RESEARCH ARTICLE

The CodY-dependent clhAB2 operon is involved in cell shape, chaining and autolysis in *Bacillus cereus* ATCC 14579

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Abstract

The Gram-positive pathogen *Bacillus cereus* is able to grow in chains of rod-shaped cells, but the regulation of chaining remains largely unknown. Here, we observe that glucose-grown cells of *B. cereus* ATCC 14579 form longer chains than those grown in the absence of glucose during the late exponential and transition growth phases, and identify that the clhAB2 operon is required for this chain lengthening phenotype. The clhAB2 operon is specific to the *B. cereus* group (i.e., *B. thuringiensis*, *B. anthracis* and *B. cereus*) and encodes two membrane proteins of unknown function, which are homologous to the *Staphylococcus aureus* CidA and CidB proteins involved in cell death control within glucose-grown cells. A deletion mutant (ΔclhAB2) was constructed and our quantitative image analyses show that ΔclhAB2 cells formed abnormal short chains regardless of the presence of glucose. We also found that glucose-grown cells of ΔclhAB2 were significantly wider than wild-type cells (1.47 μm ± CI95% 0.04 vs 1.19 μm ± CI95% 0.03, respectively), suggesting an alteration of the bacterial cell wall. Remarkably, ΔclhAB2 cells showed accelerated autolysis under autolysis-inducing conditions, compared to wild-type cells. Overall, our data suggest that the *B. cereus* clhAB2 operon modulates peptidoglycan hydrolase activity, which is required for proper cell shape and chain length during cell growth, and down-regulates autolysin activity. Lastly, we studied the transcription of clhAB2 using a lacZ transcriptional reporter in wild-type, ccpA and codY deletion-mutant strains. We found that the global transcriptional regulatory protein CodY is required for the basal level of clhAB2 expression under all conditions tested, including the transition growth phase while CcpA, the major global carbon regulator, is needed for the high-level expression of clhAB2 in glucose-grown cells.
Introduction

In *Staphylococcus aureus*, the CidR regulon is necessary for optimal survival in late stationary phase cultures and during biofilm development in the presence of excess glucose (~0.6%) [1–3]. Under these growth conditions, the CidR regulon is involved in the down-regulation of acetate production, which reduces cytoplasmic acidification and ultimately limits cell death and lysis [1,2]. The CidR transcriptional regulator activates the expression of two operons including *cidABC* and *alsSD* that display pro- and anti-death functions, respectively [3]. The *cidABC* operon encodes the CidA and CidB integral membrane proteins and the CidC pyruvate oxidase and the expression of *cidABC* is activated in glucose-grown cells and in the presence of acetate [4,5]. Recently, it was revealed that CidA and CidB modulate cell death through the direct control of these overflow metabolic enzymes: CidC (pyruvate:menaquinone oxidoreductase) involved in acetate production and Als (α-acetolactate synthetase) and AlD (α-acetolactate decarboxylase) involved in acetoin production [3]. This was a surprising conclusion, because Bayles and collaborators had previously hypothesized that CidA and a structural homolog named LrgA were functionally similar to bacteriophage holin/anti-holin proteins [6]. In this previous model, under stress conditions (such as acidification), the holin-like CidA may collapse the membrane potential and change the cell-wall pH, thus triggering cell-wall associated peptidoglycan hydrolases (PHs) activity and lysis [6]. According to Bayles, much about the Cid/Lrg family of cell-death modulators largely present in Gram positive and in Gram negative bacteria remains unknown; specifically the identification and characterization of PHs involved in this cell death phenomena remain largely unknown [7].

Recent work has found that YsbA protein—a LrgA homolog—is not involved in cell death control but in cell growth with pyruvate as the sole carbon source in *Bacillus subtilis* [8]. The *ysbAB* operon is highly expressed after glucose exhaustion and when *B. subtilis* grows in defined medium with pyruvate as only carbon source. In these growth conditions, the two-component system LytS/T is involved in the *ysbAB* expression while in the presence of glucose, the carbon regulator CcpA directly represses *ysbAB* expression [8].

The *Bacillus cereus* group includes *B. anthracis* and *B. cereus*, two well-known spore-forming pathogens of mammals [9]. The former is the organism that causes anthrax while the latter is frequently associated with food-borne infections that result in gastroenteritis [10]. These bacteria possess *cidAB*, *lrqAB* and *alsSD* operons and the *cidR* gene but not *cidC* [11]. Bayles and collaborators studied the CidR regulon in *B. anthracis* and found that in glucose-grown cells (~0.6%) and a *clhAB*2-inactivated mutant presents complex phenotypes, including cell morphological and survival changes in the late stationary growth phase and reduced spore production [12]. The means by which expression of *clhAB*2 is regulated in *B. cereus* species and how it influences bacterial growth and sporulation are unknown.

Members of the *B. cereus* group are unique in possessing two additional *cid* paralogs, the *clhAB1* and *clhAB2* operons, which have overall similarity scores that are >50% to the *cidAB* operon [12]. The *clhAB2* operon encodes two putative integral membrane proteins, ClhA2 and ClhB2. In *B. anthracis*, expression of *clhAB2* is repressed in glucose-grown cells (~0.6%) and a *clhAB2*-inactivated mutant presents complex phenotypes, including cell morphological and survival changes in the late stationary growth phase and reduced spore production [12]. The means by which expression of *clhAB2* is regulated in *B. cereus* species and how it influences bacterial growth and sporulation are unknown.

Here, we investigated the expression and the physiological role of *clhAB2* in *B. cereus* ATCC 14579 in the presence and absence of glucose. We first determined how *clhAB2* responded to...
glucose signal and investigated the transcriptional regulators involved in the regulation of clhAB2 expression. Then, we constructed a clhAB2 deletion mutant and found that sporulation was not impaired, but clhAB2 mutant exhibited morphological changes during the late-exponential and early-transition growth phases. To analyse these morphological changes, the wild-type, the clhAB2 mutant and the complemented strains were imaged with FM4-64 dye and quantitative image analysis was performed. We found that cell shape, chain length and intra-chain cell arrangement of clhAB2 mutant were significantly different from wild-type chain in the presence but not in the absence of glucose.

Materials and methods

Bacterial strains, growth conditions, and glucose assay

B. cereus strains (Table 1) were grown at 37˚C, with the exception of the growth rate analysis (see below), which was conducted at 30˚C. Exponentially growing cultures of B. cereus were inoculated into standard LB medium [13] or LB supplemented with 0.35% glucose (LBG) at a final optical density (OD) of 0.05. Catabolizable amino acids are plentiful in LB broth, presumably in the form of oligopeptides [13]. Glucose was added to LBG cultures at the onset of growth. The glucose concentrations in B. cereus cultures were determined using filtered supernatants and the Glucose (GO) Assay Kit (Sigma). Culture pH was monitored by pH electrode. With the exception of the growth rate analysis, all cultures were grown in flasks with an aeration ratio of 10 on a rotary shaker at 175 rpm. The onset of the transition growth phase (t0) was defined as the breakpoint in the slope of the log phase growth curve, and tn is the number of hours before (-) or after time zero [14]. Quantitative image analyses were performed using t0 cultures and three independent cultures were performed for each experimental condition.

Growth rate determination

Bacterial growth analysis was performed using a micro-plate reader system (Tecan Infinite F200PRO, Magellan software) with flat transparent 96-well plates (Greiner). The “Growth-Rates v2.0” program was employed for growth rate determinations using output files of OD610 values from the microplate reader [17].

Stationary phase survival and sporulation test

Viable cells were enumerated at the onset of the culture, t0, 24 h, 48 h, and 72 h using the serial dilution method. Sporulation test was performed as described in [14].

DNA manipulation techniques

Chromosomal DNA was extracted from B. cereus cells with the Puregene DNA Purification kit (Gentra Systems, USA). Plasmid DNA was extracted from E. coli using QIAprep spin columns (QIAGEN, France). Restriction enzymes (New England Biolabs, USA) and T4 DNA ligase (New England Biolabs, USA) were used in accordance with manufacturer’s recommendations. Oligonucleotide primers were synthesized by Sigma-Proligo (Paris, France). PCR was performed in an Applied Biosystem 2720 Thermal cycler (Applied Biosystem, USA). Amplified fragments were purified with the QIAquick PCR Purification Kit (QIAGEN, France). Digested DNA fragments were extracted from gels with the QIAquick Gel Extraction Kit (QIAGEN, France). Nucleotide sequences were determined by Cogenics (Meylan, France).
Construction of the clhAB\(_2\) deletion strain

The \(B.\) \textit{cereus} clhA\(_2\) (BC5133) and clhB\(_2\) (BC5132) genes were deleted by homologous recombination, using the pRN5101 thermo-sensitive vector as previously described [18,19]. For this mutant construct, a tetracycline cassette was used for positive selection (Table 1). A fragment containing the 5’- and 3’- end flanking regions of the target gene and tetracycline cassette was inserted between the \(\text{Hind III}\) and \(\text{Bam HI}\) sites of pRN5101. Chromosomal allele exchange was confirmed by PCR with oligonucleotide primers located upstream of the 5’ fragment of the clhAB\(_2\) construct (clhA\(_2\)Vf, 5’-CGATAGGTGATTTGTGAGG-3’) and downstream of the 3’ fragment (clhB\(_2\)Vr, 5’-CCGAAAGATAGGGGATGTA-3’).

Construction of the ccpA deletion strain

For the ccpA mutant construct, a kanamycin cassette carrying the \(\text{aphA3}\) gene was used, and the overlapping PCR method was performed to construct the ccpA-mutated fragment (Table 1). This fragment, which contained the 5’- end flanking region of ccpA, the kanamycin cassette and the 3’- end flanking region of ccpA, was inserted between the \(\text{Eco RI}\) and \(\text{Bam HI}\) sites of the pMAD heat-sensitive vector [20]. Chromosomal allele exchange was confirmed by PCR with oligonucleotide primers located upstream of the 5’ fragment of the ccpA construct (ccpAVf, 5’-agtacatcccgatccagc-3’) and downstream of the 3’ fragment (ccpAVr, 5’-agttttcaacaaactaaca-3’).

Plasmid construction

pHT304-P\(_{clhAB2}\)-lacZ was obtained by inserting the DNA region upstream (corresponding to the intergenic region) of the \(B.\) \textit{cereus} ATCC 14579 clhA\(_2\) gene between the \(\text{Pst I}\) and \(\text{Xba I}\) cloning sites of pHT304-18Z using forward primer GM38 (5’-AAACTGCAG\(\text{CACCACCTATTTGTTTATCCCGTA-3’}\)) and reverse primer GM39 (5’-GCTCTAGAGCATAATAGCAACGAGTGT-3’). The resulting plasmid was then transferred into \(B.\) \textit{cereus}\ by electroporation.

Site-directed deletion of the CodY presumed box 5’-TAAATTCAGAAAATA-3’ was performed by PCR-driven overlap extension method [21] with mutagenic primers which share complementary sequence, F-codYBm (5’-TGAAATAATAGTCTTTAAAACTTTTTATATTAG) and R-codYBm (5’-CTAATATAAAAAGTTTTAAGACTATTATTTCA) and flanking primers GM38 and GM39. pHT304-P\(_{\text{codY\(_{Bm}\)}}\)-lacZ was obtained by inserting this mutated DNA

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**Table 1. Strains used.**

| Strain | Genotype | Source or reference |
|--------|----------|---------------------|
| \(B.\) \textit{cereus} ATCC 14579 (Bc) | Wild-type reference strain | [15,16] |
| \(\Delta\text{clhAB}_2\) | Bc \(\Delta\text{clhAB}_2::\text{tet}\) | This study |
| \(\Delta\text{ccpA}\) | Bc \(\Delta\text{ccpA::kan}\) | This study |
| \(\Delta\text{codY}\) | Bc \(\Delta\text{codY}\) | [16] |
| \(\Delta\text{clhAB}_2\) \(\Omega\) \(\text{clhAB}_2\) | \(\Delta\text{clhAB}_2\), \(\text{pHT315}\) \(\Omega\) \(\text{clhAB}_2\) | This study |
| \(\Delta\text{clhAB}_2\) \(\text{pHT315}\) | \(\Delta\text{clhAB}_2\), \(\text{pHT315}\) | This study |
| \(\Delta\text{clhAB}_2\) \(\text{pHT304-18P}_{\text{clhAB2}}\)-lacZ | \(\text{pHT304-18P}_{\text{clhAB2}}\)-lacZ (locus tag BC5133-BC5132 = \(\text{clhAB2}\)) | This study |
| \(\text{clhAB2}\) \(\Delta\text{ccpA}\) | \(\Delta\text{ccpA}, \text{pHT304-18\(\Omega\)clhAB2}\)-lacZ | This study |
| \(\text{clhAB2}\) \(\Delta\text{ccpA}\) \(\text{ppHT315}\) | \(\Delta\text{ccpA}, \text{pHT315}\) | This study |
| \(\text{clhAB2}\) \(\Delta\text{ppHT315}\) | \(\Delta\text{ppHT315}\) | This study |
| \(\text{mCodYBox}\) \(\Delta\text{codY}\) | \(\text{mCodYBox}\) \(\Delta\text{codY}\) | This study |
| \(\text{mCodYBox}\) \(\Delta\text{codY}\) | \(\text{mCodYBox}\) \(\Delta\text{codY}\) | This study |

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fragment between the PstI and XbaI cloning sites of pH304-18Z and the resulting plasmid was then transferred into *B. cereus* by electroporation.

A *clhAB* complementation plasmid was constructed by amplifying a fragment (including the coding sequence of *clhA* and *clhB* and the promoter region) by PCR using the primer pair CAB2F (5'-GGGGATCCCTATCTTGTTTATC-3') and CAB2R (5'-CGGAATTCGACTTGGCGTACCT-3'). The fragment was cloned in vector pH315, which is presumably present at 15 copies per cell [22]. A *ccpA* complemented strain was constructed which harbored a copy of the *ccpA* gene integrated in the *amyE* locus. The *ccpA* gene was cloned in a modified pMAD thermo-sensitive vector [23], harboring upstream and downstream fragments of *amyE*. The recombinant plasmid was introduced into the *ccpA* mutant strain. Chromosomal allele exchange was confirmed by PCR. The pH1618KΩPxylcodY strain was constructed in a previous analysis [24].

**β-Galactosidase assay**

β-Galactosidase activity was assayed as described in [19].

**Fluorescence microscopy, image capture and analysis**

The septum of dividing cells and cytoplasmic membranes were imaged using the FM4-64 lipophilic dye (Molecular Probes). Cells were isolated from cultures (see Growth conditions paragraph) and incubated with FM4-64 at 20 μg mL⁻¹ at room temperature for 5 min. Slides were spotted with 4-μl aliquots, then cells were visualized by oil-immersion fluorescence microscopy (magnification of x100; NA of 1.4). Cell chains and individual cells were observed with a Zeiss Axio Observer.Z1 inverted fluorescence microscope equipped with a Zeiss AxioCam MRm digital camera and a custom-made filter (excitation: D510/40, beam splitter 540 DCLP, emission: D640/50). Fluorescent and phase-contrast images were processed with Zeiss ZEN 2-lite software.

**Cell width determination.** Using fluorescent digital images, cell width was measured from a traced line segment generated with the image analysis tools in FIJI software (see below for statistical analysis).

**Determination of number of cells per chain.** Using fluorescent digital images, the total number of cells per chain was counted using the “automatic particle counting” tool in FIJI software and was manually corrected.

**Determination of short and long inter-constriction cell arrangements.** Microscopy images of cell chains revealed strong constriction at some cell wall septa that connected cells within each chain. These deeper invaginations correspond to cells undergoing separation while other septa show no detectable constrictions. In our study, “short” inter-constriction arrangement contained 2–4 cells and “long” inter-constriction arrangement had 6–8 cells. Using fluorescent images, short and long cell arrangements were counted manually.

**Statistical analysis**

**Cell width measures.** An ANOVA and a Student’s t-test analysis were performed using the means of each condition using R software (version 3.1.1) (R Core Team, 2014). These analyses examined three factors: day, mutation, and glucose. Our model took into account the main effects of these factors and the interaction effects between mutation and glucose. Between 270 and 320 measures were acquired for *Bc, ΔclhAB₂*, and *ΔclhAB₂ΩclhAB₂* in both growth conditions.

**Determination of number of cells per chain.** A non-parametric Mann-Whitney test and a Two-Sample Fisher–Pitman Permutation Test using R software (version 3.1.1) (R Core Team, 2014) were performed to examine differences in the number of cells per chain in the
absence or presence of glucose for each strain. Between 90 and 130 chains were analyzed for Bc, ΔclhAB2, and ΔclhAB2ΔclhAB2 in both growth conditions.

Count of the occurrences of short and long inter-constriction cell arrangements. A quasibinomial generalized linear model (GLM) with overdispersion was used using R software (version 3.1.1, function = quasibinomial) (R Core Team, 2014). This analysis examined three factors: day, mutation, and glucose. Our model took into account the main effects of these factors and the interaction effects between mutation and glucose. Between 280 and 320 cell arrangements were counted for Bc, ΔclhAB2, and ΔclhAB2ΔclhAB2 in LB and in LBG media.

Results

Expression of clhAB2 in the presence or absence of glucose

We first investigated how expression of the clhAB2 operon responded to the presence of glucose during growth of B. cereus ATCC 14579 wild-type strain (Bc). For this, a PclhAB2- lacZ transcriptional fusion construct (Fig 1A) was introduced into the Bc strain, and the clhAB2-Z operon was studied.

Fig 1. Expression of clhAB2 in the presence or presence of glucose. (A) Genetic organization of clhAB2 locus in the Bc genome and schematic representation of the clhAB2-Z transcriptional fusion construct. (B) Bc strain clhAB2-Z was grown in LB in the presence (open circles) or absence (closed circles) of 0.35% glucose. Optical densities (OD600, in blue circles) of cell cultures and β-galactosidase specific activities (U/mg protein, in black losanges) in the presence (open losanges) or absence (closed losanges) of 0.35% glucose are shown. The levels of lacZ expression of pHT304-18’Z (background level) were around 15 U/mg protein. (C) Filtered supernatants were measured for glucose concentration (Glucose Assay Kit, Sigma, in blue squares) and for pH determination (black triangles). The start of the transition growth phase is indicated as t0 for time zero. The glucose concentration of LB medium (closed squares) was below 0.01% and pH (closed triangles) was 7 ± 0.2. The data presented are representative of four independent experiments.

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cells (log-phase cultures) were inoculated in LB [13] and LBG media. We quantified β-galactosidase activity from two hours before entry into the transition growth phase (t₀, Materials and Methods) to five hours after (Fig 1B). We also measured glucose utilization along with changes in pH (Fig 1C). We observed that clhAB₂ transcription remained at a constant low level in LB medium, but in the LBG medium, clhAB₂ expression gradually increased from one hour before t₀ to five hours after (Fig 1B). Following the increase in clhAB₂ expression, glucose concentration decreased from 0.35% to 0.01% and pH values dropped from 6.8 to 5.2 (Fig 1C). The glucose concentration was estimated to be around 0.28% at the end of exponential growth phase and decreased below 0.03% one hour later. Remarkably, activation of clhAB₂ expression was still observed despite the total glucose consumption. Furthermore, we observed similar patterns of clhAB₂ expression when different initial concentrations of glucose (ranging from 0.35% to 1%) were used, as well as when cells were grown with fructose or sucrose instead of glucose (S5 Fig). Therefore, clhAB₂ expression responded positively to the presence of glucose (as well as of two other rapidly fermented sugars), and this gene activation was concomitant with glucose consumption, decrease in pH, and entry into the transition growth phase.

In order to evaluate the influence of acidic pH on clhAB₂ expression, we have used MOPS to buffer LB medium to grow clhAB₂::Z cells. Again, we observed that clhAB₂ transcription remained constant in LB-MOPS medium, but in the presence of glucose, clhAB₂ expression gradually increased until t₁ and then reached a plateau (S1 Fig). Thus, our results show that the presence of glucose but not acidic pH was involved in the activation of clhAB₂ expression in our growth conditions.

The regulation of the clhAB₂ operon in the presence of glucose is CcpA-dependent

In low G+C Gram-positive bacteria, transcription in response to the availability of a preferred carbon source such as glucose is mainly regulated by CcpA [25]. CcpA is a member of the LacI protein family of transcription factors, and can be either a positive or a negative regulator. CcpA is a DNA-binding protein that recognizes target promoters that contain the catabolite-responsive element (CRE) site, which is a 14-bp palindromic sequence [25]. We therefore decided to investigate the role of CcpA in the glucose-dependent activation of clhAB₂ expression. A ΔccpA mutant and a genetically complemented mutant were constructed, and the P<sub>clhA-B₂</sub>-β₂-galZ fusion was introduced in these mutant strains. Cells were grown in LB and LBG media, and β-galactosidase activity was measured one hour before entry into the transition growth phase (t₁) to four hours after (t₄) (Fig 2). The deletion of ccpA abolished the glucose-induced activation of clhAB₂ expression (Fig 2). Indeed, in the ΔccpA mutant, clhAB₂ expression in the presence of glucose was similar to that observed without glucose in the wild-type strain (Fig 2). The addition of ccpA at the amy chromosomal locus successfully complemented the ΔccpA mutant such that β-galactosidase activity levels were actually similar to wild-type levels in LBG medium. This result demonstrated that CcpA control is required for glucose-activated clhAB₂ expression. However, visual analysis of the clhAB₂ promoter region did not identify any sequence that resembled the CcpA consensus sequence of <i>B. subtilis</i> or <i>B. cereus</i>, and a previous bioinformatic search had failed to identify a CRE motif in the promoter region of the clhAB₂ gene [26]. We thus hypothesized that CcpA controls clhAB₂ indirectly, through the altered expression or activity of other transcriptional regulators.

**CodY is needed for clhAB₂ expression under all the conditions tested**

CodY is a branched-chain amino acid and GTP sensor and a global regulator of transcription in low G + C Gram-positive bacteria [27,28]. CodY has been characterized in several bacterial
The CodY-dependent clhAB2 operon is involved in cell shape, chaining and autolysis in *Bacillus cereus* ATCC 14579

**Fig 2.** The glucose-activated expression of clhAB2 is abolished by the deletion of ccpA. Effect of ccpA mutation on the activation of clhAB2. Cells of Bc and isogenic mutant strains (ΔccpA, ccpA-complemented mutant), which all harbored the transcriptional P_{clhAB2}-lacZ fusion construct, were grown in LB (closed symbols) or in LBG (open symbols) media. Samples were harvested at the indicated times and were assayed for β-galactosidase specific activity. Glucose 0.35% was added, when appropriate, at the onset of the culture. t0 is the number of hours before (-) or after t0. SD bars are shown.

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pathogens [28–30] including *B. cereus* [16,24,31,32] and *B. anthracis* [33,34]. In *B. cereus* and *B. thuringiensis*, CodY is active under various rich laboratory media such as LB broth and BHI medium, during the exponential phase [16,24], as well during the early transition growth phase [16,24,32]. In Bc strain, numerous genes involved in biofilm formation, as well as amino acid transport and metabolism were upregulated, while genes associated with motility and virulence were repressed upon deletion of codY [16].

We hypothesized that CodY could positively control clhAB2 expression under our nutrient-rich growth conditions. We measured the transcriptional activity of clhAB2 promoter fused to the reporter gene lacZ in wild-type and ΔcodY cells, in LB and LBG media (Fig 3). The deletion of codY led to a strong decrease in β-galactosidase activity under all the conditions tested: the expression levels in the ΔcodY mutant were very low (around 50–60 SA) until t_{0.5}; from t_{0.5}, in LB medium the expression levels were slightly increased (two- to threefold), and in LBG
medium, the expression levels were gradually elevated (two- to eightfold); that said, all expression levels were still lower than the wild-type basal expression level (i.e., in LB medium). While complementation of the ΔcodY mutant restored the clhAB2 expression under all the conditions tested, expression levels in LB medium were actually higher than those in the wild type (Fig 3). Our result demonstrated that CodY is active and needed for the transcription of the clhAB2 operon under all the conditions tested.

We also analyzed the CodY repressor activity in LB and LBG media during both the late exponential and transition growth phases until $t_4$. We chose one of the most up-expressed genes in the Bc ΔcodY mutant, namely BC2026, which encodes an OppA-like peptide binding and transport protein [16]. We measured the transcriptional activity of BC2026 promoter fused to the reporter gene lacZ in wild-type and ΔcodY cells, in LB and LBG media (S2 Fig). As expected [16], the expression of $P_{BC2026}^{lacZ}$ was abolished in Bc (around 10–15 SA, similar to background levels), while high expression levels (400- to 100-fold increase) were observed during exponential and transition growth phases until the end of the experiment ($t_4$) in the codY mutant (S2 Fig). Again, this result demonstrated that CodY is clearly active under all the conditions tested.
**clhAB**₂ expression requires the presence of a 15-bp CodY-binding sequence with four mismatches

CodY is a unique DNA-binding protein that recognizes target promoters containing the CodY motif, which is a 15-bp consensus palindromic DNA sequence [35]. In *B. anthracis*, CodY target genes were identified by the genome-wide analysis of *in vitro* CodY-DNA complexes [36]. A CodY-binding fragment of 33-nt was identified upstream of the *B. anthracis clhAB₂* operon [36]. The CodY DNA-binding sequence, 5’-TAAATTCAGAAAATA-3’, which has four mismatches with respect to the CodY-binding consensus motif, AATTTTCWGAAAATT, was identified in this fragment [36]. We also found this CodY DNA-binding site sequence upstream of *clhAB₂*, which was localized 178 bp upstream of the *Bc clhA₂* initiation codon (Fig 4A). We thus hypothesized that CodY directly activates *clhAB₂* through the DNA binding to this CodY motif.

To address the contribution of this CodY motif in *clhAB₂* expression, we deleted the 15-bp CodY motif sequence of the P*clhAB₂* insert, resulting in the fusion of P*CodYBm’-lacZ* (see Materials and methods and Fig 4B). The expression of the P*CodYBm’-lacZ* fusion was abolished under all the conditions tested (around 10–15 Sa, similar to background levels) (Fig 4C). Thus, our results showed that *clhAB₂* expression absolutely requires the presence of this 15-nt sequence under all the tested conditions. The P*CodYBm’-lacZ* fusion was also introduced into the Δ*codY* mutant, and cells were grown in LB and LBG media. Again, the expression of the P*CodYBm’-
lacZ fusion was abolished under all the tested conditions. Overall, our results suggested that clhAB2 expression requires the presence of this 15-nt sequence.

The deletion of clhAB2 did not affect growth and sporulation in B. cereus ATCC 14579

We investigated the role of the clhAB2 operon in the Bc wild-type strain by deleting the entire operon via allelic exchange. Bc and ΔclhAB2 strains grew similarly, with comparable growth rates in LB and LBG media (Fig 5A). This finding suggested that clhAB2 is not necessary for exponential growth. We also assessed the ΔclhAB2 mutant in terms of cell viability in the absence (Fig 5B) or presence (Fig 5C) of glucose after 24, 48, and 72 hours of incubation. Small significant differences (P<0.05) were observed between ΔclhAB2 and Bc in stationary phase survival after 24 and 48 hours of growth in LBG (Fig 5C). Spore production was performed in a specific sporulation medium, but no significant differences were observed in sporulation tests. Indeed, the median spore production of Bc at 72 h was 1.3·10^8 spores/ml (min = 7.2·10^7; max = 1.9·10^8, n = 5), while that of the ΔclhAB2 mutant was 9.5·10^7 spores/ml (min = 6.1·10^7; max = 1.7·10^8, n = 5). The fact that the ΔclhAB2 mutant did not exhibit reduced sporulation efficiency compared to wild-type stands in contrast to report from B. anthracis [12] and suggests the existence of differences in the sporulation process of these two bacteria.

clhAB2 is required for chain lengthening in the presence of glucose

In bacteria of the B. cereus species, the formation of rod-shaped cell chains of different lengths appears to be a normal aspect of growth in different media and environments [37–39]. We observed that the Bc wild-type strain (Bc) produced short chains in LB medium and long

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Fig 5. Phenotypic analyses of ΔclhAB2 isogenic mutant. (A) Box plot of growth rates in the absence (LB) or presence of 0.35% glucose (LBG). Growth rates were determined using output files of OD610 values from the microplate reader (see Materials & Methods). NS—no statistical significance. (B, C) Stationary phase survival of Bc and ΔclhAB2 isogenic mutant in LB and in LBG. Cell viability tests were performed at 0, 4, 24, 48, and 72 h. Significance is based on Mann & Whitney test with a P<0.05.

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chains and occasionally serpentine chains in LBG medium during exponential (t₁, S3 Fig) and early transition growth (t₀, Fig 6A; t₂, S3 Fig). Instead, during the same growth period, the ΔclhAB₂ mutant bacteria grew as a population of short chains in both growth conditions; in LBG medium, chains were wide and occasionally curved (Fig 6A, S3 Fig). The complemented ΔclhAB₂ strain carrying the clhAB₂ operon on a plasmid showed an absence of this mutant chaining phenotype while the clhAB₂ strain carrying the empty plasmid showed the mutant chaining phenotype in LBG medium (Fig 6A). To further analyze changes in chain
morphology, fluorescent images of cell membranes and septa were examined at the onset of the transition phase (t₀, Fig 6B). Aberrant septa locations were never observed in ΔclhAB2 cells (Figs 6B and 7A), suggesting that the clhAB2 operon is not involved in cell division. In addition, both wild-type and ΔclhAB2 mutant chains exhibit peritrichous flagella using transmission electronic microscopy, suggesting that the clhAB2 mutation did not alter neither the structure nor the implantation of flagella (S4 Fig).

We quantified the total number of cells per chain in Bc, ΔclhAB2, and ΔclhAB2ΔclhAB2 populations in the absence and presence of glucose, and displayed these data as box-and-whisker plots in order to show the shape of the distribution, its central value and the variability of the chain length (Fig 6C). Bc chains lengthened significantly in the presence of glucose (P < 0.01). The median chain length of Bc in LB medium was 8 cells per chain and in LBG medium was 12 cells (interquartile ranges [IQRs]; LB vs. LBG, 5 vs. 8 cells). In sharp contrast, glucose-induced chain lengthening was not observed in the ΔclhAB2 population: the median chain length of ΔclhAB2 was 8 and 9 cells per chain respectively in LB and LBG media, with remarkably small dispersion in LBG media (IQRs: LB vs. LBG, 5 vs. 4 cells). ΔclhAB2ΔclhAB2 chains lengthened significantly in the presence of glucose (P < 0.01), demonstrating that the expression of clhAB2 in trans was sufficient for restoration of the wild-type chaining phenotype. The median chain length of ΔclhAB2ΔclhAB2 strain in LB medium was 9 cells per chain (IQR: 6) and in LBG medium was 15 cells (IQR: 9) (Fig 6C). This quantitative analysis revealed that Bc chains lengthened in the presence of glucose and clhAB2 is required for glucose-dependent chain lengthening. In other words, our data showed that clhAB2 down-regulates cell separation process during chain production in glucose-grown cells.

clhAB2 is required for long inter-constriction cell arrangement in the presence of glucose

Microscopy images of wild-type, ΔclhAB2, and ΔclhAB2ΔclhAB2 chains revealed deep invaginations or constrictions at cell wall septum connecting cells undergoing separation (Fig 6A and 6B). We repeatedly observed in LB medium that constrictions occurred frequently at septa that were spaced 4 cells apart in Bc, ΔclhAB2, and ΔclhAB2ΔclhAB2 populations (Fig 7A). The frequency of 4-chained-cell arrangements in Bc, ΔclhAB2, and ΔclhAB2ΔclhAB2 populations were similar (56, 52, and 57%, respectively, N = 150 cell arrangements, see Materials and methods). In LBG medium, we were intrigued by two observations: the occurrence of 4-cell arrangements decreased compared to that found in LB medium, and constrictions also often occurred at septa that were spaced 8 cells apart in the Bc population (Fig 7A). In Bc, the frequencies of 4- and 8-cell arrangements were 29% and 23%, respectively in LB medium (N = 150, see Materials and methods). Instead, in the ΔclhAB2 population, the frequency of the 4-cell arrangement did not decrease (48%) and the 8-cell arrangement was observed at a very low frequency (4%) compared with that found in Bc. In ΔclhAB2ΔclhAB2 the frequencies of 4- and 8-cell arrangements were 31% and 35%, respectively, demonstrating that clhAB2 expression was sufficient for restoration of the wild-type inter-constriction cell arrangement pattern.

In order to have a meaningful picture of these inter-constriction cell arrangements in both growth conditions, we examined the distribution of “short” (<4 chained cells) versus “long” (>4 chained cells) cell arrangements in a larger Bc, ΔclhAB2, and ΔclhAB2ΔclhAB2 populations (N > 250 cell arrangements) at the onset of transition phase (t₀) (Fig 7B). In the absence of glucose, the percentages of short and long cell arrangements were 74% and 26%, respectively, but when glucose was present in the medium, these numbers swapped, to 30% short and 70% long (P < 0.01) (Fig 7B). In sharp contrast, glucose-induced long cell arrangement was not observed in the ΔclhAB2 population. In the absence of glucose, the percentages of short and long cell
Fig 7. Inter-constriction cell arrangements and cell width measures in \( Bc, \Delta clhAB_2 \) and \( \Delta clhAB_2 \Omega clhAB_2 \) populations in the presence or absence of glucose. (A) Close view of \( Bc, \Delta clhAB_2 \) and \( \Delta clhAB_2 \Omega clhAB_2 \) chains at \( t_0 \) using fluorescence microscopy. Cells were grown in LB and LB with 0.35% glucose (LBG). Division septa and cytoplasmic membranes were imaged using the FM4-64 lipophilic dye. Chains exhibited constrictions that occurred at septa spaced 4 cells apart in \( Bc, \Delta clhAB_2 \) and \( \Delta clhAB_2 \Omega clhAB_2 \) in LB medium and in \( \Delta clhAB_2 \) in LBG.
medium. They also exhibited constrictions that occurred at septa spaced 8 cells apart in Bc and ΔclhAB2ΔclhAB2 in LBG medium. (B) Distributions of “short” (≤4) and “long” (>4) inter-constriction cell types in the Bc, ΔclhAB2, and complemented mutant populations (N>250 cell arrangements). Two inter-constriction arrangement types in Bc, ΔclhAB2, and ΔclhAB2ΔclhAB2 populations were defined (see Materials and methods). The first type, containing cell arrangements with two to four cells (“short”) and the second type, including cell arrangements with six to eight cells (“long”). The significant effects of glucose (dashed line) and cell arrangements with two to four cells (“short”) and the second type, including cell arrangements with six to eight cells (“long”). The significant effects of glucose (dashed line) and clhAB2 mutation (solid line) are based on a Binomial analysis (see Materials and methods) with P<0.01** and <0.05*. (C) Cell width measures in Bc, ΔclhAB2, and ΔclhAB2ΔclhAB2 populations (N>250 bacilli) in LB and LBG media. The significant effect of clhAB2 mutation in LBG medium is based on a Student’s t test and ANOVA (see Materials and methods) with P<0.001***. A P value close to the cutoff 0.05 was considered as non significant (NS). Mean ± CI 95% is depicted.

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arrangements were 98% and 2%, respectively, and when glucose was present, these numbers were not different (91% short and 9% long) (P>0.05) (Fig 7B). The ΔclhAB2 distribution of short and long cell arrangements in the presence of glucose was significantly different from the wild-type distribution (P<0.01) (Fig 7B). A clhAB2 mutant produced short cell arrangement in the presence of glucose. Glucose also had a remarkable effect on intra-chain cell arrangement in the ΔclhAB2ΔclhAB2 population: in the absence of glucose, the percentages of short and long intra-chain arrangements were 95% and 5%, respectively, but when glucose was present in the medium, these numbers swapped, to 34% short and 66% long (P<0.01) (Fig 7B). Our results suggested that the expression of clhAB2 in trans was sufficient for restoration of Bc inter-constrictions cell arrangement in LBG medium (Fig 7B). Thus, clhAB2 is required for long inter-constriction cell arrangement in the presence of glucose. Overall, we demonstrated that Bc cells form short chains mainly associated with short cell arrangement (74%) in LB medium, but in the presence of glucose, Bc cells form significantly longer chains that are mainly associated with long cell arrangement (70%).

clhAB2 mutation produced wide cells in the presence of glucose

The cell wall has multiple functions during bacterial growth, including maintaining bacterial cell integrity and shape by resisting internal turgor pressure. Compared with other Gram-positive rod-shaped bacteria, B. cereus is large (1.0–1.2 μm by 3.0–5.0 μm) [10]. In LB medium, we observed a small but not significant enlargement in ΔclhAB2 cells (Fig 5C). In LBG medium, ΔclhAB2 cells were significantly wider (P<0.001) than wild-type cells (1.47 μM ± 0.04 CI 95% wide vs. 1.19 μM ± 0.03 CI 95% for Bc) (Fig 5C). Then, we showed that the expression of clhAB2 in trans was partially sufficient for restoration of Bc width (1.33 μM ± 0.04 CI 95%) in LBG medium. Overall, these data indicated that clhAB2 cells exhibited a decrease in cell wall resistance to the internal turgor pressure in LBG medium.

Autolytic behavior of ΔclhAB2

Certain PHs, called autolysins, destroy the peptidoglycan mesh by cleaving peptidoglycan glycan strands or cross-links of the producer strain, resulting in cell lysis [6,40]. We lastly characterized the autolytic behavior of ΔclhAB2 cells by analysing the autolytic rate. As B. cereus demonstrates a high degree of autolysis around neutral pH [41], we analyzed autolysis in PBS buffer using late exponential cells, grown in LB or LBG media (Fig 8). The ΔclhAB2 strain showed accelerated autolysis compared to its parent strain in both media, which suggests again that PH activity is deregulated in the mutant. In addition, glucose had a remarkable effect on the observed differences (Fig 8). After four hours of incubation, the ΔclhAB2 autolysis rate was 40%, compared with 10% for Bc. Instead, with glucose-grown cells, the ΔclhAB2 autolysis rate was surprisingly high, 90% versus 60% for Bc. Furthermore, the autolysis rates of Bc and the ΔclhAB2ΔclhAB2 complemented strain were not different in either medium after four
hours of incubation. Our results suggested that the expression of clhAB2 in trans was sufficient for restoration of Bc autolysis in both growth conditions. Overall, these data indicate that clhAB2 negatively controlled autolysis phenomena in B. cereus, while glucose appears to enhance autolysis phenomena.

Discussion

Roles of CodY and CcpA in the regulation of clhAB2 operon

Although the Cid/Lrg regulation network has been extensively analyzed in S. aureus, only a few studies have been performed to identify the expression and function of these genes in other bacterial species. The present study aims to investigate the expression and function of the Bc clhAB2 operon which is a cid/lrg homolog, during bacterial growth in a nutrient-rich medium with or without glucose. Here, we show that the global transcriptional regulatory protein CodY is required for the basal level of clhAB2 expression under all conditions tested while CcpA, the major global carbon regulator, is needed for the high-level expression of clhAB2 in glucose-grown cells. Our genetic evidence suggests that CcpA control is exerted in an indirect way in the presence of glucose (i.e late-exponential growth phase) and the regulatory pathway remains to be characterized. In B. subtilis, the CcpA network analysis remains complex as CcpA controls a high number of regulators directly (i.e regulation of gene expression) or indirectly (i.e modulation of activity) [25,42,43]. In B. cereus, the transcriptome analysis of the ccpA mutant showed that CcpA positively and negatively regulates several putative transcriptional regulators suggesting that the CcpA regulatory network is also complex in B. cereus [26].

Next, we further analyzed the known in vitro CodY-binding sequence (5’-TAAATTCA GAAAATA-3’) [36] localized upstream of clhAB2. We selected a straightforward mutagenesis analysis, in which the total deletion of the CodY-motif sequence was performed. Our results showed that this 15 nt-sequence is absolutely required for mediating the clhAB2 expression, as the deletion of this sequence abolished the clhAB2 expression. However, we are aware that this CodY-binding site could be located immediately upstream of the -35 region or overlap with the clhAB2 promoter. More functional analyses of the CodY-binding motif by point mutational
analysis, together with the determination of the transcriptional start of clhAB2, should enable one to conclude that CodY activates the expression of clhAB2 through DNA binding to this CodY motif.

Model of clhAB2 regulation in the presence or absence of glucose

The model in Fig 9 depicts the regulation of clhAB2 expression in LB medium and in LB medium with glucose. This model is based on our results, as well as on B. subtilis and B. cereus studies [16,25–27,30,35]. In this regulatory model, we assume that CodY protein binds specifically to the 15-nc CodY motif localized upstream of clhAB2 (Fig 4). When Bc cells grow in a nutrient-rich medium such as LB medium, the uptake of exogenous ILV is enough to maintain ILV homeostasis. CodY integrates the ILV signal as ILV is a major effector molecule of CodY protein. ILV-bound CodY binds to the CodY binding sequence and assists RNA polymerase with transcribing the clhAB2 operon. When glucose is available, CcpA integrates the glucose

Fig 9. Two models for B. cereus clhAB2 regulation. Left: regulation of clhAB2 expression in an amino acid-rich medium. In a nutrient-rich medium (such as LB medium [13]), ILV uptake is sufficient to maintain the endogenous pool of ILV. The CodY global regulatory protein displays enhanced affinity for its DNA target when bound to ILV[27]. ILV-bound CodY binds to the CodY binding sequence upstream of clhAB2, and assists RNA polymerase with transcribing the clhAB2 operon (Figs 3 and 4). The expression level of clhAB2 is constant and moderate (i.e. basal level) during the late exponential and transition growth phases (Fig 1).

Right: regulation of clhAB2 expression in an amino acid-glucose-rich medium. In LB medium with 0.35% glucose, ILV-CodY binds to the CodY motif and activates clhAB2 expression, but the expression profile is different (Fig 1). CcpA plays a positive role by indirectly regulating the transcription of clhAB2 (Fig 2) and this regulatory pathway remains to be characterized. The role of known or hypothetical effector molecules is depicted with a dashed arrow. In the left part, the ILV effector [27] is depicted with a green dashed arrow; in right part, the Fru-6-P [25] and unknown glycolytic intermediate effectors are depicted with blue dashed arrows. Moderate and constant clhAB2 expression is depicted by three identical gray arrows. High and gradual clhAB2 expression is depicted by three non-identical gray arrows. The intracellular ILV pool is depicted as a green box. The CodY motif sequence is depicted as a red box. The unknown DNA-binding motif is depicted as a blue box. ?, unknown CcpA-dependent signaling pathway; ?, unknown transcriptional regulator. Fru-6-P, fructose-6-phosphate; Glc-6-P, glucose-6-phosphate; ILV, isoleucine, leucine, valine. CodY, CodY transcriptional regulator; CcpA, CcpA global carbon regulator; PTS, phosphotransferase system. The CodY-dependent clhAB2 operon is involved in cell shape, chaining and autolysis in Bacillus cereus ATCC 14579
signal through the control of glucose uptake (via the PTS system) and the control of glycolysis. In parallel, CodY integrates the ILV signal. As discussed above, it remains unclear how CcpA controls clhAB$_2$ expression in the presence of glucose. CcpA could control directly or indirectly an additional transcriptional regulator directly involved in the transcription of clhAB$_2$ operon.

Is there a regulatory link between the CcpA and CodY signaling pathways in the regulation of clhAB$_2$ expression? The answer will require further analyses, which are beyond the scope of this study. Interconnections between the CcpA and CodY regulons in carbon and nitrogen metabolism have been well documented in _B. subtilis_ [43–46], but these connections have not yet been addressed in detail in the _B. cereus_ group. The fact that clhAB$_2$ expression is controlled by such important major regulators provides evidence of the important role of clhAB$_2$ products in the physiology of _B. cereus_.

Role of clhAB$_2$ in the regulation of peptidoglycan hydrolase activity

The _Bc_ genome presents 42 putative and largely unknown peptidoglycan hydrolases (PHs) [47]. PHs are involved in fundamental aspects of bacterial physiology: peptidoglycan growth, daughter cell separation during cell division and remodeling of the peptidoglycan sacculus to determine cell shape [40,48,49]. In addition, some PHs are members of the bacterial autolytic system involved in cell death phenomena (autolysis) [40]. Furthermore, bacteria in general can have a large number of PHs with redundant functions, while a particular PH can have more than one enzymatic activity (different substrates). For example, some PHs play an important role in rod shape maintenance and daughter cell separation or daughter cell separation and autolysis [40,49]. The precise mechanisms by which PHs are controlled are largely unknown due to the complexity of the systems involved [40,49].

Here, we found that ΔclhAB$_2$ mutant cells produced abnormal short chains and wide cells in the presence of glucose during late exponential and transition growth phases (Figs 6 and 7, S3 Fig), while ΔclhAB$_2$ cells showed accelerated autolysis under autolysis-inducing conditions compared to wild-type cells (Fig 8). Overall, we obtained evidence to confirm that _Bc_ clhAB$_2$ operon modulates PH activities, which are required for proper cell shape and proper chain length during cell growth and down-regulates autolysins activity.

Chain lengthening in the presence of glucose

_B. cereus_ chaining has been addressed in different growth conditions and environments [37–39], but the underlying molecular mechanisms remain to be elucidated. Here, we show that _B. cereus_ clhAB$_2$ operon is involved in the formation of characteristic long chains in LBG growth medium at _t_$_0$. Quantitative fluorescence microscopy was used to assess chain length and inter-constriction cell arrangement differences. We have first focused on total cell number present in a chain as this simple morphological measure -chain length- has been widely used in several chaining studies [50,51]. We were able to identify short chain phenotype in the absence of glucose and long chain phenotype in the presence of glucose. However, we found that the biological variation of the chain length measure was important for all analysed bacterial populations (box plot graphs, Fig 6C and S1 Fig). Thus, we searched for an additional morphological measure for chain length evaluation. As described in _B. anthracis_ [52,53], the _Bc_ chains present several constrictions that are spaced two to eight cells apart. We were able to identify two relevant inter-constriction cell-numbers: four in the absence of glucose and eight in the presence of glucose. These observations allow us to assess two inter-constriction cell arrangements, namely "short" (2–4 cells) and "long" (6–8 cells). This new measure is an indirect way to evaluate chain length and allow us to differentiate clearly short and long chain phenotypes. In addition, data acquisition was faster and easier; indeed, inter-constriction cell
number counting requires fewer images because full chain images are not necessary and it was easier to generate large samples (N ≥ 200 arrangements vs. N ≥ 90 chains in our study). Moreover, we also demonstrated that chain lengthening was observed in glucose-grown cells in LB buffered with MOPS (S1 Fig). Our results show that the presence of glucose but not metabolic acid production was involved in chain lengthening in Bc strain. To conclude, we provide evidence that, through tight regulation by CodY and CcpA, the clhAB2 operon of B. cereus enables glucose-grown cells to maintain proper cell-chain lengths and cell size at the onset of the transition growth phase.

Supporting information

S1 Fig. Expression of clhAB2 and morphological changes of Bacillus cereus ATCC 14579 (Bc), ΔclhAB2 mutant (Δ), complemented mutant (ΔΩ) cell-chains in LB buffered with or without glucose. (A) Division septa and cytoplasmic membranes were imaged using the FM4-64 lipophilic dye. Top row, from the left: fluorescent micrographs of Bc, Δ, and ΔΩ chains at t0 in LB-MOPS medium and then in LB-MOPS medium with glucose 0.35%. Lower row: same order, phase-contrast images. Images of chains revealed strong constrictions (deeper invaginations) corresponding to cells undergoing separation. Scale bar (5 μm) is shown for each image. (B) Box plots of chain length (number of cells per chain) at t0 in Bc (blue), ΔclhAB2 (red), ΔclhAB2ΩclhAB2 (green) populations. 90 chains from two independent cultures were analysed. Median (strong line in the box), interquartile range (IQR; box), whiskers (1.5 x IQR) and outliers (dot) are presented. Significance is based on two tests, Mann-Whitney and Two-Sample Fisher-Pitman Permutation, with a P of <0.01**. (C) Distributions of “short” (≤4) and “long” (>4) inter-constriction cell types in the Bc, ΔclhAB2, and complemented mutant populations (N = 200 cell arrangements). Two inter-constriction arrangement types in Bc, ΔclhAB2, and ΔclhAB2ΩclhAB2 populations were defined (see Materials and methods). The first type, containing cell arrangements with two to four cells (“short”) and the second type, including cell arrangements with six to eight cells (“long”). (D,E) Cells of Bc, isogenic mutant strains (ΔcodY, codY-complemented mutant, ΔccpA, ccpA-complemented mutant) which all harbored the PclhAB2’-lacZ fusion, were grown in LB-MOPS medium without (closed symbols) or with 0.35% glucose (open symbols). Exponentially growing cultures of B. cereus were inoculated into standard LB medium [13] buffered with 50mM MOPS (3-(N-morpholino-propanesulfonic acid) (pH7.7± 0.2) or LB MOPS supplemented with 0.35% glucose at a final optical density of 0.05. (TIFF)

S2 Fig. CodY represses oppA gene (BC2026) expression in the presence or absence of glucose. Cells of B. cereus ATCC 14579 (Bc) and isogenic mutant strain ΔcodY, which all harbored the PoppA (BC2026)’-lacZ fusion, were grown in LB medium without (closed symbols) or with 0.35% glucose (open symbols). Exponentially growing cultures of B. cereus were inoculated into standard LB medium [13] buffered with 50mM MOPS (3-(N-morpholino-propanesulfonic acid) (pH7.7± 0.2) or LB MOPS supplemented with 0.35% glucose at a final optical density of 0.05. Representative experiment of n = 2 experiments are shown. pH7304-PoppA’-lacZ (BC2026) was obtained by inserting the DNA region upstream (corresponding to the intergenic region) of the Bc oppA gene between the PstI and XbaI cloning sites of pH7304-18Z. The resulting plasmid was then transferred into B. cereus by electroporation. (TIFF)

S3 Fig. ΔclhAB2 mutant cells produced abnormal short chains and wide cells in the presence of glucose. Phase-contrast images of Bacillus cereus ATCC 14579 (Bc) and ΔclhAB2...
mutant (Δ) chains at $t_1$, $t_0$ and $t_2$. The onset of the transition growth phase ($t_0$) was defined as the breakpoint in the slope of the log phase growth curve, and $t_n$ is the number of hours before (-) or after time zero [14]. One hour before the start of transition phase ($t_1$), and two hours after $t_0$ ($t_2$). LBG, LB medium with glucose 0.35%. Scale bar is 10 μM. Bacterial aliquots were removed from an exponential or early stationary phase cultures and observed with a Zeiss Axio Observer.Z1 inverted fluorescence microscope equipped with a Zeiss AxioCam MRm digital camera. Phase-contrast images were processed with Zeiss ZEN 2–lite software.

S4 Fig. Images of Bacillus cereus ATCC 14579 and ΔclhAB2 mutant chains and visualization of peritrichous flagella. Flagella, septa and constrictions were visualized using transmission electronic microscopy (TEM) after negative staining of bacteria. The sequential two-droplet method was used. For each condition, 1 ml of early post-exponential cells (OD between 3 and 4) grown in LB medium with glucose 0.35% was washed 2 times by centrifugation and resuspended and concentrated in 100 μl with PBS 1X. Mesh formvar carbon coated nickel grids (Electron Microscopy Sciences, LFG distribution, France) were used and bacteria bind to grid by adsorption. Then, for staining, a 1% (w/v) phosphotungstic acid (Sigma-Aldrich, USA) was used. Observations were performed using an HT7700 transmission electron microscope (Hitachi, Japan) equipped with an 8 million pixels format CCD camera driven by the image capture engine software AMT, version 6.02, at the INRA MIMA2 microscopy platform (Jouy-en-Josas, France). Images were made at 80 kV in high contrast mode with an objective aperture adjusted for each sample and magnification.

S5 Fig. Expression of clhAB2 in the presence of three different sugars and in the presence of various concentrations of glucose. Cells of Bc which harbored the transcriptional $P_{clhAB2}$-lacZ fusion construct, were grown in LB (closed symbols) or in LB with sugar (open symbols) media. Samples were harvested at the indicated times and were assayed for β-galactosidase specific activity. (A) Fructose, glucose or sucrose and (B) different glucose concentrations (0.3%-0.6% 1%) were added at the onset of the culture. Time zero corresponds to the entry into the transition growth phase. The data presented are representative of three independent experiments.

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