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Phenotypic Analysis of New Genes Appears to be Responsible for Acid and Cold Resistance in *Bacillus cereus* ATTC-14597

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A B S T R A C T

Food-borne toxin producing bacterium *Bacillus cereus* monitor the ability of the Food-borne opportunistic pathogen. *Bacillus cereus* to survive during minimal processing of food products conditions, allowed the cells to adapt to heat and acid stresses. Mild procession a cross-protection between acid and heat occurred in *B. cereus*, resulted survival in foods. The main objective of the study to determine the relationship between the response to cold and acidity, certain genes involved in the adaptation to cold may occur in the acid stress response in *B. cereus* ATCC14579. The effect of acidity and cold on the acid-sensitive and acid-resistant mutants screening in the first part and the mutants identified as sensitive to cold: 109H2 (Δ*CshA* mutant CshA RNA helicase), 111D1 (RZC3747) 108C12 (Δ*porB* mutant pyruvate synthase), 134G8 (Δ*mutS* mutant / *porB*) and 110H1h (Δ*yqeC* mutant, 6-phospho gluconate dehydrogenase) was studied. Finding of the study shown that growths at different pH_e 7.0; 5.0; 4.8; 4.7; 4,6 and 4, 5.5 were conducted on the wild type and mutants sensitive to cold stress. The growth of mutants 111D1, 108C12, 134G8, 110H1 is altered at low pH compared to the wild-type strain and mutants 109H2 which develop more rapidly. The study suggested that the growth of the cold-sensitive mutant 109H2 (Δ*CshA* mutant) is not affected by acidity compared to the other cold sensitive's mutants (111D1, 108C12, 134G8, 110H1). Thus, the *RZC3747*, *porB* (pyruvate synthase), *mutS* / *porB* and *yqeC* (6-phospho gluconate dehydrogenase) identifed as cols sensitive mutsnts conclud that these genes can be involved also in the growth of *B. cereus* in acidic medium.

Key words: *Bacillus cereus*, Chemostat, ATR, Thermotolerance, Cross-protection, RT-PCR, HS

Bacillus cereus is a Gram-positive, facultative anaerobe, endospore-forming bacterium major food-borne pathogenic bacteria (vomiting) and diarrheal syndromes. small-molecular-weight cyclic toxin, cereulide, while the diarrheal syndrome resulted enterotoxins, hemolysin BL (HBL), non-haemolytic enterotoxin (NHE) *B. cereus* can oppose and adjust to a corrosive situation when natural acids are included for safeguarding of the defiled food or/and during the travel of this food in the stomach. *B. cereus* vegetative cells are more corrosive delicate than spores which relies upon the development pH and on the kind of food. Warmth instigated thermotolerance has been concentrated in a few food pathogens, for example, Listeria monocytogenes (Mahakarnchanakul and Beuchat 1999) and *B. cereus* (Yura *et al*. 2000). To be sure, an expansion in thermotolerance at 50°C was seen after brooding under mellow warmth conditions (37 or 40°C for a few hours heat stun proteins (HSPs) (Gottesman *et al*. 1997) assume jobs in protein collapsing, get together, and fix and counteraction of accumulation under pressure and non-stress conditions. The chaperones and proteases act together to keep up quality control of cell proteins (Abee and Wouters 1999). For a few microbes, a few HSPs are likewise instigated under different anxieties conditions other than heat, for example, presentation to ethanol, corrosive, or oxidative pressure or during macrophage endurance, may bring about expanded thermotolerance (Browne and Dowds 2002).

So as to decide the impacts of stresses forced on bacterial contaminants during food preparing and treatment of bundling material were assessed on the food pathogen

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Bacillus cereus, and comprehend Bacillus cereus ATCC 14579.

To decide the connection between the reaction to cold and the acridity, attempted to checked whether certain qualities associated with the adjustment to cold may happen in the corrosive pressure reaction in B. cereus ATCC14579 (Ando and Nakamura 2006). The effect of acidity on the mutants that were identified as sensitive to cold: 109H2 (*CshA* mutant RNA helicase): This RNA helicase was first named YdbR in the genome of *B. cereus* ATCC 14579 by homology with *Bacillus subtilis* and then renamed *CshA* (Julien *et al*. 2010) *111D1* (*RZC3747*), *108C12* (*porB* mutant pyruvate synthase), 134G8 (*mutS* mutant / porB) and 110H1 (yqeC mutant, 6-phospho gluconate dehydrogenase) (Claus and Berkeley 2010) was studied. Growths microculture at different pHe 7.0; 5.0; 4.8; 4.7; 4,6 and 4, 5.5 were conducted on the wild type and mutants sensitive to cold.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Bacillus cereus strain ATCC14579 was gotten from the American Type Culture Collection. Development medium was J-Broth (JB) (5g l-1 pepton,15g l-1 yeast separate, 3g l-1 K2HPO4 and 15g l-1 agar for plate check J Agar) (Jacques and Lereclus 2000). The pH of the medium was changed in accordance with the ideal incentive before autoclaving for 20 min at 120°C, JB medium was checked for pH esteem after cleansing and enhanced with 2g l-1 frequency sanitized glucose. *B. cereus* from stock culture was sanitized on J Agar. One state was moved in an anaerobic jar containing 100 ml of JB at pH 7, the medium was sparged with sans oxygen nitrogen gas for 15mint wipe out oxygen. Development was completed at 34°C for 15 h with fomentation at 100 rpm.

Screening of mutant library at low pH

The main objective of this section is to better understand the adaptive properties of *B. cereus* under acidic conditions. For this purpose a bank of transposition mutants (mini Tn10) was created and screened at low pH in order to identify the genes involved in the adaptation to low pH. Random mutagenesis was used to create this library, which represents the entire genome of *B. cereus*. Indeed 5000 clones were selected as much as genes present in the genome of the bacterium. For its construction the vector pIC333 was used (Fig 1).

The Mini transposon Tn10 which conveys a pUC beginning of replication utilitarian in *E. coli* and which gives the microscopic organisms protection from spectinomycin; A quality that gives protection from erythromycin and a birthplace of practical replication in Gram-positive however thermosensitive microorganisms at 32°C (not useful at >32°C). A quality encoding the transposase which will permit the transposon Tn10 to transpose into the genome of *B. cereus*.

The strain of *B. cereus* utilized is the reference strain ATCC14579 in light of the fact that its genome has been sequenced. The plasmid is acquainted all together with interfere, after transposition, the genome of the bacterium and in this way to change the statement of at least one qualities engaged with its adjustment to corrosiveness.

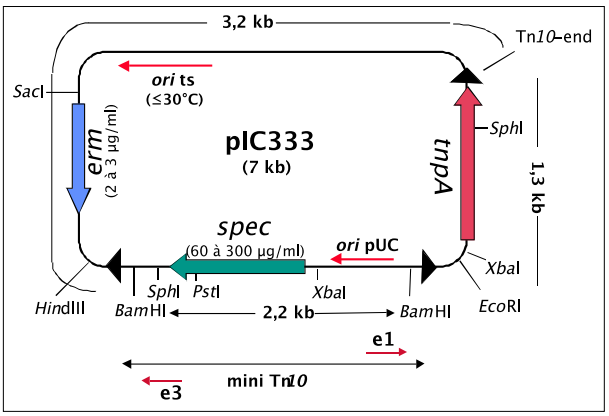

Fig 1 Physical guide of plasmid pIC333. [The dark pointed stones speak to the parts of the bargains transposon perceived by the TnpA transposase. The smaller than normal Tn10 has three unmistakable parts:

1. The starting point of replication of the plasmid pUC, practical in E. coli, and a quality giving protection from spectinomycin (70 µg/ml in E. coli and 300 µg/ml in B. cereus).
2. A quality giving protection from erythromycin (2 to 3 µg/ml) and a replication district, useful in Gram-positive microscopic organisms, yet thermosensitive from 32 ° C.
3. A quality encoding the TnpA transposase of the Tn10 transposon. The bolts assigned e1 and e3 schematize oligonucleotides situated at the parts of the bargains Tn10.

Limitation guide of plasmid pIC333. The dark pointed stones speak to the parts of the bargains transposon perceived by the TnpA transposase. The scaled down Tn10 has three particular parts: The bolts assigned e1 and e3 schematize oligonucleotides situated at the parts of the bargains Tn10].

pIC333 DNA readiness

PIC333 is fundamentally insecure in *E. coli*. Thus, the main activity is to change *B. subtilis* with an aliquot of pIC333; Select for protection from spectinomycin (60 (66) µg/ml consideration Sigma spectinomycin isn't unadulterated!) and erythromycin (1 to 2 µg/ml). Hatch at 28°C; Prepare plasmid DNA from a transformant clone and use it to change *E. coli* SCS 110. Pool the transformants, develop the cells (on 10 medium boxes) for just a couple of ages to forestall atomic improvements. Set up a supply of plasmid DNA. The cells are scratched and the Maxi-Quiagen is made straightforwardly on these microscopic organisms (the DNA is taken up in the water toward the end with the goal that it isn't important to dialyze the DNA before change).

Change of B. cereus by electroporation

As indicated by (Lehnik-Habrink *et al*. 2013). Electroporation of *B. cereus*. In Electroporation of microscopic organisms. Eynard and Teissie (Eds.) Springer Lab. Manual. For the development of the library, the plasmid pIC333 was set up in E. coli and afterward removed by a most extreme readiness of DNA before being brought

into the genome of *B. cereus* by electroporation. The plasmid duplicates a few ages at 28°C. and afterward societies of these transformants are completed at 40°C. so as to hold just the transposon and to expel the rest of the plasmid which is thermosensitive at 32°C. A confirmation step was completed in LB medium containing spectinomycin so as to check the nearness of the transposon miniTn10 in every one of the clones. Be that as it may, clones conveying the protection from erythromycin may exist, and emerge from a fractional disposal of the remainder of the plasmid. The 5000 clones are bundled in 96-well plates and afterward put away at -80°C.

Screening of the freak library under acidic conditions screening for corrosive touchy and corrosive safe

So as to recognize the qualities engaged with the low pH adjustment, the 5000 clones of freaks made in our research center were screened under corrosive conditions by a readiness of the 96-well plate societies in J-stock medium at various pH 4, 6 and 4.4 so as to acquire corrosive delicate clones which don't develop at pH 4.6 and corrosive safe which develop at pH 4.4 in the wake of confirming that the restricting pH of the development of *Bacillus cereus* ATCC14579 is pH 4.5.

Production of a corrosive touchy and corrosive safe sub-bank

Subsequent to having various corrosive touchy and corrosive safe freaks and so as to develop a library for a restricting number of corrosive delicate and corrosive safe freaks, a second screening piece of the freaks acquired during the main screen was made and a PH scope of 4.65 for corrosive touchy and 4.3 for corrosive safe.

Development of freaks chose in unregulated microplate group (Bioscreen)

The wild-type strain and freaks distinguished in our research center as cool delicate and screened as corrosive touchy and corrosive safe were utilized to examine the ramifications of the changed quality in low pH development. The wild-type strain and the freaks acquired on LB agar without ATB for the wild-type strain and with the spectinomycin at 275 µg/ml for the freaks are vaccinated into 10 ml of LB medium without for the wild and with the spectinomycin at 275 µg/ml for the freaks PH 7.0 at 34°C and 100 rpm for 16 h. 2 µl of each pre-culture are seeded in 200 µl of J-Broth medium at various pHe 7.0, 5.0, 4.8.4, 7.4, 6, 4.55, and 4.5 and hatched At 34°C. furthermore, consistent disturbance (Bioscreen, Termo-electron, Helsinki, Finland), and the optical thickness is estimated at 600 nm each 30 min.

RESULTS AND DISCUSSION

Screening of the B. cereus ATCC14579 mutant library at low pH

Screening results: In the wake of checking that the constraining development pH of *B. cereus* ATCC14579 is 4.5. We have looked to acquire corrosive delicate clones that don't develop at pH 4.6 and corrosive safe which create at pH 4.4. The screening of a freak library (developed by arbitrary mutagenesis of strain ATCC 14579) was in this way done under acidic conditions in J-stock medium at pH 4.6 and 4.4 and brooded at 34°C. for 48 hours. The development results were seen following 24 and 48 hours. From 5000 screened clones we got: 130 corrosive delicate freaks that are not created at pH 4.6 and 47 freaks corrosive safe which created at pH 4.4. A subsequent screening was completed to diminish the quantity of freaks got during the main screen. The development of these freaks was completed at pH 4.65 for corrosive delicate and at pH 4.3 for corrosive safe. This subsequent screen permitted us to choose 16 freaks; 9 Acido-delicate cells that don't create at pH 4.65: 106D11, 105H5, 108A5, 112D8, 113E6, 114C4, 142D12, 147E11, 148E12-7 corrosive safe strains which create at pH 4.3: 127A12, 126H2, 134D3, 105A1, 102A1, 103A1, 104H2.

Physiological investigation of corrosive delicate and corrosive safe freaks: The screening of the bank permitted us to segregate 9 freaks' corrosive delicate and 7 corrosives safe. The impact of corrosive culture pH on the development of these freaks was resolved so as to check whether the transformed quality in these freaks is engaged with the corrosive pressure reaction. Microplate societies of the wild strain of B. cereus ATCC14579 and 10 freaks (corrosive touchy and corrosive safe) were completed at pH 7.0, 5.0, 4.8, 4.7, 4, 6, 4, 55 and 4.5.

Physiological investigation of corrosive delicate freaks: The development consequences of the wild-type and corrosive delicate mutants (108A5, 105H5, 106D11, 112D8, 142D12) in microculture at various culture pHs (7.0, 5.0, 4.8, 4.7, 6 and 4, 55) are appeared in (Fig 2). Just the most striking outcomes are demonstrated on the grounds that at pH 7.0 and 5.0 there is no contrast between the wild-type strain and the freaks. At pH 4.8 and 4.7 we can see that the wild-type strain becomes most quickly with a dormant period of 3 hours contrasted with freaks 108A5, 105H5, 106D11, 112D8, 142D12 which build up somewhat more gradually, and whose idle stage shifts somewhere in the range of 4.5 and 8 hours relying upon the freak. At pH 4.7, two gatherings are recognized. The first involves the wild-type strain and the freaks 108A5 and 105H5 with an 8-hour inertness stage, the subsequent gathering contains the freaks 106D11, 112D8 and 142D12 with inactivity periods of 13, 17 and 19 hours separately.

For cells cultured at pH 4.6, only the wild-type strain develops after 20 hours and the mutants do not develop before 40 hours with a latent phase of about 38 to 42 hours depending on the mutants. When the mutants and the wild-type strain are cultured at pH 4.55, the wild-type strain develops after 20 hours, whereas mutants 105H5, 106D11, 112D8, 142D12 develop more slowly (latent phase 38 to 78 hours depending on mutants). Only mutant 108A5 did not develop at pH 4.55 after 100 hours. These results show that the mutants 105H5, 106D11, 112D8, 142D12 are sensitive to acidity and that mutant 108A5 is very sensitive to acidity. Mutant 108A5 is most affected by acidity, so the mutated

gene can be an essential gene for low pH growth and acid stress resistance.

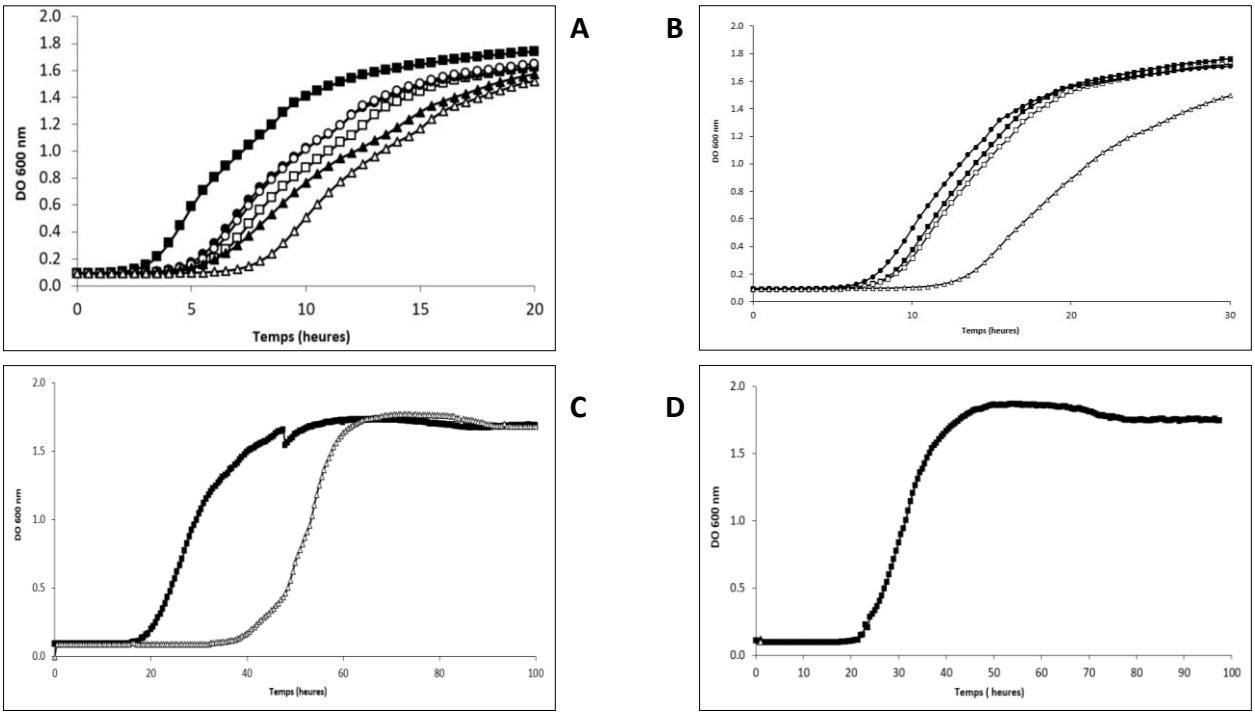

Fig 2 Growth of wild-type (CT) ATCC 14579 wild-type (WT) and mutated, cultured microculture cells at pH 4.8 (A), pH 4.7 (B), 4.6 (C) and 4.55 D): wild-type strain ATCC14579 (Δ), 108A5 (□), 105H5 (●), 106D11 (○), 112D8

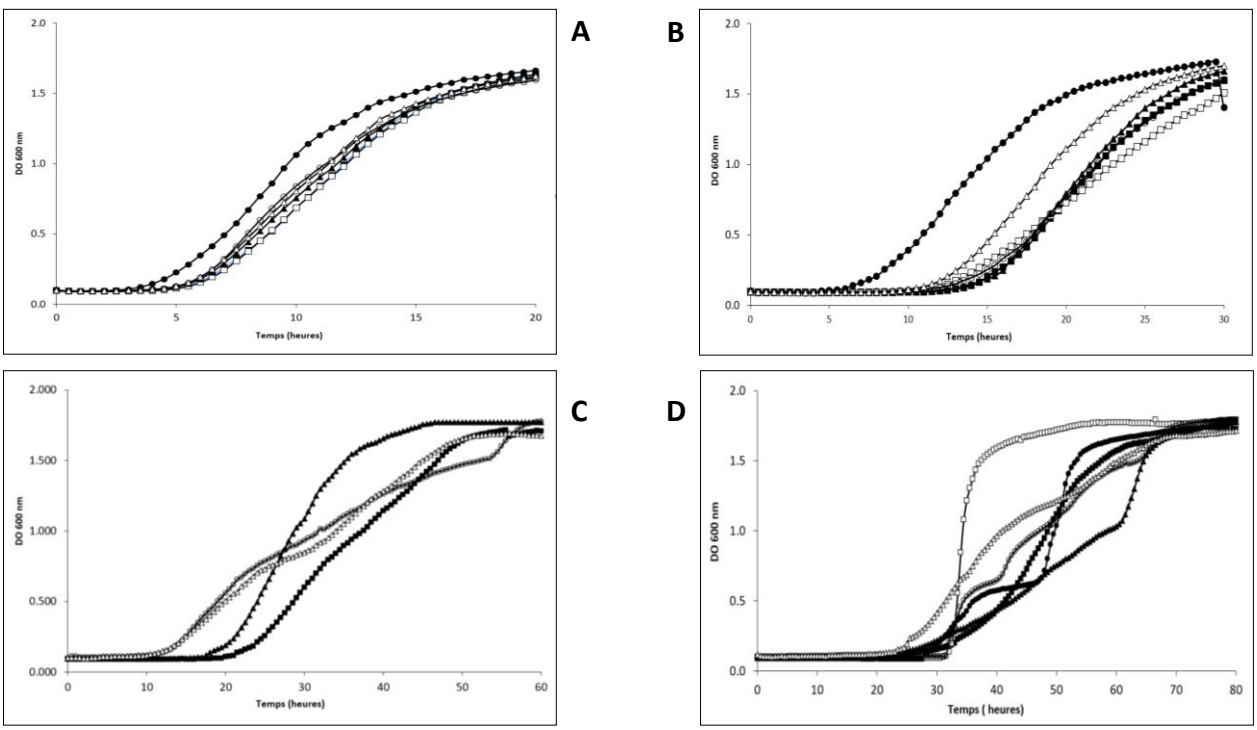

Fig 3 Growth of wild-type (CT) ATCC 14579 wild-type (WT) and mutated, cultured microculture cells at pH 4.8 (A), pH 4.6 (B), 4.55 (C) and 4.5 D): wild-type strain ATCC14579 (n), 127A12 (□), 126H2 (●), 134D3 (○), 105A1 (▲), 102A1 (Δ)

Physiological study of acid-resistant mutants: The acid-resistant mutants (127A12, 126H2, 134D3, 105A1, 102A1) were cultured in microculture at different culture pHs 7.0; 5.0; 4.8; 4.7; 4.6 and 4.55. The most striking results are shown in (Fig 3). At pH 7.0; 5.0 and 4.8 no difference was observed between the wild-type strain and the mutants. At

pH 4.6, mutant 126H2 develops more rapidly with respect to the wild-type strain and the other mutants 127A12, 134D3, 105A1, 102A1 with a latent phase of 15 hours. For the pH 4.55, the wild-type strain and the mutants 127A12, 126H2, 105A1 develop in the same way with a latent phase of 20 to 22 hours. The mutant 105A1 is characterized by a latency phase of 20 hours and a higher exponential phase with respect to the wild-type strain and the other mutants. The two mutants 134D3 and 102A1 grow faster than the wild-type strain and the other mutants with a latent phase of 13 hours.

On the other hand, when the mutants and the wild-type strain are cultured at pH 4.5, the wild-type strain and mutants 127A12, 126H2, 134D3, 105A1 develop after 29 to 32 hours depending on the mutants. The mutant 102A1 is the only one which develops a little more rapidly with a phase of latency of 25 hours. These results show that mutant 102A1 is the most resistant to acidity. The mutation of this gene can induce better growth at low pH.

Physiological study of some cold-sensitive mutants

In order to determine the relationship between cold and acidity response, we investigated whether certain genes involved in cold adaptation can intervene in the acid stress response in *B. cereus* ATCC14579. The effect of acidity on mutants that were identified in our (Ando and Nakamura 2006) as cold sensitive: 109H2 (mutant *CshA*, RNA helicase), 111D1 (RZC3747), 108C12 (mutant porB, pyruvate synthase), 134G8 / PorB) and 110H1 (mutant yqeC, 6-phospho gluconate dehydrogenase) was studied. Growth in microculture at different pHe 7.0; 5.0; 4.8; 4.7; 4.6 and 4.55 were conducted on the wild-type strain and the cold-sensitive mutants. The most striking results are shown in (Fig 4).

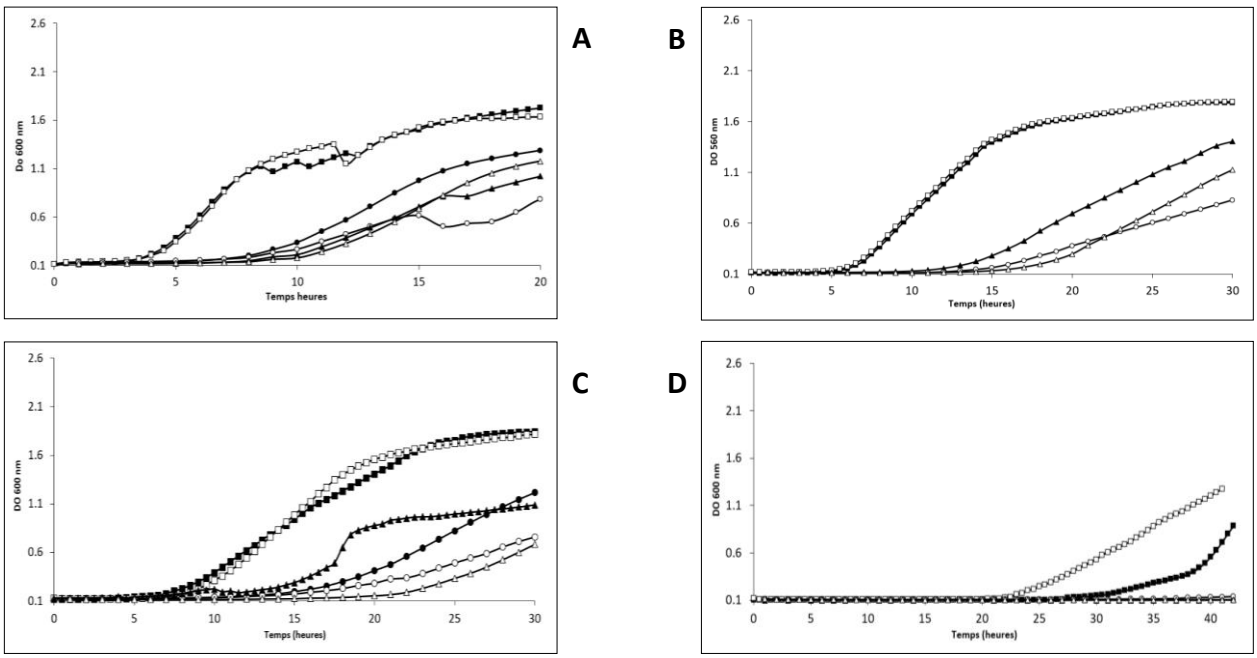

Fig 2 Growth of wild-type (CT) ATCC 14579 wild-type (WT) and mutated, cultured microculture cells at pH 4.8 (A), pH 4.7 (B), 4.6 (C) and 4.55 D): wild-type strain ATCC14579 (Δ), 108A5 (□), 105H5 (●), 106D11 (○), 112D8

At pH 7.0, no difference was observed between the wild-type strain and the mutants. At pH 4.8 and 4.7, we can see that the wild-type strain and the 109H2 mutant develop faster. Then we have a group consisting of mutants 111D1, 108C12, 134G8, 110H1 which develop a little more slowly. At pH 4.6, two groups are again distinguished: the first comprises the wild-type strain and the 109H2 mutant (CshA) with an 8-hour latent phase. The *B. cereus* CshA protein also showed high homology with CshA of *B. subtilis* (formerly named YdbR) and CsdA of *E. coli*. The *cshA* growth was also slightly impaired at 30°C but was similar to that of the WT at 37°C. Different results were reported concerning the *B. subtilis cshA* homologue: Hunger *et al*. (2006) reported that a single deletion of *cshA* or *cshB* gene did not result in a cold- specific growth defect and that a double deletion was lethal for *B. subtilis* (Hunger *et al*. 2006). Indeed, CshA is the major RNA helicase of *B. subtilis* leads to cold sensitive phynotype (Lehnik-Habrink *et al*. 2013). For *B. cereus*, deletion of *cshA*, *cshB* or *cshC* clearly led to growth defect at 10°C with a strong effect on cell morphology as revealed by both optical microscopic observations and analysis of bacterial structures on TEM. Cells of *cshA* were long and formed large aggregates as previously described for *E. coli csdA* (Jones *et al*. 1996).

The second group contains the mutants 111D1, 108C12, 134G8, 110H1 characterized by A latent phase of about 15 to 20 hours depending on the mutants. When the cells are cultured at pH 4.55, only the wild-type strain and the Δ*csdA* mutant develop after 30 hours and 20 hours, respectively. The other mutants did not develop after 40 hours. These results show that the cold-sensitive Δ*csdA* is unaffected by acidity and develops faster than the wild-type strain, the other mutants are sensitive to acidity. Previous results have been shown that the *CshA* RNA helicase may have a

stronger role in *B. cereus* temperature adaptation (Pandiani *et al*. 2010), and growth of this mutant at pH 5 show the impact of this gene to acid adaptation (Owttrim 2006). Contrary to our results of acid adaptation of this mutant DcshA at pH 4,7 to 4,5. Acid condition could modify mRNA and rRNA conformation, leading to a loss of functionality and consequently requiring the action of RNA helicase as chaperonnes (Pandiani *et al*. 2011). This suggests that *CshA* gene play an important role in the growth of *B. cereus* in acidic media.

The screening of a 5000 mutants library constructed in our laboratory (by random mutation by transposition of a mini Tn10 in the genomes of *B. cereus* ATCC14579) made it possible to isolate acid-sensitive mutants (which do not develop at pH 4.6) and acid-resistant (which develop at pH 4.4). Among these mutants, 5 acid-sensitive and acid-resistant 5 were chosen for a physiological study of growth at different culture pHs. The results of the growth showed an alteration in the growth of the acid-sensitive mutants at low pH. Thus, mutated genes in these mutants could be essential in low pH growth. For acid-resistant mutants, mutant 102A1 develops more rapidly at low pH 4.5 and 4.55 compared to other mutants and the wild-type strain. These results show that the mutation of these genes by transposition of the mini Tn10 affects the growth of these mutants at low pH or they develop more slowly compared to the wild-type strain for the acid-sensitive mutants and they develop more rapidly for the mutants Acid-resistant. Other results of growth of the wild-type strain and the mutants identified in our laboratory as cold sensitive 109H2 (YdbR), 111D1, 108C12, 134G8, 110H1 were obtained. The growth of mutants 111D1, 108C12, 134G8, 110H1 is altered at low pH with respect to the wild-type strain and mutants 109H2 which develop more rapidly. This suggests that the growth of the cold-sensitive mutant 109H2 (ydbR) is not affected by acidity compared to the other mutants (111D1, 108C12, 134G8, 110H1). Thus, the RZC3747, *porB* (pyruvate synthase), *mutS* / *porB* and *yqeC* (6-phospho gluconate dehydrogenase) genes can be involved in the growth of *B. cereus* in acidic medium.

In order to identify other genes that may be involved in growth in cold and acid conditions. This gene can be implicated in acid stress resistance in *B. cereus* ATCC 14579. The physiological approach of screening the mutant library will be deepened by an analysis of the mutated gene sequences in order to better understand the different modes of acid resistance in this bacterium. Next, stable mutant creation instead of transposition mutants will be required to study the role of each gene in acid stress response.

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