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Heat and acid resistance depend on growth pH in the food-borne pathogen Bacillus cereus ATCC 14579

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ABSTRACT:

Bacillus cereus is a toxin producing bacterium responsible for food-borne toxi-infections. To monitor the ability of the food-borne opportunistic pathogen Bacillus cereus to survive during minimal processing of food products conditions were established which allowed the cells to adapt to heat and acid stresses. Cells were grown in chemostat at different pH (7.0 and 5.5) and at a growth rate of 0,2 h^{-1}. Cells were submitted during 40 minutes to an acid shock at pH 4 or to a heat shock at 50°C. The effect of pre-adaptation at a subletal temperature (42°C -15 min) was studied. Cells grown at low pH (pH 5.5) shown an acid stress adaptation with induction of an Acid Tolerance Response (ATR).

Cross-adaptations was observed between thermotolerance and acidotolerance which suggested that a common mechanism was involved in the acid and heat stress responses in B. cereus ATCC 14579. Adaptive levels of stressor were found to induce the over expression of different genes encoding classical heat shock proteins such as dnaK, dnaJ sigB and GroEL and gene involved in internal pH homeostasis encoding ATPase, arginine decarboxylase or deiminase and lysine decarboxylase activities. These data shown that during mild processing, a cross-protection between acid and heat was occurred in B. cereus. These common mechanisms between acid and heat stress response may result in increasing survival of B. cereus cells in foods and can rising the pathogenicity of this bacteria. The virulence of this bacterium poses a major problem in public health in food poisoning.

Keywords: Bacillus cereus, chemostat, ATR, thermotolerance, cross-protection, RT-PCR, HSP (Heat shock protein).

INTRODUCTION

Bacillus cereus is one of the major food-borne pathogenic bacteria and a common contaminant of food and dairy products. It is a gram-positive, facultatively anaerobic, spore-forming bacterium that is the causative agent of two types of food poisoning by production of emetic and diarrheal forms.

B. cereus is an important cause of food poisoning in industrialized parts of the world might be because consumers required fresher and more natural food products, which results in a reduction in the intrinsic preservation of foods (Gould et al., 1995). The microbial safety and stability of most minimally processed foods are based on the

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application of combined preservative factors of which (i) mild heating which is the most common preservation technique used (Leistner, 2000) (ii) less acidic food.

Bacteria have evolved adaptive networks to face the challenges of a changing environment and to survive under stress conditions (Abee and Wouters, 1999). *B. cereus* is able to resist and adapt to an acid environment when organic acids are added for preservation of the contaminated food or/and during the transit of this food in the stomach. *B. cereus* vegetative cells are more acid-sensitive than spores which depends on the growth pH and on the type of food (Clavel et al., 2004). We have shown that *B. cereus* (strain TZ415) vegetative cells, like many other bacteria was able to induce an acid tolerance response ATR when cells were grown at low medium pH "pH_e 5.5" (Jobin et al., 2002). Similar results were founded in *B. cereus* ATCC 14597 (Senouci-rezkkalah et al., 2011; 2015).

Heat-induced thermotolerance has been studied in several food pathogens such as *Listeria monocytogenes* (Pagán et al., 1997) and *B. cereus* (Mahakarnchanakul and Beuchat, 1999). Indeed, an increase in thermotolerance at 50°C was observed after incubation under mild heat conditions (37 or 40°C for several hours). In a wide variety of bacteria, the heat shock response includes increased synthesis of a set of conserved heat shock proteins (HSPs) (Yura et al., 2000). The molecular genetics of the heat shock response has been most extensively studied in *Escherichia coli* and *B. subtilis* (Hecker et al., 1996); (Yura et al., 2000). Classical HSPs are the molecular chaperones (e.g., DnaK, GroEL, and their cohorts) or ATP-dependent proteases (e.g., ClpP). These proteins play roles in protein folding, assembly, and repair and prevention of aggregation under stress and non stress conditions. The chaperones and proteases act together to maintain quality control of cellular proteins (Gottesman et al., 1997). For several bacteria, several HSPs are also induced under various stresses conditions other than heat, such as exposure to ethanol, acid, or oxidative stress or during macrophage survival, might result in increased thermotolerance. (Abee and Wouters, 1999); (Yura et al., 2000).

Cross protection between salt, heat and hydrogen peroxide has been established in *B. subtilis*. Each stressor results in the induction of both proteins specific to that stress and proteins that can be induced by other stresses named as Heat Stress Protein (HSP) or chaperone proteins.
Moreover, Browne and Dowds showed that *Bacillus cereus* NCIMB11796 adapted to acid stress (pH 4.6) when it was pre-exposed to a non lethal, inducing pH 6.3 or to inducing heat, concentrations of ethanol, salt or hydrogen peroxide. The cross protection between stresses involves a common mechanisms of the response to a various stress (Browne and Dowds, 2002).

In order to design worst case processing conditions for foods, it is necessary to understand the response of the common bacterial contaminants to the processing stresses after various storage conditions of the raw food.

In this report, we provide evidence for acid and heat-protective response in *B. cereus* ATCC 14579, and to determine the effects of stresses imposed on bacterial contaminants during food processing and treatment of packaging material were evaluated on the food pathogen *Bacillus cereus*, and understand *Bacillus cereus* ATCC 14579 Acid Stress Response.

Chemostat cultivation allows reproducible steady-state cultivation of microorganisms (Pirt S J., 1975). In chemostat cultures, important parameters such as the specific growth rate, culture pH, and dissolved-oxygen concentration can be accurately controlled. Thus, chemostat cultivation allows physiological studies in which a single culture parameter is varied while all other conditions are kept constant (Weusthuis R A., et al., 1994). Chemostats are continuous culture systems in which cells are grown in a tightly controlled, chemically constant environment

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where culture density is constrained by limiting specific nutrients. Data from chemostats are highly reproducible for the measurement of quantitative phenotypes as they provide a constant growth rate and environment at steady state. This makes chemostat cultivation a virtually indispensable technique for genome-wide expression studies. For these reasons, chemostats have become useful tools for fine-scale characterization of physiology through analysis of gene expression and other characteristics of cultures at steady-state equilibrium.

BODY TEXT

Bacterial strains and growth conditions

Bacillus cereus strain ATCC14579 was obtained from the American Type Culture Collection.

Growth medium was J-Broth (JB) (5g l^{-1} pepton,15g l^{-1} yeast extract, 3g l^{-1} K_2HPO_4 and 15g l^{-1} agar for plate count J Agar) (Claus and Berkeley, 1986).

The pH of the medium was adjusted to the desired value before autoclaving for 20 min at 120°C, JB medium was checked for pH value after sterilization and supplemented with 2g l^{-1} filter-sterilized glucose. *B. cereus* from stock culture was purified on J Agar. One colony was transferred in an anaerobic flask containing 100 ml of JB at pH 7, the medium was sparged with oxygen-free nitrogen gas for 15 min to eliminate oxygen. Growth was carried out at 34°C for 15 h with agitation at 100 rpm.

Chemostat cultures

Chemostat cultures were performed in a 2-l bioreactor (Discovery 100 MRU, INCELETECH, Toulouse, France) using a 1-l working volume. All experiments were carried out at 34°C with agitation at 300 rpm. Culture pH was monitored and maintained at pH 5.5 ± 0.04 or 7.0 ± 0.06. During fermentation, the culture pH was continuously sparged with oxygen-free nitrogen gas to ensure anaerobiosis. Medium inflow rates (F, $1h^{-1}$) were adjusted to generate the desired dilution rate (D, $1h^{-1}$). Since the volume of the growth vessel (V, in l) is set by the operator, dilution rate was equal to specific growth rate (μ), the chemostat was operated at steady-state dilution rate of 0.2 h^{-1}. To ensure a steady-state, cells were maintained at dilution rate of 0.2 h^{-1} for at least five generations before sampling for analysis.

Cells counting

Decimal dilution in 100 mmol l^{-1} potassium phosphate buffer pH 7.0 were surface spread on J Agar plates using a spiral plate maker (Spiral systems®, Intersciences, St Nom la Bretèche, France). The cell concentration was calculated according to the manufacturer's instructions and expressed as colony forming units per ml (CFU ml^{-1}). The detection limit of the method was 200 CFU ml^{-1}.

Determination of viability loss of *B. cereus* exposed to different stresses.

One millitre of steady-state *Bacillus cereus* cells at different pH (pH 7.0 or 5.5) was diluted in 19 ml of JB acidified to pH 4 with HCl and maintained at 34°C with agitation at 100 rpm for determined their acido-tolerance. One millitre of steady-state *Bacillus cereus* cells was diluted in 19 ml of JB at equivalent grown pH and exposed to heat shock at 50 °C.

10 ml of culture of cells were pre-exposed to mild heat treatment at 42°C for 15 min, after 1 ml of this cells were submited to heat or acid shocks at 50°C and at pH 4 respectively.

Viability loss was expressed as $\log(N/N_0)_t$, where N is the viable count after 10,20 30 40 min (time t) exposure at pH 4,0 or 50 °C and N_0 is the viable count at the beginning of acid or heat shock. The time to achieve one logarithmic unit reduction in population ($T_{-1\log}$) was estimated graphically.

mRNA preparation and quantification

Quantification of mRNA was performed by real-time PCR (RT-PCR) as previously described (Duport et al., 2004). The primers used are given in Table 1.

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Table 1 PCR primers used in this study.

Primers	Function	Sequence(5'→ 3')[a]
atpB	a subunit of F_0F_1-ATPase	F:GCAATATGTTCGCCAGCTTC R:ACTCGCAGCTTAGCTCTTCG
sp*speA*	Arginine decarboxylase	F:CAGGGCATGGATCCTACATT R:GCGCCACTTGTACCTTGAAT
yaaO		F:GAACCATTTCCGAAGTCAGC R:CCAAGGAAGCCATAATTGGA
arcA	Arginine decarboxylase	F:AATTCACCCAGCCATTCAAG R:CCATCATTCCATTGTTCACG
YvdD	Arginine deiminase	F: TACGTTGGTTCATGTGTTGG R:GGAAGCGCAATAAAAGCATC
SigB	Lysine decarboxylase	F:ACCTACAACGTTCACCGAGG R:CTAAAAATGCGGCTTGCTTC
DnaJ	the alternative sigma factor	F:CATGAATTCTTCGAGCGTGA R:AGCACGTTTAAGCTTTCCGA
DnaK	Heat shock chaperone	F:GCATTAGGTGCTGCAGTTCA R:GCTTGCTCTTTGCTTGTTCC
GroEL	Heat shock chaperone 60-kDa chaperonin	F:CGACGAAGCAACAGGTATCA R:TGGTTCTGGCTTGTCAGCTA
FadS	Faty Acid Desaturase	F:GGCGGGATTAATATCGATTG' R:AAGCTTTGGCCCTGTTGATA'

[a] F forward, R reverse.

To evaluate the reproducibility of the method, three independent RNA samples were analyzed similarly for three independent cultures that were performed at different pH (7.0 and 5.5). Samples were quantified

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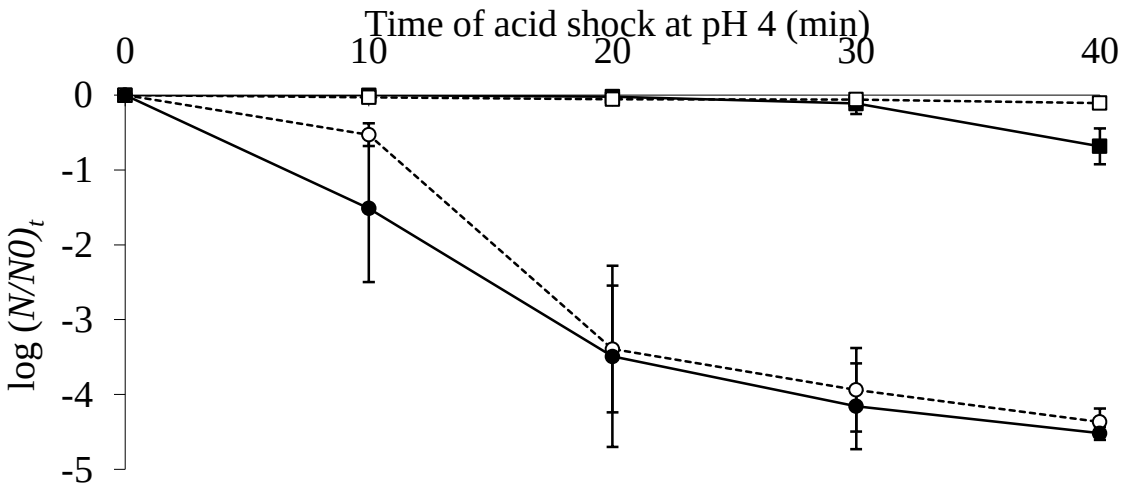
according to Light-Cycler Software version 3.5 (Roche Diagnostics, Le Meylan, France) and standardized for 16S rRNA. The mRNA level changes of each gene were normalized to the mRNA level of the unregulated gene encoding 16S RNA and quantified by using the mathematical model established by Pfaffl (Pfaffl, 2001). The standard deviation value was found to be approximately constant for the genes coded for defirents enzymes implicated in internal pH maintain (*atpB*, *speA*, *yaaO*, *arcA* and *yvdD*) and genes coded for heat shock proteins HSP (*dnaK*, *dnaJ*, *GroEL* and *Sig B*).

FIGURE LEGEND

Fig. 1 population reduction of *B. cereus* ATCC14579 cells subjected to acid shock at pH 4.0 (A) or to heat shock et 50°C (B). Steady-state cells were grown at a pH of 7.0 (●), and after pre-exposition at a non lethal temperature of 42°C during 15 min (○), or grown at pH 5.5(■) and after pre-exposition at a non lethal temperature of 42°C during 15 min (□), N_0 initial population, N population after exposure to acid shock at pH 4.0, log(N/N0) logarithm of population reduction during acid shock. Each point is the mean of 9 experimental data, three replicates of acid shock for each growth pH. Bars represent standard deviation between the 9 experimental data.

Figure 1 A



B

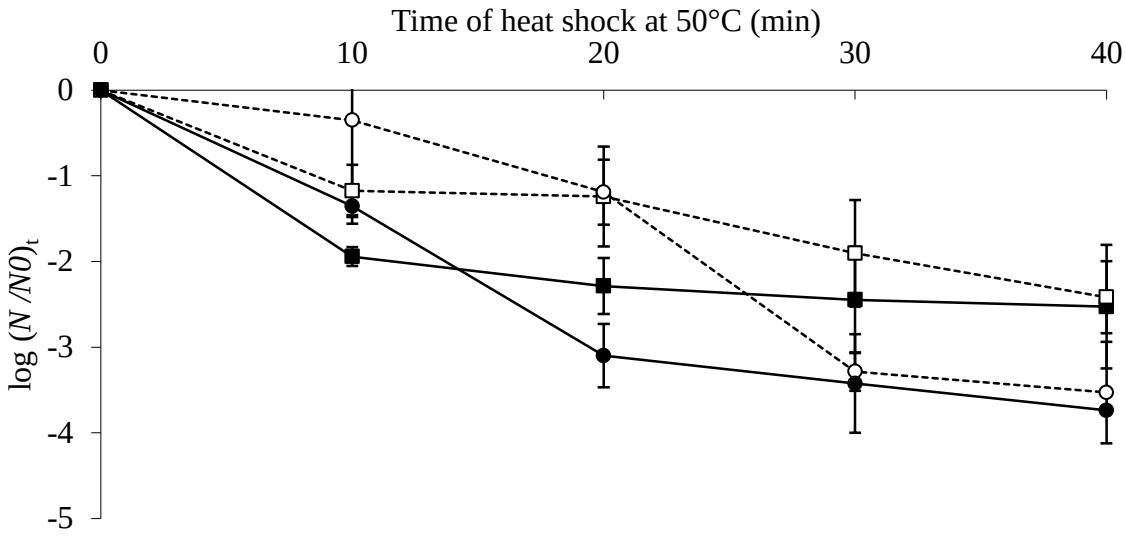


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Fig. 2 RT-PCR assays conducted on mRNA isolated in steady-state *B. cereus* un-adapted cells (grown at pH 7.0) (□) and acid-adapted cells (grown at pH 5.5) (■) grown in chemostat at a growth rate of 0.2 h^{-1}. The expression of genes (*atpB*, *speA*, *yaaO*, *arcA* and *yvdD*, *dnaK*, *dnaJ*, *GroEL* and *Sig B*) was measured (A), after 10 min of acid shock at pH 4.0 (B), after 15 min of pre-exposition at 42°C (C) and after 15 min of pre-exposition at 42°C followed by acid shock at pH 4.0 (D). Relative gene expression of *B. cereus* ATCC14579 cells grown at pH 7.0 was set at 1.0.

Figure 2

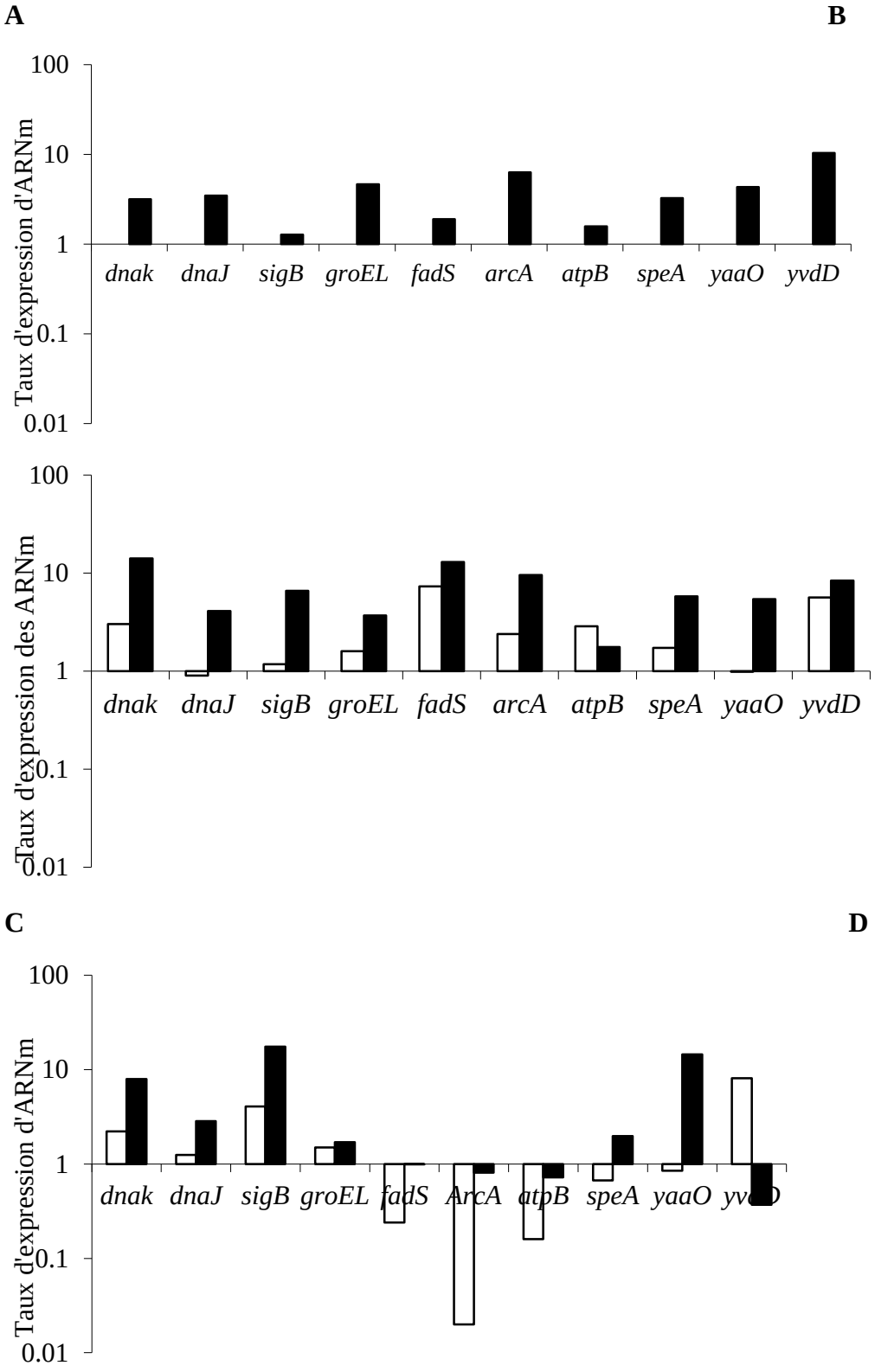

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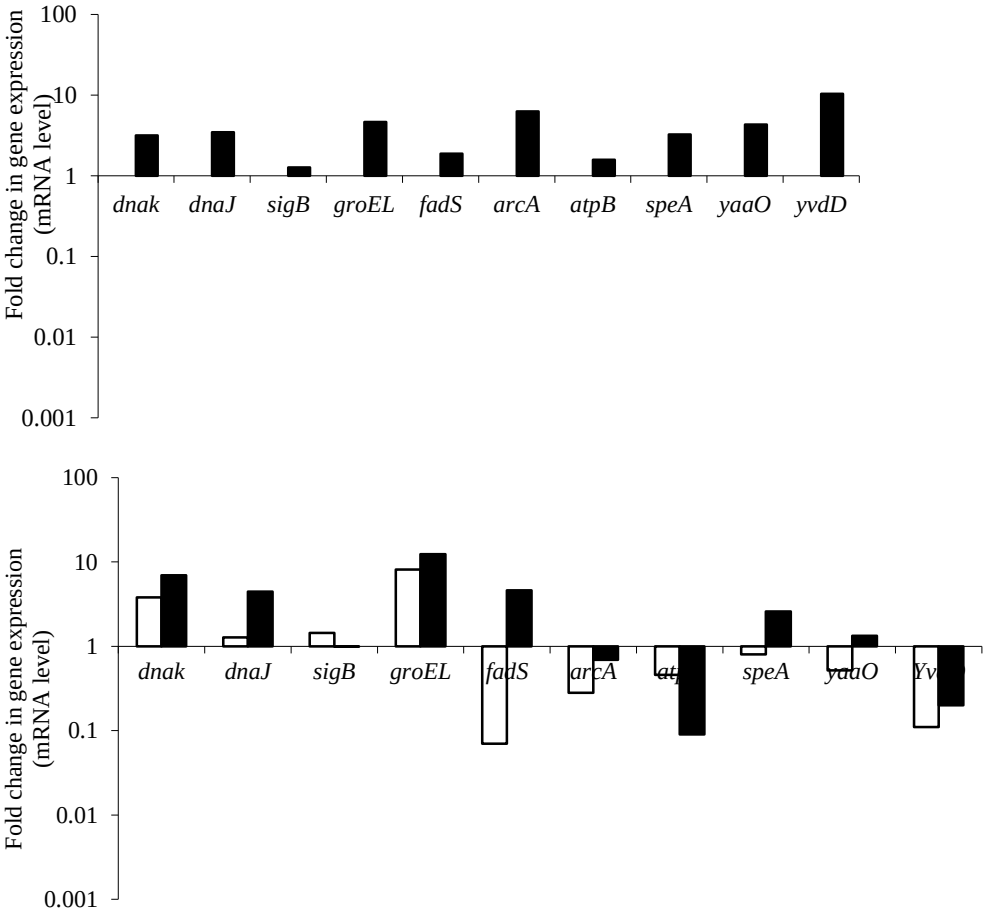
Fig. 3 RT-PCR assays conducted on mRNA isolated in steady-state *B. cereus* un-adapted cells (grown at pH 7.0) (□) and acid-adapted cells (grown at pH 5.5) (■) grown in chemostat at a growth rate of 0.2 h^{-1}. The expression of genes (*atpB*, *speA*, *yaaO*, *arcA* and *yvdD*, *dnaK*, *dnaJ*, *GroEL* and *Sig B*) was measured (A), after 10 min of heat shock at 50°C(B), after 15 min of pre-exposition at 42°C (C) and after 15 min of pre-exposition at 42°C followed by heat shock at 50°C (D). Relative gene expression of *B. cereus* ATCC14579 cells grown at pH 7.0 was set at 1.0.

Figure 3

A **B**



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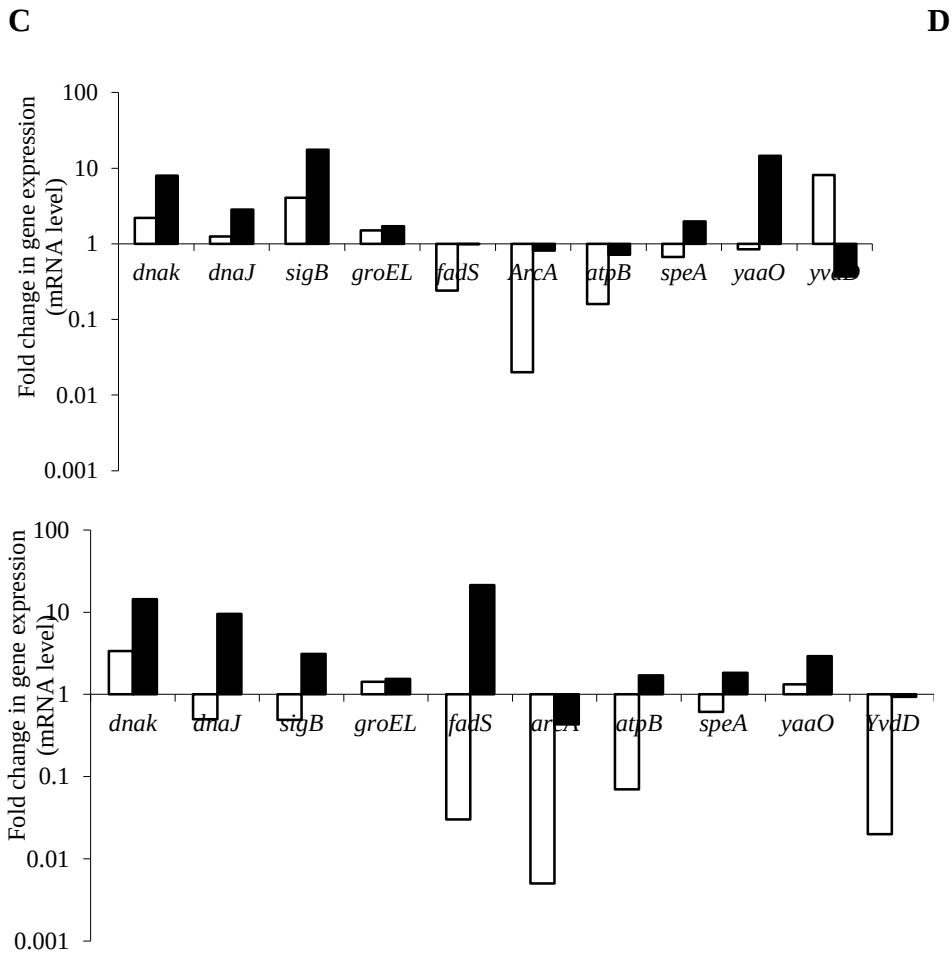
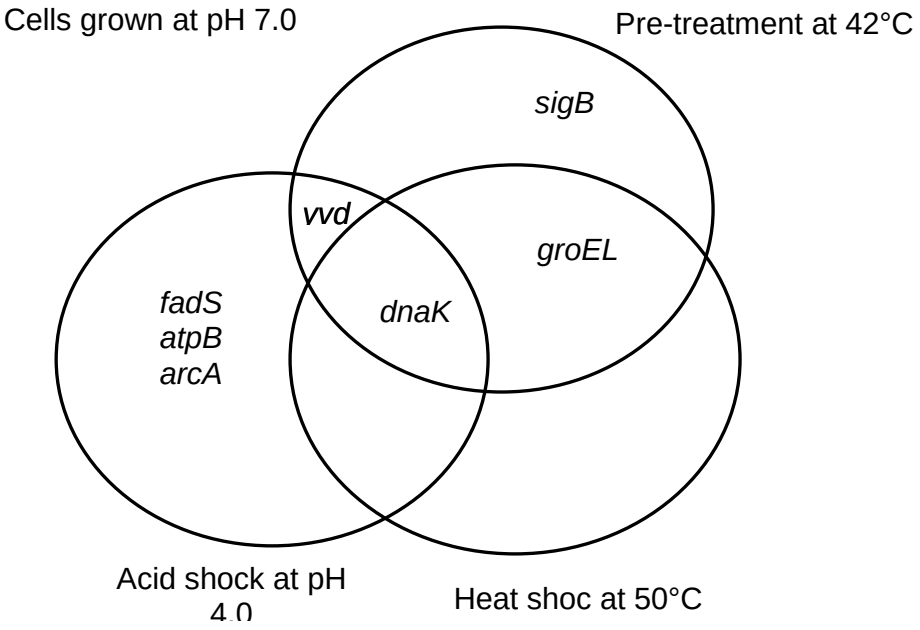


Fig. 4 Venn diagrams. A Venn diagram for *B. cereus* ATCC14579 strain grown at pH 7.0 (unadapted cells) (A), or grown at pH 5.5 (acid-adapted cells) (B).

Figure 4 A



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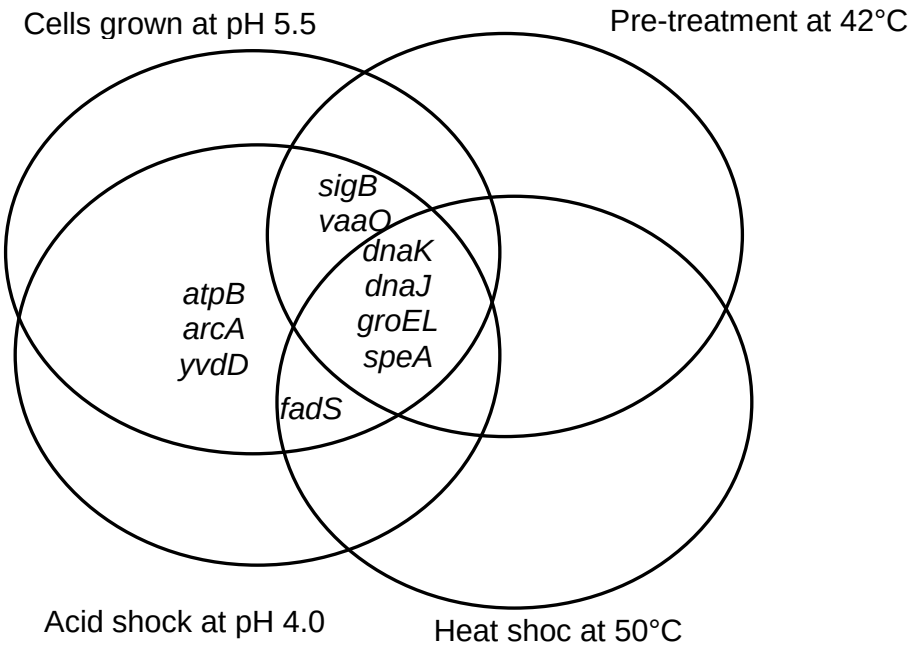
B



RESULTS AND DISCUSSION

Effect of medium pH and pre-adaptation on acidotolerance and thermotolerance

Our primary goal in this study was to determine the effect of adaptation of *B .cereus* in a non-lethal pH and temperature on the resistance at acid and heat stress. Bacteria have been known to survive otherwise lethal stress treatments when pre-exposed to mild levels of the same or different stress. This has serious implications for the food industry, the bacteria may survive the stresses imposed in them during food processing because of inappropriate processing storage conditions (Browne and Dowds, 2001).

Cells were grown in chemostat at medium pH (pHe) of 5.5 or 7.0 at growth rate of 0.2 h^{-1}. Steady-state *B. cereus* ATCC14579 cells were harvested and subjected (i) to acid shock treatment in JB at pH 4 from 10 to 40 min or (ii) to heat shock treatment at 50°C.

Likewise, the effect of the short pre-exposition of 15 min at a non-lethal temperature of 42°C on the acid and heat resistance has been measured.

Cell survival (log N/No) was determined during 10, 20 30 and 40 min of acid shock treatment (Fig. 1A) or during heat shock treatment (Fig 1B). In figure 1A we have shown that the loss in viability after 40 min of acid shock was about -0.7 and -4.36 log (N/No) for cells grown at pHe 5.5 and 7.0, respectively. In addition, for un-adapted cells or adapted-cells, about same effect on population reduction was observed between heat pre-treated cells or cells submitted to acid-shock.

This shows that *B. cereus* cells grown at low pH_e were able to adapt to acid shock and to induce a marked ATR. In addition, the pre-exposition to 42°C for 15 min shown significant resistance to acid shock after 40min in acid-adapted cells (pHe 5.5) compared to un-adapted cells (pHe 7.0). The cells was more strongly affected by growth pH, and varied from -0.11 at pH 5.5 to -4.51 at pH 7.

In Fig 1B, we shown that the population of un-adapted and acid-adapted cells to pH (pHe 7.0 and 5.5, respectively) decreased as the time of heat exposure (50°C) increased whatever the pHe.

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Moreover, the acid-adapted cells (pHe 5.5) were better resistance at heat-shock treatment (50°C) than the un-adapted cells (pH_e 7.0).

The cells of *B. cereus* were more resistance at acid stress by pre-exposure to a non-lethal temperature. The previous studies of the acid stress resistance on other strains of *B. cereus* cultivated at low pH. The results shown that the cells grown at low pH have the ability of adapted at acid stress, with the induction of the acid tolerance response ATR (Browne and Dowds, 2002); (Jobin et al., 2002), (Senouci-Rezkkalah et al., 2011; 2015).
Our study indicates that in *B. cereus* ATCC14579, the cells grown on chemostat at pH5.5 were better resistant at a choc acid at pH 4 against the cells grown at pH 7.

After 40 min of heat-shock, log (N/N_0) was –2.52 and –3.73 for pH_e 5.5 and 7.0, respectively.

The results above shown that acid-adapted cells were more acid-resistant and heat-resistant than un-adapted-cells. Moreover, a cross-shock between acid and heat stresses had been carried out. Cells grown at pHe 7.0 or pH 5.5 were submitted to heat-pretreatment (pre-incubated during 10 min à 42°C), then, heat shock (Fig 1B, broken line) was carried out.

Figure 1B shown that for un-adapted cells or un-adapted cells to pHe (pHe 5.5), about same effect on population reduction was observed between heat-pretreated cells and cells submitted to acid-shock. Adaptation of cells to pH 5.5 led to cell protection against lethal level of heat shock. However, cells were protected against the killing effect of pH 4 treatment by pre-treatment with the adaptative level of non-lethal temperature. It appears that the Cross-protection between stresses is observed when the pretreatment with low levels of one stress protects cultures against killing by high level of another stress. So, the thermo-tolerance depends on growth medium pH and pre-exposition to non-lethal temperature.

We observed that the induction of the ATR was correlated with a pH is also exist in *B. cereus*. These results confirm the ones obtained at our laboratory (Thomassin et al., 2006). *B. cereus* is also able to induce the response adaptive at the lethal temperature of 50°C. In fact; the thermo tolerance of cells is better after pre-adaptation at a non-lethal temperature of 42°C.

Other results has been obtained on other strains similar of *B. cereus*, present that the resistance at a heat choc at 50°C increase when the cells pre-exposed at a non-lethal T° at 43°C (Browne and Dowds, 2001) or 42°C (Periago et al., 2002). Furthermore, the pre-exposure at a non-lethal T° induce the acido-resistance and thermo-resistance. As well as, the cells grown at a low pH showed a better survival at the acid and heat choc.

So, we have looked that the survival and mechanisms of adaptation of *B. cereus* to acid stress, but also to heat stress. What is watch that the bacterium could not only adapt to acidic conditions when pre-exposed to the same stress but also following pre-exposure to sub lethal heat. What is more, the protection was achieved against heat stress by pre-exposure to acid. Thus, the cross protection between acid and heat choc exist in *B. cereus* ATCC14579. A cross-protection between acid and heat shock was demonstrated in *B. cereus* ATCC14579. Indeed, *B. cereus* was shown to induce a durable acid tolerance response and a marked heat-resistance when it was pre-exposed to sub-lethal acid condition, whereas pre-exposition to sub-lethal temperature conferred a short acid- or heat- protection.

Transcriptional analysis

From the above experiemnts, reverse transcription (RT)-PCR assays were caried out in order to determine the effect of growth pHe on genes involved in stress response (ATPase, arginine decaboxylase and arginine deiminase genes expression) (Table 2).

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Table 2 RT-PCR assays conducted on mRNA isolated in steady state cells adapted (grown at pH 5.5), un-adapted (grown at pH 7.0) cultivated in chemostat.

Growth pH		**pH 7**				**pH 5.5**			
Shock		**Acid**		**Heat**		**Acid shock**		**Heat shock at**	
Gene/Preadaptation		**shock at**		**shock at**		**et pH 4.0**		**50°C**	
at 42°C for 15 min		**pH 4.0**		**50°C**					
		-	+	-	+	-	+	-	+
Dnak (HSP)	10	1.0	2.2	1.0	2.2	3.16	7.95	3.16	7.95
	10	3.03	2.43	3.79	3.37	14.16	3.79	6.94	14.41
DnaJ (HSP)	10	1.0	0.95	1.0	0.92	3.46	2.84	3.46	2.84
	10	0.90	0.76	1.28	0.50	4.11	1.56	4.45	9.53
SigB (sigma		1.0	4.08	1.0	4.08	1.27	17.5	1.27	17.5
factor)	1	1.18	10.85	1.44	0.49	6.6	1.47	0.99	3.11
	0	1.0		1.0	1.5	0	1.7	4.63	1.7
GroEL (HSP)	1	1.6	1.5	8.12	1.42	4.63	0.81	12.32	1.53
	0		2.32		0.67	3.7	1.64		
	1	1.0	0.67	1.0	0.61	6.30	2.13	6.30	1.73
ArcA (Arginine	0	2.40	1.05	0.28	1.85	9.56	0.72	0.69	0.43
deiminase)	1	1.0	1.85	1.0	1.33	1.58	1.42	1.58	1.71
AtpB (ATPase)	0	2.88	0.84	0.46	8.12	1.76	1.97	0.9	1.97
	0	1.0	8.12	1.0	0.2	3.25	2.51	3.25	1.83
SpeA	1	1.73	3.19	0.80		5.81	14.53	2.60	14.53
and	0	1.0		1.0		4.32	2.14	4.32	2.91
YaaO (arginine		0.99		0.52		5.42	0.37	1.33	0.37
decarboxylase)		1.0		1.0		10.35	0.22	10.35	0.93
YvdD (lysine		5.65		0.11		8.38		0.2	
decarboxylse)									

mRNA quantification from preadaptation cells at 42°C for 15 min was realized before acid or heat shock. Relative gene expression of *B. cereus ATCC14579* cells grown at pH 7.0 was set at 1.0.

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AtpB transcripts showed an 1.5 fold increase in the acid adapted cells (grown at pH 5.5) compared to the un-adapted cells grown at pH 7.0 (Table 2). Arginine deiminase *(arcA)* and decarboxylase (speA and yaaO) genes were respectively 6-, 3- and 4-fold over-expressed in adapted *cells (grown at pH 5.5)* compared to un-adapted cells (grown at pH 7). Moreover, we have observed the over-expression of *YvdD, Dnak, DnaJ, SigB, GroEL* genes that reached to 5, 6,7,8 and 9 respectively. Thus transcription of ATPase, arginine decarboxylase and deiminase and chaperone proteins genes was induced by acid adaptation in *B. cereus*.

mRNA levels from the various experiments have been analysis using the Venn Diagrams.

As a summary of this part, we presented the results of gene expression in non-adapted cells after an acid and heat shock. The most expressed genes after an acid shock are *yvdD, dnaK, fadS, atpB and ArcA*, and after heat adaptation are *yvdD, dnaK, GroEL and sigB.* After heat shock alone *dnaK* and *GroEL* genes are over-expressed.

This suggests that the expression of *dnaK* gene is higher in non-adapted cells after an acid and heat shock, indicating the involvement of this stress protein acid in stress response heat in *B. cereus* cells. Similar results was shown an overproduction of DnaK protein after an acid shock in the strain NCIMB 11796 by Brown and Dowds, 2002 (Brown and Dowds, 2002). The results of gene expression in pre-adapted cells after heat and acid shock are presented on the venn diagram B. We observed that all genes studied are expressed before after an acid shock. Genes encoding *sigB, yaaO, dnaK, dnaJ, GorEL* and *speA* are most expressed after a heat adaptation and after heat shock with the over expression of gene encoding the desaturase enzymes *fadS* gene after heat shock. So, we can conclude the *dnaK, dnaJ, GroEL* and *speA* gene are over-expressed in the pre-adapted cells before and during an acid and heat shock. This suggests that the genes encoding stress proteins (chaperone proteins) are involved in the response to acid and heat stress in adapted cells.

CONCLUSION

It is clearly shown that cross-protection between the acid and heat shock involves common mechanisms in *B. cereus* cells. To identify other Systems responsible to acid and heat stress response, comparison and analysis of proteins by MALDI-TOF sequencing will precisely identify those who are involved in the response to acid and / or heat stress in *B. cereus* ATCC14579. The cellular role of these mechanisms (proteins) will finally be determined by the construction of targeted mutants deficient in the expression of these genes.

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