Veronika Smith

Cellular signaling and molecular regulation governing motility, biofilm formation, and virulence in the *Bacillus cereus group*

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Veronika Smith

Oslo, November 2020.

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Outline of the thesis

The Gram-positive opportunistic pathogen *Bacillus cereus* is increasingly recognized as a major source of food-borne disease, as well as of severe and sometimes fatal non-gastrointestinal infections. *B. cereus* belongs to the *B. cereus* group, a diverse group of bacteria including the mammalian pathogen *B. anthracis,* the cause of anthrax, the insect pathogen *B. thuringiensis*, which is the world's most commonly used biopesticide, as well as non-motile species such as *B. mycoides* and *B. pseudomycoides*. Although primarily a pathogen to insects, *B. thuringiensis* has also been identified as the cause in cases of human disease.

B. cereus readily forms biofilms, and research has shown that the regulation of biofilm formation, flagellar motility and virulence is often interconnected in this species. The aim of this study was to further unravel the regulatory mechanisms connecting these phenotypes, which are all of importance to the pathogenicity of the bacterium, by comparative analysis in the various species of the *Bacillus cereus* group. The plasmid-cured, *cry*-negative strain *Bacillus thuringiensis* 407 was used as a model for *B. cereus*, as it is a sequenced, highly transformable and motile strain which readily forms biofilms, and for which *in vivo* toxicity models have been firmly established. Phenotypically, the *B. thuringiensis* 407 strain is principally indistinguishable from *B. cereus.*

The second messenger c-di-GMP was previously identified in Gram-negative bacteria as a key regulator of the switch from planktonic growth to biofilm formation, as well as of expression of virulence factors. Prior to the start of this thesis work, less was known about c-di-GMP regulation in Gram-positive species. By searching the genomes of all available *B. cereus* group strains, ten conserved proteins (CdgA-J) were identified, putatively related to c-di-GMP synthesis (diguanylate cyclases; DGCs) or breakdown (phosphodiesterases; PDEs). Quantitative analyses by LC-MS/MS confirmed that *B. thuringiensis* 407 produced higher cellular levels of c-di-GMP upon overexpression of CdgF compared to a control, identifying CdgF as a main DGC in this species. Furthermore, biofilm formation, motility and/or virulence were affected by deletion and/or overexpression of the genes predicted to be involved in c-di-GMP metabolism. In general, high levels of c-di-GMP promoted biofilm formation and reduced motility and virulence. Among the putative DGCs and PDEs, only two were conserved in *Bacillus subtilis,* indicating that c-di-GMP signaling is different between the *B. cereus* and *B. subtilis* groups of bacteria. An additional conserved gene *cdgL,* encoding a protein with a degenerate DGC domain, was subjected to further functional analysis. Microscale thermophoresis experiments did however not indicate c-di-GMP binding by CdgL. The protein was nevertheless found to be essential to motility in *B. thuringiensis* 407, and a *cdgL* deletion rendered the bacterium severely reduced in flagellar gene expression, resulting in non-flagellated, non-motile cells. *cdgL* deletion also caused a delay in biofilm pellicle production, an effect which was also observed in a separate non-motile gene disruption mutant (Δ*flaAB),* in which both flagellin genes were deleted. *cdgL* resides in a three-gene operon also encoding a putative glycosyl transferase and a NupC family transporter. The operon is conserved throughout the *B. cereus* group, with the exception of non-motile *B. pseudomycoides,* potentially indicating a related functions for the three genes.

A newly identified putative transcriptional regulator in *B. thuringiensis* 407, MogR, and which is present only in *Listeria* spp. and *B. cereus* group bacteria, was also found to affect expression of flagellar genes in *B. thuringiensis* 407, similar to what was previously described for *Listeria monocytogenes* MogR. In *B. thuringiensis*, MogR appears to act as a transcriptional regulator by binding to a consensus sequence upstream of regulated genes, although the prerequisites for DNA binding seem to be different from *L. monocytogenes*. *B. thuringiensis* MogR in addition to regulating motility genes, appears to also affect the expression of genes related to virulence and biofilm formation, either directly or indirectly, and electrophoretic mobility shift assays suggested a direct repression of flagellar genes. Increased biofilm formation and attenuation of virulence upon MogR overexpression was confirmed by phenotypic assays, and the influence of MogR on both phenotypes was found to be independent of the loss of flagella. LC-MS/MS analyses and microarray experiments suggested that increased total cellular levels of c-di-GMP and higher transcriptional levels of *sinI,* respectively, could potentially play a role in this positive effect on biofilm formation.

Taken together, work conducted as part of this thesis provides the first description of a putative c-di-GMP regulatory network in the *B. cereus* group, and with the functional characterization of MogR, expands on the transcriptional regulatory network in the *B. cereus* group, previously known to involve regulators such as PlcR, NprR, and SinR. Altogether the work further develops the knowledge of the intersection between motility, virulence and biofilm formation, and the coordinated regulation of these processes in *B. cereus* group bacteria.

Abbreviations

AFM	Atomic force microscopy
AFLP	Amplified fragment length polymorphism
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
c-di-GMP	Cyclic diguanylic acid
DGC	Diguanylate cyclase
DNA	Deoxyribonucleic acid
EMSA	Electrophoretic mobility shift assay
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
EPS	Exopolysaccharide
In vivo	Within the living
In vitro	Within the glass
kb	Kilobase pairs
LC-MS/MS	Liquid chromatography–mass spectrometry/mass spectrometry
mRNA	Messenger Ribonucleic acid
MLST	Multilocus sequence typing
PDE	Phosphodiesterase
PCR	Polymerase chain reaction
pGpG	5'-phosphoguanylyl-(3',5')-guanosine
RNA	Ribonucleic acid
RT-qPCR	Quantitative reverse transcription PCR

List of publications

Paper 1

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

Annette Fagerlund, Veronika Smith, Åsmund K. Røhr, Toril Lindbäck, Marthe P. Parmer, Kristoffer K. Andersson, Leon Reubsaet, Ole Andreas Økstad.

Mol Microbiol. 2016, 101(3):471-94. doi: 10.1111/mmi.13405. Epub 2016 Jun 2.

Paper 2

MogR is a ubiquitous transcriptional repressor affecting motility, biofilm formation and virulence in the *Bacillus cereus* group.

Veronika Smith, Malin Josefsen, Toril Lindbäck, Ida K. Hegna, Sarah Finke, Nicolas J. Tourasse, Christina Nielsen-LeRoux, Ole Andreas Økstad, Annette Fagerlund.

Revised version accepted in Front. Microbiol. 2020, 11: 610650. doi: 10.3389/fmicb.2020.610650

Paper 3

CdgL is a degenerate GGDEF domain protein affecting flagellin synthesis and motility in *Bacillus thuringiensis.*

Veronika Smith, Ida Kristine Bu Nilssen, Ida K. Hegna, Bjørn Dalhus, Annette Fagerlund, Ole Andreas Økstad.

In revision, Research in Microbiology

Paper not included in thesis:

Bacillus thuringiensis CbpA is a collagen binding cell surface protein under c-di-GMP control.

Sarah Finke, Annette Fagerlund, Veronika Smith, Veronica Krogstad, Mimmi Zhang, Athanasios Saragliadis, Dirk Linke, Christina Nielsen-LeRoux, Ole Andreas Økstad.

The Cell Surface. 2019, 5:100032, DOI: https://doi.org/10.1016/j.tcsw.2019.100032

1 Introduction

1.1 The genus *Bacillus*

The genus *Bacillus* was first proposed by Ferdinand Cohn in 1872 and described as endospore-forming, Gram-positive, aerobic or facultative aerobic, rod-shaped bacteria (Cohn 1872). Over the years, a vast number of species were assigned to the genus, with as many as 146 species in 1938 (Berkeley 2002). The genus thus comprised a large and diverse group of bacteria. In 1991, however, the emergence of 16S rRNA sequencing made it possible to divide the genus into distinct clusters (Ash et al. 1991), and over the years further developments in typing methods have culminated in only two groups of species belonging to the genus *Bacillus*; the *Bacillus cereus* group bacteria and the *Bacillus subtilis* group (Bhandari et al. 2013) (Table 1), the remaining having been re-classified as new species.

Firmicutes are a phylum of bacteria, most of which have a typical Gram-positive cell wall structure. The phylum is further classified into class, order, family, genus and species, as described underneath (Table 1). The bacterial species comprising the *Bacillus* genus belong to the class III "*Bacilli*" of the phylum *Firmicutes*. The class *Bacilli* also includes the genus *Listeria*. All members of the class *Bacilli* are Gram-positive facultative anaerobe motile bacteria, but only the *Bacillus* species are capable of endospore formation. *B. subtilis* is the best studied Gram-positive bacterium to date and has been a model bacterium in laboratory work for decades, due to being non-pathogenic and easy to manipulate genetically (Cui et al. 2018; Vlamakis et al. 2013).

Table 1: Taxonomic relationship between the *B. cereus* and *B. subtilis* groups, and *L. monocytogenes.*

Phylum	Class	Order	Family	Genus	Example Species
Firmicutes	*Bacilli*	*Bacilliales*	*Bacillaceae*	*Bacillus*	*B. cereus*
					B. subtilis
			Listeriaceae	*Listeria*	*L. monocytogenes*

1.2 The *Bacillus cereus* group

1.2.1 Characteristics of the *B. cereus* group species

The *B. cereus* group (*B. cereus sensu lato*) encompasses at least seven bacterial species; *B. cereus* (*sensu stricto*), *Bacillus anthracis*, *Bacillus thuringiensis*, *Bacillus weihenstephanensis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, and *Bacillus cytotoxicus*. In recent years fourteen additional new taxa have been suggested as individual species in the *B. cereus sensu lato* group, based on a wide set of criteria such as e.g. 16S rRNA sequencing, *gyrA* and *rpoB* gene sequencing and MLST analysis (Jung et al. 2011; Jung et al. 2010; Liu et al. 2014; Liu et al. 2017; Miller et al. 2016; Peak et al. 2007), including *Bacillus toyonensis,* which has been used as a probiotic in animal nutrition for more than 30 years (Jimenez et al. 2013).

The three species of the *B. cereus* group which have been the subject of most studies, *B. cereus* (*sensu stricto*), *B. anthracis* and *B. thuringiensis*, were first described and classified at the end of the nineteenth century. ***B. cereus sensu stricto*** (Frankland 1887) is a soil bacterium which is also found in water and

dust, but their natural habitat has been suggested to be as symbionts of the invertebrate gut (Jensen et al. 2003; Saile and Koehler 2006). The bacterium is however a frequent cause of emetic disease or diarrheal gastroenteritis as well as other human opportunistic infections (Bottone 2010; Drobniewski 1993). Contamination by *B. cereus* is a persistent problem in the dairy industry (Gopal et al. 2015), and can also cause serious complications in hospital environments by colonization of medical equipment and hospital linen (Ikram et al. 2019; Hosein et al. 2013; Yamada et al. 2019). ***B. thuringiensis*** is unique in carrying genes that encode toxins that are lethal to larvae of various insect orders, and is used as a commercial biopesticide worldwide (Soberon et al. 2007). The insecticidal toxins Cry and Cyt are produced during sporulation and *the cry* and *cyt* genes are usually carried on plasmids (Schnepf et al. 1998). Although these plasmid-encoded toxins enable *B. thuringiensis* to live as a pathogen of the insect gut, specific chromosomal-encoded virulence traits allow the bacteria to also live a *B. cereus*-like lifestyle inhabiting a variety of environments, and some isolates have been shown to cause infection in humans and animals (Celandroni et al. 2014; Kim et al. 2015). ***B. anthracis*** is the causative agent of the fatal disease anthrax in humans and mammals (Mock and Fouet 2001), which is endemic in several parts of the world including e.g. parts of Africa and the USA. It was used by Robert Koch in 1876 to form postulates about the microbial origins of infection, and by Louis Pasteur in 1881 to create the first effective bacterial vaccine (Schwartz 2009). In this century, *B. anthracis* has been used in biological warfare and bioterrorism when anthrax spores in envelopes were sent through the U.S. postal system in 2001 (Canter et al. 2005). Like in *B. thuringiensis*, the main virulence factors are carried by plasmids; the anthrax toxin components encoded on pXO1 (Okinaka et al. 1999), and the antiphagocytic capsule on pXO2 (Drysdale et al. 2005).

The remaining species of the *B. cereus* group have been studied less extensively. ***B. mycoides*** was discovered in 1932 (Lewis 1932) and is along with ***B. pseudomycoides*** defined mainly by morphology, being characterized by rhizoidal growth on solid medium. *B. pseudomycoides* was later delineated from *B. mycoides* based on a different fatty acid composition (Nakamura 1998) and comprises a separate group in the *B. cereus sensu lato* phylogeny. Both species are non-motile due to severely truncated motility loci. ***B. weihenstephanensis*** is a common dairy contaminant and is psychrotolerant, i.e. able to grow at 7°C or below. The species is characterized by features of the 16S and 23S rDNA, the 16S-23S rDNA spacer region, and the gene encoding the cold shock protein CspA (Lechner et al. 1998). The thermotolerant ***B. cytotoxicus*** NVH 391-98 was discovered in 1998 as a severe food-borne pathogen which resulted in three deaths following a food poisoning incident, presumably due to the presence of the hitherto undiscovered enterotoxin CytK (Lund, De Buyser, and Granum 2000). Since then, other isolates carrying variants of the gene encoding CytK have been found, although not all are cytotoxic (Fagerlund et al. 2007). The *B. cytotoxicus* species was recognized as a member of the *B. cereus* group in 2013 based on 16 rRNA gene sequence similarity, fatty acid composition, and MLST data (Guinebretiere et al. 2013). *B. cytotoxicus* has a reduced genome size compared to the other *B. cereus* group strains and forms the most distant phylogenetic cluster of the group (group VII, see Figure 1) (Guinebretiere et al. 2013; Lapidus et al. 2008; Schmidt, Scott, and Dyer 2011).

1.2.2 Genome organization

The species in the *B. cereus* group, despite their phenotypical differences, share a common chromosomal backbone and carry several chromosomal genes encoding virulence factors, including the key virulence transcriptional regulator PlcR (Helgason et al. 1998; Helgason et al. 2000; Schmidt, Scott, and Dyer 2011; Read et al. 2003). In *B. anthracis* the *plcR* gene however contains a mutation resulting in a premature termination signal which renders the encoded PlcR protein truncated and non-active as a regulator (Agaisse et al. 1999). Moreover, nonsense mutations in genes located to the motility locus (Read et al. 2003) make *B. anthracis* non-motile, although the motility genes are still present on the chromosome. The genomes of *B. anthracis* strains also contain four conserved prophages not present in the other members of the *B. cereus* group (Sozhamannan et al. 2006).

The members of the *B. cereus* group commonly contain plasmids, with some strains containing more than 10 and with sizes ranging up to 600 kb (Zheng et al. 2015; Zheng et al. 2013). Genes carried on these plasmids may be responsible for characteristic traits such as virulence, growth temperature, morphological properties and adaptation properties. The plasmids may be self-transmissible, which may result in a transfer of these phenotypic traits within the *B. cereus* group members (Patino-Navarrete and Sanchis 2017). The species-specific virulence factors that distinguish *B. thuringiensis* and *B. anthracis* from *B. cereus sensu stricto* are located on plasmids, which may be lost or gained, and in the case of *B. thuringiensis*, loss of the plasmid carrying the insecticidal *cry* and *cyt* genes will make the bacterium phenotypically indistinguishable from *B. cereus* (Ivanova et al. 2003; Kolsto, Tourasse, and Okstad 2009). The presence of both pXO1 and pXO2 plasmids is required for full virulence in *B. anthracis*, but strains lacking one or both plasmids do exist (Busch et al. 2018; Welkos, Vietri, and Gibbs 1993). In recent years, two variants of isolates, so-called atypical *B. cereus* strains, from the *B. cereus* group have been discovered, carrying a non-anthracis chromosome, i.e. being motile and carrying the classic *B. anthracis* prophages, while at the same time carrying *B. anthracis* pXO1 and pXO2-like (or other capsule-encoding) plasmids and thus capable of causing anthrax-like disease in mammals (Hoffmaster et al. 2004; Klee et al. 2010; Baldwin 2020). Interestingly, one variant of these atypical strains have a functional non-mutates plcR gene while the other variant has a frameshift mutation in the gene, different from the non-sense mutation found in *B. anthracis plcR* (Baldwin 2020). Whole genome sequencing has been performed on four such isolates, demonstrating that these bacteria comprise a distinct clade within the *B. cereus* group, named *B. cereus* biovar *anthracis*, and the plasmids appear to have been acquired by a common ancestor (Antonation et al. 2016).

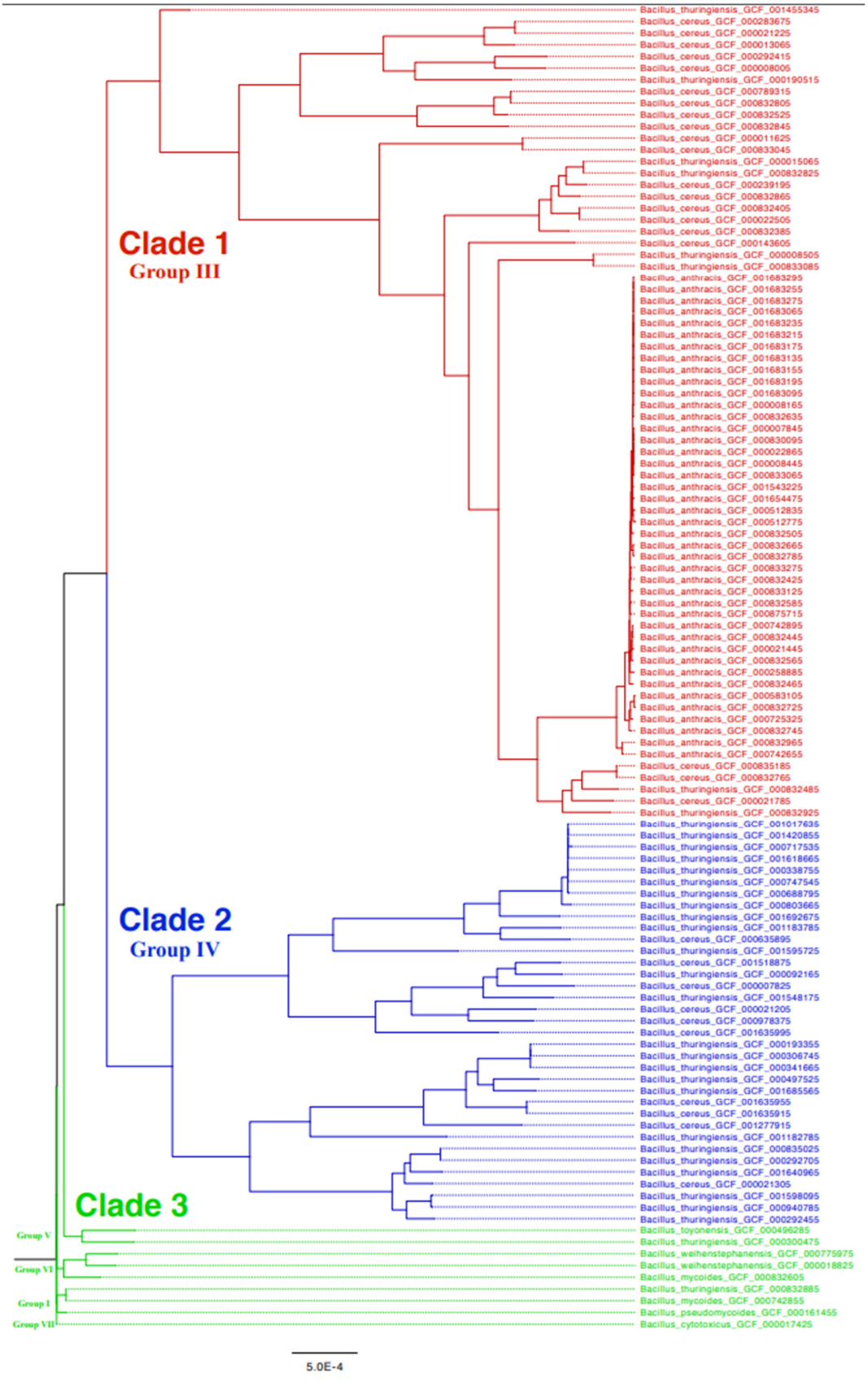
A *B. cereus* group genome typically contains around 5500 protein coding genes (Toby, Widmer, and Dyer 2014). The total complement of all genes shared by a species is known as the pan-genome. In the case of the *B. cereus* group, the extent of genes carried on plasmids, which can be lost and gained, contributes to a high genetic diversity within the species (Helgason et al. 1998). Moreover, the pan-genome of *B. cereus* is “open”, in that sequencing of new genomes will lead to the identification of new genes (Bazinet 2017; Zwick et al. 2012). The current pan-genome of the *B. cereus* group has been estimated to ~60000 genes (Bazinet 2017). Of these, around 600 genes are considered the “core genome”, which are genes that are shared by at least 99% of the taxa sampled (Bazinet 2017). Of the remaining genes,

~32000 genes can be defined as “accessory genes” (present in at least two taxa, ca 54% of all genes) and ~27000 as “unique genes” (present in only one taxon, ca 45% of all genes) (Bazinet 2017).

1.2.3 Phylogeny of the *B. cereus* group

The phylogeny and population structure of the members of the *B. cereus* group has been extensively studied using diverse typing methods such as multilocus sequence typing (MLST) of housekeeping genes, for which there are several schemes (Bohm et al. 2015; Didelot et al. 2009; Tourasse, Okstad, and Kolsto 2010; Barker, Thakker, and Priest 2005; Helgason et al. 2004), and amplified fragment length polymorphism (AFLP) analysis (Guinebretiere et al. 2008; Tourasse et al. 2011). These studies have revealed great similarities between isolates of different species regarding nucleotide sequence identity, and although the genetic diversity within this group is high (Helgason et al. 2004), analysis of whole-genome sequences of multiple *B. cereus* strains has shown great conservation in gene and operon organization (Okinaka and Keim 2016; Ivanova et al. 2003; Read et al. 2003).

The two most widely accepted classifications of the *B. cereus* group are composed of three clades and seven groups or clusters (Figure 1 and Table 2). In the three clades system (Didelot et al. 2009; Helgason et al. 2004; Kolsto, Tourasse, and Okstad 2009; Okinaka and Keim 2016; Zwick et al. 2012), clade 1 contains all *B. anthracis* strains and related *B. cereus* and *B. thuringiensis* strains, including most clinical isolates and emetic strains. In clade 2 we find mostly *B. thuringiensis* and some *B. cereus* strains. Clade 3 is the most genetically diverse and contains mostly environmental isolates including *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis,* and *B. cytotoxicus*, in addition to some strains classified as *B. cereus* and *B. thuringiensis.* The newly discovered species *B. toyonensis, B. bingmayongensis,* and *B. gaemokensis* have been assigned to clade 3, while *B. wiedmannii* has been assigned to clade 1 (Bazinet 2017). The *B. cereus* group species can be further divided into seven groups with different thermotolerance profiles, based on AFLP, ribosomal gene sequence, and *panC* and *cspA* gene sequences, where clade 1 includes groups II and III, clade 2 corresponds to group IV and clade 3 comprises groups I, V, VI and VII (Guinebretiere et al. 2008). The cytotoxicity of the bacteria has also been shown to vary according to these seven phylogenetic groups, with group VII (*B. cytotoxicus*) including some of the most cytotoxic strains and group VI the least (Table 2) (Bohm et al. 2015; Guinebretiere et al. 2010). Recently, a study using a k-mer based approach has re-analyzed all the available closed genomes in the *B. cereus* group as per 2017, and the analysis reproduced the phylogenic classification structures with three clades and seven clusters (Bazinet 2017).


Clade 1
Group III
Clade 2
Group IV
Clade 3
Group V
Group VI
Group I
Group VII
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Bacillus_cereus_GCF_000283675
Bacillus_cereus_GCF_000021225
Bacillus_cereus_GCF_000013065
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Bacillus_mycoides_GCF_000742855
Bacillus_pseudomycoides_GCF_000161455
Bacillus_cytotoxicus_GCF_000017425
5.0E-4

Figure 1: A rooted phylogenetic tree generated by maximum likelihood phylogenetic analysis using all closed *B. cereus* group genomes (114 genomes available at the time of analysis). The tree depicts the three clades and the seven groups of the *B. cereus* group phylogeny. The figure is from (Bazinet 2017) and is reprinted with permission.

Table 2: An overview of the seven earliest classified *B. cereus* group members divided into clades and phylogenetic groups (Bazinet 2017; Bohm et al. 2015; Guinebretiere et al. 2013; Guinebretiere et al. 2008; Zwick et al. 2012)

Phylogenetic group	Clade	Species	Temperature of growth	Thermotype
I	3	*B. pseudomycoides*	10°C to 43°C	mesophilic
II	1	*B. cereus* *B. thuringiensis*	7°C to 40°C	psychrotolerant
III	1	*B. cereus* *B. thuringiensis* *B. anthracis*	15°C to 45°C	mesophilic
IV	2	*B. cereus* *B. thuringiensis*	10°C to 45°C	mesophilic
V	3	*B. cereus* *B. thuringiensis*	8°C to 40°C	intermediate
VI	3	*B. mycoides* *B. weihenstephanensis* *B. thuringiensis*	5°C to 37°C	psychrotolerant
VII	3	*B. cytotoxicus*	20°C to 55°C	thermotolerant

1.2.4 Disease caused by *B. cereus* and *B. thuringiensis*

B. cereus is an opportunistic pathogen and a common cause of food-poisoning illness, including both the emetic and diarrheal syndrome (Drobniewski 1993), and is now the third most important cause of food-borne infections in Europe, after *Salmonella* spp. and *Staphylococcus aureus* (Ramarao and Sanchis 2013). Moreover, *B. cereus* can cause serious and fatal non-gastrointestinal infections, particularly in immunocompromised individuals including infants and the elderly (Glasset et al. 2018; Kelley, Onderdonk, and Kao 2013; Bottone 2010). Eye infections by *B. cereus* are especially virulent and can cause the destruction of an eye within days (Bottone 2010; Callegan et al. 2005). Recently, a study has shed light on the extent of *B. cereus* infections in hospital settings (Glasset et al. 2018), showing that these bacteria persist routine cleaning through formation of biofilms and spores which are resilient and adhesive, making them difficult to get rid of, resulting in many hospital- and food contaminations (Andersson, Ronner, and Granum 1995; Barrie et al. 1994; Sasahara et al. 2016). As *B. thuringiensis* is chromosomally indistinguishable from *B. cereus*, and thus harbor the same chromosomal virulence factors, it is not surprising that *B. thuringiensis* have been implicated in human infections, gastrointestinal as well as non-gastrointestinal (Celandroni et al. 2014; Ghelardi, Celandroni, Salvetti, Fiscarelli, et al. 2007).

1.2.5 Virulence and virulence factors

Virulence factors are characteristics and components produced by bacteria that enhance their pathogenicity, e.g. by assisting colonization of the host or by causing damage to host tissue. In a wide sense, this comprises both flagella and secreted proteins, as well as processes such as e.g. biofilm formation and sporulation. However, virulence factors will henceforth refer to toxins and degrading enzymes produced by the bacterium.

The three main enterotoxins responsible for *B. cereus* diarrheal disease are hemolysin BL (Hbl), non-hemolytic enterotoxin (Nhe) and cytotoxin K (CytK). Hbl and Nhe form three-component proteins which lyse cells by forming transmembrane pores and thereby permeating the plasma membrane of the small intestine (Beecher and Macmillan 1991; Beecher and Wong 1997; Lund and Granum 1996; Stenfors Arnesen, Fagerlund, and Granum 2008). The potency of Hbl is comparable to the cholera toxin (Beecher, Schoeni, and Wong 1995). CytK was first discovered in *B. cytotoxicus* NVH391-98 as a β-barrel pore-forming toxin (Lund, De Buyser, and Granum 2000). Later, a homologue of this toxin, was recognized in other *Bacillus cereus* group strains, but this homologue harbors only a fifth of the toxicity of the *B. cytotoxicus* NVH 391-98 homologue (Fagerlund et al. 2004), although this attenuation could also be due to different levels of transcription (Brillard and Lereclus 2004). In addition to these three toxins, several other virulence factors are present, and multiple factors are thought to act synergistically in the gastro-intestinal tract (Stenfors Arnesen, Fagerlund, and Granum 2008; Doll, Ehling-Schulz, and Vogelmann 2013). Other such virulence factors include the metalloprotease InhA which helps spores of *B. cereus* survive and escape macrophages (Ramarao and Lereclus 2005), cereolysin O, haemolysin II and III, and three phospholipases C (Stenfors Arnesen, Fagerlund, and Granum 2008). Several of these virulence factors are produced in the small intestine after the ingestion of bacteria or spores (Ramarao and Sanchis 2013). The symptoms of *B. cereus* related diarrheal syndrome normally arise 8 to 16 hours after ingestion of viable vegetative bacteria (Ramarao and Sanchis 2013; Ceuppens, Boon, and Uyttendaele 2013). In contrast, the causative agent of the *B. cereus* emetic syndrome, the emetic toxin cereulide, is produced by the bacteria in food before ingestion, causing an intoxication. The symptoms of nausea and vomiting appear after only a few hours (Ramarao and Sanchis 2013). The emetic toxin cereulide is a cyclic dodecadepepsipeptide which is produced by a non-ribosomal peptide synthase (NRPS) complex encoded by the plasmid-borne cereulide synthetase (*ces*) gene cluster (Ehling-Schulz et al. 2005). Cereulide is resistant to acid, proteolysis and heat, properties which enable the toxin to survive both re-heating of food and the harsh environments of the stomach and GI system (Stenfors Arnesen, Fagerlund, and Granum 2008). The toxicity of *B. cereus* varies between strains. Where some strains (emetic) are sufficiently potent to have caused fatalities depending on the host's immune status, others have been deemed safe to use as probiotics for mammals, such as e.g. *B. toyonensis* (Stenfors Arnesen, Fagerlund, and Granum 2008; Jimenez et al. 2013). The genes encoding the Nhe enterotoxin however are present in all strains known to date, while those encoding Hbl and CytK are present in around 50% of strains, mainly in clinical and food isolates (Guinebretiere, Broussolle, and Nguyen-The 2002). The emetic strains mainly compile a small clonal cluster of only 5% of the *B. cereus* group III /clade 1 strains (Bohm et al. 2015).

1.2.6 Regulation of virulence in the *B. cereus* group

The genes encoding extracellular virulence factors in *B. cereus* are under transcriptional control by the global regulator PlcR (Agaisse et al. 1999). *B. anthracis* is the only *B. cereus* group species (in addition to *B. cereus* biovar *anthracis*) where PlcR is non-functional, caused by a nonsense mutation in the *plcR* gene (Agaisse et al. 1999). The genes in the PlcR regulon are widely distributed on the chromosome, and a highly conserved palindromic DNA sequence (the PlcR box) upstream of the regulated genes was revealed to constitute the recognition sequence and binding site governing PlcR regulation (Agaisse et al. 1999). The PlcR regulon includes altogether 45 genes controlled by 28 PlcR boxes. The main enterotoxins Hbl, Nhe and CytK are all included in the PlcR regulon, as well as several other virulence factors such as two phospholipases and InhA (Gohar et al. 2008).

PlcR positively regulates its own expression and that of a small cell-cell signaling peptide PapR which interacts with PlcR to facilitate binding to target sites in DNA (Lereclus et al. 1996; Slamti and Lereclus 2002). PapR is secreted, and then re-imported through the oligopeptide permease system Opp (Gominet et al. 2001). These gene products make up a quorum sensing system, which enable the bacteria to communicate and determine the cellular density. *plcR* is activated when a certain bacterial density is reached, whereupon the expression of enterotoxins causes lysis of the epithelial cells of the intestinal wall allowing the bacteria to spread (Bassler and Losick 2006; Dunny and Leonard 1997). Inactivation of *plcR* significantly reduces but does not completely abolish virulence in *B. cereus* (Bouillaut et al. 2005), indicating that other regulatory factors also play a role in pathogenicity. Interestingly, PlcR also affects motility and biofilm formation in *B. cereus*. In a Δ*plcR* mutant, motility and the production of flagellin was reduced (Gohar et al. 2002; Callegan et al. 2003), and the formation of biofilm was increased compared to the wild type (Bouillaut et al. 2005; Hsueh et al. 2006), although the precise mechanisms of regulation remain to be elucidated.

Spo0A~P is a transition state regulator initiating the sporulation process and the formation of biofilm, depending on the level of phosphorylation (see sections 1.4.5 and 1.4.6 for details). Spo0A~P also acts as a negative regulator for *plcR* expression (Lereclus et al. 2000), and is predicted to act as an activator for the transcription of *abrB* and *sinI* (Fagerlund et al. 2014). Orthologues of *abrB* and *sinI* are present in *B. subtilis*, where the activation of these genes by Spo0A~P has been shown *(Shafikhani et al. 2002; Strauch et al. 1990).* SinR, along with its antirepressor SinI and AbrB, are key regulators of biofilm formation in the *B. cereus* group. In addition AbrB represses cereulide production (Lucking et al. 2009) and SinR represses the expression of a number of virulence factors, including genes encoding Hbl and InhA (Fagerlund et al. 2014; Pflughoeft, Sumby, and Koehler 2011). CodY is a pleiotropic transcriptional regulator that responds to lower cellular energy and branched chain amino acids levels (Lindback et al. 2012). CodY was found to have a positive effect on genes belonging to the PlcR regulon, through CodY acting on Opp-like proteins, thereby promoting the uptake of PapR and the resulting activating of the PlcR regulon (Slamti et al. 2015). Moreover, CodY acts independently as a repressor for *hbl, nhe, inhA* and the plasmid-borne cereulide (Bohm et al. 2016; Frenzel et al. 2012) (see Figure 2 for details).

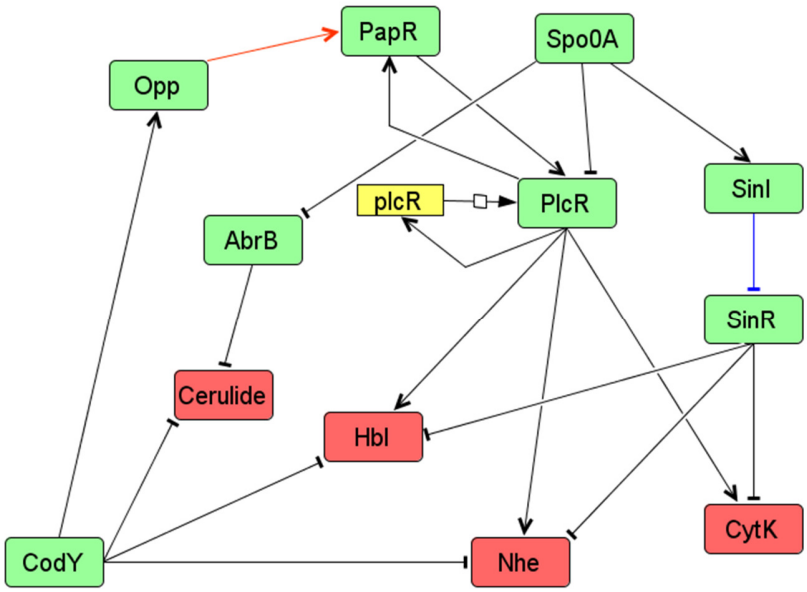

Figure 2: The main pathways of activation and repression of virulence factors in *B. cereus*. Green boxes represent regulatory proteins, the yellow box represents the *plcR* gene and the red boxes indicate virulence factors. The figure was drawn based on results from (Bohm et al. 2016; Fagerlund et al. 2014; Frenzel et al. 2012; Gohar et al. 2008; Gominet et al. 2001; Lereclus et al. 1996; Lereclus et al. 2000; Lucking et al. 2009; Pflughoeft, Sumby, and Koehler 2011; Shafikhani et al. 2002; Slamti et al. 2015; Slamti and Lereclus 2002; Strauch et al. 1990). Black lines represent transcriptional regulation while the red line represents transport regulation and the blue line shows direct protein-protein interaction. Arrows (→) indicate positive influence, ended lines (⊢) indicate negative influence. A solid arrow broken by a box indicates expression of a protein from an activated gene.

1.3 Flagellar motility

1.3.1 Motility as a virulence factor

Most species in the *B. cereus* group are motile by means of peritrichous flagella. The flagella enable the bacterium to reach the site of infection and may play a part in the initial attachment of bacteria to host cells and in the early stages of biofilm formation (Chaban, Hughes, and Beeby 2015; Berthold-Pluta, Pluta, and Garbowska 2015; Duan et al. 2013). Intestinal pathogens may use flagella to aid penetration of the intestinal mucus layer in order to reach the epithelial cells (Chaban, Hughes, and Beeby 2015). In *B. cereus* enterotoxins are produced after the flagellar attachment to ensure that the toxins are released in close proximity to the intestinal epithelial cells (Ceuppens et al. 2012; Raymond and Bonsall 2013).

1.3.2 The flagellum

Many bacteria can perform swimming motility by means of a long, rotating helical structure called a flagellum. The flagellum consists of three basic structures, the basal body, the hook, and the filament (Figure 3) (Mukherjee and Kearns 2014). The basal body anchors the flagellum to the cell envelope and contains the flagellar export apparatus and the motor which powers flagellar rotation (Chaban, Hughes, and Beeby 2015). The stator converts the power of chemiosmotic ion motive force into mechanical rotation. The proteins comprising the hook and the filament are exported through the cell membrane by way of the flagellar export apparatus (Mukherjee and Kearns 2014). The hook is flexible and converts the motor force into waves made by the filament. The filament is composed of flagellin subunits forming the long helix which drives the bacterium forward by pushing against the surrounding medium (Erhardt, Namba, and Hughes 2010; Smith and Hoover 2009).

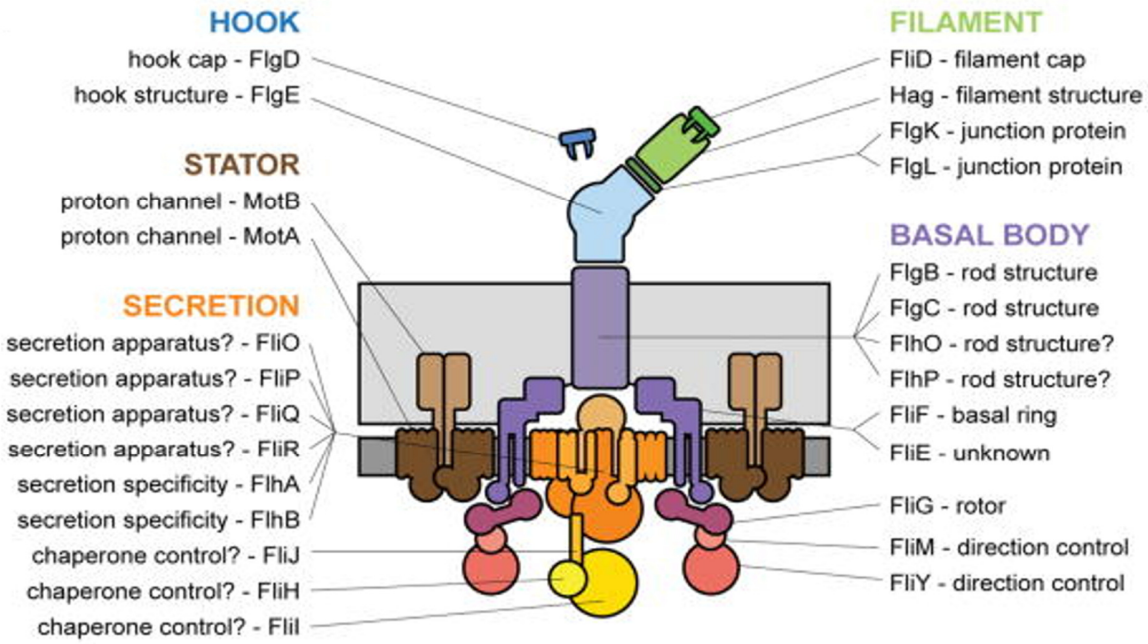

Figure 3: A model of the *B. subtilis* flagellum, illustrating the components that make up the flagellum and the proteins involved in each structure. Reprinted from (Mukherjee and Kearns 2014) with permission.

1.3.3 Regulation of flagellar motility

In most motile bacterial species, the expression of the flagellar genes follows a hierarchal cascade (Gao, Shi, and Gao 2018). In this traditional regulation paradigm, the first genes to be transcribed are regulatory proteins, so-called master regulators or class I genes. The master regulator activates the transcription of the class II genes which commonly consists of regulatory genes and genes encoding proteins associated

with the flagella export apparatus and the basal body. Subsequently, genes encoding the basal body, hook, motor proteins (class III) and lastly the flagellin subunits (class IV) are transcribed (Smith and Hoover 2009). This temporal regulation ensures that genes encoding proteins which are needed later in the flagellar assembly are not expressed unnecessarily but in an energy conserving regulation pattern.

In *B. subtilis*, this master regulator is SwrA which activates the expression of the flagellum/chemotaxis (*fla/che*) operon (Kearns and Losick 2005). This operon contains the RNA polymerase sigma factor σ^D, which in turn activates the remaining components of the flagellar apparatus, including *hag*, the flagellin gene encoding the *B. subtilis* flagellar filament, which is located outside the flagellar and chemotaxis (*fla/che)* operon (Kearns and Losick 2005; Mirel and Chamberlin 1989).

The flagellar genes in *B. cereus* however appear to be regulated differently than in *B. subtilis*. No homologues of SwrA or σ^D have been found (Smith and Hoover 2009), and no other master regulator of motility has to date been identified. Moreover, a *B. thuringiensis* deletion mutant in the export apparatus gene *flhA* could nevertheless synthesize flagellin subunits but not export them, and thereby not assemble functioning flagella (Ghelardi et al. 2002), indicating that the flagellar genes are expressed in a non-hierarchal manner. Even though the members of the *B. cereus* group are more closely evolutionary related to *B. subtilis* than to *L. monocytogenes*, it appears that the flagellar loci of *B. cereus* and *L. monocytogenes* are homologous and more similarly clustered (Chiara et al. 2015). It has therefore been speculated that there has been a lateral gene transfer of the entire flagellar pathway from a *B. cereus*-like organism to an ancestor of *L. monocytogenes*, or that both *B. cereus* and *L. monocytogenes* acquired the flagellar locus from a common or similar donor (Chiara et al. 2015).

In *L. monocytogenes* the transcription of flagellar genes is not controlled by a flagellar master regulator in the traditional sense, but by the transcriptional regulator MogR which represses flagellar genes in a non-hierarchal fashion (Grundling et al. 2004; Shen and Higgins 2006). *L. monocytogenes* is a facultative intracellular pathogen which uses flagellum-based motility to move in extracellular environments. During infection, motility genes are downregulated by the transcriptional regulator MogR upon sensing of mammalian physiological temperature (37°C) (Shen and Higgins 2006). MogR acts by binding to the consensus sequence 5' TTTTWWNWWAAAA 3' in the promoter areas of target genes, thereby directly repressing the expression of flagellar- and chemotaxis genes (Grundling et al. 2004; Shen, Higgins, and Panne 2009). The temperature sensor is the GmaR antirepressor, which by direct protein-protein interaction antagonizes MogR repression activity at temperatures below 37°C (Shen et al. 2006). The activity of GmaR is dependent on the transcriptional activation by DegU at low temperatures (Shen and Higgins 2006). The only other species besides *Listeria* spp. known to carry a MogR homolog are those in the *B. cereus* group (Grundling et al. 2004).

Most *B. cereus* group strains contain a cluster of approximately 45-50 genes with homology to flagellar-based motility- and chemotaxis genes. Figure 4 depicts the motility loci of *B. thuringiensis* 407, which consist of altogether 46 genes, 44 genes in the *fla/che* operon and the two genes encoding the flagellar motor proteins (*motA* and *motB*) located separately on the chromosome. As mentioned above, no known master regulator is known for bacteria in the *B. cereus* group, but a gene encoding a homologue

of the *L. monocytogenes* transcriptional regulator MogR (*mogR*; shown in red), is located adjacent to the flagellar genes *flaA* and *flaB* (checkered pattern) (Figure 4).

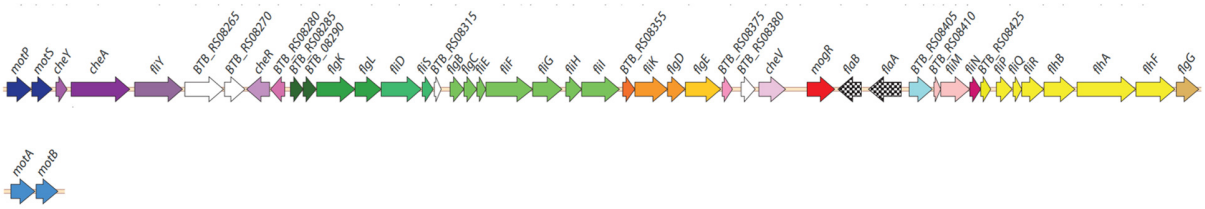

Figure 4: The genes in the motility loci of *B. thuringiensis* Bt407, comprising the *fla/che* operon (BTB_RS08240 - BTB_RS08460; top) and the *motA-motB* operon (BTB_RS22910 - BTB_RS22905; bottom), respectively. The genes have been color coded by similar function or through making up the corresponding component of the flagellum. Shown in blue are genes encoding the flagellar motor proteins MotA and MotB (H^+-coupled stator; light blue) and the paralogues MotP and MotS (Na^+-coupled stator; dark blue). Similarly, in pink/purple are genes encoding proteins pertaining to chemotaxis, e.g. the histidine kinase CheA and the flagellar motor direction control protein FliM. Genes encoding proteins comprising the basal body are shown in green while genes encoding hook-related proteins are displayed in shades of orange (e.g. FlgD and FlgE). Genes encoding proteins in the flagellar export apparatus are depicted in yellow, whereof FlhA, a protein related to secretion specificity, is the best described (Mukherjee and Kearns 2014). Genes encoding hypothetical proteins or proteins of unknown function are shown in white.

1.4 Biofilm

1.4.1 What is a biofilm?

A biofilm can be defined as a multicellular community of microorganisms attached to a surface or associated with an interface (Davey and O'Toole G 2000; Martinez and Vadyvaloo 2014). Biofilms can form on biotic and abiotic surfaces and are commonly found in nature, where they can form on surfaces such as rocks and plant roots (Costerton et al. 1995; Gao et al. 2015; Vlamakis et al. 2013). Biofilms also readily form on the surfaces of dairy tanks and food processing surfaces where they pose a large problem in the food industry (Latorre et al. 2010; Olszewska 2013). Moreover, a range of indwelling medical devices have been shown to harbor biofilms and thereby resulting in numerous hospital-acquired bacterial infections (Arciola, Campoccia, and Montanaro 2018; Veerachamy et al. 2014).

In a biofilm the bacteria are embedded in a self-produced layer of extracellular matrix. The matrix protects the bacteria from environmental challenges, such as dehydration, phagocytosis by the human immune system, and a range of antimicrobial agents (Davey and O'Toole G 2000; Hall-Stoodley, Costerton, and Stoodley 2004). The composition of the matrix will vary between bacterial species and even closely related strains, also depending on environmental conditions (Karatan and Watnick 2009). The matrix consists mainly of exopolysaccharides (EPS), proteins and sometimes extracellular nucleic acids, as well as lipids and surfactins (Branda et al. 2005; Flemming and Wingender 2010; Whitchurch et

al. 2002). In most biofilms bacterial cells take up less than 10% of the volume, whereas the extracellular matrix can account for over 90% (Flemming and Wingender 2010). Water constitutes the largest part of the matrix and provides the bacteria with a hydrated environment, protecting against drying and desiccation (Flemming and Wingender 2010).

1.4.2 Biofilm as a virulence factor

Biofilms have been claimed to account for over 80% of human microbial infections (Veerachamy et al. 2014). Biofilm is a common component in gum disease, urinary tract infections, and in chronic wounds where it was found in as many as 30 out of 50 cases (James et al. 2008). The lungs of patients with cystic fibrosis, a genetic disease causing lower respiratory malfunction, are notoriously colonized by *Pseudomonas aeruginosa* biofilm leading to a chronic pulmonary infection (Hall-Stoodley, Costerton, and Stoodley 2004). Biofilm formation can thus be considered a virulence factor by potentially spreading the infection and causing further inflammation and tissue destruction while also preventing the wound from healing (Cooper, Bjarnsholt, and Alhede 2014).

Bacteria can form biofilm on human tissue, but also on implanted devices such as intravenous catheters, prosthetic heart valves, dental implants and cardiac pacemakers. The infectious bacteria can originate from contaminated devices or from the patient itself, via bacteria from the skin or oral cavity which enter the blood stream (Hall-Stoodley, Costerton, and Stoodley 2004). Urinary tract infections, periodontal/peri-implant diseases and endocarditis are common implant-related infections, and the bacteria may also enter the blood stream causing sepsis (Donlan 2002; Lee and Wang 2010; Nguyen, Park, and Otto 2017; Scotland et al. 2019). Once formed, the biofilm is extremely difficult to remove and the infection may become chronic (Nguyen, Park, and Otto 2017; Scotland et al. 2019). The biofilm can also protect the bacteria against host defenses, both by physical shielding by the extracellular matrix and by the excretion of proteases, toxins and lipases which attack the immune cells (Hall-Stoodley, Costerton, and Stoodley 2004). Biofilms are 10 to 1000-fold more resistant to antimicrobial compounds than planktonic cells (Hoyle and Costerton 1991). This results from both protection by the biofilm matrix, dormant or slow-growing cells in the biofilm surviving antibiotic exposure, and the development of antibiotic resistance through mutations in chromosomal genes or by acquisition of external resistance genes (Taylor, Yeung, and Hancock 2014; Munita and Arias 2016). Moreover, the biofilm serves as a beneficial environment for horizontal transfer of genes, including virulence genes and antibiotic resistance genes (Solheim et al. 2013; Aguila-Arcos et al. 2017).

1.4.3 Formation of a biofilm

The formation of a biofilm starts when single free-swimming (planktonic) bacteria respond to environmental signals by forming an initial and reversible attachment to a surface (Kolter and Greenberg 2006). These signals can be mechanical by sensing of a surface by flagella, or extracellular chemical signals such as nutritional contents of the growth medium, temperature, osmolarity, pH, iron and/or oxygen (Davey and O'Toole G 2000; Karatan and Watnick 2009). Extracellular signals are sensed directly or indirectly by cellular receptors which convey the information to a target protein. Quorum sensing, a cell-cell communication system, is also important during initiation of biofilm formation (Li and Tian

2012). Upon entering the biofilm lifestyle, a large number of genes are differentially regulated compared to the planktonic stage (Davey and O'Toole G 2000). Altogether, a great number of signals and genes constitute the road to biofilm formation in bacteria, demonstrating the fundamental importance of this phenotype (Hall-Stoodley, Costerton, and Stoodley 2004).

Biofilm formation is found among both motile and non-motile bacteria. In non-motile bacteria, the production of adhesins is upregulated under biofilm promoting conditions, promoting both cell-cell adherence and cell-surface adherence (Gotz 2002). For motile bacteria, flagella may play a part in the movement of bacteria to a surface and enhances initial interaction by helping the bacteria overcome repulsive forces and can also help bacteria move along the surface (Karatan and Watnick 2009). In the first attachment stage, bacteria can still revert to the planktonic phase or move along the surface by means of pili or flagella (Davey and O'Toole G 2000; Pratt and Kolter 1998; Stoodley et al. 2002; O'Toole and Kolter 1998). If conditions are favorable, additional adhesins can be synthesized to help permanently stabilize the attachment, allowing the production of a biofilm monolayer to begin. The monolayer biofilm is characterized by cells attaching to the surface only, and flagellar genes are reported to be downregulated at this stage (Karatan and Watnick 2009). As the cells divide and multiply, the biofilm develops to a multilayer biofilm where the cells also adhere to neighboring bacteria, and the production of the extracellular matrix starts, culminating in a mature biofilm (Figure 5) (Davey and O'Toole G 2000; Hall-Stoodley, Costerton, and Stoodley 2004; Toyofuku et al. 2015).

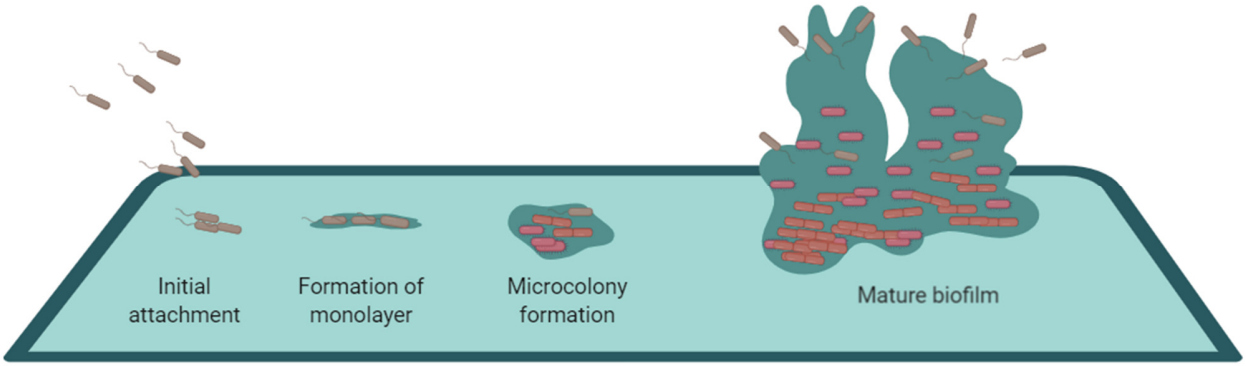

Figure 5: Stages of biofilm formation; planktonic cells form an initial attachment to a surface before synthesis of adhesins allow the formation of a monolayer. Production of extracellular matrix and multiplication of cells provide the basis for microcolony production and subsequent maturation to a three-dimensional biofilm. From the mature biofilm, cells will eventually disperse, reverting to planktonic growth.

The mature biofilm is a three-dimensional structure, composed of cells and extracellular matrix in the form of microbial cell clusters and open water channels (de Beer et al. 1994; Lawrence et al. 1991). Cells may leave the biofilm, leaving empty spaces that become part of the water channels (Stoodley et al.

2002). Biofilms are highly hydrated, and the water channels facilitate transport of nutrients to cells in the interior of the biofilm, expulsion of waste products, and distribution of signaling molecules throughout the biofilm (Wilking et al. 2013). The structure and architecture of the mature biofilm depends on both physical and nutritional conditions and can be divided into biofilms with flat topology and biofilms with irregular, mushroom-like topology (Karatan and Watnick 2009). In nature, biofilms are most often composed of several bacterial species, with single-species biofilms being more common in infections and contaminations of medical implants (Davey and O'Toole G 2000). Despite arising from a genetically clonal bacterial population, a mature biofilm is heterogenous and generally consists of several subpopulations distinguished by variable global gene expression patterns which have specialized functions, including motile cells, producers of matrix components and cells with varying metabolic activities (Boles, Thoendel, and Singh 2004; Vasudevan 2014; Vlamakis et al. 2008). In mixed biofilms, interactions between species can be antagonistic such as competition over nutrients or synergistic where the waste products of one species can be a nutritional source for the other (Elias and Banin 2012; Stewart and Franklin 2008).

Despite the effective distribution of nutrients in the biofilm, cells in the biofilm interior often become starved due to the bacteria in the outer layers consuming the nutrients and oxygen before they can reach the inner layers (Stewart and Franklin 2008). As a response, the cells in the interior downregulate their metabolism to the point where they become dormant (Werner et al. 2004). These cells have a low level of transcription, translation, genome replication and low metabolic activity, all of which are processes constituting targets for antibiotics, making these bacteria harder to kill, while enabling them to develop a tolerance for the antibiotics they are exposed to (Taylor, Yeung, and Hancock 2014). After antibiotics have killed the outer layers of the biofilm cells, the surviving dormant cells can reactivate and re-populate the biofilm with bacteria tolerant to this type of antibiotic (Stewart and Franklin 2008).

The last stage of the biofilm life cycle is dispersal, which is initiated when life in the biofilm is no longer optimal. Without the possibility to detach, a biofilm becomes a dead end for the bacteria. As opposed to passive dispersal caused by sloughing and erosion of biofilm cells, active dispersion is highly regulated and occurs in response to a number of extracellular cues such as nutrient availability, oxygen and nitric oxide as well as intracellular signals including quorum sensing and autoinducers (McDougald et al. 2011). Active dispersal is often preceded by localized cell death in the center of the macrocolony structures (Webb et al. 2003). Upregulation of motility genes and production of enzymes that break down the matrix enable the bacteria to escape and return to the planktonic state. This enables them to move to new localization for the establishment of a new biofilm under better conditions (McDougald et al. 2011).

1.4.5 Sporulation in *Bacilli:* Regulation of initiation

Spores are key structures of *Bacillus* biofilms (Lindsay, Brözel, and von Holy 2005, 2006). Like in *B. subtilis*, a *B. cereus* biofilm culminates in sporulation in a subset of cells (Verplaetse et al. 2017) and the amount of spores present is higher in biofilms than in planktonic suspension (Wijman et al. 2007; Majed et al. 2016). Endospore formation is utilized by two classes of bacteria only, the *Bacilli* and the *Clostridia*, both members of the *Firmicutes* phylum. The formation of spores, the process called sporulation, allows

the bacteria to survive particularly harsh environments (Cutting and Ricca 2014). Traditionally, sporulation genes have been classified by identifying mutants defective in sporulation (Galperin et al. 2012; Eichenberger et al. 2003), but recently phenotypical profiling has been applied in order to uncover genes which are not essential for sporulation, but whose mutants cause a developmental effect (Traag et al. 2013).

The sporulation process has been characterized in great detail in the model organism *B. subtilis* (Smith et al. 1992; Errington 2003; Higgins and Dworkin 2012). During vegetative growth the master sporulation regulator *spo0A* is continuously transcribed at low levels until a number of extracellular or intracellular signals trigger the activation of one or more histidine protein kinases (KinA-E), initiating a phosphorelay culminating in the phosphorylation of Spo0A (Figure 6) (Hilbert and Piggot 2004; Jiang et al. 2000; Predich, Nair, and Smith 1992). The sporulation regulatory network, including the *spo0A* phosphorelay activation and the sporulation sigma factors, is highly conserved in all Gram-positive endospore-forming bacteria (de Hoon, Eichenberger, and Vitkup 2010; Stephenson and Hoch 2002), including the *B. cereus* group, as described for *B. anthracis* (Bergman et al. 2006; Brunsing et al. 2005; Wang et al. 2013) and *B. thuringiensis* (Wang et al. 2013). The KinA-E kinases are typically triggered by starvation signals and subsequently transfer a phosphate group to a response regulator Spo0F which again phosphorylates the phosphotransferase protein Spo0B before the phosphate group is transferred to the transcription regulatory protein Spo0A, forming a phosphorelay (Figure 6) (Burbulys, Trach, and Hoch 1991; Trach et al. 1991). The histidine kinases are, with the exception of KinA and KinE, membrane bound (Burbulys, Trach, and Hoch 1991; McLoon et al. 2011). The initiation of the phosphorelay in *B. cereus* may be more complicated however, as there are as many as fourteen histidine kinases putatively involved in the process (de Been et al. 2006).

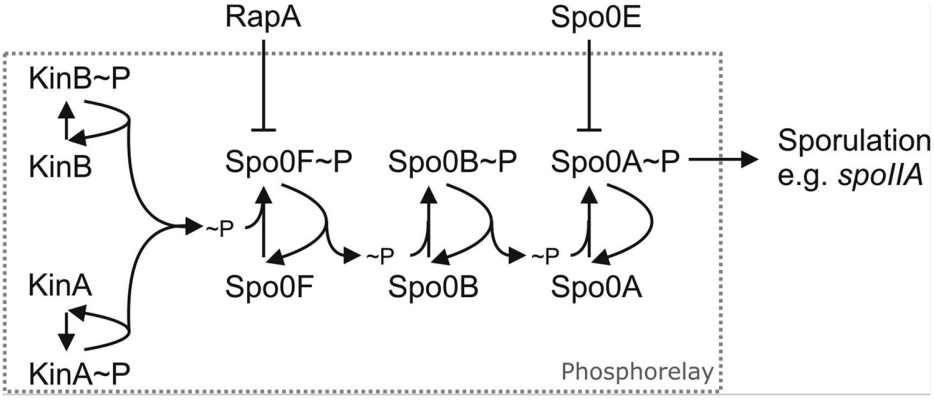

Figure 6: The *B. subtilis* phosphorelay. The sporulation process is initiated by the phosphorylation of the histidine kinases KinA and KinB, culminating in the phosphorylation of the transcription regulator Spo0A (de Jong, Veening, and Kuipers 2010), reprinted with permission.

In *B. subtilis*, the active form of Spo0A, Spo0A~P, binds to its own promoter, inducing *spo0A* transcription (Strauch et al. 1992). When a threshold level of Spo0A~P is reached, Spo0A~P not only activates its own transcription through a positive-feedback loop, but represses the transcription of *abrB* (Hamon and Lazazzera 2001; Strauch et al. 1990), a repressor of σ^H which again is an activator of *spo0A* (Smith et al. 1992; Strauch et al. 1992). The de-repression of σ^H leads to further induction of *spo0A* as well as other sporulation genes (Lopez, Vlamakis, and Kolter 2009). The Spo0A regulon comprises 121 genes which are under direct control (Molle et al. 2003), and the expression of over 500 genes is significantly influenced by the regulator (Fawcett et al. 2000). Spo0A~P accumulates gradually in the cells, and over a certain threshold biofilm formation is induced, while even higher levels of Spo0A~P induce sporulation (Fujita, Gonzalez-Pastor, and Losick 2005; Fujita and Losick 2005).

1.4.6 Biofilm formation *in B. subtilis*

Gram-positive bacteria are in general less well studied than Gram-negative bacteria regarding biofilm formation. However, the regulation of biofilm formation in the Gram-positive model bacterium *B. subtilis* has been subject to numerous studies, and *B. cereus* and *B. subtilis* share many of the same regulation mechanisms for biofilm formation and sporulation (see Figure 16, page 61 for details). *B. subtilis* forms biofilms both on solid surfaces and as floating pellicles at air/liquid interphases (Branda et al. 2001; Hamon and Lazazzera 2001). The extracellular matrix in *B. subtilis* has been shown to consist of exopolysaccharides (EPS) and proteins and is important for the structure of complex colonies (Mielich-Suss and Lopez 2015). The matrix exopolysaccharides are synthesized by the products of the 15 genes in the *epsA-epsO* operon (Branda et al. 2001; Kearns et al. 2005). Also encoded by the *eps* operon is EpsE, a protein that disables flagellar rotation by uncoupling the FliG rotor from the MotA/MotB proton channel (Blair et al. 2008). This means that motility is directly inhibited in cells undergoing biofilm formation. The major protein component of the extracellular matrix is TasA (Branda et al. 2006; Chu et al. 2006) which forms amyloid fibers that create a network that binds the biofilm together (Romero et al. 2010). The *tapA-sipW-tasA* operon is located separately from the *eps* operon in the chromosome and encodes three components that are all required for biofilm formation (Chu et al. 2006). TapA (previously YqxM) is an anchor protein that attaches TasA to the cell wall but is also involved in the assembly of the amyloid fibers (Romero et al. 2011). SipW is a bifunctional signal peptidase (Branda et al. 2004; Terra et al. 2012) which is responsible for the export of TapA and TasA out of the cell (Stover and Driks 1999b, 1999a). The non-signal peptidase role of SipW appears to be to upregulate the biofilm matrix *tapA* and *eps* operons, although the precise mechanism remains unknown (Terra et al. 2012). An additional protein component of the biofilm matrix, BslA, was more recently discovered, and forms a hydrophobic layer on top of the biofilm, creating a water-repellent barrier (Figure 7) (Hobley et al. 2015; Kobayashi and Iwano 2012).

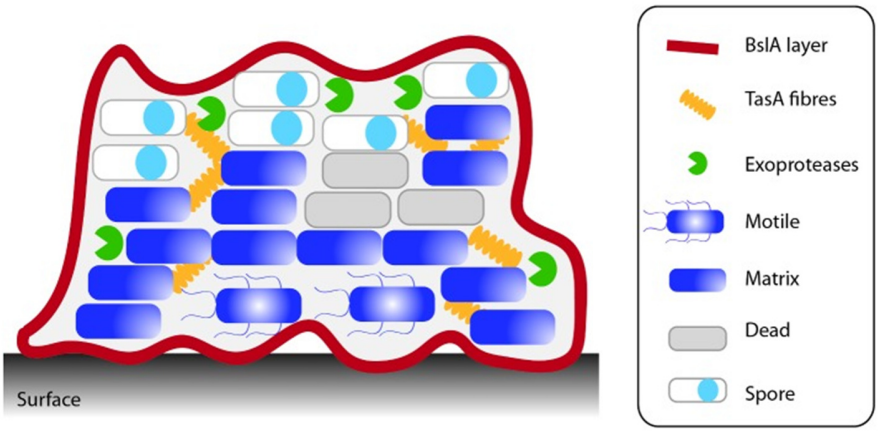

Figure 7: The *B. subtilis* biofilm is a heterogenic three-dimensional community consisting of several cell types as well as different proteins and exopolysaccharides (Hobley et al. 2015), reprinted with permission.

The key sporulation transcription factor Spo0A was revealed to affect biofilm formation also in *B. subtilis*. A mutant lacking *spo0A,* or *spo0H* which encodes σ^H, a sigma factor that controls expression of proteins involved in the early stages of sporulation, did not form pellicles (Branda et al. 2001; Hamon and Lazazzera 2001). In early exponential growth, *spo0A* is expressed continuously. As growth progresses and the phosphorelay is activated (Figure 6), phosphorylation of Spo0A increases gradually to levels high enough to promote biofilm formation. At biofilm promoting levels Spo0A~P represses the transcription of the gene encoding the transcriptional activator AbrB (Jiang et al. 2000; Strauch et al. 1990) and activates the expression of *sinI* (see Figure 16, page 61 for details) (Shafikhani et al. 2002). Mutations in *abrB* relieved the biofilm defect of a *spo0A* mutant, indicating that AbrB is a repressor of biofilm formation (Hamon and Lazazzera 2001). AbrB has been shown to repress expression of the biofilm matrix proteins encoded by the *tasA*-operon and by *bslA* (Hamon et al. 2004; Verhamme, Murray, and Stanley-Wall 2009).

The *B. subtilis* biofilm is a heterogeneous community comprising subpopulations of motile cells, matrix producing cells and sporulating cells (Figure 7) (Vlamakis et al. 2008). This heterogeneity arises due to the bistability of the transition state regulator Spo0A~P (Chai et al. 2008; Kearns 2008; Lopez, Vlamakis, and Kolter 2009). Bistability is a phenomenon where a regulatory system can switch between two alternative states, but not exist at intermediate stages (Dubnau and Losick 2006). The *B. subtilis* biofilm also contains surfactin producing cells, and “miner” and “cannibal” cells which break down extracellular proteins and kill sensitive cells, respectively, which can be used as nutrition for the rest of the cells in the biofilm (Lopez and Kolter 2010).

SinR is a DNA binding protein in *B. subtilis* constituting master transcriptional regulator governing the change from a motile to a sessile lifestyle and binds to the promoter regions of the *eps* operon (Kearns et al. 2005) and the *tapA* operon (Chu et al. 2006), both of which are essential for biofilm formation. Moreover, the full SinR regulon comprises at least 35 genes, mainly related to sporulation and

competence (Chu et al. 2006; Chu et al. 2008; Guillen, Weinrauch, and Dubnau 1989; Kallio et al. 1991; Kodgire, Dixit, and Rao 2006; Liu et al. 1996). SinR was found to bind to a consensus DNA sequence, the SinR box (Chu et al. 2006; Kearns et al. 2005). SinI is the antagonist of SinR, with which it forms a complex (Gaur, Oppenheim, and Smith 1991). At low levels of Spo0A phosphorylation, SinR can act as a repressor for the *tasA*-operon and the *eps*-operon (including the gene encoding the EpsE clutch), which leaves the cells motile and not producing matrix proteins. Interestingly, the secreted matrix protein TasA was recently revealed to be necessary for the expression of flagellar and chemotaxis genes and to activate the switch from non-motile to motile cells within a biofilm in *B. subtilis* (Steinberg et al. 2020). As the phosphorylation of Spo0A increases, SinI is produced (Shafikhani et al. 2002), sequestering the SinR repressor. Paradoxically, in a biofilm forming population, the overall expression of *sinI* was substantially lower than that of *sinR* (Chai et al. 2008). This is in accordance with the fact that *spo0A* is only expressed in a subset of cells (Chung et al. 1994). While *sinR* is expressed continuously in all cells, *sinI* is only expressed when activated by Spo0A~P (Chai et al. 2008; Vlamakis et al. 2008). Likewise, the matrix-producing operons which are under SinR regulation are only expressed in the same subset of cells (Figure 8) (Chai et al. 2008). Mutations in both *eps* and *tasA* cause a defect in biofilm formation, but only a double mutant is completely unable to form biofilm. A mixture of *eps* and *tasA* mutants will however complement each other and form a biofilm comparable to the wild type (Branda et al. 2006), indicating that components of extracellular matrix are shared as a greater good throughout the biofilm and that the bacteria in the biofilm practice a division of labor.

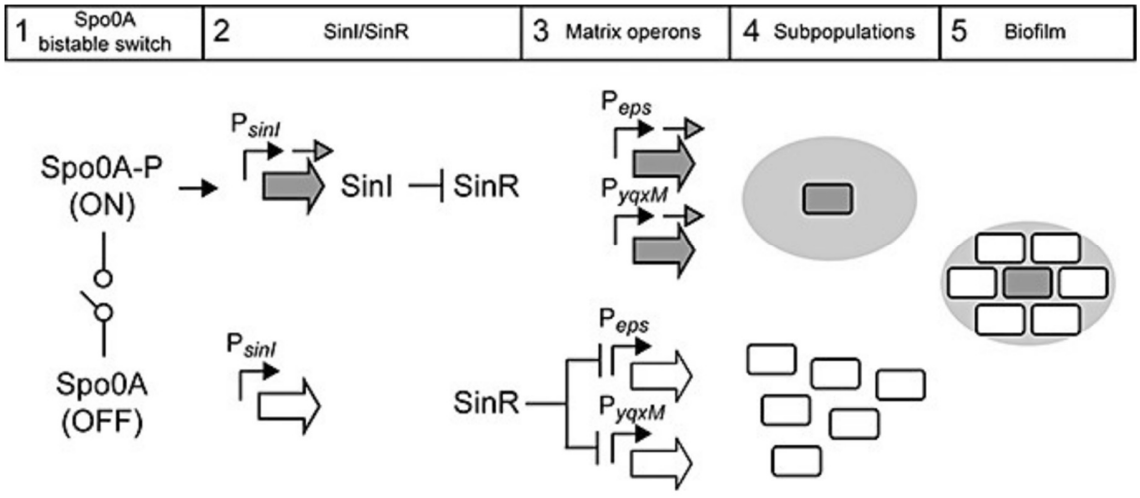

Figure 8: 1) Gene regulation by Spo0A is either in the ON or OFF state (bistable switch). 2) *sinI* is transcribed in cells where Spo0A is in the ON state, leading to antagonism of SinR by direct protein-protein interaction. 3) Matrix operons are transcribed in the subset of cells where SinR de-repression occurs. 4) Biofilm matrix (oval) is produced by this subset of cells. 5) The biofilm is heterogeneously composed of both subtypes of cells. Figure from (Kearns 2008), reprinted with permission.

Additionally, expression of flagellar genes is repressed by a complex formed by SinR and its paralogue SlrR (Chai et al. 2010; Vlamakis et al. 2013). The SinR-SlrR complex also represses autolysin genes (Chai et al. 2010), thereby impairing cell separation and causing chaining of the cells which in turn contributes to biofilm formation. Another transcriptional regulator, DegU, was also shown to play a part in regulation of biofilm and motility in *B. subtilis* (Kobayashi 2007b), and the SlrR-SinR complex was found to be a involved in the activation of the *degU* gene by DegU∼P (Ogura, Yoshikawa, and Chibazakura 2014) (see Figure 16, page 61 for details).

1.4.7 Biofilm formation in *B. cereus*

In *B. cereus* the main protein component TasA and the signal peptidase SipW are orthologous to the corresponding proteins in *B. subtilis*, but instead of the anchor protein TapA, *B. cereus* encodes two paralogues of TasA (Fagerlund et al. 2014; Pflughoeft, Sumby, and Koehler 2011), whereof one was designated CalY (Caro-Astorga et al. 2014). TasA and CalY form fibers in the biofilm similar to those of *B. subtilis,* and CalY was recently revealed to act as a cell-surface adhesin in the early stationary phase, before disconnecting and forming fibers in the biofilm stage (Candela et al. 2018). A triple deletion of *sipW/tasA/calY* prevented biofilm formation, as did deletion of *sipW* alone. Deletion of *tasA* gave a thicker biofilm at 24h which disappeared after 72h (Caro-Astorga et al. 2014). Deletion of *calY* gave the opposite result, no biofilm at 24h but a thicker biofilm after 72h. Both biofilms stuck poorly to the sides of plastic wells used to grow the biofilm. Altogether this indicates that TasA and CalY participate at different stages of biofilm formation (Caro-Astorga et al. 2014).

In the same manner as in *B. subtilis*, *B. cereus* Spo0A∼P represses the phase-transition regulator *abrB* (Dubey et al. 2009; Saile and Koehler 2002; Xu et al. 2017). Biofilm formation is promoted by Spo0A∼P and repressed by AbrB, but neither seem to affect motility (Fagerlund et al. 2014). The Spo0A∼P/SinI/SinR network in *B. cereus* appears to regulate biofilm formation and motility in an analogous manner to *B. subtilis* (Fagerlund et al. 2014; Xu et al. 2017). The Spo0A, SinI and SinR proteins of *B. cereus* share a moderate to high level of sequence identity with their *B. subtilis* orthologues (81%, 32%, and 64%, respectively) (Xu et al. 2017). Of note, all three proteins can functionally replace their *B. subtilis* counterparts in the regulation of biofilm formation in *B. subtilis* (Xu et al. 2017). Like in *B. subtilis*, the SinI/SinR antirepressor-repressor pair constitutes a switch between biofilm formation and swimming motility (Fagerlund et al. 2014). The genes in the *eps* operon of *B. cereus* share some homology with those of *B. subtilis* (Irnov and Winkler 2010; Ivanova et al. 2003), but most importantly the gene encoding the flagellar clutch EpsE is not present in *B. cereus*, nor are genes encoding orthologues to *slrR* or *degU* (see Figure 16, page 61). Thus, the connection between SinI/SinR and motility in *B. cereus* remains unknown (Fagerlund et al. 2014).

The SinR regulon of the *B. cereus* group has been studied in *B. anthracis* (Pflughoeft, Sumby, and Koehler 2011), *B. thuringiensis* 407 (Fagerlund et al. 2014) and *B. cereus* AR156 (Xu et al. 2017). Transcriptional profiling indicated that only four genes were shared between *B. anthracis* and *B. thuringiensis* 407; the three genes of the *tasA* operon (*tasA* , *calY* and *sipW*), and a gene encoding an endonuclease (Fagerlund et al. 2014; Pflughoeft, Sumby, and Koehler 2011). Consensus SinR DNA binding sites (Chu et al. 2006;

Kearns et al. 2005) were found in the promoter area of the matrix operon genes in *B. cereus* AR156 (Xu et al. 2017). There is little overlap between the SinR regulons of the *B. cereus* group bacteria and that of *B. subtilis*. Comparing the regulons of *B. anthracis* and *B. subtilis,* four genes (*tasA*, *sipW*, *spoIIE*, and *spoIIG*) were common between the two species (Pflughoeft, Sumby, and Koehler 2011). Surprisingly, *spoIIE*, and *spoIIG* do not appear to be regulated by SinR in *B. thuringiensis* 407 (Fagerlund et al. 2014) nor in *B. cereus* AR156 (Xu et al. 2017). The differences in the SinR regulons between the *B. cereus* group species, and also to *B. subtilis*, may potentially be due to dissimilarities in lifestyles and thus target genes (Chu et al. 2006; Fagerlund et al. 2014; Pflughoeft, Sumby, and Koehler 2011; Xu et al. 2017).

1.5 Cyclic diguanylic acid (c-di-GMP)

1.5.1 Second messengers and c-di-GMP

Second messengers are small molecules that convey a signal received from cell-surface receptors to an intracellular target. A variety of cyclic nucleotides function in this manner, as conductors of environmental signals to cellular responses. Cyclic adenosine 3',5'-monophosphate (cAMP) has been recognized since the early 1990s as a regulator of carbon catabolite repression in *Escherichia coli* (Kolb et al. 1993), and has in later years also been linked to bacterial virulence in host-pathogen interactions (Gao et al. 2016; Knapp and McDonough 2014). Other examples include bis-(3',5') cyclic diadenylic acid (c-di-AMP) which affects various physiological roles, mainly in Gram-positive bacteria (Fahmi, Port, and Cho 2017; Witte et al. 2008) and the more recently discovered hybrid cyclic AMP-GMP (Davies et al. 2012).

Bis-(3',5')-cyclic diguanylic acid (c-di-GMP) is a secondary messenger that was first discovered in 1987 as an activator of cellulose synthase in the Gram-negative bacterium *Gluconacetobacter xylinus* (previously named *Acetobacter xylinum*) (Ross et al. 1987). Soon after, the domain responsible for the synthesis of c-di-GMP was determined in the protein PleD in *Caulobacter crescentus,* and the GGDEF consensus c-di-GMP binding sequence of the diguanylate cyclases (DGCs) was defined (Hecht and Newton 1995). As whole-genome sequencing was speeding up in the late 1990s, this consensus sequence was found to be present in proteins in an increasing number of bacterial species. The whole genome sequence of *E. coli* K-12 was published in 1997 and encoded 19 proteins containing the GGDEF-domain (Galperin, Nikolskaya, and Koonin 2001; Blattner et al. 1997). A few years later the EAL consensus sequence of the phosphodiesterases (PDEs) was discovered, again in *A. xylinum* (Tal et al. 1998). In 2000 the COG (Clusters of Orthologous Groups of proteins) database was established (Tatusov et al. 2000), and genes for c-di-GMP synthesis (DGCs) and breakdown (PDEs) were identified in most bacteria, but not in archaea. An additional domain which breaks down c-di-GMP was discovered in 2006. The domain was designated HD-GYP, and mutation of codons for the conserved H and D amino acid residues resulted in loss of enzymatic activity (Ryan et al. 2006). As with GGDEF and EAL domains, the HD-GYP domain is widely distributed in most bacteria.

c-di-GMP has been shown to affect many processes in bacteria. DGCs synthesize c-di-GMP from two guanosine-5'triphosphate (GTP) molecules, and its breakdown to 5' GMP is catalyzed by

phosphodiesterases (PDEs) (Figure 9) (Ross et al. 1987). The activities of DCGs and PDEs are frequently controlled by N-terminal sensor domains (PAS, GAF, etc.) which may often be located in the cytoplasmic membrane and may bind ligands or proteins, constituting a two-component signal transduction system (Galperin, Nikolskaya, and Koonin 2001; Galperin 2005).

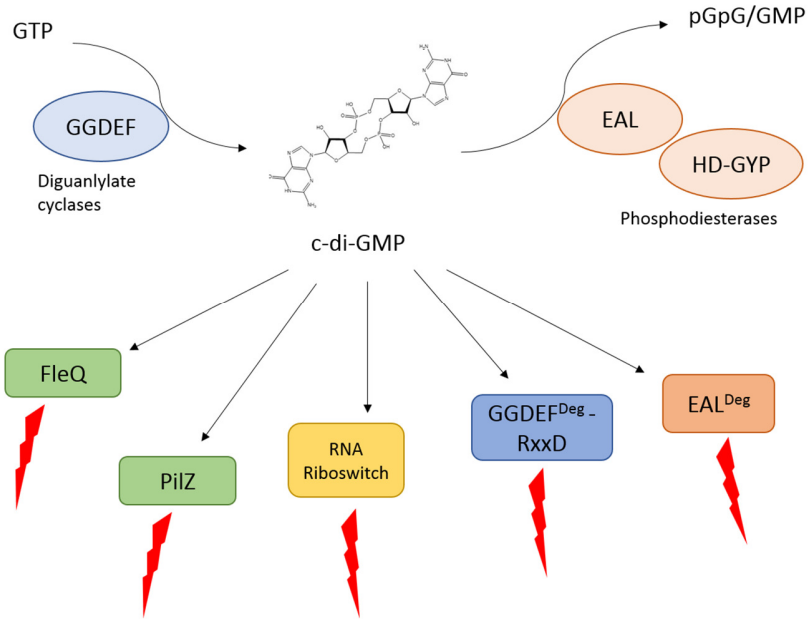

Figure 9: Synthesis of c-di-GMP from GTP by diguanylate cyclases (DGCs) (blue spheres) and degradation to GMP/pGpG by phosphodiesterases (PDEs) (orange spheres). Proteins and RNA can bind c-di-GMP and act as downstream effectors. Degenerate GGDEF and EAL domains are depicted as blue and orange boxes, respectively. The effector proteins FleQ and PliZ are shown as green boxes and the RNA riboswitch as a yellow box. High levels of c-di-GMP are commonly associated with reduction in motility and virulence and increased cellular attachment and biofilm formation.

The previously mentioned PleD protein is perhaps the best characterized DGC to date, and it was also in the GGDEF domain of this protein that the allosteric inhibition site (I-site) was revealed (Chan et al. 2004). The I-site is used to modulate the DGC activity in response to differing levels of c-di-GMP. PleD synthesizes c-di-GMP from GTP by forming a dimer at the active site GGDEF and the RxxD motif of the I-site acts as a non-competitive product inhibitor by binding c-di-GMP and thereby changing the conformation and preventing the active PleD dimer from forming (Chan et al. 2004; Christen et al. 2006). This RxxD motif was found to be conserved in the majority of DGCs (Christen et al. 2006).

c-di-GMP exerts its effects through c-di-GMP binding effector molecules which mediate the c-di-GMP phenotypic responses in intracellular signaling networks (Figure 9). Several such c-di-GMP binding effectors have been identified and are described below. In general, high levels of intracellular c-di-GMP

promotes biofilm formation by increasing the production of EPS and reducing flagella- and pilus mediated motility (Pesavento et al. 2008; Simm et al. 2004; Beyhan et al. 2006; Kuchma et al. 2007), but has also been found to have effect on other cellular processes, e.g. virulence (Christensen et al. 2013; Shahbaz Ph et al. 2020).

The first c-di-GMP downstream receptor to be discovered was the PilZ domain (Amikam and Galperin 2006), named after the *P. aeruginosa* gene *pilZ* encoding a 118 amino acid protein related to pili formation and twitching motility. The PilZ domain is found in proteins in most bacteria, but not in eukaryotes and archaea. The PilZ domain is involved in cellular signaling governing a range of different phenotypes, and may have effects on virulence (McCarthy et al. 2008; Pratt et al. 2007), motility (Christen et al. 2007; McCarthy et al. 2008; Pratt et al. 2007; Ryjenkov et al. 2006), and synthesis of EPS (Merighi et al. 2007; Ryjenkov et al. 2006).

Degenerate and thereby enzymatically inactive EAL domains have been shown to still be able to bind c-di-GMP and act as downstream effectors (Sondermann, Shikuma, and Yildiz 2012; Minasov et al. 2009; Navarro et al. 2009). In *Pseudomonas fluorescens* the effector molecule LapD harbors both an inactive GGDEF domain and an inactive EAL domain. LapD is a transmembrane protein which binds c-di-GMP via the cytoplasmic degenerate EAL domain and transfers the signal through the membrane where it inhibits the activity of the periplasmic protease LapG. LapG is then unable to degrade the adhesive molecule LapA which by staying attached to the outer membrane promotes biofilm formation (Newell et al. 2011; Newell, Monds, and O'Toole 2009). Proteins harboring degenerate and catalytically inactive GGDEF domains have also been shown to act as effector molecules, although the binding of c-di-GMP to these proteins appears to be through the presence of an active I-site (Kunz et al. 2020; Zouhir et al. 2020). An orthologue of PleD in *P. aeruginosa*, PelD, was revealed to have a degenerated active GGDEF domain and instead acted as an effector molecule of the regulation of Pel polysaccharide biosynthesis which was dependent on the allosteric binding of c-di-GMP to the active I-site (Lee et al. 2007; Whitney et al. 2012). Additionally, degenerate and inactive GGDEF domains coupled to an active EAL domain have been shown to nevertheless bind GTP and thereby allosterically activating the EAL domain (Christen et al. 2005).

FleQ is the master regulator of flagella gene expression in *P. aeruginosa* (Arora et al. 1997), and also negatively regulates a number of genes that encode exopolysaccharides (Hickman and Harwood 2008). FleQ is an AAA+ ATPase enhancer-binding protein which binds c-di-GMP through its AAA+ domain (Baraquet and Harwood 2013; Matsuyama et al. 2016). Binding of c-di-GMP causes a conformational change which converts FleQ from a repressor of the genes encoding EPS to an activator of the same genes (Baraquet et al. 2012). The effect on the flagellar genes is also by binding of c-di-GMP, but here c-di-GMP acts as a competitor of ATP and dampens the activity of FleQ, thereby causing down-regulation of flagella gene expression (Baraquet and Harwood 2013).

Riboswitches are regulatory components in the leader sequences of an mRNA molecule that can bind small molecules and turn the corresponding genes on and off accordingly (Nudler and Mironov 2004). Upon binding, the RNA structure is altered, which can induce or prevent the formation of terminators or ribosomal binding sites by forming alternative structures in response to this ligand binding. In 2008, the

first c-di-GMP binding riboswitches were discovered in *Vibrio cholerae, Clostridium difficile* and *B. cereus* (Sudarsan et al. 2008; Lee, Sudarsan, and Breaker 2010). These were found to be associated with regulation of genes related to virulence, flagellar biosynthesis and pilus formation, respectively. Another class of c-di-GMP binding riboswitches was discovered in 2010, also in *C. difficile* (Lee et al. 2010) which regulated self-splicing of the mRNA by ribozymes. Both classes of c-di-GMP riboswitches contain the highly conserved RNA GEMM domain.

In recent years, several new receptors for c-di-GMP have been discovered, such as MshE-type ATPases (Wang et al. 2016), VpsT/VpsR in *Vibrio cholerae (Krasteva et al. 2010)*, and a consensus sequence in *P. aeruginosa* protein PA3740 (Duvel et al. 2016). Additionally, c-di-GMP has been shown to bind at protein interfaces and facilitate or disrupt protein-protein interactions for transcriptional factors (Chou and Galperin 2016). Given the ubiquitousness of this second messenger and the many bacterial functions it affects, several authors expect that more c-di-GMP receptors will be revealed in the future (Chou and Galperin 2016; Hengge et al. 2019; Jenal, Reinders, and Lori 2017).

1.5.2 c-di-GMP signaling in Gram-positive bacteria

Since the discovery of c-di-GMP in 1985, an increasing interest in c-di-GMP signaling has resulted in numerous studies, mainly in Gram-negative bacteria. The first recorded instance of c-di-GMP signaling in Gram-positive bacteria was not identified until 2010, when c-di-GMP was found to influence developmental processes in *Streptomyces coelicolor* (Figure 10) (den Hengst et al. 2010; Hull et al. 2012; Tran et al. 2011).

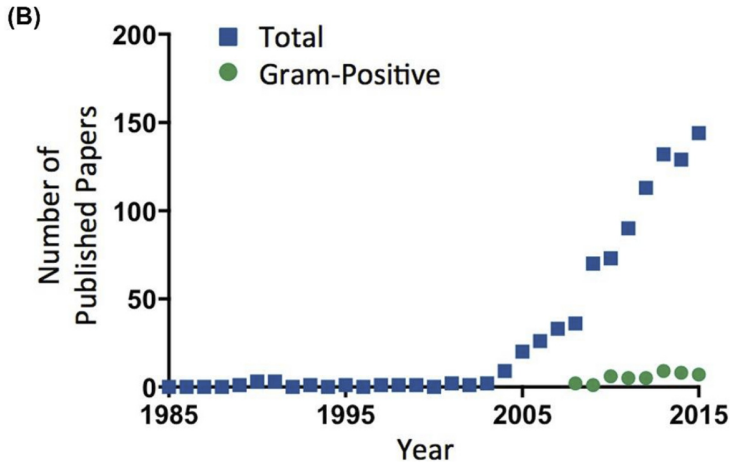

Figure 10: Number of published papers involving c-di-GMP from its discovery in 1985, showing total number and findings in Gram-positive bacteria, respectively. From (Purcell and Tamayo 2016), reprinted with permission from Oxford University Press.

The genome of the enteric pathogen *C. difficile* encodes a plethora of c-di-GMP signaling molecules. As many as 37 proteins containing either a GGDEF or an EAL domain are encoded, and enzymatic activity has been determined for one DGC and two PDEs (Bordeleau et al. 2011). Furthermore, *C. difficile* contains one PilZ domain protein and 16 predicted c-di-GMP riboswitches (Bordeleau et al. 2015; Lee et al. 2010; Sudarsan et al. 2008). In *C. difficile*, c-di-GMP appears to be mainly involved in the transition from the motile to sessile state.

Recently, cyclic di-GMP networks have been discovered in *L. monocytogenes* and *B. subtilis*. In *L. monocytogenes*, a total of three functioning DGCs (Dgc A-C) and three PDEs (PdeB-D) are present in the bacterium along with a protein with a degenerate GGDEF domain but with an intact I-site (PssE) (Chen et al. 2014). DgcA and DgcB generate a pool of local c-di-GMP which binds to PssE and stabilizes the PssE-PssC complex which is responsible for exopolysaccharide (EPS) synthesis (Koseoglu et al. 2015). The increase in EPS production promotes cell aggregation but does not affect biofilm formation. A reduction in motility is seen at higher levels of cellular c-di-GMP, but this was found to be caused by the aggregation followed by the increase in EPS (Chen et al. 2014). Elevated c-di-GMP levels also impairs *L. monocytogenes* invasion of mammalian cells (Chen et al. 2014), and it was recently discovered that this is due to downregulated expression of the master virulence regulator *prfA* (Elbakush, Miller, and Gomelsky 2018). Synthesis of large amounts of c-di-GMP diminish the levels of GTP which is a cofactor for CodY, enhancing its DNA binding abilities (Handke, Shivers, and Sonenshein 2008). With CodY being an activator for *prfA*, the connection to c-di-GMP was established (Elbakush, Miller, and Gomelsky 2018). A summary of c-di-GMP related proteins in *L. monocytogenes* is provided in Table 3.

Table 3: Summary of proteins related to c-di-GMP turnover and binding in *L. monocytogenes EGD-e.*

Gene	Locus tag	Domain	Activity *in vivo*	Deletion phenotype	Overexpression phenotype	Reference
dgcB	lmo1912	GGDEF	DGC	Decreased EPS production and aggregation	Reduced motility Increased EPS production	(Chen et al. 2014; Koseoglu et al. 2015)
dgcC	lmo2174	GGDEF	DGC	Nd	Reduced motility Increased EPS production	(Chen et al. 2014)
pdeB	lmo0131	EAL	PDE	None (but a triple pdeBDC deletion reduced motility and increased EPS production)	Na	(Chen et al. 2014)
pdeC	lmo1914	EAL	PDE	Nd	Na	(Chen et al. 2014)
pdeD	lmo0111	EAL	PDE	Nd	Na	(Chen et al. 2014)
pssE	lmo0531	GGDEF*/RxxD	c-di-GMP binding	Decreased EPS production and aggregation	Na	(Chen et al. 2014)

* degenerated domain
Na: not analysed
Nd: not detected

The genome of *B. subtilis* is predicted to encode four proteins with GGDEF domains, two proteins with EAL domains, one protein with both a GGDEF and an EAL domain, and one PilZ-domain protein. While wild type *B. subtilis* did not synthesize detectable levels of c-di-GMP, overexpression of DGCs and mutant strains of PDEs produced measurable amounts of c-di-GMP *in vivo* (Gao et al. 2013). Of the five proteins with GGDEF domains, only three (DgcK, DgcP and DgcW) were able to act as functional DGCs, while the other two, YdaK and YybT, have degenerate GGDEF sites. Of the three proteins with EAL domains, only one (PdeH) exhibited PDE activity (Gao et al. 2013). YkuI has a degenerate EAL domain but is nevertheless able to bind c-di-GMP (Minasov et al. 2009), and DgcW contains both GGDEF and EAL domains, but was found to primarily act as a DGC (Gao et al. 2013). Binding of c-di-GMP to the degenerate GGDEF domains has also been investigated, and of the two, only YdaK was able to bind c-di-GMP to a significant degree (Gao et al. 2013; Rao et al. 2010) although this was found to be via the domain's intact I-site (Kunz et al. 2020). Surprisingly, the GGDEF domain of YybT, which does not harbor an I-site, was revealed to possess unprecedented ATPase activity rather than DGC activity (Rao et al. 2010).

The primary effect accredited to c-di-GMP in *B. subtilis* is on motility. Though lacking PDE activity, the deletion mutant of *ykuI* showed a slight reduction in motility (Minasov et al. 2009). Furthermore, mutation of *pdeH* gave a strong defect in swarming motility. The effect on motility was revealed to occur through the actions of the PilZ-domain protein DrgA, which acts as a downstream c-di-GMP receptor for PdeH and binds to the MotA protein of the flagellar motor acting as a flagellar clutch (Chen et al. 2012; Gao et al. 2013; Subramanian et al. 2017). As opposed to most c-di-GMP turnover proteins, PdeH does not have a sensing domain. Instead, two Spo0A~P binding sites were found upstream of *pdeH*, and it was confirmed that *pdeH* is under negative control of Spo0A~P (Chen et al. 2012). The three proteins with functional DGC domains (DgcK, DgcP and DgcW) all showed reduction in motility when overexpressed (Chen et al. 2012).

The effect of c-di-GMP on biofilm in *B. subtilis* has been unclear. While (Gao et al. 2013) could find no effects on biofilm, Chen and co-workers found that deletion mutants of the PilZ receptor protein DrgA formed a more robust biofilm than the wild type, and that the matrix operons *eps* and *tasA* were upregulated (Chen et al. 2012). Recently, the operon *ydaJKLMN* encoding the c-di-GMP binding protein YdaK was found to be a producer of EPS (Bedrunka and Graumann 2017a, 2017b). Previous studies have indicated that this operon is essential in *B. subtilis* (Chen et al. 2012). A summary is provided in Table 4.

A close relative to *B. subtilis*, *Bacillus amyloliquefaciens*, harbors an orthologue to the PdeH protein and shows reduced and increased motility upon deletion and overexpression, respectively. Interestingly, no orthologue to the PilZ-domain protein DrgA is present, so a different mechanism of regulation must occur here. Orthologues to DgcK and DgcP are also present in *B. amyloliquefaciens* and show typical effects on motility when mutated and overexpressed (Yang et al. 2018).

Table 4: Summary of proteins related to c-di-GMP turnover and binding in *B. subtilis 168*.

Gene	Locus tag	Domain	Activity *in vivo*	Deletion phenotype	Overexpression phenotype	Reference
yhuX/pdeH	BSU31740	EAL	PDE	Reduced motility	Na	(Chen et al. 2012)
ykuI	BSU14090	EAL*	c-di-GMP binding	slightly reduced motility	Nd	(Minasov et al. 2009)
ypfA/drgA	BSU22910	PilZ	c-di-GMP binding	Increased motility More robust biofilm morphology and delayed biofilm dispersion	Reduced motility	(Chen et al. 2012)
ykoW/dgcW	BSU13420	GGDEF/EAL	DGC	Nd	Reduced motility	(Chen et al. 2012)
ytrP/dgcP	BSU29650	GGDEF	DGC	Nd	Reduced motility	(Chen et al. 2012)
yhcK/dgcK	BSU09120	GGDEF	DGC	Nd	Reduced motility	(Chen et al. 2012)
ydaK	BSU04280	GGDEF*/RxxD	c-di-GMP binding	Na	Increased EPS production	(Bedrunka and Graumann 2017b; Chen et al. 2012; Kunz et al. 2020)
yybT	BSU40510	GGDEF*	ATP-ase	Nd	Na	(Rao et al. 2010)

* degenerated domain
Na: not analysed
Nd: not detected

2 Aim of the study

The overall goal of this study was to expand our knowledge on the regulation of biofilm formation, motility and virulence in the *B. cereus* group, and how this regulation is coordinated in *B. cereus* group species.

The discovery of a c-di-GMP binding riboswitch in *B. cereus* (Sudarsan et al. 2008) led us to hypothesize that c-di-GMP signaling might play a part in the regulation of important phenotypes in the *B. cereus* group. We therefore aimed to perform a systematic study of genes encoding putative c-di-GMP signaling proteins in the *B. cereus* group. Realizing that these phenotypes can also be under the control of transcriptional regulators, and that it had been recently established that a homolog of the *Listeria* spp. motility repressor MogR exists in *B. cereus* group bacteria, we also aimed to characterize the putative role for this protein in regulating motility and related phenotypes in the *B. cereus* group.

We therefore proposed the following questions:

- Are there genes present in the *B. cereus* group genomes coding for proteins putatively related to c-di-GMP turnover? If so, what are the molecular functions of the proteins encoded by these genes?
- Which phenotypes are regulated by c-di-GMP in the *B. cereus* group?
- Can effector molecules of c-di-GMP be identified, and their cellular function(s) characterized?
- Is MogR a key regulator of motility in the *B. cereus* group?
- Does MogR also affect other phenotypes in *B. cereus* group bacteria?

3 Summary of papers

Paper 1: Cyclic diguanylate regulation of *Bacillus cereus* group biofilm formation.

Genes potentially involved in c-di-GMP turnover in the *B. cereus group* were identified by searching 90 available genome sequences. Ten genes (*cdgA-F, cdgH-J* and *cdgL*) were found to be highly conserved in the group while another three (*cdgG*, *cdgM* and *cdgK*) were less conserved. *cdgA-J* were subjected to further analyses. Of these, six proteins harbor both GGDEF and EAL domains (CdgD-I), three contain a GGDEF domain only (CdgA-C) and one contains a single EAL domain (CdgJ). Selected phenotypes were investigated in deletion mutants and overexpression strains, and the ability to produce c-di-GMP was measured. Wild type *B. thuringiensis* 407 did not synthesize detectable levels of c-di-GMP, but overexpression strains of putative diguanylate cyclases (DGCs) and deletion mutants of putative phosphodiesterases (PDEs) produced measurable amounts of c-di-GMP. The greatest effect was seen upon overexpressing CdgF, the only bifunctional protein known to act as a DGC or a PDE in *B. cereus*, depending on oxidation level. Under the examined conditions, CdgF was revealed to be the main DGC, producing ~500 fold more c-di-GMP when overexpressed compared to an empty vector control strain. Both overexpression and deletion of this gene showed phenotypes in line with those expected from c-di-GMP; increased biofilm formation and impaired motility at high levels of c-di-GMP and the inverse at low c-di-GMP levels. In addition, cytotoxicity paralleled with secretion of the enterotoxins Nhe and CytK were strongly reduced upon overexpressing CdgF. In conclusion, this work firmly established that a c-di-GMP signaling network is active in the *B. cereus* group and affects biofilm formation, motility and virulence.

Paper 2: MogR is a ubiquitous transcriptional repressor affecting motility, biofilm formation and virulence in the *Bacillus cereus* group

The transcriptional regulator MogR regulates motility in a temperature-dependent manner in *Listeria monocytogenes* by binding directly to a conserved DNA sequence upstream of target genes. The only other known species carrying a homologue to MogR are those of the *Bacillus cereus* group. MogR was found to act independent of temperature in *B. cereus*, in a growth-phase dependent manner. Overexpression of MogR abolished expression of flagellar genes and rendered the bacteria non-motile. A microarray assay revealed that 89 additional genes are affected by MogR expression, including genes affecting virulence and biofilm formation, either directly or indirectly. To correct for effects directly caused by the lack of flagella in the MogR overexpression strain, biofilm studies were performed on strains overexpressing MogR in a non-flagellated Δ*flaAB* background. Upon overexpression of MogR, biofilm formation was significantly increased after 48 and 72 hours compared to an empty vector control, showing that MogR has a positive influence on biofilm formation. Likewise, virulence and

toxicity assays showed the MogR overexpression strain to be severely attenuated with regard to virulence compared to the control. Introducing point mutations in the predicted DNA binding sites on the overexpressed MogR protein (MogR$^{QN\rightarrow AA}$) restored all phenotypes to those of the empty vector control. Moreover, gel shift assays showed *in vitro* binding of purified MogR protein to predicted intergenic consensus sequences in *B. thuringiensis* DNA, which was not observed for MogR$^{QN\rightarrow AA}$, potentially reflecting the DNA binding nature of *B. thuringiensis* MogR. Genomic analyses of the motility loci of all available sequenced strains in the *B. cereus* group show that in the non-motile species the motility genes are mutated (*B. anthracis*) or severely reduced in number (*B. mycoides*), but *mogR* is retained. In *B. pseudomycoides mogR* is the only gene retained in the motility locus and is moreover conserved in all *B. pseudomycoides* strains fully sequenced in GenBank. Taken together, this may suggest that MogR exerts other functions than regulation of motility in the *B. cereus* group.

Paper 3: CdgL is a degenerate GGDEF domain protein affecting flagellin synthesis and motility in *Bacillus thuringiensis*

As a putative downstream effector of c-di-GMP, we investigated the function of CdgL, which is a protein with a degenerate GGDEF domain and a transmembrane domain. Despite the presence of a degenerate GGDEF domain, no binding of c-di-GMP could be detected under our tested conditions, perhaps related to the lack of an intact I-site in the GGDEF domain. Deletion of *cdgL* in *B. thuringiensis* 407 resulted in the downregulation of transcription of the entire motility locus and subsequent loss of flagella and motility, as demonstrated by qPCR, Western blot analyses and atomic force microscopy. No known regulators of motility in *B. cereus* were affected in expression levels by the deletion of *cdgL*, with the possible exception of *sinI,* encoding the antirepressor of SinR, which was slightly upregulated. We found that contrary to earlier theories, while the absence of flagellar motility lead to a decrease in biofilm formation at 24 hours, no difference in biofilm mass could be detected at later timepoints relative to the wild type strain, indicating a delay rather than a defect in biofilm formation. No other effects on biofilm formation could be found with regards to deletion or overexpression of *cdgL* compared to controls. The gene encoding CdgL appears to be part of a three-gene operon which is conserved throughout the *B. cereus* group with the exception of the non-motile species *B. pseudomycoides*. The two other genes of the operon encode a NupC-like transporter and a glycosyl transferase, respectively. The proteins encoded by all three genes are membrane bound, allowing potential protein interactions and a possibility for the genes in this operon all being connected to the functional regulation of flagellar motility in *B. thuringiensis* 407.

4 Main results and discussion

4.1 *Bacillus thuringiensis* 407 (*cry-*)

The experimental work for this thesis was done using the Cry-negative strain *B. thuringiensis* 407 (*cry-)* as a model for *B. cereus. B. thuringiensis* 407 was originally isolated in Brazil by Dr Sergio Batista Alves from an insect larva (*Anagasta kuhniella*), and was cured of its Cry toxin-encoding plasmid by Olivia Arantes (Lereclus et al. 1989), and is thus genetically indistinguishable from *Bacillus cereus* (Helgason et al. 1998; Helgason et al. 2000; Schmidt, Scott, and Dyer 2011). *B. thuringiensis* 407 contains nine other plasmids ranging in size from 2062 to 501911 bp.

4.2 c-di-GMP signaling in the *B. cereus* group

Prior to conducting the current work, only very few studies had been conducted on c-di-GMP in Gram-positive bacteria, and little was known about c-di-GMP in *L. monocytogenes* and *B. subtilis*. Parallel to the current work, cyclic di-GMP networks have been discovered in both species (Bedrunka and Graumann 2017a, 2017b; Chen et al. 2014; Chen et al. 2012; Koseoglu et al. 2015; Minasov et al. 2009; Rao et al. 2010). In paper 1 we systematically uncovered genes governing c-di-GMP production in *B. thuringiensis* 407 upon overexpression of DGCs in whole cell *in vivo* experiments using LC-MS/MS analysis. Overexpression of CdgB and in particular CdgF produced high levels of c-di-GMP (Paper I; Table 5), while c-di-GMP levels in wild type *B. thuringiensis* 407 was below the limit of detection under our experimental conditions, something that was also observed for wild type *B. subtilis* (Gao et al. 2013). No c-di-GMP production could be detected in any of the deletion mutants of putative PDEs in our study (Table 5).

In a study from 2018 c-di-GMP signaling was investigated in a separate strain, *B. thuringiensis* BMB171 (Fu et al. 2018). A triple deletion mutant of three putative PDEs (*cdgF*, *cdgH* and *cdgE*) produced substantially elevated levels of c-di-GMP compared to the wild type as detected *in vivo* using LC-MS/MS analysis. *In vivo* DGC activity was detected in CdgA by a dual-fluorescence reporter system (Fu 2018; Zhou H, 2016). Moreover, purified recombinant proteins of the GGDEF and/or EAL domains in *B. thuringiensis* BMB171 were tested *in vitro* for their ability to synthesize or degrade c-di-GMP, respectively. Results from HPLC analysis suggested PDE activity in CdgG, CdgF, CdgH and CdgJ (Fu et al. 2018). CdgF was also found in *B. thuringiensis* 407 to exhibit PDE activity (under anaerobic conditions), and to constitute a protein where both PDE and DGC activity was present, depending on oxidation level (Paper 1).

4.2.1 Increased levels of c-di-GMP affect motility, biofilm formation and virulence in *B. thuringiensis* 407

c-di-GMP is generally connected to motility and biofilm formation in bacteria, in that high levels of c-di-GMP induce downregulation of flagellar genes and upregulation of biofilm promoting genes (Simm et al. 2004; Jenal, Reinders, and Lori 2017; Valentini and Filloux 2016). In this study, assays screening for effects on these phenotypes were performed for all deletion mutants and overexpression strains of the ten identified putative c-di-GMP turnover genes in *B. thuringiensis* 407 (Paper 1; Table 5). In general, high c-di-GMP levels correlated with increased biofilm formation and reduced motility and virulence, while the opposite was seen where low levels of c-di-GMP were predicted, such as upon overexpression of a putative PDE. Noteworthy, CdgF – the DGC which produced the highest levels of c-di-GMP in this study (Paper I) - was essential for biofilm formation in *B. cereus* ATCC 10987 in a transposon insertion mutant library screening for mutants defective in pellicle biofilm formation (Okshevsky et al. 2017). Moreover, a very recent study (Whitfield et al. 2020) made the discovery that in *B. cereus* ATCC 10987, CdgF and CdgE reciprocally regulate the production of a polysaccharide similar to the Pel polysaccharide of *Pseudomonas aeruginosa* through the binding of c-di-GMP to the degenerate GGDEF domain (intact I-site) of a PelD orthologue (Friedman and Kolter 2004), composing a minimal c-di-GMP network in this strain. The *pel* locus and hence the PelD orthologue is however conserved in only a small number of *B. cereus* group strains, none of which includes *B. thuringiensis* 407 (Whitfield et al. 2020), which implies that synthesis of c-di-GMP by CdgF in this strain most likely is connected to one or more c-di-GMP responsive networks .

A microarray experiment performed in our lab (paper 2; E-MTAB-8898) analyzing the effect of overexpression of CdgF (DGC) relative to the empty vector control strain, showed downregulation of flagellin and several other motility genes at high levels of c-di-GMP (Table 6). Moreover, the microarray experiment showed that expression of the biofilm regulator genes *spo0A*, *sinI* and *codY* are affected by high levels of c-di-GMP (Table 6), which would potentially be expected to lead to increased biofilm formation. Spo0A and SinI are well established regulators of biofilm (see section 1.6.3) and could also be linked to the downregulation of flagellar genes. Reduction in the expression of *codY* at high levels of c-di-GMP was also observed in *L. monocytogenes*, and has been linked to a decrease in GTP which is a cofactor of CodY (Elbakush, Miller, and Gomelsky 2018) and a precursor for c-di-GMP (Ross et al. 1987). With CodY being a repressor of matrix synthesis genes in *B. cereus* (Lindback et al. 2012), a downregulation may contribute to increased biofilm formation in line with the results from Paper 1. These results are in line with the finding that the triple PDE mutant in *B. thuringiensis* BMB171 showed increased biofilm formation and reduced motility (Fu et al. 2018).

In Paper 1, increased levels of c-di-GMP were found to be related to a reduction in Vero cell cytotoxicity and a clear reduction in the levels of production of the secreted enterotoxins Hbl, Nhe and CytK. Reduced virulence in response to increased c-di-GMP is consistent with earlier studies (Hall and Lee 2018; Ryan 2013; Römling, Galperin, and Gomelsky 2013; Elbakush, Miller, and Gomelsky 2018), and the microarray experiment analyzing overexpression of CdgF also shows transcriptional downregulation of *cytK* and several other putative virulence factors upon overexpression of CdgF (Table 6). The global

virulence activator *plcR* is also significantly downregulated, along with the gene encoding the signaling peptide PapR, which is functionally associated with PlcR (Table 6).

Table 5: Description of proteins putatively related to c-di-GMP turnover or binding in *B. thuringiensis* 407 (Paper 1).

Gene	Locus tag	Domain (motif)	Activity *in vivo*	Deletion phenotype	Overexpression phenotype	Reference
cdgA	BTB_RS27620	GGDEF (GGEEF)	Nd	Increased cytotoxicity.	Reduced cytotoxicity Slightly increased biofilm formation/reduced motility	Paper 1
cdgB	BTB_RS20530	GGDEF (GGEEF)	DGC	Increased cytotoxicity	Reduced cytotoxicity Slightly increased biofilm formation/reduced motility	Paper 1
cdgC	BTB_RS20295	GGDEF*/RxxD	Nd	Early onset sporulation	Toxic to cells	Paper 1
cdgD	BTB_RS27060	GGDEF*/EAL	Nd	Increased biofilm formation	Reduced biofilm formation Reduced motility	Paper 1
cdgE	BTB_RS18755	GGDEF/EAL	Nd	Increased biofilm formation	Reduced biofilm formation Increased motility	Paper 1
cdgF	BTB_RS03185	GGDEF/EAL	DGC/PDE	Reduced biofilm formation Increased cytotoxicity	Increased biofilm formation Reduced motility Reduced cytotoxicity	Paper 1
cdgG	BTB_RS17620	GGDEF/EAL	Nd	Reduced cytotoxicity	Increased biofilm formation Reduced motility	Paper 1
cdgH	BTB_RS02795	GGDEF*/EAL	Nd	Increased cytotoxicity	Reduced biofilm formation	Paper 1
cdgI	BTB_RS27295	GGDEF/RxxD/ EAL	Nd	Reduced motility Reduced cytotoxicity	Reduced biofilm formation	Paper 1
cdgJ	BTB_RS20255	EAL*	Nd	Nd	Increased biofilm formation Reduced cytotoxicity	Paper 1
cdgL	BTB_RS26690	GGDEF*	Nd	Loss of motility	Nd	Paper 3

* degenerated domain
Nd: not detected

Table 6: Genes related to motility, biofilm and virulence, which were differentially regulated in the *B. thuringiensis* 407 strain ($p_{FDR\text{-}corr} < 0.05$) upon overexpression of CdgF (microarray experiment described in Paper 2; E-MTAB-8898).

Locus tag in *B. thuringiensis* 407	Locus tag in *B. cereus* ATCC 14579	Predicted function	log_2 (fold change)
Motility genes			
BTB_RS08320	BC1641	Flagellar basal-body rod protein FlgB	-0.68
BTB_RS08325	BC1642	Flagellar basal-body rod protein FlgC	-0.71
BTB_RS08330	BC1643	Flagellar hook-basal body complex protein FliE	-0.53
BTB_RS08335	BC1644	Flagellar M-ring protein FliF	-0.64
BTB_RS08365	BC1650	Basal-body rod modification protein FlgD	-0.55
BTB_RS08400	BC1657	Flagellin	-2.16
BTB_RS08410	BC1661	Flagellar motor switch protein FliN	-0.55
BTB_RS08415	BC1662	Flagellar motor switch protein FliM	-0.52
BTB_RS08420	BC1663	Flagellar motor switch protein FliN	-0.60
BTB_RS08425	BC1664	Flagellar motor switch protein FliN	-0.59
BTB_RS08430	BC1665	Flagellar biosynthetic protein FliP	-0.58
BTB_RS08435	BC1666	Flagellar biosynthetic protein FliQ	-0.57
BTB_RS08440	BC1667	Flagellar biosynthetic protein FliR	-0.51
Biofilm genes			
BTB_RS06545	BC1283	SinI protein	0.67
BTB_RS21200	BC4170	Stage 0 sporulation protein A	0.62
BTB_RS19405	BC3826	Transcription pleiotropic repressor CodY	-0.82
Virulence genes			
BTB_RS05745	BC1110	Beta-channel forming cytolysin CytK	-1.1
BTB_RS03430	BC0670	Phospholipase C	-0.67
BTB_RS27300	BC5349	PapR protein	-0.40
BTB_RS27305	BC5350	Transcriptional activator PlcR	-0.32

Surprisingly, an increase in virulence at high levels of c-di-GMP was reported in *B. thuringiensis* BMB171, coupled with increased expression of *plcR* (Fu et al. 2018). We subsequently conducted a corresponding study, performing an RT-qPCR analysis investigating *plcR* expression upon CdgF overexpression (V. Smith, unpublished results). The samples were taken at the same point in the growth phase as in the study by Fu et al., at the point of culture where around 50% of bacteria have produced spores. Our results were strikingly different, however, and were in accordance with our own previous results; after 20 hours the expression of *plcR* was downregulated more than 70-fold in the CdgF overexpression strain compared to the empty vector control strain (Figure 11).

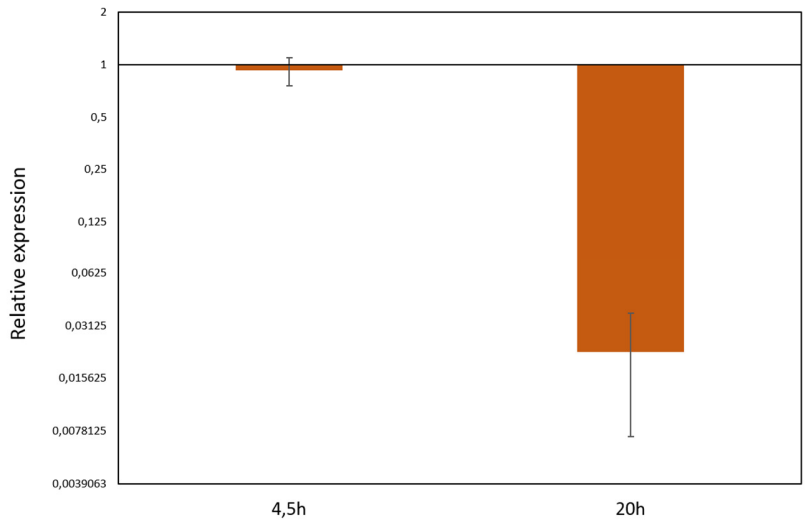

Figure 11: Relative gene expression of the *plcR* gene in the CdgF overexpression strain relative to the empty vector control strain, as analyzed by RT-qPCR. The mean and standard error of the mean for three independent experiments is shown.

4.2.2 A c-di-GMP regulatory network in *B. thuringiensis* 407

Based on predicted motifs and functional analyses, we propose a c-di-GMP network in *B. thuringiensis* 407 (Table 7). Two c-di-GMP sensing riboswitches were identified in *B. cereus* in 2008 (Sudarsan et al. 2008; Lee, Sudarsan, and Breaker 2010). The riboswitch designated Bc2 responds to increased c-di-GMP levels by acting as an "on-switch" for the downstream gene, the collagen binding cell surface protein CbpA (Tang et al. 2016; Finke et al. 2019). The gene encoding CbpA was found to be significantly downregulated upon deletion of *cdgF*, compared to the wildtype (Finke et al. 2019) and conversely upregulated in the microarray experiment analyzing the effect of overexpression of CdgF relative to the empty vector control ($p_{FDR\text{-}corr} < 0.002$; E-MTAB-8898; Paper 2). The other riboswitch, Bc1, acts as an "off-switch" for the downstream gene, encoding a methyl-accepting chemotaxis protein (McpA). In addition, among other potential downstream effector candidates that have been identified is a protein carrying two putatively c-di-GMP binding PilZ domains along with a cellulose synthase domain, as well as the three proteins harboring degenerate GGDEF or EAL domains, CdgC, CdgJ and CdgL (Table 7).

Table 7: Putative c-di-GMP binding effector molecules in *B. thuringiensis* 407.

Gene	Locus tag	Domain (motif)	Putative function	Determined function	Reference
cdgC	BTB_RS20295	GGDEF*/RxxD	Sporulation	Not determined	
cdgJ	BTB_RS20255	EAL*	Not determined	Not determined	
cdgL	BTB_RS26690	GGDEF*	c-di-GMP binding	Motility	Paper 3
cbpA	BTB_RS05575	Riboswitch	Collagen adhesion	Collagen adhesion	(Finke et al. 2019)
cspA	BTB_RS08505	PilZ	Cellulose synthesis	Not determined	
mcpA	BTB_RS02175	Riboswitch	Chemotaxis	Not determined	

* degenerate domain

Motility, cellulose synthesis, adhesion and chemotaxis are all phenotypes previously under control of cellular c-di-GMP levels (Hu et al. 2013; Orr and Lee 2016; Ross et al. 1987). While sporulation is not one of the phenotypes traditionally associated with c-di-GMP, there are reports linking this second messenger to sporulation in *Streptomyces coelicolor (Tschowri et al. 2014)* and fruiting body formation in *Myxococcus xanthus* (Skotnicka et al. 2016). None of the c-di-GMP metabolism genes tested in the current study have previously been identified as sporulation genes in *B. cereus* or in *B. subtilis* (Molle et al. 2003; Traag et al. 2013; Yan et al. 2016), and in line with this, no deletion or overexpression of any of the CDG or PDE genes produced a complete sporulation defect (Paper 1). Nevertheless, we found that deletion of *cdgC* lead to a significantly earlier onset of sporulation and higher sporulation efficacy than the wild type strain, in line with the observation that expression of *cdgC* showed an eight-fold increase between the last time point of planktonic growth to a 24 hour biofilm, an interesting finding as *Bacillus* biofilms frequently contain spores (Lindsay, Brözel, and Von Holy 2006; Wijman et al. 2007). CdgC is a protein carrying a degenerate GGDEF domain and an intact I-site and could thus potentially act as c-di-GMP binding receptor (Paper 1). In Paper 1 we also showed that overexpression of CdgC was toxic to the *B. thuringiensis* 407 cells, which were severely impaired in growth until they had accumulated inactivating transposon insertion mutations in the *cdgC* gene copy located on the overexpression plasmid. This effect was dependent on an active I-site, as a point mutation changing the RxxD motif to AxxA abolished the toxic effect (Paper 1). If indeed CdgC binds c-di-GMP, one explanation for this could potentially be that excess cellular CdgC sequesters c-di-GMP away from important functions.

4.3 CdgL is required for flagellar expression in *B. thuringiensis* 407

Based on the presence of a degenerate GGDEF domain, CdgL was originally predicted to be part of the c-di-GMP signaling network in *B. thuringiensis* 407. The CdgL protein has no obvious sensing domain such as PAS/PAC (Paper 3) but harbors a transmembrane domain which may serve as a sensor or in protein-protein interactions (Anantharaman and Aravind 2003; Galperin 2005).

Notably, deleting the *cdgL* gene left the cells completely non-motile and devoid of flagella, a phenotype that was rescued by overexpressing the *cdgL* gene *in trans*. Further analyses by RT-qPCR in Paper 3 showed that the expression of the entire motility locus was downregulated by around ten-fold upon

deletion of *cdgL*. The effect of CdgL on flagellar genes is in line with the expression *cdgL* being downregulated in biofilm relative to planktonic growth (Candela et al. 2018) and our own observation that expression of *cdgL* is at its highest point after 2,5 hours of planktonic growth where motility was found to be at its peak (Paper 2). Analysis by microscale thermophoresis however revealed that protein did not bind c-di-GMP in the experimental conditions tested in Paper 3, and has also been shown to lack DGC activity *in vitro* (Fu et al. 2018). A degenerate GGDEF domain without an intact I-site may nevertheless have other effects than c-di-GMP binding. In *B. subtilis*, the degenerate GGDEF domain of YybT was revealed to possess an unprecedented ATPase activity rather than DGC activity (Rao et al. 2010), and a GGDEF domain synthesizing a hybrid cAMP-GMP has also been discovered (Hallberg et al. 2016; Romling, Liang, and Dow 2017), so one cannot exclude the possibility that this domain has a similar or novel related function in *B. thuringiensis* 407.

It is noteworthy that *cdgL* resides in a three gene operon (Kristoffersen et al. 2012) which is conserved throughout the *B. cereus* group with the exception of non-motile *B. pseudomycoides*. The two other genes in the operon encode a putative NupC family transporter (BTB_RS26685) and a glycosyl transferase (BTB_RS26695). Glycosyl transferases catalyze the transfer of activated sugars from a donor substrate to an acceptor substrate. Donors are typically in the form of nucleotide activated sugars (Sheikh et al. 2017). Glycosyl transferases have been shown to affect biofilm formation through synthesis of EPS (Rainey et al. 2019; Pang et al. 2018). However, they may also have functional effects on motility, and glycosylation of flagellin units by glycosyl transferases has been described in a diverse number of bacterial species, in particular Gram-negative strains. In general, glycosylation is required for proper assembly of the flagellar apparatus (Logan 2006; Merino and Tomas 2014; Sulzenbacher et al. 2018), although in some bacteria such as *Pseudomonas* spp., a defect in glycosylation of flagellin units did not affect the flagellar apparatus, but the cells were impaired in host pathogenicity (Taguchi et al. 2010).

Glycosylation of flagellin units has also been found to be important for motility in some Gram-positive species. In *Clostridium difficile*, deletion of a conserved glycosyltransferase gene (CD0240) resulted in an inability to assemble functional flagella on the cell surface (Twine et al. 2009). In *Burkholderia pseudomallei* and *Burkholderia thailandensis*, deletion of a glycosyltransferase resulted in non-glycosylated flagellin units and loss of motility. In this case the flagella were exported and assembled on the cell surface, but were immotile due to defects in function of the flagellar filaments (Scott et al. 2011). Conversely, a glycosyltransferase mutant in *Paenibacillus alvei* CCM 2051 exhibited a decrease in the amount of flagellin produced and a lack of flagella assembled on the cell surface (Janesch et al. 2016). In *L. monocytogenes*, the glycosyl transferase GmaR is required for glycosylation of flagellin, but the effect it has on motility is due to also being a temperature dependent antirepressor for the transcriptional regulator MogR (Schirm et al. 2004; Shen et al. 2006). The glycosyl transferase genes known to effect motility in Gram-positive bacteria have in the cases mentioned above been located in close proximity to the genes encoding flagellin proteins. However, no glycosyl transferases are annotated near the motility locus of *B. thuringiensis* 407 (BTB_RS08240 - BTB_RS08460). In fact the whole *cdgL* containing operon is located completely separate from the motility genes on the chromosome (BTB_RS26685 - BTB_RS26695).

It is tempting to theorize that the three genes in the *cdgL* operon have connected functions, especially since all three proteins are membrane bound. One could speculate that if CdgL affects the activity of the

glycosyl transferase, a deletion of *cdgL* could potentially lead to failure of glycosylation of flagellin units which in turn might influence the flagellin synthesis and assembly of the flagellar apparatus. Such an indirect effect correlates with the observation that the genes in the motility locus are only downregulated around ten-fold (paper 3). The NupC transporter, which is known to transport purine and pyrimidine nucleosides (Patching et al. 2005), could potentially constitute the importer of the precursors of the putative nucleotide binding to the nucleotide cyclase superfamily domain of CdgL.

4.4 Loss of flagella causes a delay in biofilm formation in *B. thuringiensis* 407

B. cereus forms biofilms at the air-liquid interface, but can also form submerged biofilms (Wijman et al. 2007). While a non-flagellated mutant of *B. cereus* has been claimed to be defective in biofilm formation at the air-liquid interphase, this was not the case in a submerged or flow cell biofilm assay (Hayrapetyan et al. 2015; Houry et al. 2010). In this assay a biofilm comparable to the biofilm produced by the wild type strain was formed, indicating that flagella might play a part in the formation of biofilms where movement to a surface is required, but are not imperative in a non-static assay (Hayrapetyan et al. 2015; Houry et al. 2010).

In the current work we tested biofilm formation in the *cdgL* deletion mutant in a standard crystal violet microtiter plate assay alongside the non-flagellated Δ*flaAB* strain as a control. Compared to the wild type strain, a lack of biofilm formation by Δ*cdgL* and Δ*flaAB* at 24 hours could be seen with the naked eye. At later time points however, the biofilm made by both non-flagellated strains was comparable to that of the wild type (Paper 3) (Figure 12). In contrast, the *cdgF* deletion mutant which is a strain expected to produce reduced levels of c-di-GMP and shown to have increased motility compared to the wild type strain (Paper 1), remained defective in biofilm formation also after 48 and 72 hours (Paper 1) (Figure 12). The same delay in biofilm formation observed for the *B. thuringiensis* Δ*cdgL* and Δ*flaAB* strains has also been found in flagellar mutants of *B. subtilis* (Kobayashi 2007a). While the *B. subtilis* mutants had not formed a pellicle by 24 hours, after 48 hours pellicle formation was comparable to the wild type. The flagella may aid the bacteria in reaching the air-liquid interphase, and thus form a biofilm more rapidly than a non-flagellated mutant, but once there, the non-motile bacteria clearly have no trouble forming a biofilm. This corresponds with the findings that expression of flagellar genes are normally downregulated during the transition to biofilm formation (Guttenplan and Kearns 2013). Moreover, biofilms were shown to form faster and in greater quantities in a flow cell assay by non-motile mutants of *L. monocytogenes* (Todhanakasem and Young 2008). It has been speculated that presence of flagella may interfere with cellular attachment, resulting in the non-motile strains surpassing the wild type in biofilm mass (Lemon, Higgins, and Kolter 2007).

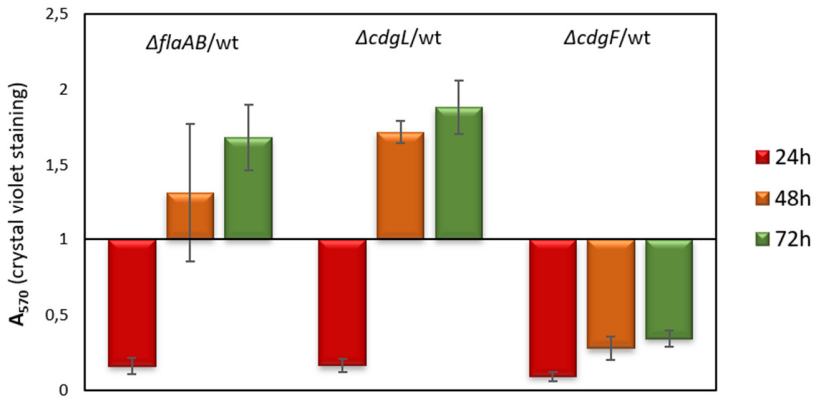

Figure 12: Biofilm formation in microtiter plates after 24, 48 and 72 hours relative to the *B. thuringiensis* 407 wild type, in *ΔflaAB*, *ΔcdgL* and *ΔcdgF* strains. The mean and standard error of the mean for three independent experiments is shown.

4.5 MogR is a repressor of motility genes in *B. thuringiensis* 407

In Gram-positive bacteria there are several patterns of regulation of motility (see also section 1.3.2). For most Gram-positive bacteria, except those in the *B. cereus* group and *Listeria* ssp., flagellar genes are regulated in a hierarchical manner (Smith and Hoover 2009). Moreover, c-di-GMP is a common regulator of motility and biofilm (Purcell and Tamayo 2016), which was shown in Paper 1 to also be the case in *B. thuringiensis* 407. Lastly, motility can be regulated by transcriptional regulators, such as MogR in *L. monocytogenes* (Grundling et al. 2004; Shen and Higgins 2006).

In Paper 2, we establish MogR to be a DNA binding repressor of motility genes in *B. thuringiensis* 407 in the same manner as in *L. monocytogenes*. A *B. thuringiensis* 407 strain overexpressing MogR was found to be completely non-motile. Further analysis with RT-qPCR and microarray experiments revealed that the expression of flagellar- and motility genes were significantly downregulated. Three putative DNA binding sites for MogR as predicted from the *L. monocytogenes* consensus binding sequence (Shen, Higgins, and Panne 2009) were identified in the intergenic region upstream of the flagellin (*flaAB*) operon of *B. thuringiensis* 407. Electrophoretic mobility shift assays (EMSAs) performed with purified MogR protein and purified DNA fragments constituting the *flaAB* promoter region containing these binding sites, supported the binding of the *B. thuringiensis* 407 MogR protein to the DNA fragments. A control strain overexpressing a MogR variant with two mutations in the predicted DNA-binding domain (Shen, Higgins, and Panne 2009) (MogR$^{QN\rightarrow AA}$) did no longer bind to the DNA (Paper 2), establishing MogR as a DNA binding repressor of motility genes in *B. thuringiensis* 407, like in *L. monocytogenes*.

4.5.1 Microarray analysis and EMSA experiments reveal genes affected by MogR

Due to numerous failed attempts to construct a *mogR* deletion mutant, the microarray experiment in Paper 2 was performed with the strain overexpressing MogR relative to the corresponding empty vector control. The aim was to investigate whether MogR influenced motility at the gene expression level and if other genes than those governing motility were affected. Interestingly, as many as 110 genes (Paper 2) were either down- or upregulated upon overexpression of MogR – either directly or indirectly, many of which were genes involved in phenotypes such as virulence, biofilm formation and stress response.

Expression of genes may depend on growth conditions and sampling times of the bacterial cultures. The samples compared in the microarray experiment were harvested in the mid-exponential phase (3 hours), at the point where motility and flagellin expression had been shown to be at its highest point in the wild type strain (Paper 2). Both flagellin genes, *flaA* and *flaB*, were strongly downregulated in the MogR overexpression strain, along with several other motility genes. Unfortunately, this was also the time point at which the expression of *mogR* itself was around its peak. The difference in the level of expression of *mogR* between the overexpression strain and the empty vector strain in this study may therefore not have been as substantial as if the samples had been taken at a at a different time point. In addition, many of the genes which are potentially regulated by MogR based on phenotypical analyses are not expressed until later in the growth cycle. Although a microarray experiment is very useful to compare differences in gene expression between two strains, the true regulon of a transcriptional regulator is more complicated to consolidate. The analysis is conservative and may likely not find all candidates, especially not genes that are weakly expressed, nor does it take into account if genes are directly or indirectly regulated. Moreover, due to the employment of an overexpression strain rather than a deletion mutant, false negative results may arise from genes already repressed by MogR at the timepoint and conditions at which the samples were taken. Overexpression of MogR may also have caused the protein to be induced to higher expression levels than would occur naturally, also due to the number of plasmid copies per cell.

In *L. monocytogenes*, MogR appears to bind as a dimer in a very specifical manner to the TTTWWNWWAAAA consensus sequences in the upstream promoter area of target genes, allowing for very little mismatch (Shen, Higgins, and Panne 2009). In addition, the binding sequences adhere to the rule of being positioned one to three helical turns apart along the double-stranded DNA, covering the -10 and/or -35 areas of promoters, thus allowing MogR to form a tetramer and thereby act as a steric repressor of the promoter (Shen and Higgins 2006; Shen, Higgins, and Panne 2009). Binding of *B. thuringiensis* MogR to *B. thuringiensis* 407 DNA was in Paper 2 shown to be dependent on the same amino acids in the active site of the protein, as those established for *L. monocytogenes* MogR. The consensus sequence and conditions to which MogR binds in *B. thuringiensis* 407 however appears to deviate from those in *L. monocytogenes*. Among the genes where expression levels have been shown to be affected and binding to the promoter areas have been determined by EMSA analysis (Paper 2), only the promoter area of the *flaAB* operon seems to meet the *L. monocytogenes* criteria of consensus sequences and helical turns, not accounting for that the transcriptional start site in *B. thuringiensis* 407 remains unknown. In the case of *hbl*, the putative MogR binding sequences in the promoter area fall outside the classical criteria, but nevertheless appear to bind MogR *in vitro* (Paper 2). This divergence

from the criteria set for *L. monocytogenes* regarding the level of mismatch from the consensus sequence and placement of the sequences is seen in most of the genes predicted to be regulated by *B. thuringiensis* MogR and where putative binding sequences have been found in the promoter area, indicating that MogR may have a different binding specificity in *B. thuringiensis* 407. Moreover, for many *B. thuringiensis* genes that appear to be regulated by MogR as shown in the microarray experiment or indicated by phenotypical assays, no potential binding sequences could be found, suggesting that they may be indirectly regulated.

4.6 MogR positively affects biofilm formation in *B. thuringiensis* 407 independent of flagellar motility

As opposed to a non-motile strain where only the expression of motility genes is affected, overexpression of MogR appears to affect many different genes with differing functions (Paper 2). Whereas an observable delay in biofilm formation was seen after 24 hours in the *cdgL* and *flaAB* deletion mutants relative to wild type (Paper 3), this was not as apparent in the MogR overexpression strain compared to the empty vector control (Paper 2). There might be a bias in that both the MogR overexpression strain and the empty vector control strain carry a xylose inducible plasmid vector which makes the direct comparison to the wild type difficult. The results from the microtiter plate assays nevertheless indicated that the MogR overexpression strain produced less biofilm at 24 hours than the empty vector control strain and the MogR$^{QN^{-}AA}$ overexpression strain, although the reduction was less severe than that observed for the *ΔcdgL* and Δ*flaAB* deletion strains. After 48 and 72 hours, the amount of biofilm formed by the MogR overexpression strain was comparable to that of the empty vector control strain and the MogR $^{QN^{-}AA}$ overproducing strain (Paper 2; Figure 13A). We wanted to investigate if the effect of MogR on biofilm formation was indeed caused by the overexpression of MogR, or simply an effect of the subsequent lack of flagella in this strain compared to the control strains. The effect on biofilm formation from MogR overexpression was therefore investigated in a *ΔflaAB* background, and a clear effect of MogR on biofilm formation could be observed, independent of effects caused by the lack of flagella (Paper 2). In a *ΔflaAB* background, MogR overexpression resulted in production of significantly ($p<0.05$) more biofilm after 48 and 72 hours compared with in the empty vector control. After 48 hours of growth, a more than five-fold increase in biofilm formation was seen compared to the *ΔflaAB* -empty vector strain (Figure 13B). In the Δ*flaAB* -MogR$^{QN\rightarrow AA}$ control, no detectable difference in biofilm formation compared to the empty vector strain was observed (Paper 2; Figure 13B). It seems apparent that the overexpression of MogR, independent of flagellar function, clearly confers an increase in biofilm formation. The increase in biofilm formation observed in the *B. thuringiensis* 407 *ΔflaAB* MogR overexpression strain could be explained by a regulatory effect on biofilm-associated genes by MogR, directly or indirectly.

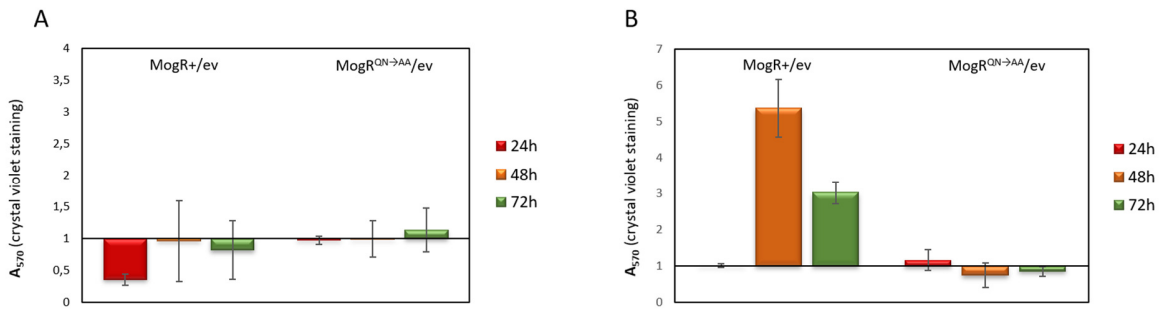

Figure 13: Biofilm formation in a microtiter plate assay, after 24, 48 and 72 hours growth, comparing MogR and MogR$^{QN\rightarrow AA}$ overexpression strains relative to the empty vector strain in (A) wild type and (B) *ΔflaAB* genetic backgrounds. The mean and standard error of the mean for three independent experiments is shown. The data are the same as provided in paper 2, however presented as relative values.

Microarray experimental results (Paper 2) showed upregulation of the gene coding for the SinR antagonist SinI following MogR overexpression. SinR has been shown to repress biofilm formation both in *B. subtilis* and in *B. thuringiensis* 407, while the antirepressor SinI had the opposite effect (Fagerlund et al. 2014). Although no changes in expression of the genes encoding matrix proteins were detected in the microarray experiment, upregulation of *sinI* would be expected to lead to increased biofilm formation through de-repression of the *sipW-tasA-calY* operon (Fagerlund et al. 2014; Pflughoeft, Sumby, and Koehler 2011).

Overexpression of MogR also lead to detectable cellular levels of c-di-GMP, as opposed to levels below the limit of detection in the wild type strain and empty vector control (Paper 2), and thus putatively affects expression and/or activity of c-di-GMP turnover genes, directly or indirectly. This corresponds well with the results from the microarray experiment in Paper 2, where the gene encoding the collagen adhesion protein CbpA carrying an upstream c-di-GMP responsive “on” riboswitch (Finke et al. 2019) was upregulated, and the gene encoding a protein with a c-di-GMP responsive “off” riboswitch (McpA) was downregulated upon MogR overexpression. Also, high levels of c-di-GMP have been shown to increase biofilm formation in *B. thuringiensis* 407 (Paper 1) as in a range of different bacteria (Jenal, Reinders, and Lori 2017; Opoku-Temeng and Sintim 2017; Tolker-Nielsen 2015). No genes related to c-di-GMP turnover were detected in this microarray experiment, but previous studies have shown that the activity of DGCs and PDE are most often regulated post-transcriptionally in response to sensory input (Jenal and Malone 2006). This is also in line with our own observations from Paper 1 where the expression of the c-di-GMP turnover genes appeared to not be affected by growth phases.

Biofilm formation is a complex life form, and it is not surprising that in addition to the main regulators, many other genes may also affect this process. In *B. cereus*, it appears that genes involved in purine synthesis are upregulated during biofilm formation (Okshevsky et al. 2017; Yan et al. 2017) which might be linked to increased production of c-di-GMP. It is however difficult to consolidate a “biofilm regulon”,

in that there is little overlap between genes in the studies that have been performed, and that the composition of a *B. cereus* biofilm will vary depending on individual species (and possibly strains) and with the growth environment (Majed et al. 2016). Genes affecting biofilm formation include those involved in amino acid and carbohydrate metabolism, replication and modification of RNA and DNA, sporulation and fermentation, and transporters of various kinds are also upregulated in biofilm formation (Okshevsky et al. 2017; Yan et al. 2017). Examples of all these types of genes are also present in the list of differentially expressed genes upon overexpression of MogR (Paper 2), with the exception of sporulation genes, but no overlap with specific genes affecting biofilm formation in other studies was found. Interestingly, *mogR* itself was found upregulated in biofilm formation in *Bacillus cereus* ATCC 10987 (Yan et al. 2017).

Due to sporulation being an integral part of the *Bacillus* biofilm lifestyle (Huang, Flint, and Palmer 2020; Lindsay, Brözel, and Von Holy 2006), we wanted to investigate whether MogR could affect sporulation in *B. cereus*. Sporulation efficacy was measured in the MogR overexpression strain relative to the empty vector strain, employing the same assay used for the *cdg* genes in Paper 1 (V. Smith, unpublished results). Both results from this assay and visual observation in the microscope indicated that sporulation was reduced after 24 hours in the MogR overexpression strain, although not with statistical significance (p-value= 0.13) (Figure 14). Further observation showed that after 36 hours the number of spores in the two strains were similar, pointing to a delayed sporulation instead of a defect. A delay in sporulation could be caused by MogR affecting genes involved in the sporulation process.

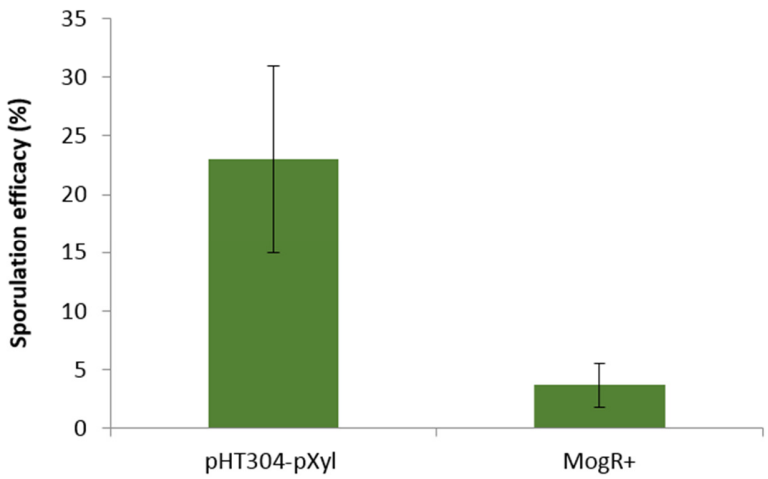

Figure 14: Sporulation efficacy in empty vector strain and MogR overexpression strain after 24 hours. Results from five individual experiments are shown, error bars denote the standard error of the mean.

The samples for the microarray experiment (Paper 2) were taken at a time point too early in the growth phase for sporulation genes to be expressed (Bergman et al. 2006). Not surprisingly, no known sporulation genes were affected in the microarray except for the gene encoding the transition state

regulator NprR, which has been linked to sporulation through the phosphorylation of Spo0A (Bongiorni et al. 2006; Verplaetse et al. 2015). Additionally, *flhA*, a gene encoding a protein of the flagellar type III export apparatus which has been shown to lead to loss of sporulation when mutated (Bouillaut et al. 2005) was downregulated along with rest of the genes in the motility locus.

4.7 MogR attenuates virulence in *B. thuringiensis* 407

Several regulators affect the expression of virulence factors in *B. cereus*. The genes encoding the enterotoxins Hbl, Nhe and CytK are mainly activated by PlcR (Gohar et al. 2008), but the *hbl* and *nhe* genes are also under activation by the redox regulators ResDE and Fnr (Zigha et al. 2007; Esbelin et al. 2009; Esbelin et al. 2008). Furthermore, *hbl* and *nhe* are repressed by the catabolite control protein CcpA (van der Voort et al. 2008) along with the transcriptional regulator CodY (Bohm et al. 2016). In addition, *hbl* is independently repressed by SinR (Bohm et al. 2016; Fagerlund et al. 2014). See Figure 15 for details. The microarray experiment in Paper 2 showed that six genes encoding virulence factors that are also included in the PlcR regulon were downregulated by MogR, either directly or indirectly (Serine protease, Phospholipase C, Sphingomyelase, InhA and Hbl components B and L1). The fact that expression of *plcR* itself was not altered and that only six out of the 45 genes comprising the PlcR regulon were affected makes it unlikely that MogR influences these genes through PlcR.

Considering that several studies have shown a relationship between the loss of flagella and reduced virulence in *B. cereus* (Ghelardi, Celandroni, Salvetti, Ceragioli, et al. 2007; Mazzantini et al. 2016; Salvetti et al. 2011; Zhang et al. 1993), we wanted to make sure the attenuation of virulence by overexpression of MogR was not simply due to the absence of flagella. Experiments were therefore additionally performed with the overexpression of MogR and control vector clones in a Δ*flaAB* background, where all strains involved were non-flagellated (Paper 2). The Δ*flaAB* strain overexpressing MogR exhibited attenuated virulence both in an oral infection model using *Galleria mellonella* larvae and in a Vero cell cytotoxicity assay, and Western blots revealed a reduction in production of the enterotoxins Hbl, Nhe and CytK compared to the control strains, indicating that this effect is independent of the presence of flagella. This coincides with earlier findings that the main enterotoxins are secreted though the Sec translocation pathway and that any reduction in virulence upon loss of flagella is thus due to a regulatory connection between motility and virulence (Fagerlund, Lindback, and Granum 2010).

MogR downregulates the gene encoding the flagellar export apparatus protein FlhA by almost three-fold (Paper 2). Mutation of this gene in *B. cereus* resulted in non-flagellated cells due to flagellar proteins not being secreted (Bouillaut et al. 2005). Moreover, the mutant was shown to have reduced production of virulence factors such as Hbl, Nhe and CytK (Bouillaut et al. 2005; Fagerlund, Lindback, and Granum 2010; Ghelardi et al. 2002) independently of PlcR, the expression of which was not affected (Bouillaut et al. 2005). Cytotoxicity and larvae mortality were also attenuated in a Δ*flhA* mutant (Bouillaut et al. 2005). Reduction of virulence upon deletion of *flhA* has also been reported in other bacterial species

(Carrillo et al. 2004; Fleiszig et al. 2001). Although *hbl* was indicated in Paper 2 to be possibly directly repressed by MogR, the possibility that the effect by MogR on other virulence factors is due to downregulation of *flhA* or other motility genes should be considered. Repetition of the virulence assays with MogR overexpressed in a Δ*flhA* background could be a possibility, although it would be difficult to discern if any attenuation of virulence was due to overexpression of MogR independently, the absence of *flhA*, or by downregulation of *flhA* by MogR.

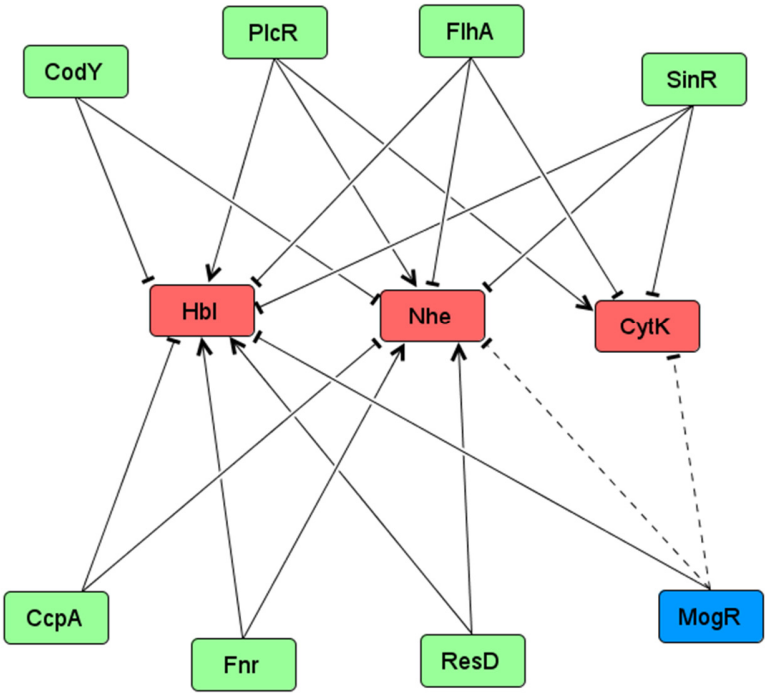

Figure 15: Co-regulation of virulence factors in *B. cereus* and *B. thuringiensis*. The figure is based on experiments performed as part of this thesis (Paper 2) and on results from: (Bohm et al. 2016; Esbelin et al. 2009; Esbelin et al. 2008; Fagerlund et al. 2014; van der Voort et al. 2008; Zigha et al. 2007; Bouillaut et al. 2005; Fagerlund, Lindback, and Granum 2010; Gohar et al. 2008). Arrows (→) indicate positive influence, ended lines (⊢) indicate negative influence. Solid lines indicate where direct regulation has been shown while dotted lines show indirect regulation.

4.8 The role of MogR in the *B. cereus* group

B. anthracis has been shown to be non-motile due to nonsense mutations throughout the genes in the motility locus rendering them unfunctional (Read et al. 2003). We were therefore intrigued to see that MogR is conserved in *B. anthracis*, with a pairwise identity of ~ 85% to the orthologue in *B. thuringiensis* 407, and expressed in the late exponential phase in *B. anthracis* (Bergman et al. 2006), in contrast to the

motility genes . Of the two other non-motile strains in the *B. cereus* group*, B. pseudomycoides* har the most severely reduced motility locus, retaining only the *mogR* gene (~ 55% pairwise identity in the corresponding protein, to *B. thuringiensis* 407 MogR). The motility locus of *B. mycoides* has retained around half of the motility genes that are present in motile *B. cereus* strains, frequently corresponding to the genes directly upstream and downstream of *mogR*. The MogR protein in *B. mycoides* shows a ~ 83% pairwise identity to the corresponding orthologue in *B. thuringiensis* 407.

It is apparent that the role of MogR in *B. cereus* is different than in *L. monocytogenes*. Apart from the differences in binding affinity and genes regulated is the fact that expression of *mogR* is growth related in *B. cereus* and not temperature regulated like in *L. monocytogenes* (paper 2). The temperature regulation of MogR in *L. monocytogenes* is dependent of the GmaR antirepressor (Shen et al. 2006) and the transcriptional regulator DegU (Shen and Higgins 2006). No orthologues to DegU have been found in *B. cereus* group bacteria, and an orthologue of GmaR is found only in the thermotolerant *B. cytotoxicus,* with the exception of *B. cereus* VD184, an environmental soil isolate from the United Arab Emirates (Dubai) (Hoton et al. 2009). Moreover, the conservation of *mogR* in spite of the loss of all other motility genes in the strains of group I, may be indicative of the importance of this protein in *B. cereus* group bacteria, and thus potentially related to our inability to create *mogR* deletion mutants, which were successfully created in *L. monocytogenes*. At the current stage however, MogR appears to be an intriguing pleiotropic protein which should be subject to further studies.

4.9 Motility, biofilm formation and virulence are co-regulated in B. cereus and *B. thuringiensis*

Regulation of biofilm formation, sporulation, virulence and motility are often interconnected (Abee et al. 2011; Gueriri et al. 2008). At the start of this project, only a few regulators of these phenotypes were known in *B. cereus*. The regulation of biofilm formation in *B. subtilis* has been described in detail, and several of these regulators and proteins are also conserved in *B. cereus*, but not all. Figure 16 depicts the current knowledge of regulation of biofilm formation in *B. subtilis* and *B. cereus*.

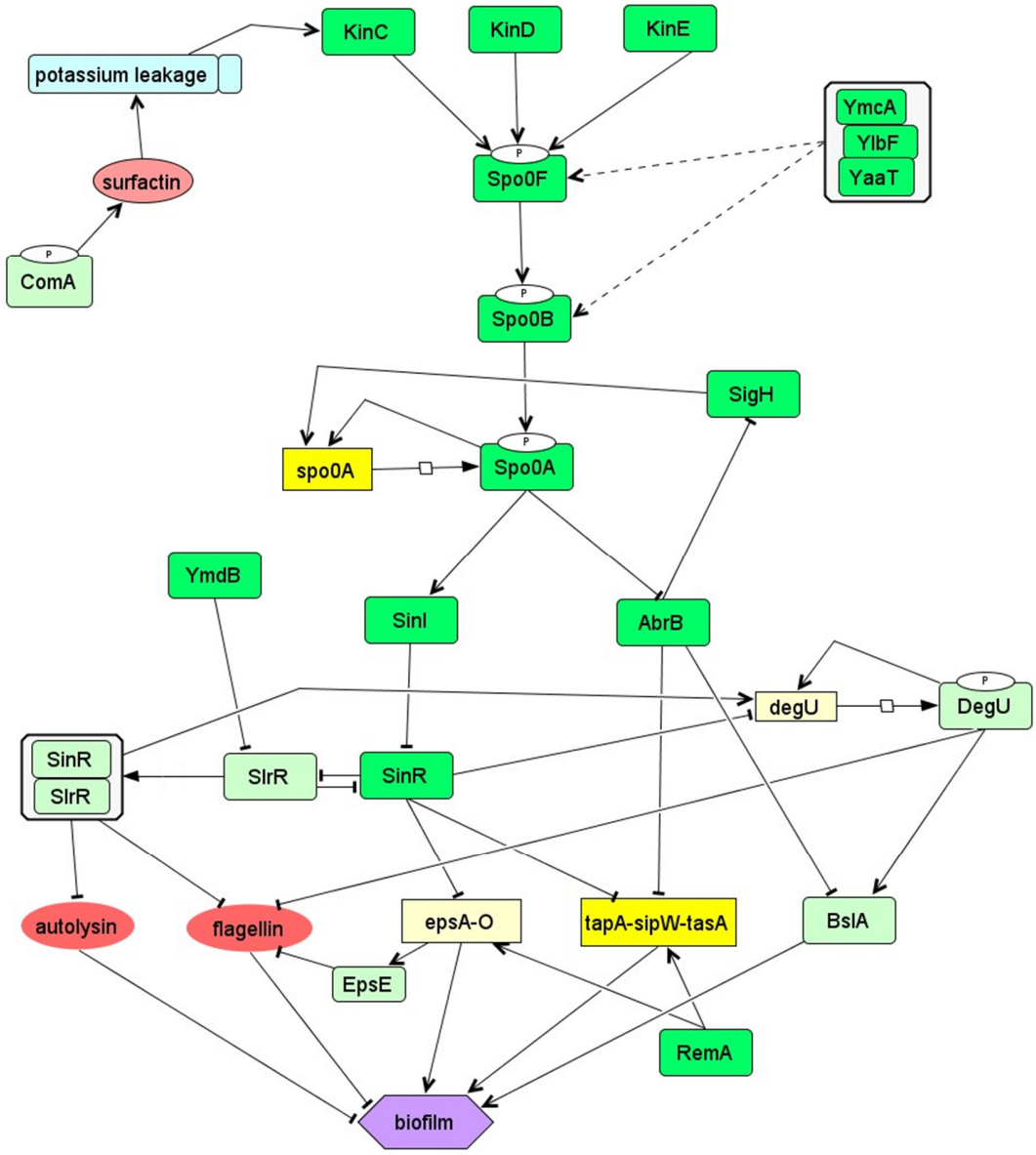

Figure 16: A flowchart depicting the regulation of biofilm in *B. subtilis* and *B. cereus/B. thuringiensis*. Boxes in dark colors (dark green and yellow) represent proteins and genes, respectively, present in both *B. cereus* and *B. subtilis (gene content and organization of the tapA operon is slightly different in these species)*. Boxes in light colors (pale green and yellow) represent proteins and genes, respectively, present in *B. subtilis* only. Figure based on results from (Branda et al. 2004; Carabetta et al. 2013; Chu et al. 2006; de Jong, Veening, and Kuipers 2010; Dubnau et al. 2016; Gaur, Oppenheim, and Smith 1991; Kearns et al. 2005; Blair et al. 2008; Chai, Kolter, and Losick 2010; Chai et al. 2010; Chai, Kolter, and Losick 2009; Diethmaier et al. 2011; Hamon et al. 2004; Lopez and Kolter 2010; Lopez, Vlamakis, and Kolter 2009; Ogura, Yoshikawa, and Chibazakura 2014; Verhamme, Murray, and Stanley-Wall 2009; Vlamakis et al. 2013; Winkelman et al. 2013). Arrows (→) indicate positive influence, ended lines (⊦) indicate negative influence. Solid lines indicate where direct regulation has been proven while dotted lines show indirect or unproven regulation. A solid arrow broken by a box indicates expression of a protein from an activated gene.

Flagellar motility and biofilm formation are reciprocally regulated in several species, as flagella have been shown to be downregulated in order to allow the bacteria to form multicellular aggregates (Guttenplan and Kearns 2013). Co-regulation of flagella and virulence factors is common in enteropathogenic bacteria, as virulence factors are normally produced after flagella-mediated attachment to intestinal cells (Raymond and Bonsall 2013). In *B. cereus*, impaired motility has been linked to reduced secretion of virulence factors and attenuation of virulence *in vivo* (Mazzantini et al. 2016; Zhang et al. 1993), and the gene encoding the enterotoxin Hbl is upregulated in hypermotile swarm cells (Ghelardi, Celandroni, Salvetti, Ceragioli, et al. 2007; Salvetti et al. 2011). Several regulators known to affect more than one of these phenotypes are mentioned below, and the regulatory network is summarized in Figure 17. Other factors affecting parts of this network exist, but are not further discussed, including CwpFM (Tran et al. 2010), σ^{54} (Hayrapetyan et al. 2015), and ComER (Yan et al. 2016).

In addition to the three genes in the matrix producing *sipW/tasA/calY* operon, twenty-nine genes are repressed by SinR in *B. cereus*, including the genes involved in the biosynthesis of kurstakin, a lipopeptide surfactant required for proper biofilm formation in *B. cereus* (Dubois et al. 2012; Fagerlund et al. 2014; Gelis-Jeanvoine et al. 2017). Motility assays have found a *sinR* deletion mutant to be defective in swimming motility and a *sinI* deletion mutant to be hypermotile, although the mechanism of regulation remains to be elucidated (Fagerlund et al. 2014), as *B. cereus* lacks the EpsE flagellar clutch, SlrR (and thus the SinR-SlrR complex) and DegU which connects SinI/SinR to regulation of motility in *B. subtilis* (Figure 16). Moreover, several virulence factors are also repressed by SinR, including the enterotoxin *hbl* genes and *inhA* (Fagerlund et al. 2014; Pflughoeft, Sumby, and Koehler 2011).

Downregulation of *codY* at high levels of c-di-GMP has previously been reported and linked to diminishing levels of GTP in *B. subtilis* (Handke, Shivers, and Sonenshein 2008). As mentioned (see section 1.2.6), *B. cereus* CodY has a positive effect on PlcR by activating the Opp proteins (Slamti et al. 2015), and independently represses several virulence genes including cereulide and the enterotoxins *hbl* and *nhe* (Bohm et al. 2016; Frenzel et al. 2012). Furthermore, CodY has a negative effect on biofilm formation, mainly by downregulating the transcription of the matrix producing *sipW/tasA/calY* operon (Lindback et al. 2012). Finally, CodY is further connected to the PlcR-loop by repressing the expression of *nprR* (Figure 17) (Dubois et al. 2013). For entomopathogenic strains the transition state regulator NprR is activated after the insect's death and induces transcription of digestive enzymes which allow the bacteria to use the contents of the cadaver as nutrients (necrotrophism) and survive or sporulate (Dubois et al. 2016).

Interestingly, the global virulence regulator PlcR also affects both motility and biofilm formation. Despite not having an upstream PlcR box, the precursors for the flagellar filament, flagellin, are downregulated in a Δ*plcR* mutant, the mechanism by which remains unknown (Gohar et al. 2002), and motility is therefore reduced (Callegan et al. 2003). Biofilm formation is also affected by PlcR, as a *plcR* deletion mutant formed considerably more biofilm than the wild type (Bouillaut et al. 2005; Hsueh et al. 2006). Biofilm formation in *B. cereus* also appears to be supported by an optimal range of bio-surfactant production. This surfactant is negatively regulated by PlcR, possibly indirectly (Hsueh et al. 2006). More recently, PlcR was discovered to activate the expression of *nprR* (Dubois et al. 2013) and thereby to

repress the phosphorylation of Spo0A by way of the NprR-NprX necrotrophism system and the Rap-Phr system which controls the phosphorylation cascade that leads to the activation of the sporulation regulator Spo0A, making this an interconnected loop fine-tuning the pathogen to the surroundings (Figure 17) (Bongiorni et al. 2006; Verplaetse et al. 2015).

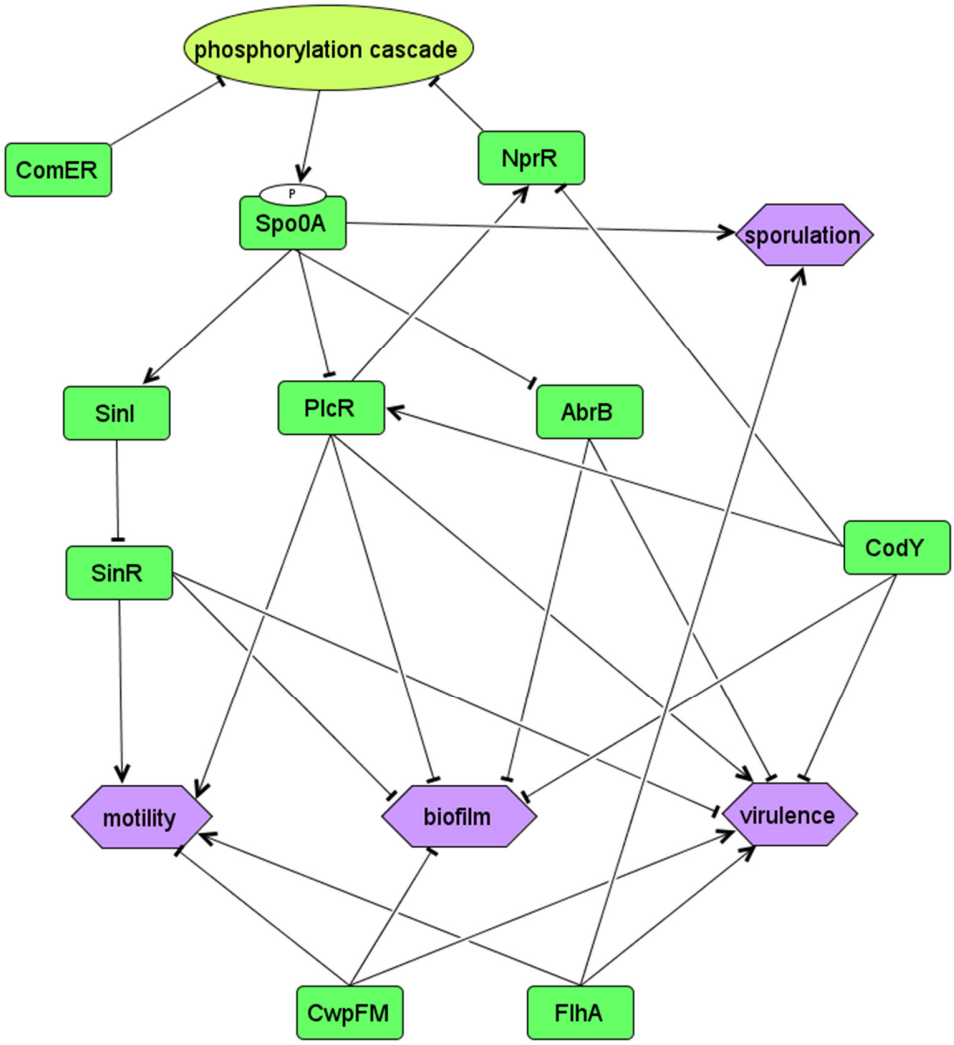

Figure 17: A flowchart illustrating the co-regulation of biofilm, motility, virulence and sporulation in *B. cereus* and *B. thuringiensis*. The figure is based on results from: (Agaisse et al. 1999; Bohm et al. 2016; Bongiorni et al. 2006; Bouillaut et al. 2005; Callegan et al. 2003; Dubois et al. 2013; Fagerlund et al. 2014; Fagerlund, Lindback, and Granum 2010; Frenzel et al. 2012; Fujita, Gonzalez-Pastor, and Losick 2005; Fujita and Losick 2005; Ghelardi et al. 2002; Gohar et al. 2002; Hsueh et al. 2006; Lindback et al. 2012; Lucking et al. 2009; Pflughoeft, Sumby, and Koehler 2011; Shafikhani et al. 2002; Slamti et al. 2015; Slamti and Lereclus 2002; Strauch et al. 1990; Tran et al. 2010; Verplaetse et al. 2015; Yan et al. 2016). Arrows (→) indicate positive influence, ended lines (⊢) indicate negative influence.

The work in this thesis has provided some clear expansions to the network connecting biofilm formation, motility and virulence in the *B. cereus* group, as well as provided results which require further work to provide conclusive evidence (Figure 18). Biofilm formation, motility and virulence were found to be influenced by the second messenger c-di-GMP in *B. thuringiensis* 407, and a functional investigation was performed to map the individual roles of proteins putatively involved in c-di-GMP synthesis and breakdown (Paper 1). Further studies will be needed to provide a complete view of the effector proteins which mediate downstream effects, but preliminary studies involving microarray experiments and RT-qPCR suggest that c-di-GMP may possibly affect the activity and/or synthesis of Spo0A, SinI, PlcR and CodY, which could in turn regulate the observed phenotypes (Figure 17; Figure 18). Moreover, the recent discovery of c-di-GMP binding to a PelD ortholog with a degenerate GGDEF domain and an active I-site and the subsequent effect on Pel polysaccharide synthesis and biofilm formation in *B. cereus* ATCC 10987 (Whitfield et al. 2020) as well as previously identified c-di-GMP riboswitches (Finke et al. 2019; Lee, Sudarsan, and Breaker 2010; Sudarsan et al. 2008; Tang et al. 2016), altogether indicates that c-di-GMP networks are indeed present in *B. cereus* group bacteria.

Additional studies will have to be undertaken in order to uncover the functional and mechanistic relationship between CdgL and flagellar motility, but as a preliminary starting point it is interesting to note that RT-qPCR analysis showed that *sinI* was transcriptionally upregulated three-fold in the Δ*cdgL* mutant (Paper 3). While the SinR regulator positively affects motility in *B. thuringiensis* 407, this effect is alleviated by SinI binding, and *sinI* transcription is in turn activated by Spo0A (Figures 16 and 17). A *spo0A* deletion mutant was however found to have no effect on motility compared to the wild type (Fagerlund et al. 2014), potentially indicating that other regulators may also affect motility either through *sinI* or via other means. In *B. subtilis,* transcription of *sinI* is regulated both by Spo0A~P and by the protease production regulatory protein Hpr (Shafikhani et al. 2002). Interestingly, an orthologue of *B. subtilis* Hpr appears to be conserved throughout the *B. cereus* group with identities ranging from 94% to >99%. The *B. thuringiensis* 407 Hpr protein shows a 64% pairwise identity to its *B. subtilis subsp. subtilis* str. 168 orthologue*,* by BlastP analysis.

The transcriptional regulator MogR was however found to repress the expression of motility genes and bind to the promoter region of motility genes *in vitro* (Paper 2), providing an explanation for the loss of flagella and motility upon overexpressing this protein. Direct binding to the promoter area of the genes encoding the enterotoxin Hbl was also shown *in vitro*, suggesting potential direct repression of these genes and perhaps other virulence genes. The substantial effect of MogR on biofilm formation could not be explained by the results of the microarray experiment, possibly due to the samples being taken in the early exponential phase. Nevertheless, *sinI* and *abrB* upstream regions matched the MogR binding criteria (based on the *L. monocytogenes* consensus) in *in silico* searches. *sinI* was however upregulated in the microarray experiment. MogR was also found to induce the synthesis of a small amount c-di-GMP, potentially providing a link to increased biofilm formation (Figure 18).

The expression of *mogR* was found to be turned on in late exponential phase / early stationary phase (Paper 2). In *L. monocytogenes*, MogR is regulated by two promoters, one putative P2 and one P1 SigB box (Toledo-Arana, A et al, 2009). The regulation of *mogR* in *B. cereus* has not been investigated, but interestingly a putative PlcR box is located in the intergenic region upstream of *mogR*. In a previous

study establishing the PlcR regulon, MogR (BC1655) was identified as a potential PlcR-regulated gene by *in silico* analyses, but in the microarray experiment analysing differential expression between a *plcR* deletion mutant and wild type, *mogR* did not meet the cut-off criteria established in the study (relative expression ratio greater than 2.5 and $p_{FDR\text{-}corr} < 0.2$) and was therefore not considered part of the PlcR regulon (Gohar et al. 2008). Interestingly, NprR was not registered as part of the PlcR regulon in that study either. Like *mogR*, the *nprR* upstream intergenic region carries a potential PlcR binding site, but did not come out as differentially expressed in the microarray experiment (Gohar et al. 2008). NprR was nevertheless shown to be directly regulated by PlcR in a later study (Dubois et al. 2013), showing that some PlcR-regulated genes were not identified in the microarray study alone. Furthermore, some additional genes detected as differentially expressed by the microarray analysis were further investigated with *lacZ* transcriptional fusions. The gene encoding the flagellar basal body rod protein FlgB was found not to be affected by PlcR deletion, even though the whole operon *flgB* was downregulated in a *ΔplcR* mutant in the microarray experiment (Gohar et al. 2008). Neither *nprR* nor *mogR* have been tested by *lacZ* transcriptional fusion analyses (Gohar et al. 2008). Also, an earlier study showed that the genes encoding flagellin are downregulated in a *ΔplcR* mutant despite not carrying an identifiable upstream PlcR box, in addition to motility being reduced in the mutant compared to wild type cells (Gohar et al. 2002), potentially indicating indirect regulation.

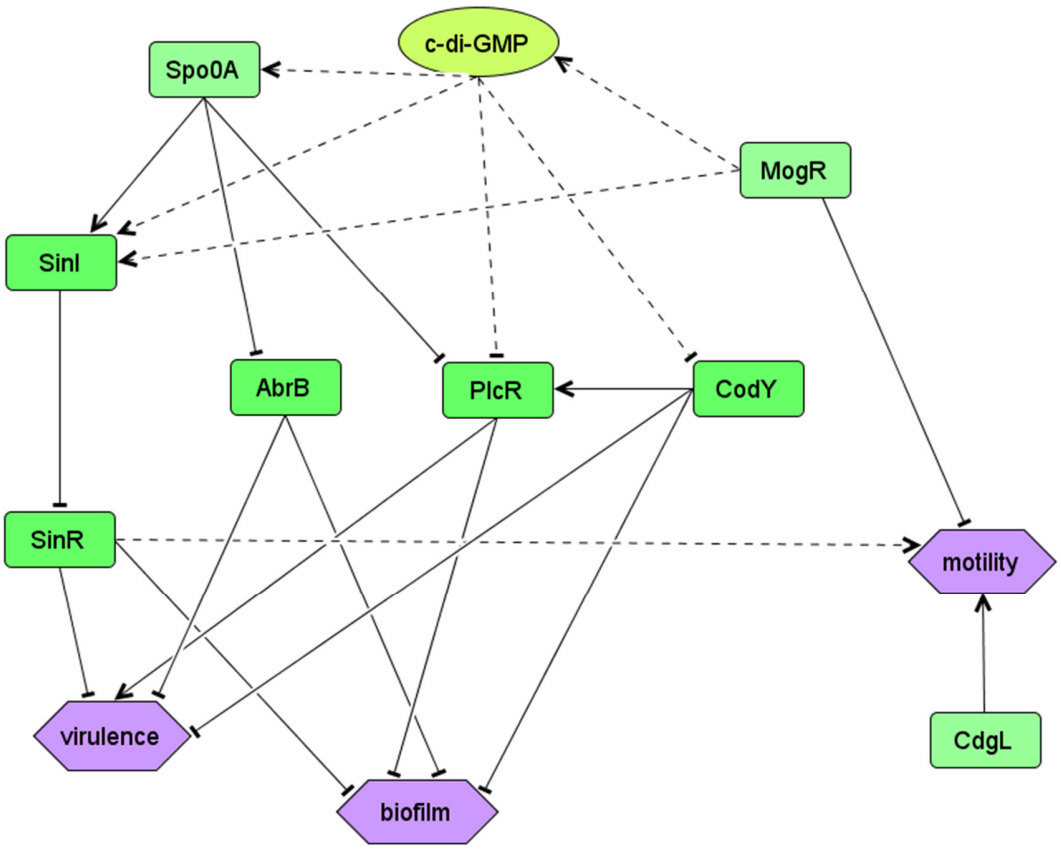

Figure 18: A flowchart illustrating the co-regulation of biofilm, motility and virulence in *Bacillus cereus*. Figure based on findings in Paper 1, Paper 2 and Paper 3 along with results from microarray experiment E-MTAB-8898 and additional experiments performed during the work described in this thesis in addition to results from: (Fagerlund et al. 2014; Hamon and Lazazzera 2001; Hsueh et al. 2006; Lereclus et al. 2000; Lindback et al. 2012; Pflughoeft, Sumby, and Koehler 2011; Shafikhani et al. 2002; Slamti et al. 2015; Strauch et al. 1990; Elbakush, Miller, and Gomelsky 2018; Lucking et al. 2009; Bohm et al. 2016; Frenzel et al. 2012). Arrows (→) indicate positive influence, ended lines (⊢) indicate negative influence. Solid lines indicate where direct regulation has been proven while dotted lines show indirect or putative regulation.

For many pathogenic bacteria, e.g. such as *P. aeruginosa*, *L. monocytogenes*, *E. coli* and *Helicobacter pylori*, motility, biofilm formation and virulence are found to be co-regulated (Chaban, Hughes, and Beeby 2015; Duan et al. 2013; Josenhans and Suerbaum 2002). This is beneficial in order to avoid proteins being expressed unnecessarily, such as e.g. toxins being released far from their target site. Flagellar motility is important both in the initial phases of biofilm formation, for cellular dispersal from a biofilm, and for the bacteria to reach target cells for colonization of host tissues (Chaban, Hughes, and Beeby 2015; Duan et al. 2013). As biofilm is a sessile lifestyle, the majority of bacteria in a biofilm are non-motile, and expression of flagella is commonly downregulated upon biofilm formation (Guttenplan and Kearns 2013). Biofilms are generally associated with chronic rather than acute infections (Rodrigues

et al. 2018), and toxin production is downregulated while the cells in the biofilm are more or less dormant (Koo et al. 2017). In a biofilm, the bacteria are protected from the immune system and antibiotics, and active infection can recur when cells dispatch from the biofilm by upregulation of motility and virulence factors (Chaban, Hughes, and Beeby 2015). Being opportunistic pathogens, e.g. of the human and/or insect gut, it is not surprising that *B. cereus* and *B. thuringiensis* also co-regulate these phenotypes. Although much has been gained during the past decade in obtaining an understanding of the mechanisms with which this co-regulation occurs, much however remains to be learned in order to obtain more complete molecular view of the coordinated regulation.

5 Conclusions and further perspectives

The main findings in this study are as follows:

- Bacteria in the *B. cereus* group encode ten conserved proteins containing domains related to c-di-GMP synthesis or breakdown. Among these, six proteins harbor both GGDEF and EAL domains (CdgD-I), three contain a GGDEF domain only (CdgA-C), and one contains a single EAL domain (CdgJ). CdgF was identified as a main DGC, producing increased cellular levels of c-di-GMP when overexpressed. In general, we found that increased c-di-GMP levels in *B. thuringiensis* 407 promotes biofilm formation and reduces motility, in addition to reducing cytotoxicity.

- Several candidate c-di-GMP effector molecules could be identified, including CdgL, which however carries a degenerate GGDEF domain that did not bind c-di-GMP. The deletion of *cdgL* nevertheless lead to downregulation of flagellar gene expression in *B. thuringiensis* 407, and non-motile deflagellated cells.

- MogR is a DNA binding regulator of motility genes in *B. thuringiensis* 407, functionally similar to *L. monocytogenes*, but is regulated in a growth-dependent manner rather than by temperature.

- In addition to motility, MogR positively affects biofilm formation and attenuates virulence when overexpressed in *B. thuringiensis* 407, either through direct regulation or by indirect mechanisms.

- The functional role of MogR in processes other than motility functions, is supported by the fact that *mogR* is expressed in non-motile *B. anthracis*, as well as by *mogR* being the only gene retained in the highly reduced motility locus in all sequenced strains of non-motile *B. pseudomycoides*.

- Finally, both MogR and c-di-GMP can be regarded as key regulators of motility, biofilm formation and virulence in *B. thuringiensis* 407. Further work is required to elucidate the precise downstream effectors and associated molecular mechanisms.

In order to close the gaps and provide further understanding of the coordinated regulation of motility, virulence and biofilm formation the following follow-up studies may be considered:

While preliminary investigations suggest that c-di-GMP affects biofilm formation, motility and virulence through the transcriptional regulators SinR (and its associated antirepressor SinI), Spo0A, CodY and PlcR, the c-di-GMP binding downstream effectors facilitating this functional link are yet to be elucidated. Considering the number of new studies identifying additional c-di-GMP binding domains, a new

screening of the currently available *B. cereus* group genomes and corresponding functional analyses could uncover new putative members of the *B. cereus* c-di-GMP signaling network.

The effect on motility by CdgL should be explored further. Binding to other cyclic nucleotides such as e.g. c-di-AMP should be investigated. Additional studies would also be needed in order to elucidate the relationship and possibly coordinated function(s) between CdgL and the two other genes in the three-gene operon. The genes encoding glycosyl transferases, which are known to affect glycosylation of flagellin in Gram-positive bacteria, are located in the vicinity of the motility locus (De Maayer and Cowan 2016; Janesch et al. 2016; Twine et al. 2009; Scott et al. 2011). This is however not the case for the *cdgL* operon in *B. thuringiensis* 407. It would be particularly interesting to uncover the physical location of CdgL in the bacterial cell, as well as of the putative glycosyl transference and the NupC membrane transporter encoded by the same operon. Using fluorescent protein fusions (Inaba et al. 2017) one could investigate whether the CdgL protein and/or the other proteins encoded in the *cdgL* operon are located in the vicinity of the flagellar apparatus, which could potentially give insight to the relationship between CdgL and the regulation of flagellar synthesis and function. Moreover, RNA sequencing could be used to investigate if other genes are differentially expressed upon deletion of *cdgL*.

In order to identify the *B. thuringiensis* MogR regulon, follow-up studies should be undertaken, e.g. through the use of chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) (Galagan, Lyubetskaya, and Gomes 2013; Myers et al. 2015) which would be an extremely valuable tool to reveal genes directly regulated by MogR *in vivo*. This could also provide answers to the question whether MogR potentially regulates virulence genes indirectly through the downregulation of motility locus genes such as *flhA* and *flhF*, or whether the regulation is through direct repression. The hypothesis that MogR might be under regulation by PlcR should also be considered and could be investigated by *lacZ* transcriptional fusions as described by Gohar and co-workers (Gohar et al. 2008).

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Scientific papers 1-3

Paper 1

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Cyclic diguanylate regulation of *Bacillus cereus* group biofilm formation

Annette Fagerlund,[1†] Veronika Smith,[1,2§] Åsmund K. Røhr,[3‡§] Toril Lindbäck,[4§] Marthe P. Parmer,[5] K. Kristoffer Andersson,[3] Leon Reubsaet[5] and Ole Andreas Økstad[1,2*]

[1]*Laboratory for Microbial Dynamics (LaMDa), Section for Pharmaceutical Biosciences, School of Pharmacy, University of Oslo, PB1068, Oslo 0316, Norway.*
[2]*Centre for Integrative Microbial Evolution (CIME), Faculty of Mathematics and Natural Sciences, University of Oslo, Oslo 0316, Norway.*
[3]*Department of Biosciences, University of Oslo, PB1066, Oslo 0316, Norway.*
[4]*Department of Food Safety and Infection Biology, Norwegian University of Life Sciences, PB8146 Dep, Oslo 0033, Norway.*
[5]*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.

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: †Nofima, PB210, 1431 Ås, Norway; ‡Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, PB5003, 1432 Ås, Norway. §These authors contributed equally to this work.

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 *Clostridum 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 –

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 YkuI, 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 *ykuI*, 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 pseudomycoides* 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. pseudomycoides* DSM 12442 (cluster I in Fig. 2) is

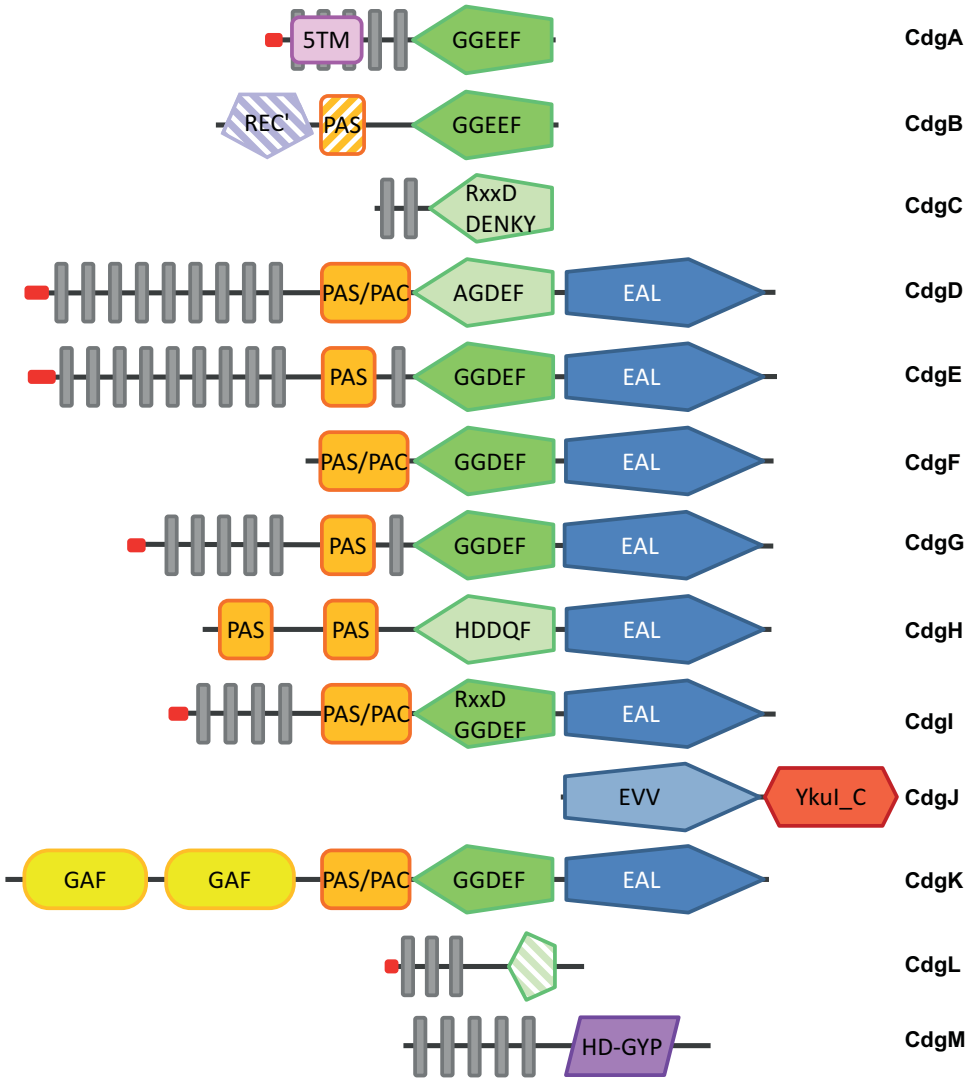

Fig. 1. Domain organization of predicted proteins from *B. cereus* group genomes containing putative c-di-GMP signalling domains. GGDEF domains (green), EAL domains (blue), PAS or PAS/PAC domains (orange), GAF domains (yellow), the YkuI_C domain (red) and the 5TM-5TMR-LYT domain (in pink, indicated by «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

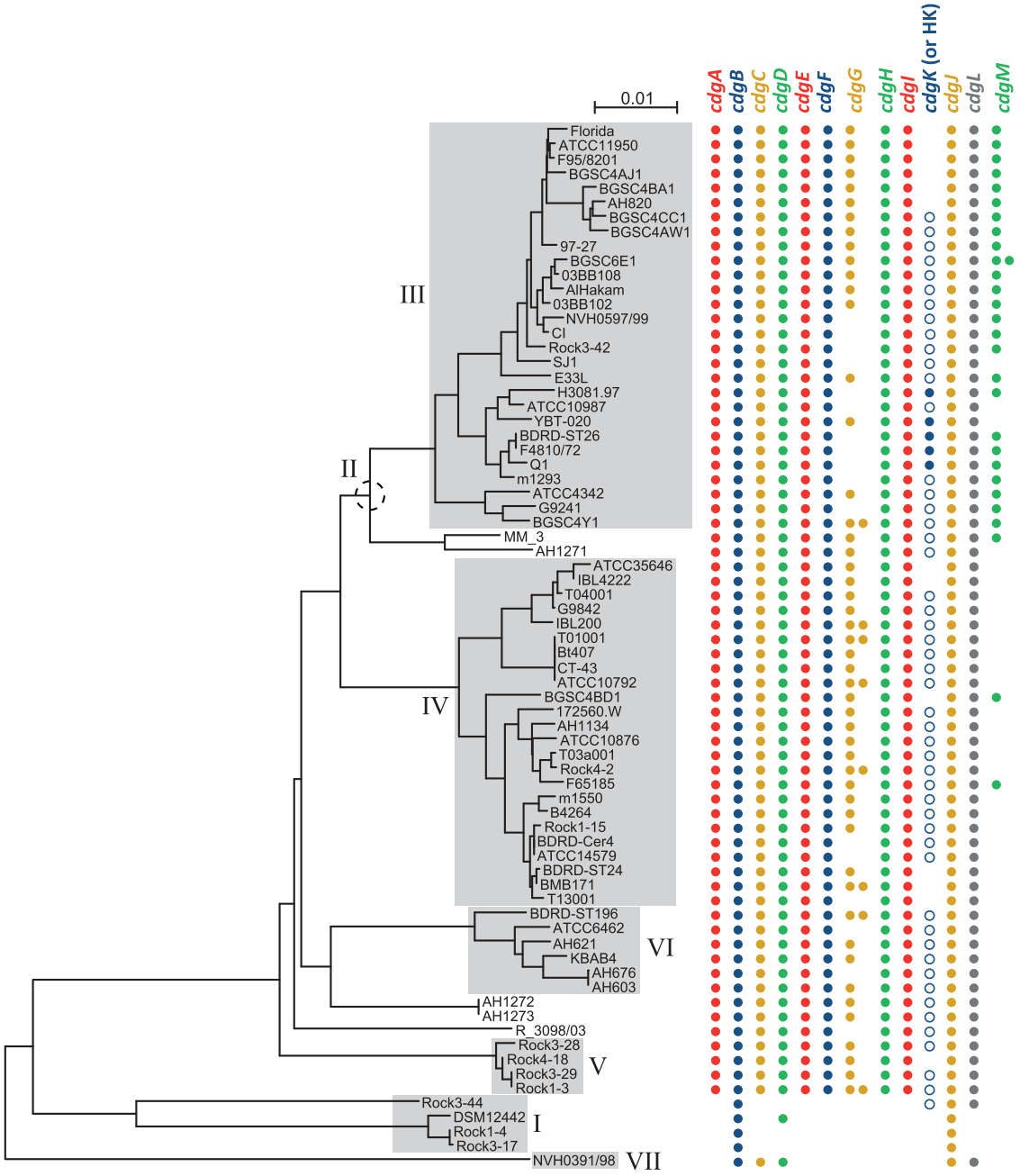

Fig. 2. Distribution of *cdg* genes within the *B. cereus* group population. A phylogenetic tree of sequenced strains representing the *B. cereus* group population was constructed as described (methods). The seven phylogenetic clusters within the *B. cereus* group, referred to as group I–VII, are marked. 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 YkuI_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* YkuI 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 YkuI_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 *chinensis* 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, *chinensis* 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. Noteably, *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-Pxyl 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.

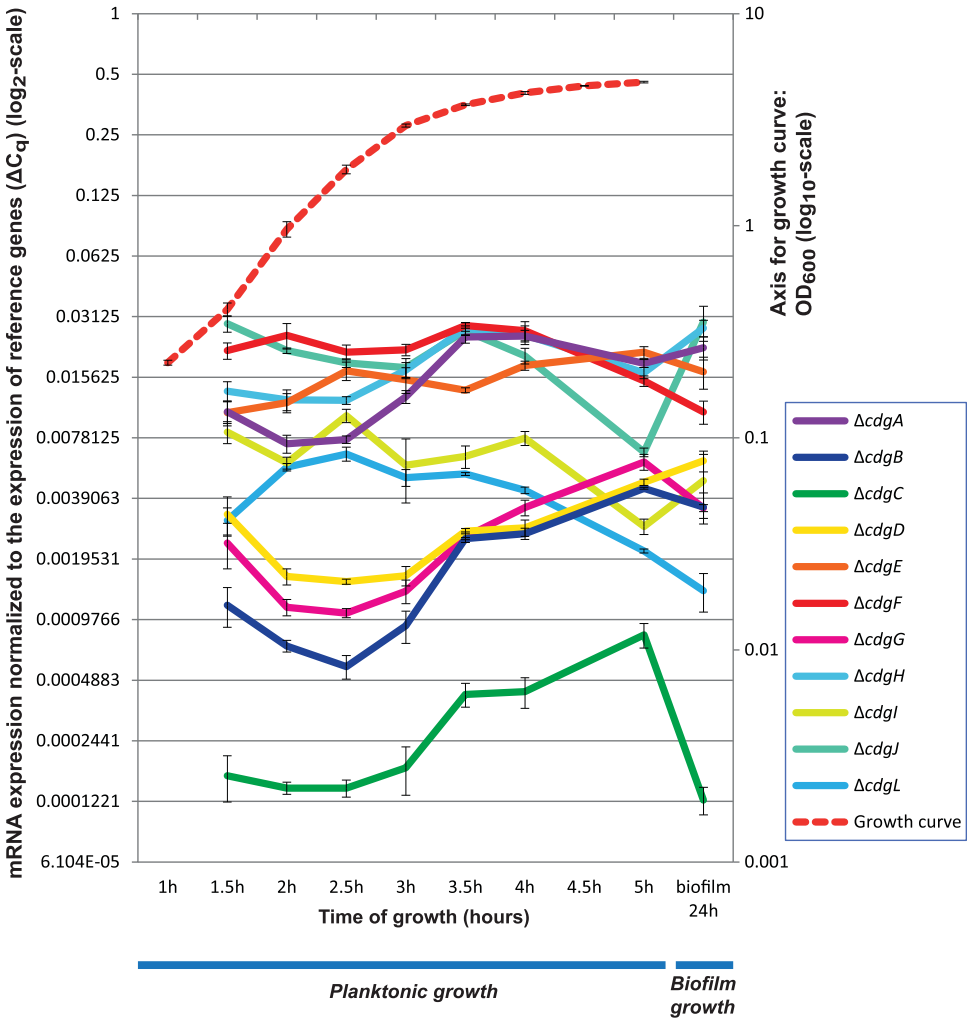

Fig. 3. RT-qPCR based expression profiles of the *B. thuringiensis* 407 *cdg* genes. Levels of mRNA transcripts for each *cdg* gene were determined by RNA sampling throughout the growth curve, and after 24 h growth in biofilm. Relative transcript expression levels for each target gene were determined 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 TnpI 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 ^{134}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^{-1}; Limit of quantitation, LOQ: 3.5 ng ml^{-1}). 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).

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[a] (results for two or three independent replicates listed)	
Gene deleted or overexpressed	Deletion mutant	Overexpression strain
cdgA	< LOD	< LOD
	< LOD	7 ng ml^{-1}
	< LOD	
cdgB	1 ng ml^{-1}	58 ng ml^{-1}
	< LOD	16 ng ml^{-1}
	< LOD	
cdgC	< LOD	*NA*[b]
	< LOD	
	< LOD	
cdgD	< LOD	2.2 ng/ml
	< LOD	6 ng/ml
	< LOD	
cdgE	2.3 ng ml^{-1}	< LOD
	2.7 ng ml^{-1}	< LOD
	< LOD	
cdgF	< LOD	621 ng ml^{-1}
	< LOD	422 ng ml^{-1}
	< LOD	404 ng ml^{-1}
cdgG	1.1 ng ml^{-1}	< LOD
	0.9 ng ml^{-1}	5 ng ml^{-1}
	< LOD	
cdgH	1.6 ng ml^{-1}	<LOD
	1.3 ng ml^{-1}	3.1 ng ml^{-1}
	< LOD	
cdgI	1 ng ml^{-1}	<LOD
	0.9 ng ml^{-1}	3.1 ng ml^{-1}
	< LOD	
cdgJ	< LOD	< LOD
	2 ng ml^{-1}	< LOD
	< LOD	
wild type	< LOD	
	< LOD	
	< LOD	
Empty vector control (wt pHT304-Pxyl)		< LOD
		6 ng ml^{-1}

a. Limit of detection (LOD) was 0.8 ng ml^{-1}; Limit of quantitation (LOQ): 3.5 ng ml^{-1}

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

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 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

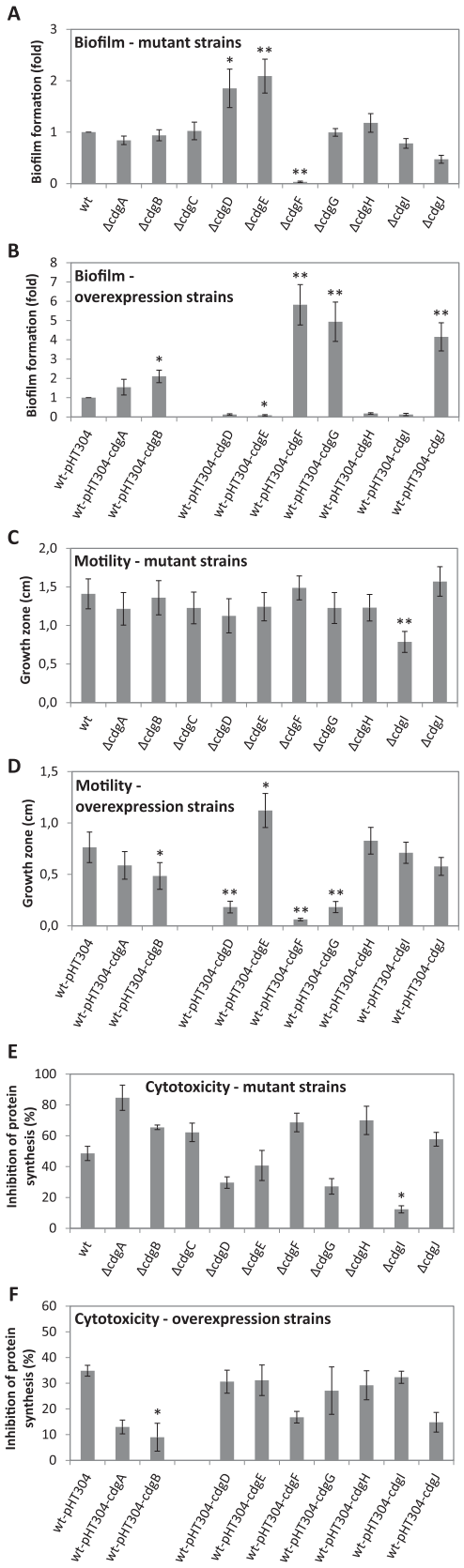

Fig. 4. Effect of *cdg* gene deletion and overexpression on *B. thuringiensis* 407 biofilm formation, motility, and Vero cell toxicity of culture supernatant. The assays were performed on *B. thuringiensis* 407 cells deleted for (A,C,E) or overexpressing (B,D,F) specific putative c-di-GMP signalling (*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-Pxyl 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 ^{221}GGDEF motif of CdgF to GGAAF ($CdgF^{GGDEF \rightarrow GGAAF}$). Overexpression of $CdgF^{GGAAF}$ from the pHT304-Pxyl 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	*In silico* predicted enzymatic activity	Enzymatic activity predicted from c-di-GMP measurements	Effect of gene deletion (Δ) or overexpression (OE) on phenotype, relative to wild type or vector control respectively							
			Biofilm formation		Motility		*In vitro* cytotoxicity		Sporulation	
			Δ	OE	Δ	OE	Δ	OE	Δ	OE
cdgA	DGC		–	(↑)	–	–	↑	↓	–	–
			$P = 0.22$	$P = 0.28$	$P = 0.13$	$P = 0.053$	$P = 0.09$	$P = 0.11$	$P = 0.43$	$P = 0.36$
									$P = 0.58$	$P = 0.88$
cdgB	DGC	DGC	–	(↑)	–	(↓)	(↑)	↓	–	↓
			$P = 0.87$	**$P = 0.02$**	$P = 0.70$	**$P = 0.02$**	$P = 0.06$	**$P = 0.02$**	$P = 0.45$	$P = 0.39$
									$P = 0.96$	$P = 0.06$
cdgC	none (GGDEF domain I-site)		–	*not tested*[a]	–	*not tested*[a]	(↑)	*not tested*[a]	↑	*not tested*[a]
			$P = 0.86$		$P = 0.30$		$P = 0.055$		**$P = 0.002$**	
									$P = 0.104$	
cdgD	PDE		↑	↓↓	(↓)	↓	↓	–	–	↓
			$P = 0.04$	$P = 0.06$	$P = 0.05$	**$P = 0.0009$**	$P = 0.10$	$P = 0.15$	$P = 0.18$	$P = 0.28$
									$P = 0.28$	$P = 0.48$
cdgE	DGC/PDE	PDE	↑	↓↓	–	↑	–	–	–	–
			$P = 0.0006$	**$P = 0.0498$**	$P = 0.10$	**$P = 0.012$**	$P = 0.70$	$P = 0.59$	$P = 0.55$	NA
									$P = 0.29$	0.92
cdgF	DGC/PDE	DGC	↓↓	↑	–	↓	(↑)	↓	–	↓
			$P = 0.002$	**$P = 0.0009$**	$P = 0.46$	**$P = 0.0009$**	$P = 0.43$	$P = 0.08$	$P = 0.20$	$P = 0.114$
									$P = 0.18$	$P = 0.091$
cdgG	DGC/PDE		–	↑	–	↓	↓	–	–	↓
			$P = 0.54$	**$P = 0.01$**	$P = 0.29$	**$P = 0.002$**	$P = 0.052$	$P = 0.27$	$P = 0.06$	NA
									$P = 0.50$	$P = 0.07$
cdgH	PDE		–	↓↓	–	–	(↑)	–	–	↓
			$P = 0.37$	$P = 0.06$	$P = 0.30$	$P = 0.62$	$P = 0.13$	$P = 0.35$	$P = 0.90$	$P = 0.39$
									$P = 0.47$	$P = 0.37$
cdgI	DGC (both A-site and I-site)/PDE		–	↓↓	↓	–	↓	–	–	↓
			$P = 0.35$	$P = 0.07$	**$P = 0.0004$**	$P = 0.68$	**$P = 0.014$**	$P = 0.054$	$P = 0.27$	NA
									$P = 0.54$	$P = 0.36$
cdgJ	none		(↓)	↑	–	–	(↑)	↓	↓	(↑)
			$P = 0.07$	**$P = 0.01$**	$P = 0.14$	$P = 0.12$	$P = 0.56$	$P = 0.06$	$P = 0.30$	$P = 0.06$
									$P = 0.25$	$P = 0.16$

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 elevated 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

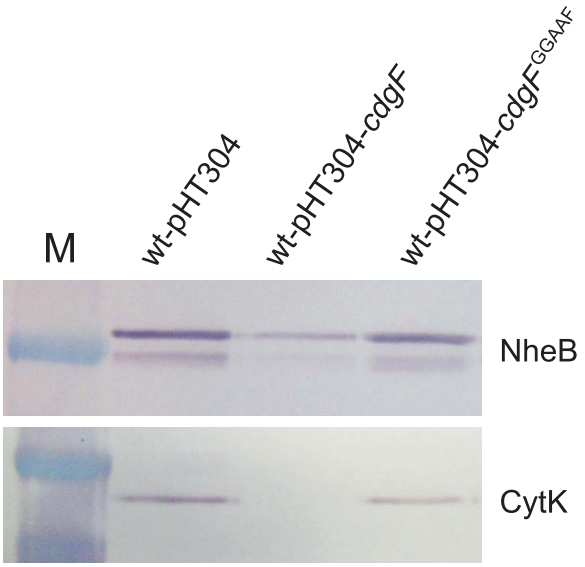

Fig. 5. Overexpression of CdgF leads to a decrease in secreted NheB and CytK toxin components, and is dependent on an intact CdgF GGDEF motif. Western immunoblot analysis of culture supernatants from the *B. thuringiensis* 407 empty vector control strain, the CdgF overproducing strain, and from the strain overproducing CdgF^{GGAAF}, 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 Δ*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 YkuI 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* YkuI 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

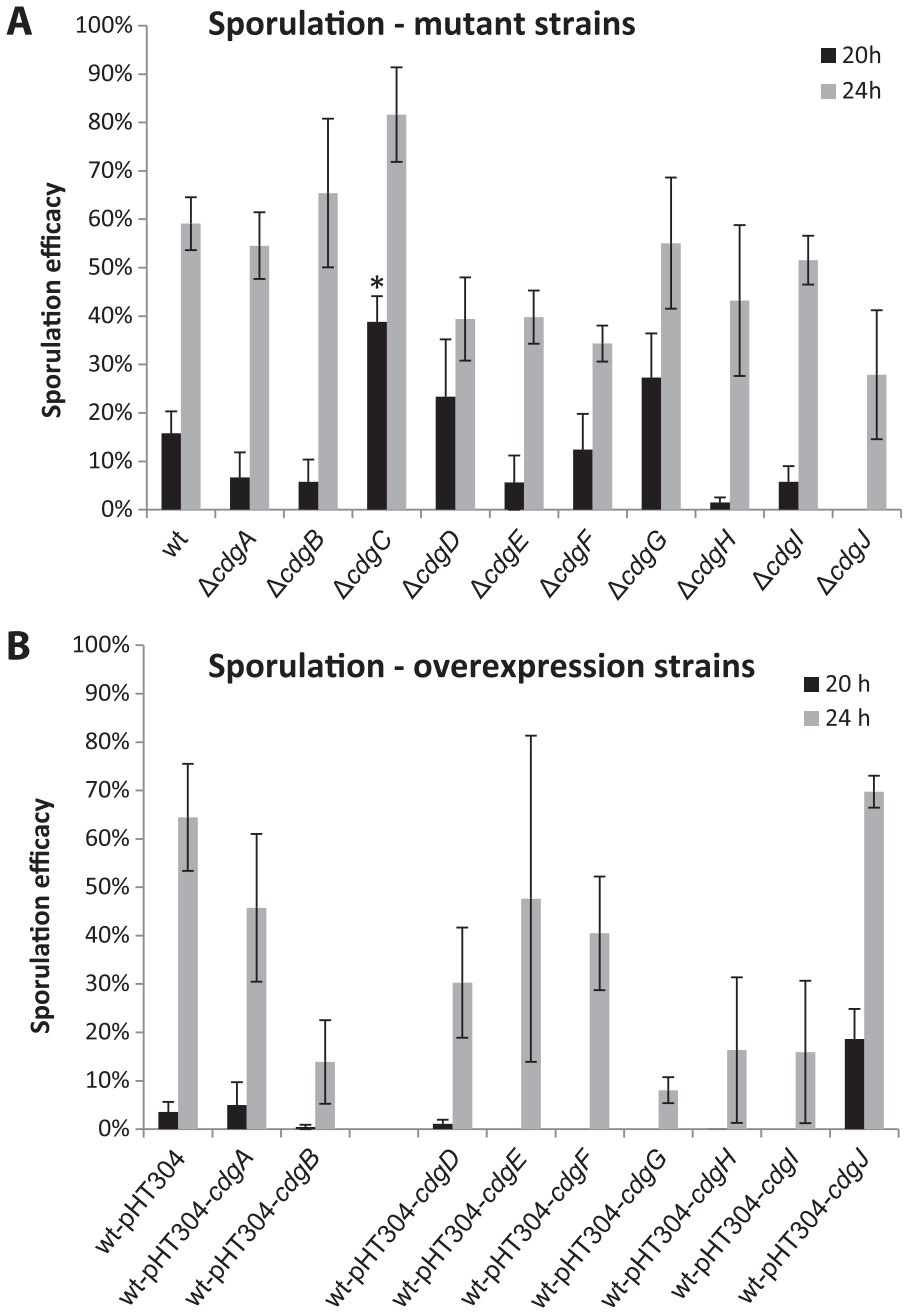

Fig. 6. Effect of *cdg* gene deletion and overexpression on *B. thuringiensis* 407 sporulation efficiency. Sporulation efficiency was determined in cultures of *B. thuringiensis* 407 deleted for (A) or overexpressing (B) specific putative c-di-GMP signalling (*cdg*) genes. Sporulation was assayed after 20 and 24 h of growth in four independent experiments for each time point and strain tested (twelve independent experiments performed for: wt, ΔcdgC, 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

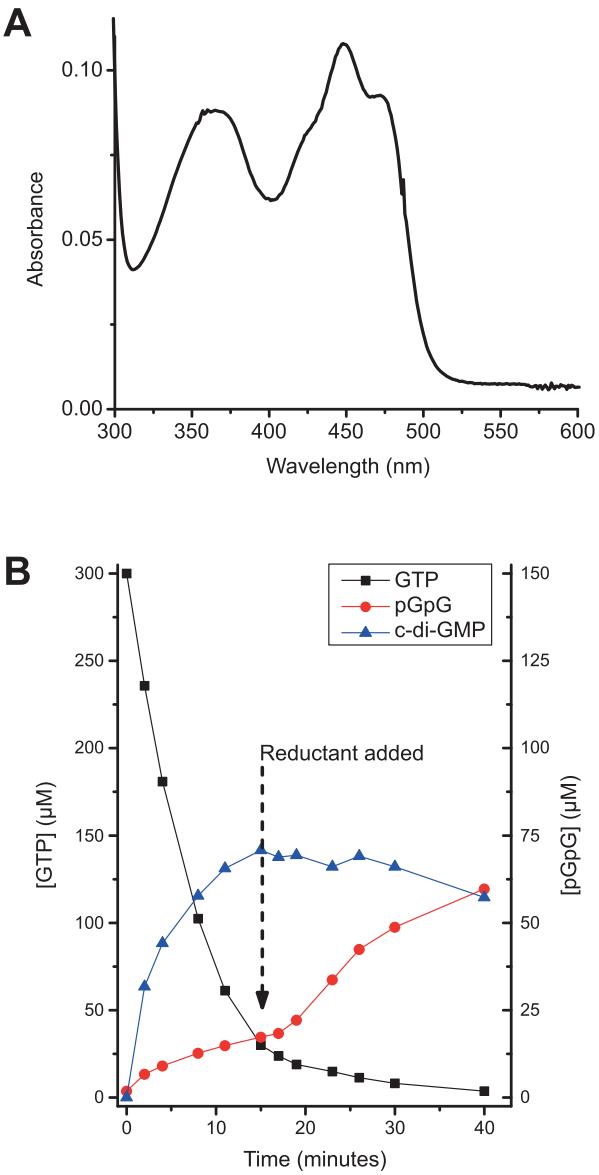

Fig. 7. Biochemical characterization of purified CdgF protein.
A. UV-Vis spectrum of purified CdgF protein from *Bacillus cereus* ATCC 14579 (BC_0628) following protein overexpression in *E. coli*, showing a spectrum characteristic of flavin-containing proteins.
B. Enzymatic assay showing that recombinant purified CdgF has functional PAS, GGDEF and EAL domains.
The reaction mixture, holding purified enzyme, initially contained 300 μM 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 (Guinebretiere *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 $CdgF^{GGAAF}$ 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 $CdgF^{GGAAF}$ 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, (Guinebretiere *et al.*, 2008; Lapidus *et al.*, 2008). Whether other Cdg proteins, e.g. such as the universally conservered 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, *ykuI*; 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-d-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) as of May 2011 using the global alignment algorithm GGSEARCH at 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 (Guinebretiere *et al*., 2008, 2010; Tourasse *et al*., 2011), were labelled.

B. cereus *group strains and culture conditions*

The acrystalliferous *B. thuringiensis* strain 407 Cry^- (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 bactopeptone medium (1% w/v bactopeptone, 0.5% w/v yeast extract, 1% w/v NaCl). Erythromycin at 10 $\mu g\ ml^{-1}$ 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_{600} 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 bactopeptone 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 bactopeptone 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^{-1} 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 Light Cycler 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^{Cq} (Pfaffl, 2001). For each target gene in each of the biological replicates, the gene expression was normalized to the geometric mean of the E^{Cq} values obtained for the three reference genes (delta-Cq-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 *xylR* and the *xylA* 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-*Sce*I (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-*Sce*I 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 *Mlu*I, 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 *Mlu*I-sites, cloned into pCR 2.1-TOPO vector (Invitrogen) and transferred to pMAD-I-*Sce*I. 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^{-1} 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_2O containing 20 ng ml^{-1} 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 (anionexchange 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^{-1} 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^{-1} 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^{-1} 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 bactopeptone 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^{-1} 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^{14}-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 $OD_{600} = 2.4$) were applied to the assay in duplicate. For overexpression strains, 150 μl culture supernatants harvested at $OD_{600} = 2.4$ 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 ($CdgF^{GGDEF \rightarrow GGAAF}$), 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_{600} of approximately 0.8. For strains carrying pHT304-Pxyl plasmid constructs, erythromycin was added to 10 μg ml^{-1}. This pre-culture was again diluted 1:100 in LB (added 1 mM xylose and 10 μg ml^{-1} 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 AATAATTTTGTTTAACTTTAAGAAGGAGATATACAT ATGC-TAGAACAGAGAGGTCATGC-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^{-1} ampicillin. The overnight culture was diluted in 1 L TB-medium containing 100 μg ml^{-1} ampicillin, and incubated in a shaker at 30°C until $OD_{600} = 1.8–2$. The culture was then cooled to 20°C on ice while purging the culture medium with N_2 (g) for 5 min. The culture was added 25 mM glucose and 20 mM $NaNO_3$ 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 my centrifugation at 30 000 x g for 30 min. Proteins in the resulting supernatant were precipitated by adding 0.43 g ml^{-1} ammonium sulphate followed by centrifugation at 30 000 x g for 30 min. The protein pellet was carefully dissolved 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 $MgCl_2$ 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% MetOH. 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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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Paper 2

MogR is a ubiquitous transcriptional repressor affecting motility, biofilm formation and virulence in the *Bacillus cereus* group

Veronika Smith[1,2], Malin Josefsen[1], Toril Lindbäck[3], Ida K. Hegna[1], Sarah Finke[1,2], Nicolas J. Tourasse[4], Christina Nielsen-LeRoux[5], Ole Andreas Økstad[1,2*], Annette Fagerlund[1,6*]

[1]Laboratory for Microbial Dynamics (LaMDa), Section for Pharmacology and Pharmaceutical Biosciences, Department of Pharmacy, University of Oslo, Oslo, Norway

[2]Centre for Integrated Microbial Evolution (CIME), Faculty of Mathematics and Natural Sciences, University of Oslo, Oslo, Norway

[3]Department of Paraclinical Sciences, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Oslo, Norway

[4]CNRS, INSERM, ARNA, UMR 5320, U1212, University of Bordeaux, Bordeaux, France

[5]INRAE, AgroParisTech, Micalis, Jouy-en-Josas, Paris-Saclay University, France

[6]Nofima, Norwegian Institute of Food, Fisheries and Aquaculture Research, Ås, Norway

*** Correspondence:**
Annette Fagerlund
annette.fagerlund@nofima.no
Ole Andreas Økstad
aloechen@farmasi.uio.no

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Abstract

Flagellar motility is considered an important virulence factor in a range of different pathogenic bacteria. In *Listeria monocytogenes* the transcriptional repressor MogR regulates motility in a temperature-dependent manner by directly repressing flagellar- and chemotaxis genes. The only other bacteria known to carry a *mogR* homolog are members of the *Bacillus cereus* group, which includes both motile and non-motile species. Furthermore, the motility locus in *B. cereus* group bacteria appears to be more closely related to *L. monocytogenes* than to *Bacillus subtilis*. In this study we show that in *Bacillus thuringiensis*, overexpression of MogR results in non-motile host cells devoid of flagella. Global gene expression profiling showed that 110 genes were differentially regulated by MogR overexpression, including flagellar motility genes, but also genes associated with virulence, stress response and a biofilm lifestyle. Accordingly, phenotypic assays showed that MogR also affects cytotoxicity and biofilm formation in *B. thuringiensis*. Overexpression of a MogR variant mutated in two key amino acids within the putative DNA binding domain restored phenotypes to those of a wild type empty vector control strain. In line with this, the introduction of these mutations resulted in complete loss in the ability of MogR to bind to its putative target site within the flagellar motility locus *in vitro*. In contrast to *L. monocytogenes*, MogR appears to be regulated in a growth-phase dependent and temperature-independent manner in *B. thuringiensis* 407. Interestingly, *mogR* was found to be conserved also in non-motile *B. cereus* group species with highly reduced motility loci, such as *Bacillus mycoides* and *Bacillus pseudomycoides*, and is expressed in non-motile *Bacillus anthracis*, providing further indications of an expanded and unexpected set of functions for MogR in *B. cereus* group species, in addition to motility regulation. In conclusion, MogR constitutes a novel *B. cereus* group pleiotropic transcriptional regulator, acting as a repressor of motility genes, and affecting the expression of a variety of additional genes involved in biofilm formation and virulence.

1 Introduction

In many bacterial species, flagella have been demonstrated to be important to virulence functions, including reaching the optimal host site, colonization or invasion, maintenance at the infection site, post-infection dispersal, protein secretion and more (Chaban et al., 2015). Temperature-dependent regulation of motility in the human pathogen *Listeria monocytogenes* has been described in a series of studies published by Higgins and co-workers, in which motility was shown to be regulated by the transcriptional repressor MogR and its anti-repressor GmaR (Gründling et al., 2004; Shen and Higgins, 2006; Shen et al., 2006; Kamp and Higgins, 2009; Shen et al., 2009; Kamp and Higgins, 2011), the genes for which are widely distributed among different *Listeria* spp. (Smith and Hoover, 2009). *L. monocytogenes* is a foodborne facultative intracellular pathogen, which uses flagellum-based motility when present in its extracellular environmental niche. During mammalian infection however, motility genes are downregulated by MogR-dependent repression upon sensing of mammalian physiological temperature (37 °C in the human). In this system, the GmaR anti-repressor functions as the temperature sensor, by antagonizing MogR repression activity at temperatures below 37 °C (Shen et al., 2006). The activity of GmaR is dependent on the transcriptional activation by DegU at low temperatures, and a temperature-dependent, post-transcriptional mechanism limits GmaR production to temperatures below 37 °C (Kamp and Higgins, 2009). This system allows *L. monocytogenes* to switch from an environmental and extracellular motile bacterium to an intracellular pathogen. Inside host cells, flagella are not required as *L. monocytogenes* cells instead move by actin-based motility, and downregulation of flagella during infection is thought to aid bacterial evasion of the host innate immune system (Hayashi et al., 2001; Li et al., 2017).

The only other known bacteria carrying a homolog to *Listeria* spp. *mogR* are species from the *Bacillus cereus* group (*B. cereus sensu lato*) (Gründling et al., 2004; Fagerlund et al., 2010), which is a group of closely related Gram-positive spore-forming bacteria of considerable medical and economic importance. The group comprises at least seven species, including *B. cereus* (*sensu stricto*), *Bacillus anthracis*, *Bacillus thuringiensis*, *Bacillus weihenstephanensis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, and *Bacillus cytotoxicus*, which, like *L. monocytogenes*, can be isolated from the environment, e.g. soil, air and water. In contrast to *L. monocytogenes* however, *B. cereus* is an extracellular opportunistic pathogen. The majority of strains of *B. cereus sensu stricto*, *B. thuringiensis*, *B. weihenstephanensis* and *B. cytotoxicus* are motile by peritrichous flagella, while *B. anthracis*, *B. mycoides*, and *B. pseudomycoides* are described as non-motile (Twine et al., 2009; Guinebretière et al., 2013). *B. cereus* group bacteria can cause foodborne disease (emetic or diarrhoeal syndrome) and serious opportunistic infections in man, but also encompasses strains that are used as probiotics (Stenfors Arnesen et al., 2008; Bottone, 2010; Cutting, 2011), some of which have been suggested to have separate species status (e.g. *Bacillus toyonensis;* Jimenez et al., 2013). The obligate mammalian pathogen *B. anthracis* is the cause of anthrax disease, being endemic in several parts of the world, and has been used as a biological terror agent. *B. thuringiensis* is an entomopathogenic bacterium, frequently used as a biopesticide to protect crops against insect pests, however this species also carries virulence factors shared with *B. cereus sensu stricto* (Damgaard, 1995; Gaviria Rivera et al., 2000; Swiecicka et al., 2006; Celandroni et al., 2014; Kim et al., 2015) and has caused human infections similar to those caused by *B. cereus* (Samples and Buettner, 1983; Jackson et al., 1995; Damgaard et al., 1997; Hernandez et al., 1998; Ghelardi et al., 2007b). Although strains in the *B. cereus* group to a certain degree follow ecological diversification patterns through evolutionary time (Guinebretière et al., 2008), *B. cereus* and *B. thuringiensis* isolates are intermingled in global MLST- or *k*-mer-

based phylogenetic analyses of a high number of non-biased isolates (Kolstø et al., 2009; Bazinet, 2017), while the thermotolerant species *B. cytotoxicus* forms a separate and phylogenetically remote clade within the *B. cereus* group population.

The bacterial flagellum is a complex molecular structure made up of about 25 different proteins. In most bacteria studied to date, expression of flagellar genes is subject to hierarchical regulation to ensure the sequential expression required for proper flagellum assembly (Smith and Hoover, 2009; Erhardt et al., 2010). In contrast, *L. monocytogenes* and *B. cereus* group strains appear to lack this transcriptional cascade control of flagellar biosynthesis (Smith and Hoover, 2009; Chiara et al., 2015). *B. cereus* group bacteria also lack σ^D, a common key regulator of motility genes in bacteria, indicating a different mode of transcriptional regulation for the motility genes. In this study, we aimed to investigate functional roles of the MogR homolog identified in *B. cereus* group bacteria, and whether motility regulation in the *B. cereus* group more closely resembles that of *L. monocytogenes* rather than other *Bacillus* species.

2 Materials and Methods

2.1 Sequence analysis

Motility proteins that were orthologs between species were identified by amino acid sequence searches (BLASTP) performed using BLAST v.2.6.0+ (Altschul et al., 1990; Altschul et al., 1997) between all pairwise combinations of the motility loci from *B. thuringiensis* 407, *L. monocytogenes* EGD-e, and *B. subtilis* 168 (RefSeq accession numbers NC_018877.1, NC_003210.1, and NC_000964.3, respectively; Glaser et al., 2001; Barbe et al., 2009; Sheppard et al., 2013). Results for reciprocal best BLASTP hits between each pair were included in Supplementary Table S1 if the BLASTP alignments had a percentage of identical matches above 20%, a bit score greater than 30 and covered at least 25% of each amino acid sequence. Comparisons between the motility locus in different *B. cereus* group strains were obtained using the Integrated Microbial Genomes (IMG) browser (Markowitz et al., 2010; http://img.jgi.doe.gov), by searching for orthologous genome neighborhoods to genes from the *B. thuringiensis* 407 motility operon. Annotations for the *B. cereus* ATCC 10987 motility cluster and the *B. mycoides* DSM 2048 *mogR* gene were corrected using EasyGene (Larsen and Krogh, 2003). To perform an exhaustive comparative analysis of the genetic structure of the motility locus, genome sequences of 106 subgroup I strains of the *B. cereus* group that had been sequenced to a minimum of scaffold level were downloaded from NCBI (November 25, 2019), and a local BLAST database was indexed from the corresponding subgroup I proteomes. The corresponding protein sequence from each of the 50 genes in the main *B. thuringiensis* 407 motility locus (genes with locus tags ranging from AFV17362.1 to AFV17411.1, found within coordinates 1608289 to 1653756 in the closed *B. thuringiensis* 407 genome sequence, accession number CP003889) were used as queries in BLASTP searches (parameters: -num_threads 10 -evalue 1.0e-05 -seg no -outfmt 0) for homologous proteins among the subgroup I strains. Output files were parsed using custom-made shell scripts, and *sed/awk*. Candidate MogR binding sites in the *B. thuringiensis* 407 genome were identified by searching the intergenic regions upstream of candidate genes using CLC Main Workbench (Qiagen), with the *L. monocytogenes* consensus MogR binding sequence (TTTTWWNWWAAAA [IUPAC nucleotide codes]; Shen et al., 2009) as query, allowing for up to two mismatches to identify candidate hits.

2.2 Strains and growth conditions

The strains used in this study are presented in Table 1. *B. thuringiensis* 407 Cry$^-$ (also sometimes referred to as Bt407) is an acrystalliferous strain cured of its *cry* plasmid (Lereclus et al., 1989). It is genetically close to the *B. cereus* type strain ATCC 14579 (Tourasse et al., 2006).

Unless otherwise stated, *B. thuringiensis* 407 cultures were inoculated with 1% of an overnight culture and grown at 30 °C and 200 rpm in Luria Bertani (LB) broth or in bactopeptone medium (1% w/v bactopeptone, 0.5% w/v yeast extract, 1% w/v NaCl). For cloning and expression in *Escherichia coli*, ampicillin at 50 or 100 µg ml^{-1}, kanamycin at 50 µg ml^{-1} and/or erythromycin at 400 µg ml^{-1} was used. Erythromycin at 10 µg ml^{-1} was used to maintain the pHT304-P$_{xyl}$ plasmid constructs in *B. thuringiensis* 407. For induction of gene expression from the *xylA* promoter on pHT304-P$_{xyl}$, xylose was added to the growth medium at 1 mM or as otherwise stated. For induction of MBP-MogR in *E. coli* BL21(DE3), 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was used. Growth curves were prepared in bactopeptone medium, pH 7.0 with cultures grown with shaking at 220 rpm, using 50 ml culture volumes in 250 ml baffled flasks.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
PLASMIDS		
pHT304-P$_{xyl}$	Low copy number expression/shuttle vector; *xylA* promoter (Ap^r, Ery^r)	(Arantes and Lereclus, 1991; Salamitou et al., 1997)
pHT304-P$_{xyl}$-*mogR*	wild type *mogR* in pHT304-P$_{xyl}$	This study
pHT304-P$_{xyl}$-*mogR*$^{QN\rightarrow AA}$	Q119A, N120A mutant *mogR* in pHT304-P$_{xyl}$	This study
pMAL-p5X	Expression vector for production of MBP fusions, IPTG promoter (Ap^r)	New England Biolabs
pMAL-p5X-*mogR*	wild type *mogR* in pMAL-p5X	This study
pMAL-p5X-*mogR*$^{QN\rightarrow AA}$	Q119A, N120A mutant *mogR* in pMAL-p5X	This study
STRAINS		
***B. cereus* group**		
407	*B. thuringiensis* 407 Cry$^-$	(Lereclus et al., 1989)
407/pHT304-P$_{xyl}$	pHT304-P$_{xyl}$ in 407	This study
407/MogR$^+$	pHT304-P$_{xyl}$-*mogR* in 407	This study
407/MogR$^{QN\rightarrow AA}$	pHT304-P$_{xyl}$-*mogR*$^{QN\rightarrow AA}$ in 407	This study
407Δ*flaAB*	*B. thuringiensis* 407Δ*flaA*Δ*flaB* (Km^r)	(Houry et al., 2010)
407Δ*flaAB*/pHT304-P$_{xyl}$	pHT304-P$_{xyl}$ in 407Δ*flaAB*	This study
407Δ*flaAB*/MogR$^+$	pHT304-P$_{xyl}$-*mogR* in 407Δ*flaAB*	This study
407Δ*flaAB*/MogR$^{QN\rightarrow AA}$	pHT304-P$_{xyl}$-*mogR*$^{QN\rightarrow AA}$ in 407Δ*flaAB*	This study
E. coli		
BL21(DE3)	*E. coli* BL21(DE3)	New England Biolabs
BL21/MogR$^+$	pMAL-p5X-*mogR* in BL21(DE3)	This study
BL21/MogR$^{QN\rightarrow AA}$	pMAL-p5X-*mogR*$^{QN\rightarrow AA}$ in BL21(DE3)	This study

2.3 Reverse transcription quantitative PCR (RT-qPCR)

For analysis of *mogR*, *flaA* and *flaB* expression throughout the bacterial growth phase, RT-qPCR was performed essentially as described by Fagerlund et al. (2016). Briefly, cells grown in bactopeptone medium at 30 °C were incubated in an equal volume of ice-cold methanol for 5 minutes before harvesting by centrifugation. Cells were lysed using a Precellys 24 Tissue Homogenizer (Bertin) and RNA was isolated using the RNeasy Mini or Midi Kits (Qiagen). After treatment with DNase and further purification, cDNA synthesis was performed in

duplicate for each sample using SuperScript III Reverse Transcriptase (Invitrogen). For all samples, a negative control reaction without reverse transcriptase was included. RT-qPCR was carried out with a LightCycler 480 Real-Time PCR System (Roche) using primers listed in Supplementary Table S2. The three genes *gatB*, *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. The second derivative maximum method in the LightCycler 480 software (Roche) was utilized to obtain a quantification cycle (Cq) value for each reaction. The expression of each target gene in each biological replicate was converted into E^{Cq} values (Pfaffl, 2001) and then normalized to the geometric mean of the E^{Cq} values obtained for the three reference genes. Finally, averages and standard deviations were calculated from the normalized expression values from the biological replicates.

For analysis of *flaA* and *flaB* expression in the *B. thuringiensis* 407 strains overexpressing MogR and $MogR^{QN\rightarrow AA}$ (see below), the same procedure was used except that *gatB* and *rpsU* were used as reference genes.

2.4 Cloning of *mogR* for expression in *B. thuringiensis* 407

The low-copy number *E. coli*/*Bacillus* shuttle vector pHT304-P_{xyl}, in which *xylR* and the *xylA* promoter from *B. subtilis* was inserted into the pHT304 cloning site (Arantes and Lereclus, 1991) allowing xylose-inducible expression of downstream cloned genes, was used for overexpression studies. The sequence encoding MogR from *B. thuringiensis* 407 (BTB_RS08390) was PCR-amplified using primers 5'-gtcggatccgaattgtgaaaggatgagg-3' and 5'-taaggtacctcttccttcttcggaacg-3' and genomic DNA as the template. The PCR product was inserted into pHT304-P_{xyl} using the primer-incorporated *Bam*HI and *Kpn*I restriction sites (underlined), creating pHT304-P_{xyl}-*mogR* and placing *mogR* under transcriptional control of the *xylA* promoter.

The plasmid pHT304-P_{xyl}-$mogR^{QN\rightarrow AA}$, containing *mogR* with amino acid substitutions in two of the six conserved amino acid residues predicted to make base-specific contacts with the MogR recognition site (Shen et al., 2009) was created by site-directed mutagenesis of pHT304-P_{xyl}-*mogR* using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) and primers 5'-tccaaaaacagaaagtcaattggcagctacgtattataaattgaaaaaacgtg-3' and 5'-cacgttttttcaatttataatacgtagctgccaattgactttctgtttttgga-3' (mutated bases underlined). The introduced mutations were Q119A and N120A.

The plasmids were verified by sequencing. The constructed pHT304-P_{xyl} plasmids and the empty vector were introduced by electroporation into *B. thuringiensis* 407 (Masson et al., 1989).

2.5 Motility assay

Swimming ability was determined on 0.3% LB soft agar plates with 1 mM xylose and 10 µg ml^{-1} erythromycin added. A 5 µl drop of culture grown in LB broth overnight at 30 °C was spotted on each agar plate. The plates were wrapped in plastic and incubated for 7 hours at 30 °C. Each independent assay was performed with two or three technical replicates.

2.6 Atomic force microscopy (AFM)

AFM imaging and analysis was performed using a Nanowizard AFM microscope (JPK Instruments). Bacterial cell culture was grown in LB broth with erythromycin (10 µg ml^{-1}) and xylose (10 mM) at 37 °C to an OD_{600} of 3. One ml of culture was washed three times and finally resuspended in 0.9% NaCl. Cells were then diluted (15:50) in a 10 mM magnesium/Tris buffer, pH 7.5. Ten µl was applied onto freshly cleaved mica surfaces mounted on a glass slide, and allowed to adhere for 10 minutes followed by washing (10×100 µl) using sterile dH_2O. Excess water was carefully removed, and the slide gently dried using a nitrogen gas jet stream. Images were recorded in intermittent-contact mode at room temperature in air using a MicroMasch NSC35/AIBS cantilever. AFM images were analyzed using The NanoWizard IP Image Processing Software (JPK Instruments).

2.7 SDS-PAGE and Western immunoblotting

For detection of flagellin, for each strain analyzed, two parallel bacterial culture samples (10 ml LB broth) were harvested by centrifugation (4100 × *g*, 4 °C) after 3.5 hours growth at 30 °C (OD_{600} ~1.2) – one for extraction of surface proteins, and one for whole cell protein extraction. The cell pellets were resuspended in 1 ml PBS (pH 7.1) and kept on ice. For extraction of surface proteins, the washed cells were centrifuged for 5 minutes at 16,000 × *g* and 4 °C and resuspended in an equal volume of 2×SDS-PAGE sample buffer before incubation at 95 °C for 5 minutes. The supernatant was collected by centrifugation as before. The whole cell fraction was prepared by washing the cell pellet in PBS followed by centrifugation for 5 minutes at 16,000 × *g* and 4 °C. The pellet was then resuspended in 500 µl PBS and lysed using a Precellys 24 Tissue Homogenizer (Bertin). The supernatant was collected by centrifugation for 8 minutes as before. Whole cell supernatant (21 µl) with 7 µl 4×SDS-PAGE buffer and 5 µl of the surface protein fraction were separated on 12% SDS-PAGE gels as described below.

For detection of Hbl, Nhe and CytK, cultures were harvested after 4.5 hours growth. Supernatant samples were collected by centrifugation, concentrated 40-fold by precipitation with four volumes of ice-cold acid acetone:methanol (1:1 v/v), stored at -20 °C overnight, and harvested by centrifugation for 30 minutes at 16,000 × *g* and 4 °C. Then, pellets were left to evaporate at 4 °C overnight and resuspended in 2×SDS-PAGE sample buffer. Samples were diluted 20-fold with MQ H_2O. Concentrated supernatant samples (10 µl) were separated on 10% SDS-PAGE gels.

SDS-PAGE was carried out using a Bio-Rad Mini-Protean II Dual Slab Cell, using 10 µl Prestained Protein Marker, Broad range (New England Biolabs) as the molecular weight marker. Western blot analysis was performed using Immun-Blot PVDF membranes (Bio-Rad) according to standard protocols (Harlow, 1988). Blocking was performed for 1 hour in 5% non-fat dry milk in TBST. Flagellin proteins were detected using a rabbit antiserum raised against flagellin from *Bacillus mojavensis*, used at a 1:300 dilution, and a HRP-conjugated donkey-anti-rabbit antibody (Santa Cruz Biotechnology) diluted 1:10,000 as secondary antibody. CytK was detected using rabbit antiserum (Fagerlund et al., 2004) diluted 1:2000, followed by HRP-conjugated donkey anti-rabbit (Santa Cruz Biotechnology) diluted 1:5000. Hbl B and NheA were detected using monoclonal antibodies 2A3 against Hbl B (Dietrich et al., 1999) and 1A8 against NheA (Dietrich et al., 2005) (both diluted 1:15), followed by HRP-conjugated AffiniPure Goat-anti-mouse IgG (H+L) (Jackson Immuno Research Laboratories) at 80 µg ml^{-1}. SuperSignal West Femto Substrate (Pierce) was used to develop the blots. Western blots were photographed in a Chemi Genius Bio Imaging System (Syngene), and sub-saturation images were saved as jpeg files in ImageJ (Abràmoff et al., 2004).

2.8 Microarray analysis

Cultures of *B. thuringiensis* 407 harboring either pHT304-P_{xyl} or pHT304-P_{xyl}-*mogR* were grown in LB broth containing 10 mM xylose at 37 °C for 3 hours, and then incubated in an equal volume of ice-cold methanol for 5 minutes before pellets were harvested by centrifugation at 2800 × *g* for 20 minutes. For extraction of RNA, cells were lysed using a Precellys 24 Tissue Homogenizer (Bertin) and RNA was isolated using the RNeasy Mini Kit (Qiagen) and subjected to on-column DNase-treatment using the RNase-Free DNase Set (Qiagen). cDNA synthesis, labelling and purification was performed as described (Gohar et al., 2008). Microarray slides were printed at The Microarray core facility of the Norwegian University of Science and Technology (NTNU). Design, printing, prehybridization, hybridization and scanning of the slides and analysis of the data was performed as described (Gohar et al., 2008). Each microarray experiment was based on four slides, all biological replicates. *P*-values were computed using a false discovery rate (FDR) of 0.05.

The microarray slides contain 70-mer oligonucleotide probes designed to detect open reading frames (ORFs) in the following strains: *B. anthracis* Ames, *B. anthracis* A2012, and *B. cereus* ATCC 14579, in addition to selected genes from *B. cereus* ATCC 10987 (Kristoffersen et al., 2007). All probe sequences on the microarray were analyzed by BLAST for hits to the annotated genes of a *B. thuringiensis* 407 draft genome sequence (as of April 30, 2009; the gene lists are based on the GenBank annotations as of this date; accession no. ACMZ00000000.1). Only probes with 93% identity or greater to a transcript/feature sequence of *B. thuringiensis* 407 were included in the analysis. Of the predicted *B. thuringiensis* 407 genes, 1719 genes, most of which were hypothetical genes, did not have corresponding probes on the array (these include the 761 genes on contigs 00213 and 00060, which appear to be plasmid-borne). COG categories were obtained for the analysed genes as reported in the IMG database (http://img.jgi.doe.gov).

2.9 Cytotoxicity assay

The Vero cell cytotoxicity assay was performed as described by Lindbäck and Granum (2006), and measures the percentage inhibition of C^{14}-leucine incorporation in cells due to the cells being subjected to toxins, calculated relative to a negative control where cells were not subjected to toxin sample. Samples of early stationary phase cultures grown to an OD_{600} of 2.4 were collected and 100 µl or 150 µl samples (for deletion mutants and overexpression strains, respectively) were applied to the cytotoxicity assay. The assays were performed for three independent biological replicates with two technical replicates in each assay.

2.10 *In vivo* toxicity in insect larvae

The virulence-related properties of MogR were assessed by comparing the killing effect of the *B. thuringiensis* 407 MogR overexpression and empty vector control (pHT304-P_{xyl} plasmid) strains, in both wild type and Δ*flaAB* backgrounds, by infection (force feeding) in 5th instar *Galleria mellonella* larvae. *G. mellonella* eggs were hatched at 28 °C and the larvae reared on beeswax and pollen. For infection experiments, groups of 20 to 25 *G. mellonella* larvae, weighing about 200 mg each, were used. As MogR overexpression is activated from the pHT304-P_{xyl} promoter by the addition of xylose, we tried for the first time to evaluate if activation could occur in the insect intestine as well. Therefore, xylose (20 mM) was both added to the LB growth medium of the four strains, as well as to the bacterial inoculums and the toxin alone control (Cry1C) at time zero (time point of force feeding). Larvae were force fed a second time with 10 µl 20 mM xylose 5 hours later (in order to again activate MogR expression from the plasmid). Infections were otherwise performed as previously described

(Fedhila et al., 2006), by force feeding with 10 µl of a mixture containing 4-5×10^6 of vegetative bacteria (exponential growth OD_{600} ~1 in LB) with 20 mM xylose and 3 µg of activated Cry1C toxin to overcome the *B. thurinngiensis* 407 strain being *cry* negative. The larvae in the control group were fed PBS buffer or Cry1C toxin and xylose in corresponding amounts to the samples containing bacterial inocula. The chosen dose was expected to result in about 70% (± 5%) mortality at 37 °C after 48 hours.

2.11 Biofilm assays

The ability to form biofilms was determined using a glass tube screening assay (Houry et al., 2010). Briefly, exponential phase cultures were diluted into HCT medium (Lecadet et al., 1980; Lereclus et al., 1982) to an OD_{600} of 0.01, and 2 ml was inoculated into sterile 6 ml glass tubes. The tubes were incubated for 72 hours at 37 °C. The biofilm was subsequently collected by removing the culture medium with a Pasteur pipette and thoroughly vortexing in 2 ml PBS before measuring the OD_{600} of the suspension of biofilm cells. Each strain was tested in five biological replicates, each with 3 or 4 technical replicates.

The ability to form biofilms in polyvinylchloride (PVC) microtiter plates was determined using a crystal violet biofilm screening assay (Auger et al., 2006). Briefly, fresh bactopeptone medium was inoculated with 0.5% exponential phase culture, transferred to 96-well plates (Falcon #353911) and incubated for 24, 48 and 72 hours at 30 °C. The biofilm was subsequently washed using PBS, stained using 0.3% crystal violet, solubilized with acetone:methanol (1:3 v/v), and transferred to flat-bottomed microtiter plates (Falcon #353915) for determination of the absorbance of the solubilized dye at 570 nm. Each strain was tested in triplicate. Statistical analyses were performed separately for each time point, as described under "Statistical analyses" below.

2.12 Expression of MBP-tagged MogR in *E. coli*

The sequence encoding MogR from *B. thuringiensis* 407 (BTB_RS08390) was PCR amplified using primers 5'-atgtatcaccacacagcaattaatgtattag-3' and 5'-gcgcggatcc<u>ttattactgtgttacggtcataacttgtcc</u>-3' and genomic DNA as the template. The PCR product was cloned into the pMAL-p5x vector (New England Biolabs) using the *Xmn*I and *Bam*HI restriction sites (underlined) according to the manufacturer's instructions, allowing expression of MBP-MogR. A construct expressing the MBP-$MogR^{QN\rightarrow AA}$ protein was created by site-directed mutagenesis of pMAL-p5x-*mogR* using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) with the same primers as for pHT304-P_{xyl}-$MogR^{QN\rightarrow AA}$. Constructs were transformed into *E. coli* BL21(DE3) cells. The plasmids were verified by sequencing. MBP-MogR and MBP-$MogR^{QN\rightarrow AA}$ proteins were expressed in *E. coli* BL21(DE3) and purified using the manufacturer's manual "Method I – Total cell extract". In short, 100 ml LB broth was inoculated with a 1 ml overnight culture containing cells harbouring fusion plasmid. Cells were grown at 37 °C with shaking to an OD_{600} of ~0.5. IPTG was added to a final concentration of 0.3 mM and cells were induced for 2 hours. The cells were harvested by centrifugation at 4500 × *g* for 10 minutes and frozen at -20 °C overnight, sonicated in an ice-water bath, and then centrifuged at 20,000 × *g* for 20 minutes at 4 °C. The supernatants were diluted 1:6 in Column Buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA). MBP-MogR proteins were purified by amylose affinity chromatography (Poly-Prep Chromatography column, 0.8 × 4 cm, Bio-Rad). The identity and purity of proteins was confirmed by SDS-PAGE (Coomassie stain) and the quantity determined by Bradford assay.

2.13 Electrophoretic mobility shift assay (EMSA)

DNA probes were generated by PCR using chromosomal DNA from *B. thuringiensis* 407 and the following primer combinations: 5'-cgggtgtcaactaaaaattcg-3' / 5'-caactatcataatatcacctttcgg-3' (P_{flaA} probe), 5'-ttacagaaatgaaatttacggataac-3' / 5'-ccttatcctttctgtctggtc-3' (P_{hblC} probe), 5'-tccgtatgtaattccgtttcaaga-3' / 5'-aatttcctgcttgacccctt-3' (P_{ctrl1} probe). Corresponding 5' biotinylated probes were generated for *flaA* and *hblC* with the same primers with biotin labelling. ctrl1 was chosen as a control for binding to *B. thuringiensis* DNA and is a PCR product from inside a randomly chosen gene from *B. thuringiensis* 407 (BTB_RS08990 encoding an elongation factor G-binding protein). A commercial EMSA kit (LightShift Chemiluminescent, Thermo Scientific) was used to detect the binding of MogR protein to DNA following the manufacturer's instructions. Unlabelled Epstein Barr nuclear antigen DNA supplied with the kit was used as negative non-*Bacillus* DNA control. DNA binding reactions were conducted in 20 ml of 10×Binding Buffer (100 mM TrisHCl, 500 mM KCl, 10 mM DTT, pH 7.5) with 50 ng μl^{-1} polydeoxyinosinicdeoxycytidylic acid (poly dI-dC). 1 µg of MBP-MogR or MBP-$MogR^{QN\rightarrow AA}$ were added to approximately 2 fmol biotinylated DNA probe and incubated for 20 minutes at room temperature. A total of 20 ml of each binding reaction was loaded on a 5% SDS-PAGE gel (0.5×TBE) and resolved for 1 hour at 100 V.

2.14 Quantification of cyclic di-GMP by LC-MS/MS

LC-MS/MS analysis was based on a method described by Spangler et al. (2010), with some modifications, as previously described (Fagerlund et al., 2016), using a Thermo Scientific LTQ XL Linear Ion Trap Mass Spectrometer (Thermo Scientific) and separation on a 5 mm × 1 mm I.D. Nucleodur C18 Pyramid precolumn and a 50 mm × 1 mm I.D. Nucleodur C18 pyramid analytical column (both from Marchery-Nagel), with an electrospray ionization (ESI) source operated in the positive ionization mode to interface the HPLC and the MS.

2.15 Statistical analyses

Minitab v.17 software was used for statistical analysis. Unless stated otherwise, all data were analyzed using analysis of variance (ANOVA) followed by Tukey's *post hoc* test for pairwise comparisons. *P*-values <0.05 were regarded as significant.

3 Results

3.1 The motility gene loci in *B. thuringiensis* and *L. monocytogenes* are closely related

A cluster of approximately 45-50 genes with homology to flagellar-based motility- and chemotaxis genes is present in most *B. cereus* group strains. Although *B. cereus* group bacteria are phylogenetically more closely related to *B. subtilis* than to *L. monocytogenes*, some aspects of organization of the *B. thuringiensis* 407 motility gene cluster were found to more closely resemble that of *L. monocytogenes*. Bidirectional BLAST analysis showed that a greater number of motility protein orthologs is shared between *B. thuringiensis* 407 and *L. monocytogenes* EGD-e (41 orthologous proteins, average alignment percentage identity 46%) than between *B. thuringiensis* 407 and *B. subtilis* 168 (31 orthologous proteins, 37% average sequence identity) (Supplementary Table S1). Furthermore, only other *Listeria* spp. and species of the *B. cereus* group are known to have orthologs to the *L. monocytogenes* motility gene repressor MogR (Gründling et al., 2004; Smith and Hoover, 2009), potentially indicating that elements of motility regulation may be shared between these species (Figure 1 and Supplementary Table S1).

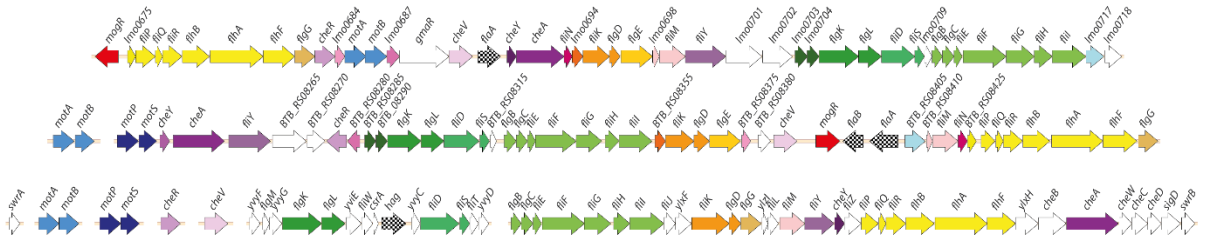

Figure 1. Comparison of genomic loci encoding flagellar-based motility genes in *L. monocytogenes, B. thuringiensis* and *B. subtilis.* Top: *L. monocytogenes* (*Lm*) EGD-e (single chromosomal locus). Middle: *B. thuringiensis* (*Bt*) 407 (two chromosomal loci). Bottom: *B. subtilis* (*Bs*) 168. The *Bs* 168 gene annotations are in accordance with gene or protein names for the corresponding RefSeq entries. Where orthologs to the *Bs* 168 genes are present in *Bt* 407 and/or *Lm* EGD-e, as determined by reciprocal best BLAST hits, these are given the same gene names, except for *fliS, fliE, fliH,* og *hag/flaA/flaB* (see Supplementary Table S1). Annotation for the following genes lacking *Bs* 168 orthologs is according to the specified references: *mogR* (Grundling et al., 2004), *gmaR* (Shen et al., 2006), *flgE* (NP_464224 and ZP_04138734), and *fliN* (ZP_04138744). Hypothetical genes in *Lm* EGD-e and *Bt* 407 are indicated by locus tags. Protein orthologs or blocks of colinear orthologs are indicated by identical coloring in two or three strains. Genes shown in white do not have orthologs in any of the two other strains.

3.2 Non-motile *B. anthracis* and *B. pseudomycoides* have retained mogR

As has previously been noted (Klee et al., 2010), comparative alignments between the motility loci of different *B. cereus* group strains indicate that the flagellin gene locus is an evolutionary hotspot, as the number of flagellin genes in each strain varies between one and five copies, and several strains also contain additional sets of genes in this locus. Isolates belonging to the *B. cereus* group may be divided into seven phylogenetic subgroups (I to VII) and for which, with the exception of *B. pseudomycoides* (group I) and *B. cytotoxicus* (group VII), species designations do not strictly follow the phylogenetic group designations. Instead, isolates belonging to the different phylogenetic subgroups have distinct ecotypes and preferred growth temperature ranges (Guinebretière et al., 2008). An analysis of *B. cereus* group genomes revealed striking differences between the motility gene clusters in strains belonging to the various phylogenetic groups (Figure 2). The motility gene clusters of strains in phylogenetic subgroups III, IV and V were overall highly similar to that of *B. thuringiensis* 407, with a few exceptions mainly related to insertion of transposable elements and variation in the number of flagellin genes. Of note, strains of *B. anthracis*, which constitutes a highly clonal lineage within subgroup III, all carry an 11.5 kb insertion in place of the second flagellin gene (*flaB*), as well as nonsense mutations in a series of the conserved motility genes (*motP*, *cheA*, *flgL*, *fliF*, BAS1560 [ortholog to BTB_RS08355], *fliK*, BAS1570 [ortholog to BTB_RS08380], *cheV*, BAS1584 [ortholog to BTB_RS08410], *fliM*, and *flhF*). *B. anthracis* strains are thus non-motile, however still carry an intact *mogR* gene (Figure 2).

Phylogenetic subgroup I corresponds to the species *B. pseudomycoides*, but also includes strains for which an original identification as *B. cereus* or *B. mycoides* has not yet been revised (Guinebretière et al., 2008). BLASTP searches were performed against available subgroup I proteomes using the corresponding protein sequences for the genes from the motility locus of *B. thuringiensis* 407 as query (Figure 2). Among six fully sequenced strains (single scaffold per replicon or closed genome) belonging to subgroup I (219298, BTZ, Rock 3-44, DSM 12442, Rock 1-4 and Rock 3-17), all contained a severely reduced motility gene cluster, and strikingly, the only conserved motility-related gene in the motility locus of these strains is *mogR* (Figure 2). The absence of all motility genes except *mogR* in all six fully sequenced group I strains analyzed, as well as in all except for one (AFS092012) of the 100 additional group I strains annotated as *B. pseudomycoides* at the NCBI and for which a

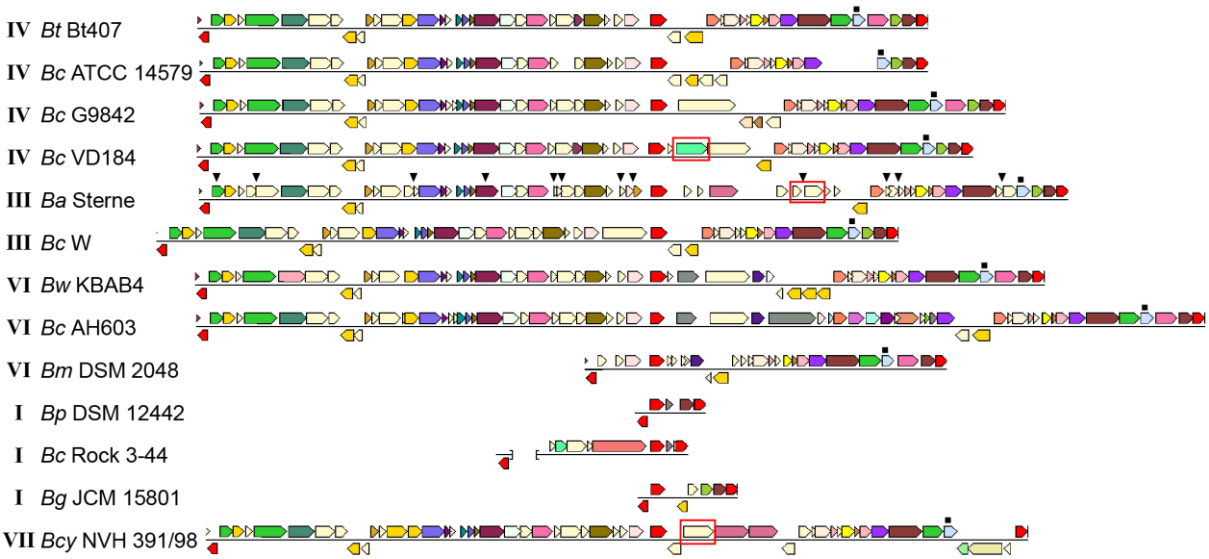

Figure 2. Comparison of motility gene loci in selected *B. cereus* group strains. Loci from different strains are aligned with respect to *mogR*, shown in red in the central part of the alignment. For each strain, the first and last gene (shown in red) are conserved genes outside of the motility gene locus itself (orthologs to BTB_RS08235 and BTB_RS08480, respectively). These are included to indicate that the flanking regions and location of the motility locus are generally conserved between strains. The last gene of the intact locus with a predicted function mapping within motility, *flgG* (light blue), is indicated by a filled square above the gene. For genes shown in a color other than red, identical color indicates that genes are from the same orthologous group (top COG hit), except light yellow which indicates no COG assignment. The *gmaR* gene is indicated with a red box. In the *B. anthracis* strain, the filled triangles above genes indicate the location of nonsense or frameshift mutations. The brackets in the representation of the *Bc* Rock 3-44 sequence indicates that this locus is split between two contigs. Roman numerals next to the strain names indicate the phylogenetic subgroup within the *B. cereus* group to which the strain belongs (Guinebretière et al., 2008). *Bm* Rock 1-4, *Bm* Rock 3-17, *Bm* 219298 og *Bp* DSM 12442 from subgroup I had indentical loci; only *Bp* DSM 12442 is shown. The figure was assembled from images obtained from the Conserved Neighbourhood Viewer of the Integrated Microbial Genomes system (Markowitz et al., 2010), using *B. thuringiensis* 407 proteins BTB_RS08235, BTB_RS08390 (*mogR*), and BTB_RS08480 as query sequences. Abbreviations: *Bc*, *B. cereus*; *Bt*, *B. thuringiensis*; *Ba*, *B. anthracis*; *Bw*, *B. weihenstephanensis*; *Bm*, *B. mycoides*; *Bp*, *B. pseudomycoides*; *Bcy*, *B. cytotoxicus*; *Bg*, *Bacillus gaemokensis*.

genome sequence at scaffold level was available, indicates that absence of motility is likely a general characteristic of this genetic group, in line with the description of *B. pseudomycoides* as a nonmotile species (Nakamura, 1998). The predicted MogR proteins of the *B. pseudomycoides* group show ~56% and ~30% pairwise identity to the corresponding orthologs in *B. thuringiensis* 407 and *L. monocytogenes* EGD-e, respectively. A multiple sequence alignment demonstrated that the *L. monocytogenes* MogR residues predicted to make base-specific DNA contacts in *L. monocytogenes* (Shen et al., 2009) have been conserved in the MogR proteins from all examined *B. cereus* group strains, including those belonging to non-motile species (Supplementary Figure S1). Interestingly, an ortholog of *gmaR*, encoding a temperature-controlled antirepressor of MogR in *L. monocytogenes* (Kamp and Higgins, 2009), is found in *B. cytotoxicus* strains (cluster VII), but is absent in most *B. cereus* group strains belonging to other phylogenetic clusters.

3.3 Flagellin gene expression drops following a sharp increase in *mogR* expression

B. thuringiensis 407 produces two flagellins, structural proteins building the flagellum main filament, from chromosomal genes *flaA* and *flaB* (Lövgren et al., 1993). Expression of *mogR* (BTB_RS08390), *flaA* (BTB_RS08400) and *flaB* (BTB_RS08395) was followed at the transcriptional level throughout the bacterial growth phase using RT-qPCR. In contrast to

previous proteomics studies which indicated that in *B. thuringiensis* 407 only the first flagellin gene (*flaA*) was expressed in early stationary phase (Gohar et al., 2005), results showed that both *flaA* and *flaB* are indeed expressed at the RNA level, although expression of *flaA* was approximately four-fold higher than that of *flaB* (Figure 3). The two *fla* genes reached maximum expression at 3.5 hours, at the transition between exponential and stationary growth phase, in agreement with corresponding data obtained using a transcriptional fusion between *lacZ* and the *flaA* promoter (Houry et al., 2010). These data also corresponded well with microscopic observations of cultures sampled throughout the growth phase. The expression of *mogR* increased 64-fold between the 2.5 hour and 3.5 hour time-point (Figure 3), peaking at 4 hours, at which time *flaA* and *flaB* expression was rapidly decreasing. This is in parallel with the expression pattern found for *mogR* in *B. anthracis* (Bergman et al., 2006). While downregulation of motility-related genes upon entry to the stationary phase is common in both Gram-negative and Gram-positive bacteria (Ramirez Santos et al., 2005; Han et al., 2017), it was nevertheless interesting to note that expression of the *flaA* and *flaB* genes dropped after 3.5 hours, subsequent to the sharp increase in *mogR* transcription (Figure 3).

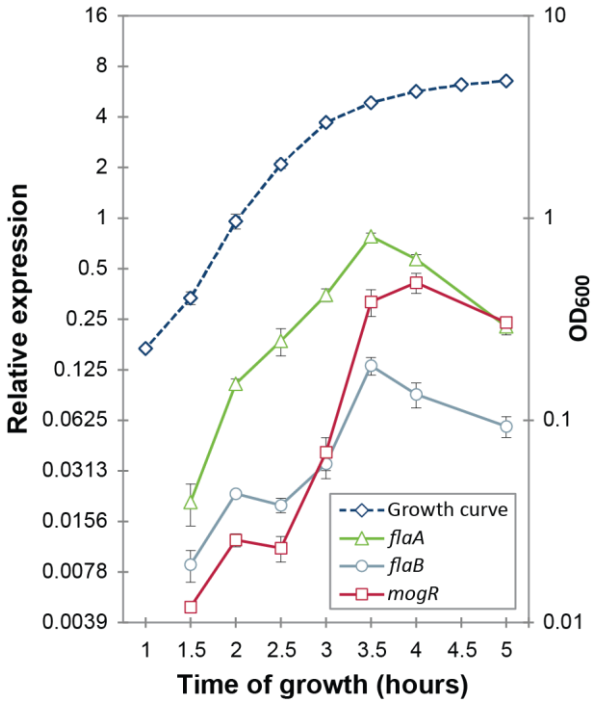

Figure 3. Gene expression analysis of *mogR* and flagellin genes *flaA* and *flaB* by RT-qPCR. The expression of each gene at each time-point was normalized to the expression of the reference genes *gatB*, *rpsU* and *udp*. Cultures were grown at 30 °C in bactopeptone culture medium, with the growth curve plotted on the right-hand vertical axis. Averages and standard errors of the means from three independent experiments are shown.

3.4 Overexpression of MogR renders *B. thuringiensis* 407 non-flagellated and non-motile

A MogR overexpression strain was constructed using the pHT304-P_{xyl} expression vector carrying a xylose-inducible promoter, as well as an isogenic strain overexpressing a version of MogR where two mutations were introduced: Q119A and N120A (plasmids and strains used are listed in Table 1). In the mutated protein (referred to as $MogR^{QN\rightarrow AA}$), alanine substitutions were made for two of the six amino acids corresponding to residues in *L. monocytogenes* MogR that were shown to make base-specific contacts with DNA, in cognate DNA recognition sites positioned upstream of *L. monocytogenes* genes that were subject to direct MogR transcriptional repression (Shen et al., 2009; Supplementary Figure S1). Cellular growth rates were not affected by MogR or $MogR^{QN\rightarrow AA}$ overexpression (relative to an empty vector control) at 25 °C, 30 °C or 37 °C (Supplementary Figure S2).

Motility of the MogR overexpression strain was investigated by swimming assays employing 0.3% LB agar plates, showing that cells overexpressing MogR were non-motile, and that the repression of motility was dependent on the two amino acid residues (Q119 and N120) mutated in MogR$^{QN\rightarrow AA}$, at 30 °C (Figures 4A,B), as well as at 25 °C and 37 °C. Swimming

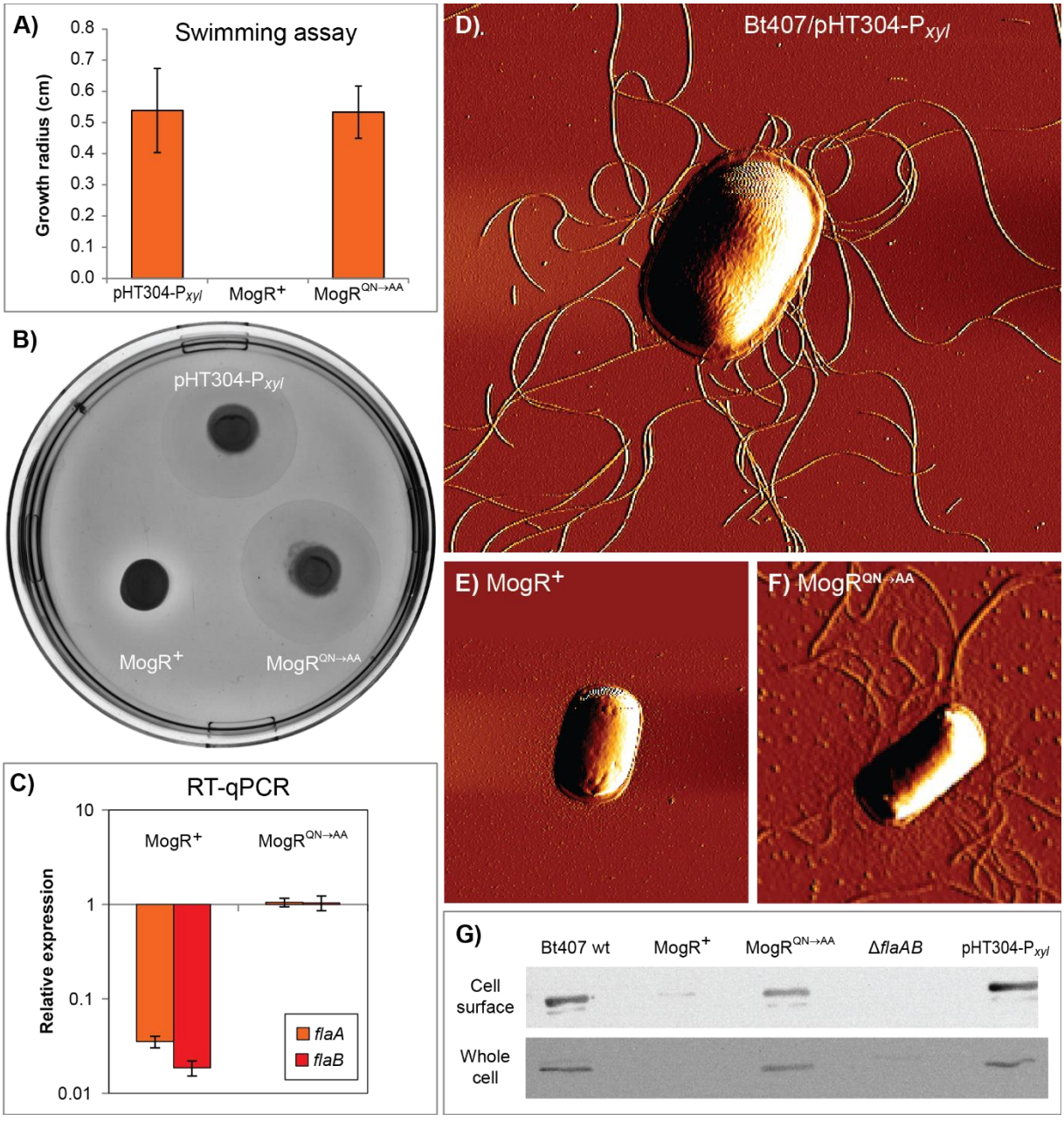

Figure 4: Analysis of motility and expression of flagella upon MogR overexpression in *B. thuringiensis* 407. Assays were conducted using the following strains: *B. thuringiensis* 407 pHT304-P$_{xyl}$ empty vector control strain (pHT304-P$_{xyl}$); *B. thuringiensis* 407 pHT304-P$_{xyl}$-*mogR* (MogR^{+}), overexpressing MogR; and *B. thuringiensis* 407 pHT304-P$_{xyl}$ -*mogR*$^{QN\rightarrow AA}$ (MogR$^{QN\rightarrow AA}$), overexpressing a mutated form of MogR (see Table 1). Expression was induced with 1 mM xylose and cells were grown at 30 °C. **(A,B)** Swimming motility was determined following growth on 0.3% LB agar for 7 hours. In **(A)**, the mean and corresponding standard error values obtained from three independent experiments are shown. **(C)** Gene expression analysis of *flaA* and *flaB* by RT-qPCR. The relative expression of *flaA* and *flaB* in the MogR or MogR$^{QN\rightarrow AA}$ strains was normalized to the expression level of each respective gene in the empty vector control strain. Averages and standard errors of the means from three experiments are shown. **(D,E,F)** Atomic Force Microscopy images of bacterial cells grown to an OD_{600} of 3. **(G)** Western immunoblots showing the level of flagellin protein present in cell surface and whole cell extracts. Here, the *B. thuringiensis* 407 wild type (wt) and *B. thuringiensis* 407 Δ*flaAB* strains were included as controls.

motility was also monitored by light microscopy and found to be completely lost upon MogR overexpression at all time points and temperatures examined (every half hour between 1 and 7 hours of cultivation, at 25 °C, 30 °C and 37 °C). To determine whether the reduced motility was due to a loss of flagellar structure or flagellar rotation, strains were analyzed by Atomic Force Microscopy (AFM). AFM analyses showed that the MogR overexpression strain was completely devoid of flagellar structures, while overexpression of $MogR^{QN\rightarrow AA}$ restored the empty vector control phenotype (Figures 4D,E,F). In accordance, a Western blot of the MogR overexpression strain using anti-flagellin antibodies showed that flagellin proteins were below the level of detection in whole cell extracts and severely attenuated in outer cell surface extracts compared to in the vector control strain, while being expressed at comparable levels in the *B. thuringiensis* 407 wild type strain and in *B. thuringiensis* 407 overexpressing $MogR^{QN\rightarrow AA}$ (Figure 4G). In a Δ*flaAB* negative control strain in which the genes encoding the FlaA and FlaB flagellins had been deleted, no flagellin proteins could be detected neither in the whole cell extract nor on the cell surface. In *L. monocytogenes*, flagellin genes were previously found to be directly repressed by MogR, and in order to investigate if MogR function was conserved in *B. thuringiensis* 407, the expression of flagellin genes was investigated in the MogR overexpression strain relative to the empty vector and $MogR^{QN\rightarrow AA}$ control strains, by quantitative RT-PCR. A 30-fold (*flaA*) and 50-fold (*flaB*) reduction in transcription, respectively, was seen in the MogR overexpression strain relative to the empty vector control, while no difference was observed relative to the empty vector strain when overexpressing $MogR^{QN\rightarrow AA}$ (Figure 4C).

3.5 Overexpression of MogR affects the expression of genes not related to motility functions

Global microarray-based transcriptional profiling was used to identify genes whose expression was affected by MogR overexpression in *B. thuringiensis* 407 relative to the empty vector control strain. Samples were taken in the early stationary growth phase (3 hours), and a total of 110 genes were identified to be differentially regulated by MogR (FDR-corrected $p<0.05$), either directly or indirectly. Selected genes are listed in Table 2 (see Supplementary Table S3 for complete list). Of the differentially expressed genes, 87 were repressed by MogR overexpression, while 23 genes exhibited higher transcriptional levels compared to the control. In total, 21 motility genes were downregulated by MogR overexpression, including flagellin genes *flaA* and *flaB* (Supplementary Figure S3). Overexpression of MogR affected genes widely dispersed in the motility loci, including the *motAB* operon located separately in the chromosome. Interestingly, the microarray analysis revealed that MogR also affected the expression of 89 non-motility-related genes, including six virulence genes which were downregulated. The latter genes included *inhA* encoding immune inhibitor A, *hblA* and *hblD* encoding enterotoxin binding component B and lytic component L1, respectively, *sfp* encoding a subtilase family serine protease, as well as genes encoding phosphatidyl-choline specific phospholipase C and sphingomyelinase. Also downregulated were five stress-related genes: the *groES*, *groEL* and *grpE* chaperonin genes, *sigB* encoding the σ^B alternative sigma factor, and *hrcA*. The gene encoding the pleiotropic transcriptional regulator NprR (Dubois et al., 2012) was also downregulated. Among the 23 genes upregulated in the MogR overexpression strain were *sinI*, encoding a protein inducing biofilm formation by direct interaction and sequestration of the biofilm repressor SinR, and a putative capsular polysaccharide biosynthesis protein-encoding gene (BTB_RS26935) corresponding to BC5278 in *B. cereus* strain ATCC 14579. The genes BC5267 to BC5278 constitute a conserved locus in *B. cereus* and *B. thuringiensis* and are homologous to the *epsAO* locus of *B. subtilis* involved in the synthesis of the exopolysaccharide component of the biofilm matrix (Branda et al., 2001; Ivanova et al., 2003; Kearns et al., 2005; Fagerlund et al., 2014). Genes

in this locus have been found to be implicated in biofilm formation in *B. cereus* in a pellicle biofilm model (Hayrapetyan et al., 2015; Okshevsky et al., 2017). Another upregulated gene was *cbpA*, encoding a collagen adhesion protein positively regulated by c-di-GMP (Finke et al., 2019).

Table 2. Genome-wide gene expression analysis of *B. thuringiensis* 407 overexpressing MogR relative to an empty vector control strain using oligonucleotide microarrays

Gene	Locus tag in *B. thuringiensis* 407	Locus tag in *B. cereus* ATCC 14579	Predicted function	$\log_2$(fold change) in MogR overexpression strain relative to control
Motility-related gene function				
cheA	BTB_RS08255	BC1628	Chemotaxis protein, histidine kinase CheA	-1.80
flgK	BTB_RS08295	BC1636	Flagellar hook-associated protein 1, FlgK	-1.67
fliS	BTB_RS08310	BC1639	Flagellar biosynthesis protein FliS	-0.68
flgC	BTB_RS08325	BC1642	Flagellar basal-body rod protein FlgC	-2.87
fliF	BTB_RS08335	BC1644	Flagellar M-ring protein FliF	-2.31
fliG	BTB_RS08340	BC1645	Flagellar motor switch protein FliG	-1.09
fliK	BTB_RS08355	BC1649	Flagellar hook-length control protein FliK	-1.47
cheV	BTB_RS08385	BC1654	Chemotaxis signal transduction protein CheV	-1.80
flaB	BTB_RS08395	BC1656	Flagellin FlaB	-1.04
flaA	BTB_RS08400	BC1657	Flagellin FlaA	-3.45
fliN	BTB_RS08425	BC1661	Flagellar motor switch protein (fliN-homolog)	-0.99
fliP	BTB_RS08430	BC1665	Flagellar biosynthesis protein FliP	-1.86
fliQ	BTB_RS08435	BC1666	Flagellar export apparatus protein FliQ	-1.23
fliR	BTB_RS08440	BC1667	Flagellar biosynthesis protein FliR	-1.02
flhB	BTB_RS08445	BC1668	Flagellar biosynthesis protein FlhB	-1.60
flhA	BTB_RS08450	BC1669	Flagellar biosynthesis protein FlhA	-1.43
motB	BTB_RS22905	BC4512	Flagellar motor protein MotB (H^+-coupled stator)	-3.01
motA	BTB_RS22910	BC4513	Flagellar motor protein MotA (H^+-coupled stator)	-1.74
mcpA	BTB_RS02175	BC0422	Methyl-accepting chemotaxis protein (carrying upstream "off" c-di-GMP riboswitch)	-1.31
Virulence- related gene function				
nprR	BTB_RS03040	BC0598	Transcriptional activator NprR (necrotrophism regulator	-0.89
inhA	BTB_RS03410	BC0666	Immune inhibitor A	-1.03
plcB	BTB_RS03430	BC0670	Phospholipase C (PC-PLC)	-2.32
sph	BTB_RS03435	BC0671	Sphingomyelinase C (Smase)	-1.47
hblA	BTB_RS12545	BC3102	Hbl component B	-1.13
hblD	BTB_RS12540	BC3103	Hbl component L1	-1.59
sfp	BTB_RS18825	BC3762	Serine protease (subtilase family)	-0.84
cbpA	BTB_RS05575	BC1060	Collagen adhesion protein (carrying upstream c-di-GMP "on" riboswitch)	3.02
Putative biofilm-related gene function				
sinI	BTB_RS06545	BC1283	SinI biofilm repressor antagonist	1.08
ywgC	BTB_RS26935	BC5278	Putative capsular polysaccharide biosynthesis protein	0.71

3.6 Q119 and N120 are critical for MogR binding to specific sites in the *B. thuringiensis* 407 genome *in vitro*

DNA regions upstream of genes differentially expressed in the *B. thuringiensis* 407 MogR overexpression strain were investigated for candidate MogR binding sites, based on sequence searches using the *L. monocytogenes* consensus MogR binding sequence (TTTTWWNWWAAAA; Shen et al., 2009). Three putative binding sites were predicted in the intergenic region upstream of the flagellin (*flaAB*) operon, two potential sites upstream of *cheY*, while four putative binding sites were predicted in the promoter region of the *hbl* operon (Lindbäck et al., 1999), all overlapping the -10 promoter element (Figure 5A). Among

the other genes downregulated by MogR overexpression, multiple binding sites were found upstream of *inhA*, as well as upstream of the biofilm-promoting gene *sinI* and *mogR* itself.

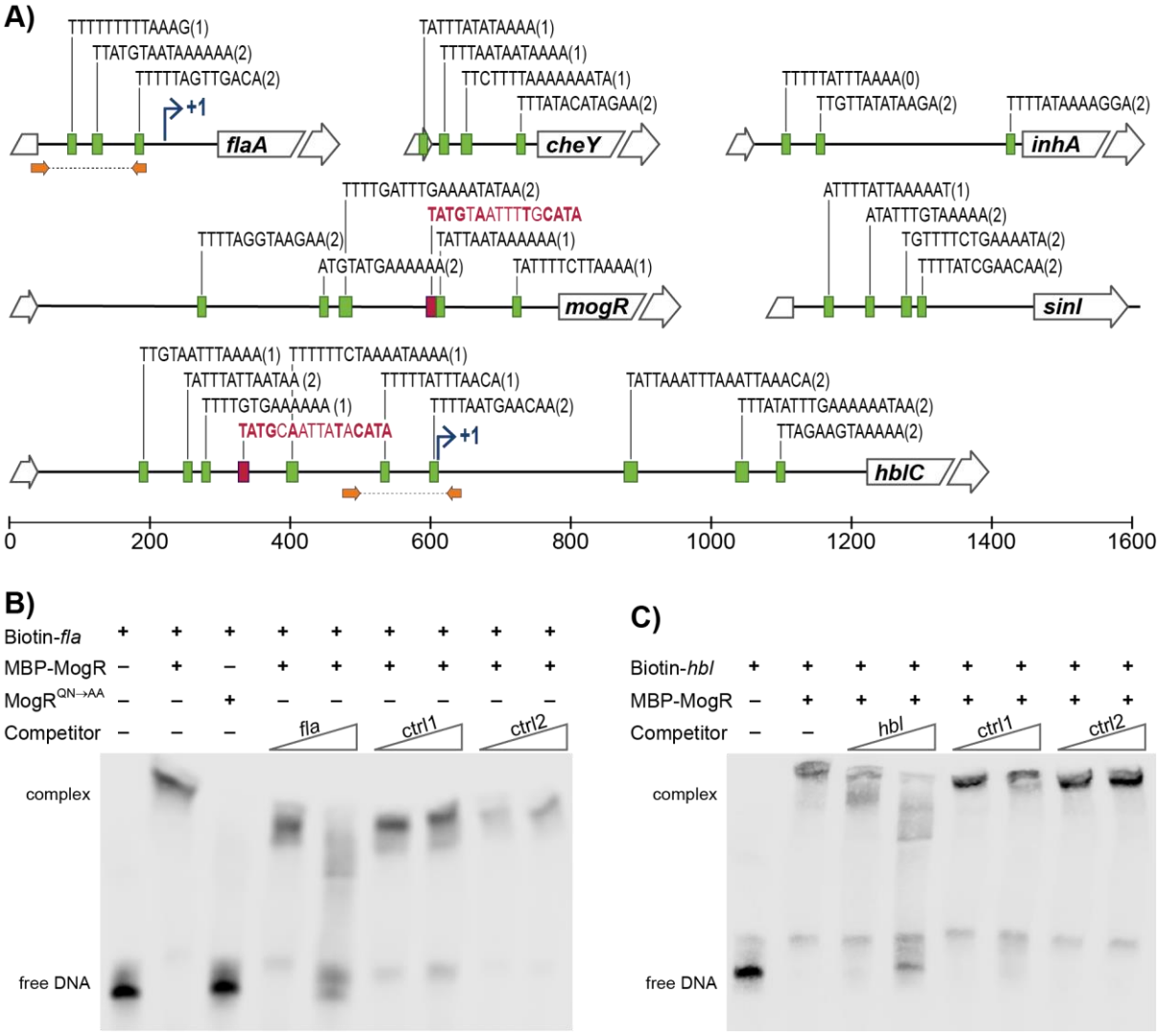

Figure 5: MogR binding to promoter regions in *B. thuringiensis* 407. (A) Putative MogR binding sites in the intergenic regions of *flaA, cheY, inhA, mogR, sinI* and *hblC* genes, allowing up to two mismatches from the sequence TTTTWWNWWAAAA, with the number of mismatches in parentheses. Putative PlcR boxes, TATGNANNNNTNCATA, are included in red (only perfect matches included). Broken arrows indicates transcriptional start sites where applicable. Location of primers used for generating *fla* and *hbl* promoter sequence probes for the electrophoretic mobility shift assay (EMSA) in **(B,C)** are indicated with orange arrows. **(B,C)** EMSAs of purified MBP-MogR fusion protein binding to purified PCR products representing the promoter regions of *flaA* **(B)** and *hblC* **(C)**, as indicated in **(A).** Biotin-labeled *flaA* or *hblC* DNA fragments (2 µM) were incubated with 1 µg purified MBP-MogR protein, in the presence or absence of unlabeled competitor DNA (50× or 1000×): *flaA* or *hblC* fragment, ctrl1 (*B. thuringiensis* 407 BTB_RS08990), and ctrl2 (gene encoding Epstein Barr virus nuclear antigen). In **(B)**, MBP-MogR$^{QN\rightarrow AA}$ was included as an extra control of binding of the mutated protein to the promoter DNA.

To investigate whether MogR may affect the expression of candidate genes in *B. thuringiensis* 407 by directly binding to upstream regulatory regions, we carried out electrophoretic mobility shift assays (EMSA) with a purified maltose-binding protein (MBP)-MogR fusion protein, and purified DNA fragments constituting PCR-amplified candidate regulatory regions (Figure 5A). Results showed that MogR could bind the promoter regions upstream of both

flaAB and the *hbl* enterotoxin locus *in vitro* (Figures 5B,C), while for MogR$^{QN\rightarrow AA}$ DNA binding was abolished (Figure 5B), demonstrating the essentiality of the Q119 and N120 residues for DNA-binding. The binding reactions to which competing cold DNA were added (labelled ctrl1 and ctrl2 in Figures 5B,C) indicated that MogR exhibits sequence specific binding to *flaAB* and *hbl* upstream regions *in vitro*.

3.7 Cytotoxin production and *in vivo* virulence are reduced upon MogR overexpression

Western blot analyses were performed to measure the effect of MogR overexpression on the amount of secreted enterotoxins. Hbl, CytK and Nhe enterotoxins were all present but at clearly reduced levels in the culture supernatant from the MogR overexpression strain, compared to that of empty vector and MogR$^{QN\rightarrow AA}$ control strains (Figure 6A). To further assess the effect of MogR on virulence capacity, supernatants collected from mutant and overexpression strains grown to exponential phase were tested in an *in vitro* Vero cell toxicity assay (Lindbäck and Granum, 2006). Supernatants from the MogR overexpressing strain, which synthesized lower amounts of the Hbl, Nhe and CytK toxins (Figure 6A), were less cytotoxic than those of the empty vector control (p=0,059) and the MogR$^{QN\rightarrow AA}$ strain (Figure 6B). In order to more confidently assess the effect of MogR on virulence, an *in vivo* model was used, where the strains were tested in an oral infection model using *Galleria mellonella* larvae. In accordance with the *in vitro* toxicity data, the MogR overexpressing strain was severely attenuated in virulence compared to the empty vector control strain *in vivo* (p=0.02; Figure 6C).

3.8 Biofilm formation is increased upon MogR overexpression in a *flaAB* negative background

The MogR overexpression strain was compared to the empty vector and MogR$^{QN\rightarrow AA}$ strains both in glass tube and microtiter plate biofilm assays (Figure 7A,B). The glass tube assay showed similar development of a biofilm pellicle at the air-liquid surface in all three strains: After 72 hours the pellicle in the MogR overexpression strain was only slightly (14%) thicker than that of the empty vector and MogR$^{QN\rightarrow AA}$ strains (Figure 7A). In the microtiter plate assay, no significant effect of MogR overexpression on biofilm formation was observed at 48 or 72 hours, with a slight decrease in biofilm formation at 24 hours compared to MogR$^{QN\rightarrow AA}$ and empty vector controls (Figure 7B). Overexpression in a Δ*flaAB* background however allowed examination of the effect of the MogR protein on biofilm formation, independently of any effect conferred through flagella. Interestingly, in the Δ*flaAB* host, overexpression of MogR led to a substantial increase in biofilm formation relative to the MogR$^{QN\rightarrow AA}$ and empty vector controls (Figure 7C), while biofilm formation in the control strain overexpressing MogR$^{QN\rightarrow AA}$ was comparable to that of the strain carrying empty vector. Intriguingly, LC-MS/MS quantitation of the biofilm-related second messenger cyclic di-GMP (c-di-GMP) in cellular extracts indicated slightly increased levels of c-di-GMP in the MogR overexpression strain (wild type background; three measurements: 8.38 ng ml^{-1}; 6.0 ng ml^{-1}; 9.0 ng ml^{-1}) compared to the *B. thuringiensis* 407 empty vector strain (two measurements: < LOD; 6.0 ng ml^{-1}) (Limit of detection, LOD: 0.8 ng ml^{-1}; Limit of quantitation, LOQ: 3.5 ng ml^{-1}).

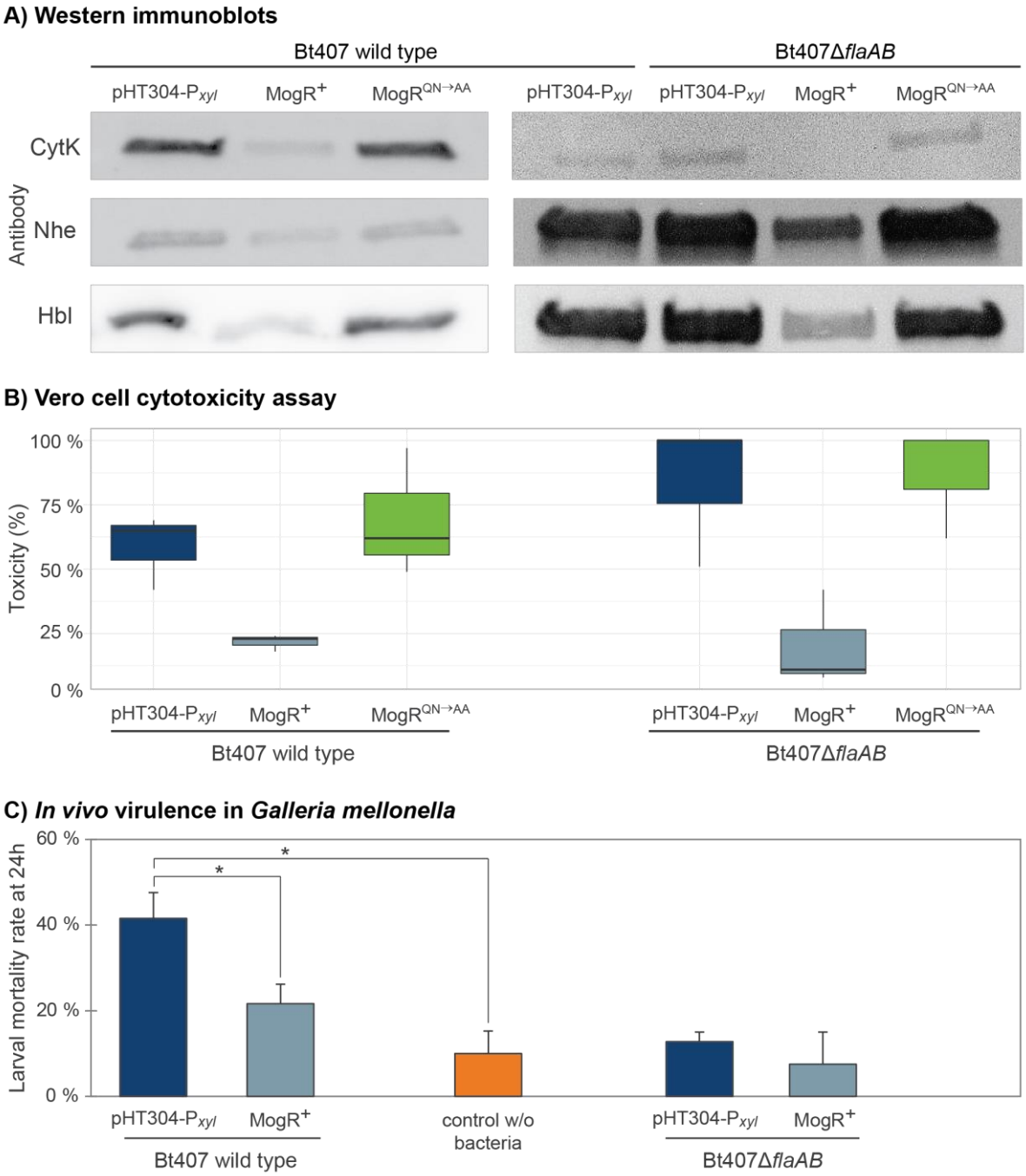

Figure 6. Analysis of the effect of overexpression of MogR on virulence and toxicity phenotypes. (A) Immunoblots against CytK, Nhe and Hbl enterotoxins using culture supernatants from *B. thuringiensis* 407 carrying the empty vector (pHT304-P*xyl*), a MogR overexpression strain (MogR^{+}), and a strain overexpressing a mutated form of MogR (MogR$^{QN\rightarrow AA}$). Constructs were analysed in *B. thuringiensis* 407 wild type and *flaAB* deletion (Δ*flaAB*) backgrounds. Cultures were grown for 4.5 hours with 1 mM xylose induction prior to harvest. **(B)** Vero cell cytotoxicity assay using supernatants from cultures of the same strains as in **(A)**. The median and first and third quartiles (25th percentile and 75th percentile) of three independent experiments are shown, as well as whiskers indicating the data variability outside the upper and lower quartiles. **(C)** *In vivo* toxicity in *Galleria mellonella* insect larvae (oral force-feeding infection model), using empty vector and MogR overexpression strains, in *B. thuringiensis* 407 wild type and Δ*flaAB* backgrounds, respectively. Bacteria were in all cases mixed with Cry 1C toxin and xylose. Also, a control with no bacteria but fed with Cry toxin and xylose was included. The means and standard errors of the mean (bars) of seven independent experiments are shown. Statistically significant differences (Tukey's *post hoc* test, $p<0.02$) are indicated with asterisks.

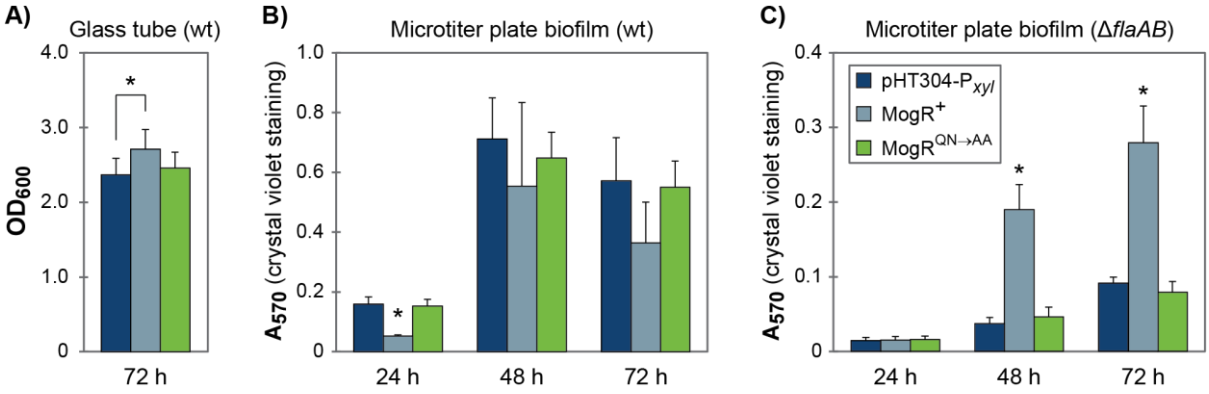

Figure 7. MogR overexpression affects biofilm formation. (A) Biofilm formation in glass tubes after 72 hours of growth. **(B,C)** Biofilm formation in a crystal violet microtiter plate assay after 24, 48, and 72 hours of growth. The legend shown in **(C)** also applies to figures **(A,B)**. The means and standard error of the means for **(A)** five or **(B, C)** three independent experiments is shown. Statistically significant differences (within each time point; Tukey's *post hoc* test, $p<0.05$) are indicated with an asterisk; in **(B,C)** the asterix indicates that the MogR$^+$ strain demonstrates a statistically significant difference to both the empty vector control strain and the overexpression of a mutated form of MogR (MogR$^{QN\rightarrow AA}$). In **(A)**, only the MogR$^+$ and empty vector control strains (pHT304-P$_{xyl}$) were significantly different.

4 Discussion

Overexpression of MogR leads to repression of a range of motility genes in *B. thuringiensis* 407, including the flagellins, rendering the bacteria non-flagellated and non-motile. In addition, amino acids in *B. thuringensis* MogR which correspond to those shown to constitute DNA backbone-interacting residues in *L. monocytogenes* MogR are clearly essential for *B. thuringiensis* MogR DNA binding *in vitro*. In accordance, overexpression of the mutated variant of MogR restores phenotypes to those of a vector control strain. Collectively these results, and analyses of MogR binding to predicted regulatory sites *in vitro*, strongly support the role of *B. thuringiensis* MogR as a DNA-binding transcriptional regulator and that the role of MogR as a key motility regulator in *B. thuringiensis* is conserved with that in *L. monocytogenes*. Global gene expression profiling however showed that in *B. thuringiensis* a large number of non-motility genes were also affected by MogR overexpression, including genes involved in stress response, virulence and biofilm formation. This was paralleled by an observed decrease in cytotoxin production, Vero cell toxicity and *in vivo* toxicity, indicating that MogR can affect additional phenotypes in *B. cereus* group bacteria. This is in apparent contrast to *L. monocytogenes*, where the primary function of MogR during intracellular infection is to repress flagellar motility gene expression, and where deletion of the flagellin gene (*flaA*) in a *mogR* deletion strain (producing Δ*mogR* Δ*flaA*) restores the LD$_{50}$ values to wild type levels in a mouse model of infection (Shen and Higgins, 2006).

In bacteria, motility and virulence can be subject to coordinated downregulation, e.g. as is observed for intracellular signaling networks involving the second messenger c-di-GMP (Fagerlund et al., 2016). Flagellar motility and virulence appears to be co-regulated in *B. cereus* group bacteria (Zhang et al., 1993; Ghelardi et al., 2002; Bouillaut et al., 2005; Ghelardi et al., 2007a; Fagerlund et al., 2010; Salvetti et al., 2011; Mazzantini et al., 2016), and a *B. cereus* strain deleted in *flhA*, which encodes a component of the flagellum-formation apparatus, is impaired in adhesion (Ramarao and Lereclus, 2006), expression of virulence factors (Ghelardi et al., 2002; Fagerlund et al., 2010), and in cytotoxicity and *in vivo* virulence independently of PlcR (Bouillaut et al., 2005). Similarly, deletion of the gene encoding the

signal recognition particle-like GTPase FlhF, located directly downstream of *flhA*, resulted in a marked reduction in the number of flagella, and a reduction in secretion of several virulence-related proteins with attenuation of *in vivo* virulence (Salvetti et al., 2007; Mazzantini et al., 2016; Mazzantini et al., 2020). A link between synthesis of the flagellar apparatus and the production of virulence factors has also been observed for *flhA* deletion mutants in other bacteria that do not carry a *mogR* ortholog (Fleiszig et al., 2001; Carrillo et al., 2004). The observed effects of MogR on toxin production and virulence-related phenotypes may thus be conferred through direct regulation, as potentially indicated through *in vitro* binding to the *hbl* upstream region, or indirectly through repression of genes in the motility locus, such as e.g. *flhA* and/or *flhF*, or both. MogR appears to function independently of the virulence gene activator PlcR, as *plcR* expression was not affected by MogR overexpression, and only six out of the 28 genes comprising the PlcR regulon were differentially expressed in the microarray analysis. Clearly, the effect on toxin production observed following deletion of *flhA* or *flhF* was not replicated in the *flaAB* deletion mutant, showing that not all deletions in the motility locus affect toxin synthesis and/or transport.

When employing a Δ*flaAB* genetic background to compare the effect of MogR overexpression on biofilm formation, relative to empty vector and $MogR^{QN\rightarrow AA}$ overexpression controls, a significant increase in biofilm formation was observed by MogR overexpression. In line with this, the transcriptional profiling experiments showed upregulated expression of the biofilm anti-repressor *sinI,* and BTB_RS26935 located in a putative polysaccharide locus previously shown to be implicated in the formation of the biofilm matrix in some *B. cereus* strains (Hayrapetyan et al., 2015; Okshevsky et al., 2017). Moreover, LC-MS/MS quantitation indicated a slight increase in cellular c-di-GMP upon MogR overexpression, which is linked to increased biofilm formation in *B. thuringiensis* 407 (Fagerlund et al., 2016; Fu et al., 2018). A microarray experiment analyzing the effect of *B. thuringiensis* 407 overexpressing the c-di-GMP synthesizing protein CdgF relative to an empty vector control strain (Fagerlund et al., 2016; A. Fagerlund, unpublished data deposited in the ArrayExpress database, accession no. E-MTAB-8898) showed that the expression of the biofilm regulators *spo0A* and *sinI* were upregulated and the transcriptional regulator *codY* was downregulated at high levels of c-di-GMP, all paralleling an increase in biofilm formation (Pflughoeft et al., 2011; Lindbäck et al., 2012; Fagerlund et al., 2014). Taken together, these experiments provide strong indication that MogR, directly or indirectly promotes biofilm formation in *B. thuringiensis* 407, a phenotype in line with the role of the protein in promoting sessility and repressing toxicity.

In *L. monocytogenes,* MogR activity is controlled by its anti-repressor GmaR (Kamp and Higgins, 2009). GmaR expression is halted when *L. monocytogenes* experiences 37 °C, which allows MogR to repress flagellar synthesis during human infection. The *gmaR* gene is not present in most *B. cereus* group genomes, in line with *mogR* appearing to be regulated in a growth-dependent but temperature-independent manner in *B. thuringiensis* 407. It is however interesting to note that a *gmaR* ortholog is found in at least twelve sequenced *B. cereus* group strains, including all hitherto characterized strains of the thermotolerant species *B. cytotoxicus*, perhaps reflecting a need for strains in this phylogenetic group (group VII) to regulate flagellin synthesis at different temperatures. Most *B. anthracis* strains also harbor a copy of *gmaR*, which however is truncated and rendered non-functional due to mutation. Uncharacteristically, an apparently full-length *gmaR* copy was identified in the genome of *B. cereus* VD184, an environmental soil isolate belonging to phylogenetic cluster IV (Hoton et al., 2009; Jalasvuori et al., 2013), as well as *B. cereus* strains RU36C, F528-94, MB.22, B4118 and FSL W8-0932, and *Bacillus wiedmannii* FSL P4-0569, for which optimal growth temperatures are not known.

In conclusion, MogR is a key regulator of motility in *B. thuringiensis* 407, a function which is probably conserved across the motile species in the *B. cereus* group. Most importantly however, both microarray experiments, protein expression analyses, biofilm screening results, and the fact that the *mogR* gene is evolutionary retained in the non-motile *B. mycoides*, *B. pseudomycoides*, and the highly virulent *B. anthracis*, as well as expressed in *B. anthracis*, speaks to the MogR transcriptional regulator serving additional functions in *B. cereus* group bacteria, beyond those identified in *L. monocytogenes*. In this respect it is interesting to note that all attempts to produce a gene deletion mutant in *mogR* in *B. thuringiensis* 407 were unsuccessful, and we cannot rule out the possibility of *mogR* constituting an essential gene in the *B. cereus* group. From the analyses performed in this study, it is tempting to speculate that MogR may serve as part of a molecular system coordinating motility, virulence, and biofilm formation, and possibly additional phenotypes. Biofilm formation and motility are reciprocally regulated in a number of bacterial species, including *B. cereus* group bacteria, by the second messenger c-di-GMP (Hengge, 2009; Fagerlund et al., 2016; Jenal et al., 2017; Fu et al., 2018), and several transcriptional regulators in both the *B. cereus* group and *L. monocytogenes* are known to have overlapping functions in regulation of virulence and biofilm formation (Hsueh et al., 2006; Lemon et al., 2010; Pflughoeft et al., 2011; Frenzel et al., 2012; Lindbäck et al., 2012; Fagerlund et al., 2014; Slamti et al., 2015; Böhm et al., 2016). It thus appears that, in the *B. cereus* group, there is substantial cross-regulation between motility, virulence, and biofilm formation, involving a multitude of regulatory networks based on both c-di-GMP and transcription factors (PlcR, MogR, NprR).

5 Data availability statement

The microarray data comparing overexpression of MogR with the empty vector control is available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-7633.

6 Author Contributions

OØ and AF contributed to conception and design of the study. VS performed EMSA, motility assays, Western immunoblotting, and biofilm assays. VS and AF performed RT-qPCR. VS, MJ and AF performed cloning and mutagenesis. MJ performed the microarray experiment, SF performed growth experiments, IH performed AFM, TL performed cytotoxicity assays, and CN performed the *in vivo* toxicity assays. AF performed bioinformatic, microarray, and statistical analyses and NT performed bioinformatic analysis of the subgroup I genomes. VS, OØ, and AF wrote the first draft of the manuscript. TL, IH, and CN wrote sections of the manuscript. All authors contributed to data analysis, read and approved the final manuscript.

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8 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be interpreted as a potential conflict of interest.

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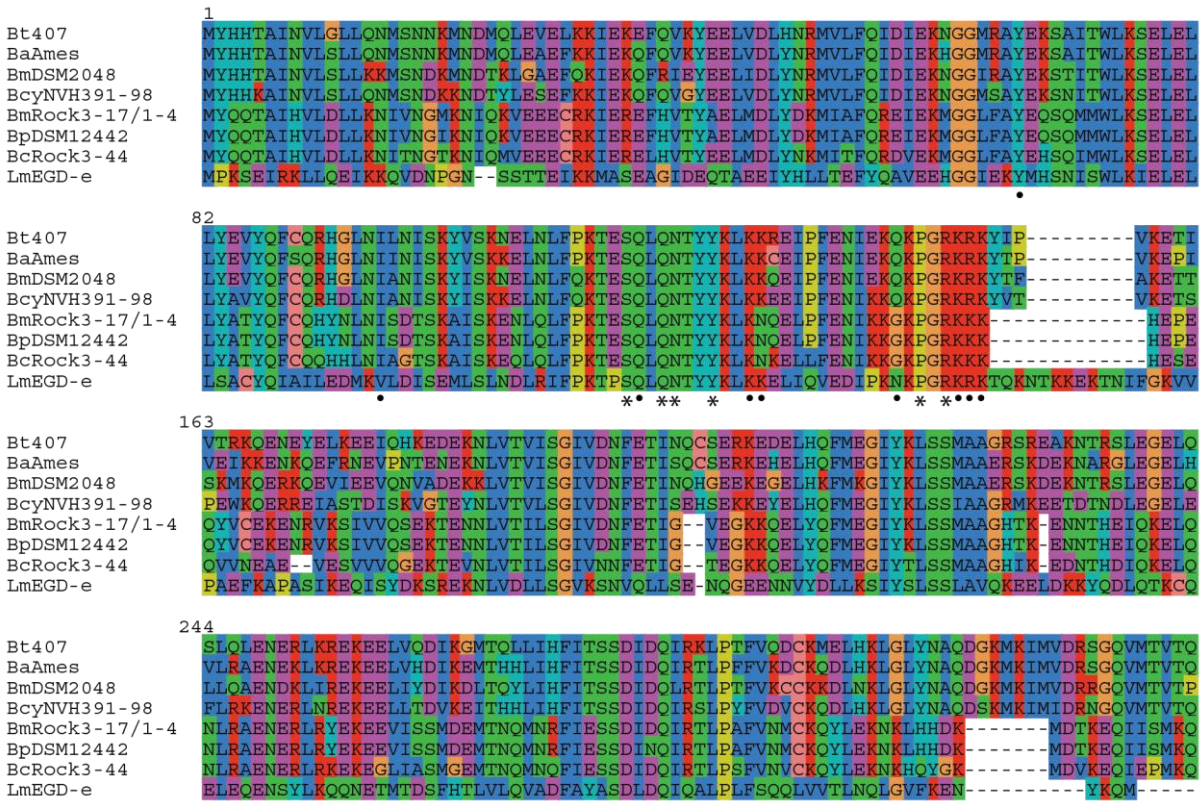
Supplementary Material

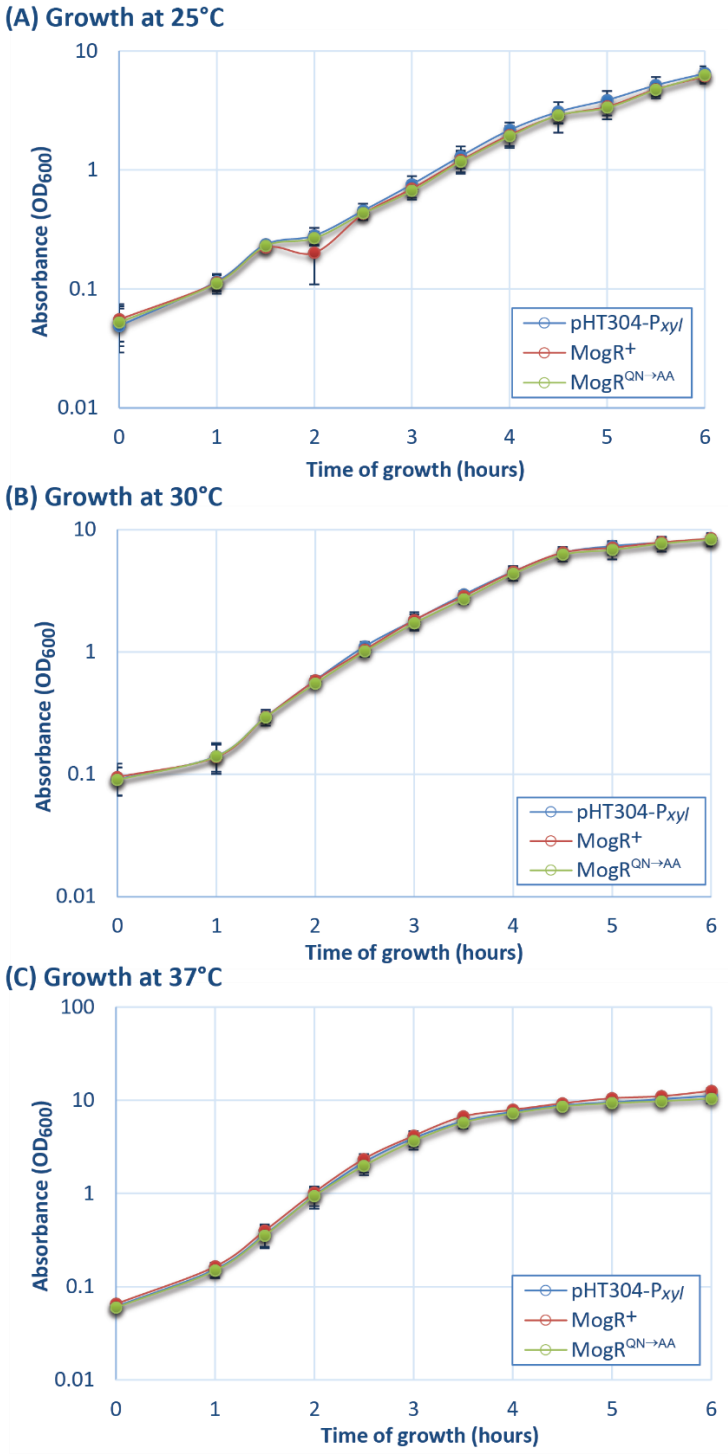
MogR is a ubiquitous transcriptional repressor affecting motility, biofilm formation and virulence in the *Bacillus cereus* group

Veronika Smith, Malin Josefsen, Toril Lindbäck, Ida K. Hegna, Sarah Finke, Nicolas J. Tourasse, Christina Nielsen-LeRoux, Ole Andreas Økstad, Annette Fagerlund

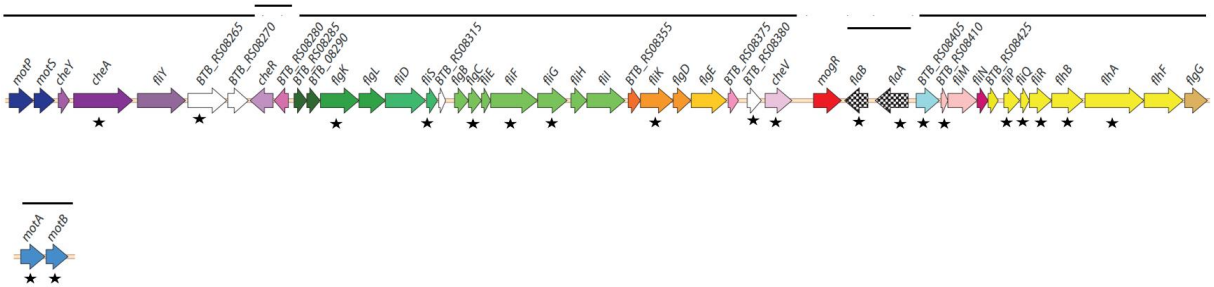
1 Supplementary Figures



Supplementary Figure S1. Multiple sequence alignment of MogR protein sequences. MogR sequences were extracted from *L. monocytogenes* strain EGD-e (locus tag: lmo0674), *B. cytotoxicus* NVH 391/98 (Bcer98_1372), and *B. thuringiensis* 407 (BTB_RS08390), in addition to the following predicted non-motile strains: The group I strains *B. cereus* Rock 3-44 (bcere0022_13670), *B. pseudomycoides* DSM 12442 (bpmyx0001_15070) and *B. mycoides* Rock 3-17 and Rock 1-4 (bmyco0002_14720 and bmyco0003_14260, which are identical), the *B. anthracis* Ames strain (BA_1692), and the group VI strain *B. mycoides* DSM 2048 (bmyco0001_14970). The annotated start site of the MogR protein from *B. mycoides* DSM 2048 was adjusted to fit the start site predicted by EasyGene (Larsen and Krogh, 2003). The N-terminal amino acid sequence MLSGSEYK of MogR from the group VI *B. mycoides* strains and *B. pseudomycoides* DSM 12442 were trimmed from the alignment. Residues making DNA backbone contacts and those making base specific DNA contacts, as determined for *L. monocytogenes* MogR by Shen et al. (2009), are marked with a solid dot and a star, respectively.



Supplementary Figure S2. Growth curves at A) 25 °C, B) 30 °C and C) 37 °C. Assays were conducted using *B. thuringiensis* 407 pHT304-P_{xyl}, an empty vector control strain (pHT304-P_{xyl}); *B. thuringiensis* 407 pHT304-P_{xyl}-*mogR* (MogR^{+}), a strain overexpressing MogR from the pHT304-P_{xyl} plasmid vector; and *B. thuringiensis* 407 pHT304-P_{xyl}-*mogR*$^{QN\rightarrow AA}$ (MogR$^{QN\rightarrow AA}$), a strain overexpressing a mutated form of MogR from the pHT304-P_{xyl} vector.



Supplementary Figure S3. Graphic representation of genes in the motility loci from *B. thuringiensis* 407. Solid lines above genes indicate operon structures, predicted from RNA-Seq data obtained from *B. cereus* strains ATCC 10987 and ATCC 14579 (Kristoffersen et al., 2012). Annotated genes are indicated with gene names positioned above the arrows. Genes affected by MogR overexpression are indicated by an asterisk.

2 Supplementary Tables

Table S1. Reciprocal best BLAST hit analysis for motility proteins.
Genomes of the following strains were subject to analyses: *B. thuringiensis* 407, *L. monocytogenes* EGD-e, and *B. subtilis* 168.

		Locus tags and seqIDs. Reference proteins are in shown in bold			Reciprocal best Blast hits*		
Function / gene product	**gene**	**Bt407**	**EGDe**	**Bs168**	**Bt407 vs EGDe**	**Bt407 vs Bs168**	**EGDe vs Bs168**
Flagellar motor protein MotP (Na^+-coupled stator)	*motP*	**BTB_RS08240**		BSU29730_ ytxD	no	yes (37%/92%)	no
Flagellar motor protein MotS (Na^+-coupled stator)	*motS*	**BTB_RS08245**		BSU29720_ ytxE	no	yes (39%/95%)	no
Chemotaxis protein, response regulator CheY	*cheY*	**BTB_RS08250**	**lmo0691_cheY**	**BSU16330_cheY**	yes (75%/98%)	yes (68%/100%)	yes (69%/95%)
Chemotaxis protein, histidine kinase CheA	*cheA*	**BTB_RS08255**	**lmo0692_cheA**	**BSU16430_cheA**	yes (56%/105%)	yes (44%7103%)	yes (45%/103%)
Flagellar motor switch FliY	*fliY*	**BTB_RS08260**	**lmo0700**	**BSU16320_fliY**	yes (42%/99%)	yes (35%/76%)	yes (38%/65%)
hypothetical protein		**BTB_RS08265**			no	no	
hypothetical protein		**BTB_RS08270**			no	no	
Chemotaxis protein, methyltransferase CheR	*cheR*	**BTB_RS08275**	**lmo0683**	BSU22720_cheR	yes (44%/98%)	yes (48%/98%)	yes (47%/98%)
hypothetical protein		**BTB_RS08280**	**lmo0687**		yes (43%/100%)	no	no
Protein of unknown function (DUF327)		**BTB_RS08285**	**lmo0703**	BSU00300_yaaR	yes (48%/93%)	yes (25%/89%)	yes (24%/55%)
FlgN-like superfamily protein		**BTB_RS08290**			no	No	
Flagellar hook-associated protein FlgK	*flgK*	**BTB_RS08295_flgK**	**lmo0705_flgK**	**BSU35410_flgK**	yes (35%/62%)	yes (27%/47%)	yes (22%/106%)
Flagellar hook-associated protein FlgL	*flgL*	**BTB_RS08300_flgL**	**lmo0706_flgL**	**BSU35400_flgL**	yes (30%/101%)	yes (27%/93%)	yes (28%/69%)
Flagellar hook-associated protein FliD	*fliD*	**BTB_RS08305_fliD**	**lmo0707_fliD**	**BSU35340_fliD**	yes (28%/97%)	yes (24%/81%)	yes (24%/107%)
Flagellar protein FliS	*fliS*	**BTB_RS08310**	**lmo0708**	**BSU35330_fliS**	yes (54%/97%)	no	yes (21%/96%)
hypothetical protein		**BTB_RS08315**	**lmo0709**		no**	no	no
Flagellar basal-body rod protein FlgB	*flgB*	**BTB_RS08320_flgB**	**lmo0710_flgB**	**BSU16180_flgB**	yes (42%/101%)	yes (27%/83%)	yes (31%/95%)
Flagellar basal-body rod protein FlgC	*flgC*	**BTB_RS08325**	**lmo0711_flgC**	**BSU16190_flgC**	yes (58%/100%)	yes (44%/103%)	yes (41%/103%)
flagellar hook-basal body protein FliE	*fliE*	**BTB_RS08330_fliE**	**lmo0712_fliE**	**BSU16200_fliE**	yes (52%/84%)	No	yes (26%/71%)
Flagellar MS-ring protein FliF	*fliF*	**BTB_RS08335**	**lmo0713_fliF**	**BSU16210_fliF**	yes (39%/97%)	yes (22%/99%)	yes (24%/77%)
Flagellar motor switch protein FliG	*fliG*	**BTB_RS08340**	**lmo0714_fliG**	**BSU16220_fliG**	yes (46%/95%)	yes (34%/99%)	yes (32%/96%)
Flagellar assembly protein H	*fliH*	**BTB_RS08345_fliH**	**lmo0715_fliH**		yes (37%/104%)	no	no
Flagellum-specific ATP synthase FliI	*fliI*	**BTB_RS08350_fliI**	**lmo0716_fliI**	**BSU16240_fliI**	yes (70%/99%)	yes (44%/96%)	yes (44%/97%)
hypothetical protein		**BTB_RS08355**	**lmo0694**		yes (34%/99%)	no	no
hypothetical protein with C-terminal domain of FliK		**BTB_RS08360**	**lmo0695**		yes (34%/27%)	no	no
Flagellar basal body rod modification protein FlgD	*flgD*	**BTB_RS08365_flgD**	**lmo0696_flgD**	**BSU16280_flgD**	yes (45%/78%)	yes (31%/60%)	yes (33%/96%)
Fagellar hook-basal body protein, FlgE/F/G	*flgE/F/G*	**BTB_RS08370**	**lmo0697_flgE**	**BSU16290_flgG**	yes (47%/102%)	yes (37%/40%)	yes (39%/50%)
Protein of unknown function DUF3964		**BTB_RS08375**	**lmo0684**		yes (41%/101%)	no	no
flagellar motor switch FliG superfamily protein		**BTB_RS08380**			No	no	
Chemotaxis signal transduction protein CheV	*cheV*	**BTB_RS08385**	**lmo0689**	BSU14010_cheV	yes (64%/102%)	yes (49%/100%)	yes (49%/99%)
Motility gene repressor MogR	*mogR*	**BTB_RS08390**	**lmo0674_mogR**		yes (35%/89%)	no	no
flagellin	*fla/hag*	**BTB_RS08395**	**lmo0690_flaA**	**BSU35360_hag**	yes (33%/104%)	yes (30%/109%)	yes (39%/105%)
flagellin	*flaA*	**BTB_RS08400**			no	no	
Putative lytic murein transglycosylase		**BTB_RS08405**	**lmo0717**	BSU11570_yjbJ	yes (49%/82%)	yes (46%/69%)	yes (59%/56%)
Flagellar motor switch FliN	*fliN*	**BTB_RS08410**	**lmo0698**		yes (50%/95%)	no	no
Flagellar motor switch protein FliM	*fliM*	**BTB_RS08415**	**lmo0699_fliM**	**BSU16310_fliM**	yes (49%/98%)	yes (26%/98%)	yes (25%/101%)
Flagellar motor switch FliN	*fliN*	**BTB_RS08420**	**lmo0693**		yes (57%/70%)	no	no
Putative flagellar motor switch FliN		**BTB_RS08425**	**lmo0675**		yes (32%/97%)	no	no
Flagellar biosynthesis protein FliP	*fliP*	**BTB_RS08430**	**lmo0676_fliP**	**BSU16350_fliP**	yes (62%/84%)	yes (38%/93%)	yes (34%/89%)
Flagellar biosynthesis protein FliQ	*fliQ*	**BTB_RS08435**	**lmo0677_fliQ**	**BSU16360_fliQ**	yes (49%/98%)	yes (33%/93%)	yes (44%/60%)

Fflagellar biosynthesis protein FliR	*fliR*	**BTB_RS08440**	**lmo0678_fliR**	**BSU16370_fliR**	yes (47%/100%)	yes (24%/98%)	yes (24%/93%)
Flagellar biosynthesis protein FlhB	*flhB*	**BTB_RS08445**	**lmo0679_flhB**	**BSU16380_flhB**	yes (48%/100%)	yes (33%/97%)	yes (34%/98%)
Flagellar biosynthesis protein FlhA	*flhA*	**BTB_RS08450**	**lmo0680_flhA**	**BSU16390_flhA**	yes (65%/100%)	yes (37%/100%)	yes (36%/101%)
Flagellar biosynthesis regulator FlhF	*flhF*	**BTB_RS08455_flhF**	**lmo0681**	**BSU16400_flhF**	yes (33%/67%)	yes (29%/67%)	yes (34%/47%)
Flagellar basal-body rod protein FlgG	*flgG*	**BTB_RS08460_flgG**	**lmo0682_flgG**		yes (42%/98%)	no	no
Flagellar motor protein MotA (H^+-coupled stator)	*motA*	BTB_RS22910	**lmo0685**	BSU13690_motA	yes (43%/96%)	yes (51%/99%)	yes (49%/91%)
Flagellar motor protein MotB (H^+-coupled stator)	*motB*	BTB_RS22905_motB	**lmo0686_motB**	BSU13680_motB	yes (45%/97%)	yes (48%/95%)	yes (44%/97%)
Glycosyltransferase. In *L. monocytogenes*: Flagellin glycosyltransferase and MogR antirepressor GmaR**	*gmaR****	BTB_RS06215***	**lmo0688**	BSU21450_yolJ***	yes (33%/42%)	yes (27%/54%)	yes (27%/53%)
hypothetical protein			**lmo0701**		no		no
hypothetical protein			**lmo0702**		no		no
hypothetical protein			**lmo0704**		no		no
hypothetical protein			**lmo0718**		no		no
Flagellar assembly protein H	*fliH*			**BSU16230_fliH**		no	no
Flagellar export protein FliJ	*fliJ*			**BSU16250_fliJ**		no	no
flagellar motor switch protein FliG superfamily protein				**BSU16260_ylxF**		no	no
Flagellar hook-length control protein FliK	*fliK*			**BSU16270_fliK**		no	no
flagellar protein				**BSU16299_ylzI**		no	no
Flagellar basal body-associated protein FliL	*fliL*			**BSU16300_fliL**		no	no
Flagellar biosynthesis protein, FliO	*fliO*			**BSU16340_fliZ**		no	no
flagellum site-determining protein YlxH				**BSU16410_ylxH**		no	no
Chemotaxis protein, response regulator CheB	*cheB*			**BSU16420_cheB**		no	no
Chemotaxis protein CheW	*cheW*			**BSU16440_cheW**		no	no
CheY-P phosphatase CheC	*cheC*			**BSU16450_cheC**		no	no
Chemoreceptor glutamine deamidase CheD	*cheD*			**BSU16460_cheD**		no	no
RNA polymerase sigma-D factor	*sigD*			**BSU16470_sigD**		no	no
swarming motility protein SwrB	*swrB*			**BSU16480_ylxL**		no	no
Swarming motility protein SwrA	*swrA*			**BSU35239-BSU35230_swrA********		no	no
ribosome hibernation promoting factor protein	*yvyD*	BTB_RS26450	lmo2511	**BSU35310_yvyD**	yes (63%/101%)	yes (67%/102%)	yes (60%/101%)
hypothetical protein				**BSU35319_yvzG**		no	no
flagellar protein FliT	*fliT*			**BSU35320_fliT**		no	no
FlaG family protein YvyC				**BSU35350_yvyC**		no	no
Carbon storage regulator CsrA	*csrA*			**BSU35370_csrA**		no	no
flagellar assembly factor FliW	*fliW*			**BSU35380_yviF**		no	no
hypothetical protein				**BSU35390_yviE**		no	no
FlgN-like family protein				**BSU35420_yvyG**		no	no
negative regulator of flagellin synthesis	*flgM*			**BSU35430_flgM**		no	no
Flagellar operon protein YvyF	*yvyF*			**BSU35440_yvyF**		no	no

* In parentheses: Percentage of identical matches in alignment / Percentage overlap of best hit, calculated as length of alignment relative to query protein length (both calculated as averages obtained from the forward and reciprocal blast)

** Score below threshold, bitscore 26.6 (87%/26%)

*** Although these proteins are reciprocal BLAST hits to *Lm* EGD-e GmaR, they are not considered true GmaR orthologs, as they lack the GmaR-specific C-terminal MogR-binding anti-repressor domain.

**** Laboratory strains of *B. subtilis* are known to contain an single “A” insertion mutation giving a frameshift in the *swrA* gene (McLoon et al., 2011). The protein used as query in this analysis was in-frame.

Table S2: Primers used in RT-qPCR

Gene name	Locus tag	Forward primer (5'-3')	Reverse primer (5'-3')	Product size	E	r^2
gatB	BTB_RS21880	agctggtcgtgaagaccttg	cggcataacagcagtcatca	175 bp	1.93	0.9968
rpsU	BTB_RS21885	aagatcggtttctaaaactggtaca	tttcttgccgcttcagattt	102 bp	1.87	0.9957
udp	BTB_RS26495	actagagaaacttggaaatgatcg	gacgcttaattgcacggaac	101 bp	1.85	0.9992
mogR	BTB_RS08390	gggatgcgagcatatgaaaa	aatgtttaaaccgtgacgttgac	102 bp	1.95	0.9992
flaB	BTB_RS08395	ctgcgaacggtacaaattca	aactcagtcgtctcgccaat	100 bp	1.96	0.9983
flaA	BTB_RS08400	ccgtgcaacactaggtgcta	cgtcttcgatttgagaagca	104 bp	1.94	0.9983

E, PCR efficiency. r^2, square of Pearson's correlation coefficient calculated from serial dilutions.

Table S3: Genome-wide gene expression analysis. Differentially regulated genes identified by microarray-based transcriptional profiling of the MogR overexpression strain relative to an empty vector control. All genes with FDR-corrected *p*-values <0.05 are listed (ordered by locus tag number).

Genes downregulated in the MogR overexpression strain:

Locus tag in *B. thuringiensis* 407	**Locus tag in *B. cereus* ATCC 14579**	**Predicted function**	**log_2 (fold change)**	***p*-value (FDR-corrected)**
BTB_RS01475	BC0294	10 kDa chaperonin	-1.06	0.00
BTB_RS01480	BC0295	60 kDa chaperonin	-0.58	0.05
BTB_RS02175	BC0422	Methyl-accepting chemotaxis protein (with upstream "off" c-di-GMP riboswitch)	-1.31	0.00
BTB_RS02990	BC0587	Acetyltransferase	-1.21	0.00
BTB_RS03005	BC0590	Uncharacterized conserved protein YjgD	-0.73	0.03
BTB_RS03040	BC0598	Helix-turn-helix domain protein/NprR	-0.89	0.00
BTB_RS03260	BC0643	Amino-acid permease rocC	-0.63	0.02
BTB_RS03410	BC0666	Immune inhibitor A	-1.03	0.00
BTB_RS03430	BC0670	Phospholipase C (PC-PLC)	-2.32	0.00
BTB_RS03435	BC0671	Sphingomyelinase C (Smase)	-1.47	0.01
BTB_RS04285	BC0791	Coenzyme A disulfide reductase Cdr	-1.54	0.00
BTB_RS04490	BC0834	Hypothetical protein	-1.95	0.00
BTB_RS04760	BC0888	N-acetylmuramoyl-L-alanine amidase CwlH	-1.50	0.00
BTB_RS05200	BC1000	Hypothetical protein	-1.01	0.00
BTB_RS05215	BC1003	Serine-protein kinase rsbW	-0.91	0.00
BTB_RS05220	BC1004	RNA polymerase sigma-B factor	-1.05	0.00
BTB_RS05375	BC1030	Hypothetical protein	-0.51	0.05
BTB_RS05580	BC1061	Hypothetical protein	-0.82	0.02
BTB_RS05760	BC1113	Sigma-M negative effector	-1.61	0.01
BTB_RS06105	BC1185	Dipeptide-binding protein DppE	-0.93	0.00
BTB_RS06140	BC1193	Oligoendopeptidase F	-2.39	0.00
BTB_RS06705	BC1316	Hypothetical protein	-0.66	0.03
BTB_RS07240	BC1424	Ferredoxin--nitrite reductase NirA	-1.62	0.00
BTB_RS07935	BC1570	Xanthine permease PbuX	-1.21	0.02
BTB_RS08255	BC1628	Chemotaxis protein, histidine kinase CheA	-1.80	0.00
BTB_RS08265	BC1630	Hypothetical protein	-0.92	0.03
BTB_RS08295	BC1636	Flagellar hook-associated protein 1, FlgK	-1.67	0.02
BTB_RS08310	BC1639	Flagellar (assembly) protein FliS	-0.68	0.00
BTB_RS08325	BC1642	Flagellar basal-body rod protein FlgC	-2.87	0.00
BTB_RS08335	BC1644	Flagellar MS-ring protein FliF	-2.31	0.00
BTB_RS08340	BC1645	Flagellar motor switch protein FliG	-1.09	0.00
BTB_RS08360	BC1649	Flagellar hook-length control protein FliK	-1.47	0.00
BTB_RS08380	BC1653	Hypothetical protein	-1.37	0.00
BTB_RS08385	BC1654	Chemotaxis signal transduction protein CheV	-1.80	0.00
BTB_RS08395	BC1656	Flagellin FlaB	-1.04	0.00
BTB_RS08400	BC1657	Flagellin FlaA	-3.45	0.00
BTB_RS08405	BC1660	Putative lytic murein transglycosylase	-0.75	0.00
BTB_RS08410	BC1661	Flagellar motor switch protein (fliN-homolog)	-0.99	0.00
BTB_RS08430	BC1665	Flagellar biosynthesis protein FliP	-1.86	0.02
BTB_RS08435	BC1666	Flagellar biosynthesis protein FliQ	-1.23	0.00
BTB_RS08440	BC1667	Flagellar biosynthesis protein FliR	-1.02	0.00
BTB_RS08445	BC1668	Flagellar biosynthesis protein FlhB	-1.60	0.00
BTB_RS08450	BC1669	Flagellar biosynthesis protein FlhA	-1.43	0.00
BTB_RS09080	BC1789	Transcriptional regulatory protein	-1.27	0.00
BTB_RS10150	BC1991	Putative murein endopeptidase	-0.80	0.00
BTB_RS10480	BC2056	Hypothetical protein	-0.74	0.02
BTB_RS10590	BC2076	Acetyltransferase	-1.47	0.00
BTB_RS12170	BC2379	Transcriptional regulator	-1.54	0.05
BTB_RS12675	BC2478	ABC transporter, ATP-binding protein	-3.19	0.00
BTB_RS12935	BC2529	Zn-dependent alcohol dehydrogenase	-1.97	0.00
BTB_RS13545	BC2659	Glutamate-rich protein grpB	-1.72	0.00
BTB_RS14470	BC2832	Putative aldehyde dehydrogenase DhaS	-2.10	0.00
BTB_RS14520	BC2842	Hypothetical protein	-0.73	0.00
BTB_RS14700	BC2881	Uncharacterized protein YhbF	-2.10	0.00
BTB_RS14970	BC2925	Nucleotidyltransferase	-2.27	0.03
BTB_RS15135	BC2959	Putative malate:quinone oxidoreductase Mqo	-0.67	0.00

BTB_RS15165	BC2964	Transcriptional regulator LsrR	-2.12	0.00
BTB_RS12545	BC3102	Hemolysin BL-binding component HblA	-1.13	0.02
BTB_RS12540	BC3103	Hbl component L1	-1.59	0.00
BTB_RS17315	BC3461	putative deacetylase YojG	-1.33	0.00
BTB_RS17995	BC3586	Dipeptide-binding protein DppE	-1.15	0.00
BTB_RS18825	BC3762	Serine protease, subtilase family Sfp	-0.84	0.02
BTB_RS19450	BC3835	Ribonuclease HII	-1.24	0.00
BTB_RS19615	BC3867	Putative coenzyme A biosynthesis bifunctional protein CoaBC	-1.12	0.00
BTB_RS20130	BC3966	Hypothetical protein	-2.57	0.02
BTB_RS20410	BC4020	Hypothetical protein	-0.98	0.02
BTB_RS21125	BC4154	Hypothetical protein	-1.47	0.00
BTB_RS21330	BC4198	Xaa-Pro dipeptidase PepQ	-0.51	0.01
BTB_RS21660	BC4260	Glucokinase	-1.85	0.01
BTB_RS21815	BC4293	Putative phosphotransferase	-0.71	0.04
BTB_RS21885	BC4307	30S ribosomal protein S21	-0.81	0.00
BTB_RS21915	BC4313	protein GrpE	-0.75	0.00
BTB_RS21920	BC4314	Heat-inducible transcription repressor hrcA	-0.67	0.01
BTB_RS22300	BC4389	Helicase, RecD/TraA	-1.53	0.00
BTB_RS22905	BC4512	Flagellar motor protein MotB (H+-coupled stator)	-3.01	0.01
BTB_RS22910	BC4513	Flagellar motor protein MotA (H+-coupled stator)	-1.74	0.00
BTB_RS23315	BC4594	Citrate synthase 2	-0.63	0.00
BTB_RS23780	BC4683	putative ribosomal N-acetyltransferase YdaF	-1.06	0.00
BTB_RS24165	BC4756	GDP-mannose-dependent alpha-mannosyltransferase MgtA	-1.58	0.00
BTB_RS24190	BC4762	Phosphoenolpyruvate carboxykinase	-1.26	0.02
BTB_RS22105	BC4861	hypothetical protein	-1.60	0.03
BTB_RS25940	BC5048	Ferritin	-1.66	0.00
BTB_RS26545	BC5211	putative permease IIC component YwbA	-1.23	0.02
BTB_RS26650	BC5228	glycolate permease GlcA	-0.84	0.00
BTB_RS26730	BC5243	hypothetical protein	-0.87	0.01
BTB_RS26945	BC5280	3R)-hydroxymyristoyl-[acyl-carrier-protein] dehydratase FabZ	-0.68	0.00
BTB_RS27450	BC5380	putative siderophore-binding lipoprotein YfiY	-0.84	0.02

Genes upregulated in the MogR overexpression strain:

Locus tag in *B. thuringiensis* 407	**Locus tag in Bc ATCC 14579**	**Predicted function**	**log_2 (fold change)**	**p-value**
BTB_RS02515	BC0492	Pyruvate formate-lyase-activating enzyme PflA	0.92	0.00
BTB_RS04390	BC0813	Cell wall-binding protein YocH	0.96	0.00
BTB_RS05575	BC1060	Collagen adhesion protein with upstream c-di-GMP "on" riboswitch CbpA	3.02	0.00
BTB_RS06050	BC1174	3-oxoacyl-[acyl-carrier-protein] synthase 2	0.96	0.00
BTB_RS06215	BC1208	Glycosyl transferase, group 2	1.23	0.00
BTB_RS06545	BC1283	SinI	1.08	0.05
BTB_RS07850	BC1553	Penicillin-binding protein 1A/1B	0.62	0.01
BTB_RS08390	BC1655	MogR	0.75	0.01
BTB_RS09115	BC1794	Dipeptide-binding protein DppE	2.01	0.00
BTB_RS09415	BC1853	Hypothetical protein	2.00	0.00
BTB_RS10950	BC2164	Isoleucine--tRNA ligase IleS	1.00	0.04
BTB_RS11420	BC2238	Hypothetical protein	1.95	0.00
BTB_RS12700	BC2483	Putative acyl-CoA dehydrogenase YngJ	1.81	0.00
BTB_RS20160	BC3972	Pyruvate dehydrogenase complex E1 component, beta subunit	0.71	0.01
BTB_RS20730	BC4083	Putative adenine permease PurP	0.73	0.03
BTB_RS20740	BC4085	Pyrimidine-nucleoside phosphorylase Pdp	0.80	0.02
BTB_RS20745	BC4086	Purine nucleoside phosphorylase 1	0.87	0.01
BTB_RS24745	BC4870	L-lactate dehydrogenase 2	0.66	0.02
BTB_RS26190	BC5138	Phosphoglycerate kinase, Pkk	0.83	0.00
BTB_RS26200	BC5141	Central glycolytic genes regulator, CggR	0.94	0.00
BTB_RS26300	BC5158	Integral membrane protein	1.58	0.02
BTB_RS26705	BC5238	Glycine betaine transporter OpuD	1.10	0.05
BTB_RS26935	BC5278	Putative capsular polysaccharide biosynthesis protein YwqC	0.71	0.02

Supplementary References

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