JOURNAL OF BACTERIOLOGY, July 2007, p. 4850–4859 Vol. 189, No. 13
0021-9193/07/$08.00+0 doi:10.1128/JB.01942-06

Predation by *Bdellovibrio bacteriovorus* HD100 Requires Type IV Pili▽†

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Received 22 December 2006/Accepted 30 March 2007

Early electron microscopy and more recent studies in our laboratory of *Bdellovibrio bacteriovorus* cells indicated the presence of narrow fibers at the nonflagellar pole of this unusual predatory bacterium. Analysis of the *B. bacteriovorus* HD100 genome showed a complete set of genes potentially encoding type IV pili and an incomplete gene set for Flp pili; therefore, the role of type IV pili in the predatory life cycle of *B. bacteriovorus* HD100 was investigated. Alignment of the predicted PilA protein with known type IV pilins showed the characteristic conserved N terminus common to type IVa pilins. The *pilA* gene, encoding the type IV pilus fiber protein, was insertionally inactivated in multiple *Bdellovibrio* replicate cultures, and the effect upon the expression of other pilus genes was monitored by reverse transcriptase PCR. Interruption of *pilA* in replicate isolates abolished *Bdellovibrio* predatory capability in liquid prey cultures and on immobilized yellow fluorescent protein-labeled prey, but the mutants could be cultured prey independently. Expression patterns of *pil* genes involved in the formation of type IV pili were profiled across the predatory life cycle from attack phase predatory *Bdellovibrio* throughout the intraperiplasmic bdelloplast stages to prey lysis and in prey-independent growth. Taken together, the data show that type IV pili play a critical role in *Bdellovibrio* predation.

Bdellovibrio bacteriovorus are small, highly motile, gram-negative deltaproteobacteria that prey upon other gram-negative bacteria such as *Escherichia coli*, *Salmonella*, and *Proteus* species (11, 31). This is accomplished through collision and attachment to prey, followed by invasion and establishment of the predator within the prey periplasm; the prey cell is modified by the predator to form an osmotically stable structure called the bdelloplast. Within the bdelloplast, the predator proceeds to degrade host macromolecules into their constituent monomers and transport them into the growing *Bdellovibrio* filament, where they are reassembled as required. When the prey cell is exhausted, the filament septates, and progeny *Bdellovibrio* grow flagella and lyse the prey ghost to escape.

Bdellovibrio organisms also have the ability to survive in the absence of prey species in a growth phase known as the prey- or host-independent (HI) state, and these cells are isolated from prey- or host-dependent (HD) cells (27). HI *Bdellovibrio* is extremely pleomorphic and can be facultatively predatory or obligately HI; no one mutation has been found to cause this exact phenotype (2), but the phenomenon is well known in *Bdellovibrio* biology (27). The HI state is a useful tool for culturing and subsequent analysis of mutations that have adverse effects on predation, which would be lethal in the HD state (7). In this study and in previous mutational work (17), we independently derived several lines of HI strains from an isogenic merodiploid HD population containing the target inactivated gene. This allows us to generate a single gene disruption in the chromosome and to study the phenotypic effects that the gene disruption has upon *Bdellovibrio* predation, without any concern about background variations in HI cell behavior.

The genome sequence of *B. bacteriovorus* HD100 (22) shows genes encoding a full set of type IV pilus genes dispersed around the chromosome and an incomplete set of genes encoding Flp pili, which are a specific subset of type IVb pili found in diverse bacterial and archaeal species (14). We along with others proposed that *Bdellovibrio* may use pili as a mechanism of entering the prey cell, possibly via attachment to cell wall through a previously generated pore in the prey outer membrane (22). It can be seen on electron micrographs of invading *Bdellovibrio* (4) (Fig. 1A and B) that the pore formed in the outer membrane is small and a "tight fit" for the invading *Bdellovibrio*. Thus, a significant force may be required for prey entry by predator. Attachment of *Bdellovibrio* to prey cells could not be disrupted by either vortexing or brief sonication (4), indicating a strong interaction between predator and prey. Type IV pili in other bacterial species such as *Myxococcus*, *Neisseria*, and *Pseudomonas* are well characterized and have been shown to be involved in many functions, including host cell adherence and invasion, twitching motility, and fruiting body formation (reviewed in references 5 and 20). Type IV pili have also been demonstrated to have considerable retractile forces of greater than 100 pN (18), which would provide the significant force required to facilitate prey cell entry by *Bdellovibrio*.

In this work, we discuss the rationale for and inactivation of the type IVa pilus fiber protein, PilA, in *B. bacteriovorus* HD100. The resulting mutant strains were incapable of prey entry and therefore predation; as such, they had to be grown HI. Reverse transcriptase-PCR (RT-PCR) analysis of *pilA* and other type IV pilus-associated genes in the HD100 genome showed logical differential expression at different time points over the *Bdellovibrio* predatory life cycle and, for some of the type IV genes, in the *pilA* mutant strain. We therefore conclude that expression of the *Bdellovibrio pilA* gene, and by

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† Supplemental material for this article is available at http://jb.asm.org/.

▽ Published ahead of print on 6 April 2007.

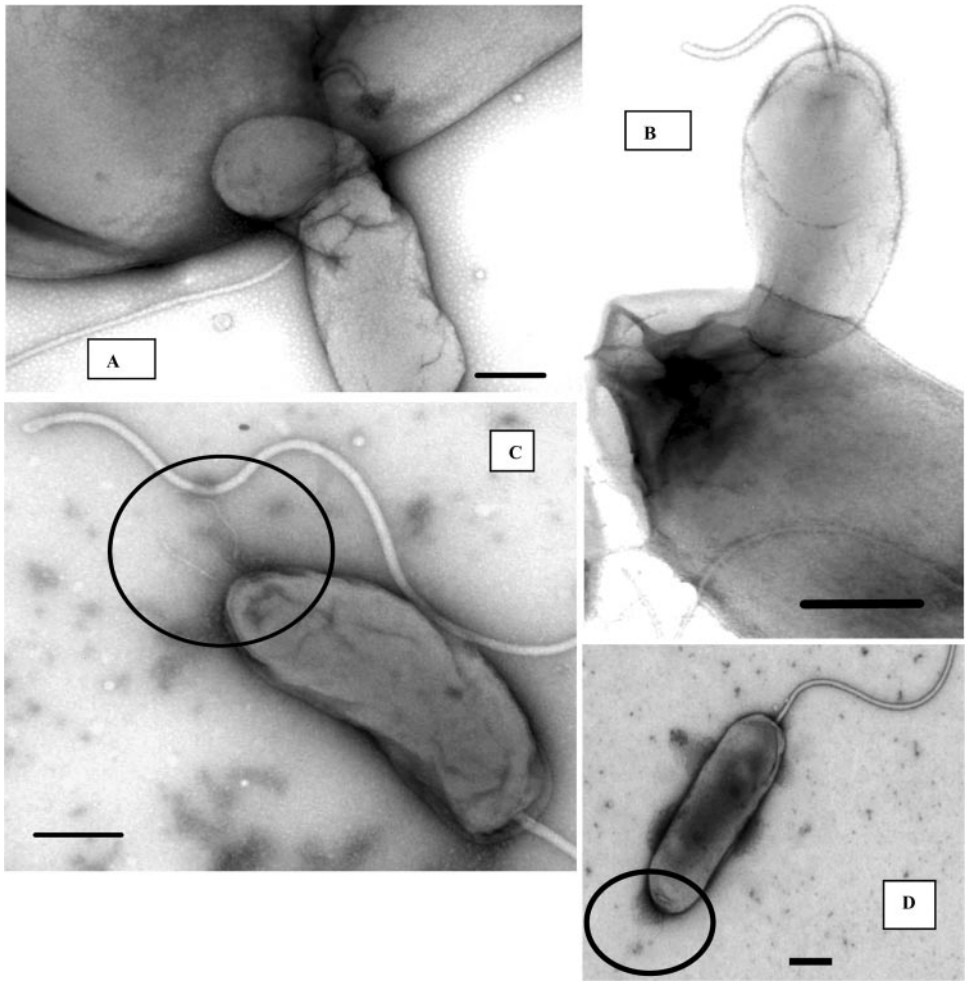

FIG. 1. Transmission electron micrographs of *Bdellovibrio* invasion and anterior fibers. (A and B) Wild-type HD100 *Bdellovibrio* invading *E. coli* S17-1 prey; prey invasion is accomplished through polar entry of the *Bdellovibrio* into the prey periplasm. (C) Anterior pilus-like fibers on HD100. The circle indicates the position of fiber attachment to the cell. Pilus-like fibers were seen on approximately 30% of cells examined ($n >$ 1,500). (D) Anterior pilus-like fibers on *B. bacteriovorus* strain 109J. The circle indicates the position of fiber attachment to the cell pole. Pilus-like fibers were seen at roughly the same frequency as in HD100 cultures ($n >$ 1,000). Bar, 0.2 μm.

inference, type IV pili, is essential to the predatory life style of *B. bacteriovorus* HD100.

MATERIALS AND METHODS

***Bdellovibrio* culturing on prey and prey independently.** Bacterial strains used in this study are listed in Table 1. Predatory (HD) cultures of *B. bacteriovorus* HD100 were grown as described previously (16) on *E. coli* S17-1, containing the pZMR100 kanamycin resistance plasmid as appropriate, in Ca^{2+}-HEPES buffer at 29°C, with shaking at 200 rpm. Prey-independent growth and isolation of HI strains were carried out as described previously (17). Briefly, HI strains were derived several times (see Fig. S10 in the supplemental material) from *pilA*/*pilA*::Km prey-dependent merodiploid HD100 strains by filtration through a 0.45-μm-pore-size filter to separate out prey cells; and cells were then plated on rich peptone-yeast extract (PY) medium containing kanamycin to retain selection for the interrupted *pilA* gene. Subsequent HI growth was axenic, without prey, in PY broth or on PY agar plates as described previously (28). *E. coli* strains were grown as standard in yeast extract-tryptone broth with appropriate antibiotics at 37°C with shaking at 200 rpm.

Insertional inactivation of the *pilA* gene. The wild-type *pilA* gene (accession no. CAE79186, EMBL BX842649.1, and gi 39574009) was amplified from HD100 genomic DNA by PCR using KOD high-fidelity DNA polymerase in buffer 2 for genomic DNA (Novagen). The full-length gene was cloned with 1 kb flanking DNA on either side using the *pilA* cloning primers (Table 2). The resulting 2.3-kb fragment was gel purified and digested with BamHI and XbaI using unique sites within the genomic sequence and then ligated into pUC19 digested with the same enzymes (24). A 1.3-kb kanamycin resistance cassette, released from pUC4K (16) by digestion with HincII, was gel purified and blunt ligated into a blunted XcmI unique site 204 bp into the coding sequence of *pilA* (37). This construct was cloned into conjugative plasmid pSET151 (3) and used to insertionally inactivate (16) the cognate *pilA* gene in the *Bdellovibrio* HD100 genome. For the conjugation, 40 ml of donor *E. coli* S17-1 containing the pSET151 construct pKJE102 was grown to an optical density at 600 nm (OD_{600}) of 0.2 to 0.4, concentrated by centrifugation to 100 μl, and added to 10 ml of overnight attack phase *B. bacteriovorus* HD100 similarly concentrated onto a nylon filter on a PY plate. After overnight incubation at 29°C, the cells were resuspended from the filter into yeast extract-tryptone medium, and various dilutions were plated onto YPSC (yeast extract, peptone, sodium acetate, calcium chloride,) overlay plates to give single exconjugant plaques for purification. Resulting merodiploid exconjugants were subject to screening for prey-dependent (HD) growth by plaquing on kanamycin-resistant prey *E. coli* S17-1:pZMR100 (2) and subsequent continual subculturing in standard Ca^{2+}-HEPES and *E. coli* S17-1:pZMR100 prey liquid lysate cultures in the presence of 50 μg ml^{-1} kanamycin sulfate (16) The HI growth mode of *Bdellovibrio* was also employed to allow growth of mutant strains that might be nonpredatory (see details in previous section). HI growth was used in case the interruption of the *pilA* gene gave a mutation that interfered with normal HD predatory growth. Several lines of cultures were passed through these treatment regimens for both

TABLE 1. Strains used in this study

Strain	Description	Reference or source
E. coli S17-1	*thi pro hsdR*$^-$ *hsdM*$^+$ *rec*A; integrated plasmid RP4-Tc::Mu-Km::Tn7	30
E. coli S17-1:pZMR100	Plasmid vector used to confer Km^r on S17-1; used as prey for Km^r *Bdellovibrio* strains	23
E. coli S17-1:pKJE102	pSET151 suicide plasmid containing *pilA*::Km^r and BamHI and XbaI fragments in conjugation donor strain S17-1; used for gene interruption in *Bdellovibrio*	3; this study
E. coli S17-1:pSB3000, pZMR100	YFP (Clontech) cloned into plasmid pDEST3 cotransformed with pZMR100; used as luminescent prey for Km^r *Bdellovibrio* strains	P. J Hill, University of Nottingham (unpublished)
B. bacteriovorus HD100	Type strain; genome sequenced	22, 31
B. bacteriovorus HD100 *pilA*/*pilA*::Km	Merodiploid HD100 with wild-type and kanamycin-interrupted *pilA*	This study
B. bacteriovorus $pilA_{HI}$/*pilA*::Km	Merodiploid HI derivative of HD100 with wild-type and kanamycin-interrupted *pilA*	This study
B. bacteriovorus $pilA_{HI}$::Km	Prey-independent HD100 derivative with kanamycin-interrupted *pilA* only	This study
B. bacteriovorus fliC3::Km	Prey-independent derivative of 109J *fliC3*::Km	This study

HD and HI growth to guard against any spontaneous mutational events giving rise to alterations in phenotypes (see Fig. S10 in the supplemental material).

Transmission electron microscopy. A total of 15 μl of *Bdellovibrio* and/or *E. coli* cells was applied to carbon-formvar-coated electron microscopy grids (Agar Scientific) and allowed to settle for up to 5 min. Excess cells were removed by blotting with Whatman paper, and the grids were stained with 1% uranyl acetate, pH 4, for between 30 s and 1 min. The stain was removed, and the grids were allowed to dry before observation at 100 kV using a JEOL JEM 1010 transmission electron microscope.

RNA isolation and RT-PCR. For RT-PCR analysis, synchronous predatory cultures were set up as described previously (17) with samples collected at 15, 30, 45, 60, 120, 180, and 240 min postinfection. HI cultures were grown in PY broth, and their OD_{600} values were matched (to 0.6) as described previously (17). RNA was isolated with modifications published elsewhere (17) on the Promega SV total RNA isolation kit, and RT-PCR was performed using a QIAGEN One-Step RT-PCR kit as described previously (17) with the following conditions: one cycle of 50°C for 30 min and 95°C for 15 min and then 25 cycles of 94°C for 1 min, 48°C for 1 min, and 72°C for 2 min, followed by one cycle of 72°C for 10 min and then a hold at 4°C. Twenty-five cycles of amplification were used so that the PCR did not go to extinction, allowing a semiquantitative view of mRNA levels at a particular time point. Control reactions using the same primers as for RT-PCR to test against the presence of contaminating *Bdellovibrio* DNA in the RNA samples were carried out using PCR with *Taq* DNA polymerase (ABgene) under the same PCR conditions. Inclusion of *E. coli* S17-1 RNA controlled for any cross-reactivity against the *Bdellovibrio pil* primers (none was seen). RT-PCR was carried out for various *pil* genes, including *pilA*, using the primers shown in Table 2.

TABLE 2. PCR and RT-PCR primers used in this study

Product or primers	Primer sequence
pilA plus flanking regions for chromosomal replacement wild-type gene and PCR screening of mutants	5′CGGAATTCCAGGCGAGGGTG AACTCAGG
	5′CGGAATTCTTGAGTCAAGCG ACCGGTGC
pilA RT-PCR internal primers	5′GAAAGCTCGTCAGTCCGAAG
	5′CACCCGCTAGCACAGTACAA
pilQ RT-PCR internal primers	5′TCGTCAGGTGACTCAGAACG
	5′GAACACCCACTTCACCGTCT
pilT1 RT-PCR internal primers	5′TCTGTGACCAATGCTCTTCG
	5′CAAAGTCAGAATGCCGGAGT
pilT2 RT-PCR internal primers	5′GGAACTGGATTTCGCCTACA
	5′ACCACGATCACAATCGATCA
pilD RT-PCR internal primers	5′GCTGTTTGCGCTTTCTTACC
	5′ACGTGCAGACAACAAGTCCA
hit Bd0108 RT-PCR internal primers	5′TCCTTTCCATCTTGCTGACC
	5′AGGCCTCATTAGGGTCTTCG
pilG RT-PCR internal primers	5′GCGTTGTTGATGGTGTGTTC
	5′CATCGGAGGAGGAGCTATCA
pilA QRT-PCR internal primers	5′GCGGTTTCTCGCTTGTAGAG
	5′CTTCGGACTGACGAGCTTTC

Quantitative real-time RT-PCR was carried out on Stratagene MX3005P or MX4000 machines, using the Statagene Full Velocity SYBR Green QRT-PCR kit in one-step reactions. Extensive optimization of primer and template concentrations was carried out to achieve specific amplification of the target gene, and this was confirmed by dissociation curve, agarose gel electrophoresis, and sequencing of the extracted PCR product. For each sample, serial dilutions of template were used to confirm the efficiency of the PCR, and absolute quantification of initial transcript amounts was by comparison to a standard curve using the pure transcript PCR product as a template. Control reactions with no template, with no reverse transcriptase, and with *E. coli* S17-1 RNA as a template were carried out and gave no significant amplification. Two independent experiments were carried out with a minimum of six replicate experiments on each sample.

Fluorescent assay for predatory capability. To assess the predatory capability of the *pilA*::Km mutant, constitutively yellow fluorescent protein (YFP)-expressing Km^r *E. coli* S17-1:pSB3000 pZMR100 prey grown overnight to an OD_{600} of 1.5 was challenged, on a solid PY agar surface, with $pilA_{HI}$::Km mutant and $pilA_{HI}$/*pilA*::Km merodiploid strains and also with an HID2 wild-type *Bdellovibrio* HI strain derived from strain HD100 (experiments were tried on more dilute LB agar but this could not support *Bdellovibrio* viability during the experiment). Repeat experiments were carried out using independently derived HI *pilA*::Km mutant strains, paired with their merodiploid parental strains and chosen for their diverse morphologies (see Fig. S1 in the supplemental material). The idea of this approach was that, as the HI phenotype has not been precisely defined by investigators in the *Bdellovibrio* field (2) and appears to involve phenotypic variation in different HI derivatives (e.g., cell size and shapes vary for individual HI derivatives of any *Bdellovibrio* strain), we wished to test the effects of *pilA* interruption in morphologically diverse HI strains that we had derived during our mutant screening. If a common phenotype was seen for all, we were satisfied that this was due to the *pilA* mutation.

To test predatory properties, the $pilA_{HI}$ strains (merodiploids and mutants) were grown for 3 days in PY medium with 50 μg ml^{-1} kanamycin at 29°C to an OD_{600} of 0.8 ± 0.05, and YFP prey were grown on 50 μg ml^{-1} ampicillin and 50 μg ml^{-1} kanamycin. A total of 50 μl of prey and 50 μl of *Bdellovibrio* culture were mixed, spotted onto PY agar plates supplemented with 50 μg ml^{-1} kanamycin, and incubated at 29°C for 24 h. For the same test using the HID2 strain, cells were grown for 3 days in PY broth only, at 29°C with shaking at 200 rpm, to the same OD as for $pilA_{HI}$ strains, and mixed with YFP prey that had been grown on 50 μg ml^{-1} ampicillin alone to select for the YFP-expressing plasmid (HID2 has no Km^r gene, and so its growth would be inhibited by the presence of kanamycin). This cell mixture was then spotted onto PY-only agar plates and again incubated for 24 h. At this time, the cells were scraped from the surface of the plate, resuspended in 1 ml of Ca^{2+}-HEPES buffer (17), pelleted by centrifugation for 1 min at 13,000 rpm in a benchtop centrifuge, and resuspended in a final volume of 100 μl. Five-microliter samples were agar mounted and examined under phase-contrast microscopy and YFP optics (excitation, 500 nm) on a Nikon Eclipse E600 epifluorescence microscope; images were taken using a Hamamatsu Orca ER camera and analyzed using IPLab, version 3.6. *E. coli* uninfected prey and infected bdelloplast numbers were counted ($n > 2{,}500$ *E. coli* cells per experiment).

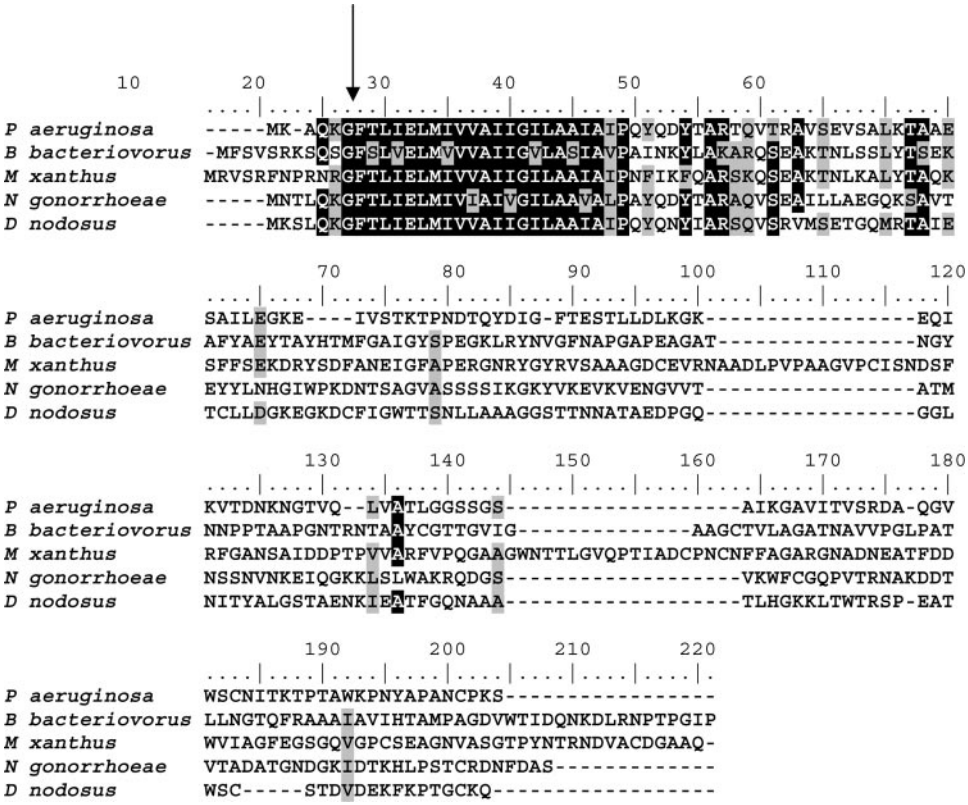

FIG. 2. Alignment of type IVa pilin proteins (8) showing *Bdellovibrio bacteriovorus pilA* to have the highly conserved N-terminal sequence homology common among type IVa pilins and phenylalanine that forms the N-terminal residue in the mature protein (indicated by the arrow). The processed *Bdellovibrio* peptide is predicted to be 179 amino acids in length. *N. gonorrhoeae*, *Neisseria gonorrhoeae*; *D. nodosus*, *Dichelobacter nodosus*. Black shading indicates identity of conserved residues; grey shading indicates conservatively substituted homologous residues. The N terminus of the mature protein is indicated by the arrow.

RESULTS

In this study we aimed to test any predatory role for the type IVa pilus fiber, the *pilA* gene homologue, from the *B. bacteriovorus* HD100 genome and to assay the expression of type IV pilus genes found distributed around the chromosome. Type IVa pili are used for twitching motility in *Pseudomonas* and cell-cell contact leading to social motility of *Myxococcus* and adherence in *Neisseria* (reviewed in reference 20). In a previous study Schwudke and coworkers (26) noted varying expression over a predatory cycle of a putative type IVb *flp-1* gene homologue in *Bdellovibrio* (22), which encodes the pilus fiber of Flp pili. In other bacteria these are a specific subset of type IVb pili (14) used for tight adherence to surfaces and are best characterized in *Actinobacillus actinomycetemcomitans*, the causative agent of juvenile periodontitis (14, 35; reviewed in reference 12).

However, we believe that Flp pili are unlikely to be functional in *B. bacteriovorus* HD100 as our BLAST analysis of the HD100 genome has shown that, while there is a full set of genes that would encode type IV pili (*pil* genes), only some of the genes present would encode Flp pili (see Fig. S6 and S7 in the supplemental material). Furthermore, the proteomic approaches employed by Schwudke and coworkers (26) failed to find any Flp-1 protein in cell envelope preparations of *B. bacteriovorus* HD100 but did find PilA, the type IVa pilus fiber protein.

Bioinformatic analyses revealed a candidate pilus fiber gene in HD100, *pilA* and its associated full complement of *pil* genes which, in other bacteria, are sufficient to assemble functional pili. PilA proteins are found in many gram-negative bacteria and show characteristic sequence homologies (8). The HD100 predicted PilA (Bd1290; CAE79186) sequence showed the greatest homology to *Myxococcus xanthus* PilA; a BLAST search of the HD100 PilA sequence through the NCBI database (25) brought up *M. xanthus* with an E value of 9e-09, with 28% identity over the whole length of the protein. Figure 2 shows an alignment of *Bdellovibrio* with other type IVa pilins, illustrating the conserved N-terminal and divergent C-terminal regions characteristic of this family of proteins. In *M. xanthus*, PilA has been shown to be the fiber-forming protein of retractile type IV pili, mediating the cell-cell interactions required for social motility (36). It therefore seemed possible that PilA and the other Pil proteins found in the genome may operate as a retractile apparatus in some aspect of the *Bdellovibrio* life cycle.

Electron microscopy shows pilus-like fibers on the nonflagellar pole. Pili may, however, have many other adhesive roles in the lifestyle of a bacterium (reviewed in references 8 and 20). Thus, to ascertain whether the type IV pili were expressed in attack phase predatory *Bdellovibrio*, electron microscopic observations of both HI-grown *Bdellovibrio* and bacteria freshly liberated from host-dependent predatory growth

were carried out (Fig. 1 and Fig. 3D, frame i). Atomic force microscopy studies of strain 109J by Nunez and colleagues (21) did not show any pilus-like fibers, but their work focused mainly on 109J predation in whole bacterial communities rather than detailed, high-magnification studies of individual cells.

Our electron microscopy studies confirmed the early work of Shilo (29) and Abram and Davis (1), in which small, polar fibers were shown on *Bdellovibrio*. Pilus-like fibers were seen in approximately 30% of host-dependent HD100 cells (Fig. 1C) in uranyl acetate-stained preparations. Agreeing with the findings of Abram and Davis (1), broken fibers were often seen lying in close proximity to the cells, indicating that the shear forces associated with staining seem to affect the proportion of *Bdellovibrio* organisms seen with intact fibers. Attack phase *B. bacteriovorus* 109J was also examined under the same conditions and found to have pilus-like fibers at the nonflagellate pole at roughly the same frequency as attack phase HD100 (Fig. 1D), showing that different strains of *Bdellovibrio* have the same fibers. The low percentage of wild-type *Bdellovibrio* cells found with electron microscopically visible pili could be attributed to two things. First, pili are retractile organelles, with PilA subunits being held under the cytoplasmic membrane and polymerized into pilus fibers in a posttranslationally regulated process (15). Thus, many of the cells will contain retracted pili that do not appear on the surface. Probably, pili are only extruded during prey interactions as they would be sheared, if permanently extruded, in such a fast-swimming bacterium as *Bdellovibrio*; HD100 organisms swim at speeds of up to 160 $\mu m\ s^{-1}$ (17). Second, although pili are strong and withstand immense retractile forces when under tension, they can also break during experimental handling, such as washing and staining for microscopy. Touhami and coworkers (33) note in their paper that "sample washing with water could detach type IV pili from bacteria"; these investigators were also careful to avoid shear forces that would break or deform pili throughout their research. Any stimulus for pilus extrusion in *Bdellovibrio* is unknown, but we hypothesize that it would be futile to have pili permanently extruded on the surface of such a highly motile bacterium; so possibly contact with a prey cell outer membrane or cell wall could provide that stimulus.

Insertional inactivation of the HD100 *pilA* gene. Supplemental Fig. S10 shows the scheme for inactivation of the *pilA* gene in *B. bacteriovorus* HD100. Multiple lines of both prey-dependent and -independent *pilA*/*pilA*::Km HD100 organisms were subcultured as HI and HD strains (see Fig. S10 in the supplemental material) and screened by PCR and Southern blotting for loss of the wild-type chromosomal copy of the gene and gain of the interrupted *pilA*::Km form (see Fig. S3 and S4 in the supplemental material). This resulted in nine *pilA*::Km mutants, all of which were derived from the HI culture stream (24 HI cultures in total) in contrast to the HD culture stream (also 24 cultures), which was always *pilA*/*pilA*::Km merodiploid. Continued screening of these prey-dependent (HD) derivatives showed no loss of the wild-type gene, indicating that it was not possible to obtain this mutation under conditions of prey dependency.

All $pilA_{HI}$::Km strains were unable to grow in predatory liquid cultures, and when spotted onto soft agar overlays of *E. coli* S17-1 prey, no zones of clearing were seen, unlike those for *pilA*/*pilA*::Km merodiploid strains (see Fig. S2 in the supplemental material). Three independent $pilA_{HI}$::Km isolates with diverse morphologies (see Fig. S1 in the supplemental material) were chosen and assayed for predatory capabilities on YFP-labeled immobilized prey S17-1:pZMR100 *E. coli* (Table 1), compared to a parental merodiploid *pilA*/*pilA*::Km HI strain for each mutant strain; this was a fair control as each isolate had been subject to the same culture regime. The *Bdellovibrio* $pilA_{HI}$/*pilA*::Km merodiploid strains all gave typically 40 to 50 bdelloplasts per 1,000 *E. coli* cells. The *Bdellovibrio* $pilA_{HI}$::Km mutant was not able to predate the immobilized *E. coli* host, and no bdelloplasts were seen (Fig. 3A and B). HID2, a wild-type HD100-derived HI strain was assayed as a further control in the same YFP prey assay and found to form similar numbers of bdelloplasts (40 to 50/1,000 *E. coli* cells) as the parental merodiploid strains (Fig. 3C; see Fig. S8 in the supplemental material). These results indicated that the loss of *pilA* results in the inhibition of predatory capability.

Extensive electron microscopy analysis of the $pilA_{HI}$::Km strains ($n > 1{,}500$ cells) revealed no piliated *Bdellovibrio* organisms in the $pilA_{HI}$::Km mutant (Fig. 3 D, frames ii and iii) strain compared to the normal, 20 to 30% piliation of $pilA^+$ HI cells seen by examination of the $pilA_{HI}$/*pilA*::Km merodiploid strain (Fig. 3D, frame i).

RT-PCR and qRT-PCR analysis of *Bdellovibrio* HD100 *pilA* and RT-PCR analysis of expression of other *pil* genes in the wild-type predatory cycle. To further analyze any role for pili in predation, expression of the single *Bdellovibrio pilA* gene and other *pil* family genes during the predatory cycle of *Bdellovibrio* was studied with RT-PCR. RNA was isolated from different time points across the predatory life cycle as described in Materials and Methods. Using 25-cycle semiquantitative RT-PCR (17) (Fig. 4C), the *pilA* gene shows constitutively high expression at all time points across the *Bdellovibrio* life cycle. Attack phase cells have high levels of *pilA* mRNA, which does not decrease greatly during bdelloplast formation in attack phase cells and after predatory invasion and maturation (Fig. 4C). The *pilA* RT-PCR analysis was validated by real-time quantitative RT-PCR (qRT-PCR) carried out on the *pilA* gene for samples from the attack phase and 30 min postinfection, and this showed that transcript levels of *pilA* remained approximately constant throughout penetration of the prey cell and establishment of the bdelloplast (Fig. 4D). Because all predatory cycle RNA preparations were made from cultures that began with the inoculation of identical numbers of predatory *Bdellovibrio* cells equivalent to those in the attack-phase-only sample, it was possible to compare time points across the life cycle. Any changes in expression observed would be the result of changes in the expression of the *Bdellovibrio* organisms that had entered prey cells and begun their developmental cycle as any excess attack phase cells would continue to express their genes at the same level as the attack-phase-only control sample. Consistently high transcript levels show abundance, and probably stability, of the *pilA* mRNA, which would facilitate rapid protein synthesis of a supramolecular fiber structure as necessary in the predatory life cycle. Standard methods of matched RNA/cDNA amounts are not appropriate in *Bdellovibrio* predation studies as the addition of the prey dilutes the proportion of predatory RNA in total RNA by up to 10-fold,

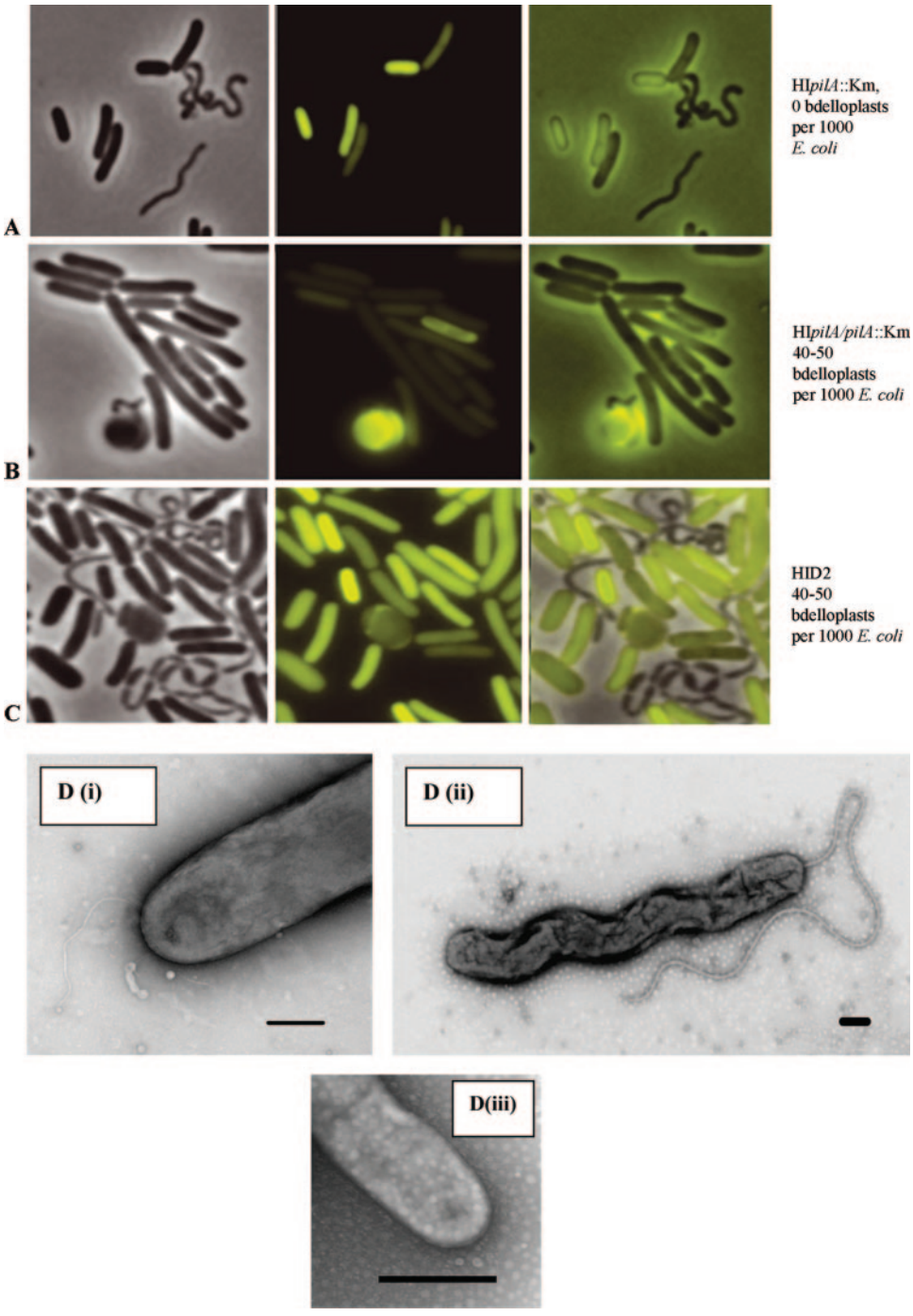

FIG. 3. (A and B) Predation tests of $pilA_{HI}$::Km and $pilA_{HI}$/*pilA*::Km on immobilized YFP-labeled *E. coli* S17-1 prey cells on plates of PY medium supplemented with 50 μg ml^{-1} kanamycin (17). The $pilA_{HI}$::Km mutant was seen to be nonpredatory, while the merodiploid $pilA_{HI}$/*pilA*::Km strain showed rounded bdelloplasts containing *Bdellovibrio* and thus normal predation. (C) HID2, a wild-type HD100-derived HI strain, predating on immobilized YFP host grown with no kanamycin and given the same number of bdelloplasts as the merodiploid $pilA_{HI}$/*pilA*::Km strain ($n > 2{,}500$) *E. coli* cells observed per strain after incubation for 24 h. (D) Transmission electron microscopy of $pilA_{HI}$/*pilA*::Km (frame i). Polar pilus fibers were seen at a frequency of approximately 20 to 30%, matching the frequency seen in wild-type $pilA^+$ HID2 ($n > 700$). Transmission electron micrographs of $pilA_{HI}$::Km strains (frames ii and iii). No polar fibers were seen in the samples examined ($n > 1{,}500$). All samples were stained with 1% uranyl acetate. Bar, 0.2 μm.

resulting in an apparent, artifactual 10-fold reduction of expression in the *Bdellovibrio* genes being studied.

In the *B. bacteriovorus* HD100 genome, there are three annotated *pilQ* genes. BLAST analysis showed the best *pilQ* homologue to be Bd0867 (CAE78812). PilQ is known in other bacteria to form the dodecameric outer membrane pore through which PilA fibers are extruded (6). In terms of gene organization conserved between different bacterial species,

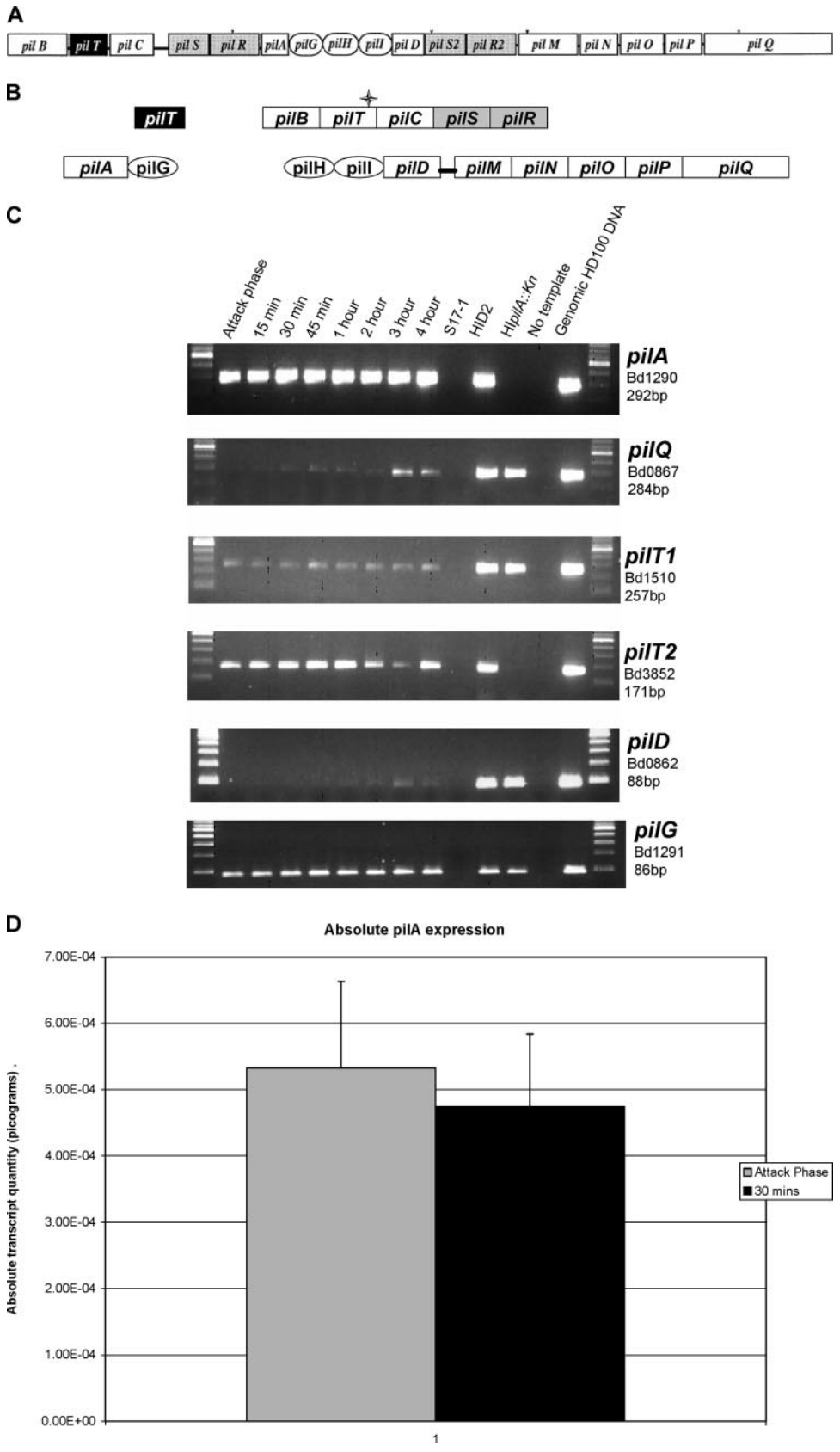

FIG. 4. Comparison of the operon structure of *pil* genes in *M. xanthus* and *Bdellovibrio*. Some cognate genes are highlighted in different colors and shapes to facilitate comparison. (A) Organization of *Myxococcus pil* genes, taken from Touhami et al. (33). (B) *pil* genes are scattered around

Bd0867 seems the more likely candidate since it is in the same operon as other *pil* genes. Reciprocal BLAST (25) homology searches bring up this gene as the strongest candidate *pilQ* in the *Bdellovibrio* genome. As shown by 25-cycle RT-PCR, *pilQ* shows lower expression in attack phase with transcript levels gradually increasing, peaking at 3 h, and dropping slightly at 4 h. This can be interpreted as the beginning of septation of the growing *Bdellovibrio* filament within the bdelloplast and the formation of new cell poles with completed pilus basal structures (including PilQ); pilus fibers have been observed to be only polar rather than lateral (Fig. 1). This also means that *pilQ* expression data would be useful as a marker for late bdelloplast stages and filament septation. BLAST analysis found two good PilT homologues. Bd1510 (CAE79390), named PilT1, has good homologies to other characterized PilT proteins, and the gene organization is conserved with that of *M. xanthus* (34). PilT in other bacteria is the ATPase needed for retraction of the PilA pilus fiber (20). *Bdellovibrio pilT1* shows a steady level of expression throughout the developmental cycle from attack phase through to late bdelloplast.

The other PilT homologue in the *B. bacteriovorus* genome, Bd3852 (CAE81209), is an even better match than PilT1 to the PilT proteins of other bacterial species. PilT2 is an orphan in the genome but is an extremely good candidate protein. In expression pattern, *pilT2* is somewhat unusual, with transcript levels increasing slightly in the early bdelloplast stages, showing the highest level of expression between 45 min and 1 h, dropping slightly at 2 h, reaching its lowest level at 3 h, and then returning to high level at 4 h. As mentioned above, PilT provides the retractile force of the type IV pilus motor, being a hexameric ATPase of the AAA^+ family (13) which is held at the base of the type IV pilus under and probably in association with the cytoplasmic membrane.

PilD, the prepilin peptidase that cleaves and methylates the immature PilA fiber protein so that it can be exported and polymerized, is associated with the cytoplasmic membrane of bacteria that utilize type IV pili (reviewed in references 5 and 20). *Bdellovibrio pilD* shows virtually no expression in attack phase cells, with transcripts starting to appear 45 min into bdelloplast formation (Fig. 4C). A gradual increase in expression is seen over the time course, peaking at 3 h and decreasing again at 4 h. The expression pattern of *pilD* is reminiscent of that of *pilQ*, which is logical, since the peak of expression of both these genes coincides with the beginning of pole formation.

PilG forms part of an ABC-type transporter required for PilA export and functional pilus biogenesis in *M. xanthus*, with these genes having no known homologues in other type IV pilus-producing species, which indicates a possible restriction to the deltaproteobacteria (36). *Bdellovibrio bacteriovorus* HD100 has good homologues of all three genes, *pilGHI* (Bd1291, Bd0860, and Bd0861). Wu et al. conclude that PilGHI may be required for outer membrane localization or export of PilA as mutants do not shed PilA into the surrounding medium and do not produce functional pilus fibers. This could account for the lack of *Bdellovibrio* genes for minor pilin proteins, which may perform a similar role in other bacteria, such as the *Pseudomonas aeruginosa pilE*, *pilV*, *pilW*, *pilX*, and *fimU* genes which have no counterparts that can be found through homology searches of both the *Bdellovibrio* and *Myxococcus* genome sequences. The *pilE*, *pilV*, *pilW*, *pilX*, and *fimU* genes are required for pilus assembly in *P. aeruginosa* (reviewed in reference 20) just as the *pilGHI* genes do in *M. xanthus* and would be expected to do in *Bdellovibrio* (9). The *pilG* gene was chosen for transcriptional assay (Fig. 4C) as it lies directly downstream of *pilA*. It must be remembered that the *pilA*::Km strains had to be grown as HI strains; thus, it was not possible to directly compare the abundance of transcripts between HD and HI strains. This is because the diverse cell lengths and morphologies seen in HI cultures do not allow their comparison to the numbers of uniform short attack phase *Bdellovibrio* cells. However, examination of transcripts by RT-PCR in the $pilA_{HI}$::Km strain and in the wild-type control HI strain HID2, allowed a simple nonquantitative examination of the effects of *pilA* disruption on other *pil* gene expression.

RT-PCR analysis of *pilA* and other *pil* genes in the $pilA^+$ HID2 strain and in the $pilA_{HI}$::Km strain. As expected the presence of *pilA* mRNA is abolished in the $pilA_{HI}$::Km mutant strain; there was no polar effect on *pilG* transcription caused by insertion of the kanamycin resistance cartridge into *pilA*. It seems, therefore, that *pilG* also has its own promoter even though it seems to lie in an operon with the *pilA* gene. All other *pil* genes examined showed no difference in expression levels between the wild-type and *pilA* mutant RNA samples (which came from cultures matched by OD_{600} values) except that *pilT2* expression was virtually abolished in the *pilA* knockout strain compared to the $pilA^+$ wild-type HID2.

DISCUSSION

We conclude that pili are essential for predation by *Bdellovibrio* as the interruption of the pilus fiber *pilA* gene rendered independently derived HI strains nonpredatory. Due to the strong homology between the *Bdellovibrio* HD100 pilus genes and the type IV pilus genes of other bacteria, we suggest that ratcheting or twitching of the pili may be an active and important mechanism for prey entry, although proving the ratcheting hypothesis requires intensive, high-resolution, atomic force microscopic studies on live invading cells, which is beyond the scope of this paper. The extent to which type IV pili may be present on the surface of attack phase *Bdellovibrio* or extruded when a prey contact is made is difficult to determine by con-

the *Bdellovibrio* genome, but a *Myxococcus*-like, possibly ancestral, organization can be seen with the gene order being conserved. The starred annotated *pilT* within the operon is a good homologue, but Bd3852 on the left is a better homologue, suggesting a duplication event. *Bdellovibrio* does not have significant homologues of *pilR2*/*S2*. (C) RT-PCR on RNA isolated from different time points during the HD100 life cycle and HI growth using primers designed to amplify internal fragments of HD100 *pil* genes. Lanes are as labeled; markers are NEB 100-bp ladder. *Taq* PCR controls were performed for each template and were negative for DNA contamination of RNA (data not shown). (D) qRT-PCR on RNA isolated from attack phase and 30 min post-prey infection using primers designed to amplify an internal fragment of the *pilA* gene.

ventional microscopy as it is a feature of type IV pili that pilus subunits are held under the membrane for rapid appropriate assembly (5, 33).

HI-grown *pilA*$^+$ *Bdellovibrio* organisms were also found to express pilus-associated genes; but this is not surprising as the *pilA*$^+$ HI strains studied retain predatory capability. Possibly, the HI growth state mimics intraperiplasmic growth of *Bdellovibrio* within prey, and thus pili may have a secondary role, post prey-entry, in anchoring the *Bdellovibrio* in the periplasm, perhaps involving adherence to prey peptidoglycan.

The *Bdellovibrio* genome contains genes whose products could act as engines of pilus assembly and retraction. These include three annotated *pilQ* homologues of which one, CAE78812, shows the best homology to other bona fide *pilQ* genes, such as that in *Myxococcus* (24% identity at the protein level). In addition, two *pilT* homologues are seen, both of which have extensive homology (*pilT1*, CAE79390, shows 24% identity and *pilT2*, CAE81209, shows 51% identity at the protein level) to *pilT* of *Myxococcus*. PilTs are known to provide motive force for type IV pilus retraction (19), and PilQ forms a functional outer membrane pore through which the pilus is extruded or retracted (10). However, both *pilT* genes and the *pilQ* gene CAE78812, are again expressed across all HD time points of infection of prey, as determined by RT-PCR (Fig. 3). Thus, the potential to extrude a type IV pilus is retained by *Bdellovibrio* in all predatory growth stages, possibly indicating roles for the pilus in the bdelloplast after initial prey entry.

Whether type IV pili play important roles anchoring the *Bdellovibrio* in the developing bdelloplast or providing, by retraction, the immense forces presumably required to squeeze *Bdellovibrio* organisms through the remarkably small pore generated in the prey cell outer membrane (Fig. 1A and B), we have definitively shown that interruption of the type IV pilus fiber protein-encoding gene, *pilA*, in *Bdellovibrio* abrogates the ability of the bacterium to predate entirely. This indicates that type IV pili provide a mechanism that is essential for prey entry or that type IV pili are vital to the productive *Bdellovibrio*-prey attachments required for intraperiplasmic predatory growth.

ACKNOWLEDGMENTS

We thank Karen Morehouse, Chi Aizawa, and Rob Till for helpful discussions and Mike Capeness, Marilyn Whitworth, and Kath Cheatle for technical assistance. We thank Mitchell Singer and Renate Lux for the gift of the anti-*Myxococcus* PilA antiserum.

K.J.E. was supported by a BBSRC quota Ph.D. studentship. This work was supported in part by Wellcome grant AL/067712 and by Human Frontier Science Programme Grant RGP57/2005 to R.E.S.

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JOURNAL OF BACTERIOLOGY, Sept. 2007, p. 6507 Vol. 189, No. 17
0021-9193/07/$08.00+0 doi:10.1128/JB.01018-07

ERRATUM

Predation by *Bdellovibrio bacteriovorus* HD100 Requires Type IV Pili

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Volume 189, no. 13, p. 4850–4859, 2007. Page 4852: In Table 1, the last entry in the first column should be "*B. bacteriovorus* HID2," and the last entry in the second column should be "Prey-independent derivative of HD100."