

# Cyclic diguanylate regulation of *Bacillus cereus* group biofilm formation

Annette Fagerlund,<sup>1†</sup> Veronika Smith,<sup>1,2§</sup>  
Åsmund K. Røhr,<sup>3†§</sup> Toril Lindbäck,<sup>4§</sup>  
Marthe P. Parmer,<sup>5</sup> K. Kristoffer Andersson,<sup>3</sup>  
Leon Reubsaet<sup>5</sup> and Ole Andreas Økstad<sup>1,2\*</sup>

<sup>1</sup>Laboratory for Microbial Dynamics (LaMDa), Section for Pharmaceutical Biosciences, School of Pharmacy, University of Oslo, PB1068, Oslo 0316, Norway.

<sup>2</sup>Centre for Integrative Microbial Evolution (CIME), Faculty of Mathematics and Natural Sciences, University of Oslo, Oslo 0316, Norway.

<sup>3</sup>Department of Biosciences, University of Oslo, PB1066, Oslo 0316, Norway.

<sup>4</sup>Department of Food Safety and Infection Biology, Norwegian University of Life Sciences, PB8146 Dep, Oslo 0033, Norway.

<sup>5</sup>Bioanalytics, Section for Pharmaceutical Chemistry, School of Pharmacy, University of Oslo, PB1068, Oslo 0316, Norway.

## Summary

Biofilm formation can be considered a bacterial virulence mechanism. In a range of Gram-negatives, increased levels of the second messenger cyclic diguanylate (c-di-GMP) promotes biofilm formation and reduces motility. Other bacterial processes known to be regulated by c-di-GMP include cell division, differentiation and virulence. Among Gram-positive bacteria, where the function of c-di-GMP signalling is less well characterized, c-di-GMP was reported to regulate swarming motility in *Bacillus subtilis* while having very limited or no effect on biofilm formation. In contrast, we show that in the *Bacillus cereus* group c-di-GMP signalling is linked to biofilm formation, and to several other phenotypes important to the lifestyle of these bacteria. The *Bacillus thuringiensis* 407 genome encodes eleven predicted proteins containing domains (GGDEF/EAL)

related to c-di-GMP synthesis or breakdown, ten of which are conserved through the majority of clades of the *B. cereus* group, including *Bacillus anthracis*. Several of the genes were shown to affect biofilm formation, motility, enterotoxin synthesis and/or sporulation. Among these, *cdgF* appeared to encode a master diguanylate cyclase essential for biofilm formation in an oxygenated environment. Only two *cdg* genes (*cdgA*, *cdgJ*) had orthologs in *B. subtilis*, highlighting differences in c-di-GMP signalling between *B. subtilis* and *B. cereus* group bacteria.

## Introduction

Cyclic dinucleotide derivatives are used as cellular signalling molecules in a wide variety of living organisms, spanning several kingdoms of life (Schaap, 2013). The second messenger cyclic diguanylate (c-di-GMP) has been widely described as a universal mediator of biofilm formation, motility, cell toxicity and other phenotypes in a range of Gram-negative bacteria (Povolotsky and Hengge, 2012; Römling *et al.*, 2013; Fazli *et al.*, 2014). Elevated levels of intra-cellular c-di-GMP are usually associated with increased biofilm formation, while reduced levels are associated with an increase in motility and virulence, and c-di-GMP has in several bacteria been shown to suppress swimming motility (Wolfe and Visick, 2008; Boehm *et al.*, 2010; Purcell *et al.*, 2012; Fazli *et al.*, 2014). Although the role of c-di-GMP as a key second messenger seems to be conserved across the bacterial domain, c-di-GMP has been less studied in Gram-positive bacteria. It has however been shown to be important in biofilm intra-cellular signalling and regulation in *Clostridium difficile* and *Listeria monocytogenes* (Bordeleau *et al.*, 2011; Purcell *et al.*, 2012; Chen *et al.*, 2014). Strikingly, c-di-GMP-signalling seems to be involved in control of swarming motility but not in biofilm formation in *Bacillus subtilis* (Chen *et al.*, 2012; Gao *et al.*, 2013). Also, in contrast to many other bacteria, the *B. subtilis* genome seems to carry a rather limited c-di-GMP-linked gene repertoire, encoding only three active diguanylate cyclases (*DgcP*, *DgcK*, and *DgcW* –

Accepted 23 April, 2016. \*For correspondence. E-mail aloechen@farmasi.uio.no; Tel. +47 22 85 47 89; Fax +47 22 84 49 44. Present addresses: <sup>†</sup>Nofima, PB210, 1431 Ås, Norway; <sup>‡</sup>Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, PB5003, 1432 Ås, Norway. <sup>§</sup>These authors contributed equally to this work.

formerly YtrP, YhcK, and YkoW, respectively), one active c-di-GMP phosphodiesterase (PdeH – formerly YuxH), and one known c-di-GMP receptor (DgrA – formerly YpfA; (Gao *et al.*, 2013)).

The *Bacillus cereus* group encompasses seven bacterial species, several of which are of substantial clinical and financial importance. This includes *Bacillus anthracis*, the cause of the acute and severe disease anthrax in humans and warm-blooded animals, the opportunistic human pathogen *Bacillus cereus* which is a frequent cause of two types of bacterial gastroenteritis as well as a range of other human opportunistic infections (Drobniewski, 1993; Bottone, 2010), and the entomopathogenic species *Bacillus thuringiensis*, a bacterium which constitutes the world's most widely used biological pesticide. *B. thuringiensis* does however in general carry the same chromosomally encoded virulence factors as *B. cereus* [reviewed in (Rasko *et al.*, 2005)]. Phylogenetic analysis of the genus *Bacillus* shows that the *B. cereus* and *B. subtilis* groups are among the most closely related subgroups (Priest, 1993), and like *B. subtilis*, *B. cereus* group bacteria efficiently form biofilms, although preferentially at air-liquid interfaces under the tested conditions (Wijman *et al.*, 2007). In *B. subtilis*, which is largely considered apathogenic, biofilm formation is controlled by a set of transcriptional regulators (Hamon and Lazazzera, 2001; Kearns *et al.*, 2005; Lemon *et al.*, 2008). Several of these regulators are genetically and functionally conserved in the *B. cereus* group, including the master transcriptional repressor SinR (Kearns *et al.*, 2005; Pflughoeft *et al.*, 2011; Fagerlund *et al.*, 2014). Here we show that in addition to transcription factor-based biofilm control (Fagerlund *et al.*, 2014), and in apparent contrast to *B. subtilis*, the majority of *B. cereus* group bacteria carry a set of ten genes (*cdgA-J*; cyclic diguanylate) encoding enzymes putatively involved in c-di-GMP synthesis and/or breakdown (diguanylate cyclases, DGCs, and phosphodiesterases, PDEs, respectively), some of which are essential for effective biofilm formation in an oxygenated environment. We assess, by systematic construction of gene deletion mutants and overexpression strains, the impact of each gene on biofilm formation, motility, cell toxicity and sporulation, and functionally characterize a putative master diguanylate cyclase severely affecting biofilm formation in *B. cereus* group bacteria.

## Results

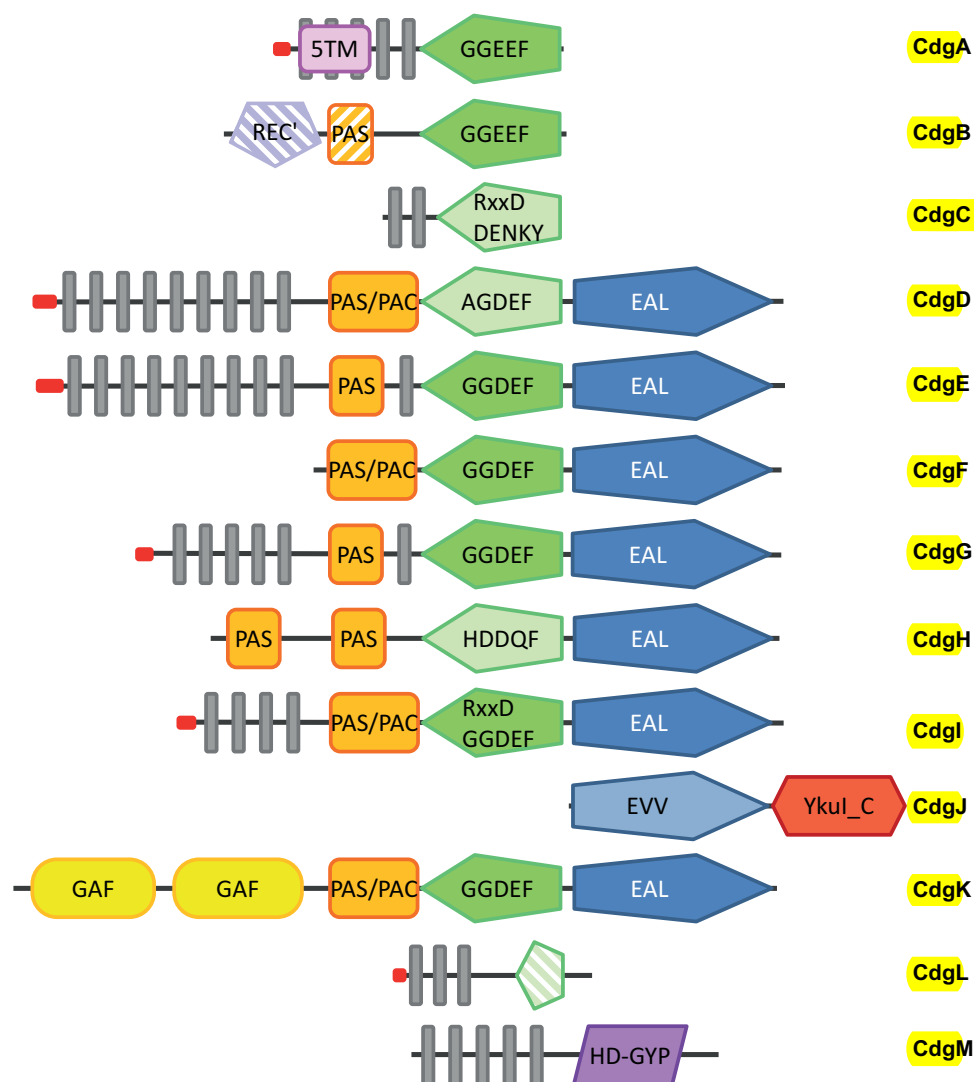
### Identification of putative DGC and PDE encoding genes in *Bacillus cereus* group genomes

Genes potentially involved in c-di-GMP turnover in *B. cereus* group strains were identified by searching 90 available genome sequences for open reading frames

encoding products with GGDEF or EAL domains (Cdg proteins). Three genes encoding proteins with a GGDEF domain (*cdgA-C*), one ortholog (*cdgJ*) to the *B. subtilis* gene encoding Ykul, carrying an EAL domain, and seven genes encoding tandem GGDEF/EAL domain proteins (*cdgD-I*, *cdgK*) were identified, with significant matches to the corresponding Pfam (Finn *et al.*, 2014) domains PF00990 and PF00563, respectively (Fig. 1). In addition, one gene (*cdgL*; locus tag BTB\_c54300 in *B. thuringiensis* 407, BC5236 in *B. cereus* ATCC 14579) was predicted to encode a highly degenerate GGDEF domain protein, and a second gene (*cdgM*) to encode a putative PDE carrying a HD-GYP domain (Galperin *et al.*, 1999). Several of the proteins were predicted to harbour transmembrane domains and/or known signalling (PAS/PAC) domains (see section below for a detailed analysis) in their N-terminus (Fig. 1). Altogether, ten *cdg* genes (*cdgA-cdgF*, *cdgH-J*, *cdgL*) were highly conserved in *B. cereus* group organisms (being present in 85 or more out of the 90 genomes analyzed, including 19 strains of *B. anthracis*, which is a highly clonal species), while *cdgG* was found in 67, and *cdgM* in 46 of the 90 analyzed genomes (Fig. 2). *cdgK* was found only in five closely related strains, within or close to the cluster of monoclonal emetic *B. cereus* strains. The presence/absence pattern of each gene in the analyzed strains, along with the locus tags (where assigned), are listed in Supporting Information Table S1. Interestingly, among the *cdgA-M* genes, only two (*cdgA*, *cdgJ*) had orthologs in *B. subtilis* (*dgcK*, formerly known as *yhcK*, locus tag BSU6051\_09120; and *ykul*, locus tag BSU6051\_14090, respectively), based on bidirectional best hits from reciprocal BLASTP (Altschul *et al.*, 1997) searches against the proteomes of strains 168 and NCIB 3610, and conservation of protein domains (Supporting Information Fig. S1).

### Phylogenetic distribution of predicted DGC and PDE encoding genes among *B. cereus* group strains

The distribution of predicted c-di-GMP signalling genes varied between different *B. cereus* group strains and phylogenetic clusters (Fig. 2). Of the identified *cdg* genes, only *cdgB* and *cdgJ* were present in all 90 analyzed *B. cereus* group strains. Furthermore, the analysis showed that the two most distant phylogenetic lineages within the group, phylogenetic groups I and VII comprising *Bacillus pseudomycolides* and *Bacillus cytotoxicus* strains, respectively, contain a strongly reduced set of *cdg* genes. Although this is reflected in a generally smaller chromosome for strains within the *B. cytotoxicus* cluster (Lapidus *et al.*, 2008), the chromosome of *B. pseudomycolides* DSM 12442 (cluster I in Fig. 2) is

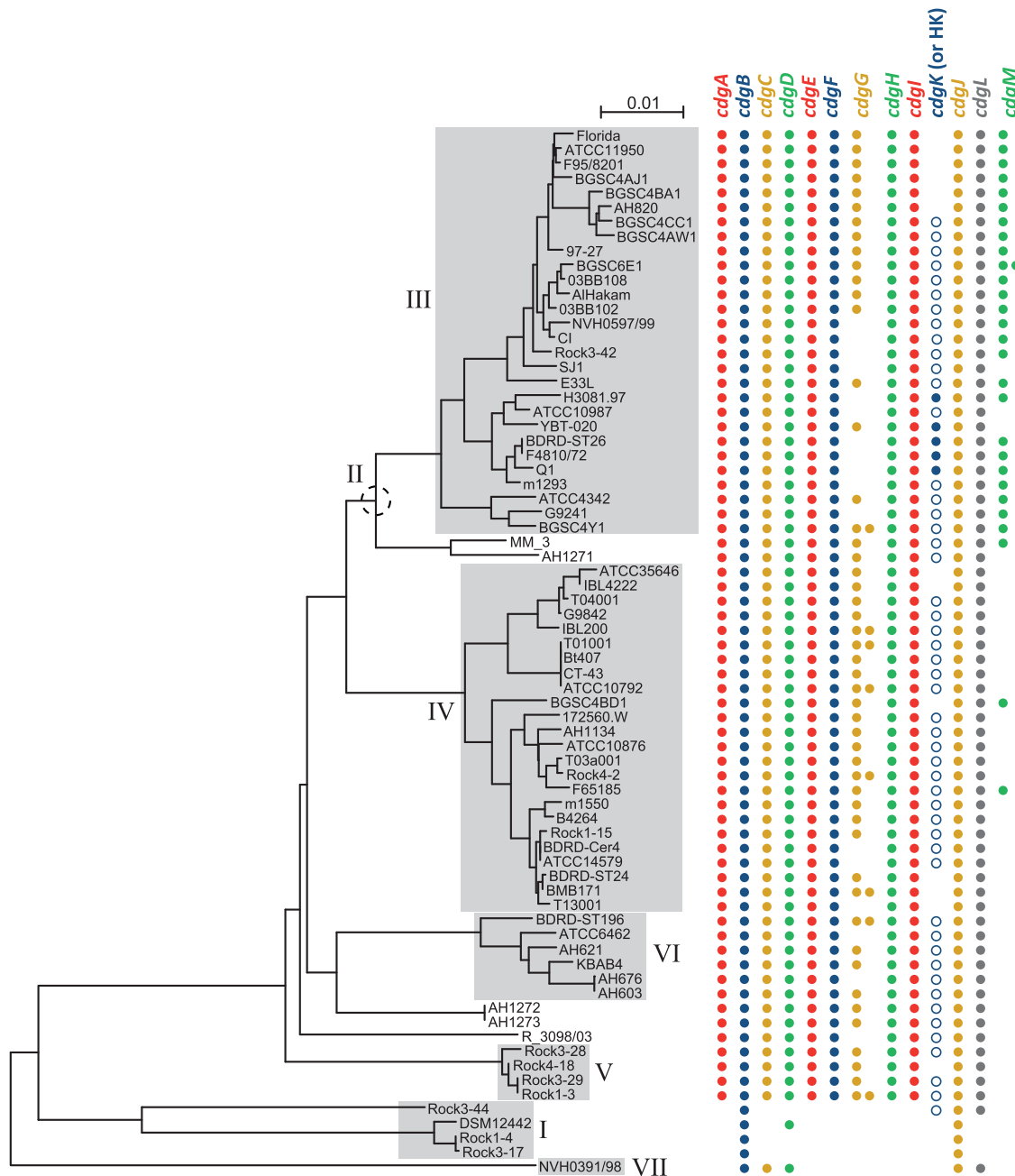


**Fig. 1.** Domain organization of predicted proteins from *B. cereus* group genomes containing putative c-di-GMP signalling domains. GGDEF domains (green), EAL domains (blue), PAS or PAS/PAC domains (orange), GAF domains (yellow), the YkuI\_C domain (red) and the 5TM-5TMR-LYT domain (in pink, indicated by «5TM»), were assigned according to SMART and/or Pfam. The degenerate REC domain in **CdgB**, indicated by «REC'», was identified through BLAST analysis and Phyre structure prediction. The HD-GYP domain (purple) is a Pfam HD domain containing the HD-GYP signature sequences. The PAS domain in **CdgB** is shown in diagonal stripes to indicate a partial match to the Pfam profile. Transmembrane segments (in grey) were predicted using the TMHMM2 program, and signal peptides (in red) were determined by SignalP. For GGDEF domains, the amino acid sequence at the A-site is indicated, along with «RxxD» if the I-site is present, except for **CdgL** where the GGDEF domain was highly degenerate. GGDEF domains predicted by amino acid sequence analysis to show **DGC** activity, and predicted enzymatically inactive GGDEF domains, are in dark and light green, respectively. For EAL domains, «EAL» indicates a domain predicted to show **PDE** activity, while the EAL domain indicated by «EVV» on light blue background (**CdgJ**) lacks conserved active site residues and is thus predicted to be enzymatically inactive. Locus tags for the genes are as follows, from *B. thuringiensis* 407 (BTB numbers) and *B. cereus* ATCC 14579 (BC numbers), unless for those proteins absent in these strains, in which case locus tags from *B. cereus* AH187 (BCAH187 numbers) are given): **CdgA**: BTB\_c56140, BC\_5414 (351 aa); **CdgB**: BTB\_c41790, BC\_4044 (415 aa); **CdgC**: BTB\_c41320, BC\_3997 (217 aa); **CdgD**: BTB\_c55030, BC\_5302 (909 aa); **CdgE**: BTB\_c38210, BC\_3747 (906 aa); **CdgF**: BTB\_c06420, BC\_0628 (563 aa); **CdgG**: BTB\_c35900 (785 aa); **CdgH**: BTB\_c05620, BC\_0547 (689 aa); **CdgI**: BTB\_c55500, BC\_5348 (735 aa); **CdgJ**: BTB\_c41240, BC\_3989 (405 aa); **CdgK**: BCAH187\_A0409 (925 aa); **CdgL**: BTB\_c54300, BC\_5236 (251 aa); **CdgM**: BCAH187\_A1141 (374 aa). Predicted protein lengths in amino acids are included, as indicated in parentheses.

5.8 Mb (Zwick *et al.*, 2012), a size typical for *B. cereus* group bacteria.

Relative to the majority of the **cdg** genes, **cdgG** and **cdgM** showed a highly variable distribution across the phylogenetic tree (Fig. 2). In some strains two homologs

were identified for these genes (Supporting Information Table S1 and locus tags therein), and investigation of genome organization showed that when present their genomic position was also variable between strains. Furthermore, in *B. thuringiensis* BMB171 one of the two



**Fig. 2.** Distribution of *cdg* genes within the *B. cereus* group population. A phylogenetic tree of sequenced strains representing the *B. cereus* group population was constructed as described (methods). The seven phylogenetic clusters within the *B. cereus* group, referred to as group I–VII, are marked. The gapped circle indicates where Cluster II strains would emerge, by extrapolation of phylogenetic data from the HyperCAT database (<http://mlstoslo.uio.no>). For *cdgK*, a filled symbol indicates presence of the GGDEF/EAL gene in the respective strain, while open symbols indicate the presence of a histidine kinase (HK) with the same GAF domains/N-terminal as *cdgK*. Two symbols in one column indicate that two copies (paralogs) of the gene in question were identified in the same strain. The horizontal bar indicates a nucleotide difference of 1%.

*cdgG* homologs is located on a plasmid, pBMB171. Except for the second *cdgM* homolog found in *B. cereus* BGSC 6E1, *cdgM* was present and the gene neighborhood conserved among most cluster III strains (Fig. 2). These strains encompass many of the human clinical isolates and human pathogenic strains. Conversely, in

the two isolates harbouring *cdgM* among cluster IV strains, the gene was located in a different genomic position. *cdgK* seems to be a very rare gene among *B. cereus* group organisms, and was found only in five closely clustered strains, three of which were known to be emetic (H3081.97) and/or belong to the

main emetic sequence type, ST26 (F4810/72 and BDRD-ST26) (Fig. 2).

#### Sequence analysis of enzymatic domains in identified DGC and PDE proteins

Structural and biochemical studies have determined highly conserved residues that are essential for the catalytic activities of GGDEF, EAL and HD-GYP domains. Based on sequence alignments, the EAL domains of all seven tandem GGDEF/EAL domain proteins (CdgD, CdgH, CdgI, CdgK; Fig. 1) were found to contain the signature motif sequences known to be required for PDE activity (Tchigvintsev *et al.*, 2010). These proteins are thus predicted to be capable of hydrolyzing c-di-GMP. Likewise, the single identified *B. cereus* group protein with an HD-GYP domain (CdgM) may be a functional c-di-GMP PDE as it contains all residues conserved in HD-GYP domain proteins including those predicted to be required for catalytic activity (Galperin *et al.*, 1999; Lovering *et al.*, 2011). In contrast, CdgJ, which is composed of a modified EAL domain followed by the Ykul\_C domain, has a degenerate catalytic motif, and is, therefore, predicted to lack c-di-GMP-specific PDE activity. CdgJ is an ortholog with 55% amino acid sequence identity to the functionally and structurally characterized *B. subtilis* Ykul protein, which has been shown functionally able to bind, but unable to hydrolyze, c-di-GMP (Minasov *et al.*, 2009).

GGDEF domains with GG[D/E]EF active-site (A-site) motifs are predicted to be active DGC enzymes. Among the seven composite GGDEF-EAL proteins, five contained the GGDEF signature sequence (CdgE-CdgG, CdgI, CdgK; Fig. 1). Of the remaining proteins containing GGDEF domains, CdgA and CdgB contain the conserved GGEEF motif. Altogether, these seven proteins are potentially capable of c-di-GMP synthesis. In contrast, the GGDEF domains of CdgC and CdgH harbour the degenerate sequence motifs DENKY and HDDQF, respectively, at the location of the A-site motif. In addition, CdgL clearly contained the most highly degenerate GGDEF domain, with an insignificant (below threshold) match to the GGDEF Pfam family (Fig. 1). These three GGDEF domain proteins are predicted to lack DGC activity. The CdgD GGDEF domain has a non-canonical AGDEF motif at the A-site. However, as the first position of the GG[D/E]EF motif has been shown to tolerate a conservative substitution (e.g. the *Pectobacterium atrosepticum* ECA3270 GGDEF domain protein with an SGDEF motif which showed DGC activity (Perez-Mendoza *et al.*, 2011)), *in silico* prediction of whether or not this domain may retain catalytic activity is difficult. The conserved RxxD motif (I-site), located five residues

upstream of the A-site, was identified in the GGDEF domain of CdgC, which is predicted to be enzymatically inactive, and in the tandem GGDEF/EAL domain protein CdgI. This motif has been shown to bind c-di-GMP and may serve as a non-competitive allosteric inhibitory site (Christen *et al.*, 2006).

Thus, out of the 13 proteins represented in Fig. 1, only three (CdgC, CdgJ, and CdgL) are predicted to be enzymatically inactive. Two proteins (CdgA and CdgB) are predicted DGCs, while three (CdgD, CdgH, and CdgM) are predicted PDEs. The remaining five proteins, CdgE, CdgF, CdgG, CdgI, and CdgK, harbour both GGDEF and EAL domains with intact active site motifs. For these, it is difficult to predict whether they may act as DGCs, PDEs, or may, although this is less common (Hengge, 2009), be bifunctional enzymes.

#### Sequence analysis of sensory and signal transduction domains

As in other bacteria, a high proportion of the *B. cereus* group proteins containing GGDEF, EAL and HD-GYP domains also contained additional sensory domains potentially implicated in ligand binding. These domains included GAF (named after some of the proteins it is found in: cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA) and Per-ARNT-Sim (PAS) which are cytoplasmic domains with the potential to bind a wide range of ligands and which frequently constitute sensory domains in signalling proteins such as those involved in c-di-GMP turnover (Galperin, 2005; Henry and Crosson, 2011). The most prevalent domain identified was the PAS domain (Pfam clan CL0183), present in the N-terminus of all seven identified tandem GGDEF/EAL domain proteins (Fig. 1). Furthermore, the central domain (residues 129-166) of CdgB contained a partial Pfam match to a PAS domain (PF13188), while CdgK contained tandem N-terminal GAF domains in addition to the PAS domain (PF13492 and PF01590). PAS and GAF domains, and the C-terminal Ykul\_C domain in CdgJ (PF10388), all share a similar structural fold (Ho *et al.*, 2000; Minasov *et al.*, 2009). Furthermore, eight of the *B. cereus* group Cdg proteins were predicted to contain multiple transmembrane segments (Fig. 1). Such regions, including the domain of the 5TM-5TMR\_LYT type (PF07694) identified in CdgA, likely represent integral membrane domains which may also serve as sensors (Anantharaman and Aravind, 2003; Galperin, 2005). For CdgB, tertiary structure prediction methods indicated that the N-terminal 128 residue region is structurally similar to the response regulator receiver (REC) domain, although the conserved active site residues required for phosphoryl transfer activity in functional



REC domains (Bourret, 2010) were lacking. c-di-GMP signalling is transmitted through various downstream effector proteins, commonly forming a c-di-GMP responsive signalling network. As proteins harbouring degenerated catalytic domains, *CdgC*, *CdgJ* and *CdgL* are all candidates for constituting c-di-GMP binding effector proteins. In addition, c-di-GMP can mediate downstream effects through binding to GEMM riboswitches located upstream of effector genes (Sudarsan *et al.*, 2008). Using predicted c-di-GMP responsive GEMM riboswitches (Sudarsan *et al.*, 2008; Zhou *et al.*, 2016) we mined 23 *B. cereus* group genomes for downstream effector genes (including three representatives for the *B. anthracis* cluster, covering the A-, B-, and C- phylogenetic branches). The analysis revealed that each mined genome carried 1-3 gene loci putatively responsive to c-di-GMP through a GEMM riboswitch, and including genes encoding a putative methyl-accepting chemotaxis protein [orthologs to BC0422 in *B. cereus* ATCC 14579; c-di-GMP off-riboswitch (Lee *et al.*, 2010)], a collagen adhesion protein (orthologs to BC1060 *B. cereus* ATCC 14579; c-di-GMP on-riboswitch (Lee *et al.*, 2010)), and/or a cell surface protein (orthologs of CT43\_CH4799 in *B. thuringiensis* subspecies *chinesis* CT-43; c-di-GMP on-riboswitch (Zhou *et al.*, 2016)) (Supporting Information Table S2). Interestingly, the chemotaxis protein with its upstream riboswitch was found in all 23 genomes, while six genomes carried two riboswitch loci (*B. cereus* strains 03BB108, AH1134, ATCC 14579, G9241; *B. thuringiensis* Al-Hakam, *B. weihenstephanensis* KBAB4), and five genomes carried all three loci (*B. cereus* strains B4264 and G9842; *B. thuringiensis* strains var. *israelensis* ATCC 35646, *chinesis* CT-43 and Bt407, which was used as a model strain in this study) (Supporting Information Table S2).

#### Expression patterns of putative *DGC* and *PDE* encoding genes in *B. thuringiensis* 407 during growth

The presence of genes putatively involved in c-di-GMP synthesis and breakdown suggested that *B. cereus* group species could possess a c-di-GMP signalling network potentially impacting traits such as biofilm formation and motility in response to different environmental cues. Not all *B. cereus* group strains form biofilms under conditions tested (Wijman *et al.*, 2007; Auger *et al.*, 2009), and not all *B. cereus* group strains are motile. However, the motile strain *B. thuringiensis* 407 is often used as a model strain in biofilm studies, as it forms a robust biofilm (Houry *et al.*, 2010) and additionally is amenable for genetic manipulation. *B. thuringiensis* 407 harboured all identified c-di-GMP signalling genes except for *cdgM* and *cdgK* (Figs. 1 and 2). Expression

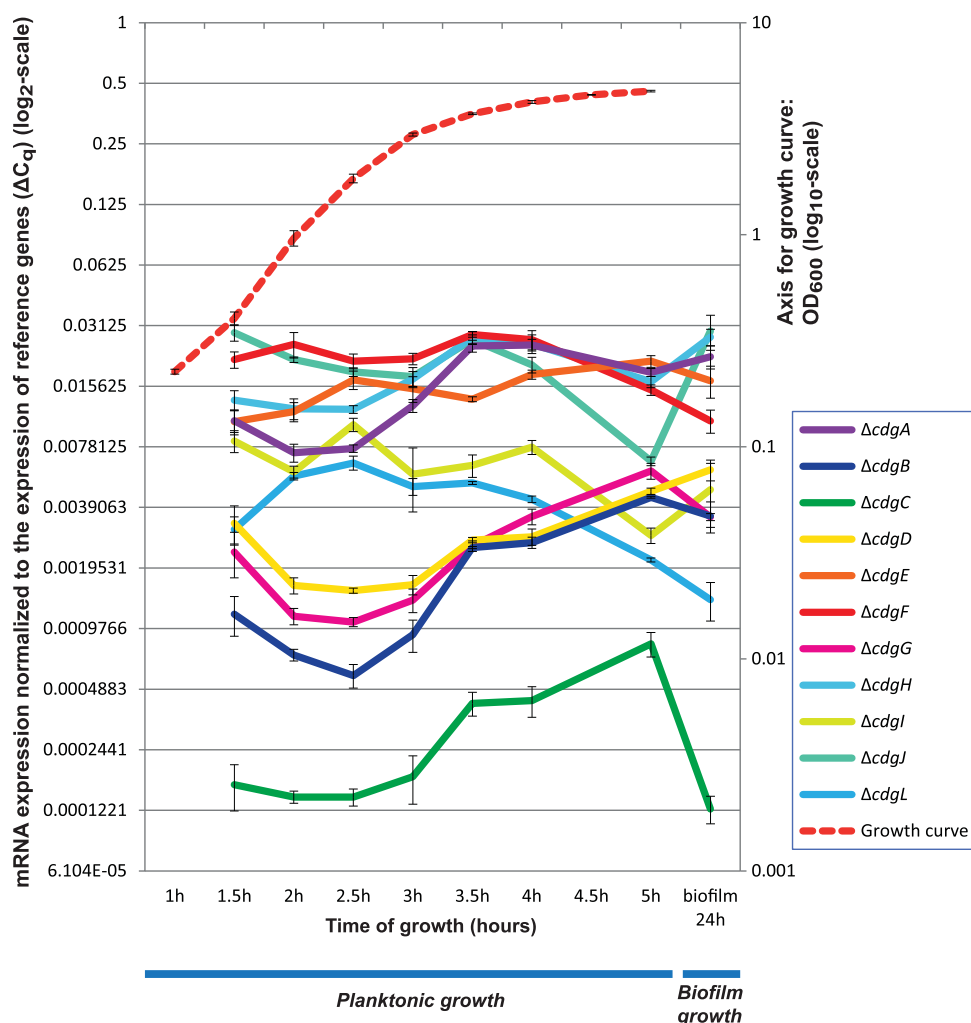
patterns of the *B. thuringiensis* 407 c-di-GMP signalling genes were examined using RT-qPCR throughout bacterial growth; RNA levels for the *cdg* genes were determined in cultures of *B. thuringiensis* 407 grown at 30°C, at seven different time points (1.5 h, 2 h, 2.5 h, 3 h, 3.5 h, 4 h and 5 h growth), and after growth in biofilm for 24 h (Fig. 3). The variation in expression level for any single gene during the course of planktonic growth was limited, although some genes showed differential expression between different time points within a factor of 4–8. The maximum difference in expression for any of the genes was an ~8-fold increase of *cdgB* expression between 2 and 5 h of growth. Notably, *cdgC* showed the lowest expression level among the *cdg* genes. Furthermore, *cdgC* and *cdgJ* showed a marked change in expression when going from the last time point of planktonic growth to 24 h biofilm, *cdgC* showing an ~8-fold decrease and *cdgJ* a ~4-fold increase respectively.

#### Creation of a library of *cdg* deletion mutants and overexpression strains in *B. thuringiensis* 407

In order to study functions of the c-di-GMP signalling network in *B. thuringiensis* 407, a library of in-frame single gene deletion mutants was constructed for all the ten putative *cdg* genes present in this strain, which were predicted to encode proteins with above-threshold Pfam matches to GGDEF and/or EAL domains (genes *cdgA*–*cdgJ*; Fig. 2). The 10 *cdg* genes were also each cloned into the low-copy shuttle vector pHT304-P<sub>xyl</sub> and introduced into strain *B. thuringiensis* 407, creating a library of overexpression strains for which *in trans* expression of each gene was inducible by the addition of xylose. The *cdgL* gene, which was present in the *B. thuringiensis* 407 strain but carried the highly degenerate GGDEF domain, was not included in further analyses.

#### Overproduction of *CdgC* carrying an intact I-site motif was toxic to native *B. thuringiensis* 407 host cells

Growth curves of all overexpression and mutant strains showed that the only strain significantly affected in growth was the strain overexpressing *cdgC*, which natively was the lowest expressed gene in wild type *B. thuringiensis* 407 cells (Fig. 3). *cdgC* encodes a protein which carries a C-terminal GGDEF domain but is predicted to be enzymatically inactive as it has a degenerate A-site motif. The *CdgC* protein does however contain a consensus RxxD I-site, and is thus predicted to be able to bind c-di-GMP. The strain overexpressing *cdgC* showed a distinct and severely prolonged lag phase compared with the empty vector control strain.



**Fig. 3.** RT-qPCR based expression profiles of the *B. thuringiensis* 407 *cdg* genes. Levels of mRNA transcripts for each *cdg* gene were determined by RNA sampling throughout the growth curve, and after 24 h growth in biofilm. Relative transcript expression levels for each target gene were determined by normalization to the geometric mean of the three internal control genes *gatB/yqeY*, *rpsU* and *udp*. The bacterial growth curve is represented by the dotted red line (log scale). The mean and standard error values from four independent experiments are shown.

The prolonged lag phase was dependent on induction of *CdgC* expression by xylose (Supporting Information Fig. S2A). A sample was taken from the delayed exponential growth phase of this strain (in cultures where *CdgC* expression was induced by 1 mM xylose) and reinoculated. A comparison of growth curves showed that while the original *CdgC* overexpression strain reproduced the prolonged lag phase, the re-inoculated overexpression strain exhibited the same growth pattern as the empty vector control. DNA sequence analysis of the cloned, plasmid-borne *CdgC* copy from three independent biological replicates of a re-inoculated *cdgC* overexpression strain showed that in each case, a copy of the *Tnpl* integrase gene and the *TnpA* transposase gene from the same transposon (Tn4430) had inserted into independent locations in the *CdgC* gene. The insertions were found inside the coding sequence (one case) or between the cloning vector-borne xylose-inducible promoter and the *CdgC* translation start site (two cases), in all cases presumably obstructing *cdgC* transcription. A

copy of the DNA fragment that had inserted into each clone is found both on plasmid BTB-15p and plasmid BTB\_78p, which are native plasmids to the *B. thuringiensis* 407 host strain. Thus, *CdgC* overproduction appears to be toxic to the *B. thuringiensis* 407 cell, since the bacteria were able to sustain growth only following transposon inactivation of the plasmidic *cdgC* copy. Also notable is the fact that following site-directed alanine-replacement mutagenesis, changing the <sup>134</sup>RxxD motif of *CdgC* to AxxA, allowed for *CdgC* overexpression from the same plasmid in the 407 strain without affecting the growth pattern (Supporting Information Fig. S2B). This strongly suggests that the toxic effect of *CdgC* overexpression is dependent on an intact RxxD motif, and one may speculate whether *CdgC* when overexpressed could act as a c-di-GMP sink, titrating most or all available c-di-GMP in the cell. As overproduction of *CdgC* was incompatible with growth, the *cdgC* overexpression strain was not subjected to further study.

### Increased levels of intracellular c-di-GMP upon overexpression of *CdgF* and *CdgB*

To determine whether alterations in the intracellular level of c-di-GMP could be detected in the strains in which *cdg* genes were deleted or overexpressed, the library of *cdg* deletion mutants and overexpression strains was assayed for whole-cell c-di-GMP content by LC-MS/MS analysis as previously described (Spangler *et al.*, 2010). For many of the *B. thuringiensis* 407 derivative strains, wild type *B. thuringiensis* 407 included, c-di-GMP content was below the level of detection (LOD, 0.8 ng ml<sup>-1</sup>; Limit of quantitation, LOQ: 3.5 ng ml<sup>-1</sup>). This has also been observed during vegetative growth in *B. subtilis* (Gao *et al.*, 2013), although detectable levels of c-di-GMP were identified by Diethmaier and co-workers (Diethmaier *et al.*, 2014). However the *CdgB* and in particular *CdgF* overexpression strains showed highly increased c-di-GMP content, indicating that they have DGC activity under the selected experimental conditions (Table 1; the full data set for all strains is described in Supporting Information Table S3).

### Phenotypic analysis of *cdg* deletion mutants and overexpression strains

In a range of bacteria, increasing levels of c-di-GMP, governed by the activity of the GGDEF domains of DGCs, mediate increased biofilm formation and reduced motility, while reduction in cellular c-di-GMP concentrations resulting from the action of the EAL or HD-GYP domains of PDEs, generally results in lower levels of biofilm formation and an increase in motility (Römling *et al.*, 2013). Furthermore, c-di-GMP has been shown in several bacteria to affect virulence gene expression, and usually increased c-di-GMP levels are associated with a decrease in virulence (Tamayo *et al.*, 2007; Ryan, 2013). To determine if any of the *cdg* genes affect formation of biofilm in *B. thuringiensis* 407, all mutant and overexpression strains were analyzed in a microtiter plate screening assay. Furthermore, all strains were tested for motility on swimming agar (0.3%) plates and for production of extracellular virulence factors by an *in vitro* Vero cell cytotoxicity assay (Lindbäck, 2006). Results from the phenotypic assays are shown in Fig. 4 and summarized in Table 2, identifying several *cdg* genes affecting multiple phenotypes, e.g. *cdgE* and *cdgF* which conferred opposite effects on biofilm formation and motility. A more detailed discussion of *cdg* gene function is included below.

### *CdgF* acts as a main DGC controlling biofilm formation and swimming motility

In the c-di-GMP quantitation experiments by LC-MS/MS, the strain overexpressing the tandem GGDEF/EAL domain protein *CdgF* was estimated to produce at least

**Table 1.** c-di-GMP concentrations in test samples isolated from *B. thuringiensis* 407 (wild type) and derivative strains, as measured by LC/MS-MS.

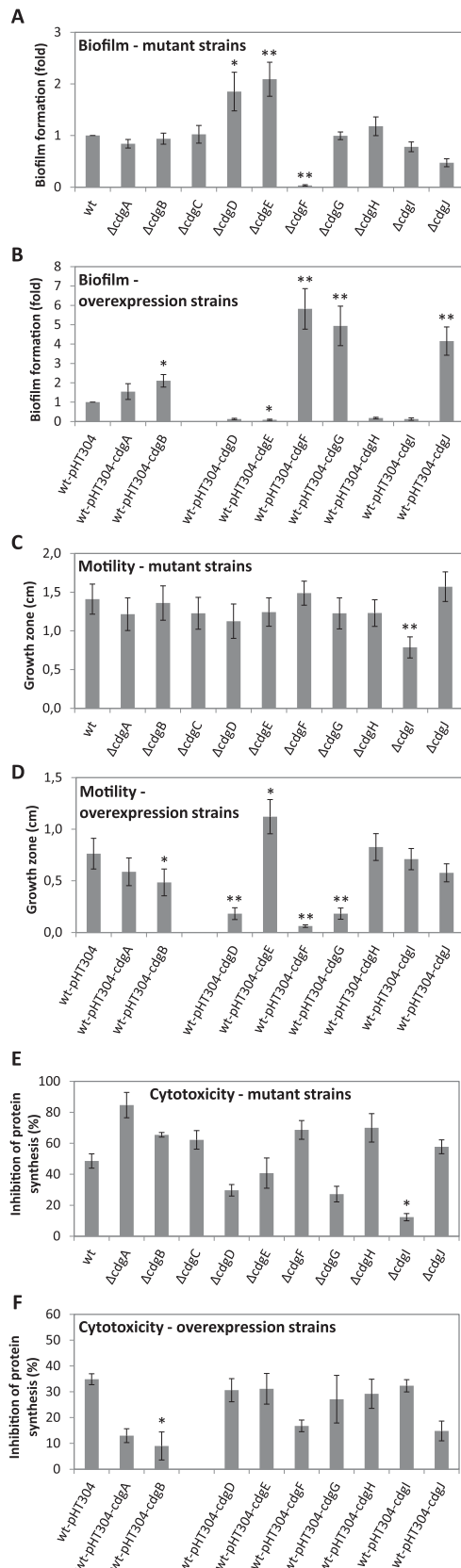
		Estimated c-di-GMP concentration in test samples <sup>a</sup> (results for two or three independent replicates listed)
Gene deleted or overexpressed	Deletion mutant	Overexpression strain
<i>cdgA</i>	< LOD < LOD < LOD	< LOD 7 ng ml <sup>-1</sup>
<i>cdgB</i>	1 ng ml <sup>-1</sup> < LOD < LOD	58 ng ml <sup>-1</sup> 16 ng ml <sup>-1</sup>
<i>cdgC</i>	< LOD < LOD < LOD	NA <sup>b</sup>
<i>cdgD</i>	< LOD < LOD < LOD	2.2 ng/ml 6 ng/ml
<i>cdgE</i>	2.3 ng ml <sup>-1</sup> 2.7 ng ml <sup>-1</sup> < LOD	< LOD < LOD
<i>cdgF</i>	< LOD < LOD < LOD	621 ng ml <sup>-1</sup> 422 ng ml <sup>-1</sup> 404 ng ml <sup>-1</sup>
<i>cdgG</i>	1.1 ng ml <sup>-1</sup> 0.9 ng ml <sup>-1</sup> < LOD	< LOD 5 ng ml <sup>-1</sup>
<i>cdgH</i>	1.6 ng ml <sup>-1</sup> 1.3 ng ml <sup>-1</sup> < LOD	< LOD 3.1 ng ml <sup>-1</sup>
<i>cdgI</i>	1 ng ml <sup>-1</sup> 0.9 ng ml <sup>-1</sup> < LOD	< LOD 3.1 ng ml <sup>-1</sup>
<i>cdgJ</i>	< LOD 2 ng ml <sup>-1</sup> < LOD	< LOD < LOD
wild type	< LOD < LOD < LOD	
Empty vector control (wt pHT304-Pxyl)		< LOD 6 ng ml <sup>-1</sup>

a. Limit of detection (LOD) was 0.8 ng ml<sup>-1</sup>; Limit of quantitation (LOQ): 3.5 ng ml<sup>-1</sup>

b. Not tested, as overexpression of *cdgC* was toxic to the cells (see text).

more than 60 times the normal level of c-di-GMP as compared to the corresponding empty vector control strain (Table 1 and Supporting Information Table S3), strongly indicating that *CdgF* acts as a DGC under the tested conditions. In line with this, deletion of *cdgF* completely abolished biofilm formation in the microtiter plate biofilm formation assay, while *cdgF* overexpression increased biofilm formation almost sixfold (Fig. 4A and B; Table 2). The *cdgF* deletion mutant was the only mutant strain in the library in which biofilm formation was abolished. Complementation of the *cdgF* deletion





**Fig. 4.** Effect of *cdg* gene deletion and overexpression on *B. thuringiensis* 407 biofilm formation, motility, and Vero cell toxicity of culture supernatant. The assays were performed on *B. thuringiensis* 407 cells deleted for (A,C,E) or overexpressing (B,D,F) specific putative c-di-GMP signalling (*cdg*) genes. (A, B) Relative biofilm formation determined after 24 hour biofilm growth in a microtiter plate. Biofilm formation was quantified by crystal violet staining, followed by measurement of  $A_{429nm}$ . Results were normalized with respect to the results for *B. thuringiensis* 407 wild type (A) or empty vector (B) strains, respectively. The mean and standard error values obtained from at least three independent experiments are shown, using a minimum of four technical replicates per strain for each experiment. (C, D) Swimming motility determined following growth on 0.3% LB agar for 7 h. Shown are the mean and corresponding standard error values obtained from at least ten independent experiments. Each assay was performed with three technical replicates. (E, F) Percentage inhibition of protein synthesis in Vero cells by culture supernatants of deletion mutants and overexpression strains. Mean and standard error values were obtained from at least three replicate experiments. For all experiments and phenotypes tested experimentally (A-F), a two-tailed paired student's *t*-test was performed (\* $P < 0.05$ ; \*\* $P < 0.01$ ) to test for statistical significance, comparing deletion mutants to wt and overexpression strains to the empty vector control strain, respectively.

mutant with *cdgF* overexpressed *in trans* from the xylose-inducible expression vector pHT304-P<sub>xyI</sub> corrected the biofilm formation defect of the *cdgF* deletion mutant (Supporting Information Fig. S3A), verifying that the biofilm phenotype was indeed caused by loss of *CdgF*. In line with *CdgF* acting as a *DGC* under the conditions studied, motility was strongly reduced when the *CdgF* overexpression strain (and the *in trans* complemented mutant) were tested in the motility assay on swimming agar (0.3%) plates (Fig. 4D and Supporting Information Fig. S3B respectively). Furthermore, an opposing cytotoxicity pattern was observed in the *in vitro* cell cytotoxicity assay for the *cdgF* gene deletion mutant and overexpression strain (Fig. 4E and F; Table 2), in which virulence was increased in the mutant and reduced upon *CdgF* overexpression. This was paralleled by the finding that levels of the B component of the Nhe enterotoxin (NheB), as well as those of Cytotoxin K (CytK), were strongly reduced in the *CdgF* overexpression strain (Fig. 5). In summary, these results strongly indicate that *CdgF* acts as a major *DGC* controlling the switch between a biofilm and a motile lifestyle under our experimental conditions, and also negatively affects cytotoxicity, possibly through regulation of toxin expression.

To confirm that the biofilm and motility phenotypes of *cdgF* depend on the *DGC* activity of the GGDEF domain, site-directed alanine-replacement mutagenesis was performed, changing the <sup>221</sup>GGDEF motif of *CdgF* to GGAAF (*CdgF*<sup>GGDEF→GGAAF</sup>). Overexpression of *CdgF*<sup>GGAAF</sup> from the pHT304-P<sub>xyI</sub> vector in the wild type background did not produce biofilm levels above those obtained with an empty vector control strain (Supporting Information Fig. S3A), nor did it lead to cellular

**Table 2.** Effects of mutation or overexpression of *cdg* genes from *B. thuringiensis* 407 on biofilm formation, motility, *in vitro* toxicity and sporulation.

Gene deleted or overexpressed	<i>In silico</i> predicted enzymatic activity	Enzymatic activity predicted from c-di-GMP measurements	Effect of gene deletion ( $\Delta$ ) or overexpression (OE) on phenotype, relative to wild type or vector control respectively							
			Biofilm formation		Motility		<i>In vitro</i> cytotoxicity		Sporulation	
			$\Delta$	OE	$\Delta$	OE	$\Delta$	OE	$\Delta$	OE
<b>cdgA</b>	DGC		– $P = 0.22$	(↑) $P = 0.28$	– $P = 0.13$	– $P = 0.053$	↑ $P = 0.09$	↓ $P = 0.11$	– $P = 0.43$ $P = 0.58$	– $P = 0.36$ $P = 0.88$
<b>cdgB</b>	DGC	DGC	– $P = 0.87$	(↑) <b><math>P = 0.02</math></b>	– $P = 0.70$	(↓) <b><math>P = 0.02</math></b>	(↑) $P = 0.06$	↓ <b><math>P = 0.02</math></b>	– $P = 0.45$ $P = 0.96$	↓ $P = 0.39$ $P = 0.06$
<b>cdgC</b>	none (GGDEF domain I-site)		– $P = 0.86$	not tested <sup>a</sup>	– $P = 0.30$	not tested <sup>a</sup>	(↑) $P = 0.055$	not tested <sup>a</sup>	↑ <b><math>P = 0.002</math></b> $P = 0.104$	not tested <sup>a</sup>
<b>cdgD</b>	PDE		↑ <b><math>P = 0.04</math></b>	↓↓ $P = 0.06$	(↓) $P = 0.05$	↓ <b><math>P = 0.0009</math></b>	↓ $P = 0.10$	– $P = 0.15$	– $P = 0.18$ $P = 0.28$	↓ $P = 0.28$ $P = 0.48$
<b>cdgE</b>	DGC/PDE	PDE	↑ <b><math>P = 0.0006</math></b>	↓↓ <b><math>P = 0.0498</math></b>	– $P = 0.10$	↑ <b><math>P = 0.012</math></b>	– $P = 0.70$	– $P = 0.59$	– $P = 0.55$ $P = 0.29$	– NA 0.92
<b>cdgF</b>	DGC/PDE	DGC	↓↓ <b><math>P = 0.002</math></b>	↑ <b><math>P = 0.0009</math></b>	– $P = 0.46$	↓ <b><math>P = 0.0009</math></b>	(↑) $P = 0.43$	↓ $P = 0.08$	– $P = 0.20$ $P = 0.18$	↓ $P = 0.114$ $P = 0.091$
<b>cdgG</b>	DGC/PDE		– $P = 0.54$	↑ <b><math>P = 0.01</math></b>	– $P = 0.29$	↓ <b><math>P = 0.002</math></b>	↓ $P = 0.052$	– $P = 0.27$	– $P = 0.06$ $P = 0.50$	↓ NA $P = 0.07$
<b>cdgH</b>	PDE		– $P = 0.37$	↓↓ $P = 0.06$	– $P = 0.30$	– $P = 0.62$	(↑) $P = 0.13$	– $P = 0.35$	– $P = 0.90$ $P = 0.47$	↓ $P = 0.39$ $P = 0.37$
<b>cdgI</b>	DGC (both A-site and I-site)/PDE		– $P = 0.35$	↓↓ $P = 0.07$	↓ <b><math>P = 0.0004</math></b>	– $P = 0.68$	↓ <b><math>P = 0.014</math></b>	– $P = 0.054$	– $P = 0.27$ $P = 0.54$	↓ NA $P = 0.36$
<b>cdgJ</b>	none		(↓) $P = 0.07$	↑ <b><math>P = 0.01</math></b>	– $P = 0.14$	– $P = 0.12$	(↑) $P = 0.56$	↓ $P = 0.06$	↓ $P = 0.30$ $P = 0.25$	(↑) $P = 0.06$ $P = 0.16$

a. Not tested, as overexpression of *cdgC* was toxic to the cells (see text).

A dash (–) denotes no difference relative to control (wt or empty vector, for gene deletion or over-expression clone, respectively).

Upward arrow indicates increase in phenotype under test condition (gene deletion or overexpression) relative to control (wild type or empty vector control, respectively).

Downward arrow(s) indicate decrease in phenotype under test condition (gene deletion or overexpression) relative to control (wild type or empty vector control, respectively).

Arrows in bold indicate relatively stronger effect, arrows in parenthesis indicate relatively weaker effect.

Double arrows (↓↓) indicate phenotype abolished (i.e. no biofilm or no spores formed under the tested conditions).

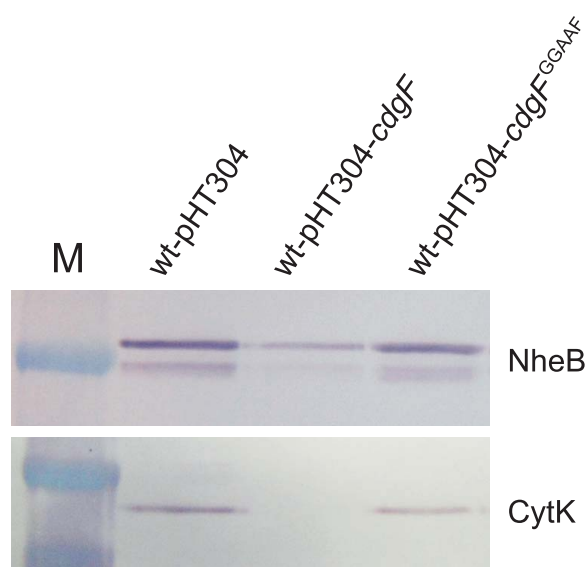
*P*-values are from a two-tailed paired students *t*-test comparing test condition (gene deletion or overexpression) to control (wild type or empty vector control, respectively).

c-di-GMP levels above the limit of detection (Supporting Information Table S3). Furthermore, the D223A and E224A substitutions also alleviated the inhibitory effects on motility and cytotoxicity (Supporting Information Fig. S3B and C), as well as alleviating the inhibition of NheB and CytK expression, observed from native *CdgF* overexpression (Fig. 5). Taken together, these results demonstrate that the effects of *CdgF* on biofilm formation, as well as its diguanylate cyclase activity, depends on an intact GGDEF motif, and strongly suggests that ele-

vated levels of c-di-GMP positively regulates biofilm formation and negatively regulates motility and cytotoxicity in *B. thuringiensis* 407.

*CdgA* and *CdgB* may act as DGC enzymes repressing cytotoxicity

Out of the *cdg* genes in *B. thuringiensis* 407, *cdgA* and *cdgB* both encode proteins with a GGDEF domain predicted to be capable of DGC activity, and no EAL domain



**Fig. 5.** Overexpression of *CdgF* leads to a decrease in secreted NheB and CytK toxin components, and is dependent on an intact *CdgF* GGDEF motif. Western immunoblot analysis of culture supernatants from the *B. thuringiensis* 407 empty vector control strain, the *CdgF* overproducing strain, and from the strain overproducing *CdgF*<sup>GGAAF</sup>, in which the GGDEF active site motif had been modified by alanine-replacement mutagenesis. Immunoblotting was performed using monoclonal antibodies directed against non-hemolytic enterotoxin component NheB (upper panel) and Cytotoxin K (lower panel), respectively.

(Fig. 1). For *CdgB*, the ability to produce c-di-GMP was confirmed in the assay of whole-cell c-di-GMP content by LC-MS/MS analysis, although *CdgB* overexpression produced c-di-GMP levels ten-fold lower than those resulting from *CdgF* overexpression (Table 1 and Supporting Information Table S3). The *cdgA* and *cdgB* deletion and overexpression strains showed a similar pattern in the biofilm, motility, and cytotoxicity assays: For both genes, overexpression resulted in a slight increase in biofilm formation (Fig. 4B; Table 2) and a slight decrease in motility (Fig. 4D; Table 2). Similar to that observed for *cdgF*, an opposing cytotoxicity pattern was observed for the *cdgA* and *cdgB* deletion and overexpression strains, where overexpression clones were less cytotoxic than vector control, and gene deletion mutants more cytotoxic than the wild type control (Fig. 4E and F; Table 2). Among the set of deletion mutants tested, the  $\Delta$ *cdgA* derivative was actually the strain showing the largest increase in virulence in the cell cytotoxicity assay. These results indicate that *CdgA* and *CdgB* generally contribute to a downregulation of cytotoxicity, while they have only a weak effect on the biofilm/motility 'switch'.

Also, a search for orthologs to *CdgB* outside of the *B. cereus* group yielded a match to Aflv\_1936 in *Anoxybacillus flavithermus* WK1. This protein has a domain architecture reminiscent of the PleD and WspR protein families (Wassmann *et al.*, 2007; De *et al.*, 2008), with

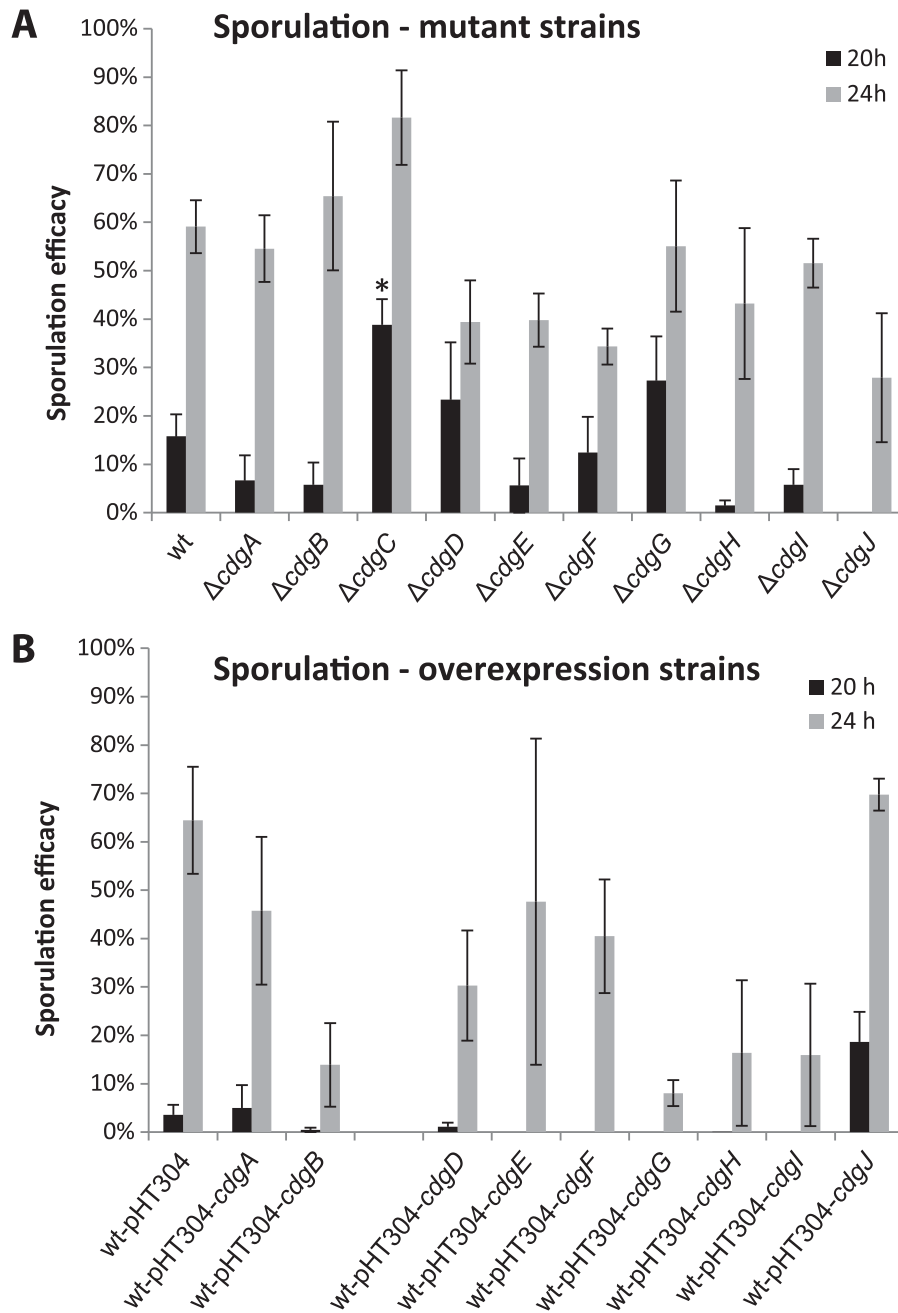
an N-terminal REC domain (Pfam PF00072; residues 10-120), a putative central PAS domain (Pfam PF13188; residues 137-204), and a C-terminal GGDEF domain (residues 263-416), indicating that Aflv\_1936 is a response regulator with a GGDEF output domain. The gene immediately upstream of Aflv\_1936 encodes a histidine kinase protein, suggesting that Aflv\_1936 is part of a two-component signal transduction system. A corresponding sensor kinase gene is, however, lacking in *B. cereus* group strains, consistent with the prediction that the *CdgB* REC domain has lost the ability to participate in phosphoryl transfer. However, it is not clear whether the apparent evolutionary divergence of the *CdgB* REC domain coincides with functional adaptations similar to those observed for the degenerate REC adapter domain in *Caulobacter crescentus* PleD, which has been shown to mediate allosteric regulation of the C-terminal GGDEF domain (Wassmann *et al.*, 2007).

#### Phenotypic assays for the EAL-domain protein *CdgJ* shows a functional pattern similar to proteins with DGC activity

*CdgJ*, the ortholog of the Ykul protein of *B. subtilis*, carries an N-terminal EAL domain with a predicted degenerate active site (Fig. 1). No detectable PDE activity was observed upon examination of the *B. subtilis* Ykul ortholog, although it was shown to be able to bind c-di-GMP (Minasov *et al.*, 2009). The *CdgJ* protein may thus be expected neither to have DGC nor PDE activity. Surprisingly, however, opposing patterns of biofilm formation and cytotoxicity was observed for the *cdgJ* deletion mutant and overexpression strains, suggesting that *CdgJ* may serve to increase biofilm formation and reduce cytotoxicity (Fig. 4; Table 2). In addition, a weak decrease in motility may be observed for the *cdgJ* deletion strain (Fig. 4). The *cdgJ* strains thus show a pattern of biofilm, motility, and toxicity phenotypes similar (although weaker in effect) to those seen for *cdgF* (Table 2), but without any observable effects on cellular c-di-GMP levels (Table 1 and Supporting Information S3). These results could potentially suggest that *CdgJ* somehow may play an indirect role in promoting DGC activity in the cell or act as a c-di-GMP receptor.

#### Characterization of *CdgD*, *CdgE*, *CdgH* and *CdgI* indicates potential PDE activity

Biofilm formation was completely abolished in our assay upon overexpression of four genes: *cdgD*, *cdgE*, *cdgH* and *cdgI* (Fig. 4B). All four genes encode tandem GGDEF/EAL domain proteins with EAL domains predicted to be capable of PDE activity, while three of the



**Fig. 6.** Effect of *cdg* gene deletion and overexpression on *B. thuringiensis* 407 sporulation efficiency. Sporulation efficiency was determined in cultures of *B. thuringiensis* 407 deleted for (A) or overexpressing (B) specific putative c-di-GMP signalling (*cdg*) genes. Sporulation was assayed after 20 and 24 h of growth in four independent experiments for each time point and strain tested (twelve independent experiments performed for: wt, Δ*cdgC*, wt-pHT304 and wt-pHT304-*cdgF*), and determined by comparing the number of spores giving rise to single colonies on plate following heat treatment relative to a non-treated control. A two-tailed paired student's *t*-test was performed ( $*P < 0.05$ ) to test for statistical significance, comparing deletion mutants to wt and overexpression strains to the empty vector control strain, respectively.

proteins may also be capable of **DGC** activity, as only **CdgH** contains a GGDEF domain with a degenerate (HDDQF) A-site (Fig. 1). For two of the genes, *cdgD* and *cdgE*, a strong increase in biofilm formation was observed for the deletion mutants (Fig. 4A). The opposing pattern observed for biofilm formation for the *cdgD* and *cdgE* gene deletion mutants and overexpression strains (Table 2) indicates that **CdgD** and **CdgE** may act as **PDEs** involved in biofilm regulation. Also supporting

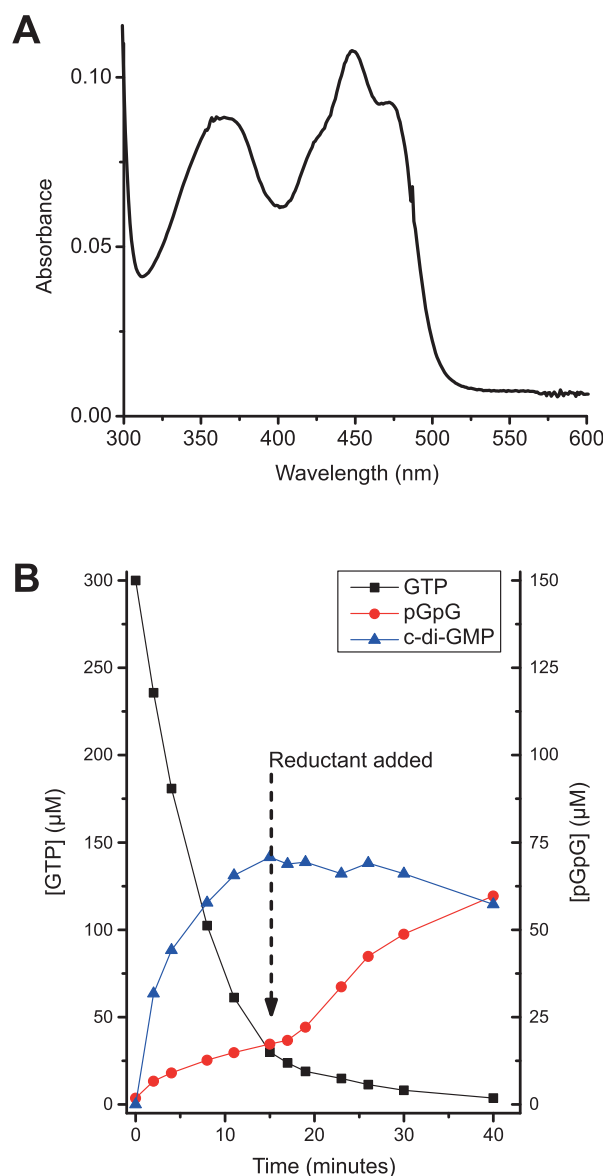
the role of **CdgE** as a **PDE** is the observation of a substantial increase in swimming motility upon *cdgE* overexpression (Fig. 4D). Furthermore, results from the measurement of whole-cell c-di-GMP content by LC-MS/MS showed detectable levels of c-di-GMP in the *cdgE* deletion strain (Table 1 and Supporting Information Table S3). For **CdgI**, a role as a **PDE** is supported by the observation that *cdgI* deletion appeared to decrease motility and cytotoxicity (Fig. 4C and E respectively).

### Effects on sporulation by *cdg* gene deletion or overexpression

Sporulation is a process which in *B. subtilis* has been shown to be linked to biofilm formation, both with respect to the transcriptional regulator Spo0A taking part in both processes, and spores constituting natural parts of a developing *B. subtilis* biofilm (reviewed in (Lopez and Kolter, 2010)). However to our knowledge, the effect of c-di-GMP-based signalling on sporulation has not previously been addressed. To examine whether sporulation was affected in any of the *cdg* strains, samples from bacterial cultures were harvested after 20 and 24 h of growth in LB medium, heat-treated at 70°C, and plated out on LB agar. None of the overexpression strains showed a statistically significant effect on sporulation (Fig. 6B; Table 2). However, among the two *cdg* genes predicted to encode enzymatically inactive proteins (*cdgC*, *cdgJ*), deletion of *cdgC*, carrying a degenerate GGDEF motif, resulted in a premature onset of sporulation ( $P = 0.002$ ), and the relative number of spores after 20 h was more than twice that of the wild type control (Fig. 6A). *CdgJ* seemed to show an opposite trend; upon *cdgJ* deletion no sporulation was observed at 20 h (Fig. 6A). Conversely, *CdgJ* overexpression seemed to lead to an early onset of sporulation ( $P = 0.06$ ; Fig. 6B; Table 2).

### *CdgF* is a redox regulated bifunctional enzyme carrying a flavin cofactor bound to its PAS domain

As the *CdgF* protein appeared to act as a main regulator of biofilm formation in *B. thuringiensis* 407, and to exhibit **DGC** activity under the experimental conditions tested, we further characterized the activities of this protein using biochemical assays. The *CdgF* ortholog from *B. cereus* ATCC 14579 (BC\_0628), which differs in only one amino acid from the *B. thuringiensis* 407 enzyme (166E/166K), was overexpressed and purified from *Escherichia coli*, and showed a UV-Vis spectrum characteristic of a flavoprotein (Fig. 7A). Thus *CdgF* could carry a functional sensory N-terminal flavin-binding PAS domain (Fig. 1) that potentially regulates the activity of the GGDEF and EAL domains. An activity assay of recombinantly expressed and purified *CdgF* showed that the protein is capable of both **DGC** and **PDE** activity, and is thus a bifunctional enzyme (Fig. 7B). The **DGC** activity of the GGDEF domain was most prominent when the flavin cofactor was in the oxidized state (as isolated, assayed under an argon atmosphere; Fig. 7B). During the course of the assay, at a point where about 90% of the GTP had been converted to c-di-GMP (Fig. 7B), the reaction mixture was added dithionite to induce reducing conditions. This increased *CdgF* **PDE** activity



**Fig. 7.** Biochemical characterization of purified *CdgF* protein. A. UV-Vis spectrum of purified *CdgF* protein from *Bacillus cereus* ATCC 14579 (BC\_0628) following protein overexpression in *E. coli*, showing a spectrum characteristic of flavin-containing proteins. B. Enzymatic assay showing that recombinant purified *CdgF* has functional PAS, GGDEF and EAL domains. The reaction mixture, holding purified enzyme, initially contained 300  $\mu\text{M}$  GTP, which is first converted to c-di-GMP by the GGDEF domain and further hydrolyzed to pGpG by the EAL domain, showing that both domains are catalytically active. At the time indicated (15 min) the reductant dithionite was added, and as a result, the rate of c-di-GMP hydrolysis carried out by the EAL domain increased, while diguanylate cyclase activity and c-di-GMP synthesis from GTP halted. This indicates that the *CdgF* protein is red-ox regulated, and may respond to oxygen by means of its flavin co-factor.

(Fig. 7B), potentially indicating that the EAL domain activity is upregulated when the PAS domain flavin cofactor is reduced. It can be noted from Fig. 7B that



the stoichiometry of the reaction (relative peak integrals) cannot be clearly observed because of c-di-GMP oligomerization, leading to hypochromicity. Stacking of cyclic dinucleotides has previously been observed both for c-di-GMP and c-di-AMP (Gentner *et al.*, 2012; Gundlach *et al.*, 2015). When the experiment was initiated at reducing conditions, resulting in immediate reduction of the PAS domain flavin cofactor, the enzyme activity profile changed drastically: The relative amounts of substrate and products after a 40 minute incubation in reducing conditions were 86.1% GTP, 2.1% c-di-GMP, and 11.8% pGpG, while the aerobic control showed 3.7% GTP, 90.7% c-di-GMP, and 5.6% pGpG. These results indicate that the DGC activity of the CdgF GGDEF domain decreases when the PAS domain flavin cofactor is reduced, and that any c-di-GMP that is formed is presumably immediately hydrolyzed to pGpG by the EAL domain, which has gained increased activity.

## Discussion

Cyclic-di-GMP has been shown to act as a near ubiquitous messenger molecule in the regulation of a number of bacterial phenotypes. Here we show that among the ten *B. thuringiensis* 407 proteins analyzed here, carrying full or partial matches to c-di-GMP related diguanylate cyclase and/or phosphodiesterase motifs (CdgA-J; Fig. 1), many were shown to affect *B. thuringiensis* biofilm formation and other classical phenotypes under c-di-GMP control. Most of the putative DGC and PDE enzymes are conserved in representative strains of *B. cereus* across the *B. cereus* group population structure, and except for the highly variable *cdgK*, *B. anthracis* carries a full complement of orthologs to the *cdg* genes identified in the *B. cereus* group (Fig. 2; Supporting Information Table S1), providing further support for *B. anthracis* biofilm formation capacity (Lee *et al.*, 2007). Organisms in clades I and VII (Guinebreiere *et al.*, 2008), including strains of *B. pseudomycoides* and *B. cytotoxicus*, respectively, were lacking the majority of the identified *B. cereus* group *cdg* genes (Fig. 2), universally retaining only *cdgB* and *cdgJ*. Notably, these clades represent outgroups in the *B. cereus* group population structure, and the difference in *cdg* gene profile may reflect different evolutionary processes from the main *B. cereus* clades (II–VI, Fig. 2). While clade VII is unusual in representing moderately thermophilic strains occasionally associated with food poisoning, and for which the NVH 391-98 strain has an unusually small genome (4.1 Mb; (Lapidus *et al.*, 2008)), clade I contains soil isolates typed as *B. cereus* and *B. pseudomycoides*, and for which genome sizes are typical for the *B. cereus* group (5.0–5.8 Mb). Notably,

*B. cytotoxicus* NVH 391-98 has been found in two studies to be an inefficient biofilm former under the conditions investigated (Wijman *et al.*, 2007; Auger *et al.*, 2009). However more data on other strains from clusters I and VII is needed to conclude whether the highly reduced complement of *cdg* genes harboured by these strains correlate with reduced biofilm formation efficiency.

### Phenotypic effects of *B. thuringiensis* 407 *Cdg* proteins

As for many other bacteria with multiple c-di-GMP genes (Holland *et al.*, 2008; Bordeleau *et al.*, 2011; Newell *et al.*, 2011b; Spurbeck *et al.*, 2012), not all *B. cereus* group *cdg* gene products appeared to be enzymatically active. Out of the six putative diguanylate cyclases in *B. thuringiensis* 407 with an intact GGDEF domain, only CdgF and CdgB produced measurable amounts of c-di-GMP under the tested conditions. Interestingly, CdgB is one of the two *cdg* genes universally conserved in the *B. cereus* group, possibly indicating that all strains are capable of c-di-GMP synthesis. While CdgF was shown to be a master DGC, with marked effects on biofilm, motility, and cytotoxicity both in overexpression strains and deletion mutants, CdgB showed limited effects on biofilm and motility when overexpressed (Fig. 4; Table 2). However, a strong reduction in cytotoxicity was seen in the overexpression strain, with a corresponding slight toxicity increase in the deletion mutant compared to wild type. Regarding the putative phosphodiesterases, both CdgD and CdgE showed an inhibitory effect on biofilm, and CdgE also increased motility. None of the other putative DGCs or PDEs appeared to produce or degrade c-di-GMP to levels required for identification. We can however not rule out the possibility that phenotypic effects for some *cdg* mutants may have been masked by the dominant DGC activity of CdgF. RT-qPCR analysis showed only very little variation in transcription of the *cdg* genes during different growth phases (Fig. 3), in line with previous studies in other bacteria which have shown that the activity of c-di-GMP signalling proteins is most often regulated post-translationally as a response to sensory input (Jenal and Malone, 2006).

### CdgF is a redox regulated dual-function enzyme capable of diguanylate cyclase and phosphodiesterase activity, affecting multiple biofilm-related phenotypes in *B. thuringiensis* 407

Although exhibiting a fairly even expression pattern across the growth curve, including the biofilm state, *cdgF* showed profound effects on *B. thuringiensis* 407 biofilm formation. This could indicate that the protein is regulated post-transcriptionally, and activity studies of

purified CdgF indeed showed that the protein is capable of both DGC and PDE activities (GGDEF and EAL domain, respectively), and that the enzymatic activity dominating at any one time is strongly regulated by the red-ox state of the protein, probably through its associated flavin co-factor. In connection to this it is interesting to note that a CdgF<sup>GGAAF</sup> overexpression strain carrying a version of the *cdgF* gene mutated in its active site, was considerably more motile and more cytotoxic than the empty vector control strain (Supporting Information Fig S3B and C), despite NheB and CytK levels being comparable to a wild type control carrying an empty expression plasmid (Fig. 5). This may potentially be an effect of increased activity from the EAL domain of the CdgF<sup>GGAAF</sup> mutant. Under aerobic conditions, where the purified enzyme was shown biochemically to act primarily as a diguanylate cyclase, CdgF strongly stimulated biofilm formation, while inhibiting bacterial cell motility and toxicity to Vero cells *in vitro*. In line with this, *cdgF*-overexpressing cells under aerobic conditions contained highly elevated levels of intracellular c-di-GMP, and was found to express reduced levels of both the NheB enterotoxin component as well as Cytotoxin K, the latter effect being dependent on a functional GGDEF motif governing the diguanylate cyclase activity. Conversely, the *cdgF* deletion strain was completely abolished in its biofilm formation capability, showing that *cdgF* is essential for *B. thuringiensis* 407 biofilm formation under these experimental conditions.

From these data, CdgF should be considered a master diguanylate cyclase governing biofilm formation under aerobic growth in *B. thuringiensis* 407. Protein domain analysis showed that CdgF, unlike many of the other Cdg proteins, does not carry transmembrane domains, nor an N-terminal signal sequence, and is probably a cytoplasmic protein. Perhaps surprisingly, the CdgF protein is not universally conserved in the *B. cereus* group, as it is lacking from the most remote phylogenetic clusters (cluster I and VII), hosting *B. pseudomycoides* and *B. cytotoxicus* strains, respectively, (Guinebreteire *et al.*, 2008; Lapidus *et al.*, 2008). Whether other Cdg proteins, e.g. such as the universally conserved CdgB, a putative DGC, may fully or partially compensate for the lack of CdgF in these strains, currently remains speculation.

#### CdgE, a PlcR regulated putative phosphodiesterase

*cdgE* was previously shown to be a part of a large regulon under positive control of the transcriptional regulator PlcR (Gohar *et al.*, 2008), a positive regulator of extracellular virulence factors in *B. cereus* and *B. thuringiensis* (Agaisse *et al.*, 1999), potentially providing a link

between c-di-GMP signalling and virulence in these bacteria. As shown in Fig. 4 and Table 2, CdgE acts as a putative phosphodiesterase under our experimental conditions, inhibiting biofilm formation and stimulating motility. Interestingly, PlcR has previously been reported to affect these phenotypes (Hsueh *et al.*, 2006; Slamti *et al.*, 2004), which are potentially important for *B. cereus* and *B. thuringiensis* during its infectious cycle (Dubois *et al.*, 2012; Slamti *et al.*, 2014), where motility could be important for spread of the pathogen within its host (Zhang *et al.*, 1993; Callegan *et al.*, 2005). It is indeed conceivable that these effects may be contributed through the action of CdgE. *cdgE* was however the only gene where no difference in cell cytotoxicity phenotypes were observed in neither the mutant nor the wild-type strain (Fig. 4E and F). This could potentially indicate that CdgE mediates specific effects of the PlcR regulon towards motility and biofilm formation rather than regulation of cytotoxicity, which is known to be governed by direct regulation by the PlcR transcriptional activator of a set of phospholipases, proteases, the HBL and Nhe enterotoxin operons, and a range of other factors (Gohar *et al.*, 2008).

#### Sporulation, a novel c-di-GMP responsive phenotype?

Sporulation is a hitherto unexplored response to c-di-GMP signalling. Spores are however a common component of *B. subtilis* biofilms (Branda *et al.*, 2001; Veening *et al.*, 2006), and both sporulation and biofilm formation responses in *B. subtilis* are known to be governed by the degree of phosphorylation of the key transcriptional regulator Spo0A [reviewed in (Lopez *et al.*, 2009)]. Despite neither CdgC nor CdgJ harbouring DGC or PDE domains predicted to be functional, both proteins exerted effects on sporulation when deleted and/or overexpressed. It is well known that Cdg proteins harbouring degenerate DGC or PDE domains may be able to bind c-di-GMP and thus act as receptor molecules mediating downstream effects (Holland *et al.*, 2008; Bordeleau *et al.*, 2011; Newell *et al.*, 2011b; Spurbeck *et al.*, 2012). Notably, *cdgJ* expression increased four-fold from planktonic growth (5 h time point) to biofilm formation after 24 hours (Fig. 3), and *cdgJ* overexpression accordingly lead to an increase in biofilm formation in the microtiter plate screening assay. In addition, the CdgJ overexpression strain showed earlier onset sporulation compared to the isogenic empty vector control, while the *cdgJ* deletion mutant exhibited reduced biofilm formation and later onset, reduced level sporulation. Conversely, CdgC showed opposite trends to CdgJ regarding expression profile and sporulation, in that *cdgC* expression decreased drastically from planktonic

growth to biofilm after 24 h (Fig. 3), coupled with a more than double increase in sporulation in the *cdgC* deletion mutant after 20 h compared to the wild type strain (Fig. 6). Although a separate study would be needed to explain the effects of these proteins on sporulation and discern molecular mechanisms potentially involved, it is tempting to speculate that *CdgC* and *CdgJ* may act as c-di-GMP binding effector proteins, mediating downstream effects on sporulation and other phenotypes. It is also interesting to note that *cdgJ*, which seems to exert effects on both sporulation and biofilm formation, is conserved throughout all strains included in our analyses, from all seven subclusters and all seven species of the *B. cereus* group (Fig. 2), in addition to *B. subtilis* (both strains 168 and NCIB 3610; see text below).

#### *c-di-GMP regulation of B. cereus group biofilm formation*

In summary, although having a more limited set of *cdg* genes compared to many Gram-negative bacteria such as *Vibrio cholerae* and *E. coli*, most *B. cereus* group bacteria (those outside population clusters I and VII; Fig. 2) carry a wider range of genes encoding c-di-GMP metabolizing enzymes compared to its Gram-positive relative *B. subtilis* (Chen *et al.*, 2012; Gao *et al.*, 2013). Notably, BLASTP searches of derivative protein sequences from the thirteen *cdg* genes found in *B. cereus* group genomes, in combination with investigation for conserved protein domain structure, returned only two potential orthologs in the biofilm model strain *B. subtilis* NCIB 3610 (ATCC 6051; (Chen *et al.*, 2012; Gao *et al.*, 2013)). These were orthologs for *cdgA* (BSU6051\_09120, *dgcK*, formerly called *yhck*; 32% amino acid identity, 98% sequence coverage) and *cdgJ* (BSU6051\_14090, *ykuJ*; 55% amino acid identity, 99% sequence coverage) (Supporting Information Table S4 and Fig. S1). Also, the functionality of the *B. thuringiensis* 407 c-di-GMP regulatory network seems to reflect what is generally observed in Gram-negative species, as well as in Gram-positive *C. difficile* and *L. monocytogenes* (Bordeleau *et al.*, 2011; Chen *et al.*, 2014). Thus, in contrast to what is reported in *B. subtilis*, c-di-GMP serves to regulate biofilm formation in *B. thuringiensis*, possibly through a network of c-di-GMP response genes that may include *cdgC* and *cdgJ*. *Bacillus* constitutes a highly diverse genus, which has recently been suggested to be re-organized with regards to nomenclature, retaining only the *B. subtilis* group and *B. cereus* group clades and renaming all other *Bacillus* members (Bhandari *et al.*, 2013). Results in the current paper may reflect a possible diversification of the role of c-di-GMP signalling even between two of the major subgroups

among Bacilli, the *B. cereus* group and the *B. subtilis* group. Constituting common bacteria of the soil environment, and which are also capable of causing infections in insect hosts (Jensen *et al.*, 2003) as well as opportunistic infections in mammals (Bottone, 2010), one may speculate that a wide range of sensory inputs are needed to regulate and/or fine tune the lifestyle and mode of growth of *B. cereus* group bacteria in these highly variable growth environments. It is interesting in this regard to note that many of the *cdg* genes in the *B. cereus* group genomes encode putative sensory domains that could serve to adjust c-di-GMP levels, many also including putative N-terminal transmembrane domains targeting the protein to the bacterial cell membrane. This is in line with earlier findings that different c-di-GMP-regulated phenotypes can be controlled by distinct DGCs, possibly by the formation of local c-di-GMP pools within subcellular microenvironments (Newell *et al.*, 2011a; Massie *et al.*, 2012; Lindenberg *et al.*, 2013). Downstream effector functions under riboswitch control included a putative chemotaxis protein for which transcription is turned off in the presence of c-di-GMP, and various putative peptidoglycan-linked cell surface proteins (five-gene operon) and putative collagen adhesin for which c-di-GMP in both cases turns transcription on (Lee *et al.*, 2010; Zhou *et al.*, 2016). Future dissection of the molecular functions and macromolecular interactions of the various *Cdg* proteins and their putative downstream effectors could provide further links between c-di-GMP signalling and phenotypes classically important for *B. cereus* group bacteria in their different habitats, including sporulation, motility, cytotoxicity and biofilm formation.

## Experimental procedures

### Bioinformatics analyses

Sequences of the ten *B. thuringiensis* 407 proteins containing significant matches to the Pfam GGDEF (PF00990), and EAL (PF00563) domain protein families were downloaded from the Pfam 25.0 server (<http://pfam.xfam.org/>). These sequences were used to search the 90 *B. cereus* group genomes represented in the Bacteria subset of the UniProtKB protein database ([www.uniprot.org](http://www.uniprot.org)) as of May 2011 using the global alignment algorithm GGSEARCH at [www.ebi.ac.uk/Tools/sss/](http://www.ebi.ac.uk/Tools/sss/). This includes the 19 *B. anthracis* strains sequenced at the time of the analysis. To identify any additional proteins containing these domains in *B. cereus* group strains, protein sequences containing significant matches to the Pfam GGDEF and EAL protein families were downloaded for all *B. cereus* group genomes in the Pfam 25.0 server as of May 2011, and compared with the protein sequences identified in the initial GGSEARCH analysis. Thus, two additional proteins were identified (*CdgK* and *CdgL*). In addition, an analysis performed by Galperin and co-workers (Galperin, 2005) showed that one HD-GYP domain protein was present in *B. cereus* group strains. The

GGSEARCH analysis was then repeated on the 90 genomes using the following protein sequences representative of the three additional identified proteins as input sequences: BCAH187\_A0409 (CdgK), BTB\_c54300 (CdgL), and BCAH187\_A1141 (CdgM). The presence of genes encoding a histidine kinase with an N-terminal orthologous to that of BCAH187\_A0409 prompted a corresponding GGSEARCH analysis using the BCE\_5355 protein as the query sequence. For each *B. cereus* group strain in which an ortholog to one or more of the above listed query proteins was not identified using GGSEARCH, the individual genome sequences in question were subjected to a new search using TFASTX and BLASTN to search for unannotated genes encoding these proteins. The results of the analysis are presented in Supporting Information Table S1.

The occurrence of functional domains was predicted using InterProScan Sequence Search (Hunter *et al.*, 2012) and SMART analysis (Letunic *et al.*, 2012). Using SMART, signal peptides were predicted using SignalP 4.0 and transmembrane segments were predicted using TMHMM2 v. 2.0. CdgB, for which the 128 amino acid long N-terminal fragment was not assigned to any known domain using the above methods, was further analysed using BLAST (Altschul *et al.*, 1997) and the protein structure prediction server Phyre2 (Kelley and Sternberg, 2009).

In cases where there was variation in the position of the annotated start codon and/or the protein length between orthologs in the different strains, all orthologs to which a locus tag had been assigned and which did not appear to be truncated due to missense or frameshift mutations, putative sequencing errors, or location at a contig terminus, were compiled and subjected to ClustalW alignment. The most likely correct start codon, and thus corresponding predicted length, for the proteins shown in Fig. 1, was assigned based on the multiple sequence alignments, the analysis of functional domains (including signal peptide predictions), and inspection of the nucleotide sequence (potential alternative start codons and the presence of a putative ribosome binding sites at a correct location with reference to the start codon). In the case of CdgB, homology modelling and alignment with homologs outside the *B. cereus* group identified by BLAST was also included in the analysis.

The search for the presence of orthologs to *B. cereus* group GGDEF, EAL, and HD-GYP domains encoding genes in species outside the *B. cereus* group was performed on the sequences available as of November 2011 in the Integrated Microbial Genomes (IMG) browser (Markowitz *et al.*, 2010). Genes which were detected as orthologs by IMG and showed conserved gene ortholog neighbourhoods were considered orthologs in the analysis. These analyses matched a complementary analysis performed by reciprocal BLASTP analyses in combination with observation for conserved protein domains between putative orthologs (NCBI conserved domains database (CDD) analysis performed in combination with the BLASTP searches; <http://blast.ncbi.nlm.nih.gov/>).

### Phylogenetic tree construction

A phylogenetic tree representing the *B. cereus* group population was constructed employing the distance-based

Neighbour-Joining-like method BioNJ (Gascuel, 1997). Pairwise distances were computed as the percentage of nucleotide differences between the 2658 bp concatenation of the seven alleles from the Tourasse-Helgason MLST scheme [(Helgason *et al.*, 2004); <http://mlstoslo.uio.no>] for each strain. Distance computations, tree building and visualization was done using SEAVIEW4 (Gouy *et al.*, 2010). The seven phylogenetic clusters within the *B. cereus* group, referred to as group I to VII (Guinebreteire *et al.*, 2008, 2010; Tourasse *et al.*, 2011), were labelled.

### *B. cereus* group strains and culture conditions

The acrySTALLIFEROUS *B. thuringiensis* strain 407 Cry<sup>-</sup> (Gominet *et al.*, 2001) (also sometimes described as strain Bt407) is genetically close to the *B. cereus* reference strain ATCC 14579 (Tourasse *et al.*, 2006), but while *B. thuringiensis* 407 forms thick biofilms, *B. cereus* ATCC 14579 is a poor biofilm producer under the tested conditions. Strain 407 is phenotypically indistinguishable from the *B. cereus* species due to loss of the plasmids encoding insecticidal crystal toxins (Helgason *et al.*, 2000; Lereclus *et al.*, 1989).

Unless otherwise stated, *B. cereus* cultures were inoculated with 1% of an overnight culture and grown at 30°C and 200 rpm in bacto-peptone medium (1% w/v bacto-peptone, 0.5% w/v yeast extract, 1% w/v NaCl). Erythromycin at 10 µg ml<sup>-1</sup> was used to maintain the pHT304-Pxyl plasmid constructs. Unless otherwise specified, 1 mM xylose was added to the growth medium for induction of gene expression from the *xylA* promoter on pHT304-Pxyl. Growth curves were obtained by measuring OD<sub>600</sub> in cultures grown in 500 ml baffled Erlenmeyer flasks.

### RNA isolation and reverse transcription quantitative PCR

Biofilm cells to be sampled for RNA isolation were grown on glass wool essentially as described (Oosthuizen *et al.*, 2001), by inoculating a culture containing 1.0 g dry-sterilized glass wool per 100 ml bacto-peptone medium with a 0.5% inoculum of an overnight culture, and incubating for 24 h under gentle shaking (50 rpm). To remove planktonic cells, the glass wool was quickly washed twice in fresh pre-warmed bacto-peptone medium. The glass wool was transferred to ice-cold 60% methanol, and cells were removed from the glass wool by vigorous shaking and collected by centrifugation.

Samples from planktonic cultures to be used for RNA isolation were incubated in an equal volume of ice-cold methanol for 5 min before harvesting by centrifugation. For extraction of RNA, cells were lysed using a Precellys 24 Tissue Homogenizer (Bertin), and RNA was isolated using the RNeasy Mini or Midi Kits (Qiagen). For RT-qPCR, RNA was treated with TURBO DNase (Ambion) as described and purified using the RNA Cleanup protocol from the RNeasy Mini Kit (Qiagen). cDNA synthesis was performed in duplicate for each sample, using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturers protocol, with 0.1 U µl<sup>-1</sup> SUPERase-In (Ambion) instead of RNaseOUT, and using 1.32 µg RNA and 90 ng random



hexamer (Applied Biosystems) in 15 µl reactions. Negative control reactions without reverse transcriptase were included for all samples. RT-qPCR reactions were performed on a LightCycler 480 Real-Time PCR System (Roche) in a 96-well microtiterplate format and a final volume of 10 µl using 0.1 µl cDNA, 5 µl LightCycler 480 SYBR Green I Master (Roche), and 0.5 µM of each primer. The primers used are listed in Supporting Information Table S5. The three genes *gatB/yqeY*, *rpsU*, and *udp*, shown to be stably expressed throughout the *B. cereus* life cycle (Reiter *et al.*, 2011), were used as reference genes, and were included for each sample and on each plate. Cycling conditions were 95°C for 5 min followed by 45 cycles at 95°C for 10 s, 58°C for 10 s, and 72°C for 8 s, and a melting curve analysis, which resulted in single product specific melting temperatures for all samples. Amplification of all negative control reactions without reverse transcriptase with two of the three reference gene primers confirmed the absence of amplification of contaminating DNA. The quantification cycle ( $C_q$ ) values were determined using the second derivative maximum method using the LightCycler 480 software (Roche). PCR efficiencies ( $E$ ) and Pearson's correlation coefficient ( $r$ ) for each primer pair (Supporting Information Table S5) was determined as previously described (Pfaffl, 2001) using triplicate samples of pooled cDNA diluted 1:3, 1:10, 1:30, 1:100, 1:300, and 1:1000. For gene expression analysis, the  $C_q$  values for the two technical replicates from each sample were averaged and transformed into linear scale expression quantities using the formula  $E^{C_q}$  (Pfaffl, 2001). For each target gene in each of the biological replicates, the gene expression was normalized to the geometric mean of the  $E^{C_q}$  values obtained for the three reference genes (delta- $C_q$ -method). Averages and standard errors were then calculated from the normalized expression values from the four biological replicates.

#### Construction of overexpressing strains and deletion mutants

The low-copy number *E. coli/Bacillus* shuttle vector pHT304-Pxyl, in which *xyIR* and the *xyIA* promoter from *B. subtilis* have been inserted into the pHT304 cloning site (Arantes and Lereclus, 1991) allowing xylose-inducible expression of downstream cloned genes, was a kind gift from Dr. Didier Lereclus (INRA, France). Ten *cdg* genes (*cdgA-cdgJ*) were PCR amplified from *B. thuringiensis* 407 using primers listed in Supporting Information Table S6 and inserted into pHT304-Pxyl using primer-incorporated restriction sites. The plasmids containing *cdgF* and *cdgC* with amino acid substitutions in the conserved GGDEF and RxxD sites, respectively, were created by site-directed mutagenesis of the respective overexpression plasmids using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) and the primer pairs listed in Supporting Information Table S6. The plasmids were verified by sequencing and introduced by electroporation into *B. thuringiensis* 407 (Masson *et al.*, 1989).

Mutants in the 10 *cdg* genes (*cdgA-cdgJ*) were generated via homologous recombination using a markerless gene replacement method (Janes and Stibitz, 2006). To

enable facilitated detection of transformants using blue-white screening on X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) plates, the pMAD shuttle vector (Arnaud *et al.*, 2004), encoding a constitutively expressed β-galactosidase gene, was used as the integrative plasmid. To allow cleavage of the integrated pMAD allelic exchange construct by the pBKJ223-encoded homing restriction enzyme I-SceI (Janes and Stibitz, 2006) promoting the second homologous recombination event, pMAD was modified to contain an I-SceI restriction site using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) and primers listed in Supporting Information Table S6. The final vector was confirmed by I-SceI digestion as well as by sequencing.

Each mutant allele was designed to contain the start and stop codons of the deleted gene separated by the ACGCGT recognition sequence of *MluI*, thus creating in-frame deletions which are not expected to exert polar effects on surrounding genes. For each gene to be deleted, approximately 800 bp of DNA sequence homologous to the upstream and downstream region of the gene was PCR amplified using the primers listed in Supporting Information Table S6. The up- and downstream fragments were fused using the primer-incorporated *MluI*-sites, cloned into pCR 2.1-TOPO vector (Invitrogen) and transferred to pMAD-I-SceI. The constructs were introduced into *B. thuringiensis* 407 by electroporation (Masson *et al.*, 1989) and allelic exchange was performed essentially as described (Janes and Stibitz, 2006). All mutant alleles were verified by sequencing of PCR products generated with primers designed to anneal outside of the sequences used for homologous recombination (Supporting Information Table S6).

#### Extraction of c-di-GMP

Nucleotides were extracted as described by Spangler and co-workers (Spangler *et al.*, 2010), with modifications as follows: Cells were cultured in bactopeptone medium at 30°C and 200 rpm, with added 10 µg ml<sup>-1</sup> erythromycin and 1 mM xylose for strains containing the pHT304-Pxyl plasmid constructs. Samples were withdrawn after 5 hours and quickly centrifuged (13000 rpm, 1 min, 4°C) to collect bacteria. The cell pellet from each sample was suspended in 1 ml ice-cold extraction solvent (40% acetonitrile/40% methanol/20% dH<sub>2</sub>O containing 20 ng ml<sup>-1</sup> of the internal standard cXMP (Cat. no. X001-05, Biolog) and transferred to Precellys tubes (VK01, Bertin). The cells were lysed in a Precellys 24 Tissue homogenizer (Bertin) for 2 cycles of 5800 rpm for 30 s, with a 20 second pause between runs. The tubes were then placed at -20°C. The cells were lysed again in the Precellys machine, centrifuged (13000 rpm, 1 min, 4°C) and 700 µl supernatant was withdrawn from the tube. A second extraction with 700 µl ice-cold extraction solvent (without cXMP) was performed after another run in the Precellys machine. The solvent of the combined supernatants (1.4 ml) was then evaporated until dryness in a Speedvac (Thermo Electron Corporation).

The pellets from the evaporation were suspended in 100 µl sterile Milli-Q water and filtered through a 0.22 µm



Millipore filter (12000 x g, 4 min). Ion exchange columns were made from layers of ion exchange filter paper (anion-exchange SR – 47 mm; Phenomenex) and conditioned by washing with 100 µl acetone, methanol, Milli-Q water, 1 M NaOH and Milli-Q water again, before adding the filtrated samples to the columns along with 100 µl 50 mM Tris buffer pH 8.5. Five spiked (25 ng ml<sup>-1</sup> c-di-GMP; Biolog, Cat. no. C057-01) and two blank (Milli-Q water) samples were included for each sample run. The columns were washed with methanol before eluting with a total of 150 µl 0.5% FA 500 mM ammonium acetate. Eluted samples were evaporated until dryness in a Speedvac. The samples were suspended in 50 µl Milli-Q water before analysis by LC-MS/MS.

#### Quantification of **cyclic di-GMP** by LC-MS/MS

LC-MS/MS analysis was based on a method described by Spangler and co-workers (Spangler *et al.*, 2010), with some modifications. The chromatographic system comprised a Dionex UltiMate 3000 WPS 3000 TSL autosampler, a LPG 3300 pump and a SRD 3300 degasser connected to a Thermo Scientific LTQ XL Linear Ion Trap Mass Spectrometer (all from Thermo Scientific, Sunnyvale, CA, USA). Data acquisition and processing was performed using Xcalibur version 2.1 software from Thermo Scientific. The chromatographic separation was accomplished with a 5 mm x 1 mm I.D. Nucleodur C18 Pyramid precolumn and a 50 mm x 1 mm I.D. Nucleodur C18 pyramid analytical column (both from Marchery-Nagel). Both columns had an average pore size of 110 Å, and particle size of 3 µm. The mobile phases consisted of A: 10mM ammonium acetate 0.1% formic acid and B: 100% methanol. The flow rate was set to 40 µl min<sup>-1</sup> and the injection volume was 15 µl. A linear gradient was run up to 50% mobile phase B in 10 min using 100% mobile phase A as starting point. After these 10 min, the mobile phase composition was kept constant for 8 min. Subsequently, the column was flushed with 100% mobile phase A for 12 min at a flow rate of 100 µl min<sup>-1</sup> prior to the next injection.

An electrospray ionization (ESI) source operated in the positive ionization mode was used to interface the High-performance liquid chromatograph and the mass spectrometer. Analyses were performed with selected reaction monitoring (SRM) using He as a collision gas and 22% collision energy. The sheath gas was set to 25 units, capillary temperature 300°C, and the spray voltage to 4 kV. The quantifier SRM transition (619.17 → 540.19) was used to quantify c-di-GMP while the qualifier transition (691.17 → 248.07) was used as confirmatory signal if necessary. Quantification was carried out using a single-point calibration.

#### Biofilm assay

The ability to form biofilms in polyvinylchloride microtiter plates was determined using a crystal violet biofilm screening assay (Auger *et al.*, 2006). Briefly, fresh bacto-peptone medium was inoculated with 0.5% exponential phase culture, transferred to 96-well plates (Falcon 353911) and incubated for 24 h at 30°C. The biofilm was subsequently washed using phosphate buffered saline (PBS), stained

using 0.3% crystal violet solubilized in 25%/75% acetone/ethanol, and transferred to flat-bottomed microtiter plates (Falcon cat no 353915) for determination of the absorbance of the solubilized dye at 570 nm. The measurements in each individual assay were normalized so that the wild type or empty vector samples were set equal to 1. Each strain was tested in at least three independent experiments, employing at least four technical replicates per strain.

#### Motility assay

Swimming motility was analyzed by inoculating 5 µl of an overnight culture (grown at 30°C, 220 rpm in LB medium) in the center of a 0.3% LB agar plate. For strains carrying a pHT304-Pxyl plasmid construct, 1 mM xylose and 10 µg ml<sup>-1</sup> erythromycin were added to the medium. Plates were incubated at 30°C for 7 h and the radius of the mobility zone was measured. The assay was performed with at least 10 independent experiments, each with three technical replicates per strain tested.

#### Cytotoxicity assay

The Vero cell cytotoxicity assay was performed as described (Lindbäck, 2006), measuring the percentage inhibition of C<sup>14</sup>-leucine incorporation in proteins, by exposure of Vero cells to *B. thuringiensis* culture supernatants. Samples (100 µl) of early stationary phase culture supernatants of gene deletion mutants and wild type cells (grown to OD<sub>600</sub> = 2.4) were applied to the assay in duplicate. For overexpression strains, 150 µl culture supernatants harvested at OD<sub>600</sub> = 2.4 were applied to the assay, and an empty vector control was included. The cytotoxicity assays were performed on at least three replicate experiments, except for the experiment overexpressing the mutant form of **CdgF** (**CdgF**<sup>GGDEF→GGAFF</sup>), which was performed in duplicate independent experiments.

#### SDS-PAGE and Western immunoblotting

Culture supernatants were applied to NuPAGE Novex Bis-Tris gel system (Invitrogen) using SeeBlue Plus2 Pre-Stained Standard (Invitrogen) as a molecular weight marker. Western blot analysis was performed according to standard protocols (Harlow and Lane, 1988). Monoclonal antibody 1C2 against NheB (Dietrich *et al.*, 1999) was a kind gift from Dr. Erwin Märtlbauer (Ludwig-Maximilians-Universität, Munich, Germany), and 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).

### Sporulation assay

Sporulation efficacy was determined by comparing the number of spores counted after heat-treatment relative to an untreated control. A 1:100 dilution of an overnight culture was grown in fresh LB to an OD<sub>600</sub> of approximately 0.8. For strains carrying pHT304-Pxyl plasmid constructs, erythromycin was added to 10 µg ml<sup>-1</sup>. This pre-culture was again diluted 1:100 in LB (added 1 mM xylose and 10 µg ml<sup>-1</sup> erythromycin for the plasmid-containing strains) and grown at 30°C with shaking (200 rpm). Samples were taken at 20 and 24 h and sonicated (SONOREX RK 100, Bandelin) for 1 min before diluting in PBS. The final dilutions were plated on LB agar, both as untreated samples and after heat treatment at 70°C for 30 min. Plates were incubated at 30°C overnight and colonies counted. The sporulation assay was performed with four independent biological replicates of each strain.

### Production of recombinant CdgF

**Cloning.** Genomic DNA was isolated from *B. cereus* ATCC 14579 using the DNeasy kit (Qiagen). The gene encoding BC\_0628 (CdgF ortholog) was amplified by PCR, and at the same time added 5'-end and 3'-end restriction sites for *Xba*I and *Bam*HI, using the primers 5'-CCCTCTAGA AATAATTTGTTTAACTTTAAGAAGGAGATATACAT ATGCTAGAACAGAGAGGTCATGC-3' and 5'-GCCGGATCCTTA AAAATCTGTAATTAAC-3' respectively. The amplified product was cloned into the pET22b vector resulting in the pET22b-BC\_0628 expression vector, which was used to transform competent *E. coli* BL21 (DE3) cells and confirmed by DNA sequencing.

**Protein expression.** *E. coli* BL21 (DE3) cells transformed with the pET22b-BC\_0628 expression vector were grown over night in 50 ml LB-medium containing 100 µg ml<sup>-1</sup> ampicillin. The overnight culture was diluted in 1 L TB-medium containing 100 µg ml<sup>-1</sup> ampicillin, and incubated in a shaker at 30°C until OD<sub>600</sub> = 1.8–2. The culture was then cooled to 20°C on ice while purging the culture medium with N<sub>2</sub> (g) for 5 min. The culture was added 25 mM glucose and 20 mM NaNO<sub>3</sub> before the flask was capped with an air-tight rubber plug and incubated in a shaker at 20°C. After 30 min, IPTG was added to a final concentration of 1 mM without exposing the culture to oxygen. The *E. coli* cells were harvested by centrifugation after 16 h anaerobic incubation at 20°C in a shaker incubator.

**Protein purification.** All of the following procedures were performed at 5–8°C. Typically, 30 g of bacterial paste was lysed using an X-press. The resulting paste of lysed cells was dissolved in 100 ml 50 mM Tris-HCl pH 7.5, 200 mM NaCl, and 1 mM DDT. The lysate was clarified by centrifugation at 48 000 x g for 1 h. In order to precipitate DNA, streptomycin sulphate was added to the supernatant to a final concentration of 2%. Precipitated DNA was removed by centrifugation at 30 000 x g for 30 min. Proteins in the resulting supernatant were precipitated by adding 0.43 g ml<sup>-1</sup> ammonium sulphate followed by centrifugation at 30 000 x g for 30 min. The protein pellet was carefully dis-

solved in 10 ml 50 mM Tris-HCl pH 7.5, 200 mM NaCl, and 1 mM DDT. The dissolved protein solution was desalted using a 70 mM G-25 fine column (column material purchased from GE Life Sciences) equilibrated with 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM DDT, and 10% glycerol.

The desalted protein solution was applied to a 60 ml HP Q ion-exchange column (column material purchased from GE Life Sciences) equilibrated with 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM DDT and 10% glycerol, and separated in a 0–0.5 M KCl gradient. The BC\_0628 protein was detected at 450 nm due to its yellow flavin cofactor and the protein weight was verified by SDS-PAGE (Novex, Invitrogen). Protein purity was estimated to 90–95% by visual inspection on the SDS-PAGE gel.

### Activity assays for CdgF

**Enzymatic assay.** The DGC and PDE assays were carried out in a 50 mM Tris-HCl pH 7.6, 0.5 mM EDTA, 50 mM NaCl, 10 mM MgCl<sub>2</sub> buffer. In all experiments the initial concentration of GTP was 300 µM and the protein concentration was varied between 0.1 and 0.3 µM. When the protein was assayed under reducing conditions, all solutions were repeatedly flushed with Argon (g) and evacuated using a vacuum line and kept in sealed vials. Reducing conditions were introduced in the experiments by adding aliquots of 150 mM sodium dithionite, buffered in 0.5 M Tris-HCl, pH 7.6, to a final concentration of 5 mM. At the end of each such experiment the red-ox indicator methyl viologen was added to confirm reducing conditions.

Samples from the enzymatic assays were filtered using a 0.22 µm centrifugal filter (Millipore) and mixed 1:1 with HPLC buffer A, consisting of 0.1 M phosphate buffer (pH 7) and 4 mM tetrabutylammonium bisulfate. Substrate and products were separated using an Äkta purifier (GE Life Sciences) equipped with a C18 column employing a linear gradient of HPLC buffer A:HPLC buffer A, 42% MeOH. Peak analysis was carried out using the Unicorn software (GE Life Sciences).

**UV-Vis spectroscopy.** Light absorbance spectra of BC\_0628 were collected using a HP8454 spectrophotometer (Agilent).

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