Downregulation of the motA gene delays the escape of the obligate predator *Bdellovibrio bacteriovorus* 109J from bdelloplasts of bacterial prey cells

Ronald S. Flannagan, Miguel A. Valvano and Susan F. Koval

**INTRODUCTION**

Bdellovibrios are small and highly motile Gram-negative bacteria commonly found in terrestrial and aquatic environments (Jurkevitch et al., 2000). They comprise at least two genera: *Bdellovibrio* and *Bacteriovorax* (Baer et al., 2000). Members of the genus *Bdellovibrio* have a remarkable lifestyle characterized by obligate predation of other Gram-negative bacteria. Thus, bdellovibrios may play an important role in controlling bacterial populations in nature, including plant and human pathogens. The lifecycle of *Bdellovibrio bacteriovorus* is biphasic, alternating between an extracellular flagellated and highly motile non-replicative attack-phase cell and a periplasmic non-flagellated growth-phase cell. The prey bacterium containing periplasmic bdellovibrios becomes spherical but osmotically stable, forming a structure known as the bdelloplast. After completing the growth phase, newly formed bdellovibrios regain their flagellum and escape the bdelloplast into the environment, where they encounter more prey bacteria. The obligate predatory nature of *B. bacteriovorus* imposes a major difficulty in introducing mutations in genes directly involved in predation, since these mutants could be non-viable. This work reports the cloning of the *B. bacteriovorus* 109J motAB operon, encoding proteins from the flagellar motor complex, and a genetic approach based on the expression of a motA antisense RNA fragment to downregulate motility. Periplasmic bdellovibrios carrying the plasmid expressing antisense RNA displayed a marked delay in escaping from bdelloplasts, while the released attack-phase cells showed altered motility. These observations suggest that a functionally intact flagellar motor is required for the predatory lifecycle of *B. bacteriovorus*. Also, the use of antisense RNA expression may be a useful genetic tool to study the *Bdellovibrio* developmental cycle.

*Bdellovibrio bacteriovorus* is a Gram-negative bacterium that preys on other Gram-negative bacteria. The lifecycle of *B. bacteriovorus* alternates between an extracellular non-replicative attack-phase cell and a periplasmic non-flagellated growth-phase cell. The prey bacterium containing periplasmic bdellovibrios becomes spherical but osmotically stable, forming a structure known as the bdelloplast. After completing the growth phase, newly formed bdellovibrios regain their flagellum and escape the bdelloplast into the environment, where they encounter more prey bacteria. The obligate predatory nature of *B. bacteriovorus* imposes a major difficulty in introducing mutations in genes directly involved in predation, since these mutants could be non-viable. This work reports the cloning of the *B. bacteriovorus* 109J motAB operon, encoding proteins from the flagellar motor complex, and a genetic approach based on the expression of a motA antisense RNA fragment to downregulate motility. Periplasmic bdellovibrios carrying the plasmid expressing antisense RNA displayed a marked delay in escaping from bdelloplasts, while the released attack-phase cells showed altered motility. These observations suggest that a functionally intact flagellar motor is required for the predatory lifecycle of *B. bacteriovorus*. Also, the use of antisense RNA expression may be a useful genetic tool to study the *Bdellovibrio* developmental cycle.

To date, the exact mechanism of *B. bacteriovorus* prey cell invasion and the factors contributing to prey susceptibility have not been characterized. Also, very little is known about the genetic networks regulating the developmental changes in *B. bacteriovorus*. Since attack-phase bdellovibrios are highly motile and cannot complete the lifecycle unless they encounter and invade prey bacteria, it is possible that motility and chemotaxis may be critical for the survival of this predator in its natural habitat. Previous studies investigating *B. bacteriovorus* chemotaxis in a facultative predatory strain UK2 (now reclassified as *Bacteriovorax stolpii* UK2) suggested that *B. bacteriovorus* is attracted to high concentrations of prey cells (Straley & Conti, 1977) and also demonstrated chemotaxis toward some amino acids.
acids (LaMarre et al., 1977), and aerotaxis (Straley et al., 1979). Recent work in our laboratories, using an mcp-specific oligonucleotide probe, demonstrated that at least 13 mcp (methyl-accepting chemotaxis protein) genes are present in B. bacteriovorus 109J (Flannagan, 2003). Attack-phase bdellovibrios may benefit from a large repertoire of chemotaxis proteins to sense multiple chemical signals that would ensure efficient prey location. However, this redundancy may also complicate the interpretation of experiments where individual mcp genes are inactivated, especially if only a moderate effect on predation is observed. Lambert et al. (2003) reported that a B. bacteriovorus mcp mutant is a less efficient predator, but in general the role of motility during Bdellovibrio predation has not been examined in detail. One of the major difficulties hampering genetic manipulations in Bdellovibrio is the prey dependence of the bacteria. Thus, it may be difficult to introduce mutations in Bdellovibrio genes directly involved in predation as well as in genes regulating the physiological transitions of the lifecycle without the potential risk of compromising the viability of the mutant cells. In this work, we used a genetic strategy to investigate the role of motility in Bdellovibrio predation by functionally disrupting the flagellar motor through the expression of a motA antisense RNA.

**METHODS**

**Bacterial strains and culture conditions.** B. bacteriovorus strain 109J was propagated on Escherichia coli ML35 (E. coli B. lacY1) as previously described (Koval & Hynes, 1991; Ruby, 1992; Thomashow & Rittenberg, 1978c). Co-cultures containing E. coli ML35 and B. bacteriovorus 109J were incubated at 30 °C for 18–36 h with vigorous aeration. When appropriate, B. bacteriovorus was propagated on E. coli ML35(pBR325) in culture medium with 15 μg chloramphenicol ml⁻¹. We used E. coli DH5α [pBR322 lacZAM15 recA1 endA1 gyrA96 thi-1 hsdR17(1Tc mK16) supE44 relA1] as a recipient for recombinant plasmids, and E. coli DH5α [pBR322 lacZAM15 recA1 endA1 gyrA96 thi-1 hsdR17(1Tc mK16) supE44 relA1] as a recipient for recombinant plasmids, and E. coli DH5α containing equal amounts (approx. 10⁹ bacteria ml⁻¹) of E. coli prey cells and B. bacteriovorus 109J were incubated in 125 ml side-arm flasks in a final volume of 20 ml HM buffer [3 mM HEPES (Sigma), pH 7.6, with 1 mM CaCl₂ and 0.1 mM MgCl₂] (Thomashow & Rittenberg, 1978c). The number of B. bacteriovorus 109J cells was determined microscopically as described below. Co-cultures were incubated at 30 °C with agitation, and predation was measured as a decrease in culture turbidity over time using a Klett-Summerson photoelectric colorimeter with a green filter.

**Molecular biology techniques.** DNA manipulations were performed by standard methods (Sambrook et al., 1989). Southern blot hybridizations were done on positively charged Biotrans nylon membranes at 60 °C in a standard hybridization buffer for digoxigenin (DIG)-based probes, as described by the manufacturer (Roche Diagnostics). After hybridization the membranes were washed at room temperature in 2× SSC, 0.1% SDS and at 60 °C in 0.5× SSC, 0.1% SDS. Hybridization was detected by chemiluminescence using CSPD (Roche Diagnostics). PCR amplifications were done with a HYBAID Omnisense thermal cycler or an MJ Research PTC-200. DNA sequencing was performed at the DNA Sequencing Facility of the J. P. Robarts Research Institute (University of Western Ontario), and the sequences were examined with the program BLAST-X (http://www.ncbi.nlm.nih.gov/).

**Table 1.** Properties of the plasmids used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Description*</th>
<th>Reference/source</th>
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<tbody>
<tr>
<td>pUC19</td>
<td>ColE1 replicon, high-copy E. coli cloning vector, Ap⁰</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>pMMB206</td>
<td>IncQ, broad-host-range expression plasmid, Ptaclac, Cm⁰</td>
<td>Morales et al. (1991)</td>
</tr>
<tr>
<td>pFPV25</td>
<td>ColE1 replicon, promoter trap vector containing a promoterless gfpmut3a gene, Ap⁰</td>
<td>R. H. Valdivia, Stanford University, CA, USA</td>
</tr>
<tr>
<td>pBR325</td>
<td>ColE1 replicon, E. coli cloning vector, Cm⁰ Te⁰</td>
<td>Bolivar (1978)</td>
</tr>
<tr>
<td>pRF4</td>
<td>pBluescript containing a 6-1 kb Apal fragment from B. bacteriovorus 109J chromosomal DNA</td>
<td>This study</td>
</tr>
<tr>
<td>pRF10</td>
<td>pMMB206 carrying the gfpmut3a coding region cloned as an EcoRI/blunt-ended PCR fragment</td>
<td>This study</td>
</tr>
<tr>
<td>pRF11</td>
<td>pMMB206 containing the 5' 251 nucleotides of the B. bacteriovorus motA gene cloned in the reverse orientation as an EcoRI/blunt-ended fragment</td>
<td>This study</td>
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*Te⁰, tetracycline resistance; Ap⁰, ampicillin resistance; Cm⁰, chloramphenicol resistance.
carries an mcp gene, as demonstrated by Southern blot hybridization with an mcp-specific oligonucleotide probe (Flanagan, 2003). The DNA sequence of pRFI also revealed an incomplete open reading frame homologous to the motility gene motB. To isolate the motAB operon from B. bacteriovorus 109J, a motB-specific probe was generated as follows. A 724 bp motB DNA fragment was amplified from pRF1 by PCR with Taq DNA polymerase (Roche Diagnostics) using the primers 5'-TAAGTCGAAAGAACATGAGAAGCATGAGAT-G-3' and 5'-TTAGTGACACTAAATTTCACTTGGAAACTGTC-3'. The PCR product was DIG-labelled using the PCR DIG-labelling mix (Roche Diagnostics). Thermal cycling conditions for the amplification of the motB-specific probe were: 5 cycles at 95 °C for 2 min, 53 °C for 1 min, 72 °C for 1 min, followed by 25 cycles at 95 °C for 1 min, 54 °C for 45 s and 72 °C for 1 min, and a final cycle at 95 °C for 1 min, 54 °C for 45 s, and 72 °C for 5 min. This probe was used to identify genomic DNA fragments of B. bacteriovorus which were sufficiently large to carry the entire motB gene and upstream sequences (data not shown). A control experiment showed that the motB probe was specific for B. bacteriovorus as it did not hybridize to chromosomal DNA of the E. coli K-12 strain RP437. B. bacteriovorus chromosomal DNA fragments digested with Apal, which were between 5 and 7 kb, were purified from the agarose gel with the QIAquick gel extraction kit (Qiagen). The purified DNA fragments were ligated into pBluescriptII that was also digested with Apal. Transformants were pooled and screened by sequential Southern blot hybridization until a single plasmid hybridizing to the probe was isolated. The plasmid containing the 6-1 kb Apal fragment was named pRF4 (Table 1) and its DNA insert fully sequenced (GenBank accession no. AY363247; Fig. 1).

**Construction of a gfp expression plasmid.** The gfpmut3A gene was amplified from pFPV25 (Table 1) with primers 5'-TTGAGATTCAAGGGAGATTCATGTAGTAAGCAGAAAGATT-3' (EcoRI restriction site underlined) and 5'-GGCATTAAGCCTGACGTGCTCAGGAGAAT-3', and Pwo DNA polymerase (Roche Diagnostics). The thermal cycling conditions in this case were 95 °C for 2.5 min at 95 °C, followed by 25 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 55 s, and a final extension of 5 min at 72 °C. The gfpmut3A amplon was digested with EcoRI, resulting in a fragment with one overhang end and one blunt end, and ligated into plasmid pMMB206 that was previously digested with EcoRI and Asp700. The ligation mixture was transformed into E. coli DH5α and transformants examined by restriction analysis. One of the isolated plasmids (pRF10) generated the predicted restriction pattern with EcoRI and SfaI that consisted of two fragments of approximately 650 bp and 9-2 kb. This pattern corresponded to the correct cloning of a promoterless gfpmut3A gene behind the Ptaclac promoter in pMMB206.

**Construction of a motA antisense expression plasmid.** A fragment of 251 bp, encoding the first 83 amino acids of the B. bacteriovorus MotA protein (corresponding to nucleotides 4294–4544 of GenBank accession number AF363247) was amplified using primers 5'-ATGCTAAGATGGAGACTGACGATTG-3' and 5'-GTTAGTCGACAGTTCTGTTGGAGG-3' (EcoRI site underlined). PCR amplification was done with Pwo DNA polymerase using the following thermal cycling conditions: 95 °C for 2 min, followed by 26 cycles of 95 °C for 30 s, 54 °C for 30 s and 72 °C for 45 s, and a final extension at 72 °C for 2 min. The resulting amplon was digested with EcoRI and ligated into pMMB206, which was digested with EcoRI and Asp700. This strategy resulted in the cloning of the 251 bp ampon in the antisense orientation, resulting in plasmid pRF11 (Table 1). The antisense orientation of the motA DNA insert was confirmed by DNA sequencing using the sequencing primer 5'-GGCTCGTATTTTTGTTGGAATTGTG-3'.

**Microscopy techniques.** For the enumeration of B. bacteriovorus, aliquots (100 µl) of B. bacteriovorus cultures were dispensed into Eppendorf tubes and stained with 2 mM 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes) on ice for 15 min. A 20 µl aliquot of the stained cells was spotted onto a glass slide coated with 0-8% agarose that had been allowed to dry. DAPI-stained bdellovibrios were visualized with a Zeiss AxioskopII microscope at a magnification of 1000 × using a mercury lamp and a blue filter with an excitation wavelength of 365 nm and an emission wavelength of 420 nm. The mean number of B. bacteriovorus cells was determined by counting 10 random fields of view for each culture. The difference in the mean number of cells per field of view between each culture was corrected for by the volume of the culture added to prey cells to ensure that all cultures were inoculated with approximately the same number of bdellovibrios. Predation at various times during the co-culture of E. coli and B. bacteriovorus was visualized by phase-contrast and transmission electron microscopy. Optical images were captured using a QIMAGING Retiga 1300 cooled mono 12-bit camera and Northern Eclipse version 6.0 software from Empix Imaging. For transmission electron microscopy, 5 ml samples of co-cultures were centrifuged, resuspended in 1-5 ml fixative (2-5% glutaraldehyde in 0-1 M sodium cacodylate buffer, pH 7-4) and left overnight at 4 °C. Cells were then fixed with 1% (w/v) osmium tetroxide and 1% (w/v) uranyl acetate and enrobed in agar. Samples were dehydrated in an ethanol series and embedded in LR White resin. Thin sections were cut and stained with 2% uranyl acetate and lead citrate. Sections were examined with a Philips EM300 electron microscope operating at 60 kV.

**RESULTS**

**Identification of the B. bacteriovorus motAB operon.**

The motAB genes and surrounding sequences in B. bacteriovorus 109J (Fig. 1) were cloned as described in Methods. The motA gene encoded a 250 amino acid polypeptide and was located upstream of motB. A purine-rich nucleotide sequence is located 20 nucleotides upstream from the putative ATG start codon of motA, which may correspond to a ribosome-binding site. The spacing between motA and motB is 17 nucleotides and no observable transcriptional terminator is present in the...
nucleotide sequence 3' to motA. The motB gene encoded a predicted polypeptide of 248 amino acids. No obvious ribosome-binding site could be identified in the DNA sequence upstream of the motB coding region. However, a stretch of purine-rich nucleotides that is located at eight nucleotides upstream from the putative ATG start codon could correspond to an atypical ribosome-binding site (data not shown). We then concluded that the complete motA and motB genes form an operon. Other genes were identified upstream of motA. They were: orf1, encoding a membrane-associated phospholipase; orf2, encoding a 3-β-hydroxysteroid dehydrogenase/isomerase; orf3, encoding a monofunctional biosynthetic peptidoglycan transglycosylase; orf4, encoding a conserved bacterial hypothetical protein; and orf5, encoding a putative ABC transporter transmembrane permease (Fig. 1). Downstream of motB but transcribed in the opposite direction, we found a gene with homology to an N-acetylgalactosamine hydrolase from Streptomyces thermoviolaceus, which we designated hydA (Fig. 1).

**Gfp expression in B. bacteriovorus**

The identification of the motAB genes provided us with the opportunity to directly test whether flagellar activity is indeed an essential feature in the *B. bacteriovorus* lifecycle. Because of the obligate nature of this predator, we sought to use a strategy based on the functional disruption of motAB genes by antisense RNA expression. *B. bacteriovorus* can be conjugated with plasmids belonging to the incompatibility group Q (IncQ), which replicate autonomously in this bacterium (Cotter & Thomashow, 1992a). Thus, we determined whether the IncQ plasmid pMMB206, encoding chloramphenicol resistance, could be conjugated from *E. coli* into *B. bacteriovorus* 109J. For this purpose, we first transformed *E. coli* ML35 prey cells with pBR325, which confers chloramphenicol resistance but has a ColE1 replicon that cannot replicate autonomously in *B. bacteriovorus* (Cotter & Thomashow, 1992a) and is not transferable. Successful conjugation of pMMB206 into *B. bacteriovorus* was confirmed by the ability of exconjugant bdellovibrios to prey on *E. coli* ML35(pBR325) cells in the presence of chloramphenicol. Predation was determined by following the decrease in turbidity of the co-cultures due to the lysis of prey cells. Light microscopy of co-culture samples confirmed the lysis of prey cells and revealed an abundance of highly motile free-swimming bdellovibrios (data not shown). Prey cell lysis by *B. bacteriovorus* 109J(pMMB206) was delayed (occurring at 24–36 h) relative to that observed in routine maintenance cultures of *B. bacteriovorus* 109J without the plasmid (occurring at 16–18 h). To confirm that *B. bacteriovorus* exconjugants indeed carried pMMB206, total DNA from these bacteria was isolated and transformed into *E. coli* DH5α. Chloramphenicol-resistant *E. coli* DH5α transformants carrying pMMB206 were recovered, while no transformants containing pBR325 (that would have been derived from prey cells) were isolated.

Next, we placed the promoterless *gfpmut3a* gene encoding green fluorescent protein under the control of the Ptac promoter in pMMB206 to evaluate whether this promoter can function in *B. bacteriovorus* 109J. The resulting construct, pRF10, was conjugated into *B. bacteriovorus* 109J and the exconjugants were examined by fluorescence microscopy. Fluorescent bdellovibrios could be seen as intense localized green fluorescent patches within bdelloplasts (Fig. 2a–c). Control cultures of *B. bacteriovorus* with the parental plasmid pMMB206 did not fluoresce. To eliminate the possibility that the localized pattern of Gfp expression observed could be due to the infection of the plasmid donor strain during conjugation, we carried out an additional control experiment where *E. coli* SM10(pRF10) was directly used as prey without conjugation into *B. bacteriovorus*. The bdelloplasts obtained in this case did not fluoresce (Fig. 2d–f). Therefore, the observation of fluorescent *B. bacteriovorus* cells within the bdelloplasts of prey cells demonstrated that pMMB206 was functional in this bacterium and provided a visual confirmation that a plasmid had indeed been introduced into *B. bacteriovorus*. However, *gfp* expression in *B. bacteriovorus* was not regulated by the inducer IPTG. The Ptac promoter was not altered since *E. coli* DH5α cells transformed with plasmid pRF10 expressed Gfp in an IPTG-dependent manner (data not shown). Therefore, it is possible that in *Bdellovibrio* the Ptac promoter is non-functional, and another promoter from the plasmid backbone drives the transcription of *gfpmut3a*.

**A functional motA is required in the predatory lifecycle of B. bacteriovorus**

Despite the apparent lack of functionality of the Ptac promoter, a plasmid that could express foreign genes in *B. bacteriovorus* provided us with a tool to inhibit the *B. bacteriovorus* flagellar motor by attempting the expression of antisense RNA complementary to the motA transcript. We constructed the recombinant plasmid pRF11, which encodes an antisense RNA transcript derived from the 5′ terminus of the *B. bacteriovorus* motA coding region. Plasmid pRF11 and the vector control pMMB206 were conjugated into *B. bacteriovorus* and the exconjugants examined in predation assays. Predation of *E. coli* ML35(pBR325) by the *B. bacteriovorus* exconjugants carrying either pRF11 or pMMB206 was assessed by following the turbidity of the culture over time. Lysis of prey cells is associated with a measurable and progressive decrease in the turbidity of the cultures. We also determined the predation of plasmidless *E. coli* ML35 by the wild-type *B. bacteriovorus* 109J. In the negative control experiment without *B. bacteriovorus*, *E. coli* ML35 lysis did not occur, as indicated by the constant level of turbidity relative to that observed in routine maintenance cultures of *B. bacteriovorus* 109J without the plasmid (occurring at 16–18 h). To confirm that *B. bacteriovorus* exconjugants indeed carried pMMB206, total DNA from these bacteria was isolated and transformed into *E. coli* DH5α. Chloramphenicol-resistant *E. coli* DH5α transformants carrying pMMB206 were recovered, while no transformants containing pBR325 (that would have been derived from prey cells) were isolated.
due to the presence of a foreign plasmid in B. bacteriovorus, as was shown in the initial conjugation experiments described above. In contrast, B. bacteriovorus 109J conjugated with pRF11 (the motA antisense expression vector) was markedly inhibited in its ability to lyse E. coli ML35 (pBR325) (Fig. 3). Samples of the cultures inoculated with B. bacteriovorus 109J(pRF11) were examined by phase-contrast microscopy at 30 h of incubation, revealing the presence of many bdelloplasts (Fig. 4a). This suggested that B. bacteriovorus cells could penetrate the E. coli prey but were impaired in their ability to escape from the bdelloplasts. By contrast, only free-swimming bdellovibrios were observed at 30 h in culture samples of B. bacteriovorus containing the parental plasmid pMMB206 (Fig. 4b), which encodes the LacZ α-peptide fragment in place of the motA antisense fragment in plasmid pRF11. Transmission electron microscopy further supported the observation that B. bacteriovorus carrying pRF11 remained within bdelloplasts at 30 h of culture (Fig. 5a), whereas with B. bacteriovorus carrying pMMB206, only free attack-phase cells were present at this time point (Fig. 5b). Many bdelloplasts had an irregular shape (Fig. 5a), compared to those observed with E. coli ML35 infected with wild-type B. bacteriovorus 109J (data not shown). At 40 h of incubation the turbidity of the E. coli culture infected with B. bacteriovorus carrying pRF11 began to decrease. Microscopic analysis revealed attack-phase free-swimming bdellovibrios, indicating that prey cell lysis had occurred. However, many of the B. bacteriovorus cells exhibited a slow tumbling pattern that was characteristically distinct from the rapid darting swimming pattern routinely observed with the bdellovibrios present in newly lysed
cultures. Altogether, these data indicated that the presence of the motA antisense-expressing plasmid pRF11 is associated with a defect in the ability of B. bacteriovorus to escape from the bdelloplasts of prey cells and altered motility of attack-phase cells.

DISCUSSION

B. bacteriovorus invade and replicate within the periplasmic space of suitable Gram-negative bacteria and are strictly dependent on host cells for growth (Ruby, 1992). Motility is a salient feature of B. bacteriovorus attack-phase cells. We hypothesized that mutations in motility genes could alter the lifestyle of this bacterium. Unfortunately, the genetic tools to manipulate bdellovibrios are very limited (Cotter & Thomashow, 1992a, b), especially for the analysis of lifecycle genes. To overcome these limitations, we devised a strategy based on antisense RNA that would cause down-regulation of gene expression in B. bacteriovorus. Cotter & Thomashow (1992a) devised a conjugation method for transferring plasmid DNA to host-dependent B. bacteriovorus 109J and a host-independent mutant, and showed that plasmids of the IncQ incompatibility group replicated autonomously in B. bacteriovorus. We therefore used the IncQ plasmid pMMB206 as a starting point to develop an antisense RNA strategy for the functional inactivation of B. bacteriovorus genes. Plasmid pMMB206 was conjugated into B. bacteriovorus 109J, and could be stably maintained

Fig. 4. Phase-contrast microscopy of 30 h B. bacteriovorus cultures in the presence or absence of the motA antisense expression plasmid. (a) E. coli ML35(pBR325) inoculated with B. bacteriovorus 109J(pRF11) (arrows denote bdelloplasts); (b) E. coli ML35(pBR325) inoculated with B. bacteriovorus 109J(pMMB206) (arrows denote free-swimming, attack-phase bdellovibrios). Magnification ×1000.

Fig. 5. Electron microscopy of 30 h B. bacteriovorus cultures in the presence or absence of the motA antisense expression plasmid. (a) Bdelloplasts (arrows) containing B. bacteriovorus 109J(pRF11), indicated by an asterisk. (b) Attack-phase B. bacteriovorus 109J(pMMB206) (arrows). Bars, 0-5 μm.
as long as antibiotic selection and prey cells were present, but it was lost if Bdellovibrio cultures were stored in the absence of selection (R. S. Flannagan, M. A. Valvano & S. F. Koval, unpublished observations). We noticed that in the presence of pMMB206, B. bacteriovorus took longer to lyse the E. coli prey. Maintenance cultures of E. coli routinely inoculated with up to 2-week-old attack-phase B. bacteriovorus cells exhibit complete lysis after 16–20 h. However, in the presence of pMMB206 complete lysis did not occur until at least 24–36 h. The delay of B. bacteriovorus carrying pMMB206 to lyse prey may be due to the physiological constraints of having to maintain a plasmid. In E. coli, plasmids can lower the maximum specific growth rate of the host bacterium (McDermott et al., 1993). However, in B. bacteriovorus plasmid carriage may be complicated by the dependence of the predator on the prey for the provision of nucleotide precursors, and its DNA replication is a highly coordinated process that occurs during the periplasmic growth phase (Rosson & Rittenberg, 1979, 1981). Conceivably, plasmid replication in B. bacteriovorus may only be possible during the periplasmic growth phase. Additional research is under way in our laboratories to investigate in detail the replication of pMMB206 during the biphasic growth cycle of B. bacteriovorus.

To our knowledge, there are no studies reporting the expression of recombinant DNA in B. bacteriovorus. We constructed a derivative of pMMB206 with the gfpmut3A gene under the control of Ptac promoter to demonstrate whether this promoter is functional in B. bacteriovorus. Detectable fluorescence by B. bacteriovorus exconjugants containing the reporter plasmid was observed during the growth phase within bdelloplasts, but these results occurred regardless of the presence of the inducer IPTG. Therefore, it is likely that the Ptac promoter in B. bacteriovorus is not functional and another constitutive promoter from the pMMB206 backbone is driving a low-level expression of gfpmut3A. These results are consistent with the possibility discussed above that plasmid-encoded genes may be preferentially expressed during the periplasmic growth phase. However, our results clearly demonstrate that pMMB206 can be used to express recombinant genes in B. bacteriovorus.

A hallmark of the B. bacteriovorus lifestyle is the transition between the presence of a polar flagellum in highly motile attack-phase cells and the loss of this structure following entry into the prey bacterium and during most of the periplasmic growth phase. The flagellum is re-formed shortly before lysis of the bdelloplast and release of progeny attack-phase bdellovibrios. The predatory lifecycle of B. bacteriovorus revolves around its ability to swim. In E. coli or Salmonella typhimurium, disruption of the motA or motB gene results in paralysis of bacterial motility despite the presence of an intact flagellum (Blair & Berg, 1988; Silverman et al., 1976). The MotA and MotB proteins constitute the stationary part of the flagellar motor (Chun & Parkinson, 1988; De Mot & Vanderleyden, 1994; Garza et al., 1995) and form a complex that facilitates the traffic of protons from the periplasmic space into the cytosol of the bacterial cell (Blair & Berg, 1990; Braun et al., 1999; Stolz & Berg, 1991; Zhou et al., 1998), which is required for flagellar rotation (Manson et al., 1977). Thus, the B. bacteriovorus motA gene was an excellent candidate for functional gene disruption studies using antisense RNA expression. B. bacteriovorus 109 carrying plasmid pRF11, expressing motA antisense RNA, showed three abnormal phenotypes: (i) pronounced delay in the escape of progeny from the bdelloplast, (ii) tumbling swimming pattern of released attack-phase bdellovibrios, and (iii) morphological abnormalities of the bdelloplasts. None of these phenotypes were observed with B. bacteriovorus carrying the vector pMMB206. Thus, our results clearly demonstrate that the delayed lysis found with B. bacteriovorus encoding the motA antisense gene fragment was associated with an inhibition of the release of predator progeny from the bdelloplasts. The exact mechanism causing these alterations in bdellovibrios carrying pRF11 remains to be determined, but it is not likely to be due to the metabolic demand of producing a random RNA transcript, since cultures with pMMB206, which encodes the LacZz fragment in place of the motA antisense fragment in plasmid pRF11, did not show a comparable delay in prey cell lysis. The tumbling motility of attack-phase cells could be explained by a partial defect in motility due to an incomplete inhibition of motAB expression by the antisense mRNA, which may be expressed at a low level as in the case of the Gfp protein. Although production of antisense mRNA could not be directly verified, the data support the notion that expression of the cloned motA antisense gene fragment may have downregulated the function of the motA gene.

In conclusion, we have cloned the motAB operon in B. bacteriovorus. We also show that the presence of a plasmid encoding an antisense motA gene fragment is associated with a marked delay in the escape of B. bacteriovorus progeny from bdelloplasts and with altered motility of attack-phase cells, suggesting that optimum functioning of the flagellar motor may play an important role in the Bdellovibrio lifestyle. Furthermore, antisense RNA expression strategies may become a useful genetic tool to unravel the function of other components participating in the developmental cycle of this obligate predator. Additional experiments are currently under way in our laboratories to elucidate the molecular mechanism of the motA antisense effect and to identify more suitable and efficient promoters for constitutive and regulated gene expression in B. bacteriovorus.

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