amongst others *S. aureus* and *Vibrio vulnificus*, where acid resistance was found to be superoxide dismutase (and catalase) dependant (Clements and Foster, 1999; Kim *et al.*, 2005).

In conclusion, the results obtained in our study provide evidence for the origin of acid stress-induced oxidative stress. In extension to the antibiotic study of Kohanski and colleagues (2007), we now propose that in aerobic conditions, the formation of radicals such as OH· and $ONOO^-$ may be a common mechanism of cellular death in bacteria exposed to severe stress conditions.

Experimental procedures

Bacterial strains and growth conditions

B. cereus strains ATCC 14579 and ATCC 10987 were obtained from the American Type Culture Collection (ATCC). Stock cultures, grown in brain heart infusion (BHI, Becton Dickinson, France) broth, were stored at -80°C in 33% glycerol. To prepare pre-cultures, 10 ml BHI in a 100 ml Erlenmeyer flask was inoculated with a droplet from the glycerol stock and incubated overnight at 30°C, with shaking at 200 rpm.

To study the effect of pH on *B. cereus* cells and the corresponding transcriptome profiles, 100 ml BHI in a 500 ml Erlenmeyer flask was inoculated with 0.5 ml pre-culture and incubated at 30°C, with shaking at 200 rpm. Upon reaching an optical density of 0.5 measured at 600 nm (OD, Novaspec II, Pharmacia Biotech, Germany), the culture pH was measured (PHM 240 pH/ION Meter, Radiometer, Denmark) and serial dilutions were made in peptone physiological salt solution (PPS, 1g/l neutralized bacteriological peptone (Oxoid, England) and 8.5 g/l NaCl in water) and plated with a spiral-plater (Eddy Jet; IUL Instruments, Spain) on BHI agar plates (15 g/l bacteriological agar, Oxoid, England). 20 ml of the culture was used to extract RNA (sample t = 0). The remaining volume of the culture was acidified with hydrochloric acid (HCl 37%, Merck, Germany) to pH 5.4, 5.0, 4.8 or 4.5 and incubated at 30°C, with shaking at 200 rpm. At designated time points (10, 30 and 60 minutes), samples were taken to measure the OD, to determine the viable counts, and to extract RNA.

RNA isolation

RNA isolation was performed by transferring 20 ml of the cultures into a 50-ml Falcon tube (Greiner Bio-one, Germany) at the designated time points. Subsequently, the cultures were pelleted at maximum speed at 4°C for 30 s (Eppendorf centrifuge 5804 R, Eppendorf, Germany). After decanting the supernatant, the cell pellets were frozen in liquid nitrogen. Within 10 min after freezing the cell pellets, 1 ml TRI-reagent (Ambion, United Kingdom) was added to the pellets. The samples were stored at -80°C until RNA extraction. RNA was extracted as described previously (van Schaik *et al.*, 2004). Residual chromosomal DNA was removed by treating the samples with DNA-*free* (Ambion, United Kingdom). The RNA concentration was measured in 2 ml cuvettes (UVettes, Eppendorf, Germany) with a BioPhotometer (Eppendorf, Germany) by determining the OD_{260} and OD_{280}. The quality of the RNA was monitored using the RNA 6000 Nano Assay (Agilent, United States) and the Agilent 2100 Bio-analyzer (Agilent, United States) according to the provided protocol. The extracted RNA samples were stored in 70% ethanol with 83 mM sodium acetate buffer (pH 5.2) at -20°C.

cDNA synthesis, labelling and microarray hybridization and design

Complementary DNA with amino-allyl-labelled dUTP (Ambion, United Kingdom) from the extracted RNA was prepared in reverse transcription reactions using Superscript III (Invitrogen, The Netherlands). Cy3 and Cy5 labelling of the cDNAs was performed with the CyScribe Post-Labeling kit (GE Healthcare, Belgium) as previously described (den Hengst *et al.,* 2005). The labelled cDNAs were purified using the CyScribe GFX purification kit (GE Healthcare) according to the provided protocol. To conduct the microarray hybridization, the Cy5-labelled cDNA samples were combined with the corresponding Cy3-labelled t0 reference samples (1:1 ratio). The microarray experiments for the comparison of the transcriptomes of the cultures exposed to various pHs were performed in two independent biological replicates, where the replicate was performed with the dyes swapped. *B. cereus* ATCC 14579 and *B. cereus* ATCC 10987 microarrays (details below) were hybridized with 200 to 300 ng labelled cDNA following the 60-mer oligo microarray processing protocol (Agilent, United States).

The microarrays used in this study were custom-made *B. cereus* ATCC 14579 and custom-made *B. cereus* ATCC 10987 microarrays developed by Agilent Technologies (United States). The *B. cereus* ATCC 14579 microarray design was based on the 11K platform of Agilent Technologies (GEO accession number GPL7680). A total of 10,262 spots represented 5,131 chromosomal open reading frames, meaning that 98.0% of the predicted chromosomal open reading frames (NCBI accession number NC_004722) are represented on the microarray. 99.6 % of the open reading frames for which probes could be designed were represented by two non-overlapping probes on the array. The remaining 0.4% of the open reading frames was represented by a single oligonucleotide spotted in duplicate on the array. The *B. cereus* ATCC 10987 microarray design was based on the 22K platform of Agilent Technologies (GEO accession number GPL7681). A total of 17,697 spots represented 5,578 chromosomal open reading frames, 240 plasmid open reading frames and 81 putative small-RNAs, meaning that 99.6% of the predicted chromosomal and the plasmid open reading frames (NCBI accession numbers NC_003909 and NC_005707, respectively) were represented on the microarray. All features (chromosomal and plasmid open reading frames and small-RNAs) were represented by three individual spots. For 4,914 features three non-overlapping probes were designed, for 488 features two probes were designed (one probe spotted in duplicate) and for 497 only one oligonucleotide could be designed (one probe spotted in triplicate).

After hybridization at 60°C for 17 hours, the microarrays were washed with 6 × SSC (0.9 M NaCl and 0.09 M sodium citrate) supplemented with 0.005% Triton X-102 at room temperature for 10 min. Subsequently, the microarray slides were washed at 4°C with prechilled 0.1 × SSC with 0.005% Triton X-102 for 5 min and dried with nitrogen gas.

Microarray scanning and data analysis

The microarray slides were scanned using an Agilent microarray scanner (G2565BA), and data were extracted from the scanned microarrays with Agilent's Feature Extraction software (version 8.1.1.1), which includes a LOWESS (locally weighted scatterplot smoothing) normalization step for the raw data. After removal of the data for the control spots, the normalized data for each spot from the microarrays were analyzed for statistical significance using the web-based VAMPIRE microarray suite (Hsiao *et al.*, 2005). A spot was found to be differentially expressed between two samples when the false discovery rate was smaller than 0.05. Subsequently, the data for the single spots were integrated to obtain expression ratios for a corresponding feature (i.e., open reading frame or sRNA). A feature was found to be differentially expressed when all spots representing the feature were significantly differentially expressed between samples.

Hierarchical clustering (Eisen *et al.*, 1998) was performed per strain to identify groups of genes showing similar expression patterns. In Genemaths XT (version 1.6.1, Applied Maths, Belgium) genes that were significantly differentially expressed in one or more conditions were $\log_2$ transformed and clustered using the complete linkage method and the Euclidian distance matrix. The groups identified from the hierarchical clustering were based on an arbitrary cut-off value. To identify relevant biological processes significantly overrepresented in a group, the genes corresponding to a group were analyzed using FIVA (Blom *et al.*, 2007). To visualize the relation between the different acidic conditions independent of the strains, the $\log_2$ transformed expression ratios from genes present on both microarrays were hierarchically clustered using the average linkage method and the Euclidian distance matrix (Genemaths XT, 1.6.1).

Flow cytometry and radical measurements

To detect radical formation, the fluorescent reporter dye 3’-(p-hydroxyphenyl) fluorescein (HPF, Invitrogen, The Netherlands) was used (Setsukinai *et al.*, 2003). At designated time points (0, 10, 30, 60 minutes) after adding HCl to the exponentially growing cultures (OD_{600nm} ~0.5), samples were obtained by centrifuging (15,000 × *g*, 30 s) 1 ml of culture and resuspension of the cell pellet in 1 ml filtered phosphate buffer saline (PBS). The samples were washed once and diluted with filtered PBS to obtain a concentration of approximately 10^6 cells per ml, subsequently HPF was added at a final concentration of 5 mM. Samples were run on a Becton Dickinson FACSCalibur flow cytometer with the following photomultiplier tube (PMT) voltage settings: E00 (FSC), 360 (SSC) and 825 (FL1). Data were obtained from 20,000 events (cells) at medium flow rate using Cellquest Pro (version 4.0.2), subsequently analyzed with WinMDI 2.9 (Joseph Trotter, Salk Institute for Biological Studies, La Jolla, California, USA; http://facs.scripps.edu/software.html) and graphically presented using Adobe Illustrator CS2 (version 12.0.1).

Acknowledgements

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Figure legends

Fig. 1. Physiological response of *B. cereus* ATCC 14579 (filled squares) and ATCC 10987 (open diamonds) upon exposure to a range of acidity levels. The colony forming units were determined after 0 and 60 minutes of exposure, the difference between the t0 and t60 is depicted. Data points represent single experiments, indicating a large variability of the responses of ATCC 10987 exposed to pH 5.0. The grey line at 0 corresponds to no growth (growth above the line) and no inactivation (inactivation below the line). The filled area between the dotted lines depicts the pH values where ATCC 14579 displayed no growth and no inactivation, i.e., survival, and where ATCC 10987 already showed to be inactivated.

Fig. 2. Physiological response of *B. cereus* ATCC 14579 (A) and ATCC 10987 (B) upon exposure to low pH. The colony forming units determined at different time points upon exposure to pH 5.4 (squares), pH 5.0 (diamonds), pH 4.8 (triangles) and pH 4.5 (circles) are depicted. At 0, 10, 30 and 60 minutes samples were taken for microarray analysis, indicated

with arrows and the error bars represent the standard deviation between duplicate experiments.

Fig. 3. Hierarchical clustering of the transcriptome profiles of different pH exposures based on the common genes of *B. cereus* ATCC 14579 and ATCC 10987. Samples with similar expression patterns were clustered using Euclidean distance and complete linkage. The corresponding phenotypic responses are shown at the right. Samples obtained from growing cultures are depicted in black, samples from non-growing ("survival" and "inactivation" phenotypes) cultures are shown in grey. Relative distance in similarity between the branches is shown at the top and bootstrap values are indicated at each branch.

Fig. 4. Average ratios of low pH and stress associated genes from *B. cereus* ATCC 14579 (closed bars) and ATCC 10987 (open bars) upon exposure to pH 5.4 (A) and pH 4.5 (B). The global adaptive response is represented by *sigB* (BCE1086 and BC1004), *sigH* (BCE0093 and BC0114) and *codY* (BCE3869 and BC3826), encoding σ^B, σ^H and CodY respectively. Additionally, *perR* (BCE0592 and BC0518), a major oxidative stress response regulator is shown. *atpA* (BCE5432 and BC5308), *napA* (BCE1729 and BC1612), and *nhaC* (BCE1840 and BC1709) represent F_1F_0-ATPase and two proton antiporters. *gad* (glutamate decarboxylase, BCE2691), *arcA* (arginine deiminase, BCE0472 and BC0406), and *ureA* (urease, BCE3664) represent systems that are described to be involved in alkaline production. Glutamate decarboxylase and urease are specific for ATCC 10987 and are indicated with an asterisk. The general stress response chaperones and proteases are depicted by *dnaK* (BCE4395 and BC4312), *groES* (BCE0288 and BC0294), and *clpC* (BCE0079 and BC BC0100). Additionally, mechanisms involved in sorbic acid stress of *B. subtilis* (Ter Beek *et al.*, 2008), such as, multidrug transporters (*mdr1*, BCE4699 and BC4568 and *mdr2*, BCE1943

and BC1786), fatty acid biosynthesis (*fabF*, BCE1294 and BC1174), and branched-chain fatty acid biosynthesis (*bkdR*, BCE4239 and BC4165) are shown.

Fig. 5. Average ratios of selected genes associated with respiration and oxidative responses from *B. cereus* ATCC 14579 (closed bars) and ATCC 10987 (open bars) upon exposure to pH 5.4 (A), pH 5.0 (B), pH 4.8 (C) and pH 4.5 (D). *ETC-4a* and *ETC-4b* depict two distinct types of complex IV of the aerobic electron transfer chain. Cytochrome C oxidase polypeptide I gene *ctaD* (BCE3990 and BC3943) represents *ETC-4a* and cytochrome D ubiquinol oxidase subunit I gene *cydA* (BCE4949 and BC4792) represent *ETC-4b*. The genomes of ATCC 14579 and ATCC 10987 harbour four different superoxide dismutase genes and three different catalase genes, the data of *sodA* (BCE5579 and BC5445) and *katB* (BCE1261 and BC1155) are shown here. Nitric oxide synthase and nitric oxide dioxygenase are represented by the corresponding genes, *nos* (BCE5578 and BC5444) and *hmp* (BCE1571 and BC1448), respectively. Nitrate and nitrite reductases are encoded by multiple genes on the genome of ATCC 14579, therefore *narI* (BC2121), *nasD* (BC2136) and *nark* (BC2128) are shown. This nitrate and nitrite reductase cluster of ATCC 14579 is absent in ATCC 10987 and therefore indicated with an asterisk.

Fig. 6. Radical formation in *B. cereus* ATCC 14579 upon exposure to pH 5.4, pH 5.0, pH 4.8 and 4.5. Samples were taken at 0 (green), 10 (light blue), 30 (blue) and 60 (dark blue) minutes. The pH and corresponding physiological response are indicated at each graph. The shift in fluorescent signal to the right indicates the formation of hydroxyl and/or peroxynitrite radicals.

Fig. 7. Radical formation in *B. cereus* ATCC 10987 upon exposure to pH 5.4, pH 5.0, pH 4.8 and 4.5. Samples were taken at 0 (green), 10 (light blue), 30 (blue) and 60 (dark blue) minutes. The pH and corresponding physiological response are indicated at each graph. The shift in fluorescent signal to the right indicates the formation of hydroxyl and/or peroxynitrite radicals.

Fig. 8. Low pH induced oxidative stress response and radical forming mechanisms in *B. cereus* ATCC 14579 and ATCC 10987. Schematic representation of radical formation conceivably induced upon exposure to lethal acid stress. Acid stress may cause perturbation of the electron transfer chain and an excess of superoxide radicals (O_2^-) may be formed. Superoxide radicals can be converted to hydrogen peroxide and water by superoxide dismutase (*sod*) and catalase (*kat*). However, when the capacity to dismutate superoxide is not sufficient, free superoxide radicals can cause damage to iron-sulphur (Fe-S) cluster containing enzymes supplying unbound iron ions. These free iron ions and hydrogen peroxide can react (Fenton reaction) and produce hydroxyl radicals (OH·). Another possible route in forming highly damaging radicals may occur via nitric oxide. Nitric oxide can be formed by nitric oxide synthase (*nos*) and can react with superoxide radicals to form peroxynitrite ($ONOO^-$). Nitric oxide can be converted to nitrate by nitric oxide dioxygenase (*hmp*). Subsequently, nitrate can be converted to nitrite and ammonium by nitrate (*nar*) and nitrite (*nas*) reductase, respectively. Nitrite can also be transported outside the bacterial cell by a Nitrite extrusion protein (*nark*). *nar, nas* and *narK* are ATCC 14579 specific, the reactions they catalyze are indicated with dotted lines.

Supplementary material

The following supplementary material is available for this article online:

14579_Allgroups.tar. A compressed folder containing the results of the hierarchical clustering of the significant differentially expressed genes of strain ATCC 14579. Opening the htmlOutputFiva.html file shows the graphical output of all used annotation modules and can be used to navigate through the results. By clicking the cluster names, the corresponding transcriptome profiles of the groups are revealed. The size of each group is displayed in blue underneath the group name and by clicking the list of genes will appear. Numbers in each rectangle represent absolute values of occurrences. The significance of occurrences is visualized in a colour gradient and with symbols which are displayed at the bottom of the plot. On the left the categories showing over representation in one or more groups are listed with the corresponding size. Clicking the categories will reveal the genes present in the corresponding category per group. The description of each category is placed at the right and more information about the categories can be displayed by clicking the description.

10987_Allgroups.tar. A compressed folder containing the results of the hierarchical clustering of the significant differentially expressed genes of strain ATCC 10987. A detailed description is given above.

Supplementary results.pdf. Supplementary results based on the microarray data obtained for *B. cereus* strains ATCC 14579 and ATCC 10987 exposed to pH 5.4, pH 5.0, pH 4.8 and pH 4.5. The data of each strain was clustered hierarchically and different groups of genes were identified based on their common transcriptome profile. The FIVA analysis showed that several function categories were overrepresented in certain groups.

Raw and processed microarray data are available for reviewers following the link to the GEO database below.

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=hfsfreqageqakzi&acc=GSE13773

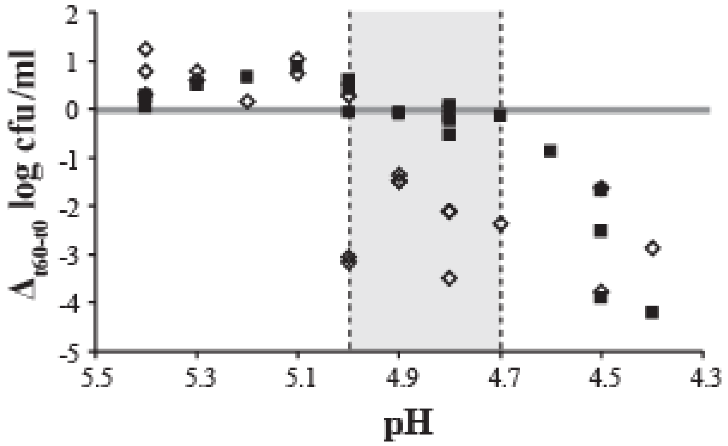

Δ_{t60-t0} log cfu/ml
2
1
0
-1
-2
-3
-4
-5
5.5
5.3
5.1
4.9
4.7
4.5
4.3
pH

Fig 1

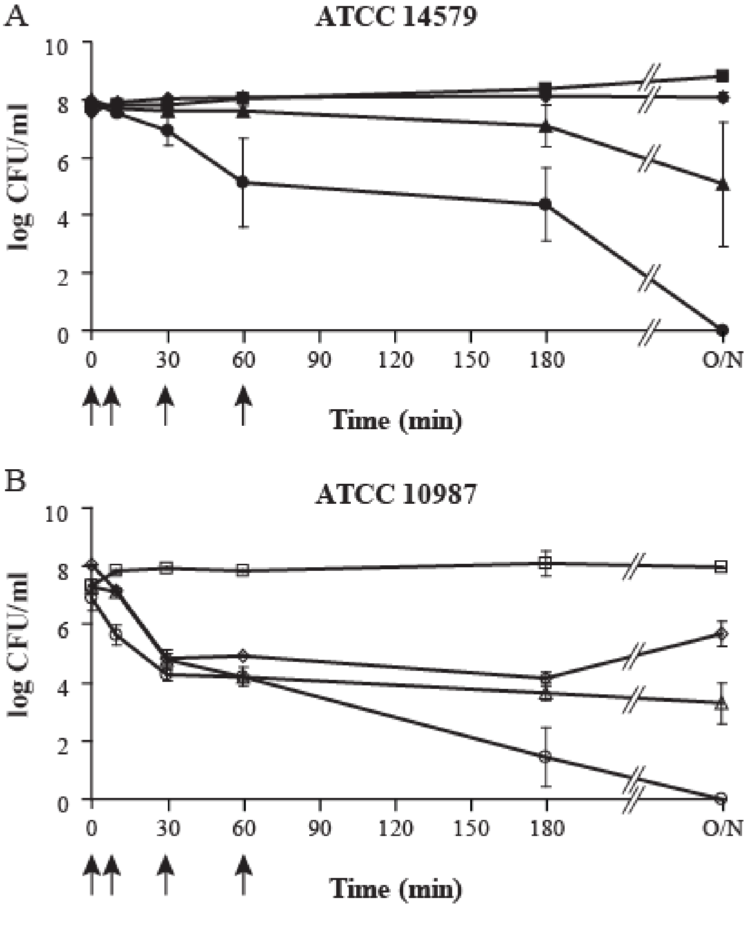
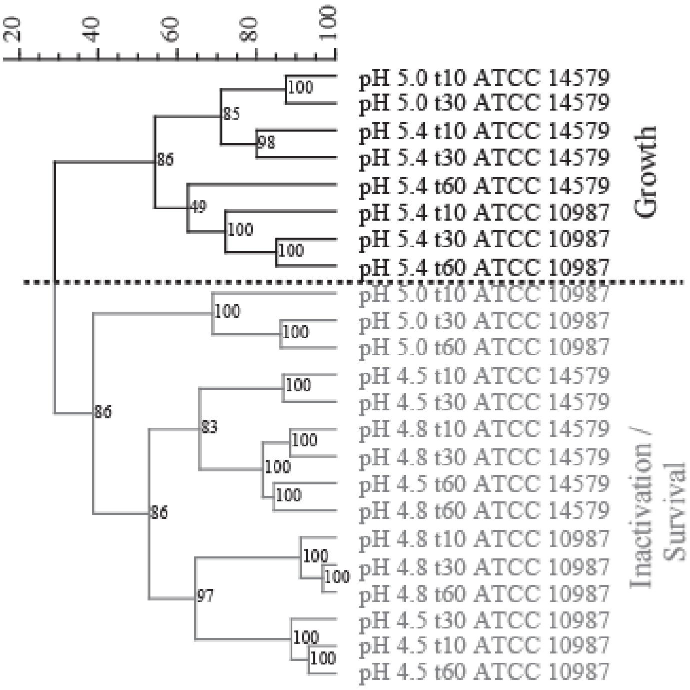

A
ATCC 14579
log CFU/ml
10
8
6
4
2
0
0
30
60
90
120
150
180
O/N
Time (min)
B
ATCC 10987
log CFU/ml
10
8
6
4
2
0
0
30
60
90
120
150
180
O/N
Time (min)

Fig 2


20
40
60
80
100
pH 5.0 t10 ATCC 14579
pH 5.0 t30 ATCC 14579
pH 5.4 t10 ATCC 14579
pH 5.4 t30 ATCC 14579
pH 5.4 t60 ATCC 14579
pH 5.4 t10 ATCC 10987
pH 5.4 t30 ATCC 10987
pH 5.4 t60 ATCC 10987
pH 5.0 t10 ATCC 10987
pH 5.0 t30 ATCC 10987
pH 5.0 t60 ATCC 10987
pH 4.5 t10 ATCC 14579
pH 4.5 t30 ATCC 14579
pH 4.8 t10 ATCC 14579
pH 4.8 t30 ATCC 14579
pH 4.5 t60 ATCC 14579
pH 4.8 t60 ATCC 14579
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pH 4.8 t30 ATCC 10987
pH 4.8 t60 ATCC 10987
pH 4.5 t30 ATCC 10987
pH 4.5 t10 ATCC 10987
pH 4.5 t60 ATCC 10987
Growth
Inactivation / Survival
100
85
98
86
49
100
100
100
100
100
86
83
100
100
100
86
100
100
97
100
100

Fig 3

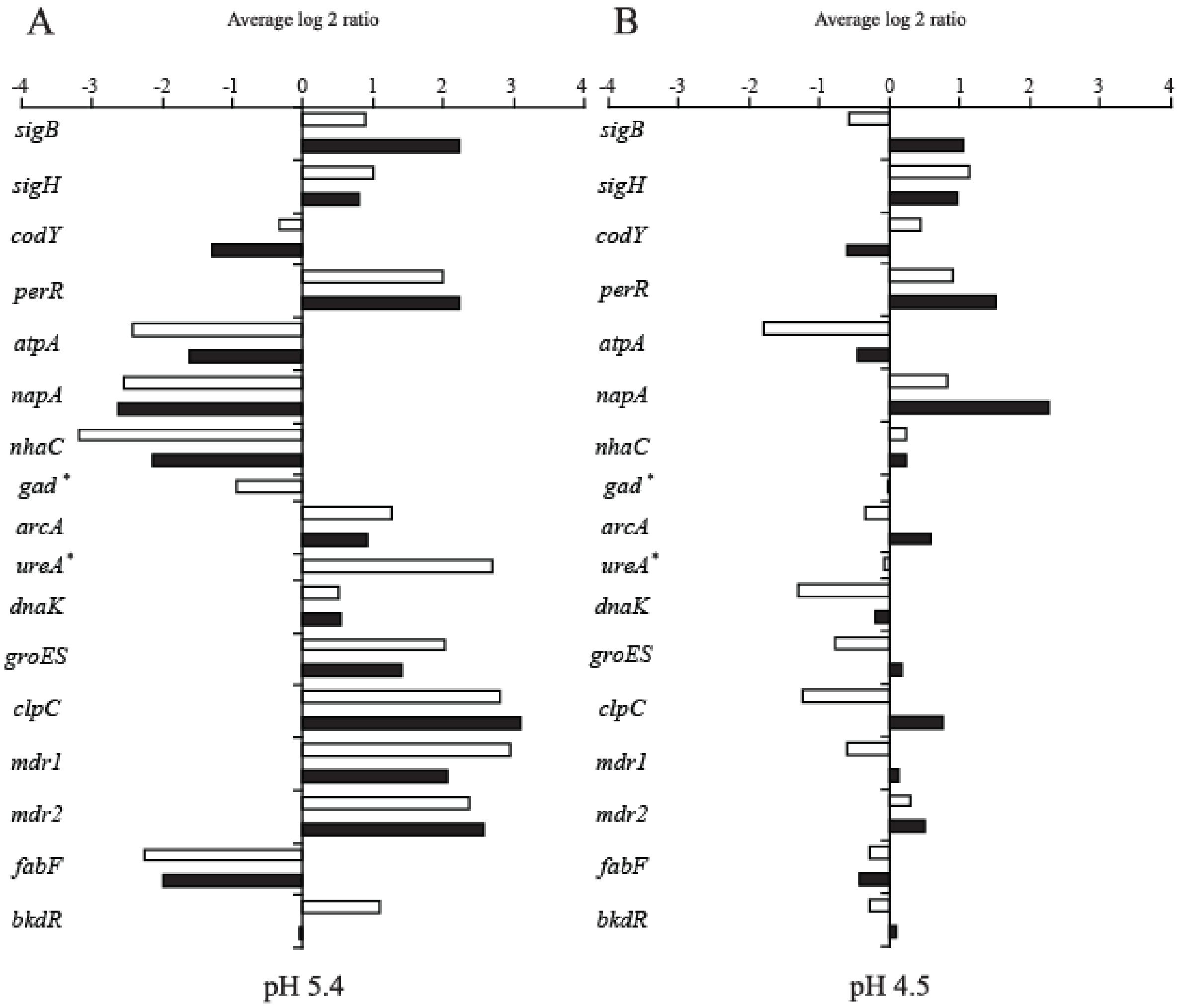

A
Average log 2 ratio
-4 -3 -2 -1 0 1 2 3 4
B
Average log 2 ratio
-4 -3 -2 -1 0 1 2 3 4
sigB
sigH
codY
perR
atpA
napA
nhaC
gad *
arcA
ureA *
dnaK
groES
clpC
mdr1
mdr2
fabF
bkdR
pH 5.4
pH 4.5

Fig 4

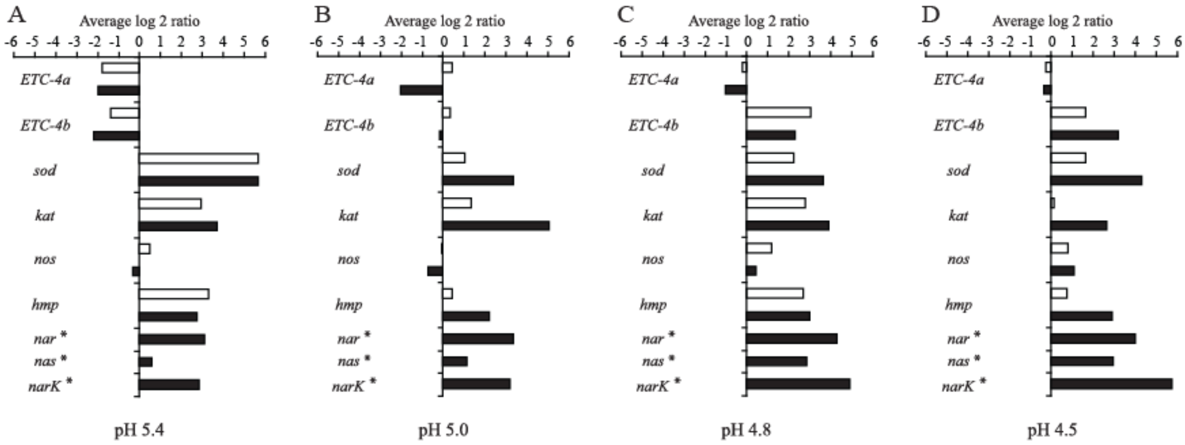
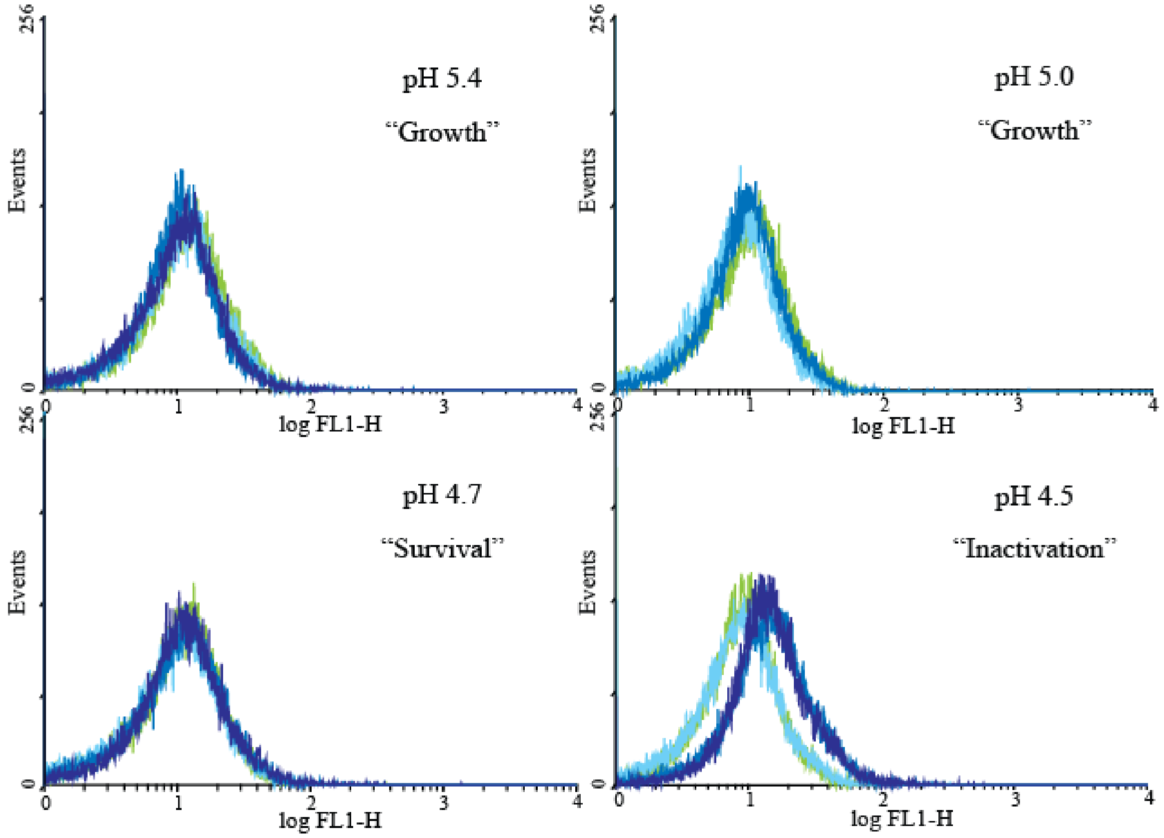

A
Average log 2 ratio
-6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6
B
Average log 2 ratio
C
Average log 2 ratio
D
Average log 2 ratio
ETC-4a
ETC-4b
sod
kat
nos
hmp
nar *
nas *
narK *
pH 5.4
pH 5.0
pH 4.8
pH 4.5

Fig 5


256
Events
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pH 5.0
"Growth"
pH 4.7
"Survival"
pH 4.5
"Inactivation"
0
1
2
3
4
log FL1-H

Fig 6

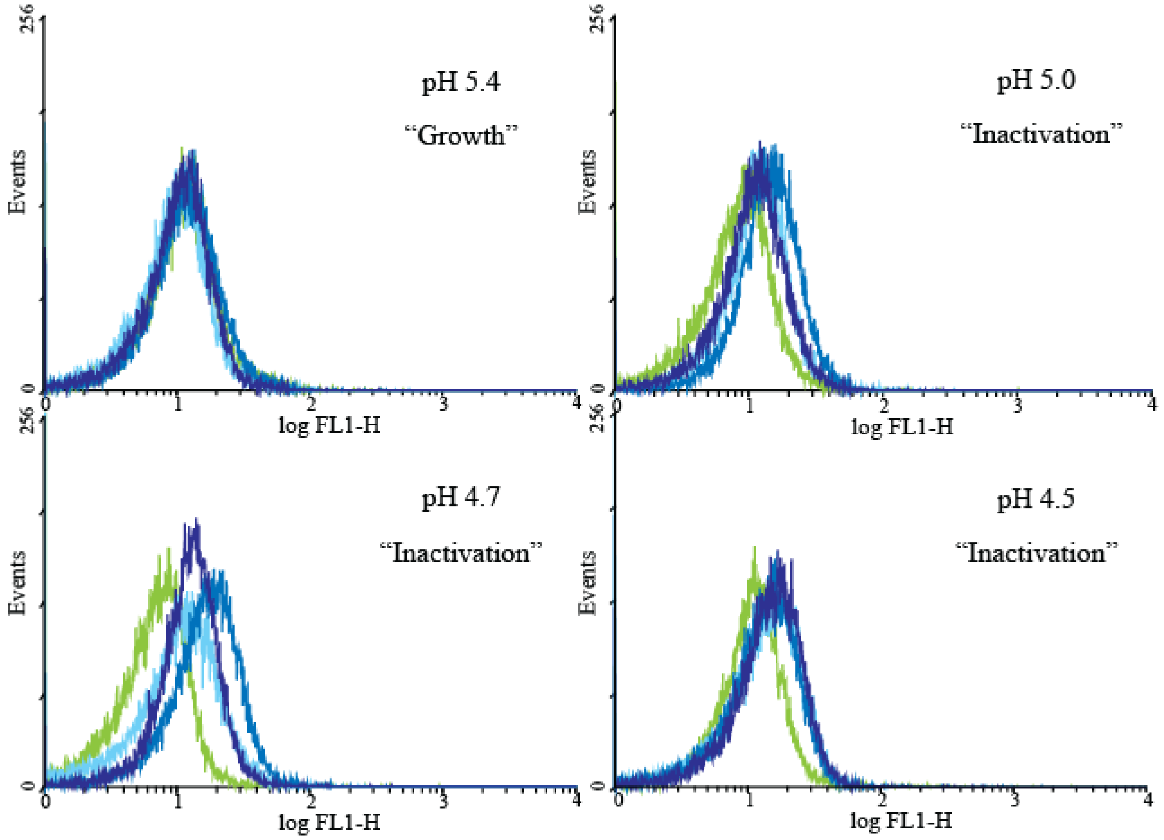
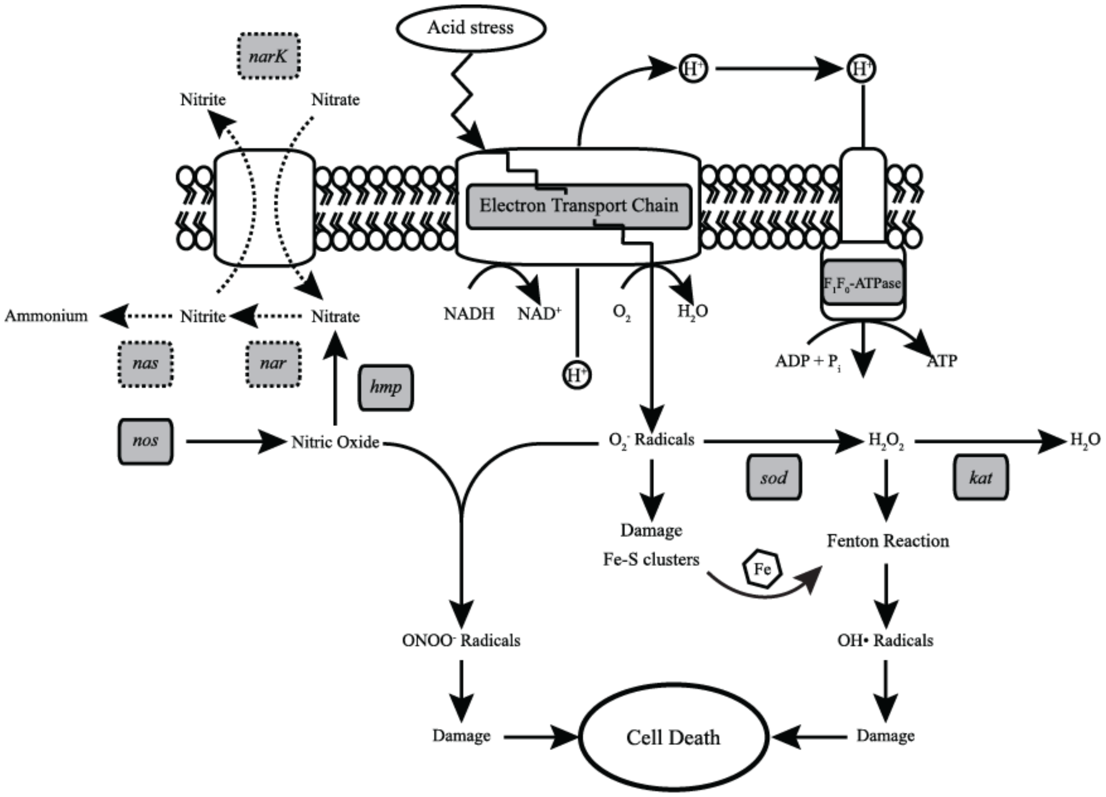

256
Events
0
pH 5.4
"Growth"
pH 5.0
"Inactivation"
pH 4.7
"Inactivation"
pH 4.5
"Inactivation"
0
1
2
3
4
log FL1-H

Fig 7



Acid stress
narK
Nitrite
Nitrate
H+
H+
Electron Transport Chain
F1F0-ATPase
Ammonium
Nitrite
Nitrate
NADH
NAD+
O2
H2O
ADP + Pi
ATP
nas
nar
hmp
H+
nos
Nitric Oxide
O2- Radicals
H2O2
H2O
sod
kat
Damage
Fe-S clusters
Fe
Fenton Reaction
ONOO- Radicals
OH• Radicals
Damage
Cell Death
Damage

Fig 8