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 (ΔcodY) 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 ΔcodY 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 mycoides and Bacillus pseudomycoïdes (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.
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, AATTTCGWGAAAATT, was studied and experimentally verified in several Gram-positive bacteria (den Hengst et al., 2005; Guedon et al., 2005; Belitsky and Sonenshein, 2008; van Schaik et al., 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 fermentation processes, including sporulation, competence development for DNA uptake, motility and biofilm formation (Slack et al., 1995; Senor 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 Streptococcus (Malke and Ferretti, 2007; Hendrikson 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 (AG of –15.6 predicted by Clone Manager 7 software). The cell morphology of wild-type and ∆codY mutant strains were examined after overnight culturing in LB medium. The wild-type culture contained single cells, while the ∆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 ∆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...
IPTG in the medium. The complemented \( \Delta \text{codY} \) 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 \( \Delta \text{codY} \) mutant

Transcriptome analyses of various Gram-positive bacteria revealed genes affected by \( \Delta \text{codY} \) mutations. In all organisms examined so far, CodY represses genes involved 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 \( \Delta \text{codY} \) 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 \( \Delta \text{codY} \) strain included the \( \text{opp} \) 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 \( \text{pep} \) 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 \( \text{codY} \) gene was deleted. Twenty-three genes of the virulence-related PlcR regulon were significantly downregulated (ratio \(< 0.33 \)) in the \( \Delta \text{codY} \) 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.
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 cytotoxin K and involved motility, respectively, were selected. Detection of fluorescence showed that the expression of BC2026, BC1396 and BC0699 was derepressed in the ΔcodY strain, while the expression of BC1110 and BC1625 was lower in the ΔcodY 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 (± 0.07) and 0.2 (± 0.03), respectively, in the ΔcodY 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 ΔcodY mutant prompted us to investigate the growth of the ΔcodY mutant in various conditions (Fig. 4). In nutrient-rich conditions the growth of the ΔcodY mutant was affected. After reaching the mid-exponential phase of growth the ΔcodY mutant proliferated a little slower than the wild type. No significant differences in growth were observed between the wild-type and the ΔcodY mutant strain when grown in M0 medium supplemented with casamino acids. When wild-type and ΔcodY 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 ΔcodY 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...
**Role of CodY of B. cereus**

**Fig. 3.** Expression profiles of CodY regulated genes. *Bacillus cereus* ATCC 14579 (solid lines) and ΔcodY 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 ΔcodY 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).
Motility genes in the microarray analysis showed decreased gene expression of motility genes in the \( \Delta \text{codY} \) deletion strain of \( B. \) cereus. Motility of the \( \Delta \text{codY} \) 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 \( \Delta \text{codY} \) 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 \( \text{codY} \) gene using an IPTG-induced Pspac promoter. However, the degree of complementation was dependent on the IPTG concentration 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 promotor used showed significant leakiness. This could explain the low, but significant, complementation in the non-induced samples compared with the \( \Delta \text{codY} \) mutant.

A previous study on a \( B. \) cereus transposon mutant of strain UW101C containing a disrupted \( \text{codY} \) gene showed a fourfold reduction of biofilm formation (Hsueh et al., 2008). The marker-less deletion of \( \text{codY} \) in the \( B. \) cereus strain ATCC 14579 resulted in elongated cells and reduced motility as mentioned above. The biofilm formation of the \( B. \) cereus \( \Delta \text{codY} \) deletion strain was examined on EPS, a low-nutrient medium (Hsueh et al., 2006). After 24 h induction, biofilm formation in EPS by the \( \Delta \text{codY} \) 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 \( \text{codY} \) was reintroduced into the \( \Delta \text{codY} \) mutant, biofilm formation was significantly reduced (\( OD_{590} \) of 0.11 ± 0.02) compared with the \( \Delta \text{codY} \) 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 \( \Delta \text{codY} \) 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 \( \Delta \text{codY} \) strain showed down-regulation of genes regulated by PlicR. The PlicR regulon includes genes that code for various enterotoxins: cytoxin 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 \( \Delta \text{codY} \) mutant strains.

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Radius in mm (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 14579</td>
<td>20</td>
</tr>
<tr>
<td>( \Delta \text{codY} )</td>
<td>20</td>
</tr>
<tr>
<td>( \Delta \text{codY} ) + pLM codY</td>
<td>20</td>
</tr>
<tr>
<td>No IPTG</td>
<td>46</td>
</tr>
<tr>
<td>0.1 mM IPTG</td>
<td>46</td>
</tr>
<tr>
<td>1 mM IPTG</td>
<td>46</td>
</tr>
<tr>
<td>10 mM IPTG</td>
<td>46</td>
</tr>
</tbody>
</table>

Fig. 5. Motility and biofilm formation of \( B. \) cereus wild-type and \( \Delta \text{codY} \) mutant strains.

A. Swimming radius of the \( B. \) cereus wild-type, \( \Delta \text{codY} \) 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, \( \Delta \text{codY} \) 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 \( \Delta \text{codY} \) deletion strain of \( B. \) cereus.
proteins decreased in the \(\Delta\text{codY}\) mutant as shown using Western blot analysis (Fig. 6). The haemolytic activity of \(B.\ cerus\) cells caused by haemolytic toxins (e.g. haemolysin BL) was assessed on sheep blood containing BH agar plates (Beecher and Wong, 1994). Beta haemolysis surrounding colonies on blood agar is a diagnostic character of \(B.\ cerus\) haemolytic activity. The \(\Delta\text{codY}\) strain showed a reduced haemolytic zone as indicative of reduced haemolysis (Fig. 7). The haemolytic activity of the \(\Delta\text{codY}\) strain with the complementation construct was slightly higher the presence of 10 mM IPTG than the \(\Delta\text{codY}\) 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 \(B.\ cerus\) sensu lato group, CodY was needed for full virulence via the plasmid-encoded AtxA regulator and its associated toxins (van Schalk et al., 2009). However,
vastly 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 ΔcodY 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 ΔcodY mutant was not significantly different in a very poor medium. The involvement of the 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* (Brandt et al., 2006; Romo et al., 2011), was also repressed by CodY. This cluster including the associated signal peptidases, were highly upregulated in the ΔcodY mutant. The sinR genes coding for the homologues of the biofilm regulators in *B. subtilis* (Kears 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 ΔcodY 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 were highly downregulated in the ΔcodY mutant. Indeed, reduced motility of the ΔcodY 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 ΔcodY 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 nbl, 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, nheB and nbl 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* ΔcodY mutant are altered. Our preliminary experiment showed that the toxicity of the *B. cereus* ΔcodY strain was lowered when examined on mammalian cell cultures (Lindbäck and Granum, 2006).

Supernatant of the *B. cereus* ΔcodY mutant, harvested at late exponential phase (OD<sub>600</sub> of 2.4), was tested for toxicity in the Vero cell cytotoxicity assay and showed around 50% reduction compared with the wild type (34 ± 10% inhibition of protein synthesis compared with the 71 ± 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* (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 ΔcodY 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 (Ratnakay-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 ΔcodY 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 K2HPO4, 3.5 mM KH2PO4, 0.8 mM MgSO4, 0.04 mM MnCl2, 0.2 mM NaCl, 0.2 mM CaCl2, 0.05 mM ZnCl2, 0.04 mM FeCl3, 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 ΔcodY mutant

An in-frame ΔcodY 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 (Finzymes, 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 to 4, Table S2) respectively. The reverse primer (oligo 2) was 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 pCRII-TOPO containing the upstream region (codY up). The complete construct (codY up and codY down) were now excised from pCRII-TOPO using EcoRI and ligated into the corresponding restriction site of the thermosensitive shuttle vector pMAD (Aarnaud et al., 2004) containing an additional I-SceI site (a kind gift from Dr Annette Fagerlund, University of Oslo, Norway). The pMAD::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 Electroporator apparatus (Eppendorf AG). Subsequently, the cells were

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DNA microarray analysis

Four independent biological replicates of exponentially growing *B. cereus* ATCC 14579 and its *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 Maceloid/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 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^−ΔΔCt method (Livak and Schmittgen, 2001).

Quantitative PCR

For quantitative PCR (qPCR) experiments, total bacterial RNA was extracted from wild-type and *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 (A260) and purity (A260/A280 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 µl reaction according to manufacturer’s protocols.

Relative mRNA levels were determined by qPCR. Five microliters of a 1:100 dilution of the cDNA reaction was used as template for qPCR amplification in 25 µl final volumes containing 12.5 µl 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^−ΔΔCt 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 *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 µl 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, Mannedorf, Switzerland). The arbitrary units presented in Fig. 3 were obtained by subtracting the signal detected in strains containing the promoter-less...
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., 1999). 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., 1999).

**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 "C-leucine (0.2 nCi µl-l) 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 × 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 ΔcodY 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 ΔcodY 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 ΔcodY 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

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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 (Mol's 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 OD_{600} 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 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-QiMc camera and Nis Elements AR software.

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References


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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 ΔcodY mutant compared with the wild-type strain. Asterisks behind the locus tag indicate the members of the predicted PoFl regulon in B. cereus ATCC 14579 (Gohar et al., 2008).

Table S2. Oligonucleotides used.

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