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► To cite this version:

Julien Brillard, Isabelle Jehanno, Claire Dargaignaratz, Isabelle Barbosa, Christian Ginies, et al.. Identification of *Bacillus cereus* genes specifically expressed during growth at low temperatures. *Applied and Environmental Microbiology*, 2010, 76 (8), pp.2562-2573. 10.1128/AEM.02348-09 . hal-01204237

HAL Id: hal-01204237

<https://hal.science/hal-01204237v1>

Submitted on 31 May 2020

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1 Identification of genes specifically expressed during *Bacillus cereus* growth at 2 low temperature

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18
19 Running title: Cold-specific *B. cereus* genes

1 Abstract

2 The mechanisms involved in the ability of *B. cereus* to multiply at low temperature were
3 investigated. It was assumed that many genes involved in cold acclimation would be up-
4 regulated at low temperature. Recombinase-based *in vivo* expression technology (IVET) was
5 adapted to the detection of the transient activation of *B. cereus* promoters during growth at
6 10 °C. Four independent screenings of a promoter library from type strain ATCC 14579 were
7 performed and 17 clones were isolated. They corresponded to 17 promoter regions that
8 displayed a reproducible elevated expression at 10 °C relative to 30 °C. This analysis revealed
9 several genes that may be important for *B. cereus* to grow successfully in the restrictive
10 conditions of cold habitats. Among them, a locus corresponding to open reading frames
11 BC5402 to BC5398 harbouring a lipase encoding gene and a putative transcriptional regulator
12 was identified three times. While a mutation in the putative regulator encoding gene did not
13 cause any particular phenotype, a mutant deficient for the lipase encoding gene showed
14 reduced growth abilities at low temperature compared with the parental strain. The mutant did
15 not change its fatty acid profiles in the same way as the wild-type when grown at 12 °C
16 instead of 37 °C. This study demonstrates the feasibility of a promoter trap strategy for
17 identifying cold induced genes. It outlines a first picture of the different processes involved in
18 *B. cereus* cold acclimation.

19

1 Introduction

2 The food-borne disease agent *Bacillus cereus* is an endospore-forming bacterium belonging to
3 the *Bacillus cereus* group (or *B. cereus sensu lato*). *B. cereus sensu lato* has recently been
4 divided into seven major phylogenetic groups (I-VII) with clearcut differences in temperature
5 growth ranges, suggesting that the genetic structure corresponds to "thermotypes" and
6 showing a multi-emergence of psychrotrophic groups within *B. cereus sensu lato* (26).
7 Temperature adaptation has thus presumably played a major role in *B. cereus* evolution.
8 *B. cereus* is also a human pathogen, causing local and systemic infections. Most cases of
9 outbreaks of foodborne poisoning have been caused by mesophilic strains (26) that can grow
10 at temperatures as low as 10 °C. These characteristics enable initially relatively low levels of
11 *B. cereus* in foods to increase greatly under commonly reported suboptimal refrigeration
12 conditions (20). Understanding the ability of *B. cereus* to grow at low temperature will help to
13 control multiplication in refrigerated food and prevent outbreaks of foodborne poisoning
14 events.

15 At low temperatures, bacteria undergo various modifications in cellular physiology, such as
16 decreased membrane fluidity or inefficient folding of proteins and secondary structures of
17 RNA and DNA (43). Bacterial responses can be divided into low temperature response (or
18 acclimation, also called low-temperature adaptation) and cold-shock response (43). Both
19 responses include a vast array of structural and physiological adjustments to cope with the
20 reduction in biochemical reaction rates induced by low temperatures. Previous studies of cold
21 response in bacteria mainly focused on cold shock response after a temperature drop (43, 51),
22 rather than on long-term adaptive response. Cold shock responses occur in many bacteria as a
23 change in the fatty acid profile of the bacterial cell membrane, to maintain optimal fluidity, or
24 in the expression of small RNA binding cold shock proteins (Csp) that mediate transcription
25 elongation and message stability, as investigated for instance in *Listeria monocytogenes* (12).

1 Studies on *B. subtilis*, reviewed by Weber *et al.* (51), account for most of the work on cold
2 adaptation in the *Bacillus* genus. Because of the psychrotrophy of many strains, *B. cereus*
3 *sensu lato* is a good model for research on the molecular mechanisms of low temperature
4 adaptation in *Bacillus* sp. and still offers wide scope for investigation. Previous work is scant
5 and piecemeal, dealing for instance with the analysis of cold-shock proteins (CSPs) (23) or
6 with changes in membrane fatty acid profile at suboptimal temperatures (21 °C or 15 °C) (29,
7 33). In fact, the establishment of the *B. cereus* in the food environment under refrigeration
8 probably requires the coordinated activity of many genes, whose identity and mode of action
9 are still largely unknown.

10 Assuming that many, if not most, of the genes involved in cold acclimation are induced or up-
11 regulated at low temperature, we hypothesised that ecologically significant genes allowing
12 *B. cereus* to survive or adapt to low temperature could be identified using a promoter trap
13 strategy to capture promoters specifically activated during growth at low temperature. We
14 therefore adapted a recombinase-based *in vivo* expression technology (IVET) to study the
15 genes activated in *B. cereus* ATCC 14579 during its growth at low temperature. The
16 advantages of the IVET approach over other genetic methods (screening of a mutant library
17 for instance) reside in its sensitivity, since it enables the detection of genes that are only
18 slightly or transiently induced, and in that it allows the selection of genes independently of
19 whether the loss of these sequences would be lethal. Thus IVET helps to recover both
20 essential and non-essential genes that contribute to the ecological success of *B. cereus* in the
21 conditions tested. Another advantage is that this approach allows the detection of promoters
22 expressed, even transiently, at any growth stage. Therefore it is well adapted to investigate
23 acclimation which may involve phenomena occurring from lag phase to the end of growth.

24 An IVET library constructed in the *B. cereus* type strain ATCC 14579 has already permitted
25 the identification of genes specifically expressed during virulence in insect larvae (22). In this

1 paper, we used this library and describe the application of IVET to detect promoters
2 specifically expressed, even transiently, during *B. cereus* growth at 10°C with the aim to gain
3 a better understanding of the mechanisms involved in its adaptive response to low
4 temperature.

6 **Materials & Methods**

7 **Strains and growth conditions**

8 All bacterial strains and plasmids used in this study are listed in Table 1. *B. cereus* cells were
9 grown aerobically in Luria broth (LB) or brain heart infusion (BHI) with vigorous shaking
10 (200 rpm) at 30 °C or 10 °C. *E. coli* cells were routinely grown in LB medium with shaking at
11 37 °C. When required, the antibiotic concentrations used for bacterial selection were:

12 erythromycin (Em) at 10 µg ml⁻¹, kanamycin (Km) at 100 µg ml⁻¹ or spectinomycin (Sp) at
13 275 µg ml⁻¹ for *B. cereus* and ampicillin at 100 µg ml⁻¹ for *E. coli*.

14 *E. coli* ET12567 *dam*⁻ *dcm*⁻ was used to generate unmethylated plasmid DNA for the
15 electrotransformation of *B. cereus*. *B. cereus* and *E. coli* strains were transformed by
16 electroporation as previously described (19, 36).

17 Growth experiments were performed with an automated turbidometer, the Microbiology
18 Bioscreen C Reader (Labsystems, Uxbridge, UK), in 100-well sterile microplates. A volume
19 of 0.1 ml of overnight cultures at 30 °C was inoculated in 10 ml fresh LB, and incubated at
20 30 °C with shaking until an OD_{600 nm} of 0.8 was obtained. These cultures were used to
21 inoculate 1 ml of fresh LB to reach a concentration of 6 × 10⁶ to 1 × 10⁷ colony-forming units
22 (CFU) per ml (depending on the experiments). Three replicate wells of the microplate were
23 filled with these dilutions of inoculated medium to a final volume of 300 µl per well. A
24 negative control was made of uninoculated LB broth. The cultures were incubated with
25 vigorous constant shaking and the OD₆₀₀ was measured at 15-min intervals at 30 °C and

1 45 °C or 1-h intervals at 12 °C over an incubation period of 48 h, or 10 days, respectively.
2 The 12°C temperature was used here instead of 10°C to avoid the flocculation of the cells
3 which sometimes occur in these culture conditions. At least three independent experiments
4 were performed for each growth condition.

5 DNA manipulation

6 Plasmid DNA was extracted from *B. cereus* and *E. coli* by a standard alkaline lysis procedure
7 using the Wizard SV miniprep purification system (Promega, Charbonnières, France), with an
8 additional incubation with lyzosome for the lysis of *B. cereus* cells as previously described
9 (9). Chromosomal DNA was extracted from *B. cereus* cells harvested in mid-log phase as
10 described previously (7). Restriction enzymes and T4 DNA ligase were used as recommended
11 by the manufacturer (Promega). Oligonucleotide primers (Table 2) were synthesised by
12 Eurogentec (Seraing, Belgium). PCR was performed in a GeneAmp PCR system 2400
13 thermal cycler (Perkin-Elmer, Coutaboeuf, France), using the Expand high-fidelity DNA
14 polymerase (Roche Applied Science, Meylan, France). Amplified DNA fragments were
15 purified using the PCR purification Kit (Roche) and separated on 0.7% agarose gels after
16 digestion. Digested DNA fragments were extracted from agarose gels with a centrifugal filter
17 device (Montage DNA gel extraction kit, Millipore, Molsheim, France). All constructions
18 were confirmed by DNA sequencing (GenomeExpress, Grenoble, France).

19 Screening of the IVET library

20 We have adapted a recombinase-based *in vivo* Expression Technology (IVET) to study the
21 genes activated in *B. cereus* ATCC 14579 during its growth at low temperature. In this IVET
22 system, constructed as previously described (22), *B. cereus* *Sau3A* chromosomal fragments
23 were fused to a promoterless resolvase *tnpI* gene from Tn 4430 harboured by a the promoter
24 trap-IVET vector pHT304-I. The reporter strain, named *B. cereus*::R2SK, carries a Km^R-res-
25 Spec^R-res-pKm cassette integrated in its chromosome at the *tetB* locus (R2SK construct).

1 This cassette carries two selectable resistance genes: a non-functional *aphA3* kanamycin
2 resistance gene (Km^R) and a spectinomycin resistance gene (Spec^R) flanked by two internal
3 resolvase recognition sequences (res). The coding sequence of the *aphA3* gene, is thus
4 separated from its promoter (pKm) by the res-Spc^R-res DNA fragment at which TnpI can
5 catalyses recombination (39). After introduction of the genomic library of chromosomal
6 fragments of *B. cereus* fused to *tnpI* in the promoter trap vector pHT304-I into *B.*
7 *cereus*::R2SK, the activation of a promoter cloned upstream of the *tnpI* gene would result in
8 TnpI production, and excision of the spectinomycin antibiotic marker from the chromosome.
9 This event also results in the restoration of a functional *aphA3* gene and marks the bacterium
10 by endowing it with an inheritable Spec-sensitive and Km-resistant phenotype. The IVET
11 library was screened as described below. Most of the promoters expressed at the reference
12 incubation temperature of 30 °C were removed using the following procedure: 1 ml of the
13 frozen library stock (22) was grown in 50 ml of BHI supplemented with spectinomycin (Sp)
14 in 250 ml flasks at 30 °C until an OD₆₀₀ = 1.0 was obtained. One percent of this culture was
15 used for inoculation of fresh BHI-Sp medium to increase the number of generations during
16 the exponential growth phase. This step was repeated four times to maximise the elimination
17 of the majority of cells for which a resolution event occurred at 30 °C (*i.e.* for which the
18 phenotype switched from Km-sensitive/Sp-resistant to Km-resistant /Sp-sensitive). Even after
19 these removal steps, 10 to 50 CFU/ml for which a resolution event had occurred (“resolution-
20 positive”) for a total population of 7×10^7 CFU/ml were usually found, corresponding to the
21 background level of this technique. The resulting depleted IVET library was frozen at -80°C
22 as 1 ml aliquots in glycerol to be used as frozen inoculum.
23 Four independent screenings at 10 °C were performed as follows: 1 ml of frozen inoculum
24 was used to inoculate 50 ml of BHI in 250 ml flasks and incubated at 10 °C with shaking. As
25 a control, an identically inoculated flask was grown at 30 °C. This step allowed the estimation

1 of the number of residual promoters activated under usual culture conditions that escaped the
2 pre-screening depletion step. During these controls, the background level of resolution-
3 positive CFU at 30 °C remained at 10-50 cfu/ml. Cultures were then diluted and spread either
4 on LB agar Em to quantify the total harvested bacterial population, or on LB agar Km to
5 isolate Km-resistant clones. Depending on the experiments, between 5×10^2 and 1×10^4
6 CFU/ml for which a resolution event had occurred were isolated from a total population
7 ranging from 5×10^7 to 9×10^7 CFU/ml. To confirm that the Km-resistant clones arose from
8 the resolution of the R2SK chromosomal cassette, selected Km-resistant clones were checked
9 for their spectinomycin sensitivity. At this stage, less than 10% of the clones were discarded.
10 To remove potential replicates of the same clone, the following procedure was used:
11 amplification of the DNA fragments cloned in pHT304-I was performed by a PCR on Km-
12 resistant /Sp-sensitive selected colonies, using the IVET-I1 and IVET-I2 primers (Table 2).
13 After electrophoresis, the clones displaying exactly the same migration profile on both 0.7%
14 and 1% agarose gels were considered to have exactly the same size and thus to be potential
15 copies of the same clone. Only one copy was kept and the others were discarded. In addition,
16 the clones for which no PCR amplification was obtained were removed. Altogether, about
17 25% of the clones were selected for further analysis during this step. The selected PCR
18 products were then purified and sequenced.
19 After DNA sequencing, two out of the remaining clones were removed: one contained
20 multiple DNA fragments originating from different chromosomal regions, and the other
21 contained an intragenic region (892 bp in the middle of a 4.3 kb open reading frame (ORF))
22 located more than 2.5 kb from the start codon of the next ORF. None of the remaining clones
23 contained an intergenic region in the wrong orientation. Three fragments identified from
24 clones isolated in three independent screenings had exactly the same sequence: only one clone
25 was kept for further analysis. Twenty clones containing an intergenic region in the correct

1 orientation, and thus likely to contain a promoter region, were finally selected and named cip
2 clones for "cold-induced promoters". For those 20 cip clones (Km-resistant /Sp-sensitive
3 phenotype), the pHT304-I derivative (*i.e.* containing a DNA fragment harbouring a promoter)
4 was extracted and reintroduced in the original *B. cereus*::R2SK strain (Km-sensitive/Sp-
5 resistant phenotype). Transformants were then grown at 30 °C and 10 °C in parallel to
6 determine the resolution frequencies at these two temperatures. Resolution frequencies were
7 determined as the ratio of numbers of Km-resistant cells to total cells (*i.e.* Em-resistant).
8 Populations of Km-resistant cells at 30 °C below the detection threshold (10 CFU/ml) were
9 arbitrarily fixed at 9 CFU/ml to have an excess estimate of the resolution frequency value.
10 Experiments were repeated twice and the mean values are presented. Clones ($n = 3$) for which
11 the resolution frequencies displayed less than 1 log difference between 30 °C and 10 °C in at
12 least one of the two experiments were discarded.

13 ***In silico* analysis**

14 Sequences were analysed using the BLAST server (NCBI, NIH) (3). Protein domains were
15 identified using SMART software (37, 42). MEME programme was used to align promoters
16 of all the cip-regions identified (6).

17 **RT-PCR experiments**

18 Total RNA was extracted from *B. cereus* ATCC 14579 (WT) cells grown at 10 °C in LB
19 broth at mid exponential phase ($OD_{600} = 0.7$), using the Tri-reagent RNA extraction solution
20 as recommended by the manufacturer (Ambion, Huntingdon, United Kingdom). cDNA
21 synthesis from 0.5 µg of total RNA was performed using AMV-RT polymerase (Titan-one-
22 tube RT-PCR kit, Roche). Specific amplifications were performed with primer couples listed
23 in Table 2 and the position of amplified regions is shown in Figure 1A. The "RT" prefix
24 designates these primers. For instance, RT-5402 -F and RT-5402 -R were used to detect the
25 mRNA transcript of the BC5402 gene; RT-5402-01-F and RT-5402-01-R were used to detect

1 the mRNA corresponding to a region overlapping BC5402 and BC5401. The reverse-
2 transcription step was followed by 30 cycles of PCR amplification with Expand-HighFidelity
3 polymerase following the manufacturer's instructions (Roche Diagnostics).

4 **Relative quantification of gene expression by real-time PCR**

5 Real-time RT-PCR was performed on a Light-Cycler instrument (Roche) as previously
6 described (9). Briefly, the QuantiFast SYBR Green RT-PCR kit (Qiagen) was used according
7 to the manufacturer's instructions, using 10 ng of total RNA as a template. RNAs were
8 extracted from cells grown at 10°C or 37°C and harvested during the middle of exponential
9 phase, at the end of exponential phase, or at the beginning of stationary phase ($OD_{600} = 0.4$,
10 1.0 and 2.0, respectively). Two independent cultures for each growth condition were
11 performed. For each RNA sample, at least two independent measurements were performed.
12 Altogether, presented results correspond to at least four measurements. The mRNA level
13 changes for each gene were normalized to the RNA level of the *ssu* gene encoding 16S RNA
14 and quantified by the $2^{-\Delta\Delta C_T}$ method as previously described (38). The coefficient of variation
15 of the ΔC_T values (where ΔC_T represents the differences in threshold cycle between the target
16 and control genes) was < 10 %. Oligonucleotides listed in Table 2 with the "LC" prefix were
17 used for Real-time PCR.

18 **Null mutant construction**

19 The BC5401 gene encoding a putative lipase, and the BC5402 gene encoding a putative
20 transcriptional regulator were interrupted in *B. cereus* ATCC 14579 by allelic exchange with
21 a cassette conferring kanamycin resistance (Km^r), as previously described (5). Briefly, DNA
22 fragments of BC5401 upstream and downstream regions were PCR-amplified using the
23 primer couples 5Up-01_Bam/3Up-01_Pst and 5Dn-01_Xba/3Dn-01_Eag, respectively (Table
24 2). Similarly, the upstream and downstream regions of BC5402 were PCR amplified with the
25 primer couples 5Up-02_Bam/3Up-02_Pst and 5Dn-02_Xba / 3Dn-02_Eag, respectively
26 (Table 2). PCR products were digested with *Bam*HI/*Pst*II and *Xba*I/*Eag*I using the primer-

1 incorporated restriction sites (Table 2). In parallel, the Km^r cassette (1.5 kb fragment
2 corresponding to the *aphA3* kanamycin resistance gene with its own promoter) was digested
3 from pHT304-Km (Table 1) with *PstI/XbaI*. pHT304-Km was obtained by cloning a 1.5 kb
4 fragment carrying the *aphA3* gene (Km^r) from the pDG783-*aphA3* plasmid (25). The three
5 digested DNA fragments were purified, and ligated in *EagI/BamHI* digested pRN5101 and
6 introduced by electroporation in *E. coli* ET 12567. Unmethylated plasmids were then
7 prepared from *E. coli* ET 12567 and the resulting recombinants plasmids pRN5101 Δ BC5401
8 and pRN5101 Δ BC5402 were transformed into *B. cereus* ATCC 14579. Transformants were
9 then subjected to allelic exchange as previously described (5). Colonies that were resistant to
10 Km and sensitive to Em arose through a double-crossover event in which the chromosomal
11 wild-type copies of BC5401 or BC5402 genes were deleted and replaced by the Km^r cassette.
12 The chromosomal allele exchange in the mutants was checked by PCR using the appropriate
13 primer couples (Km5out /5Up-01_Bam and Km3out /3Dn-01_Eag or Km5out /5Up-02_Bam
14 and Km3out /3Dn-02_Eag). PCR products were sequenced for confirmation.

15 **Extraction and quantification of fatty acid compounds by GC-MS**

16 The fatty acid (FA) profiles were determined from approximately 40 mg fresh weight of WT
17 or Δ BC5401 cells grown at 37 °C (overnight) or at 12 °C (for 21 days) on LB agar. Fatty acid
18 methyl esters (FAMES) were produced from total lipids by saponification (NaOH/methanol,
19 100 °C, 30 min.) coupled with esterification (HCl/methanol, 80 °C, 10 min, as previously
20 described (39, 40). Extraction of the FAMES by CH₂Cl₂ was followed by a washing step in a
21 0.1 M sodium hydrogencarbonate solution. Samples were then injected in a GC/MS
22 instrument (Shimadzu QP2010) equipped with an UBWAX column (length 30 m, diameter
23 0.25 mm, film thickness 0.5 μ m). Injection port temperature (splitless mode) was set at
24 250 °C. The carrier gas was helium with a linear velocity of 37 cm/s. The oven temperature
25 was held at 50 °C for 1 min, increased to 190 °C at a rate of 20°C/min, and further increased

1 to a final temperature of 230 °C at a rate of 2°C/min. For the MS, the ionisation source
2 temperature was 200 °C. The mass spectra were recorded in electron impact (70 eV) and the
3 acquisition of total ion current was between 50 and 350 u.m.a (2 scans/s). Identification of the
4 fatty acid compounds was determined by the equivalent chain length (ECL) method, and/or
5 by derivatisation methods (picolinyl derivates to determine the position of the ramifications,
6 and DMOX derivates to determine the position of unsaturations) (18, 21). The ECL of the
7 identified compounds are listed in Table 3. The area of the detected peaks (each peak
8 corresponds to distinct FA) was measured. The relative amount of one FA compound was
9 expressed as the area of each peak divided by the area sum of all FA detected (mean value +/-
10 SEM of triplicate biological samples measured twice). Significant differences in mean FA
11 peak area were determined by one-way ANOVA and Tukey's honest significance difference
12 test at the 1% level (SYSTAT version 9, SPSS, Chicago, USA). Only the most abundant FA
13 (peak area higher than 2% of the total peak area for at least one of the two strains grown at
14 either 37 °C or 12 °C) are presented.

16 **Results**

17 **Screening of transcriptional libraries for *cip* (cold induced promoters) genes**

18 Recombinase-based *in vivo* expression technology (IVET) was adapted to the investigation of
19 gene activation during low temperature growth of *B. cereus* ATCC 14579. From four
20 independent screenings performed at 10 °C, about 150 cold-induced promoter (*cip*) clones for
21 which a resolution event occurred and that switched from a Km-sensitive/Sp-resistant to a
22 Km-resistant/Sp-sensitive phenotype were isolated. After removal of potential replicates of
23 the same clone based on their insert size, or removal of a clone containing an intragenic
24 region, 20 distinct *cip* clones were finally selected.

25 **Determination of the induction profiles of the *cip* genes at 10 °C and 30 °C**

1 In the IVET system, the activity of a promoter is expressed by the frequency of resolution of
2 the R2SK cassette in the *B. cereus*::R2SK strain, leading to cells that switch from a Km-
3 sensitive/Sp-resistant to a Km-resistant/Sp-sensitive phenotype. To confirm the promoter
4 activity at low temperature of the inserts present in the 20 cip clones, their ability to induce
5 resolution in the *B. cereus*::R2SK strain was measured at 10 °C and 30 °C. For this purpose,
6 the originally isolated pHT304-I plasmids containing the *cip-tnpI* transcriptional fusions from
7 the 20 cip clones (Km-resistant/Sp-sensitive) were extracted, and reintroduced into the
8 unresolved *B. cereus*::R2SK strain (Km-sensitive/Sp-resistant). These transformants,
9 representing new clones of the 20 cip, were selected on media containing spectinomycin and
10 erythromycin. They were analysed again for resolution of their R2SK cassette, after growth in
11 LB medium at both 10 °C and 30 °C. The resolution frequencies induced by the 20 selected
12 cip were calculated and are presented in Fig. 2. The greater the relative differences in the
13 resolution frequencies obtained from clones grown at 10 °C and at 30 °C, the greater were the
14 differences in *cip* expression between the two growth temperatures.

15 Seventeen promoter regions were able to induce, reproducibly (*i.e.* in each of the two
16 independent experiments), the transcription of the *tnpI* gene at higher frequencies (at least 10-
17 fold) at 10 °C than at 30 °C. Three cip (*cip43*, *cip61* and *cip82*) induced resolution
18 frequencies with less than 1-log difference between the two growth temperatures and were
19 therefore excluded from further studies. For most of the cip-control clones ($n = 11$), no
20 expression of the promoter was detected at 30 °C as no Km-resistant colony arose from two
21 independent experiments performed with cells grown at 30 °C (*cip* marked with an asterisk in
22 Fig. 2). Consequently, resolution frequency was estimated by excess. For those 11 clones, this
23 high (and underestimated) difference in resolution frequencies strongly suggests that the *cip*
24 are specifically expressed during growth at 10 °C. For the remaining 6 cip, the higher (at least
25 10-fold) resolution frequencies they induced at 10 °C than at 30 °C suggest that these

1 promoters were expressed at a higher level at 10 °C than at 30 °C. Thus 17 selected promoters
2 in *B. cereus* ATCC 14579 seemed to be true cold-induced fusions.

3 **Analysis of DNA sequences fused to *tnpI* in the highly up-regulated cip clones**

4 Determination of the plasmid insert sizes of the remaining 17 chromosomal DNA fragments
5 (cip fragments) revealed a size range of 498 to 1, 931 bp, with an average size of 1, 070 bp
6 (Table 4). By comparing the nucleotide sequence with that of the genome of *B. cereus* ATCC
7 14579 and by considering the promoter orientation relative to the *tnpI* reporter gene, sequence
8 analysis of these 17 cip fragments inserted in the pHT304-I plasmids characterised 17
9 different putative promoter sequences, all in the 5'–3' orientation, able to allow the initiation
10 of the *tnpI* transcription. In all 17 cases, at least one putative promoter region and its
11 corresponding ORF was identified (Table 4). The corresponding genes of the 17 cip are
12 randomly located within the genome. According to their proposed COG (clusters of
13 orthologous groups) categories (44, 45), most of the identified genes (cip60, cip81, cip30,
14 cip13, cip47, cip7, cip18, cip54, cip22) encoded proteins involved in metabolism.
15 Interestingly, all metabolism COG categories were represented (energy production and
16 conversion, transport and metabolism of carbohydrate, amino acid, nucleotide, coenzyme,
17 lipid, and inorganic ion, and secondary metabolite biosynthesis, transport and catabolism).
18 Genes whose translation products are involved in information storage and processing (cip26,
19 cip39) and cellular processes and signalling (cip16, cip50, cip39) were also found. In
20 addition, three genes of unknown function (cip49, cip25, cip85) were identified. No highly
21 conserved motif was identified over the 17 promoter regions (data not shown). This strongly
22 suggests that not one but several transcriptional regulators may be implicated in the
23 expression of those promoters at low temperature.

24 **Analysis of three cip clones mapping at the same locus**

1 Among the 17 validated fusions, *cip5*, *cip22* and *cip26* sequences mapped at the same
2 chromosomal region (Table 4). Further study focused on this chromosomal region. All three
3 belonged to the 11 *cip* selected clones for which no resolution event occurred at 30 °C (Fig.
4 2). The downstream genes of *cip26*, *cip22* and *cip5* sequences are BC5402 and BC5401 and
5 BC5400, respectively. BC5402 encodes a putative transcriptional regulator, as revealed by the
6 HTH (helix-turn-helix) domain found in its N-terminal region (data not shown). BC5401
7 encodes a member of the GDSL-lipase family, as suggested by sequence homology with other
8 GDSL-lipases and by the "GDSL" (Gly-Asp-Ser-Leu) amino acid signature motif found at
9 position 63-66. Additional conserved amino acids (some of which were shown to play key
10 roles in the catalytic function of the enzyme) are present in the GDSL-lipase family proteins.
11 They were also found in the BC5401 predicted product (N at position 98, G at position 103
12 and 134, ND at position 136-137, D at position 242 and HP at position 245-246) (2). BC5400
13 was predicted to encode a Bacitracin transport ATP-binding protein BcrA. BlastN alignment
14 indicated that these BC5401 and BC5400 are found in strains of all *B. cereus* genetic groups,
15 while BC5402 in all but in the more thermotolerant strain NVH391-98 (data not shown).

16 **RT-PCR and characterisation of the *BC5402-BC5398* transcriptional unit**

17 The chromosomal locus of BC5402, BC5401 and BC5400 displays a putative operon
18 structure, including in the same orientation the two additional genes BC5399 and BC5398
19 (Fig. 1A). The five genes are also present with a conserved synteny in strains of all *B. cereus*
20 genetic groups, except for BC5402, which was absent in strain NVH391-98, as stated above.
21 Using RNA extracted from cells grown at 10 °C in exponential phase ($OD_{600} = 0.7$), RT-PCR
22 experiments revealed the presence of mRNA molecules overlapping the adjacent genes
23 BC5402/BC5401, BC5401/BC5400, BC5400/BC5399 and BC5399/BC5398 (amplicons b, d,
24 f and g on Fig. 1A and Fig. 1B), suggesting that the five genes of the locus were co-
25 transcribed. An invert-repeat with an energy level of -18.9 kcal/mol was found downstream

1 of BC5398 and may account for termination of transcription (Fig. 1A). Interestingly,
2 transcription of the BC5401 gene can be initiated both by the promoter upstream from
3 BC5402 when the two genes BC5402 and BC5401 are co-transcribed (as revealed by the
4 detected mRNA overlapping BC5402 and BC5401 using RT-PCR), and by its own promoter,
5 as revealed by the *cip22* identified fragment. Similarly, transcription of the BC5400 gene can
6 be initiated either by the promoter upstream from BC5402, or by the promoter upstream from
7 BC5401 (as revealed by the detected mRNA overlapping (i) BC5402 and BC5401 and (ii)
8 BC5401 and BC5400), and also by its own promoter, as revealed by the *cip5* identified
9 fragment. Thus the identified promoters located in this five-gene operon (BC5402 to BC5398)
10 may also be responsible for the transcription of a four-gene operon (BC5401 to BC5398) or a
11 three-gene operon (BC5400 to BC5398). The selection of three promoters in the same operon
12 after the random screening with the IVET system at 10 °C suggests that an important role is
13 played by this locus during *B. cereus* growth at low temperature.

14 **Quantification of the expression of the BC5401 and BC5402 genes**

15 The transcription level of the first two genes of this operon was analysed by quantitative RT-
16 PCR at various time during the kinetics of growth. Results presented in Table 5 confirmed
17 that the level of expression of the studied genes was significantly higher (>2-fold change)
18 when cells were grown at 10°C relative to 37°C. This was true at the three tested times of the
19 kinetics of growth for BC5402, while it was only observed during exponential phase of
20 growth for BC5401.

21 **Construction and characterisation of the BC5401 and BC5402 knockout mutants**

22 The first two genes of this operon specifically expressed at low temperature were selected for
23 mutagenesis to further investigate their contribution to *B. cereus* growth at low temperature.
24 Isogenic mutants of the *BC5402* and *BC5401* genes of *B. cereus* ATCC 14579 were
25 constructed using pRN5101 for insertional inactivation via double-crossover integration. The

1 two knockout strains (Δ BC5402 and Δ BC5401) and the WT strain were analysed for their
2 ability to grow at low temperature in comparison with 30 °C and 45 °C. The growth of
3 Δ BC5402 at 12 °C was similar to that of WT (data not shown). By contrast, the Δ BC5401
4 displayed an impaired growth ability at the three tested temperatures, only slightly at 30 °C
5 and 45 °C, but particularly clearly at 12 °C (Fig. 3). At this low temperature, a growth delay
6 was observed from the beginning of the exponential phase. A final OD₆₀₀ lower than that of
7 WT was also observed. By contrast, at 30 °C and 45 °C, growth of Δ BC5401 was only
8 slightly impaired at the end of the exponential phase, reaching the stationary phase with a
9 final OD₆₀₀ slightly lower (at 30 °C) or similar (at 45 °C) to that of the WT.

10 **Quantitative analysis of fatty acids in the BC5401 mutant and wild type strains**

11 Disruption of one of the identified cold-expressed genes (BC5401) encoding a putative lipase
12 led to a cold-impaired growth phenotype. We therefore determined the fatty acid (FA) profile
13 of both the WT and Δ BC5401 cells grown at either optimal or low temperature (37 °C and
14 12 °C, respectively) (Fig. 4). At 37 °C only slight differences were observed between the WT
15 and the Δ BC5401 strains. The only significant differences ($P < 0.01$, Tukey's HSD test), were
16 slightly lower amounts of two compounds (i16:1(2) and a17) in the mutant.
17 Several changes in the FA profiles of WT grown at 12 °C instead of 37 °C were observed. FA
18 of cells grown 12 °C changed particularly by a decreased amount of i15 and i17:1(2), and an
19 increased amount of i13, n16, i17, i17:1(1) and n18, (differences between the two
20 temperatures significant at $P < 0.01$). Three compounds (C16:1 n-11, C18:1 and C18:2) were
21 specifically found in cells grown at 12 °C. The proportion of unsaturated/saturated FA
22 increased for cells grown at low temperature: the ratio was 0.24 for cells grown at 37 °C and
23 0.32 for cells grown at 12 °C.
24 Similar changes to that in WT were observed for the Δ BC5401 strain grown at 12 °C instead
25 of 37 °C, with one major exception. The amount of i13 was not significantly different

1 between the two growth temperatures ($P > 0.01$) for the $\Delta BC5401$ strain. In addition, this
2 mutant displayed some differences ($P < 0.01$) between the two growth temperatures for i14
3 and C16:1 n-6 compounds that were not observed for WT. The ratio of unsaturated to
4 saturated FA also increased when this strain was grown at low temperature (0.21 and 0.47 for
5 cells grown at 37 °C and 12 °C, respectively). Thus during growth at low temperature, a
6 major increase of i13 was found for the WT which was not observed for the BC5401 mutant.

8 Discussion

9 Molecular microbial ecology is often hampered by the difficulty of unravelling how the
10 environment shapes bacterial physiology and enables microorganisms to respond to
11 environmental stresses. In the aim to identify genes specifically expressed at 10°C and thus
12 putatively involved in *B. cereus* low-temperature adaptation, we used an IVET approach in *B.*
13 *cereus* and identified 17 clones with elevated expression at 10°C relative to 30°C, which
14 demonstrated induced expression during growth at low temperature. To our knowledge, this is
15 the first study that demonstrates the feasibility of this approach for identifying cold-induced
16 promoters (cip) or genes in a foodborne pathogen. The screening was performed on cells
17 undergoing cold acclimation, using an approach allowing the identification of genes
18 specifically expressed (even transiently) during cold growth, at any time during the lag phase
19 or the exponential growth phase. In comparison, a conventional microarray analysis might
20 have the limitation that not all stages of the kinetics of growth would be sampled, thus
21 possibly leading to fail to isolate transiently expressed genes. The 17 cip clones could be
22 functionally divided into four categories based on the COG identity of the annotated
23 downstream gene of the captured DNA in each clone. Although most of the promoters
24 identified here (11 out of 17) were specifically expressed at low temperature, six of the
25 identified promoters were still expressed at 30 °C. In this case, the promoters were expressed

1 at a much lower level (CFU for which a resolution event had occurred were isolated at least
2 10 times less frequently at 30 °C than at 10 °C). This suggests that our growth steps at 30 °C
3 did not remove all promoters expressed at a low level. The list of genes identified here is of
4 course not exhaustive, as illustrated by a low overlap of identified clones between the four
5 independent screenings. This may be due to the relatively high stringency conditions we used
6 during the screening steps. In addition, the probable multifactorial nature of the entire cold
7 adaptation process implies that it is not governed by a few essential activated genes, but rather
8 by a large number of genes. Thus many additional genes specifically expressed at 10 °C
9 probably remain to be discovered. However, the genes identified in this screening enable us to
10 outline a first picture of the different strategies *B. cereus* uses to grow at low temperature.

11
12 One strategy may be to activate alternative metabolic pathways. Preferential metabolic
13 pathways are modified during growth at low temperature, in *B. subtilis* (32) or in *B. cereus*
14 (13, 14). Most of the genes identified in this study seem to be involved in various metabolic
15 reactions. Among them, BC0749 is predicted to code for ThiG, a protein required for the
16 biosynthesis of the thiazole moiety of thiamine (vitamin B1). Derived from thiamine, the
17 thiamin-pyrophosphate is an essential cofactor involved in central metabolism and amino acid
18 biosynthesis (35). In *B. cereus* ATCC 14579, the expression of this gene is under the control
19 (though probably indirect) of the Sigma B (σ^B) general stress response adaptation regulator
20 (48). However, no other members of the σ^B -regulon have been identified during this study, in
21 contrast to the observed induction of several σ^B -regulated genes during cold growth in *B.*
22 *subtilis* (8, 10) and in the foodborne pathogen *Listeria monocytogenes* (11). This indicates
23 that distinct roles may be played by σ^B between *B. cereus* and these other two bacterial
24 species during growth at low temperature. This hypothesis is in agreement with a recent study

1 showing that the σ^B activation pathway is highly different in the *B. cereus* group compared to
2 what is known in other low-GC Gram-positive bacteria (17).

3 Low-temperature adaptation often requires genes under the control of regulators able to sense
4 and coordinate metabolic functions during cold stress. For example, in *B. subtilis* the
5 expression of cold inducible genes responsible for membrane unsaturation is controlled by
6 DesKR, a specific two-component systems (1). Among the genes identified, one gene
7 (BC5440) was found to encode a protein of a two-component regulatory system that may
8 function to sense and perceive the environment more precisely and respond to it. The BC5440
9 gene product does not belong to the same response-regulator family as the *B. subtilis* DesR
10 (16). BC5440 encodes a response regulator probably involved in the regulation of enzymes
11 with murein hydrolase activity (LytR/AlgR family) (24). The expression of such enzymes
12 may have to be tightly controlled to allow optimal peptidoglycan turnover when bacteria are
13 grown at low temperature.

14 Modifications in the cell wall and/or membrane composition are also important for bacteria to
15 adapt to cold (e.g., changes in the ratio of saturated to unsaturated fatty acids) (29). Induced
16 expression of the products of some of the identified genes (BC1235, BC2489, BC4851),
17 presumably targets precursors for fatty acid metabolism. Their role in the fatty acid
18 metabolism might account for the modifications to the fatty acid profiles observed when cells
19 are grown at low temperature. This hypothesis is supported by the identification in *B. subtilis*
20 of an ortholog of BC4851, encoding an *o*-succinylbenzoic acid-CoA ligase, which was also
21 induced during cold growth (10).

22 One of the identified genes (BC3197) encodes a putative permease of the major facilitator
23 superfamily (MFS domain). This family includes transporters of small solutes (15, 34), and
24 some also act as osmoprotectors. In *L. monocytogenes* osmoprotectants permit better growth
25 at low temperatures (52). In addition, several of the identified genes were predicted to encode

1 hypothetical proteins (BC4246, BC4634 and BC4943). A large number of genes with
2 unknown functions have also been shown to be induced during growth at low temperature in
3 *B. subtilis* (10).

4 Finally, three promoters from the same locus (BC5402 to BC5398) were identified from
5 distinct screenings. No detection of resolution events in the three corresponding control-cip
6 clones during growth at 30 °C suggests a specific expression of this locus when *B. cereus* was
7 grown at 10 °C. The BC5402 gene was predicted to encode a regulator of the LacI family.
8 Five regulators of this family have been studied in *Erwinia* and are involved in repression of
9 adjacent genes (47). Gene BC5402 is the first of five genes occurring in an operon strongly
10 expressed at low temperature. However, the BC5402 gene, when mutated singly, does not
11 affect the growth of *B. cereus* at low temperature. This seems to indicate that this gene is not
12 coding for an essential function by itself; however, we cannot assert that this gene therefore
13 plays no role or only a very minor one in the acclimation of the bacterium to cold or in the
14 regulation of adjacent genes. Many traits contributing to ecological performance result in only
15 subtle or difficult-to-score phenotypic changes upon inactivation, and are likely to be
16 overlooked. Moreover, it is more and more frequently accepted that many regulators act in a
17 partially or fully redundant manner and so mutation of one such gene is unlikely to fully
18 abolish the activity of all the genes under its control. In the ATCC 14579 genome, two genes
19 encoding a putative lipase/acylhydrolase member of the GDSL family are located in operon
20 structures that include a putative transcriptional regulator-encoding gene. These are
21 BC5401/BC5402 and BC4123/4124. A third gene (BC2449) also displayed sequence
22 homology with BC5401, but the GDSL motif was degenerated as GDSF, and this gene was
23 not adjacent to a gene encoding a putative transcriptional regulator. These genes may have
24 similar functions, and we cannot rule out the possibility that disruption of BC5402 is

1 complemented by the expression of its paralog. A search for other possible redundant factors
2 should accordingly be pursued.

3 By contrast to the BC5402 deletion mutant, the BC5401 deletion mutant was shown to be
4 significantly impaired in its ability to grow at low-temperature. This indicates that in
5 Δ BC5402, transcription of the BC5401, encoding a member of the GDSL-lipase family, and
6 possibly of the three remaining genes located downstream BC5401 could still proceed
7 adequately, probably from the promoter found upstream from BC5401. Despite the cold-
8 induced IVET-identified promoter upstream from BC5400, a possible polar effect affecting
9 the downstream genes in the BC5401 deletion mutant cannot be discounted. Thus the possible
10 role of BC5400, BC5399 and BC5398 during growth at low temperature remains to be
11 clarified.

12 Although widely distributed in bacteria, the lipases of the GDSL family have been described
13 mostly in plants. Expression of GDSL-like lipase was up-regulated in rice overexpressing a
14 gene involved in various types of stress tolerance, including cold stress (30). In bacteria,
15 lipases of this family are generally described as lipolytic enzymes, but their physiological role
16 is not well understood (2). Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) generally
17 govern the turnover of lipids and the biogenesis of membranes in bacteria and are by
18 definition carboxylesterases, whose major substrates are long-chain triacylglycerols (\geq C₁₀),
19 releasing fatty acids and glycerol (31). They generally act at the water-lipid interface and
20 display wide substrate specificity, a property that lends the microorganisms access to diverse
21 carbon sources during plant cell wall degradation or during the recycling of lipid-containing
22 nutrients (27, 28). It has also been suggested that some of these enzymes might play a role in
23 the turnover of membrane lipids and lipid-anchored proteins, by altering cell membrane
24 composition to change cell membrane functions or adapt cell membranes to environmental
25 changes (41, 46). The main factor controlling the expression of lipase activity is therefore the

1 presence of lipid sources, although lipase production is also significantly influenced by other
2 carbon sources such as sugars, polysaccharides, amino acids and other complex compounds.
3 In addition, lipase synthesis is influenced by other physicochemical factors such as
4 temperature, pH, inorganic salts, agitation, and oxygen concentration (28). In *B. cereus*, the
5 role of BC5401 in the FA profile of *B. cereus* was investigated. As already described in many
6 other bacteria and in other *B. cereus* strains (29), both the ATCC 14579 WT strain and the
7 BC5401 mutant can adapt the fluidity of their membranes at lower growth temperature by
8 increasing the proportion of unsaturated fatty acids. Interestingly, compared with growth at
9 37 °C, the growth at low temperature led to a major increase in i13 for the WT. This is a 13-
10 carbon chain fatty acid branched at position $n-2$. No such increase was observed for the
11 BC5401 mutant. Although the role of this fatty acid compound at low temperature is not
12 known, a similar increase was observed in *Shewanella piezotolerans* grown at 4 °C versus
13 20 °C (50). However, the exact role of the BC5401 product gene on i13 biosynthesis during
14 bacterial growth at low temperature remains to be investigated.

15
16 In conclusion, we have shown the applicability of the IVET approach to gaining a fuller
17 understanding of the gene machinery specifically activated during the growth of *B. cereus* at
18 low temperature. This study gives insight into the properties required for the ecological
19 adaptation of *B. cereus* to cold environments and provides a first background for the
20 investigation of the adaptation of gene-expression mechanisms at low temperatures. The
21 different genes identified in this study certainly reflect the complexity of the processes
22 involved in *B. cereus* low-temperature adaptation (acclimation).

23 24 **Acknowledgements**

Comment citer ce document :

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(Auteur de correspondance), Nguyen The, C., Broussolle, V., Sanchis Borja, V. (2010).
Identification of *Bacillus cereus* genes specifically expressed during growth at low temperatures.
Applied and Environmental Microbiology. 76 (8). 2562-2573. . DOI : 10.1128/AEM.02348-09

Postprint

Version définitive du manuscrit publié dans / Final version of the manuscript published in : Applied and Environmental Microbiology, 2010, ahead of print, DOI: 10.1128/AEM.02348-09 - 24 -

- 1 This work was supported by INRA (*Institut National de la Recherche Agronomique*) and by a
- 2 grant from the Agence Nationale de la Recherche (ANR) (France) as part of an ANR-05-PNRA-
- 3 013 *B. cereus* contract

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1 Legend to the figures

2 Fig. 1: (A) Map of the *B. cereus* ATCC 14579 BC5398-BC5402 chromosomal region.

3 Grey arrows represent ORFs. The positions of the kanamycin resistance gene integrated in the
4 chromosome of the mutant to disrupt BC5402 or BC5401 are indicated. Small arrows
5 represent promoters. Function or putative function of gene products is indicated above the
6 ORFs. Dashed lines with small black arrows annotated by letters in brackets correspond to
7 RT-PCR amplicons (see text and Fig. 1B). The invert repeat corresponding to a predicted
8 stem-loop at the end of BC5398 is represented by two facing grey arrows. The full lines
9 correspond to the IVET identified fragments containing promoters specifically expressed at
10 10 °C identified (the cip-clones numbers are indicated).

11 (B) RT-PCR detection of BC5402 and downstream ORFs in *B. cereus* strain ATCC 12 14579.

13 RT-PCR was performed on 500 ng of RNA. Negative controls, RT-PCR on 500 ng of RNA
14 with a heat-inactivated reverse-transcriptase. Positive controls, PCR on 100 ng of genomic
15 DNA. Letters refer to the positions of the RT-PCR products on the locus, as represented on
16 Fig. 1A. Experiments were performed with the Titan One Tube RT-PCR System (Roche).
17 RNA extraction was performed on strains grown at 10 °C in LB broth and harvested in their
18 exponential phase ($OD_{600} = 0.7$).

20 Fig. 2: Resolution frequencies induced by the 20 cold-induced promoters (cip) at two 21 temperatures.

22 Transformants of the *B. cereus* R2SK strain harbouring each of the 20 selected pHT304-I
23 derivatives were grown at two temperatures. Resolution frequencies at 30 °C (grey bars) and
24 10 °C (dashed bars) were calculated as the amount of KmR CFU/ml divided by the total size

1 of the population (EmR CFU/ml). Experiments were repeated twice and the mean values are
2 presented (variability between duplicate experiments was below 15 %).

3 * Excess estimate values: at 30 °C, population of KmR CFU was below the detection
4 threshold (10 CFU/ml), and was therefore artificially set at 9 CFU/ml. For the control
5 (*B. cereus* R2SK strain harbouring the pHT304-I), no KmR CFU was detectable at both tested
6 temperatures.

7
8 **Fig. 3: Growth curves of *B. cereus* WT and Δ BC5401 at various temperatures.**

9 WT (black squares) and BC5401 mutant (white squares) strains were grown at 12 °C (A),
10 30 °C (B) or 45 °C (C). The cultures were incubated in an automated turbidometer with
11 vigorous constant shaking and the OD_{600nm} was measured at 15-min intervals (at 30 °C or
12 45 °C) or a 1 hour interval (at 12 °C). Results are the mean values with standard deviation of
13 triplicate experiments.

14
15 **Fig. 4: Fatty acid profiles of *B. cereus* WT and Δ BC5401 strains at two temperatures.**

16 WT (black) and Δ BC5401 (grey) strains were grown at 37 °C (full bars) or 12 °C (dashed
17 bars) on LB agar. The profiles of fatty acid (FA) methyl esters were determined by GC-MS as
18 previously described (39). The area of the detected peaks (each corresponding to distinct FA)
19 was measured. Each bar represents the relative amount of one FA compound, expressed as the
20 area of each peak divided by the sum of the areas of all the peaks on the chromatogram (mean
21 value +/- SEM of triplicate biological samples measured twice). Only major FA compounds
22 are presented (i.e. FA for which the area of the peak represented >2% at either 37 °C or
23 12 °C). For a given FA, the same letters above the bars indicate that no significant difference
24 between temperature and/or strain was detected (Tukey's honest significant difference test at
25 $P > 0.01$)

Table 1: Strains and plasmids used in this work

Strain or plasmid	Relevant genotype ^a	Source or reference
Strains		
<i>B. cereus</i> ATCC14579		Laboratory collection
<i>B. cereus</i> R2SK	<i>Bc::R2SK</i> , chromosomal reporter <i>B. cereus</i> strain	(22)
<i>B. cereus</i> Δ5402	ATCC14579 <i>BC5402::Km</i>	This work
<i>B. cereus</i> Δ5401	ATCC14579 <i>BC5401::Km</i>	This work
<i>E. coli</i> TG1	Δ(<i>lac-proAB</i>) <i>supE thi hsd-5 (F' traD36 proA⁺ proB⁺ lacI^q lacZΔM15)</i>	Laboratory collection
<i>E. coli</i> ET12567	<i>F dam-13::Tn9 dcm-6 hsdM hsdR recF143 zjj-202: :Tn10 galK2 galT22 ara14 pacY1 xyl-5 leuB6 thi-1</i>	Laboratory collection
Plasmids		
pHT304-I	Ap ^R and Em ^R shuttle vector used for <i>B. cereus</i> genomic library	(22)
pRN5101	Ap ^R and Em ^R shuttle vector	(49)
pRNΔ5401	Recombinant pRN5101 harboring <i>BC5401::Km</i>	This work
pRNΔ5402	Recombinant pRN5101 harboring <i>BC5402::Km</i>	This work
pHT304	Ap ^R and Em ^R cloning vector	(4)
pDG783	Ap ^R and Km ^R cloning vector	(25)
pHT304-Km	pHT304 carrying the <i>aphA3</i> Km ^r gene with its own promoter cloned between the <i>XbaI</i> and <i>PstI</i> sites of pHT304	This work

^a Km, kanamycin; Ap, ampicillin; Em, erythromycin

Table 2: Primers used in this study

Primer name	5'-3' sequence ^a	Restriction sites
5Up-01_Bam	<u>CGGGATCC</u> ATAGCGCTATTATCGAGGGG	<i>Bam</i> HI
3Up-01_Pst	AAA <u>ACTGCAGC</u> GCCCAACATAGCCAATTCC	<i>Pst</i> I
5Dn-01_Xba	GCT <u>CTAGA</u> ACAAGGAGGGGTAAAATGACG	<i>Xba</i> I
3Dn-01_Eag	CG <u>CGGCCG</u> GCCTTCTACACTCACTTCCC	<i>Eag</i> I
5Up-02_Bam	<u>CGGGATCC</u> GCGAACTATCGAATTGAAGGG	<i>Bam</i> HI
3Up-02_Pst	AA <u>ACTGCAGG</u> CTGTCTCTTTTGATATCCCC	<i>Pst</i> I
5Dn-02_Xba	GCT <u>CTAGAG</u> CGGGGATGGTTACAGAAGC	<i>Xba</i> I
3Dn-02_Eag	CG <u>CGGCCG</u> CTCTGTGTCAGGACGTACG	<i>Eag</i> I
Km5in	TCTGT <u>TCTAGAC</u> ATTTGAGGTGATAGG	<i>Xba</i> I
Km3in	GCT <u>ACTGCAG</u> ATCGATACAAATTCCTCGTAGGCG	<i>Pst</i> I
Km5out	CGGTATAATCTTACCTATCACC	
Km3out	TACTCTGATGTTTTATATCTTTTCTAA	
IVET-I1	CCCTGAACAGTGTTCTCGG	
IVET-I2	GGCGATTAAGTTGGGTAAC	
RT-5402-F	TTATTGCCACACGTAAATGA	
RT-5402-R	CCCACGTGAAAATACTTTGT	
RT-5402-01-F	ACAAGGAGTTCGTATTCCAG	
RT-5402-01-R	GCCAATTCCTTCTTTATCAC	
RT-5401-F	GATGTAGTGGCAAAGAGAA	
RT-5401-R	CAGGACGATACGTCTCTAAA	
RT-5401-00-F	AGCGCTAAACGATAAAAATG	
RT-5401-00-R	TCTGGGTTTTCAACGATACT	
RT-5400-F	CGAATGTTAGTCGGATTGAT	
RT-5400-R	TAATCCCTGCTGGATCTAAA	
RT-5400-5399-F	GAAGGCGATCGATTACTATG	
RT-5400-5399-R	CGGTTCTGAGAATCAACAAT	
RT-5399-98-F	AGGTTTATCGATGGGATTTT	
RT-5399-98-R	CAGAAAAGCTTAAACCATGC	
RT-5398-F	TTGTCATTTTAGGGCAAGAT	
RT-5398-R	CCCCACAATATGAAAATAA	
LC-BC5402-F	TTATTGCCACACGTAAATGA	
LC-BC5402-R	CCCACGTGAAAATACTTTGT	
LC-BC5401-F	CATATGGCTTTGTTTCAGG	
LC-BC5401-R	TTTGCACCACTAACTGCTAA	
LC-16S-F	GGTAGTCCACGCCGTAAACG	
LC-16S-R	GACAACCATGCACCACCTG	

^a Restriction enzyme sites are underlined

Table 3: Equivalent chain length (ECL) of the fatty acid methyl esters identified from *B. cereus* ATCC 14579

Compounds name as referred in the text	Chemical name	Retention time (min) ^a	ECL
i13	Methyl 11-methyldodecanoate	10.535	12.48
i14	Methyl 12-methyltridecanoate	11.721	13.48
n14	Methyl tetradecanoate	12.380	14.00
i15	Methyl 13-methyltetradecanoate	13.159	14.49
a15	Methyl 12-methyltetradecanoate	13.408	14.65
i16	Methyl 14-methylpentadecanoate	14.875	15.48
i16:1 (1)	Methyl 14-methyl-5-pentadecenoate	15.213	15.64
i16:1 (2)	Methyl 14-methyl-10-pentadecenoate	15.574	15.83
n16	Methyl hexadecanoate	15.842	16.00
C16:1 n-11	Methyl 5-hexadecenoate	16.195	16.15
C16:1 n-6	Methyl 10-hexadecenoate	16.629	16.33
i17	Methyl 15-methylhexadecanoate	16.932	16.48
a17	Methyl 14-ethylhexadecanoate	17.308	16.65
i17:1 (1)	Methyl 15-methyl- 5-hexadecenoate	17.300	16.65
i17:1 (2)	Methyl 15-methyl 10-hexadecenoate	17.676	16.82
n18	Methyl stearate	20.574	18.00
C18:1 n-9	Methyl oleate	21.206	18.21
C18:2	Methyl linoleate	22.567	18.68
<i>Standard C19</i>	<i>Methyl nonadecanoate</i>	<i>23.422</i>	<i>19.00</i>

^a The retention time was obtained on a GC/MS instrument (Shimadzu QP2010) equipped with an UBWAX column (see material and methods section).

Table 4: *B. cereus* genes specifically expressed during growth at low temperature identified in this study

clone reference ^a	insert size (bp)	number of potential promoter	downstream ORF ^b NCBI number	ERGO number	COG ^c	Broad COG category ^d	Assigned function ^e
cip60	719	1	BC0544 (+3)	RZC04345	C	Metabolism	iron-sulfur cluster-binding protein
cip81	711	1	BC0749 (+6)	RZC02927	H	Metabolism	Thiazole biosynthesis protein thiG
cip16	1473	2	BC0795 , BC0796	RZC01484, RZC01487	O, P	Cellular processes and signaling, Metabolism	Molybdopterin biosynthesis MoeB protein, Rhodanese-related sulfurtransferases
cip30	558	1	BC1235 (+6)	RZC03344	E	Metabolism	Indole-3-glycerol Phosph synthase
cip50	1083	1	BC1647 (+8)	RZC03052	NU	Cellular processes and signaling	Flagellar biosynthesis/type III secretory pathway ATPase
cip13	1931	1	BC2489	RZC06907	I	Metabolism	Acyl-coenzyme A synthetases/AMP-(fatty) acid ligases
cip47	1536	2	BC3197 , BC3196	RZC04150, RZC07700	I, GEPR	Metabolism, Metabolism	Permeases of the major facilitator superfamily, Biotin carboxylase
cip49	1155	3	BC4246 , BC4247 , BC4248	RZC02388, RZC02389, RZC02393	* * *	Poorly characterized, Poorly characterized, Poorly characterized	Hypothetical protein, Hypothetical protein, Hypothetical protein
cip25	1315	1	BC4634	RZC01131	*	Poorly characterized	Hypothetical protein
cip7	498	1	BC4851	RZC00481	IQ	Metabolism	O-succinylbenzoic acid--CoA ligase
cip18	1166	1	BC4866	RZC03491	G	Metabolism	glc-1P-adenyl transferase
cip85	641	1	BC4943	RZC07012	*	Poorly characterized	Hypothetical protein
cip54	963	1	BC4977 (+2)	RZC02376	F	Metabolism	5'-nucleotidase/2',3'-cyclic phosphodiesterase and related esterases
cip5	702	1	BC5400 (+2)	RZC04931	V	Cellular processes and signaling	Bacitracin transport ATP-binding protein BcrA
cip22	858	1	BC5401 (+3)	RZC04935	E	Metabolism	Lipase/Acylhydrolase with GDSL-like motif
cip26	1691	1	BC5402 (+4)	RZC04938	K	Information storage and processing	Transcriptional regulator, LacI family
cip39, cip2, cip1	1187	1	BC5440	RZC06561	KT	Information storage and processing	Autolysin response regulator

^a Three numbers indicate that the same clone was identified in 3 independent screenings

^b numbers in brackets correspond to the number of genes organized as an operon found downstream

^c COG: cluster of ortholog group, according to Tatusov *et al.*, (44). *, no COG

^d Broad functional COG categories according to the NCBI website (<ftp://ftp.ncbi.nih.gov/pub/COG/COG/fun.txt>)

^e Assigned function according to the *B. cereus* genome databases (www.integratedgenomics.com or <http://www.ncbi.nlm.nih.gov>)

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Brillard, J., Jehanno, I., Dargaignaratz, C., Barbosa, I., Ginies, C., Carlin, F., Fedhila, S. (Auteur de correspondance), Nguyen The, C., Broussolle, V., Sanchis Borja, V. (2010).

Identification of *Bacillus cereus* genes specifically expressed during growth at low temperatures.

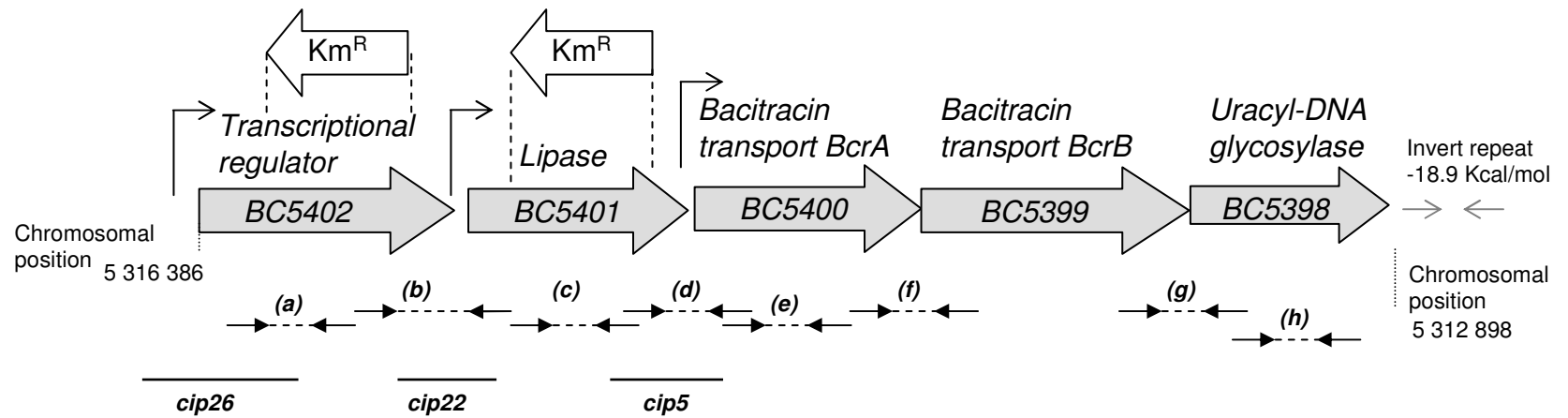
Applied and Environmental Microbiology. 76 (8). 2562-2573. DOI : 10.1128/AEM.02348-09

Table 5: Analysis by real-time RT-PCR of the level of expression of BC5402 and BC5401 at various times of the kinetics of growth

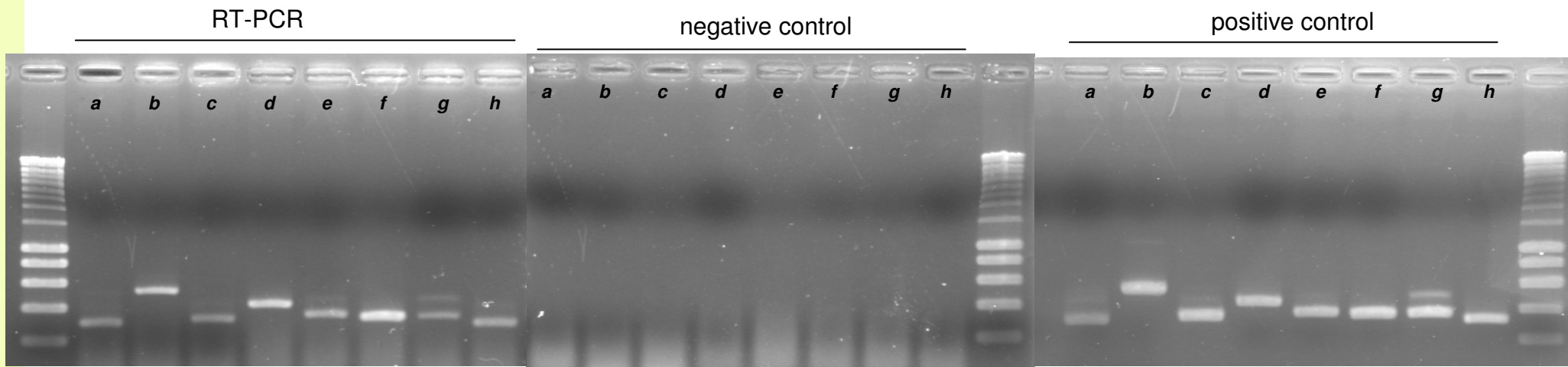
Gene	Fold change at 10°C relative to 37°C during ^a		
	Middle Exp.	End Exp.	Early Stat.
BC5402	9.46	2.69	4.42
BC5401	3.41	3.22	1.53

^a Exp., exponential phase of growth; Stat., stationary phase of growth. See material and methods section for details

Fig 1
A



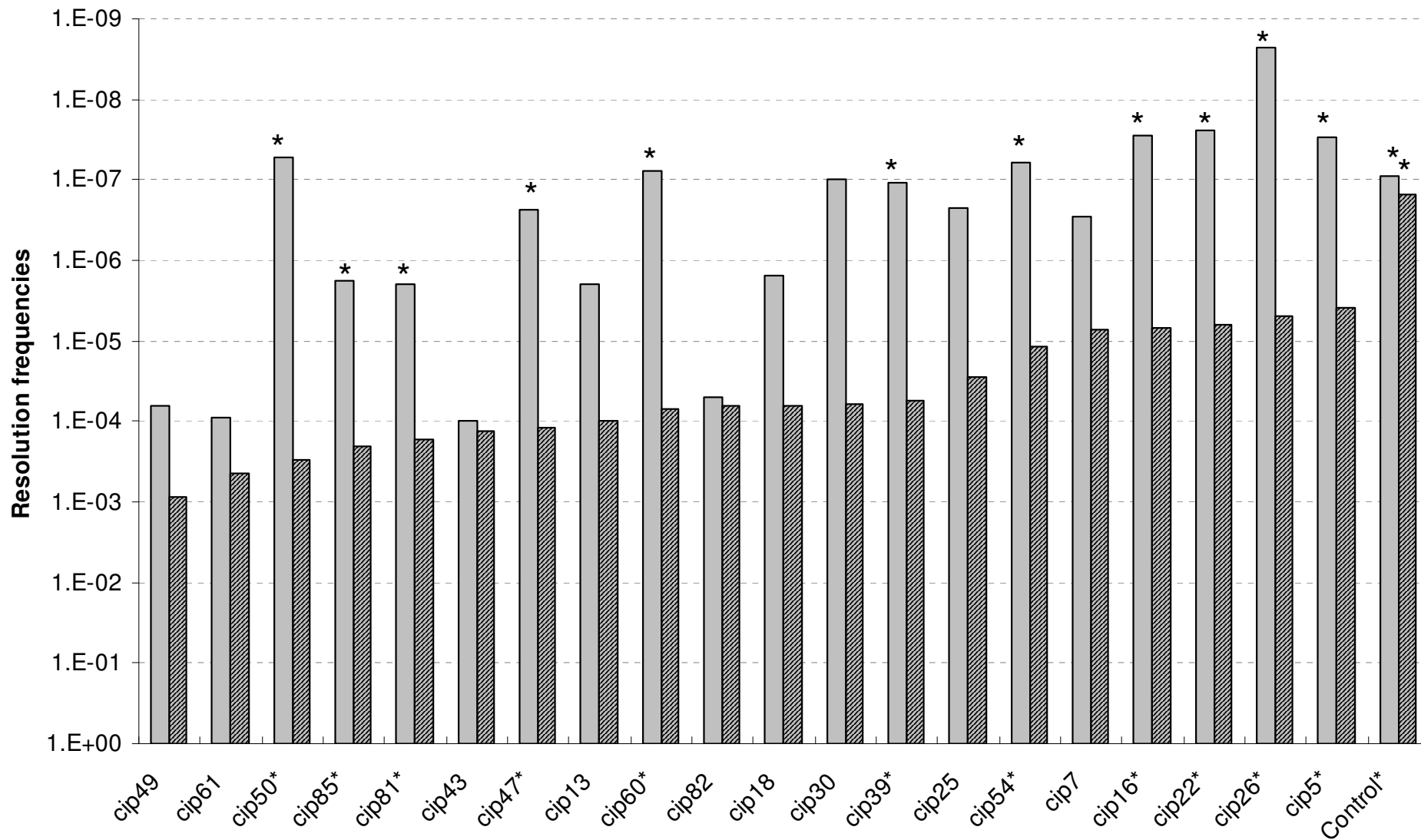
B



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Fig 2

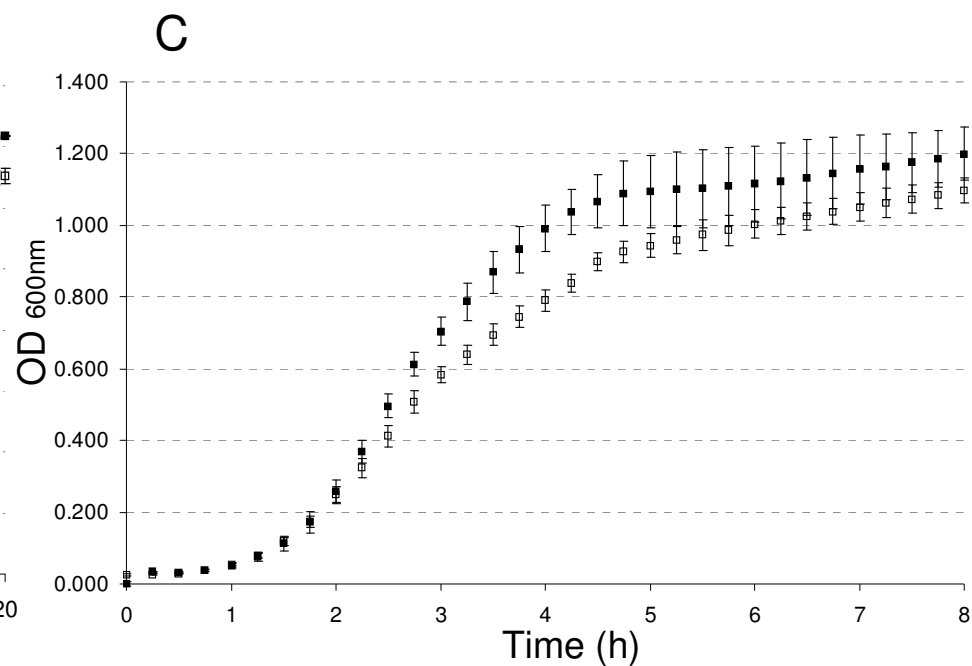
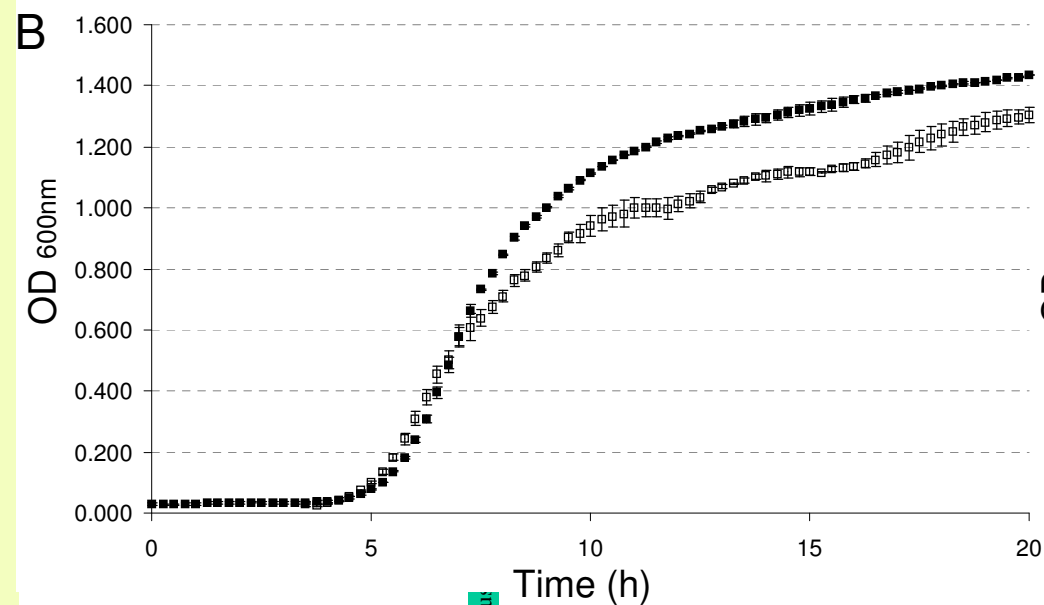
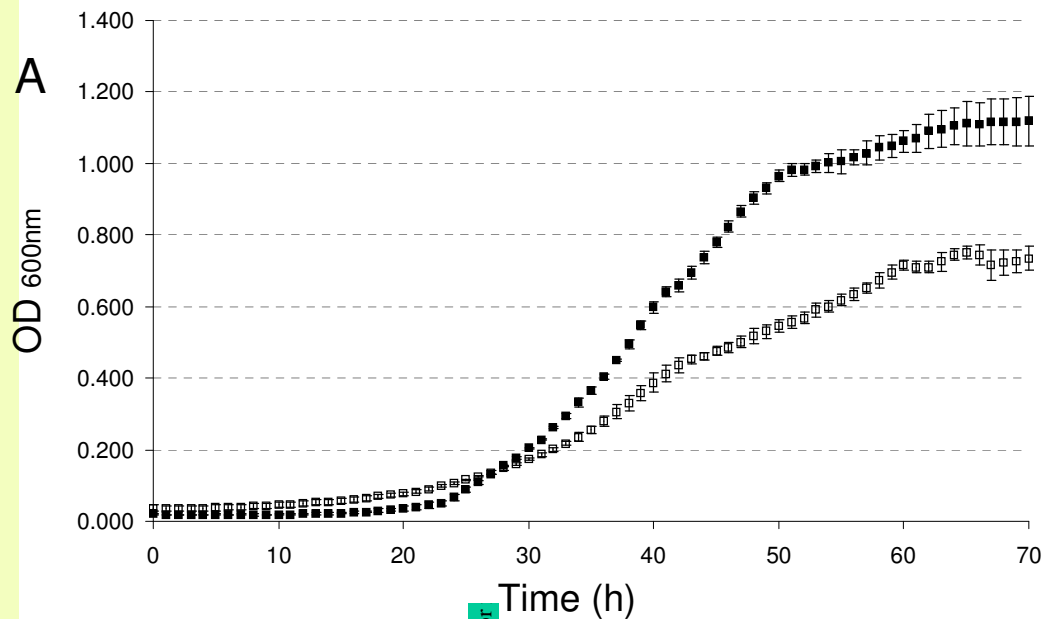


* Excess estimate values

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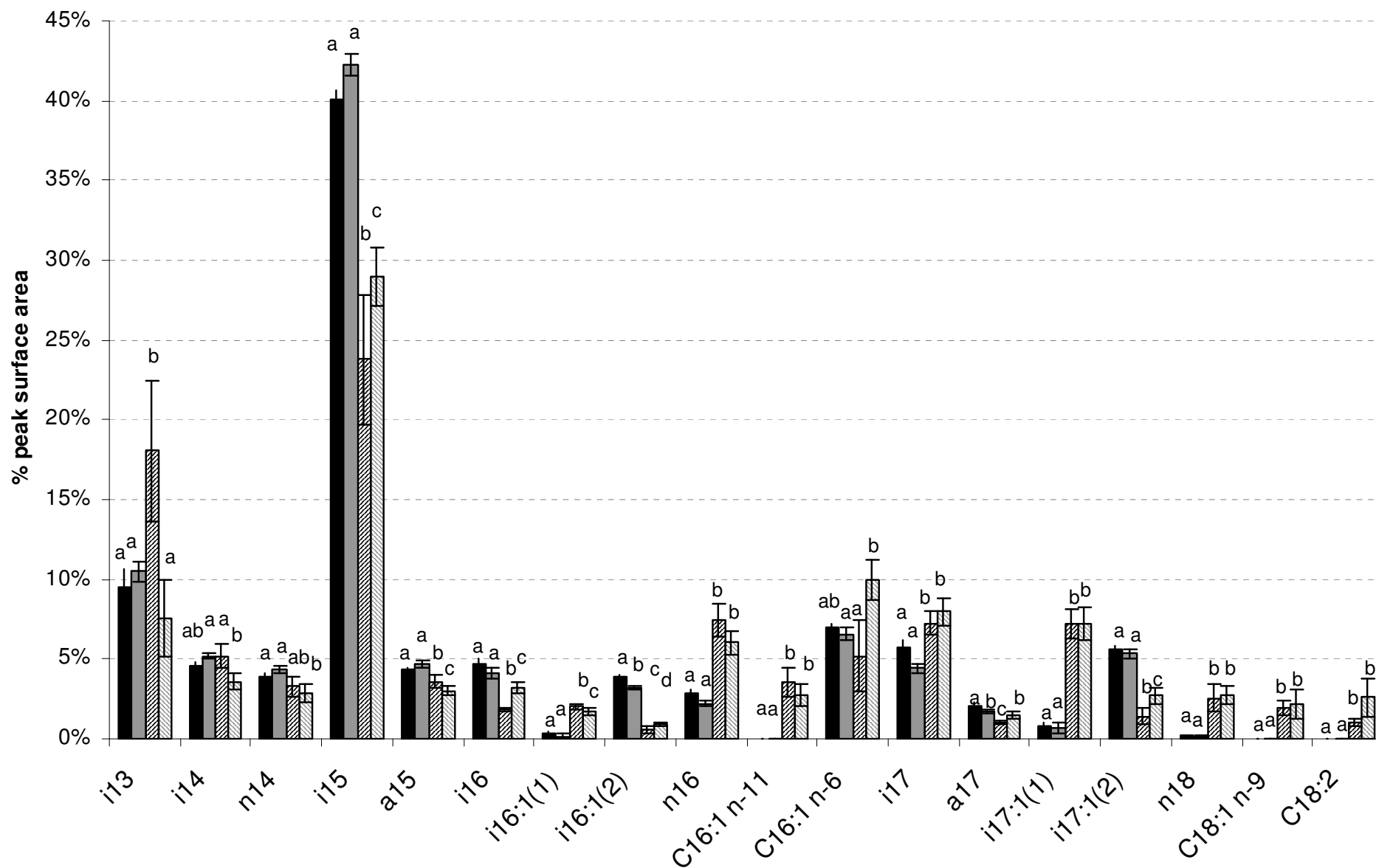
Fig 3



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Fig 4



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