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CodY, a pleiotropic regulator, influences multicellular behaviour and efficient production of virulence factors in *Bacillus cereus*

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Summary

In response to nutrient limitation in the environment, the global transcriptional regulator CodY modulates various pathways in low G+C Gram-positive bacteria. In *Bacillus subtilis* CodY triggers adaptation to starvation by secretion of proteases coupled to the expression of amino acid transporters. Furthermore, it is involved in modulating survival strategies like sporulation, motility, biofilm formation, and CodY is also known to affect virulence factor production in pathogenic bacteria. In this study, the role of CodY in *Bacillus cereus* ATCC 14579, the enterotoxin-producing type strain, is investigated. A marker-less deletion mutant of *codY* ($\Delta codY$) was generated in *B. cereus* and the transcriptome changes were surveyed using DNA microarrays. Numerous genes involved in biofilm formation and amino acid transport and metabolism were upregulated and genes associated with motility and virulence were repressed upon deletion of *codY*. Moreover, we found that CodY is important for efficient production of toxins and for adapting from nutrient-rich to nutrient-limited growth conditions of *B. cereus*. In contrast, biofilm formation is highly induced in the $\Delta codY$ mutant, suggesting that CodY represses biofilm formation. Together, these results indicate that CodY plays a crucial role in

the growth and persistence of *B. cereus* in different environments such as soil, food, insect guts and the human body.

Introduction

Bacilli belonging to the '*Bacillus cereus* group' are commonly isolated from soil (Stabb *et al.*, 1994; von Stetten *et al.*, 1999; Garbeva *et al.*, 2003; Stenfors Arnesen *et al.*, 2008). The '*B. cereus* group', also known as *B. cereus sensu lato*, is a widely used term for six genetically highly related species, including the food poisoning *B. cereus* (Bottone, 2010), the causative agent of the mammalian disease anthrax *Bacillus anthracis* (Mock and Fouet, 2001), the insect pathogen *Bacillus thuringiensis* (Aronson and Shai, 2001), the psychrotolerant *Bacillus weihenstephanensis*, and the species *Bacillus mycoides* and *Bacillus pseudomycoides* (Lechner *et al.*, 1998; Nakamura, 1998; Di Franco *et al.*, 2002). *Bacillus cereus* is believed to thrive as soil saprophyte, living in various potentially pathogenic lifestyles (Jensen *et al.*, 2003). In these different lifestyles (e.g. in soil, food, invertebrate gut, or in the human body) *B. cereus* encounters variable environments with varying resource quality and quantity. The nutrients are often present in dilute concentrations, transiently available or inaccessible due to a low water activity. To survive such variable environments, *B. cereus* has to accommodate to these conditions by expressing the most appropriate genes. Adaptation to changing environments can be achieved by harbouring a collection of genes coding for versatile metabolic pathways and by regulating these genes in response to changes in the environment. Central metabolism of *Bacilli*, including *B. cereus*, is modulated at the transcriptional level using both specific and global regulators (Sonenshein, 2007). CodY and TnrA were reported in *Bacillus subtilis* as global regulators of genes involved in nitrogen metabolism, while CodY, next to CcpA (a transcriptional regulator of carbon catabolite repression), also affects genes associated with carbon metabolism (Fisher, 1999). The intertwined actions of these regulators carefully modulate gene expression in response to changes in environmental conditions and resource availability.

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CodY, found solely in low G+C Gram-positive bacteria, is important for the adaptive response to starvation (Sonenshein, 2005). In *B. subtilis*, CodY responds to the intracellular levels of GTP and branched chain amino acids (BCAA). Binding of GTP induces dimerization of CodY. BCAA bind to the so-called GAF domain localized at the N-terminal part of the protein. In response to BCAA binding, conformational changes activate the DNA binding properties of the dimerized form of CodY. The helix–turn–helix (HTH) motif at the C-terminal region of CodY is highly conserved among various bacteria, suggesting that CodY homologues may recognize and bind similar target DNA sequences (Joseph *et al.*, 2005). The DNA motif associated with the binding of CodY, AATTTTCW-GAAAATT, was studied and experimentally verified in several Gram-positive bacteria (den Hengst *et al.*, 2005; Guedon *et al.*, 2005; Belitsky and Sonenshein, 2008; 2011). CodY is a pleiotropic transcriptional regulator that represses the transcription of numerous genes. As a response to lower energy and BCAA levels CodY triggers adaptation of bacterial cells by activating highly diverse mechanisms, such as secretion of proteases and the expression of amino acid transporters and catabolic pathways. In *B. subtilis* it has also been shown that CodY affects differentiation processes, including sporulation, competence development for DNA uptake, motility and biofilm formation (Slack *et al.*, 1995; Serron and Sonenshein, 1996; Wray *et al.*, 1997; Bergara *et al.*, 2003; Kim *et al.*, 2003; Molle *et al.*, 2003; Hsueh *et al.*, 2008). Furthermore, CodY plays an important role in the regulation of virulence factors in pathogenic bacteria, such as *B. anthracis* (van Schaik *et al.*, 2009), *Clostridium difficile* (Dineen *et al.*, 2007), *Listeria monocytogenes* (Bennett *et al.*, 2007), *Staphylococcus aureus* (Majerczyk *et al.*, 2008) and Streptococci (Malke and Ferretti, 2007; Hendriksen *et al.*, 2008; Lemos *et al.*, 2008). The role of CodY in metabolic processes and virulence in *B. cereus* is however largely unknown.

Based on the abundance of proteolytic enzymes, the multiplicity of peptide and amino acid transporters, and variety of degradation pathways of proteins and peptides, amino acids have been suggested to be potentially important nutrient sources for *B. cereus* (Ivanova *et al.*, 2003). Accordingly, *B. cereus* ATCC 14579 was shown to be able to utilize several amino acids and numerous dipeptides for growth (Mols *et al.*, 2007). Bacterial growth in soil, a natural environment for *B. cereus*, is frequently regarded as subjected to starvation due to the limited amount of resources (Foster, 1988). Therefore, the regulation of nitrogen metabolism is likely to be important for the adaptation of *B. cereus* to environments including soil and foods.

Not only metabolic pathways are regulated throughout the life cycle of *B. cereus*, also the production of toxins and other phenotypic attributes may be controlled by the

availability of nutrients and differences between environments. For example, the emetic toxin cereulide is produced in food and enteric toxins are produced in the small intestine of the host during the onset of diarrhoeal disease (Stenfors Arnesen *et al.*, 2008). *Bacillus cereus* contains a *codY* orthologous gene that may be involved in the regulation of metabolic pathways, virulence and other characteristics of *B. cereus*. To get more insight in the role of CodY in the type strain *B. cereus* ATCC 14579, we created a *codY* deletion strain and examined the effect of this mutation on the transcriptome, the growth and the virulence potential of *B. cereus*.

Results

The codY orthologue of B. cereus and its mutagenesis

A survey for the *codY* gene in the genome of *B. cereus* ATCC 14579 resulted in the identification of BC3826. BC3826 is localized in the four gene cluster (BC3829–BC3826) comprising genes coding for CodV and ClpQY homologues. The gene BC3826 is predicted to code for a protein that shows 81% identity to the *B. subtilis* CodY protein. The putative CodY protein of *B. cereus* contains highly conserved regions likely important for its activity: GTP- and BCAA-binding motifs, the amino acid residues involved in dimerization, and the HTH domain that is identical to the *B. subtilis* CodY protein (Levdikov *et al.*, 2006). The conservation of the HTH motif, which is involved in DNA binding, suggests that CodY of *B. cereus* recognizes and bind identical or very similar DNA sequences in the target promoter regions.

To examine the role of CodY in *B. cereus* ATCC 14579, the gene BC3826 was deleted from the chromosome using a pMAD-derived vector (Arnaud *et al.*, 2004) containing an additional I-SceI site (see *Experimental procedures*). Using this method (Janes and Stibitz, 2006), no antibiotic marker gene is inserted upon deletion of the *codY* gene BC3826 omitting any polar effects. Further, *codY* is the last gene of the four genes containing transcriptional unit, followed by a strong ρ -independent transcriptional termination sequence (ΔG of -15.6 predicted by Clone Manager 7 software). The cell morphology of wild-type and $\Delta codY$ mutant strains were examined after overnight culturing in LB medium. The wild-type culture contained single cells, while the $\Delta codY$ mutant strain showed long chains of cells (Fig. 1). Similar changes in morphology were not observed for cells grown overnight in BHI medium, suggesting that medium composition is affecting the role of CodY in determining cellular growth. Nonetheless, the cell size was not significantly altered in the $\Delta codY$ deletion strain. The mutation of *codY* gene in *B. cereus* was complemented by introducing an IPTG inducible copy of *codY* on plasmid pLMcodY, coupling *codY* expression to the presence of

Fig. 1. Phase-contrast microscope pictures of wild-type and $\Delta codY$ mutant strains. Phase-contrast microscopy of wild type (A), $\Delta codY$ mutant (B) and complementation strain (C–G) cells grown overnight in LB medium. The complemented strain was grown in the absence (C) or presence of 10 μ M (D), 100 μ M (E), 1 mM (F) and 10 mM (G) IPTG. Scale bar is 10 μ m.

IPTG in the medium. The complemented $\Delta codY$ mutant strain showed wild-type cell morphology after growing overnight in LB medium supplemented with 0.1–10 mM IPTG (Fig. 1E–G).

Transcriptome analysis of B. cereus ATCC 14579 and its codY mutant

Transcriptome analyses of various Gram-positive bacteria revealed genes affected by $\Delta codY$ mutations. In all organisms examined so far, CodY represses genes involved in amino acid metabolism. The genome of *B. cereus* ATCC

14579 harbours a vast amount of genes encoding enzymes related to amino acid metabolism. Furthermore, *B. cereus* can be found in wide range of environments, e.g. soil (Vilain *et al.*, 2006), plant rhizosphere (Berg *et al.*, 2005), various foods (Rosenquist *et al.*, 2005), and in faeces of healthy humans (Ghosh, 1978), where it encounters various amino acid compositions. Thus, CodY may have a pivotal role under such variable conditions. To determine which genes are affected by CodY in *B. cereus*, we compared the transcriptome profile of exponentially growing cells of wild-type and $\Delta codY$ strains in the nutrient-rich BHI medium, where CodY was suggested to exert a strong repressing activity. Analysis of the DNA microarray data of four biological replicates revealed that 248 and 176 genes were significantly (at least threefold with a *P*-value of $< 10^{-4}$) up- and downregulated respectively (Table S1).

As expected, the list of upregulated genes contained genes coding for proteins involved in amino acid transport and metabolism (Fig. 2). The most upregulated genes in the $\Delta codY$ strain included the *opp* genes coding for putative oligopeptide binding and transport proteins (BC3585 and BC2026). The BCAA metabolism coding genes (*ilv* and *leu* gene clusters) were also significantly elevated similar to the *pep* genes coding for peptidases that hydrolyse a broad range of dipeptides. CodY repressed cellular processes related to amino acid, carbohydrate, nucleotide, lipid metabolism and transport and energy production and conversion (Fig. 2).

The list of downregulated genes included genes that are associated with processes not previously shown to be affected by CodY. Genes coding for proteins involved in motility, cell lysis and toxin production were downregulated when the *codY* gene was deleted. Twenty-three genes of the virulence-related PlcR regulon were significantly downregulated (ratio < 0.33) in the $\Delta codY$ strain including genes that are involved in toxin production (indicated with an asterisk in Table S1). Also the remaining genes of the PlcR regulon were mildly downregulated (ratio 0.43–0.8).

Analysis of the upstream regions of differentially regulated genes revealed the presence of previously identified CodY-binding motifs (Guedon *et al.*, 2005; den Hengst *et al.*, 2005; Belitsky and Sonenshein, 2008) for several upregulated genes (e.g. BC0255, BC0511, BC1396, BC1836, BC2045, BC2222, BC2285, BC3085, BC4645, BC4659, BC5091 and BC5416), but only for few downregulated genes (e.g. BC0556, BC5101 and BC5387). The lack of CodY motifs in front of the downregulated genes that are related to motility, cell lysis and toxin production suggests that these genes are likely to be indirectly affected by CodY.

To validate our microarray results and the observed changes in gene expression, we selected several target genes and fused their promoter region with the promoter-

Fig. 2. Number of differentially expressed genes between wild-type and $\Delta codY$ mutant strains. Numbers of genes belonging to the clusters containing up- (black) or downregulated (white) genes upon *codY* deletion classified using COG functional categories.

less *gfp* gene. The promoter regions of the upregulated BC2026 (*oppA*), BC1396 (*bcaA*) and BC0699 (*rocE*) genes coding for proteins involved in amino acid transport and metabolism and the downregulated BC1110 (*cytK*) and BC1625 (*motA*) genes coding for cytoxin K and involved motility, respectively, were selected. Detection of fluorescence showed that the expression of BC2026, BC1396 and BC0699 was derepressed in the $\Delta codY$ strain, while the expression of BC1110 and BC1625 was lower in the $\Delta codY$ mutant (Fig. 3). In addition, the expression levels of BC1809 (*nheA*) and BC1810 (*nheB*) genes coding for the components of the non-haemolytic enterotoxin were examined using reverse transcription quantitative PCR (RT-qPCR). This RT-qPCR experiment showed that the expression level ratios of *nheA* and *nheB* genes were $0.22 (\pm 0.07)$ and $0.2 (\pm 0.03)$, respectively, in the $\Delta codY$ mutant strain compared with the wild-type strain. Thus, our microarray results were validated by promoter-GFP fusions and RT-qPCR.

Role of *CodY* in growth

The large number of genes involved in amino acid metabolism and energy production that were upregulated in the $\Delta codY$ mutant prompted us to investigate the growth of the $\Delta codY$ mutant in various conditions (Fig. 4).

In nutrient-rich conditions the growth of the $\Delta codY$ mutant was affected. After reaching the mid-exponential phase of growth the $\Delta codY$ mutant proliferated a little slower than the wild type. No significant differences in growth were observed between the wild-type and the $\Delta codY$ mutant strain when grown in M0 medium supplemented with casamino acids. When wild-type and $\Delta codY$ mutant cells were shifted during exponential growth from BHI with additional glucose to M0 medium supplemented with casamino acids the initial small difference between the two strains became much larger. The growth of the $\Delta codY$ mutant was diminished in this condition. The decreased successive growth after shifts from nutrient-rich to nutrient-limited conditions and the equal growth in nutrient-limited conditions suggest a role for *CodY* in the switch in central metabolism from glucose rich conditions to nutrient-limited conditions. Therefore, a role of *CodY*-dependent regulation in central metabolism and amino acid metabolism can be suggested and has also been found for *B. subtilis* (Sonenshein, 2007).

CodY regulates community behaviour; motility and biofilm formation

Motility and biofilm formation are related differentiation processes in bacteria (Abee *et al.*, 2011). Although motility

Fig. 3. Expression profiles of CodY regulated genes. *Bacillus cereus* ATCC 14579 (solid lines) and $\Delta codY$ mutant (dashed lines) strains, carrying fusions of the upstream region of BC2026 (A), BC1396 (B), BC0699 (C), BC1110 (D) or BC1625 (E), to *gfp* in pAD123, were grown at 30°C in BHI medium. Growth curves (grey lines) and GFP activities (black lines) are shown. Fluorescence (arbitrary units) is indicated on the left y-axis, OD_{600} is denoted on the right y-axis and time (h) is indicated on the x-axis. Four independent replicates were carried out. Error bars indicate standard deviations.

Fig. 4. Role of CodY in the growth of *B. cereus*. Wild-type (squares) and $\Delta codY$ mutant (circles) cells were grown in BHI medium supplemented with 0.5% glucose (A), M0 medium with casamino acids (B), and shifted from BHI with additional glucose upon reaching an OD of approximately 0.3 (indicated with an arrow), to M0 medium (C). The mean of three independent replicates is shown, error bars depict the standard deviation, and significant differences are indicated by asterisks (Student's *t*-test < 0.01).

Fig. 5. Motility and biofilm formation of *B. cereus* wild-type and $\Delta codY$ mutant strains.
A. Swimming radius of the *B. cereus* wild-type, $\Delta codY$ mutant and complementation strains in the absence and presence of 10 mM IPTG. Scale bar indicates 1 cm.
B. Biofilm formation of *B. cereus* wild-type, $\Delta codY$ mutant and complementation strains in the absence and presence of 10 mM IPTG in EPS medium for 24 h. Biofilm was stained with 0.3% crystal violet, washed with distilled water and biofilm-bound crystal violet was solubilized using 70% ethanol.

has been indicated to be important for the initiation of static biofilms of *B. cereus* (Houry *et al.*, 2010), biofilm generally consists of non-motile cells. Additionally, the phenotypes of motile single cells and sessile biofilm forming cells are distinct in *Bacilli* (Kobayashi, 2007). Our microarray analysis showed decreased gene expression of motility genes in the $\Delta codY$ deletion strain of *B. cereus*. Motility of the $\Delta codY$ mutant and the complementation strain was compared with the wild-type strain on swimming plates (LB medium containing 0.3% agar). The motility of the $\Delta codY$ deletion strain, recorded at 20 or 46 h after incubation, was reduced compared with that of the wild-type strain (Fig. 5 and Table 1). Motility was partially complemented by the expression of the *codY* gene using an IPTG-induced Pspac promoter. However, the degree of complementation was dependent on the IPTG concen-

tration used (Table 1). A low expression in non-induced samples of the cloned genes using the pLM5 vector was also observed in previous studies (Grande Burgos *et al.*, 2009) indicating that the promoter used showed significant leakiness. This could explain the low, but significant, complementation in the non-induced samples compared with the $\Delta codY$ mutant.

A previous study on a *B. cereus* transposon mutant of strain UW101C containing a disrupted *codY* gene showed fourfold reduction of biofilm formation (Hsueh *et al.*, 2008). The marker-less deletion of *codY* in the *B. cereus* strain ATCC 14579 resulted in elongated cells and reduced motility as mentioned above. The biofilm formation of the *B. cereus* $\Delta codY$ deletion strain was examined on EPS, a low-nutrient medium (Hsueh *et al.*, 2006). After 24 h induction, biofilm formation in EPS by the $\Delta codY$ mutant was about 3.5-fold higher (OD_{590} of 0.70 ± 0.23) than that by the wild-type strain (OD_{590} of 0.19 ± 0.09) as measured with crystal violet. When *codY* was reintroduced into the $\Delta codY$ mutant, biofilm formation was significantly reduced (OD_{590} of 0.11 ± 0.02) compared with the $\Delta codY$ mutant in the presence of 10 mM IPTG. However, biofilm formation was also reduced in the non-induced samples (OD_{590} of 0.15 ± 0.02). This is in agreement with the elevated transcription of the gene cluster (6.5–38 times upregulation in the $\Delta codY$ mutant) that codes for the putative homologues of *B. subtilis* proteins involved in the production of biofilm matrix (BC1278, BC1279 and BC1281 coding for SipW, TasA and TapA homologues respectively).

CodY is required for efficient toxin production

Microarray analysis of the $\Delta codY$ strain showed down-regulation of genes regulated by PlcR. The PlcR regulon includes genes that code for various enterotoxins: cytotoxin K (CytK coded by BC1110), non-haemolytic enterotoxin (NheABC coded by BC1809–BC1811) and haemolysin BL (HblCDBA coded by BC3104–BC3101) (Gohar *et al.*, 2008). The levels of CytK, NheA and NheB

Table 1. Motility assay of wild-type and $\Delta codY$ mutant strains.

	Incubation time (h)	Radius in mm (SD)
ATCC 14579	20	19.7 ($\pm$ 5.5)
	46	40.1 ($\pm$ 6.5)
$\Delta codY$	20	6.1 ($\pm$ 1.6)
	46	16.2 ($\pm$ 3.4)
$\Delta codY+pLMcodY$	20	9.2 ($\pm$ 2.0)
	46	21.7 ($\pm$ 2.4)
$\Delta codY+pLMcodY$ 0.1 mM IPTG	20	10.3 ($\pm$ 2.4)
	46	23.5 ($\pm$ 2.3)
$\Delta codY+pLMcodY$ 1 mM IPTG	20	12.4 ($\pm$ 0.5)
	46	27.0 ($\pm$ 1.0)
$\Delta codY+pLMcodY$ 10 mM IPTG	20	15.0 ($\pm$ 1.0)
	46	34.2 ($\pm$ 1.6)

Fig. 6. Western blots of toxin proteins in culture supernatants of wild-type (wt) and $\Delta codY$ deletion strains. Western blots of culture supernatants of wild-type and $\Delta codY$ mutant strains using antibodies against CytK (A), NheA (B) and NheB (C) as indicated. Sizes of selected bands of the SeeBlue Plus2 Pre-Stained Standard are indicated on the left side of each picture and the expected size of the target proteins is shown on the right.

proteins decreased in the $\Delta codY$ mutant as shown using Western blot analysis (Fig. 6). The haemolytic activity of *B. cereus* cells caused by haemolytic toxins (e.g. haemolysin BL) was assessed on sheep blood containing BHI agar plates (Beecher and Wong, 1994). Beta haemolysis surrounding colonies on blood agar is a diagnostic character of *B. cereus* haemolytic activity. The $\Delta codY$ strain showed a reduced haemolytic zone as indicative of reduced haemolysis (Fig. 7). The haemolytic activity of the $\Delta codY$ strain with the complementation construct was slightly higher the presence of 10 mM IPTG than the $\Delta codY$ mutant and did not show full complementation similar to the motility tests.

Discussion

The function of CodY and its regulon has been studied in detail in *B. subtilis* (Sonenshein, 2007). It is known that CodY is a pleiotropic regulator, sensing intracellular BCAA and GTP levels, and that it is present in low-GC Gram-positive organisms. Furthermore, the role of CodY in growth under certain conditions and competence has been shown in *B. subtilis*. The role of CodY in virulence and the formation of toxins have been investigated in various human pathogens, including *Streptococci*, *S. aureus* and *L. monocytogenes* (Stenz *et al.*, 2011). In *B. anthracis*, a notorious pathogen belonging to the *B. cereus sensu lato* group, CodY was needed for full virulence via the plasmid-encoded AtxA regulator and its associated toxins (van Schaik *et al.*, 2009). However,

Fig. 7. Haemolytic activity of wild-type (wt) and $\Delta codY$ mutant strains. Growth of *B. cereus* ATCC 14579, $\Delta codY$ mutant and complemented strain in the absence or presence of 10 mM IPTG on sheep blood agar. Halos around the spotted colonies represent haemolytic activity after 24 h of incubation at 30°C. Scale bars indicate 5 mm.

virtually nothing is known about the role of CodY in *B. cereus* growth and virulence, besides a transposon mutant showing a defect in biofilm formation (Hsueh *et al.*, 2008). Therefore, we made an isogenic marker-less knockout mutant and a comparative transcriptome analysis was performed using DNA microarrays.

The transcriptional profiling of our $\Delta codY$ mutant strain revealed that CodY is repressing a large number of genes, which confirms that CodY is a pleiotropic transcriptional regulator. It regulates the expression of numerous genes involved in amino acid metabolism and genes associated with energy conversion and generation. The involvement of CodY in nitrogen metabolism is corroborated by the diminished capability to adapt to nutrient-limited conditions. However, the growth of the $\Delta codY$ mutant was not significantly different in a very poor medium. The relation of CodY with central carbon- and nitrogen metabolism in *B. cereus* was expected based on the predicted protein sequence showing GTP- and BCAA-binding domains and the experimental data on the Gram-positive model organism *B. subtilis* (Sonenshein, 2007). Furthermore, a distinct gene cluster, involved in matrix formation within biofilms in *B. subtilis* (Branda *et al.*, 2006; Romero *et al.*, 2011), was also repressed by CodY. This cluster, including *tasA* and associated signal peptidases, were highly upregulated in the $\Delta codY$ mutant. The *sinIR* genes coding for the homologues of the biofilm regulators in *B. subtilis* (Kearns *et al.*, 2005) and located downstream of the operon containing *tasA*, were also found to be upregulated. Although the experimental data showing the involvement of these particular genes in biofilm formation is lacking, we do observe an increased biofilm formation in the $\Delta codY$ mutant. In contrast, in a different *B. cereus* strain with a transposon disrupting its *codY* gene a decreased biofilm forming capacity was shown (Hsueh *et al.*, 2008). This contradiction may result from the use of a different strain, polar effects of the presence of a transposon or a combination of these aspects.

Besides genes that were repressed by CodY the transcriptome analysis revealed a large set of genes to be directly or indirectly induced by CodY. The expression of flagellar and motility genes was highly downregulated in the $\Delta codY$ mutant. Indeed, reduced motility of the $\Delta codY$ mutant was shown in a swimming assay. In *B. subtilis* and *L. monocytogenes*, in contrast to *B. cereus*, CodY has been described to repress motility (Bergara *et al.*, 2003; Bennett *et al.*, 2007) in *B. subtilis* by binding to the *hag* and *fla/che* promoter regions. Next to the reduced expression of genes involved in motility, the transcription of genes involved in cell wall hydrolysis and therefore cell separation are also decreased. The tempered expression of cell separation genes may explain the observed long chain like growth of the $\Delta codY$ mutant strain and perhaps it also contributed to the reduced swimming behaviour of

the mutant cells compared with the wild type. To our surprise many, if not all, PlcR-regulated virulence factors, including *cytK*, *nheA*, *nheB* and *hbl*, were highly downregulated in the *codY* mutant suggesting a role for CodY in the expression of toxin genes and virulence. The diminished expression of *cytK*, *nheA* and *nheB* in the *codY* mutant was shown by Western blots and the reduced haemolytic activity was shown on blood agar plates. *In vivo* assays in model systems, e.g. in the insect *Galleria mellonella* could eventually demonstrate how the toxic properties of the *B. cereus* $\Delta codY$ mutant are altered. Our preliminary experiment showed that the toxicity of the *B. cereus* $\Delta codY$ strain was lowered when examined on mammalian cell cultures (Lindbäck and Granum, 2006). Supernatant of the *B. cereus* $\Delta codY$ mutant, harvested at late exponential phase (OD₆₀₀ of 2.4), was tested for toxicity in the Vero cell cytotoxicity assay and showed around 50% reduction compared with the wild type ($34 \pm 10\%$ inhibition of protein synthesis compared with the $71 \pm 21\%$ inhibition by ATCC 14579 strain). The correlation between CodY and virulence and toxin production has been shown for other Gram-positive organisms such as *L. monocytogenes*, *Streptococcus pneumoniae*, *S. aureus* and *B. anthracis* (where PlcR is non-functional) (Stenz *et al.*, 2011). In some cases, CodY regulates virulence indirectly by decreasing the transcriptional or post-transcriptional level of a regulator related to pathogenicity [e.g. the transcription of *tcdR* in *C. difficile* (Dineen *et al.*, 2007) and the protein level AtxA in *B. anthracis* (van Schaik *et al.*, 2009)]. In other bacteria, genes involved in pathogenicity are directly regulated by CodY [e.g. in *S. aureus* (Majerczyk *et al.*, 2008) and *S. pneumoniae* (Hendriksen *et al.*, 2008)]. The lower expression of *plcR* in the $\Delta codY$ mutant strain of *B. cereus* suggests that toxin production is indirectly upregulated by CodY through double repression. However, direct effects on genes related to virulence cannot be excluded [e.g. microbial collagenase (BC0556) or perfringolysin O (BC5101)].

Bacillus anthracis is a member of the *B. cereus sensu lato* group (Kolsto *et al.*, 2009) and is sometimes regarded as the same species as *B. cereus* (Helgason *et al.*, 2000). CodY is required for full virulence of *B. anthracis* in mice (van Schaik *et al.*, 2009), thus a correlation between *codY* and virulence has been shown for *B. cereus sensu lato*. However, the virulence factors associated with the pathogenicity of *B. anthracis* which were studied are all located on virulence plasmids pXO1 and pXO2. The *B. cereus* type strain ATCC 14579 does not harbour these plasmids or any pXO1-like plasmids (Ivanova *et al.*, 2003). Furthermore, the virulence factors associated with the common food-borne illness of *B. cereus* are not encoded on plasmids (Ivanova *et al.*, 2003). These toxins, phospholipases and other virulence factors are controlled by the repressor PlcR (Gohar *et al.*, 2008). This study is the first showing

that CodY regulates expression of PlcR and concomitantly the PlcR regulon, which was not functional in *B. anthracis*. Another CodY-regulated aspect in the pathogenicity of *B. cereus*, not present in *B. anthracis*, is motility. In many bacteria flagella play a crucial role in the virulence against hosts. In *B. cereus sensu lato* motility has been shown to contribute to eye infections or *Bacillus* endophthalmitis (Callegan *et al.*, 2005), and may also contribute during diarrhoeal food poisoning.

Bacillus cereus is described as a soil saprophyte, living on organic matter especially at the plant rhizosphere (Vilain *et al.*, 2006; Stenfors Arnesen *et al.*, 2008). However, it has also got a completely different life as a pathogen. In soil, nutrients are often scarce and not continuously available. However, at certain moments the nutrient availability may increase in soil, for example when decaying organic matter is present. When nutrients are available, CodY represses biofilm formation and enhances motility. This may aid in dispersal of *B. cereus* through the soil, when energy and nutrients are abundantly available. Upon depletion of nutrients, flagella are too costly and a biofilm mode of growth may be preferred. Sporulation, as a last strategy to survive starvation, is under the control of CodY in *B. subtilis* (Ratnayake-Lecamwasam *et al.*, 2001). In our study this was not observed, because the experiments were performed mostly in nutrient-rich conditions which do not allow sporulation in *B. cereus*. Furthermore, in M0 broth, sporulation initiation and spore morphology of the $\Delta codY$ mutant was similar to that of the wild type. Therefore, no indications were found that CodY also plays a role in sporulation of *B. cereus* in nutrient-limited conditions. *Bacillus cereus* is also a notorious food spoilage organism and it can reach very high cell densities in food with a high nutrient content. Another putative nutrient-rich environment for *B. cereus* is the insect gut. However, in order to liberate amino acids and other nutrients, proteases, peptidase, phospholipases and other virulence factors are required. In order to switch between high- and low-nutrient-availability environments *B. cereus* needs to govern expression of specific metabolic pathways and in some cases toxins. In this study, CodY is shown to be such primary important transcriptional regulator.

To conclude, CodY plays multiple roles in *B. cereus*. It regulates genes involved in amino acid metabolism, energy production and biofilm formation and has therefore a role in the saprophytic lifestyle of *B. cereus*. Furthermore, CodY is required for efficient production of several toxins, which indicates a direct role of CodY in the pathogenic lifestyle of *B. cereus*. Thus, CodY is a key pleiotropic regulator involved in the growth and persistence of *B. cereus* in different environments such as soil, food, insect guts and the human body.

Experimental procedures

Strains and growth conditions

Bacillus cereus ATCC 14579, the sequenced enterotoxin-producing type strain, was obtained from the American Type Culture Collection. *Escherichia coli* strains MC1061 and TOP10 were used for cloning. Strains were grown in Lennox Broth [LB (Oxoid)] or Brain Heart Infusion broth [BHI (Becton Dickinson)] for the microarray experiment and reporter studies. M0 medium (3.0 mM K_2HPO_4 , 3.5 mM KH_2PO_4 , 0.8 mM $MgSO_4$, 0.04 mM $MnCl_2$, 0.2 mM NaCl, 0.2 mM $CaCl_2$, 0.05 mM $ZnCl_2$, 0.04 mM $FeCl_3$, 2 mM glutamic acid, 20 mM glucose) supplemented with 0.02% casamino acids was used for nutrient limitation experiments (Mols *et al.*, 2007). *Bacillus cereus* strains were grown at 30°C. Haemolytic activity was tested using BHI agar plates (1.5% agar) supplemented with 4% defibrinated sheep blood (Johnny Rottier, the Netherlands).

Construction of a *B. cereus* ATCC 14579 $\Delta codY$ mutant

An *in-frame* $\Delta codY$ deletion mutant was constructed by replacing *codY* with the sequence ATGACGCGTTAA (5'–3') using the marker-less gene replacement method (Janes and Stibitz, 2006) with minor modifications. All PCRs were conducted in an Eppendorf Mastercycler gradient using DyNAzyme II DNA polymerase and dNTP Mix (Finnzymes, Finland) according to the instructions by the manufacturer. PCRs were performed using 95°C for 1 min, 30 cycles of 1 min at 95°C, 52°C for 1 min and 72°C for 1 min, and finally 5 min at 72°C. PCR products were analysed by electrophoresis on 1.0% agarose gel.

The upstream and downstream regions of *codY* were amplified by PCR using genomic DNA from *B. cereus* ATCC 14579 (oligo 1–4, Table S2) respectively. The reverse primer (oligo 2) used to obtain the upstream region and forward primer (oligo 3) used for the downstream region, contain MluI restriction sites. Amplicons were cloned into pCR 2.1-TOPO (Invitrogen) and further transformed into *E. coli* One Shot TOP10 (Invitrogen). The downstream region (*codY* down) was digested from the vector using MluI and XbaI and ligated into the MluI and XbaI sites of the pCR 2.1-TOPO containing the upstream region (*codY* up). The complete construct (*codY* up and *codY* down) were now excised from pCR 2.1-TOPO using EcoRI and ligated into the corresponding restriction site of the thermosensitive shuttle vector pMAD (Arnaud *et al.*, 2004) containing an additional I-SceI site (a kind gift from Dr Annette Fagerlund, University of Oslo, Norway). The pMAD $\Delta codY$ vector was introduced by electroporation into *B. cereus* ATCC 14579 electrocompetent cells, which were made according to Mahillon and colleagues (1989) with the following modifications. The cultures were grown in BHI at 37°C, the centrifugation steps were carried out at room temperature and resuspension of the pellets after washing was done in 40% polyethylene glycol (PEG) 6000 (Merck, Darmstadt, Germany). Electroporation was performed in electroporation cuvettes (Cat. No. 165-2086, Bio-Rad Laboratories, Hercules, CA) at 2.2 kV, 4 mS, with an Eppendorf Eporator apparatus (Eppendorf AG). Subsequently, the cells were

recovered in LB broth at 37°C, 150 r.p.m., for a minimum of 4 h. Integration of the vector plasmid (pMAD Δ codY) into the chromosome by recombination events (via homologous sequences) was performed as described by Arnaud and colleagues (2004). After verification of the transformants, pBKJ233 containing the gene for the I-SceI enzyme was introduced by electroporation. The I-SceI enzyme makes a double-stranded DNA break at the desired sequence. Subsequently, homologous recombination leads eventually to the desired genetic replacement. The deletion of *codY* was verified by PCR amplifications using oligonucleotides located upstream and downstream from *codY* (oligo 5 and 6, Table S2) on chromosomal DNA purified from clones. To confirm the deletion of *codY* deletion DNA sequencing was performed (Source BioScience Lifesciences, UK), and the sequence has been deposited in GenBank under Accession No. JN968461.

DNA microarray analysis

Four independent biological replicates of exponentially growing *B. cereus* ATCC 14579 and its Δ codY derivative were grown in BHI medium at 30°C, 225 r.p.m., until the optical density reached 1.7–1.8 at 600 nm measured with a Genesys 20 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). Pellets, harvested by centrifugation (10.397 g, 1 min, RT), were immediately frozen in liquid nitrogen and stored at –80°C until RNA isolation. Total RNA was extracted using the Macaloid/Roche protocol (van Hijum *et al.*, 2005). RNA concentrations and purity were assessed using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). RNA samples were reverse-transcribed into cDNA using the Superscript III reverse transcriptase kit (Invitrogen, Carlsbad, USA) and labelled with Cy3 or Cy5 monoreactive dyes (GE Healthcare, Amersham, the Netherlands). Labelled and purified cDNA samples (Nucleospin Extract II, Biokè, Leiden, the Netherlands) were hybridized in Ambion Slidehyb #1 buffer (Ambion Europe) at 49°C for 18–20 h to DNA microarrays containing amplicons of 5200 annotated genes from the genome of *B. cereus* ATCC 14579, where each open reading frame is represented by duplicates spots (Grande Burgos *et al.*, 2009). The arrays were constructed as described elsewhere (van Hijum *et al.*, 2003a). Slide spotting, slide treatment after spotting and also slide quality control were performed as previously reported (Kuipers *et al.*, 2002). After hybridization, slides were washed for 5 min in 2× SSC (1× SSC contains 0.15 M NaCl and 0.015 M Na₃-citrate) with 0.5% SDS, two times 5 min in 1× SSC with 0.25% SDS, 5 min in 1× SSC 0.1% SDS, dried by centrifugation (2 min, 2.000 r.p.m.) and scanned in a GenePix 4200AL scanner (Axon Instruments, CA, USA). Fluorescent signals were quantified using ArrayPro 4.5 (Media Cybernetics, Silver Spring, MD, USA) and further processed and normalized using MicroPrep (van Hijum *et al.*, 2003b). CyberT (Baldi and Long, 2001) was used to perform statistical analyses. Genes with a Bayes *P*-value of < 0.00001 and $\geq$ 3-fold differentially expressed compared with the control, were considered significantly affected. The raw and processed data have been deposited in MIAME compliant Gene Expression Omnibus database (Accession No. GSE31017).

Quantitative PCR

For quantitative PCR (qPCR) experiments, total bacterial RNA was extracted from wild-type and Δ codY mutant strains using TRIzol Reagent (Invitrogen). The cells were disrupted using Lysing Matrix B (MP Biomedicals Europe) and bead beating in a Mini-BeadBeater-8 (BioSpec) according to manufacturer's specifications. DNA was removed from each RNA preparation using Turbo DNA-free Kit (Ambion), according to manufacturer's instructions. RNA quantity (A_{260}) and purity (A_{260}/A_{280} ratio) were measured in a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized from 500 ng of RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in a 20 μ l reaction according to manufacturer's protocols.

Relative mRNA levels were determined by qPCR. Five microlitres of a 1:100 dilution of the cDNA reaction was used as template for qPCR amplification in 25 μ l final volumes containing 12.5 μ l of Power SYBR Green PCR Master Mix (Applied Biosystems) and 200 nM of each primer. Primers used for qPCR are listed in Table S2. qPCR amplification was performed using StepOne PCR software (Applied Biosystems) with thermal cycling conditions set at 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Fluorescence was monitored during each extension phase and a melting curve analysis was performed after each run to confirm the amplification of specific transcripts. Each qPCR of the RNA samples was performed in triplicate, no template was added in negative controls, and *rpoB* expression was used as control. The qPCR analysis was performed on three independent biological replicates. Slopes of the standard curves and PCR efficiency for each primer pair were estimated by amplifying serial dilutions of the cDNA template. The amount of BC1809 (*nheA*) and BC1810 (*nheB*) cDNA was normalized to the level of *rpoB* cDNA using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Generation of promoter–gfp fusions

Promoter regions of BC2026, BC1396, BC0699, BC1110 and BC1625 were amplified by PCR (Table S2) using Phusion polymerase (Finnzymes, Finland) and digested with EcoRI and BamHI (Fermentas) restriction enzymes. The resulting fragments were cloned to the corresponding sites of pAD123 vector (Dunn and Handelsman, 1999) resulting in promoter–gfp fusions as listed in Table 2. Constructs were verified by sequencing (MacroGen, the Netherlands) and electroporated into *B. cereus* ATCC 14579 and Δ codY strains using the protocol described before (Silo-Suh *et al.*, 1994).

Reporter measurements

To measure the activities of the selected promoters, *B. cereus* cells containing promoter–gfp constructs were grown at 30°C in 100 μ l of BHI in a 96-well microtitre plate under continuous shaking. The OD and fluorescence were measured every 15 min using a TECAN F200 Microplate Reader (TECAN Group, Männedorf, Switzerland). The arbitrary units presented in Fig. 3 were obtained by subtracting the signal detected in strains containing the promoter-less

Table 2. Strains and plasmids used.

Strain or plasmid	Relevant feature	Reference
Strains		
<i>B. cereus</i>		
ATCC 14579	Wild-type strain	American Type Culture Collection
$\Delta codY$	<i>codY</i> deletion mutant	This study
<i>E. coli</i>		
MC1061	F– <i>araD139</i> Δ (<i>ara-leu</i>) 7696 <i>galE15 galK16</i> Δ (<i>lac</i>)X74 <i>rpsL</i> (<i>Str^R</i>) <i>hsdR2</i> (<i>rK mK+</i>) <i>mcrA mcrB1</i>	Laboratory stock
TOP10	F– <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80/ <i>lacZ</i> Δ M15 Δ <i>lacX74 recA1 araD139</i> Δ (<i>ara-leu</i>)7697 <i>galU galK rpsL endA1 nupG</i>	Invitrogen
Plasmids		
pMAD	Shuttle vector, <i>bgaB</i> , <i>bla</i> , <i>ermC</i> , additional I-SceI site	Arnaud and colleagues (2004)
pCR 2.1-TOPO	Cloning vector, Km ^R , Amp ^R	Invitrogen
pCR 2.1 <i>codY</i> up	pCR 2.1-TOPO with the upstream region of <i>codY</i>	This study
pCR 2.1 <i>codY</i> down	pCR 2.1-TOPO with the downstream region of <i>codY</i>	This study
pCR 2.1 <i>codY</i> up/down	pCR 2.1-TOPO with the up- and downstream region of <i>codY</i>	This study
pMAD $\Delta codY$	pMAD with the up- and downstream homologue regions of <i>codY</i>	This study
pLM5	Vector containing <i>spac</i> promoter and <i>lacI</i> repressor, Km ^R	Marraffini and Schneewind (2006)
pLM <i>codY</i>	pLM5 containing <i>Pspac-codY</i> , Km ^R	This study
pAD123	Promoter-less reporter plasmid, <i>gfpmut3a</i> , <i>bla</i> , <i>cam</i>	Dunn and Handelsman (1999)
pAD <i>opp</i>	pAD123 with the promoter region of BC2026, <i>bla</i> , <i>cam</i>	This study
pAD <i>bcaA</i>	pAD123 with the promoter region of BC1396, <i>bla</i> , <i>cam</i>	This study
pAD <i>roc</i>	pAD123 with the promoter region of BC0699, <i>bla</i> , <i>cam</i>	This study
pAD <i>cytK</i>	pAD123 with the promoter region of BC1110, <i>bla</i> , <i>cam</i>	This study
pAD <i>motA</i>	pAD123 with the promoter region of BC1625, <i>bla</i> , <i>cam</i>	This study

reporter plasmid and normalized to the OD. Data were obtained from at least four independent replicates.

Western blotting

Culture supernatants were applied to NuPAGE Novex Bis-Tris gel system (Invitrogen) using SeeBlue Plus2 Pre-Stained Standard (Invitrogen) as molecular weight marker. Western blot analysis was performed according to standard protocols (Harlow and Lane, 1988). Monoclonal antibodies 1A8 against NheA and 1C2 against NheB/Hbl L1 (Dietrich *et al.*, 1999; 2005) were a kind gift from Dr Erwin Märtlbauer (Ludwig-Maximilians-Universität, Munich, Germany). The monoclonal antibodies were used in a dilution of 1:15. Rabbit antiserum for detection of CytK was used in a 1:2000 dilution (Fagerlund *et al.*, 2004). Biotin-conjugated anti-mouse antibodies (GE Healthcare) or biotin-conjugated anti-rabbit antibodies (Invitrogen) were used as secondary antibodies (1:3000). A complex of streptavidin (Bio-Rad) and biotinylated alkaline phosphatase (Bio-Rad) was used at a dilution of 1:3000, prior to development with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad).

Cytotoxicity assays

Cytotoxicity was determined using a Vero cell test (Lindbäck and Granum, 2006) that monitors the inhibition of protein synthesis by measuring the reduction of incorporated ¹⁴C-leucine in the Vero cells upon addition of toxins. Briefly, 10–30 μ l of samples of late log phase culture supernatants (OD₆₀₀ 2.4) were applied to 1 ml of low-leucine medium (Gibco, Invitrogen) covering a confluent monolayer of Vero cells. The cells were incubated for 2 h with the supernatants containing toxins, followed by washing and addition of 300 μ l

of low-leucine medium with ¹⁴C-leucine (0.2 nCi μ l^{−1}) and incubated further for 1 h. The radioactive medium was removed, the cells were washed and radioactivity (c.p.m.) in the Vero cells was counted in a scintillation counter. The percentage of inhibition of protein synthesis was calculated using the formula [(negative control – sample)/negative control] $\times$ 100. Vero cells incubated without the addition of supernatants were used as negative control. The cytotoxicity assays were performed on three independent biological replicates and two technical replicates in each assay.

Motility, biofilm and growth assays

The swimming ability of *B. cereus* ATCC 14579 and $\Delta codY$ mutant strains was determined on LB soft agar plates (0.3% agar). Two microlitres of *B. cereus* culture grown overnight in LB medium was spotted on a soft agar plate and subsequently the plate was incubated at 30°C. The diameter of resulting 'colony' was measured 20 and 46 h after inoculation.

Biofilm formation properties of *B. cereus* ATCC 14579 and $\Delta codY$ mutant strains were followed using the protocol described by Hsueh and colleagues (2008). Cells were grown overnight in LB medium and diluted 100-fold in EPS medium. One millilitre was added to wells of Cellstar 48 well cell culture multiwell plates (Greiner Bio-One) and incubated at 30°C for 24 h. Biofilm was stained with 0.3% crystal violet for 10 min, washed with distilled water. The amount of biofilm-bound crystal violet was solubilized using 70% ethanol and quantified by measuring the optical density at 590 nm.

To examine the growth capacity of the $\Delta codY$ mutant in different conditions, the mutant and the wild-type strains were cultivated overnight at 30°C, 200 r.p.m. in BHI broth supplemented with 0.5% glucose. Two hundred and fifty microlitres of these overnight cultures were used to inoculate 50 ml BHI

with 0.5% glucose in 250 ml Erlenmeyer flasks. Two hundred and fifty microlitres of the obtained overnight cultures were also used to inoculate 50 ml of 0.02% casamino acids supplemented M0 medium (Mols *et al.*, 2007) in 250 ml Erlenmeyer flasks. The flasks were incubated at 30°C, 200 r.p.m. and the optical density at 600 nm was measured regularly (Genesys 20, Thermo Fisher Scientific, USA). Upon reaching an OD₆₀₀ of approximately 0.3 the BHI cultures were centrifuged at 5600 r.p.m. (Eppendorf centrifuge 5810-R) and the supernatant was discarded. The cell pellets were resuspended in M0 medium supplemented with 0.02% casamino acids without additional washing of the cells. Growth was allowed further in Erlenmeyer flasks by incubation at 30°C, 200 r.p.m. and the OD₆₀₀ was monitored. Three independent replicates were measured and the significance of differences was tested using Student's *t*-tests with values below 0.01 regarded as significant.

Microscopy

For phase-contrast images, cells were grown overnight in LB medium and spotted on agarose to immobilize the cells. Samples were observed using a Nikon Eclipse Ti microscope and recorded with a Nikon DS-Qi1Mc camera and Nis Elements AR software.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Transcriptome analysis of gene expression in the *B. cereus* $\Delta codY$ mutant compared with the wild-type strain. Asterisks behind the locus tag indicate the members of the predicted PclR regulon in *B. cereus* ATCC 14579 (Gohar et al., 2008).

Table S2. Oligonucleotides used.

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