GTP-Dependent FlhF Homodimer Supports Secretion of a Hemolysin in Bacillus cereus

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The multidomain (B-NG) protein FlhF, a flagellar biogenesis regulator in several bacteria, is the third paralog of the signal recognition particle (SRP)-GTPases Ffh and FtsY, which are known to drive protein-delivery to the plasma membrane. Previously, we showed that FlhF is required for Bacillus cereus pathogenicity in an insect model of infection, being essential for physiological peritrichous flagellation, for motility, and for the secretion of virulence proteins. Among these proteins, we found that the L2 component of hemolysin BL, one of the most powerful toxins B. cereus produces, was drastically reduced by the FlhF depletion. Herein, we demonstrate that B. cereus FlhF forms GTP-dependent homodimers in vivo since the replacement of residues critical for their GTP-dependent homodimerization alters this ability. The protein directly or indirectly controls flagellation by affecting flagellin-gene transcription and its overproduction leads to a hyperflagellated phenotype. On the other hand, FlhF does not affect the expression of the L2-encoding gene (hblC), but physically binds L2 when in its homodimeric form, recruiting the protein to the plasma membrane for secretion. We additionally show that FlhF overproduction increases L2 secretion and that the FlhF/L2 interaction requires the NG domain of FlhF. Our findings demonstrate the peculiar behavior of B. cereus FlhF, which is required for the correct flagellar pattern and acts as SRP-GTPase in the secretion of a bacterial toxin subunit.

Keywords: Bacillus cereus, flhF, L2, hemolysin BL, virulence, flagellin

INTRODUCTION

Bacillus cereus is a Gram-positive, motile, and spore-forming rod that is ubiquitously found in a wide range of environments including soil, water and foods. Long known as cause of two types of food-borne intoxications, the diarrheal and emetic syndromes, B. cereus is also responsible for local and systemic infections in humans (Ehling-Schulz et al., 2019). Its pathogenicity is associated with the bacterial ability to adhere to and colonize host surfaces through peritrichous flagella and flagellum-driven swimming and swarming (Senesi and Ghelardi, 2010). Beside motility, the secretion of tissue-destructive exoenzymes and toxins, e.g. hemolysins, phospholipases, trimeric toxins, cytotoxins and proteases (Ramarao and Sanchis, 2013; Ehling-Schulz et al., 2019), is of crucial importance in B. cereus pathogenicity. Among these factors, the tripartite hemolysin BL (HBL), consisting of a binding component (B) and two lytic components (L1 and L2), plays a...
primary role in diarrheal food poisoning and necrotizing infections such as endophthalmitis, due to its vascular permeability, hemolytic, and enterotoxic activity (Beecher et al., 1995a,b). Flagella, swarming differentiation, and HBL secretion are intrinsically linked in *B. cereus*. In fact, HBL export is increased in hyperflagellated swarm cells (Ghelardi et al., 2007; Salvetti et al., 2011) and mutations that reduce the number of flagella impact on toxin secretion (Senesi et al., 2002; Callegan et al., 2006; Salvetti et al., 2007; Mazzantini et al., 2016). In addition, failure to secrete HBL was demonstrated in aflagellated *B. cereus* natural isolates (Ghelardi et al., 2007) and in an acrylamidiferous *Bacillus thuringiensis* (i.e. *B. cereus*) mutant for the Type-III flagellar export apparatus (Ghelardi et al., 2002).

The third signal recognition particle (SRP) GTPase, FlhF, is involved in controlling *B. cereus* flagellation and motility (Salvetti et al., 2007; Mazzantini et al., 2016). In many polarly flagellated species, this protein is required for establishing the correct place and/or quantity of flagella on the cell surface (Kazmierczak and Hendrixson, 2013; Altegoer et al., 2014; Gao et al., 2015; Schuhmacher et al., 2015; Navarrete et al., 2019), together with the MinD-like ATPase FlhG (Leipe et al., 2002). In the peritrichously flagellate *Bacillus subtilis*, FlhF and FlhG behave as antagonistic pair in regulating the symmetric grid-like pattern of flagellar basal body distribution, but do not control the number of flagella on the cell surface (Bange et al., 2011; Guttenplan et al., 2013). In *B. cereus*, no FlhG homolog was found and deletion of FlhF causes flagellar mislocalization and substantial reduction in the number of flagella (Salvetti et al., 2007).

Together with Ffh and FtsY, FlhF belongs to the SRP family of GTPases (Leipe et al., 2002). In the canonical SRP system, the FlhF/4.5S RNA complex attaches to the N-terminal signal sequence of nascent membrane protein at the ribosome, and then docks with its receptor (FtsY) on the membrane, thus resulting in the insertion of the protein into the membrane (Akopian et al., 2013; Mercier et al., 2017). SRP-GTPases are characterized by conserved N and G domains that form a structurally and functionally coupled unit (NG domain) (Grudnik et al., 2009). In all described SRP-GTPases, five nucleotide-binding signature elements (G1–G5) essential for GTP binding and hydrolysis and for SRPs dimerization have been identified in the G domain (Bange et al., 2007; Mazzantini et al., 2016). All three SRP-GTPases contain additional domains, which reflect the specific functional roles of these proteins. While Ffh presents a C-terminal methionine-rich domain (M) that is essential for signal peptide recognition and SRP-RNA binding (Janda et al., 2010; Hainzl et al., 2011), FtsY contains an unfolded N-terminal acidic domain (A) which ensures membrane attachment (de Leeuw et al., 2000; Stepanovic et al., 2011; Altegoer et al., 2014). Similar to *B. subtilis* FlhF (Bange et al., 2007), the *B. cereus* protein carries a naturally unfolded N-terminal lysine-rich basic (B) domain with unknown function (Mazzantini et al., 2016). GTP-bound FlhF forms homodimers in *Xantomonas oryzae* pv *oryzae*, *B. subtilis*, *Pseudomonas aeruginosa*, *Shewanella oneidensis*, *Campylobacter jejuni*, and *Vibrio alginolyticus* (Shen et al., 2001; Bange et al., 2007; Schniederbernd et al., 2013; Gao et al., 2015; Gulbronson et al., 2016; Kondo et al., 2018).

In addition to the flagellar defects, FlhF depletion in *B. cereus* also causes an alteration in the amount of several extracellular proteins (Mazzantini et al., 2016). Among these proteins, the L2 component of HBL was found to be consistently reduced in the extracellular proteome of a *B. cereus* ∆flhF mutant (Mazzantini et al., 2016), suggesting a potential involvement of FlhF in L2 secretion mechanisms.

In the present study, the ability of *B. cereus* FlhF to form homodimers and the effect of point mutations in FlhF putative GTP-binding elements on homodimer formation were analyzed. Functional characterization of *B. cereus* FlhF as SRP protein for L2 is reported and the requirement of the NG domain for protein contact is defined. In addition, experiments were performed to investigate the effect of different FlhF levels on *B. cereus* flagellation and L2 secretion.

**MATERIALS AND METHODS**

**Bacterial Strains and Culture Conditions**

All strains used in this study are described in Table 1. *B. cereus* ATCC 14579 wild-type (WT), its flhF mutant (ΔflhF, MP06), and flhF-overexpressed (MP08) derivatives (Salvetti et al., 2007) were grown at 37°C in Brain Heart Infusion (BHI) supplemented with 1% glucose (BHIAG). When required, 5 μg/ml erythromycin for strain ΔflhF or 30 μg/ml kanamycin for strain MP08 selection were added. MP08 cultures were also supplemented with 4 mM isopropyl-β-D-1-tiogalattopiranoside (IPTG; Merck KGaA, Darmstadt, Germany) to induce *pspac*-dependent flhF expression. *E. coli* XL1-Blue was used for general cloning strategies, *E. coli* BTH101 was used as reporter strain in the bacterial adenylate cyclase two-hybrid system (BACTH) assays, and *E. coli* BL21 (DE3) for hexa-histidine pull-down experiments. *E. coli* strains were grown at 30°C or 37°C in Luria Bertani (LB) supplemented with 100 μg/ml of ampicillin, 50 μg/ml of kanamycin, or either, when required. *E. coli* BTH101 and BL21 (DE3) cultures were also supplemented with 0.5 and 1 mM IPTG, respectively, to induce plasmid gene expression.

**Plasmids Construction**

Plasmids and primers used in this study are described in Table 1 and Supplementary Table S1, respectively. For bacterial two-hybrid experiments, the complete ORFs of *B. cereus* flhF (GenBank ID: bc1670), hblC (GenBank ID: bc3104), bc1657, and the NG encoding fragment of flhF (flhFNG) were directly amplified from genomic DNA of *B. cereus* ATCC 14579 using primer pairs flhFF1/flhFR1, bc1657F1/bc1657R1, L2F2/L2R1, NGF1/flhFR1, respectively. To produce flhFF1253Q and flhFFD391A mutant variants, site-directed mutagenesis by combined overlap extension PCR (COE-PCR) was performed (Hussain and Chong, 2016). For each variant, two primers pairs (BcflhF-T253Q-F/flhFR1 and flhFF1/BcflhF-T253Q-R, and BcflhF-D391A-F/flhFR1 and flhFF1/BcflhF-D391A-R, respectively), were used to generate two flhF mutated fragments with overlapping ends. The full-length products were obtained directly joining the two fragments by PCR using flhFF1/flhFR1 as primer pair. All amplicons were digested with BamHI/KpnI
TABLE 1 | Strains and plasmids used in this study.

| Strain | Relevant genotype or description | Source or references |
|--------|----------------------------------|---------------------|
| **B. cereus** | | |
| ATCC 14579 (WT) | Wild-type strain | ATCC, Salvetti et al., 2007 |
| ΔflhF (MP08) | Derivative of ATCC 14579, carrying a Campbell integration of pRNDflhF2 in flhF | This study |
| MP08 | Derivative of ATCC 14579, containing pDGflhF | Salvetti et al., 2007 |
| **E. coli** | | |
| XL1-Blue | endA1 gyrA96 (nalR) thi-1 recA1 relA1 lac galV44 F'::Tn10 proAB lacI21 lacZD15 hsdR17 (rK– mK–); used for subcloning | Stratagene, La Jolla, California |
| BTH101 | F-, cya-99, araD139, galE15, galK16, rpsL1 (StrR), hsdR2, mcrA1, mcrB1 | Euromedex, Soufflewessheim, France |
| BL21 (DE3) | B F- ompT gal dcm lon lacS8 (R– mB–) lambda (lacUV5-T7p07 ind1 sam7 nin5) | Thermo Fisher Scientific, France |

**Plasmids**

**For BACTH experiments**

| Plasmid | Description | Source |
|---------|-------------|--------|
| pUT18 | Derivative of pUC19, ori. E. coli CoE1, AmpR, plac-MCS (Phindl-SphI-PstI-XbaI-BamHIL-Smal-KpnI-SacII-EcoRI)-T18 | Euromedex |
| pUT18-ffH | Derivative of pUT18 expressing FlhF-T18 fusion | This study |
| pUT18-ffF NG | Derivative of pUT18 expressing FlhF NG-T18 fusion | This study |
| pUT18-bc1657 | Derivative of pUT18 expressing BC1657-T18 fusion | This study |
| pUT18-hbC | Derivative of pUT18 expressing L2-T18 fusion | This study |
| pUT18-ffH T253Q | Derivative of pUT18 expressing FlhF T253Q-T18 fusion | This study |
| pUT18-ffF D391A | Derivative of pUT18 expressing FlhF D391A-T18 fusion | This study |
| pUT18C | Derivative of pUC19, ori. E. coli CoE1, AmpR, plac-T18-MCS (Phindl-SphI-PstI-XbaI-BamHIL-Smal-KpnI-SacII-EcoRI) | Euromedex |
| pUT18C-zp | pUT18C expressing the T18-leucine zipper motif of GCN4 fusion | Euromedex |
| pUT18C-ffH | Derivative of pUT18C expressing T18-ffH fusion | This study |
| pUT18C-ffF NG | Derivative of pUT18C expressing T18-ffF NG fusion | This study |
| pUT18C-bc1657 | Derivative of pUT18C expressing T18-BC1657 fusion | This study |
| pUT18C-hbC | Derivative of pUT18C expressing T18-L2 fusion | This study |
| pUT18C-ffH T253Q | Derivative of pUT18C expressing T18-ffH T253Q fusion | This study |
| pUT18C-ffF D391A | Derivative of pUT18C expressing T18-ffF D391A fusion | This study |
| pKT25 | Derivative of pSU40, ori. E. coli p15A, KmR, plac-T25-MCS (PstI-XbaI-BamHIL-Smal-KpnI-EcoRI) | Euromedex |

**For hexa histidine pull-down experiments**

| Plasmid | Description | Source |
|---------|-------------|--------|
| pET303/CT-His | Derivative of pET303 expressing FlhF, and ligated in pET18, pUT18C, pKT25, and pKNT25 vectors, under the control of the inducible plac promoter. | Thermo Fisher Scientific, France |

(Continued)

**Quantitative Real Time PCR**

WT and ΔflhF cells were inoculated in 100 ml of BHIG broth and grown at 37°C until mid-exponential phase (OD600 of ~1.0). Bacterial cultures were centrifuged at 4,000 x g at 4°C for 15 min. Cells were washed twice with cold diethylpyrocarbonate-treated water and suspended in 350 μl of RA1 lysis buffer.
(Macherey-Nagel, Düren, Germany) supplemented with 3.5 µl of β-mercaptoethanol and 0.35 g of zirconiabeads (diameter 0.1 mm; Biospec Products, Barsteville, Okla). Bacterial lysis was performed by shaking for 4 min with a mini-bead beater (Biospec Products), alternating 0.5 min of shake with 5 min in ice bath. Residual cells and debris were removed by centrifugation for 2 min at 12,000 × g and the aqueous phases were filtered through NucleoSpin® Filters. 350 µl of 70% absolute ethanol were added, and the mixture was applied to a NucleoSpin® RNA column. After being digested with 40 units of RNase-free DNase (Macherey-Nagel), total RNA was eluted from the column following the manufacturer’s instructions. 500 ng of the purified RNA were used as a template in one-step RT-PCR with the TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (Transgenbiotech, Beijing, China), according to the manufacturers. qRT-PCRs were performed on cDNA samples using the LightCycler™ FastStart DNA Master SYBR Green I and analyzed in the LightCycler instrument (Roche, Basel, Switzerland). The rpoA (GenBank ID: bc0158) and the bc4306 genes (Reiter et al., 2011; Salvetti et al., 2011) were used as endogenous controls. qRT-PCRs were performed using the primer pairs bc1657 Fup/bc1657 Rdw, hblL up/hblL dw, rpoAup1/rpoAdw1, and gatB_Yqey up/gatB_Yqey dw (Supplementary Table S1) for bc1657, hblC, rpoA, and bc4306, respectively. The amplification conditions were optimized and the amplified fragments sequenced (Supplementary Table S1) before performing qRT-PCR experiments. Melting curve analysis was carried out in parallel to qRT-PCR to confirm the specificities of the amplification reactions. Data were analyzed using the 2−ΔΔCT method (Livak and Schmittgen, 2001). Three biological replicates were performed and for each experiment three technical replicates were carried out.

**Bacterial Adenylate Cyclase Two-Hybrid Assay**

Bacterial adenylate cyclase two-hybrid assay experiments were performed according to Battesti and Bouveret (2012). The constructed BACTH vectors (Table 1) were co-transformed in chemical competent E. coli BL21 (DE3). Bacteria were grown at 37°C in 100 ml of LB broth to OD₆₀₀ of ~0.5 and genes expression was induced by adding 1 mM IPTG. Cultures were incubated for additional 2 h (OD₆₀₀ of ~2) and centrifuged at 5,000 × g for 5 min at 4°C. Pellets were suspended in 1:1 Tris Buffered Saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.2) and Pierce Lysis Buffer (Thermo Fisher Scientific, Waltham, MA, United States), and supplemented with Halt Protease Inhibitor Cocktail, EDTA-Free 1× (Thermo Fisher Scientific). Soluble fractions were isolated by centrifugation at 12,000 × g for 5 min and stored at – 20°C until use. The correct production of FlhFHis and L2 in the respective E. coli lysates was assayed by immunoblot using mouse monoclonal Anti-His(C-term)-AP Antibody (a-His; Thermo Fisher Scientific) and rabbit polyclonal sera specific to L2 (Beecher et al., 1995b), respectively. The E. coli lysate expressing FlhFHis was incubated for 1 h at 4°C with 25 ml of settled HisPur™ Cobalt resin (Thermo Fisher Scientific), previously equilibrated according to the manufacturer’s instructions. After six washing steps with 1:1 TBS and Pierce Lysis Buffer containing 20 mM imidazole (Thermo Fisher Scientific), the E. coli lysate producing L2 was applied to the column for 2 h at 4°C. Eight wash steps with TBS containing 10 mM Imidazole were performed to remove E. coli proteins which non-specifically interact. Captured proteins were then eluted with 250 µl of 1:1 TBS and Pierce Lysis Buffer containing 290 mM Imidazole. FlhFHis and L2 identification was performed by 10% SDS-PAGE followed by immunodetection using α-His and α-L2 (Beecher et al., 1995b) antibodies, respectively. To rule out the possibility that L2 had an intrinsic affinity for the cobalt resin or non-specifically interacted with E. coli proteins able to bind the resin, the soluble fraction of cells expressing L2 was directly applied to the equilibrated resin and to the resin previously incubated with the lysate of E. coli cells containing the empty vector pET303/CT. Both control samples were treated as described above.

**Hexa-Histidine Pull-Down Experiments**

pET303/CT, pET303/flhFHis and pET303/hblC (Table 1) were independently transformed in chemical competent E. coli BL21 (DE3). Bacteria were grown at 37°C in 100 ml of LB broth to OD₆₀₀ of ~0.5 and genes expression was induced by adding 1 mM IPTG. Cultures were incubated for additional 2 h (OD₆₀₀ of ~2) and centrifuged at 5,000 × g for 5 min at 4°C. Pellets were suspended in 1:1 Tris Buffered Saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.2) and Pierce Lysis Buffer (Thermo Fisher Scientific, Waltham, MA, United States), and supplemented with Halt Protease Inhibitor Cocktail, EDTA-Free 1× (Thermo Fisher Scientific). Soluble fractions were isolated by centrifugation at 12,000 × g for 5 min and stored at – 20°C until use. The correct production of FlhFHis and L2 in the respective E. coli lysates was assayed by immunoblot using mouse monoclonal Anti-His(C-term)-AP Antibody (a-His; Thermo Fisher Scientific) and rabbit polyclonal sera specific to L2 (Beecher et al., 1995b), respectively. The E. coli lysate expressing FlhFHis was incubated for 1 h at 4°C with 25 ml of settled HisPur™ Cobalt resin (Thermo Fisher Scientific), previously equilibrated according to the manufacturer’s instructions. After six washing steps with 1:1 TBS and Pierce Lysis Buffer containing 20 mM imidazole (Thermo Fisher Scientific), the E. coli lysate producing L2 was applied to the column for 2 h at 4°C. Eight wash steps with TBS containing 10 mM Imidazole were performed to remove E. coli proteins which non-specifically interact. Captured proteins were then eluted with 250 µl of 1:1 TBS and Pierce Lysis Buffer containing 290 mM Imidazole. FlhFHis and L2 identification was performed by 10% SDS-PAGE followed by immunodetection using α-His and α-L2 (Beecher et al., 1995b) antibodies, respectively. To rule out the possibility that L2 had an intrinsic affinity for the cobalt resin or non-specifically interacted with E. coli proteins able to bind the resin, the soluble fraction of cells expressing L2 was directly applied to the equilibrated resin and to the resin previously incubated with the lysate of E. coli cells containing the empty vector pET303/CT. Both control samples were treated as described above.

**Quantification of β-Galactosidase Activity**

β-galactosidase assays were performed according to the Miller’s method (Miller, 1992). Overnight bacterial cultures were diluted in 10 ml of LB broth containing 100 µg/ml ampicillin, 50 µg/ml kanamycin, and 0.5 mM IPTG and grown at 30°C to OD₆₀₀ of ~0.5. 1 ml of each culture was centrifuged at 8000 × g at 4°C for 5 min and the pellet was suspended in an equal amount of chilled Z buffer (60 mM Na₂HPO₄ - 7H₂O, 40 mM NaH₂PO₄ - H₂O, 10 mM KCl, 1 mM MgSO₄ - 7H₂O, and 50 mM β-mercaptoethanol; pH 7.0). For cells permeabilization, 20 µl of 0.1% sodium dodecyl sulfate (SDS; Merck KGaA) and 40 µl of chloroform were added and the tubes were mixed by vortexing for 10 s. 100 µl of samples were diluted in 1 ml of chilled Z buffer and incubated with 200 µl of 4 mg/ml orto-nitrofenil-β-galactopyranoside (ONPG, Merck KGaA) at 28°C. The reaction was stopped by adding 250 µl of 1 M Na₂CO₃, β-galactosidase activity was calculated by the Miller formula (Miller units = 1000 × (OD₄₂₀ - (1.75 × OD₅₅₀)/T × V × OD₆₀₀); T, reaction time; V, volume of culture assayed in milliliter). Experiments were repeated three times in separate days and for each assay two technical replicates were carried out.
Flagella Staining and Flagellin Purification

WT and MP08 strains were grown to the late exponential growth phase in BHIG for 6 h at 37°C (OD_600 of ~2). For flagella observation, 10 μl of bacterial cells were directly stained with tannic acid and silver nitrate (Harshey and Matsuyama, 1994). Several samples were analyzed at 1,000 × magnification using an optical microscope (BH-2; Olympus, Tokyo, Japan). The extent of cell flagellation was analyzed as previously described (Calvio et al., 2005). Briefly, bacterial cultures were vigorously vortexed for 30 s, and harvested by centrifugation at 5,000 × g for 10 min at 4°C. Flagellar filaments were collected from supernatants by ultracentrifugation at 100,000 × g for 1 h at 4°C. Protein concentration was determined by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific), samples were standardized accordingly, and were subjected to 10% SDS-PAGE followed by Comassie Blue staining. Experiments were performed in triplicate in separate days and a representative result is shown.

Preparation of B. cereus Supernatants

Protein samples were prepared by growing WT and MP08 cells to the late exponential growth phase in BHIG for 6 h at 37°C (OD_600 of ~2). Cells were normalized to the same OD_600 and culture supernatants were collected by centrifugation at 10,000 × g, and added with Halt Protease Inhibitor Cocktail, EDTA-Free 1 × and 0.5 mM EDTA. Protein concentration was determined with Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). To exclude cell lysis, the activity of the cytosolic marker fructose-1,6-bisphosphate aldolase in culture supernatants was spectrophotometrically determined (Warth, 1980). Culture supernatants were concentrated using Microcon™ 10 centrifugal filter units (Merck KGaA). After being standardized for total proteins concentration, protein samples were subjected to 10% SDS-PAGE and electrotransferred on PVDF membranes for L_2 immunodetection (Beecher et al., 1995b). All experiments were performed three times in separate days and a representative result is shown.

Image Analysis

Densitometric analysis of Comassie blue stained gels and immunodetected filters were performed using a ChemiDoc™ XRS+ System with a software version 6.0.1.34 (Bio-Rad, Berkeley, California). Relative quantification was performed using the WT bands as reference.

In silico Analysis

Nucleotide and amino acid sequences in the FASTA format were retrieved from the European Nucleotide Archive¹ and the UniProt databases (The UniProt Consortium, 2015), respectively. BLASTn² was used for comparative analysis of nucleotide sequences obtained from sequencing. Compute pI/Mw tool (ExPasy Bioinformatics Resource Portal) was used for molecular weight prediction of amino acid sequences. The representation of B. cereus FlhF domains was realized using the Illustrator for Biological Sequences (IBS) tool (Liu et al., 2015).

Statistical Analysis

Data were expressed as the mean ± standard deviation (S.D.). Statistical analysis was done on GraphPad Prism version 8.0.2. For β-galactosidase quantification, the one-way analysis of variance (ANOVA) followed by the Tukey HSD test for multiple comparisons was applied. For qRT-PCR experiments, ANOVA followed by Dunnett’s multiple comparisons test was used by setting the WT values as control. For densitometric data, the two-tailed Student’s t-test for unpaired data was used. A two-sided p-value (p < 0.05) was considered significant.

RESULTS

Self-Interaction of B. cereus FlhF

The bacterial adenylate cyclase two-hybrid (BACTH) approach was applied to investigate whether FlhF forms dimers in B. cereus. The complete ORF of B. cereus flhF was fused to the T18 and T25 encoding domains of Bordetella pertussis adenylate cyclase (Cya) contained in pUT18, pUT18C, pKT25, and pKNT25 (Table 1), under the control of the plac promoter. Expression of recombinant pUT18C and pKT25 vectors lead to the production of hybrid proteins in which the adenylate cyclase domain is localized in a C-terminal position. Fusion proteins expressed by recombinant pUT18C and pKT25 possess the enzymatic domain in an N-terminal location. Four combinations of recombinant plasmids (pUT18-flhF/pKT25-flhF, pUT18-flhF/pKNT25-flhF, pUT18C-flhF/pKT25-flhF, and pUT18C-flhF/pKNT25-flhF) were co-transformed into the reporter strain E. coli BTH101, which is defective for Cya activity. Fusion proteins were tested for their ability to interact by growing co-transformed cells on M63 minimal medium plates containing maltose as unique carbon source and IPTG as inducer of protein expression. Only in the case of FlhF/FlhF interaction, Cya could be restored, cyclic adenosine monophosphate (cAMP) synthesized, and the maltose operon genes as well as the cAMP-reporter gene β-galactosidase expressed in the E. coli BTH101 strain. Colonies containing the combinations of vectors pUT18-flhF/pKT25-flhF and pUT18C-flhF/pKT25-flhF were able to grow, indicating that a physical interaction between FlhF monomers exists in vivo when fusion proteins carried the T25 domain in a N-terminal position. These qualitative observations were supported by quantification of the β-galactosidase activity that was significantly increased (p < 0.001) compared to the negative controls (Figure 1A).

GTP-dependent homodimerization of B. subtilis FlhF has principally been attributed to a threonine (T184) and an aspartic acid residue (D320) found in the G1 and G4 elements of the G domain respectively, (Bange et al., 2007). To determine whether mutations in the corresponding residues of B. cereus FlhF (i.e. T253; D391; Figure 1B) altered

¹https://www.ebi.ac.uk/ena
²http://blast.ncbi.nlm.nih.gov/Blast.cgi
FlhF Is Required for Synthesis but Not for the Export of Flagellin

Since the FlhF depletion in B. cereus causes a drastic reduction in the number of flagella (Salvetti et al., 2007), we performed a comparative analysis of the expression of the flagellin gene bc1657 in the ΔflhF mutant and in the wild-type (WT) strain. As references, we used the genes rpoA and bc4306, encoding the DNA-directed RNA polymerase subunit alpha (Salvetti et al., 2011) and the Gatb_Yqey domain-containing protein (Reiter et al., 2011), respectively. For each strain, the threshold cycle (CT) of bc1657 was separately normalized to the CTs of the reference genes. The transcription level of bc1657 was lower in the ΔflhF mutant compared to the WT (p < 0.001) using both reference genes (Figure 2A). These findings indicate that FlhF is required for full bc1657 expression in B. cereus.

To evaluate whether FlhF could directly be involved in flagellin targeting/recruitment to the plasma membrane, a physical interaction between FlhF and flagellin was checked in vivo. BACTH experiments were applied using eight combinations of recombinant vectors carrying the complete ORFs of flhF and bc1657 (Table 1). No colonies were obtained on the M63 minimal medium indicating that FlhF is unable to interact with flagellin BC1657 in vivo (data not shown).

FlhF Is Dispensable for the Expression of the L2 Encoding Gene

The low amount of the L2 component of HBL found in the extracellular proteome of the B. cereus ΔflhF strain (Mazzantini et al., 2016) prompted us to assess whether the transcription level of the L2 encoding gene (hblC) was altered in such a strain. A comparative expression analysis of hblC was performed in the WT and the ΔflhF mutant. Also in this case, rpoA and bc4306 were used as reference genes. In contrast to flagellin, no significant difference (p > 0.05) in hblC expression was found using both reference genes, indicating that FlhF depletion does not alter hblC expression (Figure 2B). Therefore, the reduced amount of L2 secreted by the ΔflhF mutant (ratio ΔflhF/WT = 0.38) (Mazzantini et al., 2016) is not the result of an expression defect, but it likely seems the consequence of altered export.
His-tagged version of B. cereus hblC containing the combinations of vectors pUT18C-flhF and L2 were obtained with clones ORFs of flhF. Eight combinations of recombinant vectors carrying the complete method to confirm FlhF/L2 interaction in vitro the combination pKNT25-flhF was significantly higher than the negative controls (p < 0.001 for the combination pUT18C-flhF/pKT25-hblC, and pUT18C-flhF/pKNT25-hblC), and p < 0.01 for the combination pKNT25-flhF/pUT18C-hblC). These findings indicate that FlhF directly interacts with L2 in vivo.

Hexa-histidine (His) pull-down was used as independent in vitro method to confirm FlhF/L2 interaction. A C-terminal His-tagged version of B. cereus FlhF (FlhFHis) and an untagged version of L2 were expressed in E. coli BL21 (DE3) to form the bait and prey proteins, respectively. The soluble fraction of cells expressing FlhFHis was applied to the HisPur™ Cobalt Resin and used as bait. The resin was washed and directly treated with elution buffer to obtain the FlhFHis fraction. In parallel, FlhFHis-bound resin was incubated with the extract of cells expressing L2 and treated with the elution buffer to obtain the FlhFHis/L2 fraction. Both fractions were subjected to SDS-PAGE. A band having a molecular weight compatible with FlhFHis was found in the FlhFHis and FlhFHis/L2 fractions (Figure 3A). A second band with a molecular weight compatible with that predicted for L2 was observed only in the FlhFHis/L2 eluted fraction. The identification of L2 and FlhFHis in the FlhFHis/L2 fraction was performed by immunoblot analysis using anti-L2 (Beecher et al., 1995b) and anti-His antibodies, respectively. As shown in Figure 3B, immunoreactive bands corresponding to L2 and FlhFHis were found in this fraction. No FlhFHis and L2 immunoreactive bands were detected in controls samples (data not shown), indicating that L2 was pulled down only in the presence of FlhFHis. Taken together, these findings indicate a direct interaction between FlhF and the L2 protein.

The NG Domain of FlhF Interacts With L2
To investigate if a physical contact between the NG domain and L2 can occur, the nucleotide sequence (flhFNG) encoding the NG domain of FlhF (Figure 1B) was amplified and cloned in the different vectors of the BACTH system (Table 1). The NG domain was tested for its ability to interact with L2 by co-transforming E. coli BTH101 with each of the above mentioned plasmids and each of the plasmids containing hblC (Table 1). The combinations of vectors pUT18-flhFNG/pKT25-hblC, pUT18-flhFNG/pKNT25-hblC, and pUT18C-flhFNG/pKNT25-hblC gave positive results on the M63 minimal medium. The β-galactosidase activity of these clones was significantly increased compared to the negative controls (p < 0.001; Figure 4). Based on these results, the NG domain of FlhF is able to interact with L2.

Point Mutations in FlhF Impair L2 Binding
To identify a possible correlation between FlhF dimerization and L2 binding, BACTH experiments were performed. All the possible combinations of vectors containing flhF391A and hblC (Table 1) were used to transform E. coli BTH101.

None of the co-transformed E. coli cells grew on the M63 minimal medium (data not shown), indicating that no interaction of FlhF391A and FlhF391A with L2 occurs and that both residues (T253 and D391) are essential for FlhF/L2 interaction. Taken together, our experiments suggest that the GTP-dependent homodimerization of FlhF is a prerequisite for its ability to interact with the L2 subunit of HBL.

Effect of FlhF Overproduction on Flagella and L2 Secretion
To evaluate the effect of FlhF overproduction on B. cereus flagellation and L2 secretion, a strain carrying a plasmid

**FIGURE 2** qRT-PCR analysis of the expression of flagellin (bc1657) and L2 flagella encoding genes in the ΔflhF mutant. (A) The level of bc1657 expression in the ΔflhF mutant (light gray bars) is presented as fold change relative to the corresponding gene in the WT strain (B. cereus ATCC 14579; white bar). (B) The level of hblC expression in the ΔflhF mutant (light gray bars) is presented as fold change relative to the corresponding gene in the WT strain (B. cereus ATCC 14579; white bar). For both figures. bc1657 and rpoA were independently used as reference genes for normalization. The relative expression was measured using β2-microglobulin and rpoA as reference genes. The expression levels were calculated using the 2(−ΔΔCt) method. The data are expressed as the mean ± S.D. from three independent experiments.

**Figure Caption:**

**A** qRT-PCR analysis of the expression of flagellin (bc1657) and L2 flagella encoding genes in the ΔflhF mutant. (A) The level of bc1657 expression in the ΔflhF mutant (light gray bars) is presented as fold change relative to the corresponding gene in the WT strain (B. cereus ATCC 14579; white bar). (B) The level of hblC expression in the ΔflhF mutant (light gray bars) is presented as fold change relative to the corresponding gene in the WT strain (B. cereus ATCC 14579; white bar). For both figures, bc1657 and rpoA were independently used as reference genes for normalization. The relative expression was measured using β2-microglobulin and rpoA as reference genes. The expression levels were calculated using the 2(−ΔΔCt) method. The data are expressed as the mean ± S.D. from three independent experiments.

**Figure Legend:**

**A** qRT-PCR analysis of the expression of flagellin (bc1657) and L2 flagella encoding genes in the ΔflhF mutant. (A) The level of bc1657 expression in the ΔflhF mutant (light gray bars) is presented as fold change relative to the corresponding gene in the WT strain (B. cereus ATCC 14579; white bar). (B) The level of hblC expression in the ΔflhF mutant (light gray bars) is presented as fold change relative to the corresponding gene in the WT strain (B. cereus ATCC 14579; white bar). For both figures, bc1657 and rpoA were independently used as reference genes for normalization. The relative expression was measured using β2-microglobulin and rpoA as reference genes. The expression levels were calculated using the 2(−ΔΔCt) method. The data are expressed as the mean ± S.D. from three independent experiments.

**Figure Title:**

**FlhF Physically Interacts With the L2 Component of HBL**

To investigate whether a physical interaction between FlhF and L2 occurs, BACTH experiments were performed using the eight combinations of recombinant vectors carrying the complete ORFs of flhF and hblC of B. cereus (Table 1). Positive interactions on the M63 minimal medium were obtained with clones containing the combinations of vectors pUT18-flhF/pKT25-hblC, pUT18C-flhF/pKT25-hblC, and pKNT25-flhF/pUT18C-hblC. The β-galactosidase activity of positive clones (Figure 1A) was significantly higher than the negative controls (p < 0.05 for the combination pUT18-flhF/pKT25-hblC; p < 0.001 for the combination pUT18C-flhF/pKT25-hblC, and p < 0.01 for the combination pKNT25-flhF/pUT18C-hblC). These findings indicate that FlhF directly interacts with L2 in vivo.
containing flhF under the control of the psac promoter (MP08) was analyzed in comparison to the WT (Salvetti et al., 2007). Light microscopy of bacteria subjected to flagellar staining suggested an increase in the amount of flagella per cell in the MP08 strain compared to the WT (Figure 5A). Therefore, the extent of cell flagellation was analyzed by quantifying the amount of filament assembled flagellin in both strains. Density of the flagellin band was 1.83 ± 0.26-fold higher in the MP08 strain compared to the WT (p < 0.01; Supplementary Figure S1).

Normalized culture supernatants from the WT and MP08 were subjected to SDS-PAGE and immunoblot analysis for the detection of L2. The protein was more abundant (2.51 ± 0.171-fold higher) in the supernatant of the MP08 strain compared to the WT (p < 0.001; Figure 5B). Overall, these results indicate that FlhF overproduction increases the number of flagella and L2 secretion in B. cereus.

DISCUSSION

In the present study, we found that B. cereus FlhF is able to self-dimerize in vivo. Two of the four combinations of recombinant plasmids used to test FlhF dimerization indicated
a positive interaction. The absence of interaction observed with the other combinations could be the consequence of a misfolding or instability of the hybrid proteins that prevent the contact, as previously suggested (Bange et al., 2011; Battesti and Bouveret, 2012).

FlhF homodimers are stabilized by hydrogen bonds in trans that involve both FlhF monomers (Bange et al., 2007). A threonine residue, conserved in all FlhF proteins with the exception of P. aeruginosa FlhF (Mazzantini et al., 2016), is believed to be essential for dimer stabilization through the interaction with the side chains of a lysine and a glutamic acid residue of the G4 element (Bange et al., 2007). However, the impact of the replacement of this residue on FlhF dimerization has never been analyzed before. Herein we demonstrate that a T253Q substitution in B. cereus FlhF completely abrogates homodimerization in vivo, indicating that this residue is essential for the interaction of monomers and/or for dimer stabilization.

In B. subtilis FlhF, specificity toward the guanosine nucleotides GDP and GTP is established by the interaction between an aspartic acid residue (D320 in B. subtilis FlhF) of the G4 element and the guanine base of the nucleotide with two hydrogen bonds. This residue is conserved in all SRP-GTPases (Bange et al., 2007). When a mutation was introduced in a residue corresponding to D320 in FtsY, the ability of the protein to bind and hydrolyze GTP was impaired, and FtsY could not form a complex with Ffh (Shan et al., 2004). A point mutation of the same aspartic acid residue strongly reduced the FlhF GTPase activity in V. cholerae and S. oneidensis (Green et al., 2009; Gao et al., 2015), and abrogated GTP binding and attenuated self-dimerization in P. aeruginosa (Schniederberend et al., 2013). The same mutation also impaired GTP load and dimer formation of FlhF in V. alginolyticus (Kusumoto et al., 2009; Kondo et al., 2018). In the present study, we found that the replacement of the corresponding D391 residue in B. cereus FlhF attenuates homodimerization, suggesting that this residue is required but not essential in this process. This result is in line with previous findings showing that a threonine residue of the G5 element (T343 in B. subtilis), conserved only in FlhF homologs, forms a third hydrogen bond with GTP in the active site, enhancing FlhF nucleotide binding compared to other SRP-GTPases (Bange et al., 2007). Since this residue is also conserved in B. cereus FlhF (T414) (Mazzantini et al., 2016), it could exert the same function maintaining a basal degree of GTP binding and FlhF dimerization.

Functionally, the main role of FlhF is supposedly related to flagellar assembly. The protein has been described to directly or indirectly, drive the synthesis of flagellar components, stimulating wild-type levels of flagellar genes expression (Niehus et al., 2004; Kim et al., 2012; Kuzmincik and Hendrixson, 2013; Schuhmacher et al., 2015; Ren et al., 2018), and/or to dictate the point of flagellar assembly by recruiting early flagellar components to the plasma membrane (Murray and Kazmierczak, 2006; Green et al., 2009; Guttenplan et al., 2013; Allegro et al., 2014). In the ΔflhF mutant of B. cereus, the amount of flagellin BC1657 was drastically reduced (ratio ΔflhF/WT = 0.22) (Mazzantini et al., 2016). In this study, qRT-PCR experiments were performed to analyze the transcriptional level of bc1657 in the WT and the ΔflhF mutant using two housekeeping genes, rpoA and bc4306. rpoA has often been used as endogenous reference for qRT-PCR in B. cereus ATCC 14579 (Grande Burgos et al., 2009; Cadot et al., 2010; Salvetti et al., 2011). Since this gene has a high coefficient of variation (about 43.0%), we also included the more stably expressed gene bc4306, which has a coefficient of variation of 8.41% (Bergman et al., 2006; Reiter et al., 2011). The finding that the transcription level of bc1657 was significantly reduced in the ΔflhF mutant, suggests that FlhF is required for the regulation of flagellar gene expression in B. cereus. In line with this hypothesis, no physical interaction between FlhF and BC1657 was found in vivo. However, we cannot exclude that B. cereus FlhF could be involved in the recruitment of early flagellar basal body components to the plasma membrane, as previously indicated for other microorganisms (Green et al., 2009; Guttenplan et al., 2013) thus indirectly influencing the expression of late flagellar genes.

The striking similarities between FlhF and FliF/FtsY might reflect similar functions in the targeting of proteins to the plasma membrane before secretion. Herein we demonstrate that the reduction in the export of the L2 component of HBL by the ΔflhF mutant (Mazzantini et al., 2016) is not due to a reduced expression of hblC and that physical interaction between FlhF and L2 occurs in B. cereus. FlhF interacts with the flagellar export chaperone Fls in Helicobacter pylori (Rain et al., 2001), with FlhG in V. alginolyticus, B. subtilis and S. putrefaciens (Kusumoto et al., 2008; Bange et al., 2011; Rossmann et al., 2015), with the pilus-twitching motility regulator XooPilL in X. oryzae pv oryzae (Shen et al., 2001), with the C ring protein FlIG and the polar scaffolding protein FimV in P. aeruginosa (Schniederberend et al., 2019). In addition, the protein is required for the recruitment of the MS ring early flagellar component FlF in V. cholerae (Green et al., 2009). This is the first report showing evidence that FlhF interacts with a protein that is not involved in flagellar biogenesis or bacterial motility.

Little is known on the role of FlhF domains and on how these domains are involved in the binding of FlhF to its interactors. The G domain of FlhF was found essential in the recruitment of FlIF to the cell pole in V. cholerae (Green et al., 2009), while the entire NG domain mediates the interaction between FlhF and FlhG in B. subtilis (Bange et al., 2011). In this study, we describe the L2 component of HBL as novel binding partner of FlhF in B. cereus. FlhF can cycle between two mutually exclusive forms: a cytoplasmic, monomeric, and nucleotide-free or GDP-bound, and a GTP-dependent, membrane-bound homodimer which represents the “ON” state of FlhF (Bange et al., 2007; Schuhmacher et al., 2015; Kondo et al., 2018). The dimeric form of FlhF has been described to be required for FlhF/FlhG contact and for the recruitment of flagellar components to the plasma membrane (Kusumoto et al., 2009; Bange et al., 2011; Schuhmacher et al., 2015; Kondo et al., 2018). Our finding that the mutant variants FlhFT253Q and FlhFp391A completely lose the ability to interact with L2 suggests that stable homodimerization of FlhF, which requires both T253 and D391, is crucial for L2 binding in B. cereus. Thus, we favor the idea that the GTP-bound homodimer of FlhF serves during secretion of the L2 subunit of HBL.
The effect of flhF overexpression on bacterial flagellation has been investigated in several microorganisms. Similar to polar flagellates (Panda et al., 2000; Correa et al., 2005; Kusumoto et al., 2008; Green et al., 2009) and different to B. subtilis (Guttenplan et al., 2013), FlhF overproduction in B. cereus results in a hyperflagellated phenotype. FlhF levels are also critical in defining the amount of one extracellularly secreted HBL component, being therefore the protein important in modulating the pathogenic potential of B. cereus.

**DATA AVAILABILITY STATEMENT**

All datasets generated for this study are included in the article/Supplementary Material.

**AUTHOR CONTRIBUTIONS**

EG and GB designed the study. DMa and RF performed the experiments and prepared the figures. All authors analyzed the data and wrote the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00879/full#supplementary-material

**FIGURE S1 |** Effect of FlhF overexpression on *B. cereus* flagellar filaments. SDS-PAGE analysis of extracellular flagellin of the WT and MP08 strains.

**TABLE S1 |** Primers used for PCR amplification and/or sequencing.

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