Characterization of type IV pili in the life cycle of the predator bacterium *Bdellovibrio*

Khaled K. Mahmoud and Susan F. Koval

Correspondence
Susan F. Koval
skoal@uwo.ca

Department of Microbiology and Immunology, University of Western Ontario, London, ON N6A 5C1, Canada

Bdellovibrio and like organisms (BALOs) are obligate prokaryotic predators of other Gram-negative bacteria. *Bdellovibrio bacteriovorus* is the most studied organism among BALOs. It has a periplasmic life cycle with two major stages: a motile, non-replicative stage spent searching for prey (the attack phase) and a stage spent inside the periplasm of the Gram-negative prey cell (the growth phase) after forming an osmotically stable body termed the bdelloplast. Within *Bdellovibrio*, there are also strains exhibiting an epibiotic life cycle. The genome sequence of the type strain *B. bacteriovorus* HD100^T revealed the presence of multiple dispersed *pil* genes encoding type IV pili. Type IV pili in other bacteria are involved in adherence to and invasion of host cells and therefore can be considered to play a role in invasion of prey cells by *Bdellovibrio*. In this study, genes involved in producing type IV pili were identified in the periplasmic strain *B. bacteriovorus* 109J and an epibiotic *Bdellovibrio* sp. strain JSS. The presence of fibres on attack-phase cells was confirmed by examining negative stains of cells fixed with 10 % buffered formalin. Fibres were at the non-flagellated pole on approximately 25 % of attack-phase cells. To confirm that these fibres were type IV pili, a truncated form of PilA lacking the first 35 amino acids was designed to facilitate purification of the protein. The truncated PilA fused to a His-tag was overexpressed in *Escherichia coli* BL21(DE3) plysS. The fusion protein, accumulated in the insoluble fraction, was purified under denaturing conditions and used to produce polyclonal antisera. Immunoelectron microscopy showed that polar fibres present on the cell surface of the predator were composed of PilA, the major subunit of type IV pili. Immunofluorescence microscopy showed the presence of pilin on attack-phase cells of *B. bacteriovorus* 109J during attachment to prey cells and just after penetration, inside the bdelloplast. Antibodies against PilA delayed and inhibited predation in co-cultures of *Bdellovibrio*. This study confirms that type IV pili play a role in invasion of prey cells by *Bdellovibrio*.

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INTRODUCTION

Bdellovibrio and like organisms (BALOs) are small Gram-negative bacteria, aerobic, highly motile with a single polar sheathed flagellum. They have the unusual property of preying on other Gram-negative bacteria (Jurkevitch, 2006). The life cycle of *Bdellovibrio bacteriovorus* is the most studied among BALOs. It has two major stages: a free swimming non-replicative stage spent searching for prey (the attack phase) and a growth stage spent inside the periplasm of the prey cell after forming a spherical, osmotically stable body termed the bdelloplast (the growth phase). Once *B.*

bacteriovorus has collided with a prey cell, it remains reversibly attached to it for a short period (recognition time), after which it becomes irreversibly anchored to the prey cell via the pole opposite the flagellum. Penetration into the prey cell is complete within a few minutes after attachment. It has been proposed that penetration involves the formation of a pore through the prey cell wall components by physical drilling as a result of the rotation or swivelling of *B. bacteriovorus* attached to the prey (Starr & Baigent, 1966; Burnham *et al.*, 1968). A mixture of hydrolytic enzymes, including glycanases and peptidases, is also applied in a locally targeted manner that prevents excessive damage to the prey and unregulated diffusion of periplasmic or cytoplasmic constituents (Thomashow & Rittenberg, 1978a, b, c). Although BALOs are obligate predators, spontaneous prey-independent mutants growing in the absence of prey cells can be isolated in the laboratory. These mutant strains maintain their predatory capabilities when regularly grown in the presence of prey cells (Varon & Seijffers, 1975; Cotter & Thomashow, 1992).

Abbreviation: BALOs, *Bdellovibrio* and like organisms.

The GenBank/EMBL/DDBJ accession numbers for the *pilA*, *pilG*, *pilT2* and *pilT1* genes of *B. bacteriovorus* 109J are GU368922, GU368924, GU368925 and GU368927, respectively. The accession numbers for the *pilM*, *pilT1*, *pilQ* and *pilT2* genes of *B. bacteriovorus* JSS are GU368928–GU368931.

Three supplementary figures are available with the online version of this paper.

A *Bdellovibrio*-like organism with a different life cycle has been isolated on lawns of *Caulobacter crescentus* (Koval & Hynes, 1991). This strain, named JSS, does not enter the periplasm of the prey cell but remains attached to the surface and utilizes the cytoplasmic contents of the prey cell from that position. No bdelloplast is formed, and the empty prey cell retains its original shape (Shemesh *et al.*, 2003). It therefore has an epibiotic, not a periplasmic life cycle. Phylogenetic studies (Davidov & Jurkevitch, 2004) and fluorescence *in situ* hybridization (Mahmoud *et al.*, 2007) showed that strain JSS belongs to the genus *Bdellovibrio* and shares 93% identity of 16S rRNA gene sequence with *B. bacteriovorus* HD100 and 109J.

The genome sequence of *B. bacteriovorus* HD100^T revealed the presence of type IV pili genes (Rendulic *et al.*, 2004). Type IV pili are thin fibres (5–8 nm in width) of variable length and relative flexibility (Craig *et al.*, 2003) and have multiple functions in bacterial cells including adherence to host cells, twitching motility, phage adsorption, DNA uptake and biofilm formation (Bradley, 1974; Mattick, 2002; Craig *et al.*, 2004). Type IV pili are distinguished from other pili by their polar location, sequence similarities among their pilins (which share an unusual N-methylated N-terminus), a conserved hydrophobic N-terminal 30-residue sequence and a carboxy-terminal disulfide bond (D-region; Craig *et al.*, 2004). The pili are composed primarily of a single protein subunit, termed PilA or pilin, assembled into a helical conformation. Although the pilus fibre is composed of a single subunit, several other proteins are also important for proper assembly and function of type IV pili, and mutations in the corresponding *pil* genes lead to non-piliation (Carbannelle *et al.*, 2005, 2006; Hansen & Forest, 2006). PilT, a hexameric ATPase associated with the inner membrane and unique to type IV pili, is required for pili retraction by disassembling the pilin subunits at the base of the fibre (Morand *et al.*, 2004). PilQ, a multimeric outer-membrane protein and member of a protein family

called secretins, forms gated pores in the outer membrane, through which the pilus fibre is extruded (Collins *et al.*, 2001; Hansen & Forest, 2006).

Polar fibres have been observed on some attack-phase cells of *B. bacteriovorus* strain 109J (Shilo, 1969; Abram & Davis, 1970) and strain HD100 (Evans *et al.*, 2007). The identification of PilA as the major subunit of fibres detected on the surface of *B. bacteriovorus* HD100 was suggested by Evans *et al.* (2007) based on the lack of pili in a *pilA* mutant. Medina *et al.* (2008) reported that *pilT* (Bd3852) was required for predation by prey-independent strains of *B. bacteriovorus* 109J in biofilms of *Escherichia coli*. In the present study, we examined the presence of type IV pili genes in *B. bacteriovorus* 109J, the strain in which most physiological studies of BALOs have been carried out. *B. bacteriovorus* 109J preys on a wide range of Gram-negative bacteria and is recognized as being an 'aggressive' predator strain among the bdellovibrios (Ruby, 1992). The presence of polar fibres on attack-phase cells of *B. bacteriovorus* 109J was visualized by electron microscopy. With the use of polyclonal antibodies against a truncated PilA, the polar fibres on *B. bacteriovorus* 109J were characterized as type IV pili. Immunofluorescence studies showed expression of pilin on *B. bacteriovorus* 109J upon attachment to prey cells and after penetration, inside the bdelloplast. Antibodies against PilA delayed and inhibited predation of *B. bacteriovorus* 109J. The presence of pilus-like fibres on the epibiotic *Bdellovibrio* sp. strain JSS is also reported. Four type IV pili genes similar to those important in the biogenesis of PilA (*pilT1*, *pilT2*, *pilQ* and *pilM*), and identical to those of strains 109J and HD 100, were identified in strain JSS. Antibodies against PilA also inhibited and delayed predation of attack-phase cells of strain JSS.

METHODS

Bacterial strains and culture conditions. Bacterial strains used in this study are listed in Table 1. *E. coli* ML35 or *Aquaspirillum serpens* VHL were used as prey cells. *E. coli* ML35 was grown overnight at

Table 1. Bacterial strains used in this study

Bacterial strain	Description	Source or reference
<i>Bdellovibrio bacteriovorus</i>		
109J	Obligate predator/periplasmic life cycle	E. G. Ruby*
109JA	Facultative predator/periplasmic life cycle	Pritchard <i>et al.</i> (1975)
HD100	Obligate predator/periplasmic life cycle; type strain, genome sequenced	Stolp & Starr (1963)
<i>Bdellovibrio</i> sp. strain JSS	Obligate predator/epibiotic life cycle	Koval & Hynes (1991)
<i>Aquaspirillum serpens</i> VHL	Prey for BALOs	Koval & Hynes (1991)
<i>Caulobacter crescentus</i> CB2A	Prey for <i>Bdellovibrio</i> sp. strain JSS	Koval & Hynes (1991)
<i>Escherichia coli</i>		
ML35	<i>lacI lacY</i> ; prey for <i>B. bacteriovorus</i> 109J and HD100	E. G. Ruby
DH5 α	ϕ 80dlacZ Δ M15 <i>recA1 endA1 gyrA96 thi1 hsdR17</i> ($r_K^- m_K^+$) <i>supE44 relA1 deoR</i> Δ (<i>lacZYA-argF</i>)U169	Invitrogen
BL21(DE3) plysS	<i>E. coli</i> B F ⁻ <i>dcm ompT hsdS</i> ($r_B^- m_B^-$) <i>gal</i> h(DE3) [plysS Cam ^r]	Stratagene

*Department of Medical Microbiology and Immunology, University of Wisconsin, Madison, WI 53706-1521.

30 °C in NB-YE medium [nutrient broth (8 g l⁻¹)-yeast extract (5 g l⁻¹), pH 7.5]. *A. serpens* VHL was grown overnight at 30 °C in PSS medium (peptone 10 g l⁻¹, succinic acid 1 g l⁻¹, (NH₄)₂SO₄ 1 g l⁻¹, MgSO₄·7H₂O 1 g l⁻¹, FeCl₃·6H₂O 0.002 g l⁻¹, MnSO₄·H₂O 0.002 g l⁻¹, CaCl₂·2H₂O 0.015 g l⁻¹, pH 6.8). For the maintenance of predators, 3 ml of prey cells and 1 ml of predators (*B. bacteriovorus* 109J or HD100) were mixed in 20 ml HM buffer [3 mM HEPES (Sigma), pH 7.6, with 1 mM CaCl₂ and 0.1 mM MgSO₄]. Co-cultures were incubated at 30 °C for 24–48 h with vigorous aeration until predation was completed. The prey-independent *B. bacteriovorus* 109JA was grown on PY medium [peptone (10 g l⁻¹), yeast extract (3 g l⁻¹), pH 7.0] for 24 h at 30 °C. For PY agar plates 1 % agar was used. *Bdellovibrio* sp. strain JSS was cultured on *Caulobacter crescentus* CB2A as described previously (Koval & Hynes, 1991). When necessary, cells of *C. crescentus* CB2A in a co-culture were separated from *Bdellovibrio* sp. strain JSS by differential centrifugation. *E. coli* DH5 α and *E. coli* BL21(DE3) pLysS (Stratagene) were grown in Luria–Bertani (LB) medium at 37 °C.

DNA manipulations and PCR conditions. Chromosomal DNA was prepared and purified from *B. bacteriovorus* 109J, 109JA and *Bdellovibrio* sp. strain JSS by extraction with phenol/chloroform and precipitation with ethanol or by using the DNeasy Blood & Tissue kit (Qiagen), following the manufacturer's instructions. Primers were designed to the regions flanking the putative coding sequences of type IV pili genes in *B. bacteriovorus* HD100 (Table 2). A 1.4 kb DNA fragment including *pilA* and *pilG* (Bd1290 and Bd1291 respectively) was amplified from chromosomal DNA of *B. bacteriovorus* strains 109J and 109JA by PCR with *Pwo* DNA polymerase (Roche Diagnostics) under the following thermal cycling conditions: 95 °C for 4 min, followed by 30 cycles of 95 °C for 45 s, 50 °C for 45 s and 72 °C for 1 min, and a final extension of 7 min at 72 °C. A 7.6 kb DNA fragment including *pilHIDMNOPQ* (Bd860–Bd867) was amplified from chro-

mosomal DNA of *B. bacteriovorus* strains 109J and 109JA by PCR with the Expand Long Template PCR System (Roche Diagnostics) under the following thermal cycling conditions: 95 °C for 4 min, followed by 30 cycles of 95 °C for 15 s, 58 °C for 45 s and 68 °C for 5 min, and a final extension of 7 min at 68 °C. An ~ 6 kb DNA fragment including *pilBTCSR* (Bd1509–1513) and a 2 kb product of *pilM* (Bd1585) were amplified from chromosomal DNA of *B. bacteriovorus* strains 109J and 109JA under the same PCR conditions as for amplification of the *pilHIDMNOPQ* cluster except that the temperature of annealing was 56 °C. *pilM* (Bd1585) was amplified from *Bdellovibrio* sp. strain JSS as described above. *pilQ* (Bd867; 2.1 kb), *pilT1* (Bd1510; 1.0 kb), and *pilT2* (Bd3852; 1.2 kb) were amplified from chromosomal DNA of *B. bacteriovorus* 109J, 109JA and *Bdellovibrio* sp. strain JSS by PCR using *Pwo* DNA polymerase (Roche Diagnostics). Thermal cycling conditions for the three genes were: 95 °C for 4 min, followed by 30 cycles of 95 °C for 45 s, 54 °C for 45 s and 72 °C for 1 min, and a final extension of 7 min at 72 °C.

Overexpression and purification of the *pilA* gene product. The first 35 amino acid residues of the annotated mature pilin PilA of *B. bacteriovorus* 109J were truncated. The DNA sequence encoding residues 36–190 was amplified by PCR from the 1.4 kb DNA fragment containing *pilA* (Bd1290; 573 bp) using forward primer pilAF1 and reverse primer pilAR1 (Table 2). The PCR was performed using Platinum *Taq* DNA polymerase high-fidelity (Invitrogen) with an annealing temperature of 56 °C. The PCR product was subcloned in the *Nco*I and *Bam*HI sites of a pET23 derivative (Newton & Mangroo, 1999) with an N-terminal histidine tag (Table 2). The construct was confirmed by restriction analysis and sequencing. *E. coli* DH5 α was transformed with the recombinant plasmid pET23/*pilA*^(36–190). Colonies of *E. coli* DH5 α transformants were picked and grown in LB broth supplemented with 100 μ g ampicillin ml⁻¹ at 37 °C. The recombinant plasmid was purified using the illustra GFX

Table 2. Plasmids and primers used in this study

F, forward; R, reverse; restriction sites are underlined. O1 primers were designed to amplify operon 1: *pilHIDMNOPQ*. O2 primers were designed to amplify operon 2: *pilBTCSR*.

Plasmid or primer	Relevant characteristics or sequence (5'–3')	Source or reference
Plasmids		
pET23 derivative	Expression vector, allows production of fusion proteins containing N-terminal His ₆ -tagged sequences, Amp ^r	Newton & Mangroo (1999)
pET23/PilA ^{36–190}	pET23 containing a 468 bp fragment of <i>pilA</i> , Amp ^r	This study
Primers		
<i>pilAF1</i>	AGGGTCCATGGGCATGTTTTCAGTTAGCAGAA	This study
<i>pilAR1</i>	GCGTCGGATCCCTATGGGATACCTGGCG	This study
<i>pilAG/F</i>	ATAGGTCGAGAGTGCTAC	This study
<i>pilAG/R</i>	TTGAAGGCGCTTGATCAG	This study
O1F	TTGACTTTGGCACGCACCAC	This study
O1R	TTAGAAGTCAGTGTTGTCCG	This study
<i>pilQF</i>	ATGAACGGATTTCATTAGATTA	This study
<i>pilQF</i>	TTAGAAGTCAGTGTTGTCCG	This study
O2F	ATGTCTTCACTCAAAATCCGG	This study
O2R	CGACTACGCCTAGATTATACT	This study
<i>pilT1F</i>	ATGAGTCTGACCGAACTCC	This study
<i>pilT1R</i>	TTAGATACCTACCTTCTTAAGA	This study
<i>pilMF</i>	CGCCTCGACCAAAATCCAG	This study
<i>pilMR</i>	TCATCTTGTACCTTCTCAAC	This study
<i>pilT2F</i>	ATGGCAACAATTGATGAATTG	This study
<i>pilT2R</i>	TTAGCCCACTTTGGTGCG	This study

Micro Plasmid Prep kit (GE Healthcare) and was transformed into the expression strain *E. coli* BL21(DE3) pLysS (Stratagene). The cells were grown to OD₆₀₀ 0.6 in LB broth supplemented with 100 µg ampicillin ml⁻¹ and 30 µg chloramphenicol ml⁻¹. Expression was induced with 1 mM IPTG. After 4 h additional growth, cells were harvested by centrifugation, lysed by French press disintegration, and cell debris was removed by centrifugation. The overexpressed protein accumulated in the insoluble fraction of *E. coli*. The PilA^(36–190)–polyhistidine fusion protein was purified by Ni-chelation (Bio-Rad) under denaturing conditions using 6 M guanidine.HCl. PilA was eluted with 300 mM imidazole. The eluate was dialysed overnight against 50 mM ammonium bicarbonate + 0.1 % SDS and concentrated with polyethylene glycol (PEG) 800.

Production of polyclonal antibodies. The purified truncated PilA (150 µg) was emulsified with complete Freund's adjuvant (Sigma-Aldrich). The preparations were injected into two male New Zealand White rabbits. Serum samples from each rabbit were collected prior to immunization for use as negative controls. After 25 days, the rabbits were boosted with a further 100 µg purified protein emulsified with incomplete Freund's adjuvant. The rabbits were bled 10 days after boosting. They were boosted again at days 53 and 82. Immune sera collected were absorbed against total cell extracts of *E. coli* BL21(DE3) pLysS to remove non-specific antibodies. The specificity of the antibody was determined by slot blots against total cell extracts of *E. coli* expressing his-PilA and purified overexpressed PilA proteins.

SDS-PAGE and Western blot analysis. *B. bacteriovorus* 109J cells were separated from prey cells present in co-cultures by filtration through a 0.45 µm pore size filter. Proteins from prey cells, from cells of *B. bacteriovorus* 109JA growing axenically, and from whole-cell lysates of attack-phase cells of *B. bacteriovorus* 109J, were separated by SDS–PAGE on 15 % polyacrylamide gels. Proteins were electrophoretically transferred to nitrocellulose membranes (0.45 µm pore size) using a Mini-Transblot cell (Bio-Rad) and the recommended buffer. Membranes were blocked with 10 % non-fat dried milk in Tris-buffered saline plus Tween (TTBS; 20 mM Tris, 500 mM NaCl, 0.05 % Tween-20, pH 7.5) and then incubated for 1 h with pre-immune serum or immune antisera (1 : 1000 dilution in TTBS, 10 % non-fat dried milk). After washing with TTBS, antibody reactions were detected using secondary antibodies IRDye 800 CW goat anti-rabbit IgG (1 : 5000 dilution in TTBS; Li-Cor).

Removal of pili. Two litres of prey-independent *B. bacteriovorus* 109JA cells were grown overnight at 30 °C. Cells were harvested and the pellet was resuspended in 20 ml PBS (pH 7.2), vortexed vigorously for 5 min, and centrifuged at 12 000 g for 10 min at 4 °C to separate the bacterial cells (pellet fraction) from the pilus-enriched supernatant (sheared fraction). The supernatant was collected, filtered twice (0.45 µm, followed by 0.2 µm filtration), and concentrated fivefold using 10 000 molecular weight cut-off Amicon Centricon filtration devices (Millipore). Proteins were precipitated from the supernatant with 15 % trichloroacetic acid (TCA) at 4 °C overnight and then washed three times with cold acetone. Protein concentration was quantified using the Bio-Rad protein assay. Pili were also removed from *B. bacteriovorus* 109JA grown for 5–6 days on PY agar plates. Colonies collected from 40 plates were resuspended in 20 ml sterile PBS and treated as described above.

Transmission electron microscopy. Cells of *B. bacteriovorus* 109J, 109JA and *Bdellovibrio* sp. strain JSS were adsorbed to Formvar–carbon-coated copper grids. Excess fluid was removed by blotting the edge of the grid with Whatman no. 2 filter paper. Cells were fixed on the grid with 10 % buffered formalin (Mahmoud *et al.*, 2007) for 15 min at 4 °C. The fixative was removed and the grids washed three times with PBS (pH 7.4) and then three times with double-distilled H₂O. Cells were stained with 1 % uranyl acetate. For negative stains of cells in a plaque, a drop of water was applied to a plaque and the Formvar–carbon-coated copper grid was floated on the bacterial

suspension for about 1 min. The grids were blotted dry and processed as described above. Samples were examined with a Philips EM410 electron microscope operating at 60 kV. For measurement of the width of the pilus fibre, a Philips CM10 electron microscope equipped with ImageJ software was used.

Immunogold electron microscopy. Cells on nickel grids were fixed as described for negative staining. After fixation, cells were blocked with 1 % BSA in PBS (pH 7.4) for 30 min at room temperature, followed by incubation on a drop of either 1 : 100 or 1 : 250 diluted anti-PilA antiserum in 1 % BSA in PBS (pH 7.4) for 1 h at room temperature. The grids were washed three times for 5 min with 1 % BSA in PBS (pH 7.4), and then incubated with the secondary antibodies [1 : 25 diluted anti-rabbit 10 nm gold conjugate (Sigma-Aldrich) in 1 % BSA in PBS pH 7.4] for 45 min at room temperature. The grids were washed with 1 % BSA in PBS pH 7.4 and double-distilled H₂O and cells were negatively stained with 1 % uranyl acetate. Controls in which pre-immune serum was applied to the cells were performed. Controls in which only the secondary antibody was applied to the cells were used to check for non-specific binding of gold particles.

Immunofluorescence. To detect type IV pili on attack-phase cells, a 300 µl volume of *B. bacteriovorus* 109J cells grown overnight on *A. serpens* VHL was fixed in 900 µl 10 % buffered formalin pH 7.4 for 3 h at 4 °C. Cells were washed three times with PBS (pH 7.4), resuspended in 30 µl HM buffer, and incubated in 0.1 % Triton in PBS for 45 min at room temperature. To examine if pilin was expressed during attachment of attack-phase cells of *B. bacteriovorus* 109J to *A. serpens*, 0.5 ml fresh attack-phase cells of the predator grown for 24 h on *A. serpens* was mixed with 5 ml washed prey cells. Attachment of *Bdellovibrio* to *A. serpens* occurred within 25–30 min of setting the co-culture. A total of 300 µl of the culture was taken and treated as described above. Cells (10 µl) were spotted into one well of a six-well CLEAR CELL slide ER-203B-2 (Erie Scientific) and incubated for 20 min in a covered Petri dish to allow the sample to dry. The cells were permeabilized by passing the slide three times through a series of different concentrations of cold ethanol (50 %, 80 % and 95 %) for 2 min each. Cells were washed three times with PBS, blocked in 2 % BSA in PBS pH 7.4 for 40 min and incubated with anti-PilA antibody (diluted 1 : 100 in 2 % BSA in PBS) for 2 h at room temperature. Cells were washed twice with 2 % BSA in PBS and three times with PBS, and incubated with sheep anti-rabbit IgG conjugated to Cy3 for 1 h at room temperature; the slides were then washed with PBS four times. Attack-phase cells incubated only with the secondary antibody were examined for non-specific binding of the secondary antibody. Controls in which the pre-immune serum was applied to attack-phase cells, and to attack-phase cells during attachment to prey, were performed as well as immunofluorescence of cells attached to prey with the permeabilization step with ethanol omitted. All samples were examined with a Zeiss Axioskop II epifluorescence microscope. Images were acquired with Northern Eclipse software version 6.0 (Empix Imaging).

Predation assays with anti-PilA antiserum. Antibodies against PilA were used to test their ability to inhibit predation by *B. bacteriovorus* strains 109J and 100HD, and *Bdellovibrio* sp. strain JSS. Before the predation assays, 10 µl volumes of bdellovibrios (10⁵ p.f.u. ml⁻¹) were incubated for 45 min at 30 °C with anti-PilA antiserum and with diluted anti-PilA antiserum (1 : 10 and 1 : 50) in triplicate. Cells incubated with pre-immune serum and anti-TolA antiserum were used as controls. *E. coli* ML35, used as the prey cell for *B. bacteriovorus* strains 109J and HD100, and *C. crescentus* CB2A, used as prey cells for *Bdellovibrio* sp. strain JSS, were grown as described above. Prey cells (50 ml) were centrifuged, washed once with HM buffer, and resuspended in 5 ml HM buffer to OD₆₀₀ 1.7 for *E. coli* and 1.2 for *C. crescentus*. Prey cells (40 µl) were added to *Bdellovibrio*

cells (preincubated with sera) in a 100-well plate (Oy Growth Curves), and the decrease in turbidity monitored over 48 h using a Bioscreen C instrument (Oy Growth Curves). This experiment was repeated three times.

RESULTS

Identification of type IV pili biosynthesis gene homologues in *B. bacteriovorus* 109J and *Bdellovibrio* sp. strain JSS

With the use of primers designed to the regions flanking the putative coding sequences of type IV pili genes in *B. bacteriovorus* HD100, the presence of these genes in *B. bacteriovorus* strains 109J and 109JA was identified. The number and chromosomal arrangement of type IV pili genes were found to be exactly the same in strain 109J as in strain HD100. DNA sequence alignment showed 97–99 % homology to the corresponding genes of strain HD100. Type IV pili genes in *B. bacteriovorus* 109J include a single *pilA* gene (GenBank accession no. GU368922), which is not located within a pilus assembly gene cluster. However, another putative monocistronic gene, *pilG* (GenBank accession no. GU368924), encoding part of an ABC transporter required for pilus export (Evans *et al.*, 2007), was found downstream of *pilA*. The putative PilA has a leader peptide of 11 residues and the putative N-methylated N-terminus of *B. bacteriovorus* 109J is phenylalanine. The characteristic pair of cysteine residues, known to be involved in formation of a disulfide bond (D-region) that is important for pilin subunit interactions (Strom & Lory, 1993; Craig *et al.*, 2004), was found in *B. bacteriovorus* 109J at positions +121 and +132. PilA of *B. bacteriovorus* 109J possesses a short D-region consisting of only 10 residues.

B. bacteriovorus 109J possesses two genes annotated as *pilT1* and *pilT2* (GenBank accession nos GU368927 and GU368925, respectively). The first one is identical to the PilT protein encoded by Bd1510 in *B. bacteriovorus* HD100 and does not contain a conserved nucleotide-binding region (Walker box A). The derived amino acid sequence of this gene shows only 20–25 % similarity to the derived amino acid sequences of PilT in other species. In contrast, the second *pilT* gene encodes a PilT that contains a Walker box A [GPTGSGK(S)T] and a highly conserved C-terminal [AIRNLIRE] motif that are important for its function (Aukema *et al.*, 2005). The derived amino acid sequence of this gene is 45–60 % identical to the derived amino acid sequences of PilT in bacteria exhibiting pili retraction and thus twitching motility. Four genes of major components of the type IV pili system [*pilQ* (GenBank accession no. GU368930), *pilT1* (GenBank accession no. GU368929), *pilM* (GenBank accession no. GU368928), and *pilT2* (GenBank accession no. GU368931)] were identified by PCR in the epibiotic *Bdellovibrio* sp. strain JSS. Taken together, these results showed a high degree of synteny and sequence similarity in these gene regions and among

different strains of the genus *Bdellovibrio*, suggesting that type IV pili are widespread within the *Bdellovibrio* group.

Visualization of pili of *Bdellovibrio*

Distinct morphological characteristics used in electron microscopy studies to define type IV pili as a specific pilus are still used in their identification (Pelicic, 2008). Chemical fixation of attack-phase cells of *B. bacteriovorus* 109J for 15 min at 4 °C with 10 % buffered formalin improved the visualization of polar fibres. A total of 5200 cells were examined by transmission electron microscopy in different trials. Fibres resembling type IV pili could be detected on approximately 25 % of attack-phase cells ($n > 1300$). Fibres were extruded only from the pole opposite the flagellum. Fig. 1(A) shows branching of the fibre as noted by Touhami *et al.* (2006) in their study of *Pseudomonas aeruginosa* type IV pili fibres. Broken and unattached fibres were also seen frequently on the grid. They often interacted laterally to form typical bundles (Fig. 1C). The presence of broken fibres could be associated with the growth of these highly motile organisms in liquid co-cultures (Evans *et al.*, 2007). In addition, the strong collision of *Bdellovibrio* with prey cells, bdelloplasts or particles present in the co-culture could result in breakage of these fibres. This may explain the low number of fibres detected on cells in negative stains. A slight increase (27 %) in the number of fibres on attack-phase cells of strain 109J grown in plaques was observed ($n > 690$). However, detached fibres near attack-phase cells were seen more frequently on the grids. This detachment could be due to the effect of water during preparation of samples for electron microscopy (Touhami *et al.*, 2006). *Bdellovibrio* sp. strain JSS also had pilus-like fibres on the cell (Fig. 1D), which seem to be more flexible than the fibres of *B. bacteriovorus* 109J. Broken fibres were also seen but at lower frequency than with *B. bacteriovorus* 109J. The width of fibres present on cells of *B. bacteriovorus* 109J, 109JA and *Bdellovibrio* sp. strain JSS as well as broken fibres on the grids ranged from 5 to 8 nm, in accordance with the reported diameter of type IV pili (see Supplementary Fig. S1A, available with the online version of this paper).

Expression of pili on the cell surface of *Bdellovibrio*

In this study, and that of Evans *et al.* (2007), pili were detected on only 25–30 % of attack-phase cells of *B. bacteriovorus*. Factors that could have an effect on the regulation and expression of pili on the cell surface of the predator are unknown. A proteome analysis of *B. bacteriovorus* 109J showed that PilR (Bd1513), a response regulator to a two-component regulatory system PilSR, is highly expressed during the attack phase of *B. bacteriovorus* 109J (Dori-Bachash *et al.*, 2008). Expression of the *pilA* gene requires the alternative sigma factor 54 as well as the products of two other genes, *pilS* and *pilR* (Hobbs *et al.*, 1993; Wu & Kaiser, 1997). This suggests that expression of

Fig. 1. Electron micrographs of negatively stained preparations of: (A, B) attack-phase cells of *B. bacteriovorus* 109J showing fibres present on the cell surface; (C) bundles of unattached and broken fibres of *B. bacteriovorus* 109J; (D) *Bdellovibrio* sp. strain JSS showing pilus-like fibres. The circles highlight the branching of the pilus in (A) and the pilus-like fibre in (D). The bars: 0.5 μm (A, B and C); 0.1 μm (D).

pili in *B. bacteriovorus* could be regulated under certain environmental conditions. Chemotaxis and aerotaxis are two important features of *B. bacteriovorus* that can affect the encounter with prey cells. Previous studies suggested that *B. bacteriovorus* is attracted to high concentrations of prey cells (Straley & Conti, 1977) and shows chemotaxis toward some amino acids (LaMarre *et al.*, 1977), and aerotaxis (Straley *et al.*, 1979). Thus to examine whether the presence of prey cells could trigger a signal to express pili on the surface of the predator, different concentrations of prey cells (10^2 – 10^6 cells ml^{-1}) were added to freshly released attack-phase cells of *B. bacteriovorus* 109J. There was no increase in the number of attack-phase cells harbouring pili on their cell surface when cells were examined by electron microscopy ($n > 1070$). The effect of aeration on the expression of pili in *B. bacteriovorus* was also examined. There was no difference between the number of attack-phase cells expressing pili on their cell surface when preying under shaking ($n > 760$) or non-shaking conditions ($n > 775$).

Truncation of PilA

It has been shown in other type IV pili systems that the first 28–30 amino acid residues extend from the rest of the protein to form an oligomerization domain that inhibits the overexpression and purification of pilin in its native form (Craig *et al.*, 2004). To facilitate purification, a

truncated form of *B. bacteriovorus* 109J PilA lacking the first 35 amino acids was engineered. The resulting protein was fused to a His₆-tag at the N-terminus and over-expressed in *E. coli*. However, the fusion protein was found in the insoluble fraction. This may be due to the presence of hydrophobic amino acids residues in the C-terminus of *B. bacteriovorus* PilA. The overexpressed fusion protein was purified under denaturing conditions and used to produce polyclonal antibodies.

Identification of pili by antibodies against truncated PilA

Western blots of whole-cell lysates of attack-phase cells of *B. bacteriovorus* 109J and cells of the prey-independent strain 109JA were performed using different dilutions of anti-PilA serum. The antibodies recognized a band of ~20.0 kDa in the Western blot (Fig. 2B). The predicted size of the mature PilA protein is 19.6 kDa. A Western blot of whole-cell lysates of attack-phase cells of *B. bacteriovorus* 109J and cells of prey-independent 109JA with pre-immune serum is shown in Fig. 2(A). The anti-PilA antiserum was also used against whole-cell lysates of prey cells (*E. coli* ML35 and *A. serpens* VHL). No bands were detected in the Western blot (data not shown).

Pili were removed and precipitated from cells of *B. bacteriovorus* 109JA, grown in absence of prey cells in PY

Fig. 2. (A, B) Immunoblots of whole-cell lysates of attack-phase cells of *B. bacteriovorus* 109J and cells of prey-independent *B. bacteriovorus* 109JA with pre-immune serum (A) and anti-PilA antiserum (B). (C) SDS-PAGE of a crude pili preparation from *B. bacteriovorus* 109JA. (D) Immunoblot of (C) exposed to anti-PilA antiserum.

medium, by mechanical shearing and precipitation with 10% TCA. Cells of *B. bacteriovorus* 109JA used for isolation of pili were first examined by electron microscopy, and 18% of cells ($n > 570$) showed the presence of broken pili at their cell surface (Supplementary Fig. S1B). Electron microscopic examination of the crude pili preparation showed the presence of vesicles, flagella, broken fibres and bundles of fibres. SDS-PAGE analysis of the preparation yielded bands of different molecular masses (Fig. 2C). However, the anti-PilA antiserum only detected the 20 kDa band, corresponding to the size of PilA, in the immunoblot (Fig. 2D).

B. bacteriovorus forms plaques when grown on prey cells on double-layer agar plates. As pili were detected on only 18% of cells of *B. bacteriovorus* 109JA grown in liquid medium, this strain was grown on 1% PY agar to see if more cells had pili on their cell surface. Colonies of strain 109JA took 4–6 days to grow. However, we were not able to isolate pili from the harvested cells. This was probably due to the fact that the prey-independent *Bdellovibrio* exhibits a mixture of both short attack-phase cells and long filamentous cells

(Barel & Jurkevitch, 2001). A large number of cells isolated from colonies were long filaments lacking pili or cells that had lost their shape and become rounded when grown for such a long time on a rich medium (Supplementary Fig. S1C). This indicated that the use of prey-independent *Bdellovibrio* strains is sometimes limited only to cells that exhibit morphologically the attack-phase features.

Immunoelectron microscopy confirmed that the fibres resembling type IV pili were composed of PilA protein. Analysis of attack-phase cells of *B. bacteriovorus* 109J with dilutions of anti-PilA antiserum from 1:50 to 1:400 and 10 nm gold-labelled secondary antibodies showed label on attached (Fig. 3A) and unattached fibres (Fig. 3B). Sheared pili in the crude preparation from *B. bacteriovorus* 109JA as well as bundles of pili assembled on the grid were also labelled after immunogold staining (Fig. 3C). The antibodies bound to the sides and the end of the pilus fibre. Thus, truncation of the first 35 amino acid residues of PilA did not affect the antibody recognition of the pilus fibre. It has been shown that this hydrophobic sequence is buried in the core of the pilus of *Neisseria gonorrhoeae* and is not

Fig. 3. Electron micrographs of negatively stained and 10 nm-immunogold-labelled type IV pili of (A, B) *B. bacteriovorus* 109J with anti-PilA serum, (C) bundles of pili from the enriched preparation. For both preparations, antiserum was diluted 1:100. Bars, 0.5 μm.

significantly immunogenic (Forest *et al.*, 1996). Clusters of gold particles sometimes seen in the background could represent small pilin aggregates or pilin subunits. Cells incubated with pre-immune serum or incubated only with secondary antibody did not show labelling of bacteria or background.

Immunofluorescence of *B. bacteriovorus* 109J

Immunofluorescence microscopy provided further insights into *B. bacteriovorus* 109J pilus expression during the life cycle of the predator. *A. serpens* VHL was used as the prey cell in these experiments as the large bdelloplast formed (Mahmoud *et al.*, 2007) facilitated the visualization of fluorescent signals. Signals were seen at the cell pole of 30 % of attack-phase cells of *B. bacteriovorus* 109J ($n > 570$; Fig. 4A), but were absent in cells incubated with pre-immune serum (Fig. 4B). Fluorescent signals detecting pilin were observed on 68 % of attack phase cells at the site of attachment to prey cells ($n > 778$; Fig. 4C and Supplementary Fig. S2A). The fluorescent signals were also detectable on attack-phase cells after invasion of the prey, inside the bdelloplast (Fig. 4D). We did not detect any fluorescent signals with cells of 109J attached to *A. serpens* with the permeabilization step omitted (Supplementary Fig. S2B). These observations confirmed that the antibody can recognize the native PilA protein. Additionally, in all cells examined, fluorescence signals were seen on only one pole, consistent with electron microscopy and immunogold observations. Cells incubated with only secondary

antibody showed no fluorescence signals (results not shown).

Inhibition of predation of *Bdellovibrio* with anti-PilA antiserum

Because the antibody can bind to pilus fibres, we examined the effect of preincubation with anti-PilA antiserum on predation of *B. bacteriovorus* 109J and HD100 in co-cultures with *E. coli*. Cells pre-incubated with anti-PilA antiserum exhibited an inhibition in predation during the first 18 h compared with cells not incubated with antiserum or cells pre-incubated with the pre-immune serum (Fig. 5A, B). Incubation of predators with polyclonal antibodies to TolA, an inner-membrane protein of *E. coli*, had no effect on predation by either strain of *B. bacteriovorus* and showed the same pattern of predation as the controls (Fig. 5A, B), indicating that inhibition of predation of *B. bacteriovorus* was specific to anti-PilA antibodies in the serum. Diluted antisera (1:10 and 1:50 respectively) showed also an inhibition of predation of both strains of *B. bacteriovorus* (Supplementary Fig. S3). Co-cultures were examined by phase-contrast microscopy at different time points. The *Bdellovibrio* cells were motile but there was no attachment to prey cells during the first 16 h in 30 fields of view examined. We did not see formation of bdelloplasts, an indication of penetration of predators into prey cells, until 18–20 h of co-culture incubation. Attack-phase cells incubated with pre-immune sera or cells incubated with anti-TolA antibodies were motile and attached to prey cells

Fig. 4. Detection of PilA in *B. bacteriovorus* 109J by immunofluorescence. Phase-contrast images (left) and epifluorescence images (right). (A) Attack-phase cells immunostained with anti-PilA serum; (B) attack-phase cells attached to *A. serpens* with pre-immune serum; (C) fluorescent signal (circled) detected at the site of attachment of attack-phase cell to *A. serpens* after incubation with anti-PilA antiserum; (D) growth-phase cell (arrowed) inside the *A. serpens* bdelloplast following penetration labelled with anti-PilA antibodies. Bars, 10 μ m.

Fig. 5. Growth curves of predators pre-incubated with undiluted pre-immune serum, anti-PilA antiserum and anti-TolA antiserum in liquid co-cultures. Predation was determined by assessing the reduction in turbidity (OD_{600}) of the co-culture over time. Data points represent the mean of three independent experiments. (A) Predation of *B. bacteriovorus* 109J on *E. coli*; (B) predation of *B. bacteriovorus* HD100 on *E. coli*; (C) predation of *Bdellovibrio* sp. strain JSS on *C. crescentus* CB2A.

after 2–4 h of incubation. A decrease in turbidity, indicating prey lysis and release of new attack-phase cells of *Bdellovibrio*, was observed after 6 h. Anti-PilA antibody also inhibited and delayed predation of the epibiotic *Bdellovibrio* sp. strain JSS on *C. crescentus* (Fig. 5C). Pre-incubation of JSS with diluted antisera (1 : 10 and 1 : 50) also inhibited and delayed predation of strain JSS on *C. crescentus* (Supplementary Fig S3). The release of new progeny cells of predator was observed after 18–20 h. Ghost cells of empty *C. crescentus* were clearly detected in the co-culture after 24–30 h of predation.

DISCUSSION

Little is known about how BALOs attack and invade their prey cells. Type IV pili are multifunctional fibres essential for adhesion, invasion of host cells and twitching motility in other bacteria. Therefore, they are candidate structures for involvement in attachment and penetration of *Bdellovibrio* into prey cells. A definitive localization study of PilA protein has not, to our knowledge, been previously reported, although mutant analysis and expression studies have suggested that PilA is the major subunit of fibres present on the cell surface of *B. bacteriovorus* (Evans *et al.*, 2007). *B. bacteriovorus* HD100 also carries genes for fimbrial low-molecular-mass protein (Flp), part of the *hit* locus, a large cluster of adhesion-related genes (Rendulic *et al.*, 2004). However, Schwudke *et al.* (2005) performed SDS-PAGE and liquid chromatography-mass spectrometry of cell envelope preparations of *B. bacteriovorus* HD100 and were unable to locate any Flp protein, the main subunit of the Flp pilus. However, PilA was found in the extracts. To confirm that the polar fibres of *B. bacteriovorus* were type IV pili, an antibody against a truncated form of PilA was generated. The antibody was specific to fibres attached to *B. bacteriovorus* 109J attack-phase cells, unattached broken fibres present near the attack-phase cells, and to fibres isolated from cells of the prey-independent strain 109JA. Thus PilA is the major subunit in *B. bacteriovorus* polar pili.

Immunofluorescence studies showed that *Bdellovibrio* could use type IV pili for adhesion to prey cells during irreversible attachment. Cultures were examined by phase-contrast microscopy a few minutes after attachment of predators to *A. serpens*, when *B. bacteriovorus* cells were

reversibly attached. Pilin was detected on predators after penetration, inside the bdelloplast and just before the initiation of the growth phase. These results confirm a previous study, which reported that the *pilA* gene is highly expressed during the *B. bacteriovorus* life cycle and that there was no significant difference in *pilA* mRNA levels of expression during bdelloplast formation, after predatory invasion and maturation (Evans *et al.*, 2007). Taken together, these data suggest that PilA also plays a role during the growth phase of the predator.

To examine the role of PilA in predation, we pre-incubated the predator with antibody against PilA and then monitored, by turbidity measurements, the growth of predators in co-cultures. Predation by *B. bacteriovorus* 109J and HD100 was delayed for almost 18 h and there was overall an inhibition of predation during the 48 h of growth compared with attack-phase cells incubated with pre-immune sera or with another non-specific polyclonal antibody. This inhibition is probably due to blocking of the attachment of predators to prey cells, as antibody binding to type IV pili can block bacterial adhesion (Rothbard *et al.*, 1985; Wu *et al.*, 2005). A *pilA* mutant of *B. bacteriovorus* strain HD100^T was shown by Evans *et al.* (2007) to be non-predacious. In *Myxococcus xanthus*, antibodies against PilA blocked pilus retraction and decreased motility (Li *et al.*, 2005). *B. bacteriovorus* could use the pilus as both a structural protein and an adhesin as in *P. aeruginosa* (Craig *et al.*, 2004; Burrows, 2005). The D-region in the C-terminus of PilA is exposed at the surface of the pilus and it binds to biotic and abiotic surfaces (Giltner *et al.*, 2006).

After irreversible attachment, the predator penetrates its prey cell to start the growth phase. The mechanism by which *Bdellovibrio* penetrates its prey cell is still unclear. When *Bdellovibrio* anchors firmly to the outer membrane of the prey cell, this results in a cascade of quick events. Electron microscopy in our lab and others (Burnham *et al.*, 1968; Evans *et al.*, 2007) showed that *Bdellovibrio* squeezes itself through a tiny pore into the prey periplasm and penetration happens quickly, within 1–2 min. *B. bacteriovorus* does not have type III or type IV secretion systems, although the predator secretes hydrolytic enzymes in a local manner to dissolve the prey's cell wall. *B. bacteriovorus* possesses type I and type II secretion systems as well as a twin arginine translocation system (TAT; Rendulic *et al.*, 2004). The type II secretion system shares a number of homologous components with type IV pili (Lory, 1998; Hansen & Forest, 2006). It has been shown that type II secretion facilitates the secretion of proteins (including toxins, proteases and lipases) from the periplasm to the extracellular milieu in Gram-negative bacteria (Sandkvist, 2001; Sandkvist *et al.*, 1997). The general secretion pathway protein D (Bd1597) is strongly expressed during the attack phase of *B. bacteriovorus* (Dori-Bachash *et al.*, 2008). Because attachment and penetration of the prey occur in a short period of time (5–20 min), an overlap between the components required for type IV pili biogenesis and

those required for type II secretion could be present in *Bdellovibrio*. *Francisella tularensis*, a pathogenic bacterium, possesses PilF, a protein essential for both type IV pili biogenesis and type-II-like protein secretion systems (Zogaj *et al.*, 2008).

During penetration into prey cells, the predator has to overcome the forces exerted by the turgor pressure of the prey cell. It has been suggested that *Bdellovibrio* could use retraction of type IV pili to squeeze itself inside the prey (Evans *et al.*, 2007; Borgnia *et al.*, 2008). Retraction of a single type IV pilus by a single PilT motor generates forces of 50–140 pN (Maier *et al.*, 2002). A study by Biais *et al.* (2008) showed that in *Neisseria gonorrhoeae*, type IV pili bundling occurs after an initial anchoring of a single type IV pilus to a surface. This results in formation of higher-order structures that retract to forces up to 8–10 times higher than the 140 pN measured for a single fibre. For *Bdellovibrio*, type IV pili are involved in attachment to prey cells. The idea that the predator could use the retractile forces exerted by type IV pili to facilitate prey entry requires further studies.

An interesting model to be considered in studying invasion of prey cells by BALOs is the epibiotic *Bdellovibrio* sp. strain JSS. This predator adheres firmly to the stalked cell of *C. crescentus* and growth occurs at the site of attachment to the prey (Shemesh *et al.*, 2003). There is no penetration of the prey cell or formation of a bdelloplast. Work in progress in our laboratory shows that strain JSS attaches to and preys on other bacteria besides *C. crescentus*, which indicates that prey are not limited to this species (S. F. Koval, unpublished data). We detected the presence of polar fibres on attack-phase cells of strain JSS, which resemble the pili detected on *B. bacteriovorus* 109J and HD100. Four type IV pili genes encoding proteins important in the biogenesis of type IV pili were found in strain JSS. Pre-incubation of anti-PilA antiserum with strain JSS also inhibited its predation on *C. crescentus*, suggesting that the fibres on its surface are composed of PilA. Further studies on pili in strain JSS are under way and should help to address the question of why this epibiotic strain does not enter its prey cell.

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