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Original Article

# CdgL is a degenerate nucleotide cyclase domain protein affecting flagellin synthesis and motility in *Bacillus thuringiensis*

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ABSTRACT

In *Bacillus subtilis*, motility genes are expressed in a hierarchical pattern – governed by the $\sigma^D$ transcription factor and other proteins such as the EpsE molecular clutch and SlrA/SlrR regulator proteins. In contrast, motile species in the *Bacillus cereus* group seem to express their motility genes in a non-hierarchical pattern, and less is known about their regulation, also given that no orthologs to $\sigma^D$, EpsE, SlrA or SlrR are found in *B. cereus* group genomes. Here we show that deletion of *cdgL* (BTB_RS26690/BTB_c54300) in *Bacillus thuringiensis* 407 (*cry*-) resulted in a six-to ten-fold down-regulation of the entire motility locus, and loss of flagellar structures and swimming motility. *cdgL* is unique to the *B. cereus* group and is found in all phylogenetic clusters in the population except for group I, which comprises isolates of non-motile *Bacillus pseudomycoides*. Analysis of RNA-Seq data revealed *cdgL* to be expressed in a three-gene operon with a NupC like nucleoside transporter, and a putative glycosyl transferase for which transposon-based gene inactivation was previously shown to produce a similar phenotype to *cdgL* deletion. Interestingly, all three proteins were predicted to be membrane-bound and may provide a concerted function in the regulation of *B. cereus* group motility.

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## 1. Introduction

The ability to receive and respond to environmental signals is imperative for the survival of bacteria. Cyclic nucleotides are key second messengers in this regard, of which early characterized examples include cyclic adenosine 3′,5′-monophosphate (cAMP) and the alarmones guanosine pentaphosphate or tetraphosphate ((p)ppGpp), which all regulate several important processes in bacteria, such as the stringent response, carbon metabolism, motility and virulence [1]. Bis-(3′,5′)-cyclic diguanylic acid (c-di-GMP) was discovered in 1987 [2], and has during later years been shown to constitute a widespread and versatile signaling molecule controlling several bacterial cellular processes, including the switch from planktonic growth to biofilm formation [3]. A more recent discovery is bis-(3′,5′)-cyclic diadenylic acid (c-di-AMP) which in 2008 was shown to affect cell division and sporulation in *Bacillus subtilis* [4], and which has later been found to be implicated in various physiological roles such as potassium homeostasis [5], biofilm formation [6], and cell wall homeostasis [7], mainly in Gram-positive bacteria (reviewed in [8]). The formation of c-di-GMP is catalyzed by diguanylate cyclases (DGCs), which commonly harbor a protein domain carrying a GGDEF consensus c-di-GMP binding sequence within a nucleotide cyclase domain [9]. The DGCs act by forming a protein dimer which can bind two guanosine-5′triphosphate (GTP) molecules, from which c-di-GMP is synthesized. c-di-GMP is subsequently degraded by phosphodiesterases (PDEs) [2] harboring either an EAL or a HD-GYP domain [10,11].

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The *Bacillus cereus* group comprises at least seven bacterial species, including *Bacillus anthracis*, the cause of the fatal disease anthrax in humans and mammals, *Bacillus cereus sensu stricto*, an opportunistic pathogen frequently associated with bacterial gastroenteritis, and *Bacillus thuringiensis*, an entomopathogenic bacterium widely used as a biological pesticide [12]. The majority of the *B. cereus* group species are motile by means of peritrichous flagella and readily form biofilms, frequently at air-liquid interfaces [13]. *B. anthracis* is however non-motile due to multiple nonsense mutations in its motility genes, while *Bacillus mycoides* and *Bacillus pseudomycoides* are non-motile due to severely reduced motility loci [14]. The motility genes of *B. cereus* group species are mainly situated in one large gene locus, encompassing 45 genes in *B. thuringiensis* 407 (BTB_RS08240 - BTB_RS08460), while the flagellar motor proteins MotA and MotB are encoded by the operon BTB_RS22910 - BTB_RS22905, located in a different part of the chromosome. The main motility locus consists of co-localized flagellar and chemotaxis genes, along with genes encoding eight proteins of unknown function and a transcriptional regulator [14]. To date, only a few transcriptional regulators of motility, biofilm and virulence have been identified in the *B. cereus* group. The SinI-SinR system is a regulatory system which is conserved between *B. cereus* and *B. subtilis,* and is composed of the transcriptional regulator SinR and its anti-repressor SinI [15,16]. SinR acts as a master transcriptional repressor governing the switch from a motile to sessile lifestyle [16]. The key transcriptional regulator of virulence genes, PlcR, also affects motility and biofilm formation [17,18]. Recently, another regulator, MogR, encoded in the motility locus, was found to repress the expression of motility genes in *B. thuringiensis* 407 [14]. In addition to motility, MogR was found to positively affect biofilm formation and attenuate virulence, and cells overexpressing *mogR* had increased intracellular c-di-GMP levels [14].

*B. cereus* group bacteria were recently shown to carry a range of c-di-GMP metabolism genes, affecting phenotypes such as motility, biofilm and virulence [19]. In addition to the DGCs and PDEs, which together determine c-di-GMP levels locally within the bacterial cell, a c-di-GMP signaling network is composed of a set of downstream c-di-GMP binding effector molecules [20,21]. The effector molecules can bind c-di-GMP through a variety of domains, including GEMM-I riboswitches and PilZ protein domains [22,23]. In addition, proteins such as PelD from *Pseudomonas aeruginosa*, which regulates the biosynthesis of exopolysaccharides [24], and PopA which promotes cell cycle progression in *Caulobacter crescentus* [25], have been shown to bind c-di-GMP through degenerate and thereby enzymatically inactive GGDEF domains, thereby acting as downstream effectors responding to changes in cellular c-di-GMP levels and mediating c-di-GMP-dependent phenotypes [21]. Degenerate GGDEF domain proteins can also harbor an allosteric inhibition site (I-site), which are common in DGCs [26], in which the RxxD motif of the I-site acts in non-competitive product inhibition by binding c-di-GMP, thereby changing DGC protein conformation and preventing the active DGC dimer from forming [26,27]. A c-di-GMP signaling network has recently been proposed in *B. thuringiensis* 407, including ten proteins related to c-di-GMP turnover and which were found to be conserved in the *B. cereus* group, as well as a PilZ domain protein [19,28]. Also putatively belonging to this network are genes downstream of two functional c-di-GMP responsive riboswitches [23,29] encoding a collagen adhesion protein [23,29] and a putative methyl-accepting chemotaxis protein, respectively. In addition, the 251 amino acid (aa) long protein CdgL (locus tag BTB_RS26690/BTB_c54300) was identified, predicted to harbor a highly degenerate nucleotide cyclase domain with no consensus I-site motif [19]. Here, we have investigated a potential functional role of the CdgL protein in motility and biofilm regulation in *B. thuringiensis* 407, a model strain for functional studies of *B. cereus* and *B. thuringiensis*.

## 2. Materials and methods

### 2.1. Strains and growth conditions

Bacterial strains and plasmids are presented in Table 1. *B. thuringiensis* 407 *cry*$^-$ is an acrystalliferous strain cured of its *cry*-encoding plasmid [30] and is genetically close to the *B. cereus* type strain ATCC 14579 [30,31]. 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). Erythromycin at 10 μg ml$^{-1}$ was used to maintain the pHT304-P$_{xyl}$ plasmid constructs. For induction of gene expression from the *xylA* promoter on pHT304-P$_{xyl}$, 1 mM xylose was added to the growth medium. *Escherichia coli* BL21 (DE3) was used for expression of the CdgL protein and grown at 37 °C with shaking.

### 2.2. Construction of deletion mutants and complementation strains

Chromosomal deletion of the *cdgL* gene was carried out via homologous recombination using a markerless gene replacement method as described [19]. Approximately 800 bp of DNA sequence homologous to the upstream and downstream region of the gene was PCR amplified using primers listed in Table 2. The mutant allele was verified by sequencing of PCR products generated with primers designed to anneal outside of the sequences used for homologous recombination (Table 2). 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 allowing xylose-inducible expression of downstream cloned genes, was used as previously described [19], for overexpression of CdgL in *B. thuringiensis* 407 and *B. thuringiensis* 407 Δ*cdgL*. The *cdgL* gene was PCR amplified from *B. thuringiensis* 407 using primers listed in Table 3.

### 2.3. Protein domain analysis

Protein domains were identified and analyzed using InterProScan at the European Bioinformatics Institute UK, with default parameters (https://www.ebi.ac.uk/interpro/search/sequence/), in protein sequences downloaded from the National Centre for Biotechnology Information (NCBI, US). Transmembrane domains were identified using TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) at DTU Bioinformatics (Copenhagen, Denmark).

### 2.4. RNA isolation and reverse transcription quantitative PCR (RT-qPCR)

For analysis of gene expression, RT-qPCR was performed essentially as described in [19]. Briefly, *B. thuringiensis* 407 cultures grown in bactopeptone medium at 30 °C were incubated in an equal volume of ice-cold methanol for 5 min before harvesting by centrifugation. Cells were lysed using a Precellys 24 Tissue Homogenizer (Bertin) and RNA 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 Table 3. The two genes *gatB* and *rpsU*, shown to be stably expressed throughout the *B. cereus* life cycle [32], were used as reference genes, and were

**Table 1**
Strains and plasmids used in this study.

| Strain or plasmid | Description[a] | Reference or source |
|---|---|---|
| ***PLASMIDS*** | | |
| pHT304-$P_{xyl}$ | Low copy number expression/shuttle vector; *xylA* promoter ($Amp^r$, $Ery^r$) | [64,65] |
| pHT304-$P_{xyl}$-*cdgL* | Wild type *cdgL* gene from *B. thuringiensis* 407 cloned in vector pHT304-P*xyl* | This study |
| pMAD-I-*Sce*I | Integrative plasmid vector used for allelic exchange and construction of gene deletion strains for *Bacillus cereus* group bacteria ($Amp^r$) | [19,66] |
| pSS4332 | Helper plasmid for gene deletion mutant construction, expressing the I-*Sce*I restriction endonuclease ($Km^r$) | [67] |
| pET-11-cdgL | GGDEF domain from the wild type *cdgL* gene in *B. thuringiensis* 407 cloned in expression vector pET-11 ($Km^r$) | Genscript |
| ***STRAINS*** | | |
| ***Bacillus cereus* group** | | |
| *Bacillus thuringiensis* 407 | *B. thuringiensis* 407 *cry*$^-$ (acrystalliferous strain cured of *cry* encoding plasmid) | [30] |
| *B. thuringiensis* 407 pHT304-$P_{xyl}$-*cdgL* | *B. thuringiensis* 407 host strain overexpressing CdgL from the xylose inducible plasmid expression vector pHT304-P*xyl* | This study |
| *B. thuringiensis* 407 Δ*cdgL* | *B. thuringiensis* 407 Cry- carrying a markerless deletion in the *cdgL* gene | This study |
| *B. thuringiensis Δcdg*L pHT304-$P_{xyl}$-*cdgL* | Complementation strain - *B. thuringiensis* 407 Δ*cdgL* strain carrying plasmid pHT304-P*xyl-cdgL* | This study |
| *B. thuringiensis* 407 Δ*flaAB* | *B. thuringiensis* 407 Cry- strain in which the *flaA* and *flaB* genes are replaced by a Kanamycin resistance cassette ($Km^r$) | [34] |
| ***Escherichia coli*** | | |
| *E. coli* BL21 (DE3) | *E. coli* BL21 (DE3) protein expression strain | New England Biolabs |
| *E. coli* BL21/CdgL | *E. coli* BL21 (DE3) expressing the *cdgL* gene from plasmid pET-11-cdgL | This study |

[a] $Amp^r$, Ampicillin resistance; $Ery^r$, Erythromycin resistance; $Km^r$, Kanamycin resistance; *cry*, *B. thuringiensis* crystal toxin.

**Table 2**
Oligonucleotides used as PCR primers for preparation of *B. thuringiensis* 407 deletion mutants and overexpression constructs.

| Forward primer | | Reverse primer | | Function |
|---|---|---|---|---|
| Designation | Sequence 5′ to 3′ | Designation | Sequence 5′ to 3′ | |
| pMAD-fwd | gcatgcatcgatagatctgtctagttaatgtgtaacg | pMAD-rev2 | caggtagatgacgaccatcagggacag | PCR amplification of pMAD-I-*Sce*I |
| Bt51010-A | cgttacacattaactagacagatctatcgatgcatgcgg ctatgcagcaatgggtat | Bt51010-B | gtttccaatttgcatagagaggattttata gaacttatc | Cloning chromosome region upstream of *cdgL* into pMAD-I-*Sce*I |
| Bt51010-C | cctctctatgcaaattggaaacaaaggacaga | Bt51010-D2 | ctgtccctgatggtcgtcatctacctgact gattctgggacgattgg | Cloning chromosome region downstream of *cdgL* into pMAD |
| pMAD-F | Ttccccctagctaattttcg | Bt51010-CD-R | tcagttgtttcagcgtgctc | Confirming *cdgL* construct in pMAD-I-*Sce*I |
| Bt51010-AB-F | Ttaggaccacacatggcaga | pMAD-R2 | gcctacaatccatgccaac | Confirming *cdgL* construct in pMAD-I-*Sce*I |
| Bt51010-OUT-FWD | Gcagaaagcttaaactcagtaacg | Bt51010-OUT-REV | tcgacactctgggcactaaa | Confirming *cdgL* deletion in *B. thuringiensis* 407 |
| pXyl-fwd | catgtgatttccccctttaaaaataaattcattcaaatacag | pXyl-rev | tcgtaatcatggtcatagctgtttcctgtg | PCR amplification of pHT304-$P_{xyl}$ |
| Bt50101-pXyl-F | ctgtatttgaatgaatttatttttaaggggaaatcacatgaaa gataagttctataaaatcctc | Bt50101-pXyl-R | cacaggaaacagctatgaccatgattacga cgagagtcatcataacaatgtctg | Cloning of *cdgL* into pHT304-$P_{xyl,}$ creating pHT304-P*xyl-cdgL* |

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 [33] and then normalized to the geometric mean of the $E^{Cq}$ values obtained for the two reference genes. For each target gene, the ratio of expression of each gene in the *cdgL* deletion mutant *vs.* in the wild type (or in the overexpression strain *vs.* in the empty vector control) was calculated for each of the three biological replicates (ΔΔCq-method; [33]), and results are presented as boxplots. For statistical analysis, gene expression values (normalized $E^{Cq}$ values) were log transformed to approach the normality assumption, and paired two-tailed *t*-tests were used to test for differences between gene expression in mutant *vs.* the wild type strain, or in overexpression *vs*. empty vector strains.

### 2.5. Motility assay

Swimming ability on soft agar was determined using 140 mm diameter 0.3% LB agar plates added 1 mM xylose. A 5 μl drop of culture grown overnight in LB medium at 30 °C was spotted on each agar plate. For the complementation strain, erythromycin and xylose were added to the overnight culture at concentrations of 10 μg/ml and 1 mM, respectively). The plates were wrapped in plastic and incubated for 7 h at 30 °C. Three independent experiments were performed, each with three technical replicates. Images were recorded with a high resolution mobile phone camera, after placing the growth plate on a blue background with the lid open, using roof lighting and carefully avoiding light reflections.

### 2.6. Atomic force microscopy (AFM)

AFM imaging and analysis was performed with a Nanowizard AFM microscope (JPK Instruments). Bacterial cell culture was harvested during exponential growth in LB medium at 30 °C and 200 rpm and washed and resuspended in 0.9% NaCl. Cells were then diluted 1:5 in a 10 mM magnesium/Tris-buffer, pH 7.5, applied onto freshly cleaved mica surfaces (Agar Scientific) mounted on a glass slide, and allowed to adhere for 10 min before being washed with deionized water (10 × 100 μl). Excess water was carefully removed, and the slides gently dried using a nitrogen gas jet stream. Images were recorded in intermittent-contact mode at room temperature in air using a MikroMasch NSC35/AlbS probe

**Table 3**
Genes subjected to qPCR analysis, and oligonucleotide primers used in the analyses.

| Gene | Function/gene product | p-value[a] (differential expression in Δ*cdgL*/wt) | Locus tag | | Sequence 5′ to 3′ | |
|---|---|---|---|---|---|---|
| | | | ATCC 14579 | Bt407 | Forward primer | Reverse primer |
| *abrB* | Transition state regulatory protein AbrB | 0.31 | BC0042 | BTB_RS00215 | gcagaaaaggacgctcttga | gcgccttctttgcttaagat |
| *nprR* | Helix-turn-helix domain protein/NprR | 0.79 | BC0598 | BTB_RS03040 | tgatgcagcaaaacaggaag | acgggatagcctcattttcc |
| *sinR* | HTH-type transcriptional regulator SinR | 0.22 | BC1282 | BTB_RS06540 | aaaaagctggcgttgctaaa | tgtgtccattcggagtctagg |
| *sinI* | DNA-binding anti-repressor SinI | 0.037 | BC1283 | BTB_RS06545 | tgactggatggcttcatacg | cagacgcactggatcaagaa |
| *cheA* | Chemotaxis protein, histidine kinase CheA | 0.025 | BC1628 | BTB_RS08255 | gaaatattccgatccgctca | tgcacatctgcaaccatctt |
| *mogR* | Motility gene repressor MogR | 0.94 | BC1655 | BTB_RS08390 | gggatgcgagcatatgaaaa | aatgtttaaaccgtgacgttgac |
| *flaB* | Flagellin | 0.0003 | BC1656 | BTB_RS08395 | ctgcgaacggtacaaattca | aactcagtcgtctcgccaat |
| *flaA* | Flagellin | 0.0001 | BC1657 | BTB_RS08400 | ccgtgcaacactaggtgcta | cgtcttcgatttgagaagca |
| *flgK* | Flagellar hook-associated protein FlgK | 0.030 | BC1636 | BTB_RS08295 | gtggaagcacagacagcaga | acttgtgtacccgcttgtcc |
| *flgC* | Flagellar basal-body rod protein FlgC | 0.034 | BC1642 | BTB_RS08325 | acgacagcgagaaagtggat | caccacactacgacgctcat |
| *fliF* | Flagellar MS-ring protein FliF | 0.0013 | BC1644 | BTB_RS08335 | gcaggacttcaagctgatcc | attccaccgaataaccacca |
| *fliH* | Flagellar assembly protein H | 0.020 | BC1646 | BTB_RS08345 | tttaactggcatcgtgcaaa | ccaaactgcagggagaaatc |
| *flgE* | Flagellar hook-basal body protein, FlgE/F/G | 0.031 | BC1651 | BTB_RS08370 | ggggaatacatggacaatgc | tggatttccaacagcatcaa |
| *flhA* | Flagellar biosynthesis protein FlhA | 0.0024 | BC1669 | BTB_RS08450 | tatgcagcgctcttctctga | ttaaagacgcgttgcacttg |
| *flgG* | Flagellar basal-body rod protein FlgG | 0.028 | BC1671 | BTB_RS08460 | caaatgctcaaacgacagga | acagcataatcgaccgaacc |
| *plcR* | Transcriptional activator PlcR | 0.24 | BC5350 | BTB_RS27305 | cgggtgcagtatacccaagt | ttccttttcagctcattcca |
| *spo0A* | Stage 0 sporulation protein A | 0.54 | BC4170 | BTB_RS21200 | tcgtcacgcgattgaagtag | gtctcagcttatccgcaacc |
| *gatB/yqeY* | GatB/Yqey domain-containing protein | – | BC4306 | BTB_RS21880 | agctggtcgtgaagaccttg | cggcataacagcagtcatca |
| *rpsU* | 30 S ribosomal protein S21 | – | BC4307 | BTB_RS21885 | aagatcggtttctaaaactggtaca | tttcttgccgcttcagattt |
| *motA* | Flagellar motor protein MotA (H + -coupled stator) | 0.042 | BC4513 | BTB_RS22910 | tggtacgtatgcaccgacat | cacccgaaatagcatgacct |

[a] p-values are based on a paired two-tailed *t*-test for the ratio of the expression level of each gene in the *cdgL* deletion mutant relative to wild type cells being different from 1.

(NanoAndMore). AFM images were analyzed using The Nano-Wizard IP Image Processing Software (JPK Instruments).

### 2.7. Biofilm assays

#### 2.7.1. Glass tube assay

The ability to form biofilms was determined using a glass tube screening assay [34]. Briefly, exponential phase cultures were diluted into HCT medium to an $OD_{600}$ of 0.01 and 2 ml was inoculated into sterile 6 ml glass tubes. The tubes were incubated for 48 h at 30 °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 suspended biofilm cells. Each strain was tested in six biological replicates, each with five to eight technical replicates. Two-tailed Student's *t*-test was used to test for differences between the level of biofilm formation obtained for the two strains.

#### 2.7.2. Microtiter plate assay

The ability to form biofilms in polyvinylchloride (PVC) microtiter plates was determined using a crystal violet biofilm screening assay [35]. Briefly, fresh bactopeptone medium was inoculated with 0.5% exponential phase culture, transferred to 96-well plates (Falcon cat. no. 353911) and incubated for 24, 48 and 72 h at 30 °C. The biofilm was subsequently washed using PBS, stained using 0.3% crystal violet, solubilized with 25% acetone/75% ethanol, and transferred to flat-bottomed microtiter plates (Falcon cat. no. 353915) for determination of the absorbance of the solubilized dye at 570 nm. Each strain was tested three times in independent experiments. One-way ANOVA and Tukey's pairwise comparison were used to test for differences between strains within each time-point for mutant and wild type strains. Two-tailed Student's *t*-test was used to test for differences between strains within each time-point, for strains with plasmid vectors.

### 2.8. SDS-PAGE and Western immunoblotting

For detection of flagellin, for each strain analyzed, two parallel bacterial culture samples (10 ml LB) were harvested by centrifugation (4100×*g*, 4 °C) after 3.5 h 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 min at 16,000 × *g* and 4 °C, and the pellet was resuspended in an equal volume of 2 × SDS-PAGE sample buffer, and incubated at 95 °C for 5 min. The supernatant was collected by centrifugation as before. The whole cell protein fraction was prepared by centrifugation of resuspended cells (PBS) for 5 min at 16000×*g* and 4 °C. The pellet was then again resuspended in 500 μl PBS and lysed using a Precellys 24 Tissue Homogenizer (Bertin). The supernatant was collected following centrifugation for 8 min as before. Twenty-one μl of whole cell supernatant added 7 μl 4 × SDS PAGE sample buffer, and 5 μl of the surface protein fraction (already added sample buffer) were separated on SDS-PAGE gels. SDS-PAGE was carried out using a Bio-Rad Mini-Protean II Dual Slab Cell, using 12% polyacrylamide gels and 10 μl Prestained Protein Marker, Broad range (NEB cat #P7708S) as the molecular weight marker. Western blot analysis was performed using a Immun-Blot PVDF membrane (BioRad) according to standard protocols. Blocking was performed for 1 h in 5% non-fat dry milk in Tris-buffered saline and Tween 20 buffer (TBST). Flagellin proteins were detected using a rabbit antiserum raised against flagellin from *Bacillus mojavensis* used at a 1:300 dilution, and an HRP-conjugated donkey-anti-rabbit antibody (Santa Cruz Biotechnology Inc.) diluted 1:10,000 as secondary antibody.

### 2.9. Purification of His-tagged CdgL protein

The sequence encoding the 145 C-terminal amino acids of CdgL, containing the putative cytoplasmic nucleotide cyclase domain, was cloned into the pET-11 expression vector using the *Nco*I and *Bam*HI restriction sites, with a C-terminal His-tag (vector clone supplied by Genscript). The plasmid construct was transformed into *E. coli* BL21 (DE3) cells. LB broth (100 ml) with kanamycin at 20 μg $ml^{-1}$ was inoculated with 1 ml of an overnight culture of *E. coli* BL21 (DE3) containing the 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 0.5 mM and protein expression was induced for

2.5 h. The cells were harvested by a centrifugation step performed at 4000×*g* for 20 min at 4 °C. Cell pellets were washed three times in TEN buffer, with the last centrifugation step performed at 9500×*g*, and frozen at −80 °C overnight. The cells were then thawed on ice and re-suspended in 16 ml phenylmethylsulfonyl fluoride (PMSF) buffer (1 mM PMSF; 50 mM $NaH_2PO_4$; 300 mM NaCl; 10 mM imidazole) added 400 μl lysozyme solution (3 mM lysozyme; 50 mM $NaH_2PO_4$; 300 mM NaCl; 10 mM imidazole), and then incubated on ice for 30 min. The suspension was sonicated 6 × 10 s while on an ice bath, and added RNase to 10 μg $ml^{-1}$ and DNase to 5 μg $ml^{-1}$, before incubation on ice for 10 min, followed by centrifugation at 12,000×*g* for 20 min at 4 °C. The suspension was filtered (0.45 μm filter) and the supernatant transferred to fresh tubes. Ni-NTA resin (1 ml; Qiagen) was added to the supernatant and incubated with shaking for 1 h at 4 °C. The solution was transferred to a chromatography column (Poly-Prep Chromatography column, 0.8 × 4 cm, BioRad) and the flow through was passed though the column twice. The column was washed twice with 4 ml washing buffer added PMSF (50 mM $NaH_2PO_4$; 300 mM NaCl; 20 mM imidazole; 1 mM PMSF) and the protein eluted with 5 ml elution buffer (1 mM PMSF; 50 mM $NaH_2PO_4$; 300 mM NaCl; 250 mM imidazole). The identity and purity of the protein was confirmed by polyacrylamide gel electrophoresis (Coomassie stain) and the quantity determined by Bradford assay.

### 2.10. Microscale thermophoresis

Microscale thermophoresis (MST) was used to test potential binding of c-di-GMP to the CdgL protein. The protein was fluorescently labelled with the amine-reactive NT-647 dye following the recommended labelling protocol (Nanotemper Technologies). His-tagged CdgL protein (20 μM) was incubated in the accompanying labeling buffer with the dye at a molar ratio of 1:3 for 30 min at room temperature in the dark. Labelled protein and unreacted dye were separated using a NAP-5 (Cytiva) desalting/size exclusion column (Fisher Scientific), and simultaneously replacing the labeling buffer with 150 mM NaCl and 10 mM HEPES, pH 7.5. The labeled protein was diluted to 50 nM in 250 mM NaCl, 10 mM Tris pH 8.0, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT and 0.05% Tween. Protein solution (10 μl) was added to 10 μl aliquots of the c-di-GMP ligand diluted beforehand in water at concentrations ranging from 3.125 mM to 24 nM. The mixtures were incubated at room temperature for a few minutes before being loaded into premium coated NT.115 MST capillaries (Nanotemper Technologies). Fluorescence was measured at 25 °C using the red channel of the Monolith NT.115 instrument (Nanotemper Technologies). The fluorescence signals were monitored for 30 s during the thermophoresis.

## 3. Results

### 3.1. CdgL is a degenerate nucleotide cyclase domain protein conserved in the B. cereus group

Analyses using TMHMM (software identifying transmembrane helices in protein sequences) and InterProScan (software identifying protein domains, motifs and signatures) predicted CdgL (locus tag BTB_RS26690 in RefSeq entry NC_018877) to contain four N-terminal transmembrane helixes (in the region aa 5-106) and a cytoplasmic nucleotide cyclase superfamily domain (IPR029787, aa 131-227; IPR043128, aa 114-242) (Fig. 1A). These domains are overlapping homologous superfamilies with the GGDEF domain; https://www.ebi.ac.uk/interpro/entry/InterPro/IPR000160/). The nucleotide cyclase superfamily domain in CdgL (Supplementary Fig. 1a) was found to be highly degenerate compared to functional domains participating in c-di-GMP binding, neither containing a conserved GGDEF motif nor a consensus I-site (Supplementary Fig. 1b). Analysis of global transcriptional profiling (RNA-Seq) data from *B. cereus* strains ATCC 10987 and ATCC 14579 [36] strongly indicated that *cdgL* was part of a three-gene operon. The first gene of the operon (BTB_RS26685) encodes a predicted NupC family nucleoside transporter, a membrane protein family known to transport purine and pyrimidine nucleosides [37], while the third gene (BTB_RS26695) encodes a putative membrane bound glycosyl transferase family protein, which are enzymes that typically catalyze the transfer of glycosyl residues to an acceptor molecule (Fig. 1B). The whole operon containing *cdgL* (BTB_RS26685-BTB_RS26695) appears to be conserved in *B. cereus* group strains (Supplementary Fig. 2), with the exception of those belonging to the phylogenetic group comprising the non-motile species *B. pseudomycoides* (subgroup I), where the whole operon is missing. Interestingly, in non-motile *B. anthracis* the operon is conserved, however both *cdgL* and the downstream glycosyl transferase gene are frameshifted (authentic frameshift mutations confirmed by sequencing of *B. anthracis* Ames Ancestor, GBAA_5476 and GBAA_5477 in AE017334; BA_5476 and BA_5477 in AE016879). No orthologs to *cdgL* were found outside the *B. cereus* group, by BLASTP analysis (NCBI), searching the UniProtKB/Swiss-Prot database using default search parameters (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins).

### 3.2. CdgL is essential for flagellar swimming motility

A markerless in-frame *cdgL* gene deletion mutant was constructed in the model strain *B. thuringiensis* 407 (*cry*-), a motile *cry*-negative (plasmid-cured) strain known to form robust biofilms [34]. Swimming assays on 0.3% agar plates revealed the *cdgL* deletion mutant to be non-motile (Fig. 2A). Complementation of the *cdgL* deletion mutant by introducing a *cdgL* gene copy expressed *in trans* from the xylose-inducible low-copy number shuttle vector pHT304-$P_{xyl}$ (*B. thuringiensis* 407 Δ*cdgL* pHT304-cdgL) restored the swimming phenotype to that of wild type *B. thuringiensis* 407, confirming that *cdgL* is required for swimming motility in *B. thuringiensis*. A non-flagellated strain Δ*flaAB* where the two genes encoding the flagellin filament (*flaA* and *flaB*) had been deleted was included as a negative control (Fig. 2A). Atomic Force Microscopy (AFM) of *B. thuringiensis* 407 Δ*cdgL* cells grown to mid-exponential growth phase, a time point where wild type cells have been shown to be motile [14], revealed that, contrary to wild type *B. thuringiensis* 407, the *cdgL* deletion strain was devoid of flagella (Fig. 2B and C), while complementation of *cdgL in trans* restored flagellated cells (Fig. 2D). As expected the *flaAB* deletion strain was non-flagellated (Fig. 2E). Overexpression of CdgL did not produce a changed swimming or flagellation phenotype relative to an empty vector control.

### 3.3. cdgL deletion affects the expression of flagellin genes

Absence of flagella can be due to loss of expression of flagellar genes or to a defect in the assembly of the flagellar apparatus. Unlike most other bacterial species, the expression of flagellar genes in *B. cereus* group strains does not seem to follow a hierarchal cascade [38], allowing the synthesis of flagellin units even in the presence of a non-functional flagellar export apparatus [39]. Whole cell protein fractions and fractions containing cell surface proteins only, were therefore compared between *B. thuringiensis* 407 wild type, the isogenic *cdgL* deletion strain (Δ*cdgL*), and the *cdgL* complemented strain (*ΔcdgL* pHT304-$P_{xyl}$-*cdgL*), in order to detect the presence of non-exported flagellin protein. A Western blot showed that no detectable amounts of flagellin subunits were present in the

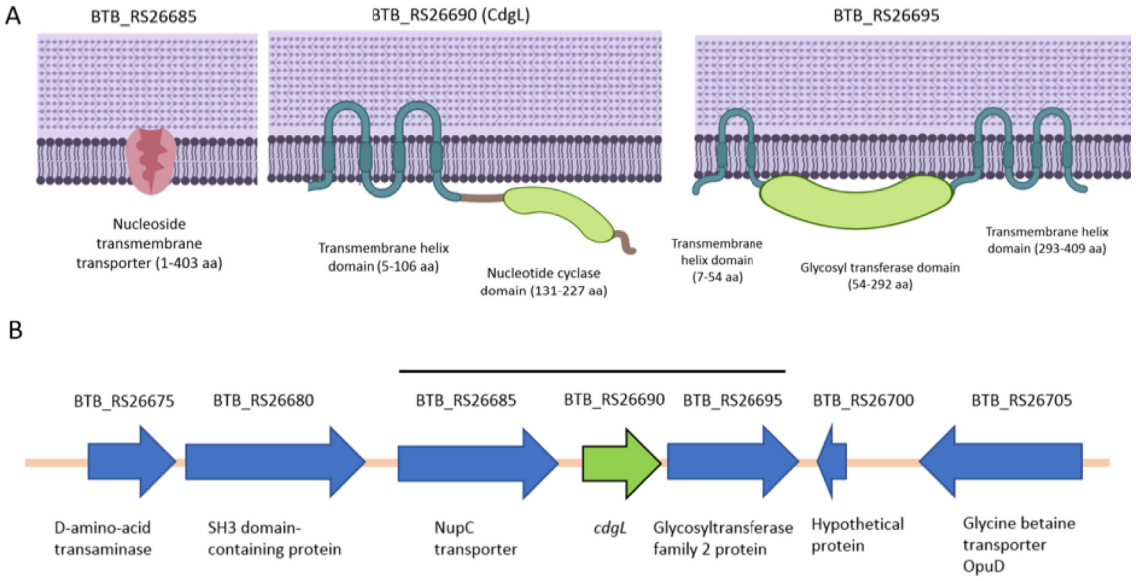


**Fig. 1.** (A) Graphic illustration of the proteins encoded in the three-gene operon containing *cdgL*, showing their predicted localization to the cell membrane. Locus tags from *B. thuringiensis* 407 and predicted protein domains are indicated. (B) Organization of the three-gene operon predicted by analysis of RNA-Seq data (indicated by the solid line), containing *cdgL* (green) and surrounding genes, specifying locus tags and predicted protein functions. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

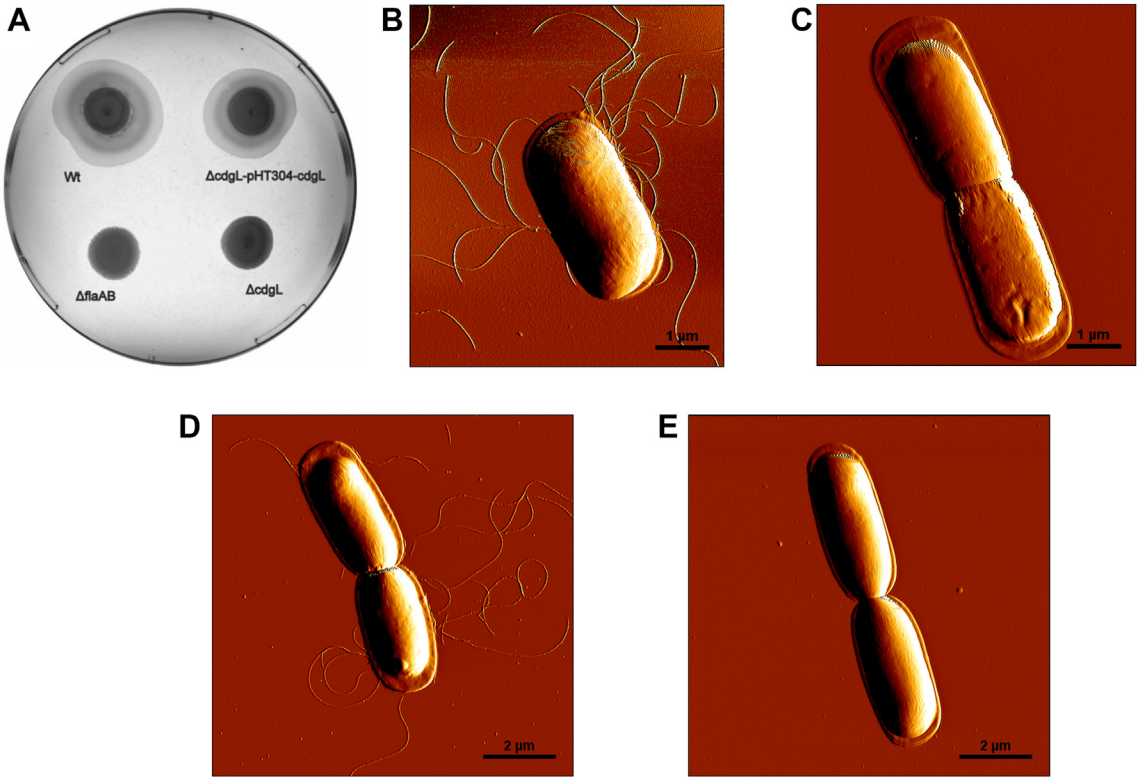


**Fig. 2. Analysis of cellular motility and presence of flagella upon deletion of *cdgL* in *B. thuringiensis* 407.** Assays were conducted using *B. thuringiensis* 407 wild type (wt) and Δ*cdgL* strains. **(A)** Swimming motility was determined following growth on 0.3% LB agar for 7 h, using a 140 mm diameter Petri dish. *B. thuringiensis* Δ*cdgL* pHT304-$P_{xyl}$-*cdgL* (*in trans* complementation strain; Table 1) was included in the analysis, along with a Δ*flaAB* strain used as a negative control. In the complementation strain, CdgL expression was induced by the addition of 1 mM xylose. The image shown was representative of three independent experiments, each performed with three technical replicates. **(B, C, D, E, F)** Atomic Force Microscopy amplitude images of bacterial cells grown to an $OD_{600}$ of 1, scanned in intermittent contact mode in air. All strains were grown at 30 °C with shaking (200 rpm). **(B)** wild type *B. thuringiensis* 407, **(C)** *B. thuringiensis* 407 Δ*cdgL* (*cdgL* deletion mutant), **(D)** *B. thuringiensis* 407 Δ*cdgL* pHT304-$P_{xyl}$-*cdgL* (*in trans* complementation strain), **(E)** *B. thuringiensis* 407 Δ*flaAB* (*flaAB* deletion mutant).

cell surface protein fraction of the *cdgL* deletion strain, and only minor amounts could be detected in the whole cell fraction (including cell surface and intracellular proteins) (Fig. 3A). Flagellin was present in both fractions from both the wild type strain and the *cdgL* complementation strain, while no flagellin was detected in the *flaAB* deletion mutant, included as a negative control. To further investigate the expression of flagellin genes in the *cdgL* deletion mutant, reverse transcriptase quantitative PCR (RT-qPCR) was performed using RNA isolated from cultures harvested in the mid-exponential growth phase. Deletion of *cdgL* resulted in an almost ten-fold downregulation of expression of the flagellin genes (*flaA* and *flaB*) compared to the wild type strain (Fig. 3B and C). Neither a *cdgL* overexpression strain nor a *cdgL* complementation strain showed significant changes in expression of *flaA* or *flaB* relative to their corresponding *B. thuringiensis* 407 empty vector control strains (Fig. 3B and C).

### 3.4. Expression of genes in the motility locus upon deletion of cdgL

Considering again the fact that the expression of flagellar genes in *B. cereus* group bacteria does not follow a hierarchical pattern, we wanted to investigate the effect of *cdgL* deletion on the expression of motility-related genes located in different operons in *B. thuringiensis* 407. Analysis of RNA-Seq data from a previous study [36] indicated that the motility genes in *B. cereus* were expressed in six operons, including the separately located *motAB* operon. Selected genes from different operons in the motility locus were included in the expression analysis (Fig. 4A; see Table 3 for gene names and annotations). RT-qPCR analysis using RNA isolated at mid-exponential growth showed that all genes tested, selected from different putative motility-related operons (including the *motAB* locus), were significantly downregulated by close to or more than ten-fold in the *cdgL* deletion strain ($0.007 < p < 0.048$) (Fig. 4B).

### 3.5. Biofilm formation is delayed in a cdgL deletion mutant

In order to examine whether *cdgL* deletion had an effect on biofilm formation, the *cdgL* deletion mutant was compared to its isogenic wild type strain in both glass tube and microtiter plate biofilm assays. In the glass tube assay, a biofilm pellicle was observed by visual inspection in the wild type strain, but not in the *cdgL* deletion strain, after 24 h. However, after 48 h, both strains had developed floating pellicles, and no difference between the strains was observed upon measurement of their pellicle biomass (Fig. 5A). In the crystal violet-based microtiter plate assay, which measures the amount of biofilm attached to the plastic well at the air-liquid interface, biofilm formation was strongly reduced in both the *cdgL* deletion strain and the *flaAB* deletion strain (control) after 24 h, relative to the wild type (Fig. 5B). After 48 and 72 h however, biofilm formation in both of these non-motile strains was comparable to that of the wild type strain (Fig. 5B). Biofilm formation was, however, not affected upon overexpression of *cdgL*, as no differences could be observed between the strain overexpressing *cdgL* and the empty vector control strain (Fig. 5C). These results indicate that *cdgL* deletion does not result in an impairment, but a delayed onset of biofilm formation. This behavior mirrors that previously observed for the non-flagellated mutant (Δ*flaAB;* [34]), possibly suggesting that the observed biofilm phenotype is a consequence of the loss of flagella in the *cdgL* mutant.

### 3.6. The degenerate CdgL GGDEF domain does not bind c-di-GMP at detectable levels

As CdgL carries a degenerate GGDEF domain, and *cdgL* deletion conferred effects on motility and biofilm formation, phenotypes which are classically controlled by c-di-GMP signaling, binding of c-di-GMP to this domain was investigated. A recombinant protein was purified, expressing an N-terminal His-tagged version of the putative CdgL c-di-GMP binding domain, but not the transmembrane domains (Supplementary Fig. 3a). Microscale thermophoresis (MST) was used to assess the binding of c-di-GMP to the purified GGDEF domain protein (25 nM) by measuring changes in fluorescence ($\Delta F_{norm}$) during 30 s of thermophoresis for a range of concentrations of the ligand, ranging from 3.125 mM down to 24 nM. However, no ligand dependent sigmoidal trend characteristic of a binding curve was observed for the collected data (two independent series) (Supplementary Fig. 3b) indicating that the CdgL degenerate nucleotide cyclase domain does not bind c-di-GMP under the tested conditions.

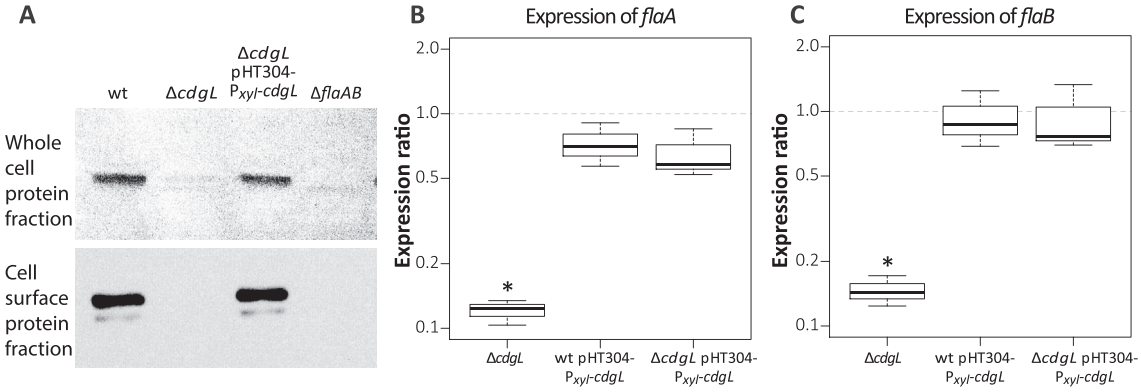


**Fig. 3. Effect of *cdgL* deletion on the presence of flagellin and expression of flagellin genes in *B. thuringiensis* 407. (A)** Western immunoblots showing the level of flagellin protein present in cell surface and whole cell protein extracts from the wild type strain, the *cdgL* deletion mutant, and the *cdgL* complementation strain, respectively. The *B. thuringiensis* 407 Δ*flaAB* strain was included as a negative control. **(B, C)** Box-plot charts demonstrating expression ratios of the **(B)** *flaA* and **(C)** *flaB* flagellin genes as determined by RT-qPCR. The gene expression data are shown as ratios relative to control strains, which were as follows: for the Δ*cdgL* mutant, the wild type *B. thuringiensis* 407 strain; for the *cdgL* overexpression strain (pHT304-$P_{xyl}$-*cdgL*), the wild type strain carrying an empty expression vector (pHT304-P*xyl*); and for the complemented strain (*B. thuringiensis* 407 Δ*cdgL*/*pHT304-*$P_{xyl}$*-cdgL*), the *cdgL* mutant carrying an empty expression vector (*B. thuringiensis* 407 Δ*cdgL*/pHT304-$P_{xyl}$). The median value of the data is indicated by the horizontal line within each box. The expression ratios of *flaA* and *flaB* in the *cdgL* mutant strain relative to wild type, were in both cases significantly different from 1 ($p < 0.003$; paired two-tailed Student's *t*-test), as indicated by the asterisk. The analysis was performed using results from three independent biological replicates, each with technical duplicates for each reaction.

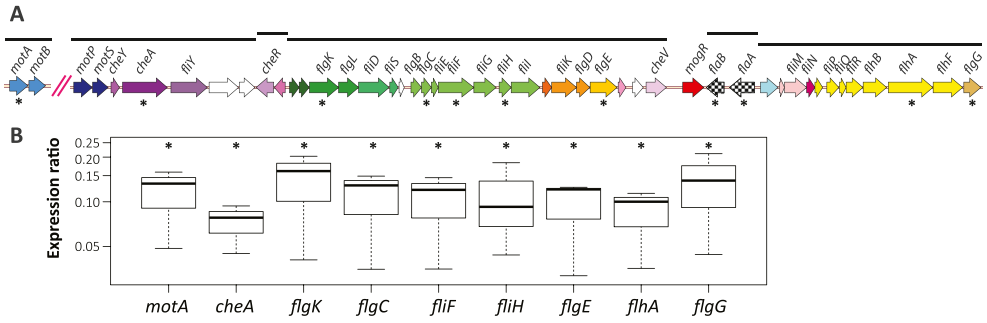


**Fig. 4. Expression of selected genes in the *B. thuringiensis* 407 motility locus following deletion of *cdgL*.** **(A)** Graphic representation of genes in the motility loci from *B. thuringiensis* 407. The solid horizontal lines indicate operon structures, predicted from RNA-Seq data from *B. cereus* strains ATCC 10987 and ATCC 14579 [36]. Annotated genes are indicated with gene names positioned above the arrows. Genes analyzed by RT-qPCR are indicated by an asterisk. **(B)** Box-plot charts demonstrating expression ratios of nine selected genes from the motility loci (as indicated by asterisks in A), in the *B. thuringiensis* 407 cdgL deletion strain relative to the wild type strain, as analyzed by RT-qPCR. Data for the relative expression of *flaA* and *flaB* are shown in Fig. 3. The median value of the data is indicated by the horizontal line within each box. Statistical analysis showed that all genes were differentially regulated (cutoff $p < 0.05$; paired two-tailed Student's *t*-test) in the Δ*cdgL* strain compared to wild type (Table 3). Experiments were performed using three independent biological replicates, each with technical duplicates for each reaction.

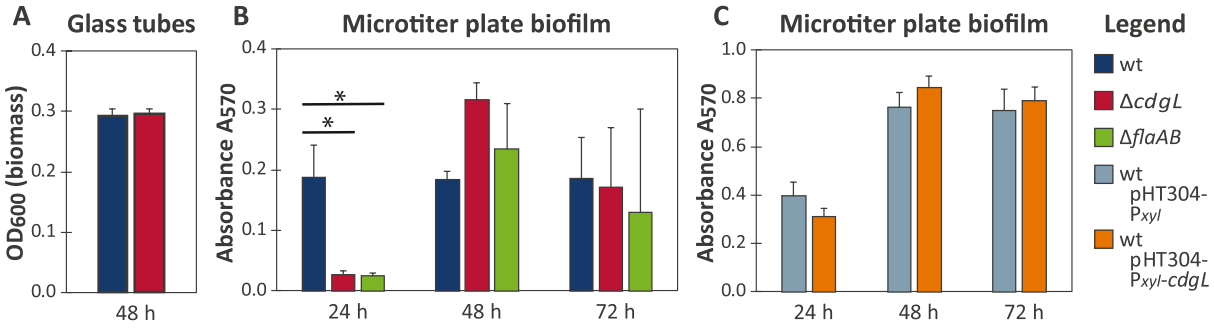


**Fig. 5. Effect of *cdgL* deletion and overexpression on biofilm formation.** Biofilm formation in **(A)** a glass tube (floating pellicle) assay after 48 h of growth, comparing wild type *B. thuringiensis* 407 to an isogenic *cdgL* deletion mutant (six independent experiments, each with five to eight technical replicates; two-tailed Student's *t*-test), and **(B, C)** a crystal violet based microtiter plate assay after 24, 48, and 72 h of growth, at 30 °C, for **(B)** wild type *B. thuringiensis* 407, its isogenic *cdgL* deletion mutant, and an isogenic *flaAB* deletion mutant, and **(C)** the *cdgL* overexpression strain compared to an empty vector control. For **(B)** and **(C)** the means and standard error of the mean for three independent experiments is shown. In **(B)** one-way ANOVA and Tukey's pairwise comparison were used to test for differences between all strains within each time-point for mutant and wild type strains. The asterisk (*) indicates $p < 0.03$. In **(C)** two-tailed Student's *t*-test was used to test for differences between strains within each time-point, for strains with plasmid vectors. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

### *3.7.* cdgL *deletion leads to modified expression of key transcriptional regulators in* B. thuringiensis *407*

Since *cdgL* deletion affected the expression of motility genes located in separate parts of the chromosome, and we found no evidence of c-di-GMP binding to CdgL, we hypothesized that CdgL could potentially act indirectly through other regulatory protein(s). The effect of the *cgdL* deletion on expression of a selected set of regulatory proteins known to affect motility and biofilm formation in the *B. cereus* group was therefore investigated, using cells harvested in the early stationary phase where key transcriptional regulators are known to be expressed [14,15,40]. Interestingly, *sinI* was significantly upregulated almost four-fold by *cdgL* deletion (p-value 0.037; Table 3), while the other regulators investigated were not found to be significantly differentially expressed relative to wild type ($0.2 < p < 0.9$) (Fig. 6; Table 3).

## 4. Discussion

### *4.1. CdgL is essential for flagellar motility in* B. thuringiensis

The CdgL protein carries a degenerate nucleotide cyclase-like domain, suggesting it could be involved in controlling phenotypes related to nucleotide-based signaling in *B. cereus* group bacteria [19]. In the current study, we show that deletion of *cdgL* results in the complete loss of flagella and flagellar motility in *B. thuringiensis* 407. The universal conservation of *cdgL* throughout the *B. cereus* group, except in the non-motile species *B. pseudomycoides*, is in line with a functional role of CdgL in motility. Several earlier studies provide data showing that expression of *cdgL* coincides with the expression of flagellar genes, and with conditions where *B. cereus* group strains are motile: In *B. cereus* ATCC 14579, *cdgL* was found to be significantly upregulated during swarming motility, which is a differentiated state characterized by hyperflagellated cells [41]. Furthermore, in *B. thuringiensis* 407, *cdgL* transcription was found to peak in the mid-exponential growth phase [19], coinciding with the peak expression of flagellar genes and the time point where the highest level of motility was observed [14]. A similar pattern was also observed in *B. anthracis*, where *cdgL* was found to be expressed during the first two hours of planktonic growth, corresponding to the mid-exponential growth phase [42]. Moreover, the expression of *cdgL* has been shown to be down-regulated in biofilms, where flagella normally are absent and motility for most cells is low [19,43]. All these findings are consistent with CdgL being functionally linked to flagellar motility.

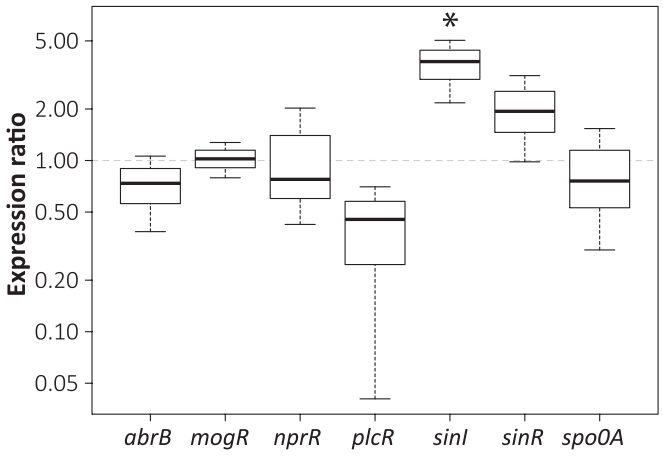


**Fig. 6.** Box-plot displaying expression ratios for transcriptional regulator genes in the *cdgL* deletion strain relative to isogenic wild type *B. thuringiensis* 407 cells, as analyzed by qPCR. The horizontal line within each box depicts the median value for each gene indicated. Results from three independent experiments are shown. Only *sinI* showed significant differential expression in the two strains ($p < 0.05$), as indicated by the asterisk ($p = 0.037$) (paired two-tailed Student's *t*-test). The experiments were performed with three independent biological replicates, each with technical duplicates for each reaction.

Deletion of *cdgL* resulted in a severe downregulation of the entire motility locus in *B. thuringiensis* 407. The repression was however not complete, with a six-to ten-fold reduction in expression of the motility genes observed upon *cdgL* deletion. This constitutes a less severe reduction than that observed upon overexpression of the motility gene repressor MogR, which results in a 30-fold and 50-fold decrease in expression of the flagellar genes *flaA* and *flaB*, respectively [14]. The observed effect of *cdgL* deletion on motility gene transcription is consistent with the detection of a small amount of flagellin protein in the whole cell fraction of the *cdgL* deletion mutant, while none was detected in the MogR overexpression strain [14]. No flagellin was detected in the cell surface fraction of the *cdgL* mutant, and the *ΔcdgL cells* were completely devoid of flagella. This could potentially suggest that CdgL also affects flagellar assembly, and clearly demonstrates the essentiality of CdgL for swimming motility in *B. cereus* group bacteria.

### 4.2. The effect of CdgL on biofilm formation may be due to loss of flagella

*B. thuringiensis* 407 readily forms robust biofilms at the air-liquid interphase, and lack of flagella has previously been claimed to negatively affect biofilm formation in this model [34]. Surprisingly, we found that while deletion of either *cdgL* or *flaAB* resulted in a delayed onset of pellicle formation, biofilm formation was not impaired in any of these strains at later time points, and neither in the non-flagellated cells resulting from overexpression of the MogR motility repressor [14]. This effect has also been observed in non-flagellated mutants of *B. subtilis* and *Listeria monocytogenes* [44–46], in line with flagella being important for movement to the site of biofilm formation and/or initial attachment. Nevertheless, flagellum-negative strains are still capable of forming surface-attached biofilm at the air-liquid interface with equal efficiency as wild type cells, given time. Taken together, the similarity of the biofilm phenotypes of the *cdgL* deletion strain and the *flaAB* mutant, which lacks only the genes encoding the flagellin subunits, as well as the missing effect of *cdgL* overexpression on biofilm formation, may suggest that *cdgL* primarily affects biofilm formation indirectly through the observed loss of flagella.

In this respect it was however also interesting to observe that expression of *sinI* was upregulated almost four-fold upon deletion of *cdgL* in *B. thuringiensis* 407. *sinI* encodes an antagonist of SinR, a transcriptional repressor of key biofilm genes in *B. cereus* and *B. subtilis* [16]. In *B. subtilis*, SinR represses biofilm formation through repression of the *tapA-sipW-tasA* [47,48] and *eps* exopolysaccharide [49] operons. SinR can also affect motility through repression of the $\sigma^D$ dependent *fla/che* operon when forming a heterodimer with its homologue SlrR [50], and through repression of *epsE*, which encodes a molecular clutch that disables flagellar motility [49]. A homologue of SinI, SlrA, aids in the anti-repression of SinR in *B. subtilis*, but has also been found to be directly involved in the repression of the *fla/che* operon, through a mechanism which is currently unknown [51]. While neither $\sigma^D$, the EpsE clutch protein, nor the SlrA/SlrR regulators are present in *B. cereus* group bacteria, the *tasA* operon is largely conserved between the *B. cereus* group and *B. subtilis* [52]. Interestingly, motility was lost in a *sinR* deletion mutant in *B. cereus*, while a *sinI* mutant was found to be hypermotile [15].

### 4.3. Function of the three-gene operon carrying cdgL

Previous work has shown that CdgL does not have the ability to synthesize c-di-GMP [28], which may be expected given that its nucleotide cyclase domain, which is essential for the catalytic activity of DGC enzymes, is poorly conserved. In the current work, we found no evidence for c-di-GMP binding to the degenerate nucleotide cyclase domain in CdgL, perhaps related to the absence of an intact I-site [53]. Thus, the effect of CdgL on motility in *B. thuringiensis* seems to be independent of the c-di-GMP regulatory network identified in this species [19,29], although the protein appears to be evolutionary related to a DGC enzyme. Clues to a function of CdgL may however perhaps be found by examining the genetic context in which the gene appears. CdgL is co-transcribed in a three-gene operon which is highly conserved in motile species of the *B. cereus* group, with genes encoding a NupC family nucleotide transporter and a putative glycosyl transferase, possibly suggesting that they are involved in a common functional pathway. All three proteins are also predicted to be located to the cell membrane. Interestingly, in a previous study of *B. cereus* ATCC 10987 genome-wide transposon mutants, revealing a large number of genes required for both biofilm formation and swimming motility [54], mutation of the glycosyl transferase gene downstream of *cdgL* resulted in a loss of both pellicle biofilm formation and motility, in line with the motility screening and biofilm results from early time points presented herein. Glycosyl transferases can be implicated in synthesis of the biofilm matrix through the formation of exopolysaccharides [55,56], but can also affect motility, as glycosylation of flagellin has been shown to affect both assembly of the flagellar apparatus and the function of flagella in both Gram-negative and Gram-positive bacteria [57–59]. In the Gram-positive bacterium *Paenibacillus alvei* CCM 2051, deletion of a glycosyl transferase resulted in complete loss of motility and extracellular flagella [60]. These observations strengthen the hypothesis that cdgL and the co-transcribed glycosyl transferase gene belong to a common functional unit. Furthermore, the first gene of the operon hosting *cdgL* encodes a NupC-like transporter, a protein family known to facilitate transport of various nucleotide-based molecules. Separate studies will be required to reveal which molecule is the substrate for this transporter and whether this molecule may have the potential to bind to the degenerate CdgL nucleotide cyclase domain. Recent studies of other proteins carrying nucleotide cyclase superfamily domains have revealed their involvement in binding and/or synthesis of nucleotides other than c-di-GMP [61–63], exemplifying a diverse range of nucleotide signaling in bacteria.

Additional work will be required to assess whether CdgL may constitute a novel effector for a nucleotide second messenger, as well as to explain the molecular links between *cdgL* deletion, and increased *sinI* transcription and repression of motility gene transcription, respectively.

In conclusion, this work clearly identifies *cdgL* as part of a three-gene operon encoding proteins that are all located to the cell membrane, and thus with a potential for molecular and functional interaction. Furthermore, CdgL constitutes a protein which is essential for the synthesis of flagellin and for flagellar motility in the *B. cereus* group, potentially through interaction with the downstream glycosyl transferase which in a separate study was shown also to be essential for motility. Further studies will be required to reveal a potential role for CdgL in nucleotide signalling in *B. cereus* group bacteria.

## Declaration of competing interest

There is no conflict of interest.

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## Appendix A. Supplementary data

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