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Involvement of motility and flagella in *Bacillus cereus* biofilm formation

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Bacillus cereus is a food-borne pathogen and a frequent contaminant of food production plants. The persistence of this pathogen in various environments results from the formation of spores and of biofilms. To investigate the role of the *B. cereus* flagellar apparatus in biofilm formation, we constructed a non-flagellated mutant and a flagellated but non-motile mutant. Unexpectedly, we found that the presence of flagella decreased the adhesion of the bacterium to glass surfaces. We hypothesize that this decrease is a consequence of the flagella hindering a direct interaction between the bacterial cell wall and the surface. In contrast, in specific conditions, motility promotes biofilm formation. Our results suggest that motility could influence biofilm formation by three mechanisms. Motility is necessary for the bacteria to reach surfaces suitable for biofilm formation. In static conditions, reaching the air–liquid interface, where the biofilm forms, is a strong requirement, whereas in flow cells bacteria can have access to the bottom glass slide by sedimentation. Therefore, motility is important for biofilm formation in glass tubes and in microtitre plates, but not in flow cells. Motility also promotes recruitment of planktonic cells within the biofilm by allowing motile bacteria to invade the whole biofilm. Finally, motility is involved in the spreading of the biofilm on glass surfaces.

INTRODUCTION

In natural environments, bacteria live mostly in biofilms (Costerton *et al.*, 1995), which are multicellular communities attached to surfaces. Attachment to surfaces involves the cell surface of the bacterium, on which may be found proteins, teichoic or lipoteichoic acids, polysaccharides, and in some species a capsule and various appendages. Appendages such as flagella or pili extend far away from the bacterial cell, up to several times the cell length (Jarrell & McBride, 2008), and are likely to be the part of the bacterium part that first makes contact with the surface on which the biofilm settles. Flagella were therefore suspected to be involved in the first step of biofilm formation and have indeed been shown to act as adhesive tools in *Listeria monocytogenes* (Vatanyoopaisarn *et al.*, 2000) and in *Aeromonas caviae* (Kirov *et al.*, 2004). In addition, flagella may promote attachment to surfaces through their role in motility, as motility might overcome repulsive forces between the cell and the surface, as was reported for *L. monocytogenes* (Lemon *et al.*, 2007) and for *Escherichia coli* (Pratt & Kolter, 1998). The involvement of flagella in biofilm formation is not limited to its initiation. Flagellum-mediated motility was suggested to be required in *L. monocytogenes* for biofilm growth through recruitment of motile cells from the planktonic phase (Lemon *et al.*, 2007), and biofilm architecture was shown to be motility dependent in *E. coli* and in *Pseudomonas aeruginosa* (Barken *et al.*, 2008; Wood *et al.*, 2006). However, the effect of flagella or motility on biofilm formation was shown to be dependent on culture conditions in *Agrobacterium tumefaciens* (Merritt *et al.*, 2007) and in *P. aeruginosa* (Parsek & Tolker-Nielsen, 2008), and this effect might be negative because of cells leaving the mature biofilm (O'Toole *et al.*, 2000). Therefore, the contribution of flagella to biofilm formation is not clear-cut but depends on several factors, including culture conditions, surface material, biofilm age, and bacterial species.

Biofilms in *Bacillus subtilis* are described as structured pellicles floating on culture media (Branda *et al.*, 2001). In this system, no adhesion to solid surfaces is required and flagella as an adhesion tool are not needed in the first step of biofilm formation. However, motility was shown to be important, but not necessary, for pellicle formation in *B. subtilis*, as deletion of flagella or construction of flagellated but non-motile mutants led to a delay in pellicle formation in a rich culture medium (Kobayashi, 2007b). This delay was suggested to be the result of feedback regulatory mechanisms mediated by components of the flagellar apparatus, rather than the consequence of a defect in

Abbreviation: CLSM, confocal laser scanning spectral microscope/microscopy.

The GenBank/EMBL/DDBJ accession number for the sequence of the *fla* locus in *B. cereus* 407 is GU246269.

motility (Kobayashi, 2007b). A high number of regulators acting at the transcriptional level were found to control biofilm formation in *B. subtilis*, including DegU/DegS, Spo0A, SinI/SinR, SlrR/SlrA and AbrB (Chai *et al.*, 2008; Hamon *et al.*, 2004; Kearns *et al.*, 2005; Kobayashi, 2007a, 2008; Verhamme *et al.*, 2007, 2009). The resulting action of these regulators is a switch between motility and exopolysaccharide matrix production, and the *B. subtilis* pellicle contains subpopulations of motile and of exopolysaccharide-producing cells. These subpopulations are not randomly arranged, but are spatially distributed from top to bottom and from centre to edges of the biofilm (Vlamakis *et al.*, 2008).

Bacillus cereus is a Gram-positive, low-GC, flagellated and spore-forming bacterium, which shares many regulatory mechanisms and metabolic pathways with *B. subtilis*, to which it is closely related. *B. cereus* is an opportunistic pathogen which has a virulence regulon of 45 genes, most of which code for extracellular toxins and degradative enzymes produced upon entry into stationary phase (Gohar *et al.*, 2002, 2008). This pathogen is frequently diagnosed in food-borne diseases but is also found in cases of endophthalmitis, meningitis or periodontitis (Helgason *et al.*, 2000; Kotiranta *et al.*, 2000). It is also a persistent contaminant of equipment in the the food industry, where it is found as spores and biofilms bound on stainless steel surfaces (Guinebretiere *et al.*, 2003; Svensson *et al.*, 2000; Wirtanen *et al.*, 1996). *B. cereus* was reported to form thick biofilms at the air–liquid interface (Auger *et al.*, 2006; Wijman *et al.*, 2007) but might also form weak biofilms on immersed glass or steel surfaces in static culture conditions (Oosthuizen *et al.*, 2001; Wijman *et al.*, 2007). The aim of this work was to investigate the role of the flagella and of flagellum-dependent motility in biofilm initiation and development in *B. cereus*. We therefore constructed a non-flagellated mutant and a flagellated but non-motile mutant, and studied biofilm formation by these mutants, at the air–liquid interface in microtitre plates or in glass tubes, and on immersed surfaces in flow cells.

METHODS

Strains and DNA manipulation. *B. cereus* strain 407 is genetically close to the sequenced strain ATCC 14579 (Tourasse *et al.*, 2006). However, strain 407 forms thick biofilms while strain ATCC 14579 is a poor biofilm producer. Therefore, strain 407 was used as an experimental model for biofilm studies, while the ATCC 14579 chromosomal sequence was used to design primers for cloning, deletion or sequencing purposes.

E. coli K-12 strain TG1 [Δ(*lac–proAB*) *supE thi hsd-5* (F′ *traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ*ΔM15)] was used as a host for cloning experiments, and *E. coli* strain ET 12567 (F$^-$ *dam-13*::Tn*9 dcm-6 hsdM hsdR recF143 zjj-202*::Tn*10 galK2 galT22 ara14 pacY1 xyl-5 leuB6 thi-1*) was used to prepare DNA for *B. cereus* transformation.

B. cereus strains 407, 407 *fla* and 407 *motA* were transformed with the plasmid pHT315Ω(*papha3-gfp*) (Daou *et al.*, 2009) to express the *gfp-mut1* gene constitutively.

Plasmid and mutant strain constructions. The entire *fla* genes locus in *B. cereus* 407 was disrupted by insertion of a kanamycin-resistance (Km^R) cassette. A 970 bp *Bam*HI–*Pst*I and a 1040 bp *Xba*I–*Hin*dIII DNA fragment, corresponding to the chromosomal DNA regions upstream and downstream of the *fla* genes locus, respectively, were generated by PCR using the primer pairs Fla79F2-Fla79R2 and Fla79F4-Fla79R4 (Table 1). The Km^R cassette was purified from plasmid pDG783 (Guerout-Fleury *et al.*, 1995) as a 1.5 kb *Pst*I–*Xba*I fragment. The amplifed DNA fragments and the Km^R cassette were cloned between the *Hin*dIII and the *Bam*HI sites of plasmid pRN5101 (Lereclus *et al.*, 1992). The resulting plasmid was used to transform the 407 wild-type strain by electroporation, and the *fla* locus was deleted and replaced with the Km^R cassette via allelic exchange by homologous recombination (Lereclus *et al.*, 1992). The corresponding mutant strain was designated *fla*. The same procedure was used to disrupt the *motA* gene, except that the upstream and downstream regions of the *motA* gene were generated by PCR as 794 bp *Bam*HI–*Pst*I and 868 bp *Xba*I–*Hin*dIII DNA fragments, using the primers pairs MotA79F1-MotA79R1 and MotA79F2-MotA79R2 (Table 1). The corresponding mutant strain was designated *motA*.

A transcriptional *pfla–lacZ* fusion was constructed by cloning the promoter region of the *flaA* gene between the *Xba*I and the *Hin*dIII sites of the replicative pHT304-18′*Z* plasmid (Agaisse & Lereclus, 1994). The *flaA* promoter region was generated by PCR as a 581 bp *Xba*I–*Hin*dIII DNA fragment using the primer pair Fla79PF5-Fla79PR5 (Table 1). The recombinant plasmid, designated pHT304-*Pfla′Z*, was introduced into the *B. cereus* 407 wild-type strain by electroporation.

***β*-Galactosidase assays.** For β-galactosidase activity measurement, biofilms were obtained from glass tubes (see below), and the biofilm ring and pellicle were washed twice with PBS to eliminate planktonic cells present in the culture medium. The bacterial cells were recovered by centrifugation from planktonic cultures and from biofilms. Cells were lysed using the FastPrep 120 system (Savant); β-galactosidase specific activities were measured as described previously (Bouillaut *et al.*, 2005), and were expressed in units of β-galactosidase per mg protein (Miller units). Two independent assays were performed for each culture condition.

Determination of the nucleotide sequence of the *B. cereus* 407 *fla* locus. The *fla* locus was amplified using the primer pair Fla79R9-Fla79F10, with strain 407 chromosomal DNA as a template. The PCR product was sent to Genome Express (Paris, France) for sequencing.

Motility assay and flagellar staining. The swimming ability of the *B. cereus* 407 mutant strains was determined on LB soft (0.3 %) agar plates. Strains were grown in LB medium at 37 °C until the culture reached an OD_{600} of 1. A 5 µl drop was spotted on an agar plate for each culture and incubated at 37 °C overnight. Flagella were stained using a silver stain method (West *et al.*, 1977).

Biofilm formation in PVC microtitre plates and in glass tubes. The ability of *B. cereus* 407 wild-type and mutant strains to form biofilms in PVC (polyvinylchloride) microtitre plates was determined as described earlier (Auger *et al.*, 2006). Briefly, cultures in the exponential phase were diluted into fresh LB medium to an OD_{600} of 0.01 and inoculated in 96-well PVC microtitre plates (Falcon 35911). The microtitre plates were incubated at 30 °C for various times, after which the amount of biofilm was measured by crystal violet staining. Absorbance of the solubilized dye was determined at 595 nm. The method used to determine biofilm formation in glass tubes was similar to that used in microtitre plates, with the following differences. UV-sterilized 6 ml glass tubes were inoculated with 2 ml of the cultures diluted to an OD_{600} of 0.01. At the end of the incubation time, the 2 ml culture medium was removed using a

Table 1. Primers used in this study

Underlined letters show restriction sites.

Primer name	Primer sequence
Fla79F2	AACTGCAGTGTATCTCCCTTTTATTTTGTACC
Fla79R2	CGGGATCCCACAGCAATTAATGTATTAGGTCT
Fla79F4	CCCAAGCTTCGCCTCCGTACTTTTTCACATTGCC
Fla79R4	GCTCTAGATGTCTTGGTTTTGTCGCATGTACTC
MotA79F1	CCCAAGCTTCCTCAACATCAATGCCGG
MotA79R1	GCTCTAGAGCCGCTATCACTATAATGAAACC
MotA79F2	AACTGCAGGCGAATATGATTGCAATCCC
MotA79R2	CGGGATCCCCGCAGCTAGCCTTTTATCG
Fla79PF5	CCCAAGCTTGCCTTCGTCTCCTTCGCAGGTTCAC
Fla79PR5	GCTCTAGATGTCTTGGTTTTGTCGCATGTACTC
Fla79R9	GCGTAGTAGCCTTCGTCTCCTTCGC
Fla79F10	GCTTTCTAGCATGGCGGCAGGGCG
Fla79R10	CGCAACCATCGGGAATGCC
Fla79F11	CGCCATGTCAGCGTCTTCG

Pasteur pipette and the OD_{600} of the biofilm, thoroughly vortexed in 2 ml PBS, was measured.

Biofilm formation in flow cells. Biofilms were cultivated at 30 °C in disposable three-channel flow cells (Stovall Life Science; channel volume 160 µl) with a sealed bottom glass coverslip. The flow cells were kept under sterile LB medium flow (2 ml h^{-1}) for 1 h until inoculation. At the time of inoculation, the flow was stopped and 1 ml of the *gfp*-expressing strain to be assayed, grown to exponential phase in LB medium and diluted to an OD_{600} of 0.01, was injected into each channel using a sterile syringe. Static conditions (no flow) were maintained for 1 h after inoculation to allow initial bacterial adhesion. The flow was then resumed at 2 ml h^{-1} using a Watson–Marlow 205S peristaltic pump. At various time points, *Z*-stacks of horizontal-plane images of biofilms were acquired using a Leica SP2 AOBS confocal laser scanning spectral microscope (CLSM) (Leica Microsystems, France) at the MIMA2 microscopy platform (http://voxel.jouy.inra.fr/mima2). The excitation wavelength used for the GFP was 488 nm, and the emitted fluorescence was recorded in the range 500–550 nm. Images were collected through a 63× Leica oil-immersion objective (NA=1.4) with a *z* step of 1 µm. Biofilm quantization (biovolume) was realized with the PHLIP Matlab routine created by J. Xavier (http://sourceforge.net/project/phlip).

Adhesion assay. Flow cells were inoculated as described above, with 1 ml of a *gfp*-expressing strain in exponential phase, grown in LB medium and diluted to an OD_{600} of 0.01. Bacteria were allowed to attach for 1 h, after which the flow was set at 2 ml min^{-1} for 20 min to eliminate unattached cells. Digital images of six fields per channel of the attached bacteria were captured using a 63× objective on the CLSM and the number of bacteria per field was counted.

Recruitment of planktonic cells into a pre-formed biofilm. The *gfp*-untagged *B. cereus* wild-type strain 407 was grown at 30 °C in LB medium in single-channel flow cells (BST FC 81, channel volume 1 ml; Biosurface Technologies Corporation) with a flow of 15 ml h^{-1} until a 24-h-old biofilm had formed. The flow was then stopped and 3 ml of *gfp*-expressing strain 407, grown in LB medium until the culture reached an OD_{600} of 1, was injected into the flow cell. After 1 h of incubation, the flow was set to 15 ml h^{-1} and *Z*-stacks of horizontal-plane images of the biofilms were acquired by CLSM. CLSM recording was done 1 h and 2 h after injection of the green fluorescent bacteria. Fluorescent bacteria detected inside the biofilm were quantified by 3D confocal imaging and their biovolume was assessed using the PHLIP Matlab routine. To show distribution of bacteria along the biofilm depth, 10 Z-sections, regularly spaced along the 3D-biofilm *y*-axis, were superimposed using Adobe Photoshop.

Biofilm spreading on glass surfaces. The *B. cereus* 407 wild-type strain or the *fla* mutant, grown in LB medium until the culture reached an OD_{600} of 1, was loaded as a 30 µl droplet at the outlet side of an empty channel of a Stovall flow cell. The droplet filled the channel until the point shown by the white arrowheads in Fig. 7. After 1 h of incubation, the flow was set to 4 ml h^{-1}, oriented from the inlet to the outlet side of the flow cell. Biofilm development along the flow axis of the flow-cell channels was assessed visually after 24 h.

RESULTS

Genetic organization of the flagellin genes locus in *B. cereus*

In contrast with *B. subtilis*, where only one flagellin gene, *hag*, is found, strains of *B. cereus* have two to five flagellin genes – the *fla* genes – all located in the same chromosomal locus in reverse direction, flanked by genes in direct orientation. In the *B. cereus* type strain, ATCC 14579, four *fla* genes are present in this locus (Fig. 1a). The three first genes, likely to be in an operon, are followed by an intrinsic terminator sequence, downstream of which is found the last *fla* gene. We have sequenced the *fla* locus in strain 407; we found two *fla* genes in this locus, separated by a terminator (Fig. 1b). The GenBank accession number for the sequence is GU246269.

Expression of flagellin genes in strain 407

Previous studies on the extracellular proteome of *B. cereus* have shown that only the first genes of the *fla* locus

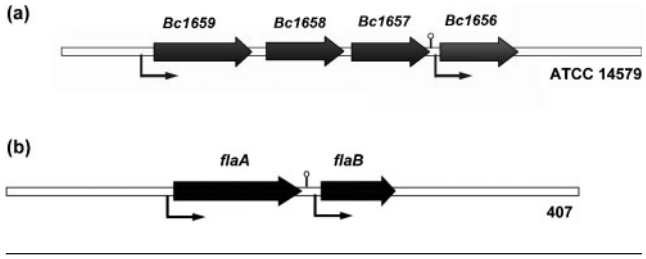

Fig. 1. Genetic organization of the flagellin genes locus in *B. cereus* strains ATCC 14579 (a) and 407 (b). The gene annotation for strain ATCC 14579 refers to the gene locus as indicated in the ATCC 14579 sequenced chromosome at NCBI (accession number NC_004722).

in strain ATCC 14579 and strain 407 are expressed in early stationary phase (Gohar *et al.*, 2005). We followed the expression of the first *fla* gene (*flaA*) in strain 407 by using a transcriptional fusion between its promoter region and *lacZ*. In planktonic cultures, *flaA* expression increased throughout the exponential phase and peaked during the transition between the exponential and the stationary phase, after which it decreased sharply (Fig. 2a). In biofilms formed at the air–liquid interface in glass tubes, *flaA* expression started at a high level in 24-h-old biofilms but decreased continuously with biofilm ageing. This decrease is likely to reflect either a decrease in the number of motile bacteria in the biofilm, or a decrease in flagellin synthesis at a decreased flow rate. However, *flaA* expression still occurred in 60-h-old biofilms (Fig. 2b).

Biofilm formation by the *fla* and the *motA* strains

The flagellar motor, located in the flagellum basal body, includes two parts, the rotor and the stator. The flagellar stator is made of two proteins, MotA and MotB (Chevance & Hughes, 2008), the genes of which form a bicistronic operon. Deletion of the *fla* locus in strain 407 led to non-flagellated and non-motile bacteria, while deletion of *motA* led to flagellated but non-motile bacteria (Fig. 3a). This result is different from what was obtained in *B. subtilis*, where deletion of *motA* resulted in a loss of most of the flagella (Ito *et al.*, 2005). The *fla* and the *motA* mutants were defective for biofilm formation in microtitre plates (Fig. 3b) and in glass tubes (Fig. 3c). However, in both assays the *motA* mutant produced more biofilm than the *fla* mutant. This result indicates that both motility and the flagella per se are involved in biofilm formation under these conditions. We then compared biofilm formation for the wild-type and for the *fla* or *motA* mutant strains in flow cells. In contrast to the marked biofilm deficiency observed in static assays, the *fla* or *motA* mutant strains formed thick and dense biofilms 24 h after inoculation, quantitatively similar to the biofilm formed by the wild-type strain (Fig. 3d). Therefore, neither flagella nor motility are required for biofilm formation under flow conditions. To further investigate the *fla* mutant biofilm phenotype in flow cells, we conducted a short-term experiment, making observations every 4 h for the first 16 h. Much to our surprise, this experiment revealed, in the first hours of biofilm growth, an increase in the rate of biofilm formation for the *fla* strain compared to the wild-type strain (Fig. 4), but no alterations in biofilm architecture (data not shown).

Role of flagella in adhesion to glass slides

In the previous experiments, the flagella per se appeared to have a positive role in biofilm formation. To understand how the flagella are involved in biofilm formation, we assessed their role in adhesion of *B. cereus* to glass.

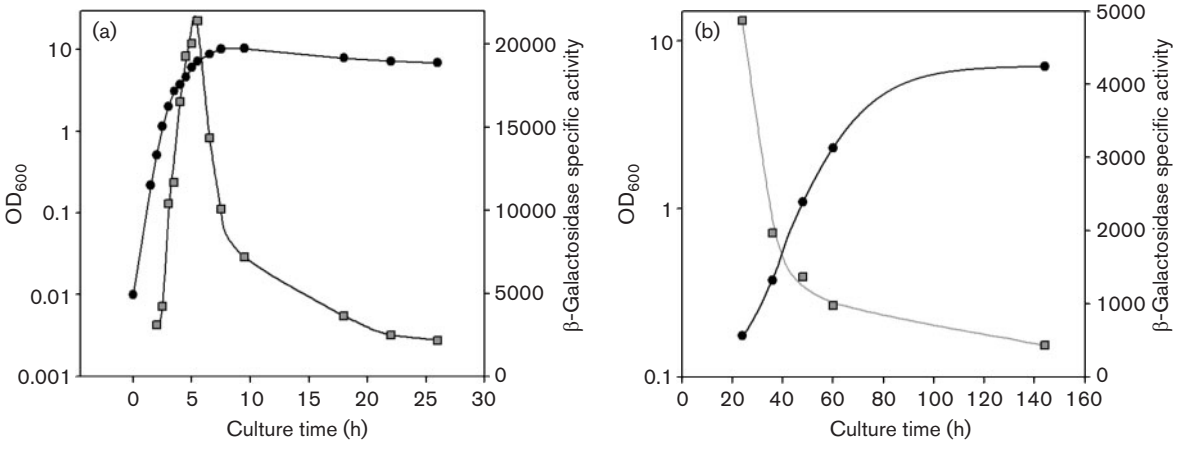

Fig. 2. Expression of the flagellin gene *flaA* in planktonic cultures (a) and in biofilms produced in glass tubes (b). *flaA* expression was measured by using a *lacZ* transcriptional fusion. Black circles, bacterial growth (OD_{600}, left axis); grey squares, transcription of the *flaA* gene as indicated by the β-galactosidase specific activity (right axis).

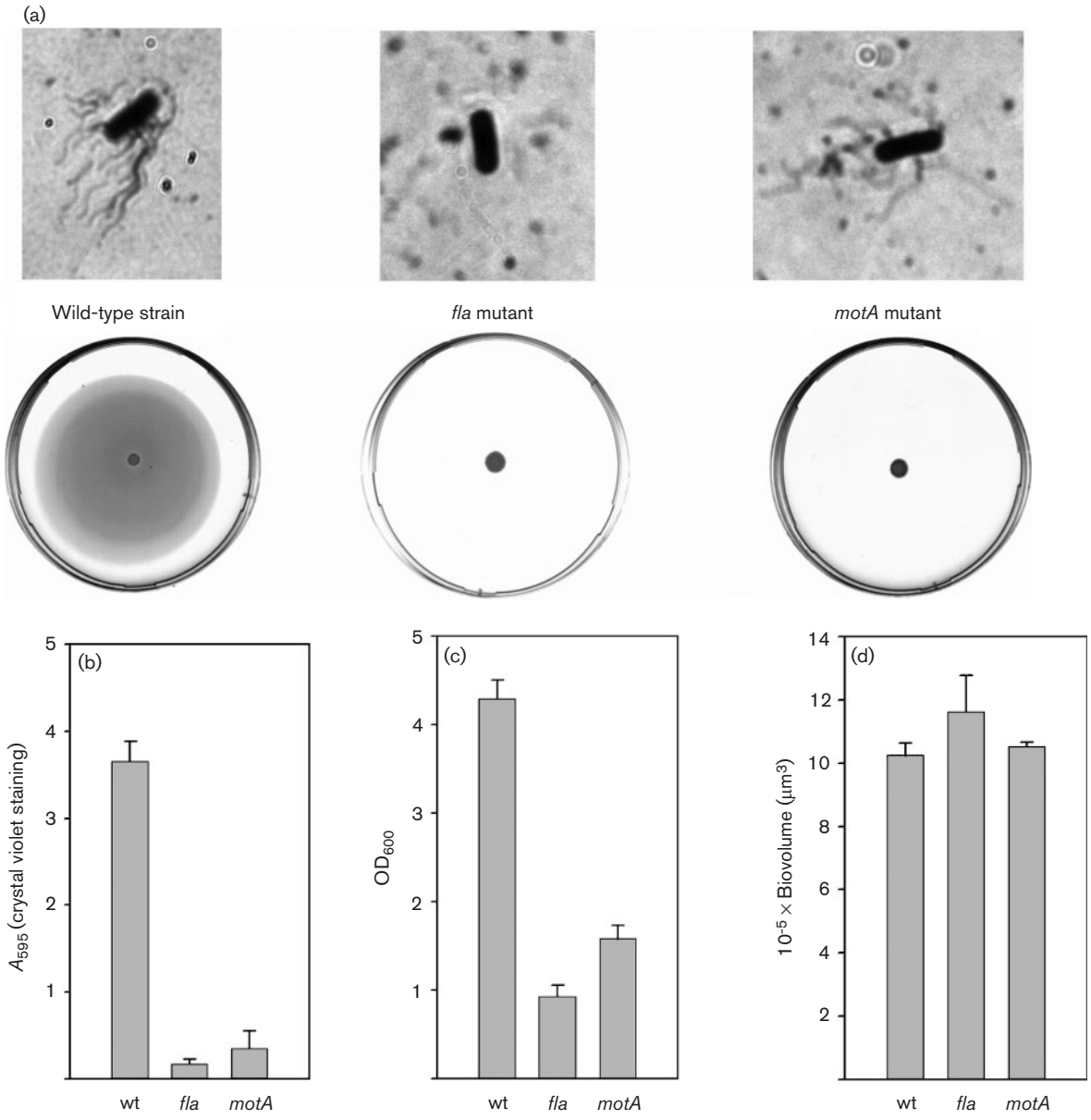

Fig. 3. Flagellation, motility and biofilm formation phenotypes in the *fla* and *motA* strains. (a) Top row, phase-contrast microscopy pictures of silver-stained flagella for the wild-type and for the mutant strains. Bottom row, soft agar plates were inoculated with the wild-type or with the mutant strains. The dark spot in the plate centre is due to bacterial colony development at the point of inoculation while the diffuse halo surrounding the colony is due to bacterial swimming. (b, c, d) Biofilm formation by the wild-type (wt), and by the *fla* and the *motA* strains: (b) PVC microtitre plates; (c) glass tubes; (d) flow cells. The data shown are the mean ± SEM of five to nine independent experiments.

Adhesion to glass was determined as the number of bacteria bound to glass after 1 h incubation, using flow cells and CLSM. Deletion of flagellin genes increased the number of bacteria adherent to the bottom glass slide of the flow cells, while this number remained unchanged in the *motA* strain relative to the wild-type strain (Fig. 5). We therefore concluded that motility is not involved in adhesion under these conditions, and that flagella play a negative role in adhesion to glass. This result is consistent with the negative role of the flagella in early biofilm formation in flow conditions.

Recruitment of planktonic cells by the biofilm

As shown in this work, motility is important for biofilm formation under static culture conditions. However, under such conditions, but not under flow conditions where planktonic bacteria are washed away by the flow, the sessile

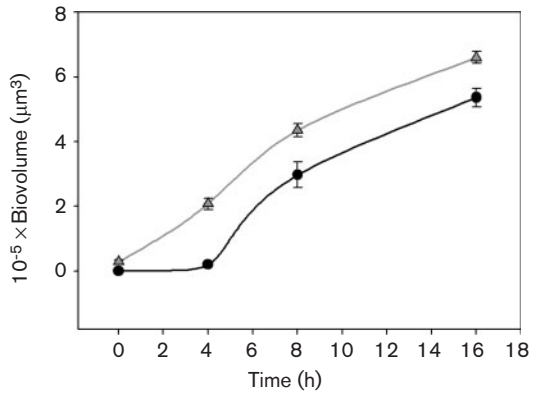

Fig. 4. Early biofilm growth in flow cells. Biofilm production by the GFP-tagged wild-type strain (black circles) and *fla* strain (grey triangles) was monitored in flow cells by CLSM. The data shown are the mean ± SEM of three experiments.

bacterial population co-exists with planktonic bacterial cells. The biofilm might therefore grow not only by division of the sessile population, but also by recruitment of the planktonic population. To determine if motility was required for biofilm growth by recruitment of planktonic bacteria, we followed the integration into a pre-formed wild-type strain biofilm of GFP-tagged wild-type and *fla* or *motA* bacteria added to the culture medium. Recruitment of wild-type bacteria in a 24-h-old pre-formed biofilm occurred rapidly, as shown by the almost unchanged volume of bacteria found inside the biofilm (from 602 to 772 μm^3) when the incubation time increased from 1 h to 2 h (Fig. 6a). These incoming bacteria did not stay at the surface of the biofilm but penetrated deeply inside it (Fig. 6c). In contrast to the wild-type strain, the *fla* and *motA* strains were not able to penetrate inside a 24-h-old biofilm, even after 2 h of incubation (Fig. 6b), and only a few were found at the surface of the biofilm (Fig. 6c). Therefore, planktonic cells of *B. cereus* are strongly and rapidly recruited by their sister cells in biofilm. Motility, by allowing planktonic cells to invade all parts of the biofilm, dramatically increases the importance of recruitment.

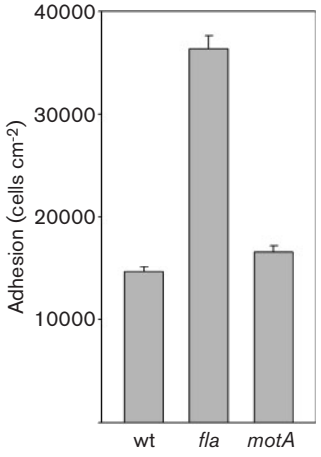

Fig. 5. Adhesion to glass of the wild-type, the *fla* and the *motA* strains. Adhesion was measured in flow cells using CLSM and GFP-tagged strains, and was determined as the number of bacteria remaining attached to glass after a 1 h incubation followed by a 20 min washing step. The data shown are the mean ± SEM of four or five independent experiments.

Role of motility in biofilm spreading

We have shown, using transcriptional fusions, that the flagellin genes are expressed in young biofilms, a situation similar to what has been described in *B. subtilis* (Stanley *et al.*, 2003; Vlamakis *et al.*, 2008). At least a proportion of the flagellated *B. cereus* cells present inside the biofilm are likely to be motile. Motile bacteria located at the biofilm edges could promote biofilm growth by extending the biofilm limits, therefore increasing the colonized surface coverage. To test this hypothesis, we inoculated the wild-type strain or the *fla* strain on one side of a flow cell. After 1 h of incubation, the flow was switched on and was directed from the side opposite the inoculation point towards the side of inoculation. After 24 h, a dense biofilm had grown on the inoculation side for both strains. The wild-type strain biofilm grew against the flow along the flow-cell length, and the newly formed biofilm showed two parts: a dense, short biofilm, and a more diffuse biofilm that extended up to the side of the flow cell opposite the inoculation point (Fig. 7a). The newly formed *fla* strain biofilm also showed the dense and short part but lacked the diffuse part (Fig. 7b). Therefore, the new dense biofilm is likely to be a consequence of biomass production pushing the bacteria ahead. In contrast, the more diffuse biofilm absent in the non-motile strain is likely to be built by motile bacteria.

DISCUSSION

In several bacterial species, the flagellum has been shown to be required for adhesion to solid surfaces (Kirov *et al.*, 2004; Vatanyoopaisarn *et al.*, 2000). Unexpectedly, we found here that deletion of the flagella in *B. cereus* increased the number of bacteria bound to glass surfaces in flow cells. This result could be due either to a higher clearance of flagellated bacteria under flow conditions or to an increased adhesion to glass surfaces upon deletion of the flagella. Adhesion of *B. cereus* on glass might involve interactions between the bacterial cell wall and the surface, hindered by the presence of inert flagella. Removal of flagella would then favour specific interactions involving other cell-surface structures. For example, *B. cereus* displays pili on its surface (Budzik *et al.*, 2007) which could interact with glass and other materials, and the presence of an S-layer in some strains

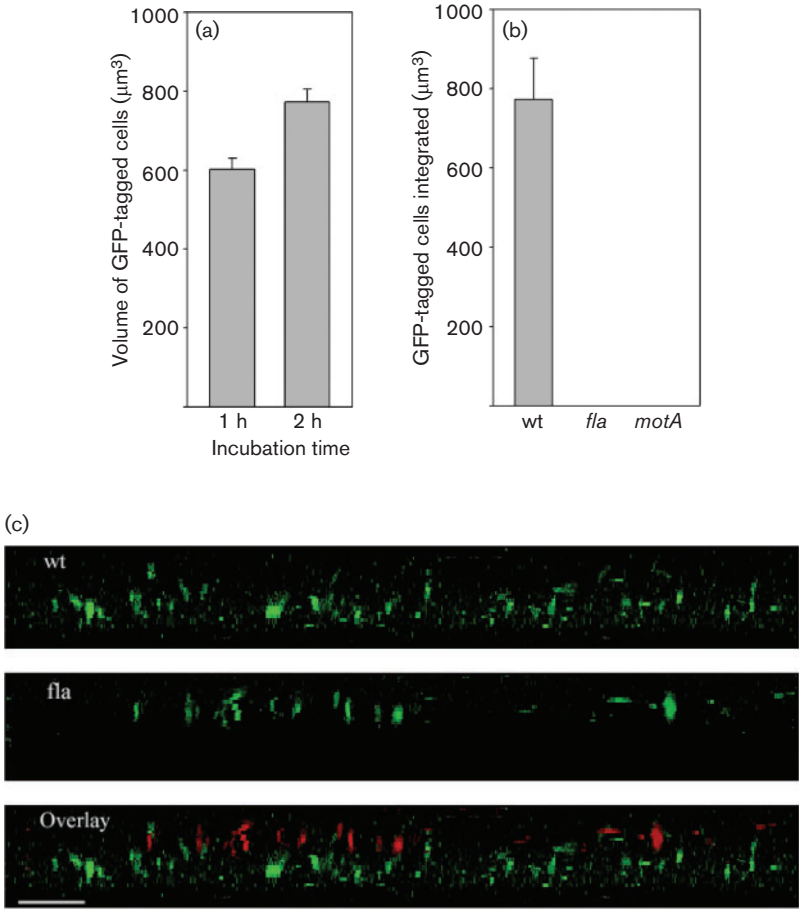

Fig. 6. Recruitment of planktonic bacteria by a 24-h-old resident biofilm. (a) The GFP-tagged *B. cereus* 407 wild-type strain in exponential phase was incubated for 1 h or 2 h with a 24-h-old untagged wild-type strain biofilm. (b) The GFP-tagged *B. cereus* 407 wild-type or *fla* mutant strains were incubated for 2 h with a 24-h-old untagged wild-type strain biofilm. The volume of GFP-tagged bacteria that had integrated into the resident biofilm was measured by CLSM. Cells located at the surface of the biofilm were not considered as integrated. The data shown are the mean ± SEM of at least three independent experiments. (c) CLSM pictures of a 24-h-old wild-type biofilm incubated for 1 h with the GFP-tagged wild-type strain (wt) or the GFP-tagged *fla* mutant strain (fla). Overlay: overlay of the two pictures, where the wild-type strain is depicted in green and the *fla* strain is depicted in red. Scale bar, 20 μm.

is negatively correlated with biofilm formation in microtitre plates (Auger *et al.*, 2009). However, while the flagella are not involved in adhesion on glass, they may play a role in adhesion on other materials and on living tissues. In *B. cereus* and in its close relative *Bacillus thuringiensis*, the flagella were reported to be involved in adhesion to epithelial cells (Bouillaut *et al.*, 2005; Ramarao & Lereclus, 2006; Zhang *et al.*, 1995).

Expression of flagellin genes peaks at the end of the exponential phase in planktonic cultures of *B. cereus*. At the end of the exponential phase *B. subtilis* enters a physiological state where it becomes able to start biofilm formation, as the sporulation master regulator Spo0A, activated by kinases at the end of the exponential phase, is required for biofilm formation (Hamon & Lazazzera, 2001). However, further increase of Spo0A phosphorylation will

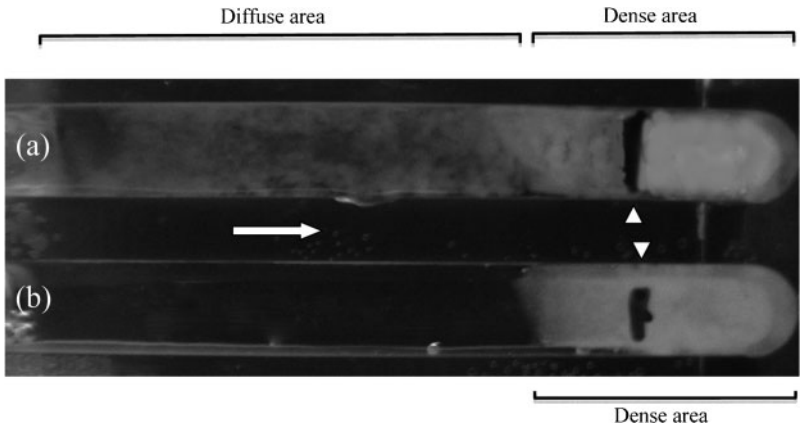

Fig. 7. Biofilm spreading in flow cells for the wild-type (a) and the *fla* mutant strain (b). The white arrowheads and black pencil lines indicate the biofilm front growth before starting the flow. The horizontal white arrow shows the flow direction. The brackets at the top and at the bottom of the figure show the dense area and the diffuse area of the biofilm.

later drive the bacterium irreversibly towards sporulation (Fujita & Losick, 2005). Therefore, the small time window when *B. subtilis* is competent for biofilm formation coincides with the time when, in *B. cereus*, flagellin expression and therefore most probably also motility are at a maximum. Once the cells are in a biofilm, flagellin expression decreases continuously but is not abolished, suggesting that at least a proportion of the bacteria in the biofilm remain motile. In *B. subtilis* too, flagellated bacteria exist in the biofilm, together with exopolysaccharide-producing bacteria (Vlamakis *et al.*, 2008). Motility is important for biofilm formation at the air–liquid interface in microtitre plates or in glass tubes, but not on immersed surfaces in flow cells. Thus it is unlikely that deletion of flagella or inactivation of the flagellar motor acts on biofilm formation by a feedback regulatory mechanism, as was suggested for *B. subtilis* (Kobayashi, 2007b) and *Vibrio cholerae* (Watnick *et al.*, 2001). Non-motile bacteria behave as particles in suspension with a density greater than that of the culture medium. Under static conditions, non-motile bacteria will therefore sediment to the bottom of the glass tube or of the microtitre plate and will have reduced access to the air–liquid interface, the only aerated place where the biofilm can form. However, the presence in the *motA* mutant of a high number of non-motile flagella surrounding the bacterium could result in a decreased sedimentation rate as compared to the *fla* mutant. This decreased sedimentation rate could explain why the *motA* mutant produces more biofilm than the *fla* mutant in static conditions. In flow cells, through which oxygenated medium is circulating and where the biofilm can form on the bottom glass slide, sedimentation of non-motile bacteria does not hinder, but on the contrary promotes, biofilm formation. Motility is therefore important for biofilm formation as a means of having access to places suitable for biofilm settlement, in the first steps of biofilm formation.

Motility is also important for planktonic bacteria to integrate into already existing biofilms. As shown in our study, this phenomenon is rapid, and motile planktonic bacteria invade all parts of a 24-h-old biofilm in less than 1 h. The contribution of the planktonic population to early biofilm growth might therefore be important under static conditions. As flagellins are expressed in the *B. cereus* biofilm, it is likely that in this species motile bacteria are present in the biofilm. These motile bacteria could leave the biofilm and return to a planktonic state, decreasing the biofilm volume (Jackson *et al.*, 2002; O'Toole *et al.*, 2000). As a result, the net flux between incoming and leaving bacteria could be negative unless the biofilm produces chemoattractants or traps motile bacteria within it. Recruitment and swarming are in general not included in the theoretical models of biofilm development (Nadell *et al.*, 2009; Xavier & Foster, 2007). The rapid and strong recruitment of planktonic bacteria by the *B. cereus* biofilm also raises the question of the importance of this phenomenon in the building of multispecies biofilms.

Another role of motility in the biofilm is its strong contribution to the spreading of the biofilm and to the colonization of new surfaces, as shown in this study. Spreading of the biofilm could result from two possibilities: either motile bacteria issuing from the biofilm first return to a planktonic state and then colonize new surfaces; or motile bacteria within the biofilm remain in it but bring it forward step by step on the uncolonized surface, thus extending the biofilm, by a process similar to swarming. The spreading of the biofilm against the flow that we observed in flow cells makes the second hypothesis more probable, as free planktonic bacteria are likely to be drained out by the flow before reaching the upstream surface. Spreading of the biofilm by motile bacteria located at the biofilm edges is also supported by the fact that, in *B. subtilis*, flagellated bacteria are in highest density at the biofilm edges (Vlamakis *et al.*, 2008).

In conclusion, we have shown in this study that, while the flagella of *B. cereus* are not directly involved in adhesion to glass surfaces, they play a major role in biofilm formation through their function in motility. Motility gives access to surfaces suitable for biofilm formation, is necessary for the recruitment of planktonic cells within the biofilm, and is required for the spreading of biofilm on non-colonized surfaces.

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