

# Swarming motility in *Bacillus cereus* and characterization of a *fliY* mutant impaired in swarm cell differentiation

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**This report describes a new behavioural response of *Bacillus cereus* that consists of a surface-induced differentiation of elongated and hyperflagellated swarm cells exhibiting the ability to move collectively across the surface of the medium. The discovery of swarming motility in *B. cereus* paralleled the isolation of a spontaneous non-swarming mutant that was found to carry a deletion of *fliY*, the homologue of which, in *Bacillus subtilis*, encodes an essential component of the flagellar motor-switch complex. However, in contrast to *B. subtilis*, the *fliY* mutant of *B. cereus* was flagellated and motile, thus suggesting a different role for *FliY* in this organism. The *B. cereus* mutant was completely deficient in chemotaxis and in the secretion of the L<sub>2</sub> component of the tripartite pore-forming necrotizing toxin, haemolysin BL, which was produced exclusively by the wild-type strain during swarm-cell differentiation. All the defects in the *fliY* mutant of *B. cereus* could be complemented by a plasmid harbouring the *B. cereus fliY* gene. These results demonstrate that the activity of *fliY* is required for swarming and chemotaxis in *B. cereus*, and suggest that swarm-cell differentiation is coupled with virulence in this organism.**

Keywords: flagellum, chemotaxis, haemolysin BL

## INTRODUCTION

Bacterial swarming is an organized form of multicellular translocation across solid surfaces (Henrichsen, 1972), which is exhibited by a wide variety of flagellated eubacteria when propagated on growth media of appropriate viscosity and composition (for reviews see Eberl *et al.*, 1999; Harshey, 1994). Bacteria capable of swarming motility produce highly organized communities initially consisting of vegetative cells, i.e. the swimmer cells, which undergo a co-ordinated surface-induced differentiation process characterized by the production of hyperflagellated, elongated, multinucleate cells, i.e. the swarm cells (Eberl *et al.*, 1996; Harshey & Matsuyama, 1994; Henrichsen, 1972; Hoeniger & Taushel, 1974; Macfarlane *et al.*, 2001). The differen-

tiated cells do not divide but possess the unique ability to migrate away from the colony in organized groups of tightly bound cells, which constitute an advancing-front movement at the rim of growing colonies. Collective swarm-cell migration (swarming motility) simultaneously stops and swarm cells dedifferentiate in unison (consolidate), reverting to the short, oligoflagellated, actively growing swimmer cells (Allison *et al.*, 1992a; Eberl *et al.*, 1999; Harshey, 1994; Harshey & Matsuyama, 1994; Henrichsen, 1972; Hoeniger & Taushel, 1974). Alternate cycles of swarming and consolidation are peculiar to the developmental behaviour of swarming colonies, which may exhibit macroscopic layered consolidation phases (terraces), forming regularly spaced concentric rings such as those produced by *Proteus mirabilis* (Henrichsen, 1972).

In contrast to other processes of differentiation in bacteria, swarming is not a starvation response and significantly varies depending on the organism and environmental growth conditions (Eberl *et al.*, 1996; Harshey, 1994; Harshey & Matsuyama, 1994). The rapid migration of swarm cells and the active

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**Abbreviations:** CI, capillary index; HBL, haemolysin BL; RAPD, randomly amplified polymorphic DNA; TBS, tris-buffered saline.

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growth of dedifferentiated swimmer cells provide swarming communities with the remarkable ability to progressively colonize the available surface. Swarming, therefore, is thought to be a successful strategy developed by flagellated micro-organisms to ensure their rapid expansion in the natural environment, where microbial activities are often associated with solid surfaces. Swarming motility was also proven to play a role in the colonization of host mucosal surfaces by infectious agents (Allison *et al.*, 1992a; Belas & Colwell, 1982). Moreover, the finding that some bacteria, such as uropathogenic strains of *P. mirabilis*, produce higher levels of specific virulence factors during their swarm-cell state (Allison *et al.*, 1992b, 1994), is of intrinsic interest as well as of great medical relevance.

Swarming differentiation has been studied mostly in Gram-negative rods and much has been learned about the regulatory mechanisms of swarming in *Serratia liquefaciens*, *Serratia marcescens*, *Salmonella typhimurium*, *Xenorhabdus nematophilus*, *Vibrio parahaemolyticus*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica* and *Escherichia coli*, as well as in *P. mirabilis* (Belas *et al.*, 1991; Eberl *et al.*, 1996; Givaudan & Lanois, 2000; Harshey & Matsuyama, 1994; O'Rear *et al.*, 1992; Rashid & Kornberg, 2000; Stewart *et al.*, 1997; Young *et al.*, 1999). Characterization of swarming-defective mutants demonstrated that molecular components of both the chemotaxis system and flagellar apparatus are essential for swarming differentiation in all the Gram-negative bacteria studied so far (Belas *et al.*, 1991, 1995; Burkart *et al.*, 1998; Givaudan & Lanois, 2000; Gygi *et al.*, 1995; O'Rear *et al.*, 1992; Young *et al.*, 1999). However, while the role of chemotaxis proteins in swarm-cell differentiation has not been clarified completely, the inability to swarm or to swarm properly exhibited by mutants defective in flagellar proteins (Belas *et al.*, 1991, 1995; O'Rear *et al.*, 1992; Young *et al.*, 1999), in motor-switch proteins (Belas *et al.*, 1995; Burkart *et al.*, 1998) or in proteins involved in the assembly of flagellar filaments (Gygi *et al.*, 1995; Young *et al.*, 1999), is consistent with the essential role played by a functional flagellum-mediated motility for the differentiation of hyperflagellated swarm cells.

Almost nothing is known about the molecular components needed for swarming differentiation in Gram-positive bacteria, although this striking behaviour has been long recognized in some species of *Clostridium* and *Bacillus* (Henrichsen, 1972; Hoeniger & Taushel, 1974). The ability to swarm has been never described in *Bacillus cereus*; the only flagellum-dependent surface motility recognized in this species is its ability to rapidly spread on the surface of solid culture media. Spreading of *B. cereus*, briefly discussed in a general review on bacterial surface translocation (Henrichsen, 1972), has been reported to be due to swimming motility only, brought about by individual cells that do not exhibit hyperflagellation or elongation.

In this report, we demonstrate that *B. cereus* is capable of swarming differentiation and that the production of

differentiated swarm cells requires the activity of *fliY* (Celandroni *et al.*, 2000), the homologue of which in *Bacillus subtilis* encodes an essential component of the flagellar-switch complex (C-ring) controlling the direction of flagellum rotation (Bischoff & Ordal, 1992). Complementation of a *fliY* deletion, identified in a motile but non-swarming and non-chemotactic spontaneous mutant of *B. cereus*, restores the ability to produce differentiated swarm cells as well as to respond to chemoattractants. Finally, evidence is produced showing that secretion of the L<sub>2</sub> component of haemolysin BL, a tripartite pore-forming necrotizing toxin from *B. cereus*, exclusively occurs during differentiation of swarm cells on solid media.

## METHODS

**Bacterial strains and growth conditions.** *B. cereus* NCIB 8122, originally obtained from the National Collection of Industrial Bacteria (Aberdeen, Scotland), has been propagated in our laboratory since 1975. Strain MP01 is a spontaneous mutant of *B. cereus* NCIB 8122, which was isolated during this study. Strain MP04 was generated from strain MP01 by complementation, as described below. *B. cereus* strains were grown with shaking (200 r.p.m.) at 37 °C in tryptone broth (TrB) containing 1% (w/v) tryptone and 0.5% (w/v) NaCl, or in Luria-Bertani (LB) broth. LB agar (1%, w/v, agar) was supplemented with 25 µg erythromycin ml<sup>-1</sup> when used for the selection of strain MP04. Tryptone agar (TrA) was used for the preparation of swim and swarm plates by varying the agar concentration, as established in the present investigation. *E. coli* strains JM109 and TOP10 (Invitrogen) were used for DNA cloning experiments. Both strains were grown in LB broth or on LB agar containing 100 µg ampicillin ml<sup>-1</sup>, when required.

**Swarming motility.** Bacteria from stationary-phase TrB cultures were seeded by spot inoculation (5 µl, 2.0 × 10<sup>8</sup> cells ml<sup>-1</sup>) onto the centre of TrA (1%, w/v, agar) plates and colony development was allowed to proceed at 37 °C in humidified chambers. Swarming migration was followed microscopically (IMT-2, Olympus) at different time intervals during growth. Formation of swarm cells was evaluated by recording the length and hyperflagellation of bacteria recovered from different portions of growing colonies. Cell length was assessed by microscopic observations of Gram-stained preparations, while flagellar staining was performed as described by Harshey & Matsuyama (1994). Since *B. cereus* flagella are very fragile, bacterial samples were taken by slide overlay of single agar blocks (5 × 5 mm) that contained different colony portions. Swarming of strain NCIB 8122 was also evaluated in TrA plates containing different agar concentrations (0.4–2.5%, w/v) and at temperature values ranging from 25 to 40 °C. The effect of mannitol on the swarming behaviour of *B. cereus* was evaluated on TrA (1%, w/v, agar) plates supplemented with mannitol (Sigma) at concentrations ranging from 0.2 to 2.0 mM.

**Cell differentiation assay.** The extent of swarm-cell differentiation was quantified by monitoring the increase in both cell length and cell-surface flagellin at fixed time intervals during growth on solid media. Differentiation assays were initiated by spreading 200 µl stationary-phase TrB cultures (2.0 × 10<sup>8</sup> cells ml<sup>-1</sup>) onto TrA (1%, w/v, agar) plates (14 cm diameter) and incubating at 37 °C. Cells were harvested by washing the entire surface of duplicate plates with 3.0 ml cold Tris-buffered saline (pH 7.4; TBS) at 2 h intervals. Cell suspensions for each

time point analysed were standardized to an OD<sub>600</sub> of 6.0 in TBS and vortexed for 5 min. Cell length was estimated by phase-contrast microscopy (BH-2, Olympus) for a total of 100 cells fixed in 1% (v/v) formaldehyde, 0.9% (w/v) NaCl. Cell-surface flagellin was recovered from supernatants of vortexed cells by centrifugation at 5000 g for 15 min. Flagellar filaments were collected from supernatants by high-speed centrifugation at 100000 g for 1 h. Flagellin was assayed by densitometry of protein stained with Coomassie brilliant blue after separation by SDS-PAGE on 12.5% (w/v) polyacrylamide gels, as described by Gygi *et al.* (1995).

**Swimming and chemotaxis assays.** Swimming in liquid media was evaluated under a phase-contrast microscope (BH-2, Olympus) by observing the smooth swimming or tumbling phenotype exhibited by bacteria suspended in a drop of LB broth. Swimming motility was also evaluated by seeding stationary phase cells (5 µl, 2.0 × 10<sup>8</sup> cells ml<sup>-1</sup>) on the centre of motility TrA plates (swim plates, 0.25%, w/v, agar). Plates were incubated at 37 °C and the diameter of developing colonies, recorded 6 h post-inoculation, was taken as a measure of bacterial migration in semisolid agar.

Chemotaxis was evaluated by using capillaries filled with attractants, as described by Ordal & Goldman (1975) with modifications. Briefly, cells were grown overnight in TrB at 37 °C and diluted 1:50 in G medium (Hanson *et al.*, 1961). After a 4 h incubation (100 r.p.m.) at 37 °C, glycerol (0.05%) was added to the cell suspensions. Cells were then harvested, diluted to OD<sub>600</sub> 0.05 in chemotaxis buffer [10 mM potassium phosphate buffer (pH 7.0), 0.3 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.14 mM CaCl<sub>2</sub>, 0.1 mM EDTA] and assayed for their ability to chemotax towards L-glutamine, L-alanine, L-cysteine, L-histidine or mannitol (50 mg each ml<sup>-1</sup> in chemotaxis buffer). The mean number of c.f.u. entering the attractant-filled capillaries (CFUa) and that entering control capillaries (CFUc) filled with chemotaxis buffer only were determined on LB agar plates by repeating each assay five times on separate days. The capillary index (CI) for each strain and attractant was calculated as follows: CI = (CFUa - CFUc)/CFUc. The ability of bacteria to chemotax in semisolid media was also determined in swim TrA plates supplemented with either glutamine or mannitol, at concentrations ranging from 0.2 to 20 mM.

**Immunoassays for haemolysin BL (HBL) and detection of *hblA* and *hblD* genes.** *B. cereus* HBL components (B, L<sub>1</sub> and L<sub>2</sub>) were assayed in supernatants of late-exponential phase cultures grown in TrB at 30 °C by Western blotting (Beecher *et al.*, 1995a). Culture supernatants were concentrated 20-fold with Millipore Ultrafree centrifugal filters. Proteins from concentrated culture supernatants were separated by SDS-PAGE on 12% (w/v) polyacrylamide gels and transferred electrophoretically to PVDF membranes (Millipore). Detection of HBL components produced on solid media was performed by seeding a liquid inoculum (5 µl stationary-phase TrB cultures) on sterile disks of nitrocellulose membrane (Millipore) layered on TrA (1%, w/v, agar) plates. The liquid inoculum was allowed to dry and after 24, 48 or 72 h at 30 °C, cells were washed from the membrane with TBS. All the membranes were soaked for 1 h at 37 °C in TBS containing 3% (w/v) BSA and probed with rabbit antisera specific to the individual HBL components (Beecher *et al.*, 1995b). Bound antibody was detected by using goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma) and the chromogenic substrate 4-chloro-1-naphthol (Sigma).

*B. cereus* was lysed and chromosomal DNA purified as previously described (Celandroni *et al.*, 2000). PCRs for

assessing the presence of the *hblA* (encoding component B) and *hblD* (encoding component L<sub>1</sub>) genes were performed using standard conditions and varying the annealing temperatures from 70 to 50 °C. PCR reactions were performed using the primers HblA1 (5'-GCTAATGTAGTTTCACCTGTAGCAAC-3')/HblA2 (5'-AATCATGCCACTGCGTGGACATATAA-3') (Prüb *et al.*, 1999) or DL1F (5'-GTAGGCAATTA-TGCATTAGGCC-3')/DL1R (5'-CAACAATAACTACCGCTCCTCC-3') for the amplification of an 883 bp and a 670 bp fragment of *B. cereus hblA* and *hblD*, respectively.

**Randomly amplified polymorphic DNA (RAPD) analysis, Southern blotting and *fliY* amplification.** PCR mixtures (50 µl) for RAPD analysis contained a single oligonucleotide primer at a concentration ranging from 1.0 to 3.0 µM, 2.5 U *Taq* polymerase (Pharmacia Biotech), 0.2 mM each dNTP, 5 µl *Taq* polymerase buffer (Pharmacia Biotech) and 10 or 30 ng *B. cereus* chromosomal DNA. The concentrations of both DNA template and primer were varied to rule out polymorphism in artefact bands. Four different PCR primers were used: HLWL74 (5'-ACGTATCTGC-3'), HLWL85 (5'-ACAACCTGCTC-3'), M13 (5'-GAGGGTGGCGGCTCT-3') and OPE06 (5'-AAGACCCCTC-3'). Cycling was as follows: 30 cycles at 94 °C for 5 s, 36 °C for 1 min and 72 °C for 1.5 min, with a 10 min extension at 72 °C for the last cycle. Amplification reactions were performed in a Gene Amp PCR System 9600 (Perkin Elmer Cetus). An amplified fragment was ligated into the pGEM-T vector (Promega) to obtain pFEM that was propagated in *E. coli* JM109. Labelling of DNA by [<sup>32</sup>P]dCTP was achieved with the Multiprime DNA labelling kit (Amersham Life Science). Southern blotting experiments were performed as described by Sambrook *et al.* (1989). Oligonucleotide primers were synthesized by Genset.

PCR amplification of *fliY* was performed with the primers YF1 (5'-CACACAAAAGGGGATACAAG-3') and YR1 (5'-ACATTTGGCGGCGTCATG-3'). Reaction mixtures (50 µl) contained 0.4 µM each primer, 2.5 U *Pfu* Turbo DNA Polymerase (Stratagene), 0.2 mM each dNTP, 5 µl *Pfu* Turbo buffer (Stratagene) and 10 ng *B. cereus* chromosomal DNA. Cycling was as follows: 30 cycles at 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, with a 10 min extension at 72 °C for the last cycle.

**Complementation of *fliY*.** *fliY* was amplified from strain NCIB 8122 using the primers 14FYU2 (5'-AGAAAAGCTT-AATGAGGAGGCAG-3') and 14FYU2 (5'-CCAAAGGAT-CCCCAAATACTC-3'), which were designed based on sequences external to the coding region of *B. cereus fliY* (Celandroni *et al.*, 2000). The primers carried synthetic *Hind*III and *Bam*HI restriction sites (underlined), respectively. PCR conditions were as described above and cycling was as follows: one cycle at 94 °C for 2 min followed by 30 cycles at 95 °C for 30 s, 60 °C for 1 min and 72 °C for 1.5 min, with a 10 min extension at 72 °C for the last cycle. After amplification, the DNA fragment was digested with *Hind*III/*Bam*HI, and inserted between the *Hind*III and *Bam*HI sites of pHT304 (Arantes & Lereclus, 1991), an *E. coli/Bacillus thuringiensis* shuttle and expression vector. The resulting vector, pHTY01, was propagated in *E. coli* TOP10 and introduced into strain MP01 by electroporation (Lereclus *et al.*, 1989) to produce strain MP04. Recombinant clones were screened on LB agar plates containing erythromycin.

**DNA sequencing and nucleotide sequence analysis.** Nucleotide sequencing was performed by the dideoxy-chain termination method on double-stranded DNA, with the ALFexpress AutoRead Sequencing Kit (Pharmacia Biotech) and the ALFexpress automatic sequencer (Pharmacia Biotech).

DNA sequences were processed using the ALF Manager version 3.02 (Pharmacia) and compared with available sequences using the BLAST programs implemented at the EMBL, GenBank and DDBJ nucleotide sequence databases. The nucleotide sequence determined in this study has been deposited in the EMBL database under the accession no. Y08031.

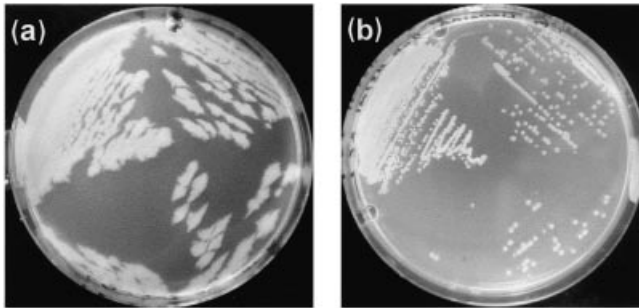
## RESULTS

### Swarming motility of *Bacillus cereus* and growth behaviour of a non-swarming mutant

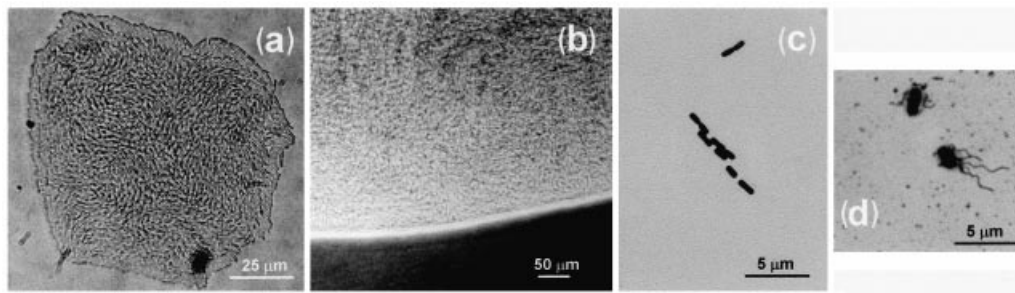
The ability to swarm was recognized in *B. cereus* NCIB 8122 following the isolation of a unique spontaneous mutant, referred to as MP01, which exhibited a colony morphotype different from that produced by the parental strain (Fig. 1). On the sporulation medium we use to achieve high rates of spore production (Senesi *et al.*, 1991), the wild-type strain developed large and rough colonies (Fig. 1a), while MP01 produced small, smooth and almost perfectly round colonies (Fig. 1b). To determine whether differences in colony morphology reflected differences in collective growth behaviour, microscopic inspections were performed for both strains during colony development. The colonies produced by strain MP01 on TrA plates (1% agar, 37 °C, humidified chambers) did not reveal any peculiar intra-colony cell organization but appeared as a homogeneous distribution of individual cells actively moving within a compact community (Fig. 2a). The rim of growing

colonies appeared separated from the surrounding milieu by a thick slime-like structure, which became evident in the late stages of colony development (Fig. 2b). Most cells, especially those nearest the colony rim, exhibited a random movement in that individual cells moved erratically, colliding with each other and changing direction very frequently. However, cells never migrated beyond the colony border in both early (Fig. 2a) and late (Fig. 2b) stages of colony development. Cell samples taken from the centre and periphery of growing colonies showed that the whole community consisted of short (Fig. 2c), oligoflagellated (Fig. 2d), rod-shaped cells, i.e. the swimmer cells, which were indistinguishable from those that grew in liquid media.

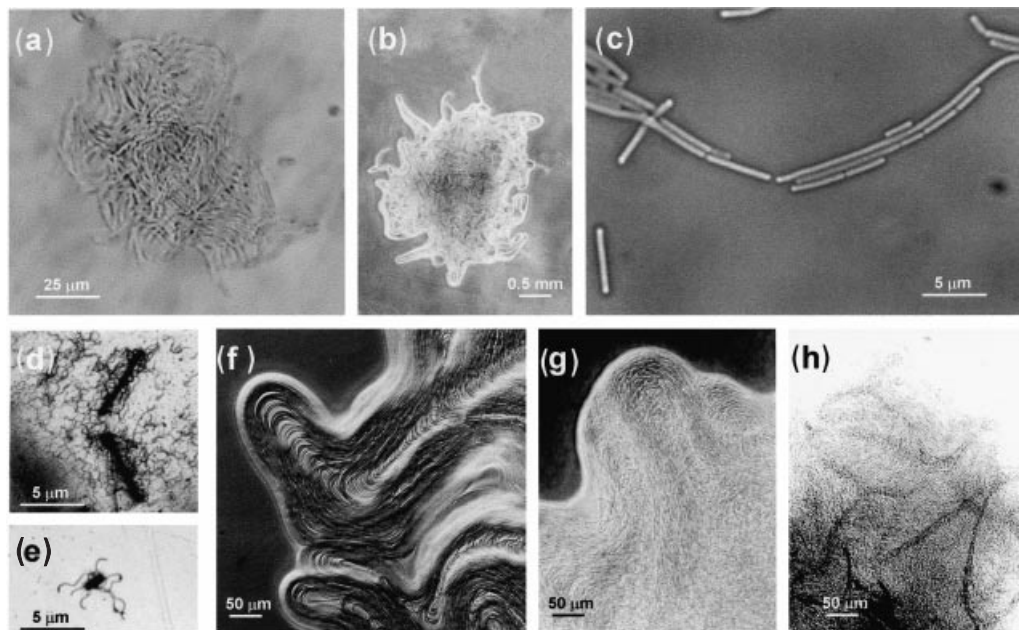
Under the same growth conditions, the wild-type strain produced more complex and organized communities that initially consisted only of short, oligoflagellated, rod-shaped cells, i.e. the vegetative swimmer cells (2–3 h post-inoculation; Fig. 3a). Thereafter, the swimmer cells at the rim of growing communities initiated a differentiation process characterized by the production of elongated and hyperflagellated cells, i.e. the swarm cells. The differentiated cells immediately started to move in organized groups of tightly bound cells, regularly aligned along their long axis, thus generating finger-like structures all around the colony rim (8 h post-inoculation; Fig. 3b). Cells from the colony rim were elongated (mean length 10 µm; Fig. 3c) and extensively flagellated (Fig. 3d), while those in the colony centre were short (mean length 3 µm), oligoflagellated and rod-shaped (Fig. 3e). The advancing bacteria were seen to move, generating curved paths that converged, thus giving rise to an outer layer co-ordinately migrating outward from the colony in a swirling fashion (Fig. 3f). While the collective movement of differentiated swarm cells continued to occur at the rim of the growing colony, the swarm cells in the inner portion of the migrating front stopped moving and dedifferentiated into swimmer cells. These cells underwent reductive divisions and produced a consistent consolidation phase inside the growing colony (Fig. 3g). Even at 24–48 h post-inoculation, the rim of colonies produced by strain NCIB 8122 was characterized by an active, although substantially reduced, migration front of swarm cells, while the colony centre appeared as a unique con-



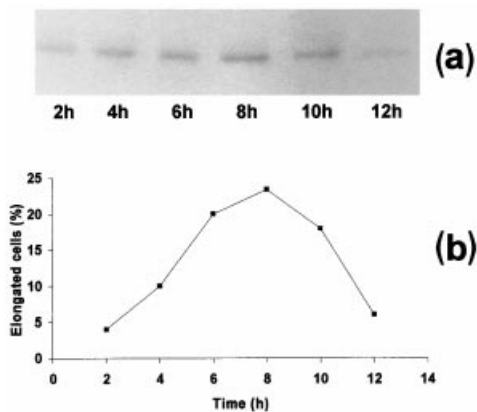
**Fig. 1.** Colonies produced by *B. cereus* strain NCIB 8122 (a) and by its spontaneous mutant MP01 (b) on sporulation medium.



**Fig. 2.** Morphological traits of *B. cereus* strain MP01. Colonies produced on TrA plates (1% agar) after 4 h (a) and 18 h (b) incubation at 37 °C. Short (c) and oligoflagellated (d) cells picked up from the colony rim.



**Fig. 3.** Morphological traits of *B. cereus* strain NCIB 8122. Colonies produced on TrA plates (1% agar) after 3 h (a) and 8 h (b) incubation at 37 °C. Elongated (c) and hyperflagellated (d) swarm cells picked up from the colony rim. Short and oligoflagellated (e) cell from the colony centre. Colony border after 12 h (f), 24 h (g) and 48 h (h) incubation at 37 °C.



**Fig. 4.** Surface flagellin and cell elongation of strain NCIB 8122 harvested during a swarm/differentiation cycle. (a) Surface flagellin separated by SDS-PAGE and stained by Coomassie blue. (b) Frequency of elongated cells observed by phase-contrast microscopy.

solidation phase, exhibiting irregular lines left behind by the pre-existent migration waves (Fig. 3h). In contrast to the swarming colonies produced by *P. mirabilis* (Henrichsen, 1972), those developed by *B. cereus* NCIB 8122 did not exhibit concentric rings of consolidation, since regularly spaced cycles of swarming motility and consolidation were not produced by this organism.

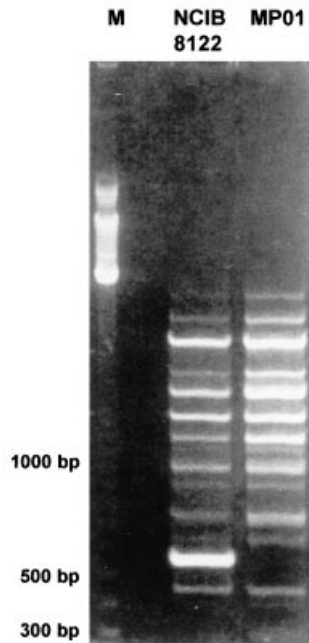
Swarm cell differentiation was better quantified by monitoring the increase in both cell length and cell-surface flagellin at fixed time intervals during growth

under the conditions established for the differentiation assays. At eight hours post-inoculation, *B. cereus* NCIB 8122 exhibited the highest amount of both surface flagellin and the maximum percentage of elongated cells (23%). This peak of swarm cell differentiation was followed by a return to near-vegetative levels after 12 h growth (Fig. 4).

Under the growth conditions we used in the present investigation, *B. cereus* exclusively exhibited swarm cell differentiation when the agar concentration was above 0.4%, while optimal swarming motility was observed at concentrations ranging from 0.7 to 1.5%. Swarming by *B. cereus* was gradually inhibited by further increasing the agar concentration and at 2.5% inhibition was complete (data not shown). In contrast to other swarming bacteria (Eberl *et al.*, 1996; Harshey & Matsuyama, 1994), the swarming response by *B. cereus* was observed at a wide range of temperatures, the optimum being between 25 and 38 °C.

#### Strain MP01 is a *fliY* null mutant

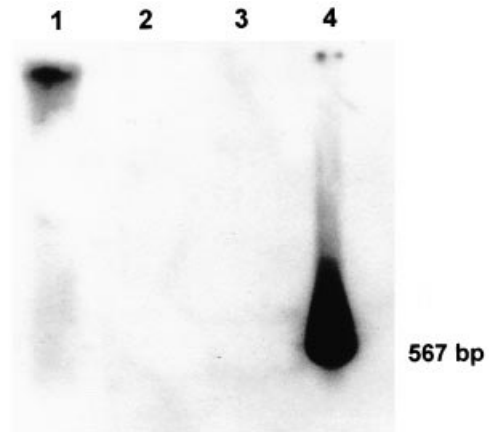
The RAPD-PCR technique is widely used for searching for sequence polymorphism exhibited by nearly identical bacterial genomes. It has been applied frequently to distinguish subgroups of closely related organisms including those showing a high degree of sequence conservation among isolates, as in the case of *Bacillus anthracis* (Henderson *et al.*, 1994). In this study, RAPD-PCR was used to fingerprint the whole genome of *B. cereus* strains NCIB 8122 and MP01. Indistinguishable DNA banding patterns were obtained with three out of



**Fig. 5.** Amplification patterns of *B. cereus* strains NCIB 8122 and MP01 obtained with RAPD-PCR and primer HLWL85. M, 100 bp ladder marker.

the four primers used (data not shown). Primer HLWL85 was the only one to generate a single polymorphic DNA fragment. A fragment of about 500 bp was amplified from strain NCIB 8122 (Fig. 5); this fragment is absent in strain MP01. The polymorphic fragment was observed under all the reaction conditions tested, including primer concentrations of 1.0 and 3.0  $\mu\text{M}$ , and DNA concentrations of 0.2 and 0.6  $\mu\text{g ml}^{-1}$ ; additional polymorphic DNA bands were never detected even among the faint bands we obtained by adopting different reaction conditions. The polymorphic fragment was cloned into pGEM-T, generating pFEM. Analysis of the nucleotide sequence of the polymorphic fragment, determined in different *E. coli* transformants containing pFEM, revealed that the fragment (567 bp) was a portion of a unique putative ORF (accession no. Y08031). The predicted amino acid sequence was most homologous to a protein fragment encoded by *fliY* of *B. cereus* ATCC 10987 (accession no. AJ272332; 98% identity in 185 overlapping amino acids, 50% of FliY), *B. cereus* ATCC 14579 (accession no. AJ272330; 88% identity in 187 overlapping amino acids, 50% of FliY) and *B. subtilis* (accession no. P24073; 40% identity in 173 overlapping amino acids, 46% of FliY). The protein encoded by *fliY* (FliY) in *B. subtilis* is an essential component of the flagellar motor-switch complex (C-ring) and represents the Gram-positive counterpart of the Gram-negative FliN (Bischoff & Ordal, 1992).

The polymorphic fragment, which was located in a central portion of *fliY* (from nt 162 to 728), was labelled and used as a probe in Southern hybridization exper-



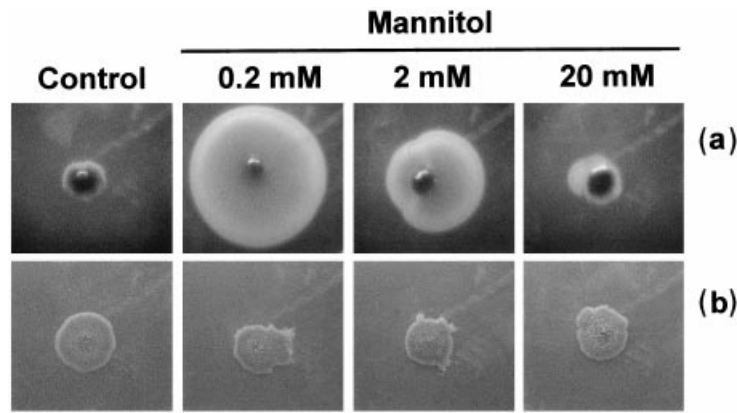
**Fig. 6.** Hybridization of a 567 bp *fliY* fragment with the chromosomal DNA derived from the strains NCIB 8122 (lane 1) and MP01 (lane 2, undigested; lane 3, digested with *EcoRI*). Positive control in lane 4.

iments with the chromosomal DNA derived from strains NCIB 8122 and MP01. Hybridization of the probe with the MP01 genome was never observed (Fig. 6), indicating that the absence of the polymorphic fragment in the RAPD-PCR profile of this strain was due to a genomic deletion rather than to a mutation occurring in one of the primer annealing sites. PCR amplification with the primers YF1 and YR1, designed on the basis of sequences located at the 3' and 5' ends of *B. cereus fliY* (Celandroni *et al.*, 2000), was performed on the genome of MP01 and on the wild-type strain, for comparison. While a PCR product of the right size (about 1131 bp) was obtained from the parental strain, no amplification was achieved with the mutant. Taken together, these results indicate that strain MP01 is a *fliY* null mutant carrying a large genomic deletion, the extension of which encompasses the 567 bp fragment and at least one flanking region at the N- or C-terminal coding sequence of *fliY*.

#### The non-swarming mutant of *B. cereus* is motile but defective in chemotaxis

While *fliY* null mutants of *B. subtilis* do not possess flagella (Bischoff & Ordal, 1992), the *B. cereus* mutant carrying a deletion in *fliY* appeared oligoflagellated and motile. However, when a suspension of MP01 in LB broth was observed microscopically, it was noticed that its swimming behaviour resulted in more tumbling than that exhibited by the wild-type. The tumbling phenotype exhibited by MP01 might account for the differences in the diameters of the colonies produced by the mutant and by the wild-type strains on swim TrA (0.25% agar) plates ( $9 \pm 1$  mm vs.  $21 \pm 2$  mm, respectively). These observations suggested that the *fliY* mutant of *B. cereus* could display a defective chemotactic response. Indeed, while the wild-type strain showed a positive chemotactic response with all the attractants tested (L-glutamine, L-alanine, L-cysteine, L-histidine and mannitol) in

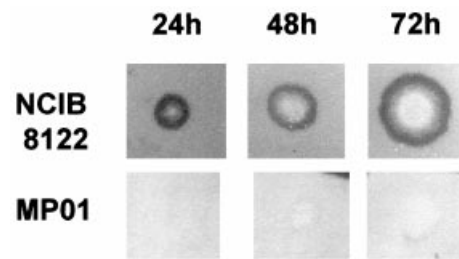




**Fig. 7.** Chemotactic and swarming response to mannitol (0.2, 2.0, 20 mM) of strain NCIB 8122 in swim (0.25% agar) (a) or swarm (1% agar) (b) TrA plates. Control plates were void of attractant.

capillary assays ( $CI_{\text{Glu}}$ , 47.1;  $CI_{\text{Ala}}$ , 23.2;  $CI_{\text{Cys}}$ , 20.4;  $CI_{\text{His}}$ , 19.8;  $CI_{\text{Mann}}$ , 60.8), the mutant did not chemotax towards any of the attractants, with the CI value obtained with each compound being  $\sim 0$ . The  $\text{Che}^-$  phenotype exhibited by MP01 also could be demonstrated by seeding the mutant strain onto semisolid TrA plates supplemented with attractants (either mannitol or glutamine) and measuring the colony diameters in comparison with those obtained with the wild-type strain. This assay is based on the assumption that bacteria growing on culture media actively metabolize the attractant added to the medium, thus generating a spatial attractant gradient allowing the bacteria to chemotax toward higher attractant concentrations (Burkart *et al.*, 1998). The colonies produced by strain NCIB 8122 in the presence of mannitol (2 mM) and glutamine (2 mM) were larger (colony diameter  $27 \pm 2$  mm and  $25 \pm 2$  mm, respectively) than those produced in the same medium without attractants (colony diameter  $21 \pm 1$  mm), indicating that the strain was able to migrate in response to attractant stimuli; in contrast, there was no increase in the diameters of colonies produced by the mutant strain following addition of the attractants to the culture medium.

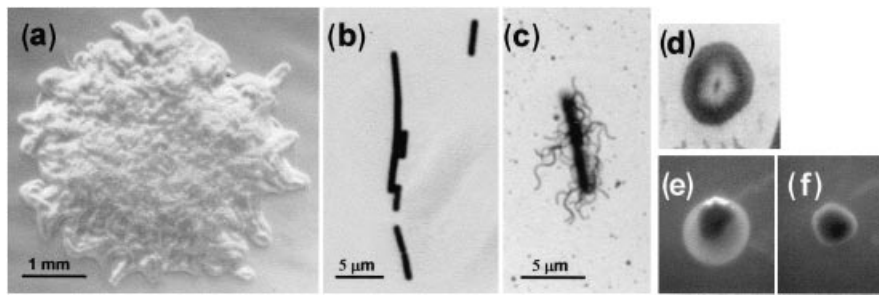
Based on the CI values recorded, mannitol ( $CI_{\text{Mann}}$ , 60.8) was the most powerful attractant for *B. cereus*. Taking into consideration the observation that high concentrations (in the millimolar range) of an attractant may saturate its own receptor and inhibit chemotaxis (Burkart *et al.*, 1998), we wondered whether sensing of a mannitol gradient could play a critical role in swarming motility other than in swimming by *B. cereus*. Fig. 7(a) shows that inhibition of the chemotactic response in strain NCIB 8122 was produced by increasing mannitol concentrations (from 2 to 20 mM), which was deduced by measuring the reduction of bacterial migration occurring in swim plates. The swarming response of this strain, on the contrary, did not exhibit any change when the concentration of mannitol in swarm TrA (1% agar) plates was varied from 0.2 to 20 mM (Fig. 7b). These results suggest that chemotaxis itself, at least toward mannitol, is unlikely to play a role in *B. cereus* swarming motility.



**Fig. 8.** Colony immunoblot assay performed with antiserum against the  $L_2$  component of HBL.

#### Secretion of the $L_2$ component of HBL is coupled with swarm cell differentiation on solid media

About 45% of the *B. cereus* isolates secrete HBL (Beecher & Wong, 1994), a membrane-lytic system composed of three antigenically distinct proteins (B,  $L_1$  and  $L_2$ ), which contributes to diarrhoeal food poisoning (Beecher *et al.*, 1995b) and necrotizing infections such as endophthalmitis (Beecher *et al.*, 1995a). To evaluate whether any difference in the amount of secreted HBL could be observed between strains NCIB 8122 and MP01, culture supernatants from both strains were screened for the presence of the individual HBL components by Western blot analysis. None of the components was detected in the culture supernatants of both strains (data not shown). In contrast, when the strains were grown on nitrocellulose filters laid on swarm TrA (1% agar) plates, only strain NCIB 8122 was capable of producing the  $L_2$  component; a positive signal with the anti- $L_2$  serum was detected starting from 24 h of incubation at 30 °C (Fig. 8). Interestingly, the positive signals were evident all around the colony rim where the migration front of swarm cells was localized. Although the production of only one or two of the three HBL components has been reported for some *B. cereus* strains (Schoeni & Wong, 1999), we wanted to assess if *hblA* and *hblD*, which encode the B and  $L_1$  components of HBL respectively, were present and not expressed in strain NCIB 8122. Therefore, PCR amplification with primers that have been already applied to screen bacteria



**Fig. 9.** Morphological traits of *B. cereus* strain MP04. Swarm colony on TrA plates (1% agar) (a). Elongated (b) and hyperflagellated (c) swarm cells. Positive colony immunoblot assay with the anti-L<sub>2</sub> serum (d). Growth on swim (0.25% agar) TrA plates containing 2 mM glutamine (e) and void of attractant (f).

for HBL genes (Prüb *et al.*, 1999) was performed. Although repeated up to eight times with different annealing temperatures (from 70 to 50 °C), PCRs never produced *hblA* and *hblD* amplification products, suggesting the absence of these genes in the strain tested.

#### *fliY* is required for swarming motility in *B. cereus*

Since strain MP01 is a spontaneous mutant of strain NCIB 8122, we wanted to determine whether the mutation in *fliY* was the only mutation responsible for the defective phenotype exhibited by strain MP01. A vector (pHTY01) containing the wild-type *fliY* under the control of the *lacZ* promoter was constructed and introduced into strain MP01, to generate strain MP04. Complementation *in trans* of the *fliY* deletion restored the ability of MP01 to develop swarming colonies (Fig. 9a) and to produce differentiated swarm cells. Cells taken from the periphery of the colonies produced by the *fliY* mutant harbouring pHTY01 were elongated (Fig. 9b) and hyperflagellated (Fig. 9c) as were those from the periphery of colonies produced by the wild-type strain. Moreover, strain MP04 was able to secrete the L<sub>2</sub> component of HBL, which, as for the wild-type strain, was the only component of HBL detectable during differentiation of swarm cells in solid media (Fig. 9d).

Restoration of the chemotactic response was also accomplished by complementation of the *fliY* mutation in MP01. Chemotaxis was evaluated by assessing the ability of strain MP04 to migrate towards attractants in both capillary assays and swim TrA plates. Positive CI values, although lower than those observed for the wild-type strain, were obtained with all the attractants tested (CI<sub>Glu</sub>, 3.4; CI<sub>Ala</sub>, 1.9; CI<sub>Cys</sub>, 1.7; CI<sub>Hys</sub>, 0.9 and CI<sub>Mann</sub>, 4.8). The diameters of the colonies produced by MP04 in swim TrA plates containing either mannitol (2 mM) or glutamine (2 mM) (Fig. 9e) were larger (21.5 and 20.4%, respectively) than those produced when MP04 was seeded onto control plates (void of attractants) (Fig. 9f). Therefore, the defective phenotype exhibited by the spontaneous mutant MP01 appeared to be due to the *fliY* deletion itself and not other mutations or polar effects caused by the mutation in *fliY*.

## DISCUSSION

*B. cereus* is a ubiquitous, spore-forming bacterium especially widespread in the soil and frequently isolated from a wide variety of commodities as a contaminant. Long recognized as a micro-organism responsible for food poisoning, *B. cereus* is now regarded as an opportunistic pathogen of increasing medical relevance, since it causes severe and sometimes fatal non-gastrointestinal infections such as endocarditis, pneumonia and endophthalmitis (Beecher *et al.*, 1995a; Miller *et al.*, 1997; Steen *et al.*, 1992; Vahey & Flynn, 1991). *B. cereus* is a motile bacterium by means of peritrichous flagella, but almost nothing is known about the involvement of flagella in its pathogenicity and even less about the molecular mechanisms responsible for its flagellum-dependent chemotactic response.

The present study demonstrates that *B. cereus* is able to exhibit two alternate forms of flagellum-driven motility, swimming and swarming, depending on whether the bacterium is grown in liquid or solid media. While swimming motility is brought about by individual swimmer cells that are short oligoflagellated rods, swarming is an organized and collective movement of differentiated swarm cells, which, in *B. cereus*, are 3–4 times longer and 40 times more flagellated than the swimmer cells. The swarming response of *B. cereus* was induced by surface sensing but different from other swarming bacteria (Eberl *et al.*, 1996; Harshey & Matsuyama, 1994; Young *et al.*, 1999), and occurred at a wide range of medium viscosity (from 0.4 to 2% agar) and temperatures (25–38 °C). Swarm-cell differentiation was also observed in minimal media, although colonies appeared small and rhizoid (data not shown). *B. cereus* swarm colonies never exhibited macroscopically layered consolidation phases due to regularly spaced cycles of swarming migration and consolidation. As also reported for *Y. enterocolitica*, *S. liquefaciens* and *Clostridium septicum* (Eberl *et al.*, 1999; Macfarlane *et al.*, 2001; Young *et al.*, 1999), *B. cereus* appeared to swarm continuously at the colony rim, while the colony centre apparently consisted of a unique consolidation phase.



An important finding emerging from this study was that a spontaneous mutant of *B. cereus* carrying a deletion in *fliY* was non-swarming and non-chemotactic, and such phenotypic defects could be complemented by a plasmid harbouring the *fliY* gene. This gene has been identified previously in *B. subtilis* (Bishoff & Ordal, 1992), where it encodes a protein that is a component of the flagellar motor-switch complex, which consists of FliG (Albertini *et al.*, 1991), FliM (Zuberi *et al.*, 1991) and FliY (Bishoff & Ordal, 1992). *B. subtilis* FliY was shown to be partly homologous (Bishoff & Ordal, 1992) to the *E. coli* and *S. typhimurium* switch proteins FliM and FliN. However, FliY differs from FliN in Gram-negative bacteria in that it appeared to act as a bifunctional protein, being able to co-operate with FliG and FliM in constituting the switch complex and also interact with other proteins in modulating intracellular chemotactic signals (Bishoff & Ordal, 1992). The *fliY* null mutant of *B. subtilis* was non-chemotactic, as was the *fliY* mutant of *B. cereus*. However, in contrast to *B. subtilis*, the *fliY* mutant of *B. cereus* was flagellated and motile. The inability of the *B. cereus* mutant to respond to chemoattractants suggests that FliY is a flagellar motor-switch protein also in this species. Such a role for FliY is supported by the observation that the *fliY* mutant of *B. cereus* was impaired in swimming motility, resulting in more tumbling than the wild-type strain.

The lack of swarming differentiation in the *fliY* mutant of *B. cereus* is consistent with the fact that all the mutants characterized by defects in any one of the flagellar genes, including those encoding the motor switch proteins (Belas *et al.*, 1995; Burkart *et al.*, 1998), are impaired in swarming migration. However, as already described for *E. coli* (Burkart *et al.*, 1998), a direct involvement of chemotaxis in swarming motility was not demonstrated, since swarming of the wild-type strain was unaffected by the addition of compounds that acted as attractants in liquid media.

Another observation pertains to the role of *fliY* in the assembly of the flagellar filament. In Gram-negative bacteria as well as in *B. subtilis*, the switch complex is required for the completion of the flagellar assembly (Bishoff & Ordal, 1992; Macnab, 1996). Therefore, the finding that the *fliY* mutant of *B. cereus* is flagellated and motile raises the question whether a peculiar regulatory pathway governing the assembly of flagellar proteins or the expression of flagellar genes acts in this species.

Finally, the demonstration that *B. cereus* strain NCIB 8122 produces the L<sub>2</sub> component of haemolysin BL only in the swarm-cell state opens new perspectives in considering swarming motility potentially coupled to the virulence of this organism. An association between swarming motility and virulence has been shown for several Gram-negative and -positive bacteria. In *P. mirabilis*, swarming has been associated with the ability to invade urothelial cells and to express higher levels of virulence factors, such as intracellular urease, extracellular haemolysin and metalloprotease (Allison *et al.*, 1992a, b, 1994). Swarming by *S. liquefaciens* was found to be accompanied by an increase in the expression of

the phospholipase gene (Eberl *et al.*, 1996), and *C. septicum* was capable of producing DNase, hyaluronidase and neuraminidase only during the swarm-cell state (Macfarlane *et al.*, 2001). The fact that the *B. cereus* mutant harbouring an expression plasmid containing *fliY* regained the ability to swarm and to produce the L<sub>2</sub> component of HBL strongly suggests that a relationship between expression of virulence factors and differentiation of swarm cells also exists in this organism.

Because bacterial motility may be crucial to seek nutrients, to rapidly colonize surfaces in the natural environment, to establish infection inside a host, and, presumably, to produce toxins and other virulence factors, the dependence of swarming, chemotaxis and L<sub>2</sub> secretion on the activity of *fliY* reveals a major role for this gene in different adaptive responses elicited by *B. cereus* to its environment.

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## REFERENCES

- Albertini, A. M., Caramori, T., Crabb, W. D., Scoffone, F. & Galizzi, A. (1991). The *flaA* locus of *Bacillus subtilis* is part of a large operon coding for flagellar structures, motility functions, and an ATPase-like polypeptide. *J Bacteriol* **173**, 3573–3579.
- Allison, C., Coleman, N., Jones, P. L. & Hughes, C. (1992a). Ability of *Proteus mirabilis* to invade human urothelial cells is coupled to motility and swarming differentiation. *Infect Immun* **60**, 4740–4746.
- Allison, C., Lai, H. C. & Hughes, C. (1992b). Co-ordinate expression of virulence genes during swarm-cell differentiation and population migration of *Proteus mirabilis*. *Mol Microbiol* **6**, 1583–1591.
- Allison, C., Emody, L., Coleman, N. & Hughes, C. (1994). The role of swarm-cell differentiation and multicellular migration in the uropathogenicity of *Proteus mirabilis*. *J Infect Dis* **69**, 1155–1158.
- Arantes, O. & Lereclus, D. (1991). Construction of cloning vectors for *Bacillus thuringiensis*. *Gene* **108**, 115–119.
- Beecher, D. J. & Wong, A. C. L. (1994). Identification of hemolysin BL-producing *Bacillus cereus* isolates by a discontinuous hemolytic pattern in blood agar. *Appl Environ Microbiol* **60**, 1646–1651.
- Beecher, D. J., Pulido, J. S., Barney, N. P. & Wong, A. C. L. (1995a). Extracellular virulence factors in *Bacillus cereus* endophthalmitis: methods and implication of involvement of hemolysin BL. *Infect Immun* **63**, 632–639.
- Beecher, D. J., Schoeni, J. L. & Wong, A. C. L. (1995b). Enterotoxigenic activity of hemolysin BL from *Bacillus cereus*. *Infect Immun* **63**, 4423–4428.
- Belas, M. R. & Colwell, R. R. (1982). Adsorption kinetics of laterally and polarly flagellated *Vibrio*. *J Bacteriol* **151**, 1568–1580.
- Belas, R., Erskine, D. & Flaherty, D. (1991). *Proteus mirabilis* mutants defective in swarmer cell differentiation and multicellular behavior. *J Bacteriol* **173**, 6279–6288.

- Belas, R., Goldman, M. & Ashliman, K. (1995).** Genetic analysis of *Proteus mirabilis* mutants defective in swarmer cell elongation. *J Bacteriol* **177**, 823–828.
- Bischoff, D. S. & Ordal, G. W. (1992).** Identification and characterization of *FliY*, a novel component of the *Bacillus subtilis* flagellar switch complex. *Mol Microbiol* **18**, 2715–2723.
- Burkart, M., Toguchi, A. & Harshey, R. M. (1998).** The chemotaxis system, but not chemotaxis, is essential for swarming motility in *Escherichia coli*. *Proc Natl Acad Sci USA* **95**, 2568–2573.
- Celandroni, F., Ghelardi, E., Pastore, M., Lupetti, A., Kolstø, A.-B. & Senesi, S. (2000).** Characterization of the chemotaxis *fliY* and *cheA* genes in *Bacillus cereus*. *FEMS Microbiol Lett* **190**, 247–253.
- Eberl, L., Christiansen, G., Molin, S. & Givskov, M. (1996).** Differentiation of *Serratia liquefaciens* into swarm cells is controlled by the expression of the *flhD* master operon. *J Bacteriol* **178**, 554–559.
- Eberl, L., Molin, S. & Givskov, M. (1999).** Surface motility of *Serratia liquefaciens* MG1. *J Bacteriol* **181**, 1703–1712.
- Givaudan, A. & Lanois, A. (2000).** *flhDC*, the flagellar master operon of *Xenorhabdus nematophilus*: requirement for motility, lipolysis, extracellular hemolysis, and full virulence in insects. *J Bacteriol* **182**, 107–115.
- Gygi, D., Bailey, M. J., Allison, C. & Huges, C. (1995).** Requirement for *FlhA* in flagella assembly and swarm-cell differentiation by *Proteus mirabilis*. *Mol Microbiol* **15**, 761–769.
- Hanson, R. S., Srinivasan, V. R. & Harvanson, H. O. (1961).** Citrate formation in *B. cereus* strain T. *Biochem Biophys Res Commun* **5**, 458–461.
- Harshey, R. M. (1994).** Bees aren't the only ones: swarming in Gram-negative bacteria. *Mol Microbiol* **13**, 389–394.
- Harshey, R. M. & Matsuyama, T. (1994).** Dimorphic transition in *Escherichia coli* and *Salmonella typhimurium*: surface-induced differentiation into hyperflagellate swarmer cells. *Proc Natl Acad Sci USA* **91**, 8631–8635.
- Henderson, I., Duggleby, C. J. & Turnbull, P. C. B. (1994).** Differentiation of *Bacillus anthracis* from other *Bacillus cereus* group bacteria with the PCR. *Int J Syst Bacteriol* **44**, 99–105.
- Henrichsen, J. (1972).** Bacterial surface translocation: a survey and a classification. *Bacteriol Rev* **36**, 478–503.
- Hoeniger, J. F. M. & Tauschel, H.-D. (1974).** Sequence of structural changes in cultures of *Clostridium tetani* grown on a solid medium. *J Med Microbiol* **7**, 425–432.
- Lereclus, D., Arantes, O., Chaufaux, J. & Lecadet, M. M. (1989).** Transformation and expression of a cloned  $\delta$ -endotoxin gene in *Bacillus thuringiensis*. *FEMS Microbiol Lett* **60**, 211–218.
- Macfarlane, S., Hopkins, M. J. & Macfarlane, G. T. (2001).** Toxin synthesis and mucin breakdown are related to swarming phenomenon in *Clostridium septicum*. *Infect Immun* **69**, 1120–1126.
- Macnab, R. M. (1996).** Flagella and motility. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, pp. 123–145. Washington, DC: American Society for Microbiology.
- Miller, J. M., Hair, J. G., Hebert, M., Hebert, L., Roberts, F. J., Jr & Weyant, R. S. (1997).** Fulminating bacteremia and pneumonia due to *Bacillus cereus*. *J Clin Microbiol* **35**, 504–507.
- Ordal, G. W. & Goldman, D. J. (1975).** Chemotaxis away from uncouplers of oxidative phosphorylation in *Bacillus subtilis*. *Science* **189**, 802–805.
- O'Rear, J., Alberti, L. & Harshey, R. M. (1992).** Mutations that impair swarming motility in *Serratia marcescens* 274 include but are not limited to those affecting chemotaxis or flagellar functions. *J Bacteriol* **174**, 6125–6137.
- Prüb, B. M., Dietrich, R., Nibler, B., Märtlbauer, E. & Scherer, S. (1999).** The hemolytic enterotoxin HBL is broadly distributed among species of the *Bacillus cereus* group. *Appl Environ Microbiol* **65**, 5436–5442.
- Rashid, M. H. & Kornberg, A. (2000).** Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **97**, 4885–4890.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schoeni, J. L. & Wong, A. C. L. (1999).** Heterogeneity observed in the components of hemolysin BL, and enterotoxin produced by *Bacillus cereus*. *Int J Food Microbiol* **53**, 159–167.
- Senesi, S., Cercignani, G., Freer, G., Batoni, G., Barnini, S. & Ota, F. (1991).** Structural and stereospecific requirements for the nucleoside-triggered germination of *Bacillus cereus* spores. *J Gen Microbiol* **137**, 399–404.
- Steen, K., Bruno-Murtha, L. A., Chaux, G., Lazar, H., Bernard, S. & Sulis, C. (1992).** *Bacillus cereus* endocarditis: report of a case and review. *Clin Infect Dis* **14**, 945–946.
- Stewart, B. J., Enos-Berlage, J. L. & McCarter, L. L. (1997).** The *lonS* gene regulates swarmer cell differentiation of *Vibrio parahaemolyticus*. *J Bacteriol* **179**, 107–114.
- Vahey, J. B. & Flynn, H. W., Jr. (1991).** Results in the management of *Bacillus* endophthalmitis. *Ophthalmic Surg* **22**, 681–686.
- Young, M. G., Smith, M. J., Minnich, S. A. & Miller, V. L. (1999).** The *Yersinia enterocolitica* motility master operon, *flhDC*, is required for flagellin production, swimming motility and swarming motility. *J Bacteriol* **181**, 2823–2833.
- Zuberi, A. R., Bischoff, O. S. & Ordal, G. W. (1991).** Nucleotide sequence and characterization of a *Bacillus subtilis* gene encoding a flagellar switch protein. *J Bacteriol* **173**, 710–719.

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