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Characterizing the flagellar filament and the role of motility in bacterial prey-penetration by *Bdellovibrio bacteriovorus*

Carey Lambert,[1] Katy J. Evans,[1] Rob Till,[1] Laura Hobley,[1] Michael Capeness,[1] Snjezana Rendulic,[2] Stephan C. Schuster,[2] Shin-Ichi Aizawa[3] and R. Elizabeth Sockett[1]*

[1]*Institute of Genetics, School of Biology, University of Nottingham, Queens Medical Centre, Nottingham NG7 2UH, UK.*

[2]*Department of Biochemistry and Molecular Biology, Center for Comparative Genomics and Bioinformatics, 310 Wartik Building, Penn State University, University Park, PA 16802, USA.*

[3]*CREST 'Soft Nano-Machine Project', Innovation Plaza Hiroshima, 3-10-23 Kagamiyama, Higashi-Hiroshima 739-0046 Japan.*

Summary

The predatory bacterium *Bdellovibrio bacteriovorus* swims rapidly by rotation of a single, polar flagellum comprised of a helical filament of flagellin monomers, contained within a membrane sheath and powered by a basal motor complex. *Bdellovibrio* collides with, enters and replicates within bacterial prey, a process previously suggested to firstly require flagellar motility and then flagellar shedding upon prey entry. Here we show that flagella are not always shed upon prey entry and we study the six *fliC* flagellin genes of *B. bacteriovorus*, finding them all conserved and expressed in genome strain HD100 and the widely studied lab strain 109J. Individual inactivation of five of the *fliC* genes gave mutant *Bdellovibrio* that still made flagella, and which were motile and predatory. Inactivation of the sixth *fliC* gene abolished normal flagellar synthesis and motility, but a disordered flagellar sheath was still seen. We find that this non-motile mutant was still able to predate when directly applied to lawns of YFP-labelled prey bacteria, showing that flagellar motility is not essential for prey entry but important for efficient encounters with prey in liquid environments.

Accepted 16 January, 2006. *For correspondence. E-mail liz.sockett@nottingham.ac.uk; Tel. (+44) 115 9194496; Fax (+44) 115 9709906.

Introduction

Bdellovibrio are small, predatory, delta-Proteobacteria. They collide with, attach to and invade Gram-negative bacterial prey by lysing a small temporary pore in the prey outer-layers (Tudor *et al*., 1990) through which they enter and establish growth in the prey periplasm in a structure known as the bdelloplast. *Bdellovibrio* attach to the cytoplasmic membrane of prey and hydrolyse and take up biomolecules for *Bdellovibrio* cell growth. *Bdellovibrio* grow in a filamentous form within the prey periplasm, and when nutrients are exhausted they septate into individual *Bdellovibrio* cells. These synthesise flagella and swim within the bdelloplast, ultimately lysing its outer membrane to release fresh, motile progeny *Bdellovibrio* for further invasion.

Bdellovibrio are highly motile, swimming at speeds from 35 $\mu m\ s^{-1}$ for strain 109J up to 160 $\mu m\ s^{-1}$ (this study) for genome-sequenced strain HD100 (Rendulic *et al*., 2004). Motility is achieved by rotation of a single polar, sheathed flagellum (Shilo, 1969). From the earliest observations and studies (Stolp and Starr, 1963; Varon and Shilo, 1968) it had been suggested that motility may well be essential to generate the forces required for attachment and/or penetration of the prey cell with Burnham and co-workers proposing that active, motile 'drilling' of the *Bdellovibrio* was responsible for prey entry (Burnham *et al*., 1968). Evidence from these studies to support this idea included the fact that inhibition of motility eliminated attachment in the liquid assay conditions used and that non-parasitic host-independent (HI) strains isolated were also non-motile. Based on electron microscopic observations of *Bdellovibrio*-infected bacterial populations, Shilo (1969) and Thomashow and Rittenberg (1979) stated that the *Bdellovibrio* flagellum is shed during attachment or immediately prior to entry to the prey cell and re-synthesized when growth and septation within prey are complete.

Thomashow and Rittenberg (1985a) studied the waveform of *Bdellovibrio bacteriovorus* 109J flagella in uranyl acetate-stained preparations of the bacteria using electron microscopy. They showed that the flagellar filament, stained in this way, had a characteristic dampened waveform with a single, non-random transition along the filament length between different helical waveforms, with a

miniature waveform of a smaller amplitude at the distal end of the filament. The complex waveform of the flagellar filament suggested that it was comprised of multiple proteins and further SDS-PAGE analysis of the filaments by Thomashow and Rittenberg (1985b) showed the presence of two major flagellin protein bands associated with the filament itself, one of approximately 29.5 K and one of approximately 28 K. Their data indicated that the cell proximal filament was comprised exclusively of the 28 K flagellin and that a 29.5 K flagellin was associated with the more distal end of the filament. Flagella, with the sheath removed, kept the same waveform, indicating that the sheath was incidental to the shape produced by the core filament.

In this study we have established that there are actually six flagellin genes expressed by *B. bacteriovorus* 109J, all giving predicted proteins ranging from 29.0 to 29.8 K. We have found that gene inactivation of all but *fliC*3 does produce motile *Bdellovibrio*, albeit with shortened flagella in the case of *fliC5*. The non-motile *fliC3* mutant has allowed us to establish that *Bdellovibrio* may enter prey, without using flagellar motility, when directly applied to them on solid media.

Results

Flagella are not always shed upon prey entry

To visualize the fate of *Bdellovibrio* flagellar filaments upon prey-penetration, the *Escherichia coli fliG* null strain DFB225 (Lloyd *et al*., 1996; Table 1), was used as prey for *Bdellovibrio* infection. This prey strain does not synthesize its own flagellar filaments because of the pleiotropic effect of the *fliG* null mutation on flagellar export and assembly, thus any flagella visualized in a *Bdellovibrio* infection are derived from the predator. Electron microscopy of wild-type *B. bacteriovorus* 109J preying upon DFB225 revealed that many minutes after attachment and invasion of these *E. coli* prey, rounded bdelloplasts could be seen from which a section of *Bdellovibrio* flagellum protruded (Fig. 1, and gallery of images in supporting online material Fig. S1). Unshed flagella in individual early-stage bdelloplasts were seen at a frequency of approximately 80% from > 100 individual bdelloplasts examined, although some bdelloplasts in a sample could not be assayed because of aggregation, so this frequency may be an overestimate for the whole population. The observation of unshed flagella should not be confused with redevelopment of a *Bdellovibrio* flagellum that occurs towards the end of the predation process. In our experiments unshed flagella were seen early during the establishment of an infection. It shows that at least a portion of the flagellum of a *Bdellovibrio* may protrude through the outer membrane of a prey cell without compromising the early stages of the infection process or apparently the integrity of the bdelloplast. The membranous sheathing of the *Bdellovibrio* flagellum may facilitate this protrusion. Thus flagellar shedding is not integral to the early invasion of prey by *Bdellovibrio* as stated previously in the literature (Thomashow and Rittenberg, 1979).

Six highly conserved flagellins are encoded by the Bdellovibrio bacteriovorus *HD100 and 109J genomes and are expressed in attack-phase growth*

Six putative flagellin genes (*fliC* 1–6) were identified (Fig. 2A) and annotated in the genome of *B. bacteriovorus* HD100 (Table 2), and their expression was examined in predatory cultures by reverse transcription polymerase chain reaction (RT-PCR). This showed for both strains 109J (Fig. 2B) and HD100 (data not shown) that all six of the *fliC* genes were expressed in the host-dependent

Table 1. Strains used in this study.

Strain	Description	Reference
E. coli S17-1	*thi*, *pro*, *hsdR*$^-$, *hsdM*$^+$, *rec*A; integrated plasmid RP4-Tc::Mu-Kn::Tn*7*	Simon *et al*. (1983)
E. coli DH5α	F′ *endA1 hsdR17* ($r_k^-m_k^-$) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacIZYA-argF*) U169 *deoR* (ϕ80d*lac*Δ(*lacZ*)M15)	Hanahan (1983)
E. coli DFB225	*E. coli fliG* null derivative of wild-type RP437 with no flagella	Lloyd *et al*. (1996)
E. coli S17-1:pZMR100	Plasmid vector used to confer Km^r on S17-1 and DFB225 that are being used as prey for Km^r *Bdellovibrio* strains	Rogers *et al*. (1986)
E. coli S17-1:pSB3000	*E. coli* constitutively expressing YFP	T. Perehinec and P.J. Hill (unpublished)
B. bacteriovorus 109J	Wild-type	Rittenberg (1972)
B. bacteriovorus 109JK	Km^r derivative of 109J	Lambert *et al*. (2003)
B. bacteriovorus HIK3	HI derivative of 109JK	This study
B. bacteriovorus fliC1	109J *fliC1::aphII*	This study
B. bacteriovorus fliC2	109J *fliC2::aphII*	This study
B. bacteriovorus fliC3	Host-independent derivative of 109J 109J *fliC3::aphII*	This study
B. bacteriovorus fliC4	109J *fliC4::aphII*	This study
B. bacteriovorus fliC5	109J *fliC5::aphII*	This study
B. bacteriovorus fliC6	109J *fliC6::aphII*	This study

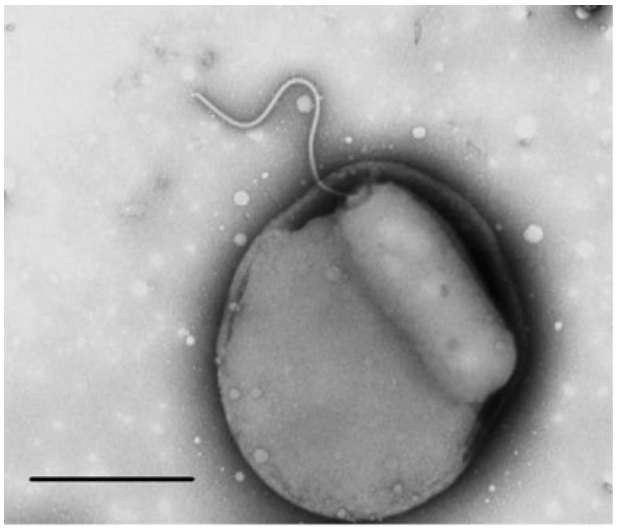


Fig. 1. Electron micrograph of *B. bacteriovorus* wild-type strain 109J inside a bdelloplast of a small *E. coli* DFB225 flagellar minus cell upon which it is preying, 20 min after predators were added to prey. The *Bdellovibrio* has modified the cell wall of the prey, which has rounded up, and it is attached to the cytoplasmic membrane of the prey, consuming its cytoplasm. The flagellum of the *Bdellovibrio* is still clearly visible protruding from the bdelloplast, 1% PTA stain. Bar = 1 μm.

(attack phase) growth mode. In the HD100 genome the *fliC1* and *2* genes were tandemly arranged as a pair on the same DNA strand with a putative transposase gene oppositely transcribed between them and with no flagellar genes flanking them. The *fliC3* and *4* genes were again tandemly arranged (but as seen later, not coexpressed) in a pair, but with a *fliD* gene, two genes downstream of *fliC4*. Both the *fliC5* and *6* genes were found singly on the genome with *fliC5* lying downstream of a *clpB* gene and *fliC6* being convergently transcribed with a putative methyl-accepting chemotaxis protein.

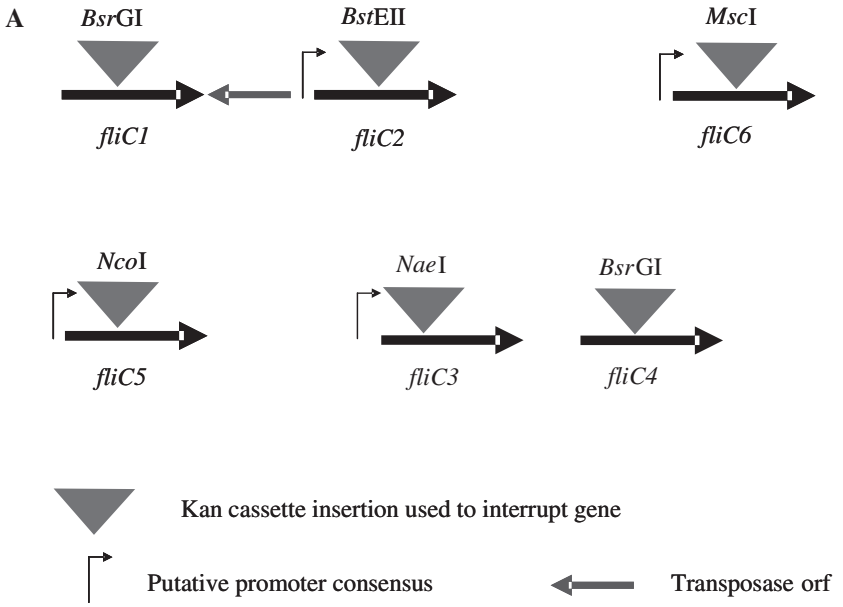
Primers made against the HD100 *fliCs* were used to clone and sequence the cognate genes from the better physiologically characterized strain *B. bacteriovorus* 109J in which flagellar filaments and motility had previously been studied by us and others (Thomashow and Rittenberg, 1985a,b; Lambert *et al*., 2003). Sequencing of these flagellins showed that they were very highly conserved between HD100 and 109J (Fig. S2). They were deposited in the EMBL database with Accession Numbers AJ748315–19 and AJ851165. For simplicity in this paper the flagellins are described simply as *fliC1–6* (Fig. S2 and Table 2 for details of corresponding gene accession numbers in both strains). Putative sigma28 sequence recognition sites, common in filament genes of other bacteria, were seen upstream of *fliC2*, *fliC3*, *fliC5* and *fliC6* (Table 2). Both *fliC1* and *fliC4* do not seem to have any recognizable sigma28 promoters and their expression may therefore be under the control of other factors. Quantitative PCR studies of *fliC* expression (for a sample of *fliCs 2*, *3*, *5*) during the predatory cycle (Fig. 2C) showed that, as predicted, *fliC* expression is high in attack-phase cells (which are highly motile) and then is greatly reduced or abolished during the 15 min and 1 h phases of infection of prey, when the *Bdellovibrio* are inside bdelloplasts. At the 4 h stage when bdelloplasts are lysed, and fresh attack-phase *Bdellovibrio* are emerging, *fliC* expression returns to a high level.

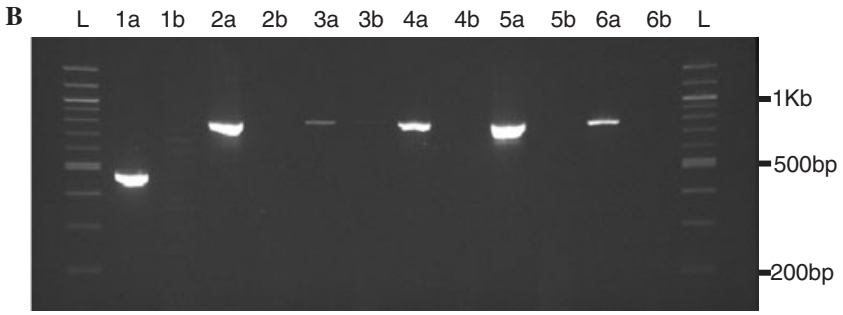
Inactivation of fliC1*,* fliC2*,* fliC4 *and* fliC6 *does not grossly affect flagellar filament morphology but inactivation of* fliC5 *causes some truncation of filaments*

Inactivation of each *fliC* gene in *B. bacteriovorus* 109J was confirmed by both Southern blotting and sequencing of DNA prepared from attack-phase cultures of putative mutants (data not shown). The inactivation of *fliC3* could not be achieved in such attack-phase cultures, presumably because motility was required for efficient predation in liquid conditions, thus selecting against inactivated mutants. Hence further HI culturing (see *Experimental procedures* and below) was used to derive and verify an HI strain with an inactivated *fliC3.* The remaining five mutants, *fliCs 1*, *2*, *4*, *5* and *6*, were characterized. Each of the five motile mutant strains was cultured in attack phase on the *fliG* null *E. coli*, DFB225, and their flagellation was examined by electron microscopy in comparison with kanamycin-resistant wild-type control *B. bacteriovorus*

Table 2. Accession numbers of *B. bacteriovorus fliC* genes plus putative sigma 28 promoter consensus regions for the flagellin genes compared with those of the *E. coli* and *Rhodobacter sphaeroides fliC* genes (Arnosti and Chamberlin, 1989; Shah *et al*., 2000).

Strain HD100 GenBank Accession No.	Strain 109J EMBL Accession No.	*Bdellovibrio* gene/ organism consensus	Putative σ^{28} consensus sequence in 109J *fliC* promoter (if any) with two known sequences for comparison
CAE78570.1	AJ851165	*fliC1*	–
CAE78572.1	AJ748315	*fliC2*	TAAA (N15) GCCGAAAG
CAE78395.1	AJ748317	*fliC3*	TAAA (N16) GCCTAGAT
CAE78397.1	AJ748319	*fliC4*	–
CAE80817.1	AJ748318	*fliC5*	TAAA (N15) GGCCATTA
CAE78149.1	AJ748316	*fliC6*	TAAA (N15) CGGCATTG
–	–	*E. coli*	TAAA (N15) CCCGATAA
–	–	*R. sphaeroides*	TAAA (N14) GCCGTTGA





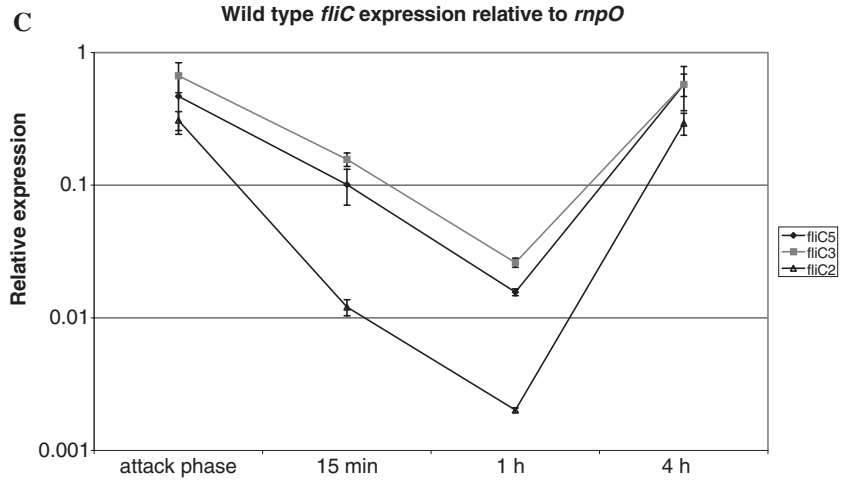


Fig. 2. A. Arrangement of *fliC* genes in both HD100 and 109J genomes, and sites used to insert an interrupting Kanamycin resistance cassette for construction of each *fliC* mutant strain.
B. Reverse transcription PCR using *fliC*-specific primers on total RNA from *B. bacteriovorus* 109J predatory culture. Products are seen for all *fliC* genes. L: 100 bp DNA ladder; 1–6: *fliC1*–*fliC6* each with no reverse transcriptase negative control in the right-side lane labelled b. Further control reactions of no template were also carried out and gave no products.
C. Quantitative PCR for expression by wild-type *B. bacteriovorus* 109J of *fliCs 2*, *3* and *5*, normalized by comparison with *rnpO* expression. Expression levels are shown for mRNA prepared from free-swimming, attack-phase *Bdellovibrio*, with no prey, and for *Bdellovibrio* during infection of prey, after 15 min (attachment and early invasion), after 1 h (establishment and growth within the bdelloplast) and after 4 h (lysis of the bdelloplast and release of attack-phase cells).

109JK (Fig. 3A). For mutants *fliC1*, *2*, *4* and *6* (Fig. 3B), no gross morphological changes could be seen in the flagellar filaments compared with wild type. All flagellar filaments, including wild-type, had a mean length of 4.0 ± 0.5 μm. For mutant *fliC5* (Fig. 3C), truncated flagellar filaments, with a mean length of 2.3 ± 0.5 μm were seen, significantly shorter than the wild type, and appearing to lack the distal, smaller-amplitude, filament waveform. When the speed of swimming motility was recorded for each strain by phase-contrast microscopy and analysed with a Hobson Tracker, it was found that for the *fliC5* mutant there was an appreciable reduction in motile speed in comparison with *B. bacteriovorus* 109JK. In the *fliC5* strain, the mean swimming speed was reduced to approximately 80% of the wild-type value of 35 μm s^{-1}, this resulted in a *P*-value of $P < 0.0005$ of reduced swimming speed. Thus the shortened flagellar filament in this strain seemed to reduce the thrust produced and lower the swimming speed. Interestingly, the *fliC4* mutant also showed a reduced swimming speed of approximately 85% of that of the wild-type ($P < 0.0005$). In addition, the *fliC1* mutant was also seen to have a slightly reduced swimming speed of approximately 92% that of the wild-type ($P < 0.005$), showing there is no simple correlation between the flagellar filament length and the swimming speed.

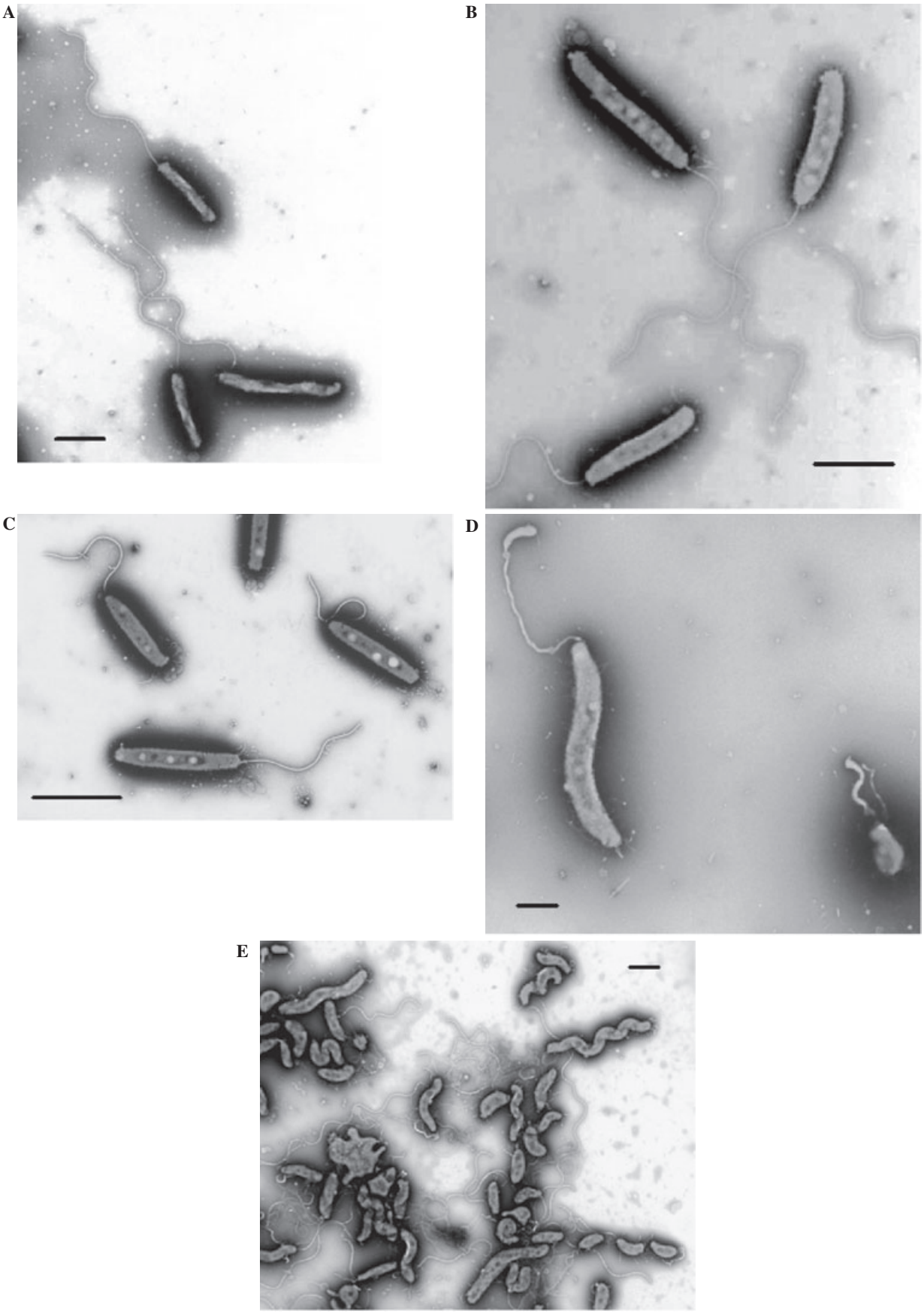
The predation efficiencies of each of the mutant *fliC* strains *1*, *2*, *4*, *5* and *6* were monitored in a luminescent prey assay in comparison with *B. bacteriovorus* 109JK. For strains *fliC5 and fliC4* a small but reproducible difference in predation efficiency was seen by this assay in comparison with the wild-type (Fig. S3), but for the other four strains no significant difference was seen (data not shown). Thus motility was reduced by changes in flagellar waveform or rigidity in the *fliC4* mutant and by changes in those parameters or filament length, for the *fliC5* mutant. These motility effects did both slightly reduce predation efficiency in a liquid environment.

Inactivation of fliC3 *abolishes motility but not flagellar sheath production*

Chromosomal inactivation of the *fliC3* gene by reciprocal recombination from a suicide plasmid construct could not be achieved in *B. bacteriovorus* 109J that were being grown in liquid culture upon *E. coli* prey, despite continued attempts that had resulted in the inactivation of all other *fliC* genes. This was probably because the desired recombinants could not productively encounter prey in liquid media. The gene was, however, successfully inactivated when the exconjugant merodiploid *Bdellovibrio* were cultured 'host independently' (HI) on rich media without prey. Single colonies of the *fliC3* HI derivative were isolated from these cultures and the chromosomal inactivation of the *fliC3* gene was verified by Southern blotting and DNA sequencing (data not shown). Liquid cultures of *fliC3* HI were examined by phase-contrast microscopy and found to be entirely non-motile in contrast to HI derivatives of the parent 109JK wild-type strain that were motile. Electron microscopy (Fig. 3D) of negatively stained samples of *fliC3* HI showed that the bacteria produced no normal flagella, compared with a flagellate wild-type HI-derivative 109J strain HIK3, which is typical of other 109JK-derived HI (Fig. 3E), but interestingly that they synthesized copious, disordered, tubular material resembling outer membrane. These we take to be disordered sheaths without a normal flagellum within. To see whether the membrane sheaths contained any flagellar filament sections at all, the membrane tubes were mechanically sheared from the bacteria and subject to SDS-PAGE analysis (Fig. 4A). We also examined, by RT-PCR, the expression levels of all *fliC* genes in the *fliC3* background (Fig. 4B).

Interestingly we found that all *fliC* genes, except the inactivated *fliC3* were transcribed in the *fliC3* mutant HI strain. Furthermore, protein bands corresponding to FliC protein sizes of c29K were seen in the *fliC3* mutant HI strain, and were of a significant abundance compared with the flagellate, motile 109J HI Kn^R strain HIK3. Thus although the *fliC3* HI strain was non-motile, it did contain disordered fragments of flagellar filaments within its sheath (microscopic data not shown). Quantitative time of flight mass spectrometry (Q-TOF MS) analysis was used to identify which FliC proteins were present within the *fliC3* flagellar sheath. The distribution of SDS-PAGE flagellin bands differed between mutant and wild-type. Q-TOF MS analysis was performed for the dominant flagellin bands: 29 and 31 K for wild-type HIK3; and 31 and 33 K for *fliC3*

Fig. 3. Morphologies of flagella from wild type and *fliC* mutant strains derived from *B. bacteriovorus* 109J.
A. Electron micrograph of *B. bacteriovorus* 109JK, a wild-type kanamycin-resistant strain, showing wild-type flagellar length and waveform. 5 k magnification, 1% PTA stain.
B. 109J *fliC6* strain; inactivation of this flagellin gene had no appreciable effect on the waveform or length of the flagellum. This is representative of strains with inactivated *fliC1*, *fliC2* or *fliC4*, which also had the same visible phenotypes in 109J. 5 k magnification, 1% PTA stain.
C. 109J *fliC5* strain. Inactivation of this flagellin resulted in shorter flagella, with the absence of the distal miniature waveform, and the strain also showed reduced mean swimming speed and reduced predation efficiencies compared with wild-type. 5 k magnification, 1% PTA stain.
D. Host-independent 109J *fliC 3* strain. Deletion of *fliC3* caused the absence of a flagellum and a non-motile phenotype but a disordered flagellar sheath was still seen. 7 k magnification, 1% PTA. stain. Bars = 1 μm.
E. Host-independent 109J HIK3 strain showing typical, normal sheathed flagella and the diversity of cell size and shape typical for an HI strain. 7 k magnification, 1% PTA. stain. Bars = 1 μm.



(the 29 K band in the *fliC3* mutant was reduced and not sufficiently abundant for accurate analysis). Results showed that, as expected, the FliC3-derived peptides were detectable in wild-type and absent in the *fliC3* mutant. Peptides from FliC5 and FliC2 were the most readily detectable from wild-type flagellar bands and these and FliC1 were found to be present in both wild-type and the disordered *fliC3* mutant sheath preparation. FliC6 and FliC3 were only detected in the 29 K band from wild type, which was insufficiently abundant to be analysed in the mutant. FliC4 peptides could not be reliably detected in this analysis in either wild type or mutant.

Having established that these *fliC3* HI bacteria were a truly non-motile strain of *Bdellovibrio*, we tested their predation potential to determine the role of flagellar motility in bacterial prey penetration.

Motility minus Bdellovibrio fliC3 *mutants predate bacteria if applied directly to them*

Cultures of the non-flagellate *fliC3*HI mutant failed to lyse liquid prey cultures in contrast to the other *fliC* mutants and a flagellate 109J-derived HI strain, HIK3, despite repeated attempts. Spotting of 100 μl of *fliC3* HI culture grown overnight in PYE broth onto a YPSC soft agar overlay containing *E. coli* prey did result in some clearing of the lawn of prey cells after 10 days, compared with 3–4 days for the wild-type HIK3. The clearing of the lawn was only partial, even up to 20 days while wild-type *Bdellovibrio* cleared the lawn completely by 5 days. Thus to visualize whether the *fliC3* mutant *Bdellovibrio* did actually invade prey and produce bdelloplasts a YFP-labelled derivitive of *E. coli* S17-1:pZMR100 was used as prey in direct predator–prey overlays. Fluorescence microscopy clearly revealed the presence of yellow *E. coli* bdelloplasts containing black *Bdellovibrio* (Fig. 5), showing that the flagellar minus *B. bacteriovorus fliC3* HI strain is capable of infecting host cells if placed in contact with them. These bdelloplasts were observed also to lyse, and release new *Bdellovibrio*, despite the absence of motility in the predator. The percentage of bdelloplasts was low, less than 1% of prey were infected, compared with approximately 20% of prey treated with motile HIK3 during the 6 h incubation.

Discussion

The *B. bacteriovorus* flagellum is a multiwaveform helical structure physically enclosed within a membrane sheath in both the *B. bacteriovorus* HD100 genome strain and the larger, more physiologically studied *B. bacteriovorus* 109J strain. In this study we have found that the flagellar filament is not always shed upon prey entry and infection establishment, although *Bdellovibrio* are immotile within their prey during the early phase of infection. The flagellum may be internalized as the coencytic *Bdellovibrio* grow within the prey and further flagella are synthesized as one long *Bdellovibrio* multinucleoid filament divides to give more motile progeny late in infection.

The genome sequence of *B. bacteriovorus* HD100 (Rendulic *et al*., 2004) revealed the presence of six potential *fliC* flagellin genes. Strain 109J was shown to also have six almost identical genes, more than the two predicted by pioneering early protein analysis of *B. bacteriovorus* 109J flagella by Thomashow and Rittenberg (1985b). All of the *fliC* genes encoded predicted proteins of similar 29 K molecular weight and similar protein sequence. Typically for flagellins, the N and C termini, which are required for flagellar export (Kanto *et al*., 1991), were the most highly conserved and the central regions between amino acids 120 and 220 were the most divergent. RT-PCR indicated qualitatively that all flagellins were expressed in free-swimming attack-phase cultures of *Bdellovibrio*. Quantitative-PCR (QPCR) for *fliCs 2*, *3* and *5* indicated that their expression was abolished or severely downregulated when inside bdelloplasts, and that it was induced upon bdelloplast lysis.

The sequence similarity between flagellins made an immunological characterization of their contribution to the multiwave flagellar filament problematic, so we took a genetic approach and insertionally inactivated each on the chromosome. Inactivation of two of the flagellin genes, *fliC 2* and *6*, did not result in striking morphological changes (Fig. 3) in the flagellar filament and had no significant effect on swimming speed or predation (data not shown). Inactivation of both *fliC1* and *fliC4* did not give an observable change in filament morphology (Fig. 3) but did significantly reduce swimming speed by 8% and 15% respectively. For *fliC4* this was accompanied by a significant reduction in predatory capability (Fig. 3); for *fliC1* predation efficiency was not distiguishable from wild type (data not shown).

The lack of obvious morphological changes may indicate redundancy of these flagellin gene products and possibly that they may be found in small quantities in the filament. These flagellins could possibly be in a mosaic central flagellar filament region indicated by Thomashow and Rittenberg as occurring at the junction of the large and small waveforms in the flagellar filament (Thomashow and Rittenberg, 1985b). This apparently minor role is a little surprising given the high conservation of FliC protein sequences seen.

Inactivation of *fliC5* caused truncated flagella apparently lacking the distal filament waveform (Fig. 3B), having a swimming speed reduced by 20% compared with wild type and significantly reduced predation efficiency (Fig. S3). As both the *fliC4* and *fliC5* mutations caused an approximately 15–20% reduction in swimming speed and an accompanying reduction in predation efficiency

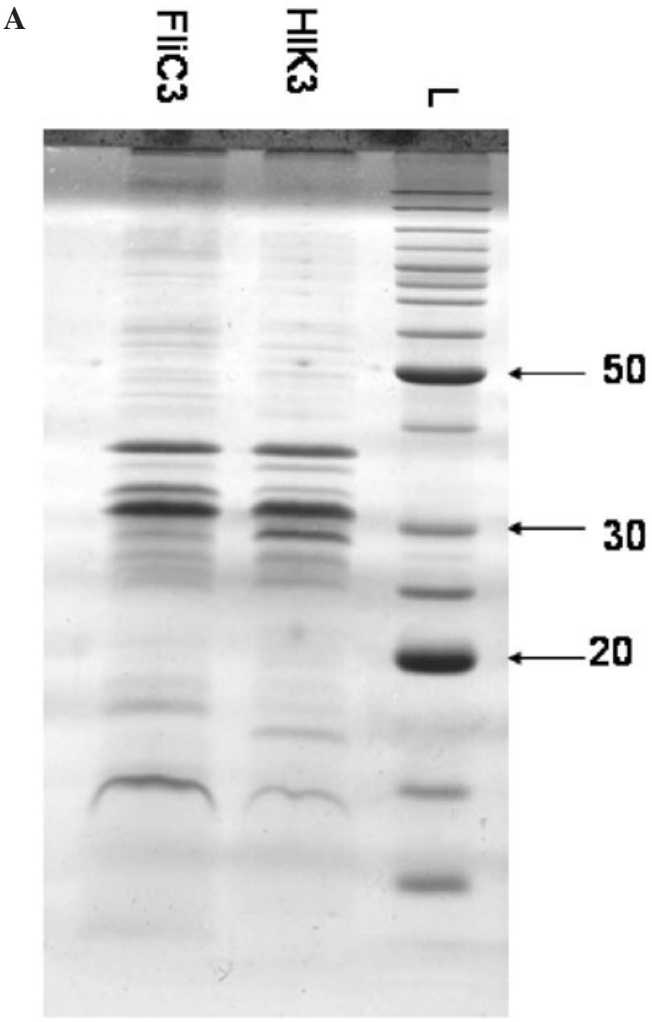


Fig. 4. A. SDS-PAGE of sheared flagella/flagellar sheath preparations, mechanically removed from the surface of *B. bacteriovorus fliC3* HI mutant (lane 1) and control 'wild-type' HIK3 (lane 2) strains. L is BenchMark protein ladder.
B. Semi-quantitative reverse transcription PCR to demonstrate approximate *fliC* expression levels using *fliC*-specific primers and 25 cycles of amplification on total RNA from *B. bacteriovorus fliC*3 host-independent culture and HIK3: a 109JK-derived host-independent culture that is wild-type for flagella. Products are seen for all *fliC* genes with the exception of *fliC*3 in the *fliC3* mutant strain. Lanes: L, 100 bp DNA ladder; 1–6: *fliC1–fliC6* primer pairs, respectively, in the HI *fliC3* mutant strain; 7–12: *fliC1–fliC6* primer pairs, respectively, in the HIK3 flagellate 'wild type' strain.

(Fig. S3) in liquid media; this indicates that flagellar motility is important for encountering prey bacteria in liquids. The smaller 8% reduction in swimming speed seen for the *fliC1* mutant was not sufficient to give a measurable drop in predation efficiency, in the conditions that we tested. As only the *fliC5* mutation, and not the *fliC4* mutation, caused a visible truncation in the flagellar filament, which might obviously cause a motility effect; one can only speculate that perhaps the rotating waveform or rigidity of a flagellar filament, in the *fliC4* mutant strain was compromised, compared with wild-type, reducing swimming speed and predation efficiency.

It had been suggested (Thomashow and Rittenberg, 1985b) that the distal waveform, altered in the *fliC5*

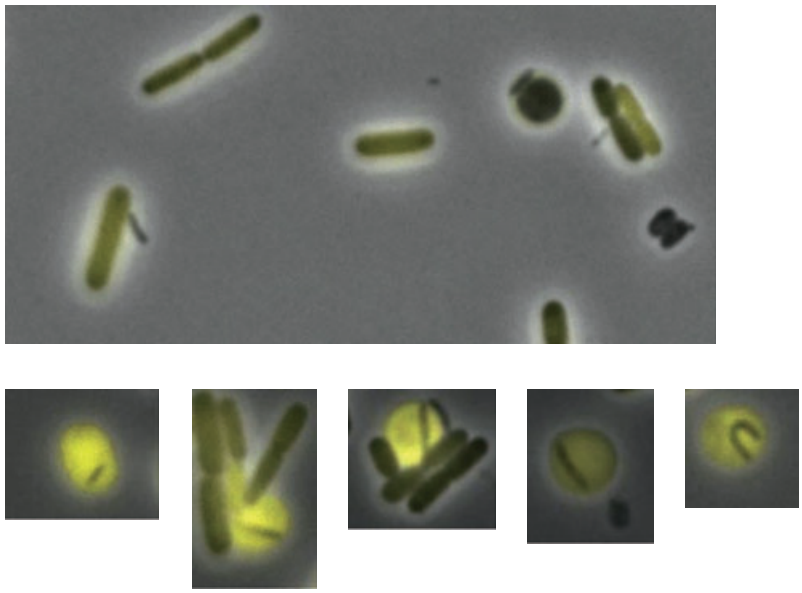


Fig. 5. Spherical bdelloplasts and rod-shaped hosts picked from solid agar overlays containing YFP-labelled *E. coli* S17-1:pZMR100 hosts incubated with *Bdellovibrio* HI *fliC3* mutant strain.
A. A typical view including a spherical bdelloplast and a HI *fliC3* cell attached to an *E. coli* prey cell.
B. A gallery of rounded, yellow, and therefore definitively *E. coli*-derived, bdelloplasts are seen, which contain a growing black coencytic HI *fliC3* mutant *Bdellovibrio*, indicating that this *fliC3* strain, although lacking a functional flagellum, will still predate when placed in close proximity to prey on a surface.

mutant, continues to be synthesized during flagellar elongation after the bursting out of *Bdellovibrio* from exhausted prey. Our QPCR data (Fig. 2C) for *fliC5* transcription cannot differentiate the precise timing of expression between *fliC* genes. The QPCR did indicate that the *fliC3* and *fliC5* genes, which gave the greatest phenotypic changes when genetically inactivated, were more highly expressed than the *fliC2* gene, which was representative of a *fliC* gene that did not give a large phenotypic effect when inactivated (Fig. 3).

Inactivation of only the *fliC3* gene gave non-motile *Bdellovibrio* that still produced membranous sheath material, containing disordered flagellar filament proteins (Figs 3D and 4A), but no functional flagellum. Surprisingly, despite the high-sequence conservation between *fliCs* in *Bdellovibrio*, there was no cross-complementation, despite the apparent increase in levels of *fliC2*, *5* and *6* transcripts observed in *fliC3* mRNA (Fig. 4B). Overexpression of the other five flagellin genes for cross-complementation experiments in *fliC3* backgrounds are currently precluded in *Bdellovibrio* by the lack of stably replicating plasmids. The fact that *fliC3* mutants still produced disordered membrane sheath material also indicates, slightly surprisingly, that intact flagellar filament synthesis is not a prerequisite for flagellar sheath formation in *Bdellovibrio*. This has also been reported (McCarter, 2001) for the flagella of *Vibrio* species.

SDS-PAGE of preparations, sheared from the whole flagella, on the outside of the motile HIK3 strain, and from disordered sheath of the *fliC3* mutant, both contained some flagellin bands, distributed between 29 and 33 K, despite the predicted molecular weights all being 29–30 K (Fig. 4A). This size variation, was also reported, for sheared *Bdellovibrio* flagella, by Thomashow and Rittenberg (1985a). The distribution of SDS-PAGE flagellin bands differed between mutant and wild-type. Q-TOF MS analysis of the dominant flagellin bands was: 29 and 31 K for wild-type HIK3; and 31 and 33 K for *fliC3* (the 29 K band in the *fliC3* mutant was reduced and not sufficiently abundant for accurate analysis). Analysis showed that, as expected, the FliC3-derived peptides were detectable in wild-type and absent in the *fliC3* mutant. Peptides from FliC5 and FliC2 were the most readily detectable from wild-type flagellar bands and these and FliC1 were found to be present in both wild type and the disordered *fliC3* mutant sheath preparation. FliC6 and FliC3 were only detected in the 29 K band from wild-type, which could not be analysed in the mutant, probably as its *fliC3* content was absent. FliC4 peptides could not be reliably detected in this analysis in either wild type or mutant.

The finding by Q-TOF MS analysis, that peptides from FliC5 and FliC2 flagellins were in the *fliC3* disordered sheath preparations is supported by electron-microscopic examination, which shows disordered flagellar filament sections (data not shown). Semi-quantitative RT-PCR studies (Fig. 4B) of mRNA prepared from *fliC3* and HIK3, with *fliC*-specific primers, helped to illuminate the expression patterns for *fliCs*. Figure 4B shows that levels of *fliC2*, *5* and *6* transcripts increased in the *fliC3* mutant with *fliC1* and *4* levels remaining approximately the same as wild-type HIK3. No *fliC3* mRNA was detectable in the *fliC3* mutant as expected, but it was detected in the wild-type. Taken together, these data do imply that FliC3 is either a core protein of the *Bdellovibrio* flagellar filament, without which the other flagellins cannot be organized into a functional filament, or a linker between hook associated protein (HAP) and filament regions. It is not possible to assign an exact position to FliC3 proteins within

the filament, without extensive immunological studies beyond the scope of this work.

The finding that the *fliC3* mutant *Bdellovibrio* cannot predate bacteria in liquid culture shows that flagellar motility is important in prey location and collision, but our finding that applying the *fliC3* mutant directly onto immobilized prey on an agar surface does result in infective bdelloplasts (Fig. 5A and B) and limited clearing of bacterial lawns, with replication of *Bdellovibrio*, shows that flagellar motility is not absolutely required for *Bdellovibrio* to enter the periplasm of their prey. This finding lends weight to suggested *Bdellovibrio* entry mechanisms that require pili (Rendulic *et al*., 2004) or enzymatic hydrolysis of the outer membrane of prey alone (Tudor *et al*., 1990). That flagellar motility is important in prey encounters in liquid media is reinforced by a correlation between swimming speed and predation efficiency seen for mutants *fliC4* and *5*. We have established however, that the active, flagellar-driven, drilling mechanism, proposed by Burnham *et al*. (1968) is not required for prey entry. This work shows that *Bdellovibrio* use their impressive motility in liquid environments to enhance the probability of productive prey encounters; but not actually to enter prey. This may be relevant to predator–prey dynamics in dilute aquatic and marine environments. Little is known of any sessile lifestyle for this famously motile predator, but this work shows that such *Bdellovibrio* cells would be capable of prey entry. This may have implications for predation in biofilms in nature and medicine.

Experimental procedures

Bdellovibrio *culturing on prey and prey-independently*

Bacterial strains used in this study are listed in Table 1. *Bdellovibrio* were routinely cultured on *E. coli* S17-1 prey cells in Ca/HEPES buffer as described previously (Lambert *et al*., 2003). HI *fliC3* were generated by growing merodiploid exconjugants on *E. coli* prey cells, then separating from the remaining prey cells by passing through a 0.45 μm filter, followed by growth on rich media (PYE) plates. HI growth was in PYE broth or on agar plates as described previously (Shilo and Bruff, 1965).

For electron microscopy purposes, 109J were grown on *E. coli* DFB225 prey, a *fliG* null RP437 derivative with no flagella (Lloyd *et al*., 1996) to ensure that all flagella imaged were only derived from *Bdellovibrio*. DFB225 were cultured as standard in YT broth at 37°C, 90 r.p.m. 109J were cultured on these prey as previously stated (Lambert *et al*., 2003) with the exception that shaking was at 90 r.p.m.

Observations to look for flagellar shedding were made from samples of synchronous culture at different time points at 10 min intervals from time 0 (time of inoculation) to 120 min. The majority of bdelloplasts tended to aggregate on grids because of the physical forces encountered when that sample and stain are removed. This resulted in the majority of bdelloplasts being in large groups, meaning that any flagella present were impossible to see, so only individual bdelloplasts were examined in eight independent experiments.

Flagellar shearing and SDS-PAGE analysis of fliC3 *mutant*

The *fliC3* HI mutant and a kanamycin-resistant 109JK-derived wild-type HI strain, HIK3, were continually subcultured from single colonies into increasing volumes of PY media containing kanamycin at 50 μg l^{-1}, over a period of 7 days at 200 r.p.m., 29°C to achieve large culture volumes. Cultures were matched to an OD_{600} of 0.7 in final volumes of 2×300 ml. Cells were harvested at 4300 *g*, 4°C for 45 min so as not to shear off sheath or any flagella present; pellets were then gently resuspended in ice-cold 10 mM HEPES pH 7.0 and combined to give a final volume of 10 ml. These were passed 18 times through 30 cm × 1 mm cannulae (Smith Medical) on ice and centrifuged at 4300 *g*, 4°C, 45 min to pellet the cell debris and leave sheath and any flagellar filaments from them in the supernatant. This supernatant was then ultracentrifuged at 115 000 *g*, 90 min, 4°C and the resulting pellet resuspended in 225 μl sterile TE (10 mM Tris-HCl, 2 mM EDTA, pH 8.0).

Analysis was carried out by electron microscopy on the initial cultures and the final pellet and by SDS-PAGE on a 12.5% gel using 5 μl BenchMark Protein Ladder (Invitrogen), loading matched total protein samples as determined using a Lowry assay (Lowry *et al*., 1951). Gels were stained overnight using Coomassie brilliant blue and destained in 30% (v/v) methanol, 10% (v/v) glacial acetic acid for several hours. The 31 K bands seen for both *fliC3* mutant and HIK3 flagellar wild-type strains corresponded to the predicted molecular weights of the six flagellin proteins; these were excised, along with a band seen at 29 K in the wild-type HIK3 preparation, and a band seen at 33 K in the *fliC3* mutant preparation, and subject to tryptic digestion and Q-TOF MS analysis, testing for the presence of peptides unique to each of the six flagellin proteins.

Electron microscopy

A 5–10 μl aliquot of *Bdellovibrio* culture was allowed to settle on a carbon/formovar grid for a period of 1 min, removed and immediately stained with 4°C 1% phosphotungstic acid (PTA) for 30 s. The stain was removed and grids allowed to dry before being imaged using a JEOL JEM 1200-EX electron microscope. Sheared flagellar filaments and sheaths were imaged from 5 μl of sample and stained for 30 s with 15 μl of 1% PTA pH 7. Magnifications are as stated in figures.

Insertional inactivation of fliC *genes*

Wild-type *fliC* genes were cloned by PCR from *B. bacteriovorus* genomic DNA of strain 109J, using cloned *Pfu* polymerase (Stratagene) for high fidelity. The full-length genes were cloned with 500 bp–1 Kb of flanking DNA. *fliC1* was amplified with a 5′ primer (5′-GCTCTAGACAGAAGAA GATGAACCTCGA) and a 3′ primer (5′-GACTTGGTTTG CGTCGTTGC) with XbaI and SalI (internal) restriction sites. *fliC2* was amplified using a 5′ primer (5′-GCTCTAGAAGCCT

GTTCTGGGATAAGGC) and a 3′ primer (5′-GCGAGCTCT TCCCTCCACCAGAAGGACG) with XbaI and SacI restriction sites. *fliC3* was amplified using a 5′ primer (5′-GCTCTA GAAGCACCTTCGTAGCGCTGGA) and a 3′ primer (5′-CGG GATCCGCCAATCTTGCCATTGATCT) with XbaI and BamHI restriction sites. *fliC4* was amplified using a 5′ primer (5′-GCTCTAGAAACGGAACTCAGTTGCTGGC) and a 3′ primer (5′-CGGGATCCGGAACAACTGAACGCCGGTC) with XbaI and BamHI restriction sites. *fliC5* was amplified using a 5′ primer (5′-GCTCTAGAGCACGTCCTGTTTGATGATA) and a 3′ primer (5′-GGAATTCCTTCGATGTCGATCATGCTG) with XbaI and EcoRI restriction sites. *fliC6* was amplified using a 5′ primer (5′-CGGGATCCAACACACAGGAAGAAGTGAA) and a 3′ primer (5′-GCTCTAGATTAAAAAAGACCGG ATTTCA) with BamHI and XbaI restriction sites.

The PCR fragments were gel purified, digested with the appropriate enzymes and ligated into the pUC19 (Vieira and Messing, 1982) cloning vector. A 1.3 Kb kanamycin resistance cassette released from pUC4K (Yanisch-Perron *et al*., 1985) by digestion with HincII was gel-purified and blunt-ligated into unique restriction sites within the flagellin open reading frames. The restriction sites used were as follows: *fliC1* BsrGI; *fliC2* BstEII; *fliC3* NaeI; *fliC4* BsrGI (excising 235 bp of the gene); *fliC5* NcoI; *fliC6* MscI. Where appropriate, a fill-in or 3′-5′ exonuclease reaction was carried out using *Pfu* polymerase to blunt the fragment for the ligation. The disrupted flagellin genes with flanking DNA were then excised from pUC19 and ligated into the pSET151 vector (Bierman *et al*., 1992). Restriction sites used for this were as follows: *fliC1* XbaI and HincII; *fliC2* EcoRI; *fliC3* SphI and BamHI; *fliC4* SphI and BamHI; *fliC5* EcoRI and XbaI; *fliC6* EcoRI and SphI. These pSET151 constructs containing the disrupted flagellin genes were conjugated into *Bdellovibrio* as described previously (Lambert *et al*., 2003). Confirmation that the genes were knocked out by Southern blotting was as described elsewhere (Southern, 1975) using probes of the kanamycin cassette that was inserted (to confirm its presence) and the pSET151 vector (to confirm its absence). The labelling and detection kit used was the Phototope-star kit (New England Biolabs). Confirmation of knockout mutants by sequencing was performed by amplifying the disrupted region of the gene by PCR with a proofreading polymerase and direct sequencing of that PCR product by MWG Biotech Ltd. The sequence data were analysed to confirm the disruption of the gene at the correct restriction site by the kanamycin cassette sequence.

RT-PCR to detect expression of fliC *genes*

RT-PCR was used to determine which of the six flagellin genes were expressed in attack-phase predatory cultures (Fig. 2B), and in the *fliC3* HI mutant strain and comparable HIK3 axenic wild type for flagella (Fig. 4B). For predatory cultures 5×10^8–5.6×10^9 of stationary-phase *E. coli* S17-1 were mixed with 5×10^8–3×10^{11} attack-phase *Bdellovibrio* (as determined by cfu and pfu respectively) in Ca/HEPES buffer. After 15 min, samples were mixed to a final 1% (v/v) phenol and 19% (v/v) ethanol, and then the RNA extracted using Promega SV total RNA isolation kit according to the IFR published protocol (http://www.ifr.bbsrc.ac.uk/Safety/Microarrays/protocols.html) with the exception that RNA was eluted in 50 μl rather than 100 μl H_2O. The RNA was further DNase-treated with the Ambion DNA-free kit according to manufacturer's instructions. Control infections of *Bdellovibrio* only and *E. coli* only were also set up. For HI strains, axenic cultures were grown for 1–2 days in PY broth and then OD_{600} of the cultures was matched (to 0.6) by diluting before being mixed (4 ml) with phenol/ethanol and treated as for predatory cultures. The strain HIK3 was derived from strain 109JK (Lambert *et al*., 2003) by standard methods described elsewhere (Seidler and Starr, 1969) and was wild-type for flagella. Quantification of RNA was by OD_{260} measurements.

Primers designed to anneal to the *fliC* open reading frames were as follows: *fliC1* 5′ primer GCATCTATCGCAGCAC AACG, 3′ primer CCGTCGAGTCGGCATCAAATTCAAA; *fliC2* 5′ primer GAACGTATCCGCTATCAACG, 3′ primer TAGTGCAGAGTTTGGCATCG; *fliC3* 5′ primer ATGCTCAG AGAGTTCTCTGG, 3′ primer AATGACTTGTTCAAGAGTCC; *fliC4* 5′ primer TCGGTACCAATGTGGCAGCG, 3′ primer ATTGTTGTGACTGGTTCGCC; *fliC5* 5′ primer GCTCAACGT AACTTGGTCGG, 3′ primer AGCCGATCAGCTTAAGAGCC; *fliC6* 5′ primer TCAGCTTTAACGCCAACTGG, 3′ primer TATT GCAGCCCTGAACGCCC.

RT-PCR was carried out using the QIAGEN® OneStep RT-PCR kit according to instructions. Thermal cycling was carried out in a Thermo hybaid MBS 0.2G thermal cycler programmed to complete one cycle of 50°C for 30 min, 95°C for 15 s, then 30 cycles of 94°C for 1 min, 48°C for 1 min, 72°C for 2 min followed by one cycle of 72°C for 10 min then 4°C hold. Where stated (Fig. 4B) 25 cycles were used to highlight a semi-quantitative difference of expression. Control reactions to test against the presence of contaminating DNA were carried out using PCR with Taq DNA polymerase (ABgene) for the same PCR conditions.

As a kanamycin-resistant but otherwise wild-type *B. bacteriovorus* 109J strain was required for the luminescent predation assays upon a kanamycin-resistant host carrying a plasmid encoded *luxCDEAB* cassette, we used a kanamycin-resistant *B. bacteriovorus* 109JK strain containing a Kn^R Tn*5* insertion into its genome. The site of this insertion was determined to be within a putative ABC transporter gene homologous to gene NP969171 from the *B. bacteriovorus* HD100 genome. We previously verified (Lambert *et al*., 2003) that interruption of this gene in *B. bacteriovorus* 109JK had no effect on predation efficiency, but here we also used RT-PCR to verify the expression level of this gene. Primers designed to anneal to the putative ABC transporter open reading frame were as follows: 5′ primer GCTGACCTATG CACTGATGG, 3′ primer ACACCATTTCGCAACGAACC and did not yield a product showing that the gene was not expressed in attack phase or during HI growth.

Conditions for quantitative RT-PCR for fliC *genes*

Overnight cultures of *E. coli* S17-1 and *B. bacteriovorus* 109J were concentrated by centrifugation and resuspended in Ca/HEPES buffer, then incubated at 29°C with 200 r.p.m. shaking for 3 h to stabilize transcription. Infections were set up at an moi of 0.5–0.75 with *Bdellovibrio*-only and *E. coli*-only controls and also incubated at 29°C with shaking at 200 r.p.m. A 4 ml aliquot of samples was taken at 15 min, 1 h and 4 h post infection and transcription was stabilized

in these samples by bringing to a final concentration of 1% (v/v) phenol 19% (v/v) ethanol at 4°C. RNA was then prepared as described above. Plaque (for *Bdellovibrio*) and colony (for *E. coli*) enumeration confirmed the replication of *Bdellovibrio* at the expense of *E. coli* throughout the infection.

cDNA preparation

cDNA was made with SuperscriptII reverse transcriptase (Invitrogen) according to the protocol of Gomelsky *et al*. (2003).

Quantitative RT-PCR

Quantitative PCR was carried out using the Bio-Rad SYBR green supermix system in a Stratagene MX4000 machine. The following temperature profile was used for cycling: one cycle of denaturation at 95°C for 10 min, 40 cycles of 95°C for 30 s, 60°C for 1 min and 72°C for 1 min. Fluorescence acquisition was during the annealing step. Melting curve analysis was from 55°C to 95°C at 1°C per cycle. Specificity of reaction was further determined by 2% agarose gels. Primers used were as follows: for *rnpO*, forward primer 3′-AAGCG ACAGTTTCCACTCGT-5′, reverse primer 3′-ACGGATAGCTT CGTTTGCAT-5′; for *fliC*s as above for RT-PCR.

Expression of the flagellin genes was presented relative to expression of the *rnpO* gene encoding the omega factor of RNA polymerase. The formula used to express this was $2^{\wedge(\text{Ct } rnpO - \text{Ct flagellin})}$. The results presented were from at least two independent experiments.

Predation efficiency of fliC *mutant strains*

Bdellovibrio bacteriovorus 109J mutants of *fliC1*, *fliC2*, *fliC4*, *fliC5* and *fliC6*, were compared with the control strain 109JK using the luminescent host assay described previously (Lambert *et al*., 2003) with the same conditions. A minimum of 10 separate experiments were carried out for each strain resulting in a minimum of 50 data points for each. Enumeration of the prey by cfu and *Bdellovibrio* by pfu at 0, 6 and 24 h confirmed the correlation between luminescence and killing of the prey cells to yield predator cells.

To assess the predatory capability of the non-motile *fliC3* mutant, YFP-tagged Kn^R*E. coli* S17-1 prey grown overnight to an OD_{600} of 1.5 were challenged, on a solid PYE agar surface, with HIK3 (kanamycin-resistant but predatory HI 'wild-type' strain) and HI*fliC3::Kn Bdellovibrio* strains grown for 3 days in PYE Kn 50 μg ml^{-1} at 29°C to an average OD_{600} of 0.8. In total, 50 μl of prey and 50 μl of *Bdellovibrio* were mixed and spotted onto PYEKn50 agar plates and incubated at 29°C for 24 h. At this time, the cells were scraped from the surface of the plate, resuspended in 1 ml Ca/HEPES and pelleted by centrifugation for 1 min, 13 000 r.p.m. in a bench-top centrifuge and resuspended in a final volume of 100 μl. A 5 μl aliquot of samples was agar-mounted and examined under phase contrast and YFP optics on a Nikon Eclipse E600 and *E. coli* prey and bdelloplast numbers were counted ($n > 2500$ *E. coli* per experiment).

Hobson tracker analysis of Bdellovibrio fliC *mutant swimming behaviours*

Hobson BacTracker analysis was used to determine the mean run speeds of each motile mutant strain in comparison with *B. bacteriovorus* 109JK wild-type. *Bdellovibrio* were grown in typical predatory lysates on *E. coli* DFB225 (pZMR100) and tracked 21–22 h after inoculation. A 16 μl aliquot of culture was dropped onto a microscope slide and covered with a 22 × 22 mm coverslip giving a chamber depth of 33 μm. Cultures were observed using a 40× phase-contrast objective on a Nikon Labophot 2A microscope with real-time computer tracking using the Hobson BacTracker 50 Hz system (Hobson Tracking Systems, Sheffield, UK). Data on mean and median run speeds were collected from a minimum of 45 sets of 50 tracks for each strain; these were then analysed using a standard two-sample Student's *t*-test between each mutant strain and the wild-type to test for a decrease in mean swimming speed, *P*-values given are for the mutant mean swimming speed being less than that of wild-type.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Gallery of images of *Bdellovibrio* within a DFB225 bdelloplast yet with flagellum protruding through the grey membrane.

Fig. S2. Sequence alignment of the six flagellin proteins from *B. bacteriovorus* HD100 and 109J. The flagellins are all highly related, with the *fliC1* sequence being the most divergent. There is very little divergence between the two strains.

Fig. S3. Luminescent prey assay for *E. coli* predation efficiency by the *fliC4 and 5* mutants versus *B. bacteriovorus* 109JK and wild-type kanamycin-resistant strain.

This material is available as part of the online article from http://www.blackwell-synergy.com

