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FlhF, a signal recognition particle-like GTPase, is involved in the regulation of flagellar arrangement, motility behaviour and protein secretion in *Bacillus cereus*

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Flagellar arrangement is a highly conserved feature within bacterial species. However, only a few genes regulating cell flagellation have been described in polar flagellate bacteria. This report demonstrates that the arrangement of flagella in the peritrichous flagellate *Bacillus cereus* is controlled by *flhF*. Disruption of *flhF* in *B. cereus* led to a reduction in the number of flagella from 10–12 to 1–3 filaments per cell in the insertion mutant MP06. Moreover, compared to the parental strain, MP06 exhibited: (i) shorter smooth swimming phases, causing reduced swimming motility but not affecting chemotaxis; (ii) complete inhibition of swarming motility, as differentiated swarm cells were never detected; (iii) an increased amount of extracellular proteins; and (iv) differential export of virulence determinants, such as haemolysin BL (HBL), phosphatidylcholine-preferring phospholipase C (PC-PLC) and non-haemolytic enterotoxin (NHE). Introduction of a plasmid harbouring *flhF* (pDG*flhF*) into MP06 completely restored the wild-type phenotype in the *trans*-complemented strain MP07. *B. cereus flhF* was found to constitute a monocistronic transcriptional unit and its overexpression did not produce abnormal features in the wild-type background. Characterization of a *B. cereus* mutant (MP05) carrying a partial *flhF* deletion indicated that the last C-terminal domain of FlhF is involved in protein export while not required for flagellar arrangement and motility behaviour. Taken together, these data suggest that *B. cereus* FlhF is a promising candidate for connecting diverse cellular functions, such as flagellar arrangement, motility behaviour, pattern of protein secretion and virulence phenotype.

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INTRODUCTION

The number and position of flagellar filaments on the surface of bacterial cells are highly conserved phenotypic traits, with the polar and peritrichous arrangements being the most frequently observed. Though the type of cell flagellation is used as an additional taxonomic key to distinguish motile bacteria, little is known about the mechanisms regulating the maintenance of the number and position of flagella. Only a few genes affecting these phenotypic traits have recently been identified in polar flagellates. *fleN* (also referred to as *flhG*) plays a role in regulating the number of polar flagella in *Pseudomonas aeruginosa* and *Vibrio* spp. (Correa *et al.*, 2005; Dasgupta *et al.*, 2000; Kusumoto *et al.*, 2006). *flhF* has been recognized to be necessary for the placement of flagella at a cell pole in *Pseudomonas putida* (Pandza *et al.*, 2000) and *P. aeruginosa* (Murray & Kazmierczak, 2006), as well as for flagellation in *Vibrio cholerae* (Correa *et al.*, 2005), *Campylobacter jejuni* (Hendrixson & DiRita, 2003) and *Helicobacter pylori* (Niehus *et al.*, 2004). In *Bacillus subtilis*, a newly identified gene, *swrAA*, governs the degree of cell flagellation (Calvio *et al.*, 2005), while the role of *flhF*, also present in this peritrichous species, remains to be clarified. In fact, depending upon the genetic background of *B. subtilis*, *flhF* disruption has been reported to differently

Abbreviations: BCA, bicinchoninic acid; HBL, haemolysin BL; NHE, non-haemolytic enterotoxin; PC, phosphatidylcholine; PC-PLC, phosphatidylcholine-preferring phospholipase C; SRP, signal recognition particle.

The GenBank/EMBL/DDBJ accession number for the *flhF* sequence reported in this study is AM180264.

affect the motility phenotype of this species (Carpenter *et al.*, 1992; Zanen *et al.*, 2004). In all bacterial species studied so far, the protein encoded by *flhF* shares substantial homology with proteins of the GTP-binding signal recognition particle (SRP) family (Carpenter *et al.*, 1992; Kim & McCarter, 2000; Pandza *et al.*, 2000), such as Ffh and FtsY. However, while Ffh and FtsY are required for targeting many secretory and membrane proteins to the plasma membrane (for a review see Halic & Beckmann, 2005), an involvement of FlhF in protein secretion has been demonstrated only in *P. putida* (Pandza *et al.*, 2000).

Despite the observation that the arrangement of bacterial flagella is conserved over generations, this feature can vary following cell adaptation to different environmental conditions. For several flagellated bacteria, surface sensing is an environmental signal inducing swarming differentiation, a specialized form of flagellum-driven motility that is exhibited by a wide range of species when transferred from liquid to solid growth media (Allison & Hughes, 1991; Ghelardi *et al.*, 2002; Kearns & Losick, 2003; McCarter, 1999; Senesi *et al.*, 2002). This surface-induced motility is dependent on the production of differentiated swarm cells that are aseptate, multinucleate and elongated, and express a higher number of peritrichously placed flagella than the corresponding oligoflagellated swimmer cells (for a review see Fraser & Hughes, 1999). The swarm cells possess the unique ability to migrate away from the colony in organized groups of tightly bound cells that coordinately move across solid surfaces, including host mucosal surfaces during colonization by swarming-proficient infectious agents (for a review see Fraser & Hughes, 1999). In *Bacillus cereus*, the ability to swarm contributes to the severity of experimental endophthalmitis in rabbits, being responsible for a very rapid invasion of the anterior segment of the eye (Callegan *et al.*, 2006).

The interest in studying bacterial flagella has been substantially increased following the demonstration that the flagellum should not be regarded only as a locomotion organelle. Indeed, it contains a sophisticated secretory apparatus, whose components share substantial homology with those of the type III secretion system described exclusively in Gram-negative bacteria (for a review see Hueck, 1998). In *Escherichia coli*, *Yersinia enterocolitica* and *C. jejuni* the flagellar export apparatus is required for the secretion of specific virulence-associated proteins (Givskov & Molin, 1993; Konkel *et al.*, 2004; Young *et al.*, 1999a). In *Bacillus thuringiensis*, the closest relative of *B. cereus* (Helgason *et al.*, 2000), the flagellar type III secretion system is required for secretion of haemolysin BL (HBL), a tripartite heat-labile toxin showing haemolytic, enterotoxic and dermo-necrotizing activities, as mutants lacking flagella do not export the intracellularly produced toxin (Ghelardi *et al.*, 2002). In this study, we describe the *flhF* gene in the peritrichous flagellate species *B. cereus* and give evidence showing its role in regulating degree of cell flagellation, motility behaviour and protein secretion.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and the plasmids used are listed in Table 1. Bacteria were grown at 37 °C in either tryptone-NaCl (TrB; 1 % tryptone, 0.5 % NaCl), brain heart infusion supplemented with 0.1 % glucose (BHIG), Luria–Bertani (LB) or HBL sheep blood agar (Beecher & Wong, 1994). Media were routinely solidified with 1 % bacteriological agar, unless otherwise specified. For selection of *B. cereus* transformants, appropriate antibiotics (5 μg erythromycin ml^{-1} or 30 μg kanamycin ml^{-1}) were added to the media. The *E. coli* strain TOP10 (Invitrogen) was used for general cloning strategies while the strain SCS110 (Stratagene) was used as an intermediary host in *B. cereus* transformation experiments. *E. coli* strains were grown at 37 °C in LB supplemented with ampicillin (100 μg ml^{-1}) when required.

Strain construction. *B. cereus flhF* insertional mutants were generated by Campbell-type recombination of pRNΔ*flhF*1 or pRNΔ*flhF*2 in *flhF* of *B. cereus* ATCC 14579, thus producing strains MP05 and MP06, respectively (Table 1 and Fig. 2c). For plasmid construction, *B. cereus* ATCC 14579 chromosomal DNA was amplified using the primers flhFEcoU1/flhFHindL2 for pRNΔ*flhF*1 and flhFBamU2/flhFHindL4 for pRNΔ*flhF*2 (Table 2, Fig. 2a). PCR fragments were purified, digested, and separately cloned in the thermosensitive plasmid pRN5101 (Lereclus *et al.*, 1995), thus producing pRNΔ*flhF*1 and pRNΔ*flhF*2, which were used to transform *B. cereus* ATCC 14579 by electroporation. Erythromycin-resistant transformants were isolated by growing cells at 28 °C on LB agar plates containing erythromycin. Transformants were pooled, transferred into LB broth, and incubated at a non-permissive temperature for plasmid replication (40 °C). *flhF* disruption was verified by PCR with the primers flhFU2 and flhFL2 (Table 2, Fig. 2a) and by DNA sequencing.

For complementation experiments, *flhF* was amplified by PCR from *B. cereus* ATCC 14579 with the primers flhFXbaU4 and flhFSphL5 (Table 2, Fig. 2a). The 1351 bp PCR product was cleaved with *Xba*I and *Sph*I and ligated into the expression vector pDG148 (Stragier *et al.*, 1988). The recombinant plasmid (pDG*flhF*), carrying the *flhF* coding region under the transcriptional control of the inducible *spac* promoter (P_{spac}), was used to transform strain MP06 or MP05. After selection for kanamycin resistance, strains MP07 and MP09 were respectively isolated. For overexpression experiments, pDG*flhF* was introduced into *B. cereus* ATCC 14579 to produce the strain MP08. P_{spac} was induced by adding IPTG (final concentration, 2 or 4 mM) to bacterial cultures.

Motility and chemotaxis assays. Swimming and swarming motility were examined as previously described (Senesi *et al.*, 2002). Briefly, for swimming motility assays, 0.5 μl of an overnight culture (approx. 2×10^8 cells ml^{-1}) was spotted onto the centre of TrB plates solidified with 0.25 % agar (TrM), and growth halo diameters were measured after 6–8 h of incubation at 37 °C in a humidified chamber. Swimming motility was also evaluated under a phase-contrast microscope (BH-2; Olympus) by measuring the time of the smooth swimming phase between two consecutive tumblings. Isolated cells (n=200) were analysed in each sample for a total of five experiments performed on separate days. Flagellar staining for light microscopy was performed as described by Harshey & Matsuyama (1994) and several samples were analysed at 1000× magnification using an optical microscope (BH-2; Olympus). The ability of bacteria to undergo chemotaxis was determined by recording, at different time intervals during incubation, the growth halo diameter (D_a) on TrM plates supplemented with 2 mM mannitol in comparison to the growth haloes measured in TrM without attractant (D_c). Each assay was repeated five times on separated days and the chemotaxis index (CI) for each strain was calculated as follows: $CI=(D_a-D_c)/D_c$.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or characteristic	Source or reference
B. cereus		
ATCC 14579	Wild-type strain	ATCC
MP05	Derivative of ATCC 14579, carrying a Campbell integration of pRNΔ*flhF*1 in *flhF*	This study
MP06	Derivative of ATCC 14579, carrying a Campbell integration of pRNΔ*flhF*2 in *flhF*	This study
MP09	Derivative of MP05, containing pDG*flhF*	This study
MP07	Derivative of MP06, containing pDG*flhF*	This study
MP08	Derivative of ATCC 14579, containing pDG*flhF*	This study
E. coli		
SCS110	*rpsL* (Str^r) *thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}*Z* ΔM15]	Stratagene
TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) *φ80lacZ*ΔM15 Δ*lacX74 deoR recA1 end*A1 *ara*Δ*139* Δ(*ara, leu*)*7697 galU galK* λ^- *rpsL*(Str^r) *nupG*	Invitrogen
Plasmids		
pRN5101	Thermosensitive Gram-positive origin of replication, *ori E. coli*; Em^r Ap^r	Lereclus *et al.* (1995)
pRNΔ*flhF*1	Derivative of pRN5101, containing a *flhF* fragment from bp +282 to +1071 relative to the translational initiation site	This study
pRNΔ*flhF*2	Derivative of pRN5101, containing a *flhF* fragment from bp +210 to +685 relative to the translational initiation site	This study
pDG148	Expression vector with IPTG-inducible *spac* promoter (P_{spac}); Km^r Ap^r	Stragier *et al.* (1988)
pDG*flhF*	Derivative of pDG148, containing the *B. cereus flhF* under P_{spac} control	This study

Assays for swarming motility were initiated by spreading 50 µl of a culture containing approximately 2×10^4 cells ml^{-1} onto TrB plates containing 1 % agar (TrA) and incubating plates at 37 °C. Formation of swarm cells was evaluated by analysing cell length and the amount of cell surface flagellin at different time intervals during growth (Senesi *et al.*, 2002). Cell length was measured by phase-contrast microscopy of bacteria stained with a solution containing 10 % (w/v) crystal violet. The extent of cell flagellation was measured as previously described by subjecting purified extracellular flagellin to protein gel electrophoresis (Calvio *et al.*, 2005). The intensity of the flagellin band, at different time intervals during growth, was measured by densitometric analysis using the Image Master 1D software (Pharmacia Biotech). For each strain the increase was calculated as follow: $IF=(I_t-I_{t0})/I_{t0}$, where I_t is the intensity of the flagellin band at different times (2, 4, 6 and 8 h) post-inoculation and I_{t0} is the intensity of the band at 2 h post-inoculation.

Table 2. Primers used for PCR and RT-PCR amplification

Primer	Sequence*	Target gene/base pair coordinates†	Restriction site
flhFU2	5′-GCGAATTAGCACCAGAAA-3′	*flhF*, from −64 to −47	None
flhFU3	5′-ATGATTACGGAAGAAGAAGTT-3′	*flhF*, from +646 to +666	None
flhFL2	5′-CATCATGCCCATAGAACCTA-3′	*flhF*, from +1398 to +1379	None
flhFEcoU1	5′-ATGGAATTCAATCAGTACT-3′	*flhF*, from +278 to +296	*Eco*RI
flhFBamU2	5′-GAAGGATCCTACAGCTCAATTT-3′	*flhF*, from +201 to +222	*Bam*HI
flhFXbaU4	5′-AGTATCTAGAGAAGTAATGGAAAGTACAGAAA-3′	*flhF*, from −16 to +16	*Xba*I
flhFHindL2	5′-CGTAAGCTTAATATAATCTGG-3′	*flhF*, from +1080 to +1060	*Hin*dIII
flhFHindL4	5′-TCAAAGCTTCCAATATGTACCTG-3′	*flhF*, from +691 to +669	*Hin*dIII
flhFSphL5	5′-TTCTGCATGCCTCTACTACGATGTTTGTAA-3′	*flhF*, from +1335 to +1306	*Sph*I
flgGU1	5′-AGGTTCTATGGGCATGATGA-3′	*flgG*, from +18 to +37	None
flgGL1	5′-AGTCATAACGCGCTGTGA-3′	*flgG*, from +726 to +709	None
flgGL2	5′-TCATTCGCTTCTTTTTCATAA-3′	*flgG*, from +761 to +741	None
fAint1	5′-CAGAAAAAGATGCACAGG-3′	*flhA*, from +476 to +501	None

*Restriction site is underlined.

†Numbers refer to nucleotide position in the indicated gene, relative to the translational initiation site.

Electron microscopy. Bacteria were grown overnight in 5 ml LB broth, diluted (1 : 250) into fresh LB medium and then grown for 6 h at 150 r.p.m. Two drops of bacterial culture were subsequently placed onto the same Formvar-coated copper grid. After 1 min, excess liquid was wicked off without completely drying the grid to avoid flagellum shearing. The grids were negatively stained with 2 % (w/v) phosphotungstic acid for 3 min and air-dried. Samples were analysed using a transmission electron microscope (JEOL 100SX). To quantify flagella per cell, 200 cells of each strain were examined; flagella were counted only in well-isolated cells.

Aldolase activity. The fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) activity was assayed in culture supernatants and cell lysates as described by Warth (1980).

RNA isolation and RT-PCR analysis. Total RNA was extracted from *B. cereus* cultures with the RNeasy Mini Kit (Qiagen) modified as previously described (Ghelardi *et al.*, 2002). The concentration, integrity and purity of RNA samples were determined spectrophotometrically and by visual inspection of agarose gels. Purified RNA (100 ng) was used as a template in one-step RT-PCR with the cMaster RT^{plus}PCR system (Eppendorf), according to the manufacturer's instructions. The primers used in different reactions, listed in Table 2, were: flhFU2/flhFL2, for the amplification of *flhF* cDNA; flhFU2/flhFHindL2, to amplify the first 1120 bp of the *flhF* cDNA; flgGU1/flgGL1, for the amplification of *flgG* cDNA; fAint1/flhFHindL4, for the amplification of a cDNA fragment containing both *flhA* and *flhF*; and flhFU3/flgGL2, for the amplification of a cDNA fragment containing both *flhF* and *flgG*. The RT reactions were carried out at 42 °C for 70 min in a thermal cycler, immediately followed by 30 cycles of PCR, consisting of 15 s at 94 °C, 20 s at 58 °C and 1 min at 68 °C, and ending with a 7 min incubation at 68 °C. To detect DNA contamination in RNA samples, reactions in which RT enzyme was replaced by RNase-free water (negative controls) were performed. Positive controls were obtained using genomic DNA as template. The RT-PCR products were analysed by agarose gel electrophoresis.

Protein samples and gel electrophoresis. Protein samples were prepared by growing bacterial cells to the late exponential growth phase in BHIG at 200 r.p.m. for 6 h at 30 °C. Cultures were normalized to the same OD_{600} and aliquots of the resulting bacterial suspensions were pelleted by centrifugation at 10 000 ***g***. Culture supernatants were collected and cell lysates prepared as previously described (Ghelardi *et al.*, 2002). Protein concentration was determined by the bicinchoninic acid (BCA) assay (Smith *et al.*, 1985), with BSA as a standard. Protein samples were suspended in NuPAGE LDS sample buffer (Invitrogen), heated at 72 °C for 10 min, and subjected to SDS-PAGE. Gels were either silver stained or electrotransferred to nitrocellulose membranes, using the XCell II Blot Module system (Invitrogen), for immunoblot analysis.

Detection of phosphatidylcholine-preferring phospholipase C (PC-PLC), HBL and non-haemolytic enterotoxin (NHE). For detection of virulence factors, whole or diluted protein samples were used. PC-PLC activity was measured by a gel-diffusion assay with a gel containing crude phosphatidylcholine (PC), as previously described (Ghelardi *et al.*, 2002). Different amounts of pure PC-PLC (Sigma-Aldrich) were used to generate a standard calibration curve. NHE was detected by using the commercial Bacillus Diarrhoeal Enterotoxin (BDE) visual immunoassay kit (Tecra Diagnostics) according to the manufacturer's instructions. Sample absorbance was measured at 405 nm (A_{405}) using a plate reader. HBL activity was assessed on sheep blood agar plates by the formation of a discontinuous zone of haemolysis around colonies (Beecher & Wong, 1994), while individual HBL components were detected by immunoblot analysis. Nitrocellulose membranes (prepared as described above) were probed with rabbit polyclonal antibodies recognizing individual HBL components, followed by incubation with a secondary antibody conjugated with horseradish peroxidase. The peroxidase activity was visualized by diaminobenzidine colorimetric reaction in accordance with standard procedures (Sambrook *et al.*, 1989).

Statistical analyses. Statistical analyses were performed with InStat (GraphPad Software). A P value <0.05 was considered significant. Values were expressed as the mean $\pm$ SD from five independent experiments.

RESULTS

In silico analysis of the *B. cereus* ATCC 14579 genome

The involvement of *fleN*/*flhG*, *flhF* and *swrAA* in the regulation of the number and/or position of flagella in different organisms (Calvio *et al.*, 2005; Correa *et al.*, 2005; Dasgupta *et al.*, 2000; Hendrixson & DiRita, 2003; Kusumoto *et al.*, 2006; Niehus *et al.*, 2004; Pandza *et al.*, 2000) prompted us to evaluate whether homologues of such genes were present in *B. cereus*. *In silico* analysis was performed on the *B. cereus* ATCC 14579 genome with the ERGO Genome and Discovery System (Overbeek *et al.*, 2003) from Integrated Genomics and the BacMap Bacterial Genomes Atlas (Stothard *et al.*, 2005). An *flhF* homologue was identified, but no homologues of *fleN*/*flhG* and *swrAA* were found.

The *B. cereus flhF* nucleotide sequence available in databases showed homologies with *flhF* of other organisms; however, a stop codon (codon 301) internal to the *B. cereus flhF* ORF was identified. We presumed that this unexpected stop codon derived from an erroneous annotation of the sequence and therefore decided to revisit the sequence of the *B. cereus flhF* ORF. A chromosomal region comprising *flhF* was sequenced from *B. cereus* ATCC 14579 and compared with the *flhF* sequence present in the database. Sequence analysis revealed that two additional base pairs were present in the annotated sequence (nucleotides 889 and 1001), thus leading to a frame-shift that produced an erroneous stop codon. The new sequence of the *B. cereus* ATCC 14579 *flhF* gene was deposited in the EMBL database (GenBank accession no. AM180264).

B. cereus flhF encodes a putative protein of 439 aa, having an estimated molecular mass of 50.2 kDa and an isoelectric point of 5.93. No predicted signal peptide sequence or transmembrane domains were found. As shown by sequence alignments and domain searches, *B. cereus* FlhF contains a conserved C-terminal and consensus GTP-binding motif (G-domain), which is typical for the proteins belonging to the SRP family (Fig. 1). The N-terminal sequence of *B. cereus* FlhF is less conserved compared to other bacteria, but a basic lysine-rich region (B-domain), described also for *B. subtilis* FlhF (Zanen *et al.*, 2004), was found from aa 125 to aa 173 (Fig. 1).

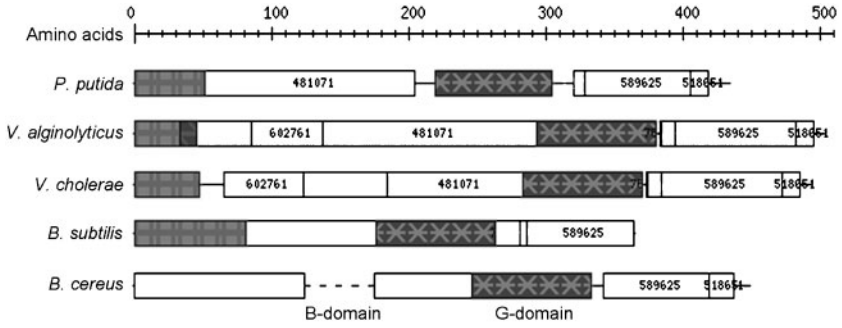


Fig. 1. Conserved domains in the amino acid sequence of FlhF. FlhF of *P. putida*, *V. alginolyticus*, *V. cholerae*, *B. subtilis* and *B. cereus* are represented schematically following ProDom analysis (Servant *et al.*, 2002). Boxes containing the same number or symbol represent sequences belonging to conserved protein domain families generated from global comparison of all available protein sequences present in the ProDom database. Unfilled boxes represent domains that are not conserved.

The *flhF* locus in *B. cereus*

flhF is contained in a region comprising genes involved in flagellum biogenesis and it maps between *flhA* and *flgG* (Fig. 2a). The *flhA* gene, which encodes a membrane protein that is part of the flagellar export apparatus (Ghelardi *et al.*, 2002; Minamino & Macnab, 1999), is located upstream of *flhF* in *B. cereus*, as well as in many other bacteria (Carpenter *et al.*, 1992; Dasgupta *et al.*, 2000; Kim & McCarter, 2000; Kusumoto *et al.*, 2006; Pandza *et al.*, 2000). In contrast, only in *B. cereus*, the gene downstream of *flhF* is *flgG*, which encodes the flagellar basal body rod protein in many micro-organisms (Minamino & Macnab, 1999; Zuberi *et al.*, 1991).

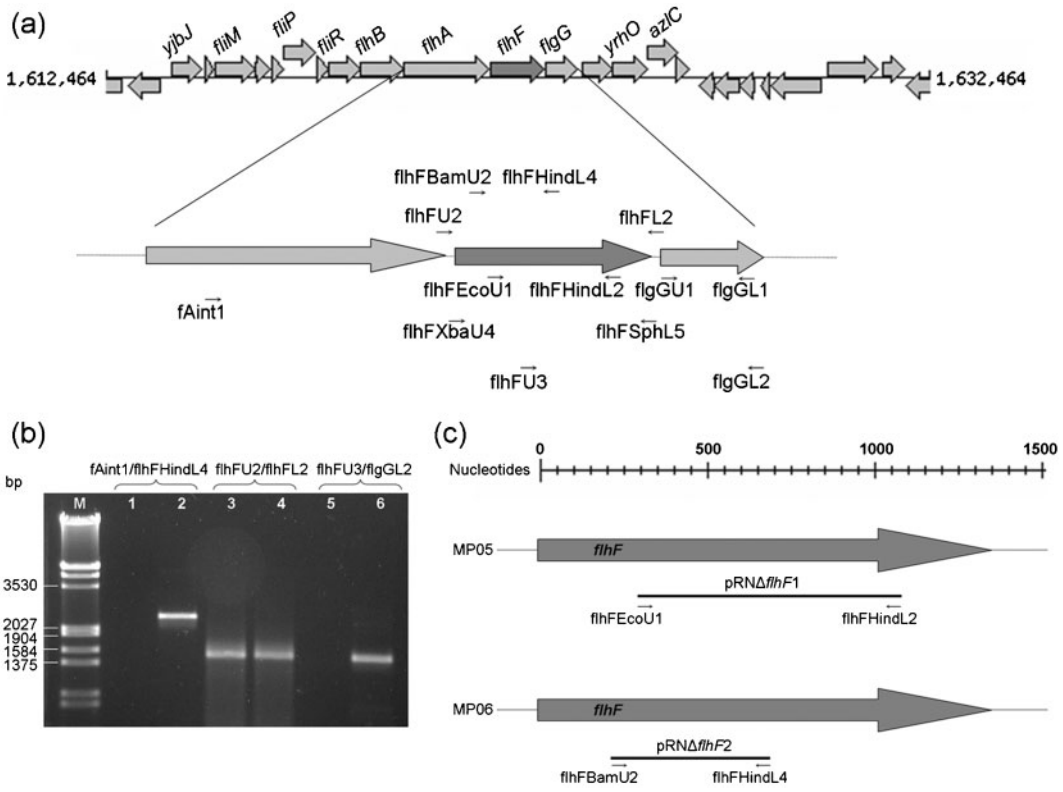


Fig. 2. Analysis of the *flhF* locus in *B. cereus* ATCC 14579. (a) Schematic representation of the locus. Positions and orientations of primers are shown in the enlarged detail. (b) Electrophoretic profiles of RT-PCR amplified products obtained from *B. cereus* ATCC 14579 total RNA (lanes 1, 3 and 5) or chromosomal DNA (lanes 2, 4 and 6). Primers used in the different reactions are shown at the top of the image. Lane M, molecular size standard. (c) Physical maps showing the locations of inserted plasmids in strains MP05 and MP06. The grey arrows represent the *flhF* ORF; inserts of plasmid clones are represented by bold horizontal lines, which are positioned at the corresponding homology regions. The primers used to produce inserts cloned in each plasmid are shown.

To evaluate whether *flhF* was part of a transcriptional unit also containing *flhA* or *flgG*, RT-PCR analysis was carried out on total RNA purified from *B. cereus* ATCC 14579. No amplification products were obtained using the primer pairs fAint1/flhFHindL4 or flhFU3/flgGL2, that were designed on the sequence of *flhA*/*flhF* and *flhF*/*flgG*, respectively (Fig. 2b, lanes 1 and 5). An amplification product of the predicted size was obtained using the primer pair flhFU2/flhFL2, designed on the sequence of *flhF* (Fig. 2b, lane 3). Thus, differently from other bacteria in which *flhF* is part of a large operon including flagellar and chemotaxis genes (Carpenter *et al.*, 1992; Kim & McCarter, 2000), the *B. cereus flhF* constitutes a monocistronic transcriptional unit.

Disruption of *flhF* causes defects in the number and localization of flagella

flhF has a crucial role in flagellar biogenesis of polar flagellate bacteria (Correa *et al.*, 2005; Hendrixson & DiRita, 2003; Kusumoto *et al.*, 2006; Niehus *et al.*, 2004; Pandza *et al.*, 2000), whereas in the peritrichous species *B. subtilis*, *flhF* has been reported to be either dispensable (Zanen *et al.*, 2004) or required (Carpenter *et al.*, 1992) for cell flagellation, mainly depending upon the genetic background of the strain. To evaluate whether *flhF* was involved in the control of cell flagellation in *B. cereus*, an *flhF* insertional mutant (MP06) and a complemented strain (MP07) carrying a plasmid harbouring *flhF* under the P_{spac} transcriptional control were generated. Disruption of *flhF* in MP06 did not cause polar effects on the expression of *flgG*, which is immediately downstream of *flhF*, as demonstrated by the presence of a *flgG* transcript detected by RT-PCR analysis with the primers flgGU1 and flgGL1 (Table 2) (data not shown).

Phenotypic analysis of bacteria propagated in liquid media revealed that strain MP06 was characterized by a remarkable reduction in the number of flagella (1–3 per cell) in comparison to wild-type (10–12 per cell) (Fig. 3a); moreover, electron microscopy showed that in mutant MP06, flagella were always localized in polar or peri-polar positions compared to the peritrichous distribution observed in the wild-type (Fig. 3c). The wild-type phenotype was completely restored in strain MP07, after induction of the P_{spac} promoter with 2 mM IPTG (Fig. 3a). The altered ability of MP06 to produce flagellar filaments was also confirmed by SDS-PAGE analysis of purified flagella: the protein band corresponding to extracellular flagellin was less abundant in the mutant than in the wild-type and complemented strains (Fig. 3b). These observations indicated that disruption of *B. cereus flhF* determined a loss in the ability to control the arrangement of flagella on the cell surface in a peritrichous bacterial species.

In several flagellate bacteria, the degree of cell flagellation has been widely reported to be linked to cell division events, although flagellum biogenesis may be influenced by environmental factors (for a review see Aizawa & Kubori, 1998). Thus we addressed the question of whether a lack of *flhF* caused metabolic or growth defects leading, directly or indirectly, to an impairment in flagella assembly. Therefore, both the biochemical profile and the ability to assemble flagella at fixed time intervals during growth were compared in *B. cereus* ATCC 14579, MP06 and MP07. No difference was revealed in the ability to use carbohydrates or amino acids for growth by using the API 50-CHB and API 20E strip tests (bioMérieux). All strains showed overlapping growth curves (Fig. 4) and comparable percentages of flagellated cells at mid-exponential, late-exponential or stationary phases (Fig. 4). Remarkably, no

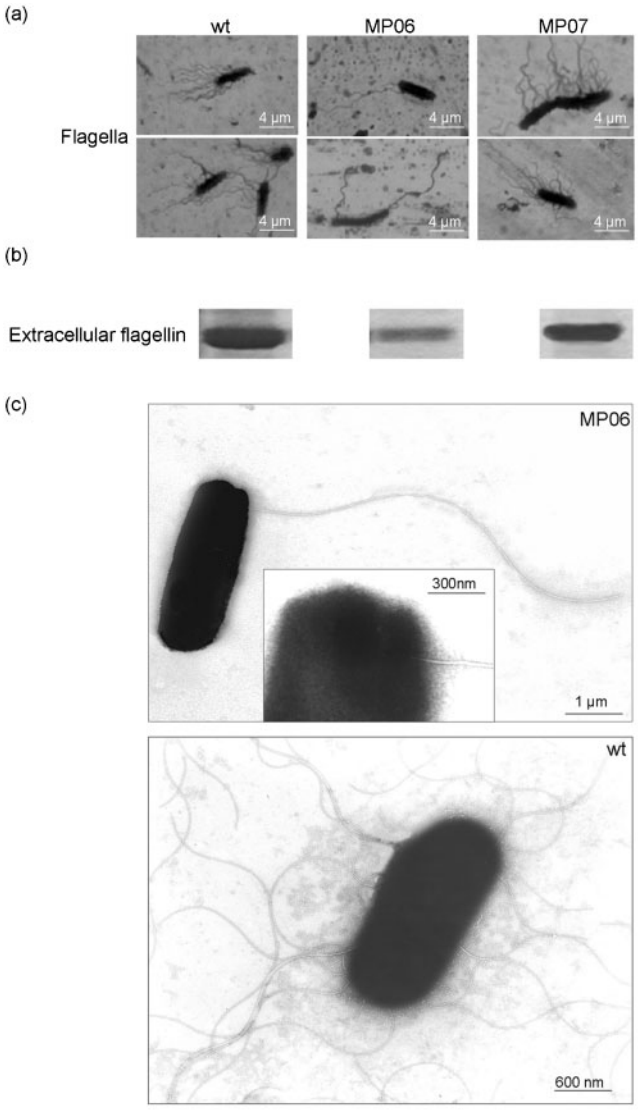


Fig. 3. (a) Light-microscopy (1000×) of flagella arrangement. (b) SDS-PAGE analysis of extracellular flagellin of *B. cereus* ATCC 14579 (wt), MP06 (*flhF* mutant) and MP07 (complemented strain). (c) Electron micrographs of the *flhF* mutant (MP06) and wild-type strain (wt); the inset in the upper panel shows a higher-magnification view of the flagellar insertion.

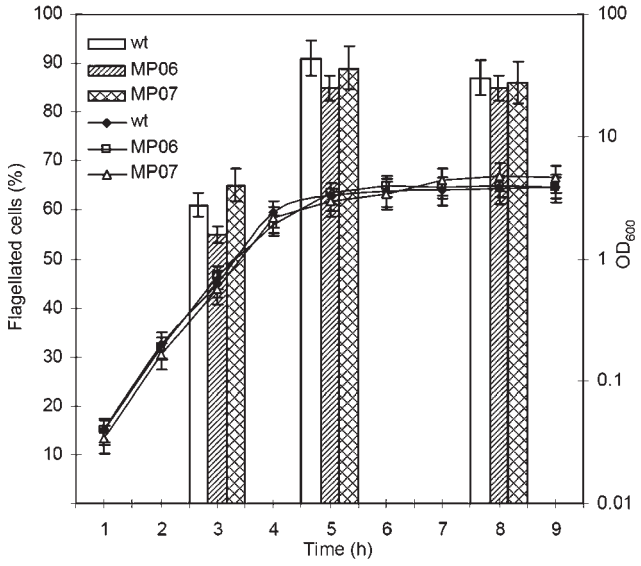
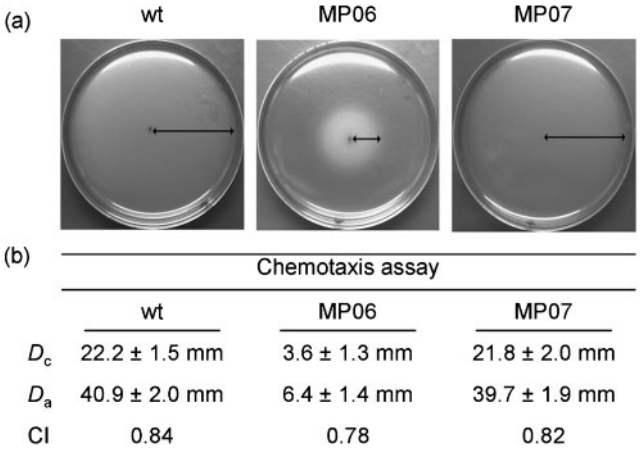


Fig. 4. Percentage of flagellated cells at various times during growth of *B. cereus* strains. Spot symbols show OD_{600} values of cultures at different time points. Bars represent the percentage of flagellated cells at 3, 5 and 8 h post-inoculation. wt, *B. cereus* ATCC 14579; MP06, *flhF* mutant; MP07, complemented strain.



	wt	MP06	MP07
D_c	22.2 ± 1.5 mm	3.6 ± 1.3 mm	21.8 ± 2.0 mm
D_a	40.9 ± 2.0 mm	6.4 ± 1.4 mm	39.7 ± 1.9 mm
CI	0.84	0.78	0.82

Fig. 5. Motility and chemotaxis of *B. cereus* strains. (a) Swimming haloes (indicated with arrows) of *B. cereus* ATCC 14579 (wt), MP06 (*flhF* mutant) and MP07 (complemented strain) on motility plates (TrM). (b) Diameters of motility haloes on TrM (D_c) or TrM supplemented with mannitol (D_a). Chemotaxis indexes (CI) were calculated as described in Methods.

cell harbouring peritrichous flagella or more than three flagella was observed in mutant MP06.

The reduced degree of flagellation observed in the *flhF* insertional mutant prompted us to evaluate whether MP06 displayed altered motility in a liquid environment. Analysis of swimming motility, performed by measuring the diameters of growth haloes on TrM agar plates, revealed that swimming was notably reduced in strain MP06 (9.7 ± 1.5 mm) when compared to the wild-type (40.7 ± 1.9 mm) and complemented strain (39.4 ± 2.0 mm) ($P<0.001$) (Fig. 5a). Microscopic examinations of bacteria by the hanging-drop method revealed that mutant MP06 maintained the directional movement exhibited by the peritrichous flagellated wild-type. However, smooth swimming phases were shorter in the mutant strain than in the wild-type, the time between two consecutive tumblings being 2.96 ± 0.35 s and 7.36 ± 1.17 s, respectively ($P=0.0013$). Despite exhibiting an altered motility phenotype, MP06 was not defective in chemotactic response, since similar chemotaxis indexes were obtained for all strains toward mannitol, which acts as a strong attractant for *B. cereus* (Fig. 5b). Taken together, these results suggest that the altered motility of MP06 was exclusively due to the reduced number and defective arrangement of flagella displayed on the cell surface.

The integrity of *flhF* is required for swarming differentiation

Swarming differentiation by *B. cereus* occurs on rich media at a wide range of viscosity and temperature. Swarming colonies of *B. cereus* are characterized by the presence of elongated and hyperflagellated swarm cells, mainly localized at the colony rim, while the colony centre is constituted by short oligoflagellated vegetative cells (Senesi *et al.*, 2002).

To evaluate whether *flhF* was required for *B. cereus* to undergo surface-induced swarming differentiation, we measured cell length and the amount of cell-surface flagellin in strains ATCC 14579, MP06, and MP07 at fixed time intervals during growth on TrA plates (Fig. 6). SDS-PAGE analysis of purified flagella revealed an increase in the level of extracellular flagellin in the wild-type and MP07 strains during the first 8 h post-inoculation (Fig. 6a). In contrast, levels of flagellin from strain MP06 remained unchanged at all time points examined (Fig. 6a). Moreover, microscopic examination of cells collected from growing colonies revealed a progressive increase in the percentage of elongated cells in both the wild-type and MP07, while the mutant never exhibited cells with an elongated phenotype (Fig. 6b). These observations indicate that MP06 is unable to undergo swarming differentiation and suggest that *flhF* is involved in a common pathway controlling the number of flagella in swimmer as well as in swarmer cells.

FlhF plays a role in protein secretion

flhF has been reported to influence protein secretion in *P. putida* (Pandza *et al.*, 2000), while in *B. subtilis*, such a gene appears to be dispensable for its secretory properties (Zanen *et al.*, 2004). To evaluate whether *flhF* could influence protein secretion in *B. cereus*, culture supernatants of *B. cereus* ATCC 14579 and MP06 (see Methods for details) were analysed by SDS-PAGE. The levels of

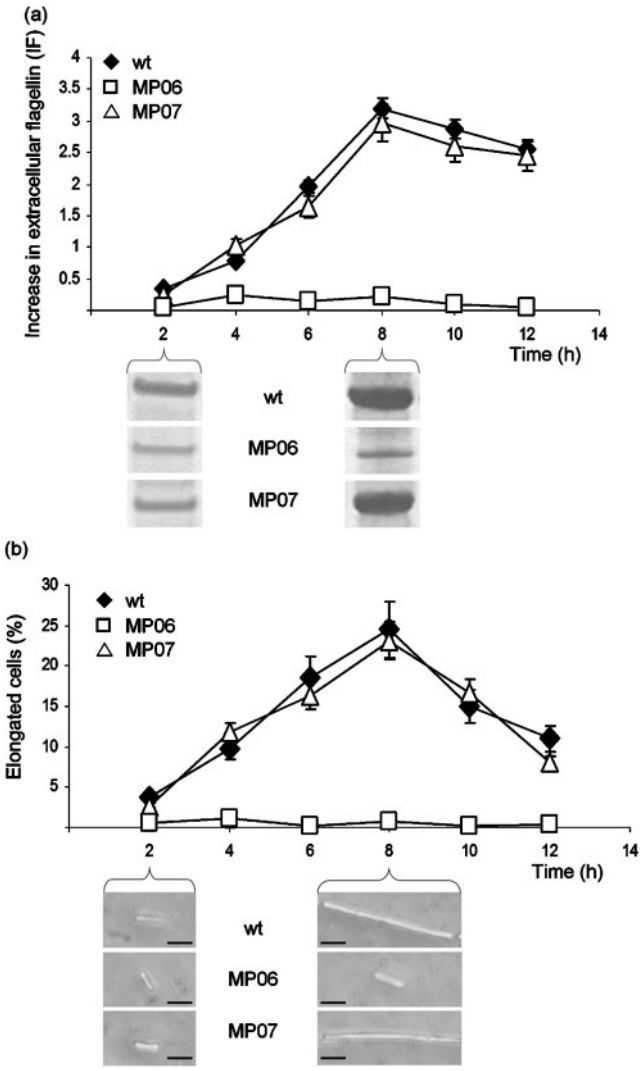


Fig. 6. Swarming differentiation of *B. cereus* strains. (a) Analysis of cell hyperflagellation. Curves show the increase in extracellular flagellin during growth on TrA plates. SDS-PAGE shows flagellin bands at 2 and 8 h post-inoculation. (b) Analysis of cell elongation. Curves show the percentage of elongated cells within growing colonies. Phase-contrast micrographs show representative cells with different length collected at 2 and 8 h post-inoculation. wt, *B. cereus* ATCC 14579; MP06, *flhF* mutant; MP07, complemented strain. Bars, 3 μm.

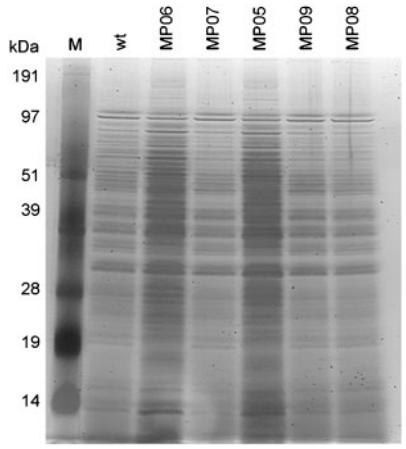


Fig. 7. Proteins secreted by *B. cereus* ATCC 14579 (wt) and its derivative strains. Culture supernatants collected from late exponential growth phase cultures normalized at the same OD_{600} were separated by SDS-PAGE and silver-stained. Lane M, molecular mass standard; MP06, *flhF* insertion mutant carrying a Campbell integration of pRNΔ*flhF*2 in *flhF*; MP07, complemented strain; MP05, *flhF* insertion mutant carrying a Campbell integration of pRNΔ*flhF*1 in *flhF*; MP09, derivative of MP05 containing pDG*flhF*; MP08, derivative of *B. cereus* ATCC 14579 containing pDG*flhF*.

proteins secreted by mutant MP06 appeared increased compared to the wild-type strain (Fig. 7). Protein quantification by the BCA assay revealed that the proteins in the supernatants of MP06 were about 20 % higher than that of the parental strain. To rule out that MP06 was subjected to cell lysis, supernatants and cell lysates of both strains were assayed for the presence of fructose-1,6-bisphosphate aldolase, a cytoplasmic enzyme that is not released by intact cells. No aldolase activity was detected in culture supernatants, while identical enzyme activity was observed in cell lysates (0.08 U ml^{-1}). This result indicates that the higher protein amount in the supernatant of MP06 was not due to cell lysis during growth.

Analysis of the *trans*-complemented strain MP07 demonstrated that disruption of *flhF* solely was responsible for the increased protein secretion in strain MP06. Indeed, after induction with 2 mM IPTG, the quantity of proteins secreted by this strain was comparable to that obtained for the wild-type (Fig. 7) and did not vary when *flhF* expression was hyper-stimulated using higher IPTG concentrations (up to 4 mM; data not shown). The effect of *flhF* overexpression was also analysed in strain MP08, obtained by introducing pDG*flhF* into strain ATCC 14579. Overexpression of *flhF* in the wild-type background had no detectable effect on protein secretion, even when IPTG was used at the highest concentration (Fig. 7). Therefore, while overexpression of *flhF* did not alter the amount of secreted proteins, under the conditions tested, lack of *flhF* led to protein hyper-secretion.

Disruption of *flhF* differently affects the export of secretory virulence factors

The higher level of proteins secreted in the *flhF* null mutant of *B. cereus* raised the question of whether the lack of *flhF* altered the export of secretory virulence factors, such as NHE, HBL and PC-PLC.

Secretion of NHE was tested in the strains ATCC 14579, MP06 and MP07 of *B. cereus*. Immunoassays of culture

supernatants showed that the level of extracellular NHE was higher in the mutant MP06 (A_{405} 1.02 ± 0.08) compared to the wild-type (A_{405} 0.78 ± 0.06) and complemented (A_{405} 0.87 ± 0.09) strains ($P<0.05$). This result was in agreement with the increase in total protein secretion observed for MP06.

Secretion of the enterotoxic tripartite HBL was evaluated by growing bacteria on sheep blood agar plates. Production of the typical HBL discontinuous pattern of haemolysis around colonies (Beecher & Wong, 1994) was revealed for the mutant, parental, and complemented strains, thus indicating that all strains were able to secrete an active toxin (data not shown). To evaluate whether different levels of HBL were secreted by the strains, immunoblot analysis of supernatants from cultures normalized as described was performed with polyclonal sera specific to the individual HBL proteins (B, L_1, L_2). Although a positive signal was obtained for each HBL component in all strains, an almost threefold reduction in the amount of HBL proteins was registered in mutant MP06, compared to the wild-type and MP07 (as shown in Fig. 8 for the L_1 component of HBL). As observed for HBL, the amount of PC-PLC activity secreted by MP06 (0.060 ± 0.016 U ml^{-1}) was also lower than that detected for the wild-type (0.124 ± 0.018 U ml^{-1}) and the complemented strain (0.134 ± 0.011 U ml^{-1}) ($P<0.001$).

The C-terminal domain of FlhF is dispensable for flagellation and motility while required for protein secretion

Analysis of the FlhF amino acid sequence (Fig. 1) showed that the C-terminal half of FlhF is highly conserved in all flagellated bacteria studied so far. However, a difference in this region emerged between *B. cereus* and *B. subtilis*, the only two peritrichous species in which *flhF* has been studied, with *B. subtilis* lacking the last C-terminal domain (referred to as domain no. 518651, as following ProDom analysis). To evaluate the functional role of such protein domain in *B. cereus*, an ATCC 14579 derivative (MP05) carrying a truncation in the 3′ end of *flhF* was constructed by Campbell-type recombination of pRNΔ*flhF*2 at the *flhF* locus. RT-PCR analysis on total RNA extracted from strain MP05 showed the absence of an *flhF* full-length mRNA (Fig. 9, lane 2); expression of a *flhF* transcript lacking the last 198 nt was demonstrated (Fig. 9, lane 4) following amplification of total RNA with the primers flhFHindL2, which was designed on a sequence immediately upstream of the pRNΔ*flhF*2 insertion site, and flhFU2 (Table 2, Fig. 2a).

Growth rate, flagellar arrangement, swimming and swarming motility were analysed in strain MP05 in comparison to the wild-type. These traits were completely unaffected in mutant MP05 (data not shown), thus indicating that the last C-terminal domain (from aa 375 to aa 439) of FlhF is dispensable for cell flagellation and motility behaviour in *B. cereus*. However, the analysis of culture supernatants revealed that the amount of proteins secreted by MP05 was higher than that detected in the wild-type and similar to that of MP06 (Fig. 7). Restoration of a wild-type pattern of protein secretion was observed in strain MP09 (Fig. 7), which was obtained after transformation of MP05 with the plasmid pDG*flhF*, harbouring a copy of wild-type *flhF* gene. Among the virulence-associated proteins analysed, NHE and PC-PLC levels were found to be respectively higher (A_{405} of 0.97 ± 0.03) and lower (0.057 ± 0.015 U ml^{-1}) in strain MP05 than in the parental strain. In contrast, no difference in the levels of secreted HBL was noted between MP05 and the wild-type strain (data not shown).

DISCUSSION

The flagellar gene *flhF* is harboured by several flagellated bacteria, but its role in flagellum biogenesis has been almost exclusively investigated in polar flagellates (Correa

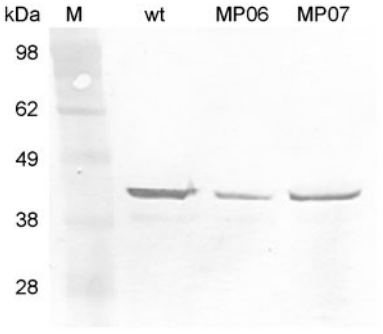


Fig. 8. HBL secretion by *B. cereus* strains. Immunoblot with polyclonal antibody to the L_1 component of HBL on culture supernatants prepared from *B. cereus* ATCC 14579 (wt), MP06 (*flhF* mutant) and MP07 (complemented strain). Lane M, molecular mass standard.

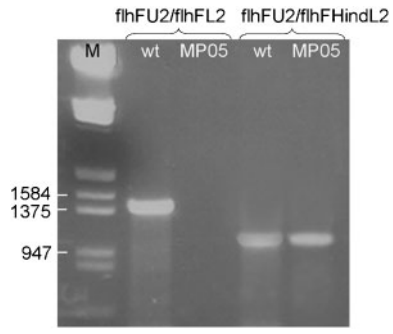


Fig. 9. Analysis of *flhF* expression in MP05. Electrophoretic separation of the amplification products obtained by RT-PCR on total RNA from MP05 strain and *B. cereus* ATCC 14579 (wt) as a control. Primers used in different reactions are indicated at the top of the image. Lane M, molecular size standard.

et al., 2005; Hendrixson & DiRita, 2003; Niehus *et al.*, 2004; Pandza *et al.*, 2000). In these bacteria, the protein encoded by *flhF* displays a high degree of sequence conservation, particularly in its C-terminal half (Fig. 1), while the functions attributed to FlhF vary in the species analysed so far. Indeed, while *flhF* mutants of *P. aeruginosa* and *P. putida* display peritrichous distribution of flagella (Murray & Kazmierczak, 2006; Pandza *et al.*, 2000), lack of FlhF produces non-flagellated phenotypes in *C. jejuni*, *H. pylori* and *V. cholerae* (Correa *et al.*, 2005; Hendrixson & DiRita, 2003; Niehus *et al.*, 2004). The absence of *flhF* homologues in *E. coli*, together with the doubtful role of *flhF* in *B. subtilis* (Carpenter *et al.*, 1992; Zanen *et al.*, 2004), addressed the question of whether this gene played a role in cell flagellation of peritrichous bacteria.

In this investigation, we demonstrate that the number and distribution of flagellar filaments in the peritrichous species *B. cereus* depend on the integrity of *flhF*. The *flhF* insertional mutant of *B. cereus* described in this study (MP06) showed a remarkable reduction in the number of flagella (from 10–12 to 1–3 per cell) (Fig. 3a) that, moreover, were localized in polar or peri-polar positions (Fig. 3c). This reduction was not due to a delay in the assembly of flagella during growth, since the percentage of flagellated cells of MP06 was comparable to that of the wild-type strain (Fig. 4) at all stages during growth. The abnormal flagellar arrangement exhibited by MP06 causes a significant reduction in its ability to swim (Fig. 5a). The motility impairment appeared to be a direct consequence of shorter directional smooth swimming phases the mutant exhibited in comparison to the wild-type strain (2.96 ± 0.35 s vs 7.36 ± 1.17 s; $P=0.0013$). It is of some interest to highlight that a non-peritrichous distribution of flagella in a naturally peritrichously flagellate species does not determine an impairment in the mechanism enabling cells to coordinate directional movement. The maintenance of directional movement by MP06 explains why disruption of *flhF* does not lead to an impairment in the ability to undergo chemotaxis (Fig. 5b).

The *B. cereus* mutant MP06 was found to be completely impaired also in swarming differentiation, and restoration of the swarming phenotype was observed in the complemented strain MP07 (Fig. 6). This finding is in agreement with a robust literature demonstrating that deletions in flagellar genes severely impair or abolish the ability to undergo swarming differentiation in swarming-proficient species (Gygi *et al.*, 1995; Harshey & Matsuyama, 1994; O'Rear *et al.*, 1992; Senesi *et al.*, 2002; Young *et al.*, 1999b). In addition, *flhF* has been recently demonstrated to be required for swarming motility in the Gram-negative species *P. aeruginosa* (Murray & Kazmierczak, 2006). Lack of hyper-flagellated cells in strain MP06 suggests that *B. cereus flhF* is involved in a common pathway controlling degree of flagellation in the swim as well as in the swarm cell-state.

The molecular mechanisms by which *flhF* governs the flagellar arrangement in *B. cereus* remain to be clarified. In many polar flagellates, such as *Pseudomonas* spp. and *Vibrio* spp., *flhF* resides in a large region concerned with flagellar synthesis, chemotaxis and motility, and FlhF has been described to act as a positive regulator for flagellar gene transcription (Correa *et al.*, 2005; Hendrixson & DiRita, 2003; Niehus *et al.*, 2004; Pandza *et al.*, 2000). In *B. cereus*, the expression of *flhF* is independent from that of the upstream (*flhA*) and downstream (*flgG*) genes (Fig. 2b), and *flhF* overexpression does not appear to influence cell flagellation in both the mutant (MP07) and wild-type (MP08) backgrounds. In *H. pylori*, the absence of FlhF leads to a downregulation of the expression of flagellar class 3 genes, which is dependent on the antisigma factor FlgM (Niehus *et al.*, 2004). In *Vibrio parahaemolyticus*, Kim & McCarter (2000) suggested that FlhF could co-operate with FlhG in determining site selection of flagellar insertion. Also in *B. subtilis*, *ylxH*, a *fleN*/*flhG* homologue, has been reported to cooperate with *flhF* for correct motility (Zanen *et al.*, 2004). All the interactions described for FlhF in other bacteria appear not to be applicable to *B. cereus* FlhF, since no homologues of *fleN*/*flhG* and *flgM* have been found. In this context, we can only suggest that the sequence predicted to encode the GTP-binding domain of FlhF is involved in the activity exerted by this protein in regulating *B. cereus* motility. Indeed, in the *B. cereus* mutant MP05, which harbours a *flhF* deletion in a region downstream of the G-domain, no defect in motility or flagellar arrangement was evidenced.

The demonstration that *flhF* mutants of *B. cereus* display increased levels of extracellular proteins (Fig. 7) further supports the involvement of FlhF in the regulation of protein secretion, as already reported for *P. putida* (Pandza *et al.*, 2000). FlhF is a member of the SRP-GTPase family (Carpenter *et al.*, 1992; Pandza *et al.*, 2000) and shows substantial homology with Ffh and FtsY, two proteins required for extracellular accumulation of proteins in *E. coli* and *B. subtilis* (Sijbrandi *et al.*, 2003; Zanen *et al.*, 2006). Similarly to *flhF* disruption in *B. cereus*, Ffh or FtsY depletion in *B. subtilis* determines an increased level of various extracellular proteins (Zanen *et al.*, 2006). However, the mechanisms whereby FlhF is involved in the regulation of protein secretion in *B. cereus* are presently not understood. Nevertheless, it appears that the lack of *flhF*, but not its overexpression, is responsible for the altered protein secretion in *B. cereus*, since overexpression of *flhF* does not apparently cause secretory defects in the wild-type background (Fig. 7).

Detection of virulence determinants known to be secreted by *B. cereus* highlighted that export of individual proteins is differently influenced by an *flhF* deletion. In fact, reduced amounts of both HBL (about 70 %) and PC-PLC (about 50 %) were observed in mutant MP06, together with an increase in the amount of secreted NHE. Notably, the increase in NHE secretion was as high as that observed for the total proteins secreted by the *flhF* mutant (about 20 %), suggesting that lack of FlhF causes a dysregulation in a secretory pathway also needed for NHE export.

The reduced secretion of HBL observed in strain MP06 was interpreted with the lower number of flagella carried by this strain. Indeed, HBL components have already been shown to be secreted only through the flagellar type III export machinery in *B. thuringiensis* (Bouillaut *et al.*, 2005; Ghelardi *et al.*, 2002), the closest relative of *B. cereus*. The reduced amount of extracellular PC-PLC activity in mutant MP06 was more difficult to interpret; in fact, it could not be considered as a consequence of the reduced number of flagella, since (i) a lower amount of PC-PLC was also detected in the MP05 mutant, which carries a wild-type flagellar arrangement, and (ii) a *B. thuringiensis* mutant completely lacking flagella was found to be able to secrete this toxin (Ghelardi *et al.*, 2002). However, whatever the mechanism accounting for the reduced amount of PC-PLC, it is interesting that a deficiency in a single flagellar gene influences the virulence phenotype exhibited by *B. cereus*.

Finally, the identification of an FlhF domain that appears to be essential for correct protein secretion in *B. cereus* is of some relevance. The mutant MP05, lacking the last C-terminal domain of FlhF, secretes an abnormal amount of proteins (Fig. 7), while it shows a wild-type flagellar arrangement and motility phenotype. The correct flagellar arrangement of MP05, together with the dependence of HBL secretion on the flagellum channel, explains why no difference in HBL export was detected between MP05 and the wild-type strain.

Taken together, the results described in this study indicate that *B. cereus* FlhF plays a critical role in regulating flagellar arrangement, motility behaviour and protein secretion, thus placing FlhF as a promising candidate for connecting motility/flagella with other cellular functions.

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