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The *Bdellovibrio bacteriovorus* twin-arginine transport system has roles in predatory and prey-independent growth

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Bdellovibrio bacteriovorus grows in one of two ways: either (i) predatorily [in a host-dependent (HD) manner], when it invades the periplasm of another Gram-negative bacterium, exporting into the prey co-ordinated waves of soluble enzymes using the prey cell contents for growth; or (ii) in a host-independent (HI) manner, when it grows (slowly) axenically in rich media. Periplasmic invasion potentially exposes *B. bacteriovorus* to extremes of pH and exposes the need to scavenge electron donors from prey electron transport components by synthesis of metalloenzymes. The twin-arginine transport system (Tat) in other bacteria transports folded metalloenzymes and the *B. bacteriovorus* genome encodes 21 potential Tat-transported substrates and Tat transporter proteins TatA1, TatA2 and TatBC. GFP tagging of the Tat signal peptide from Bd1802, a high-potential iron–sulfur protein (HiPIP), revealed it to be exported into the prey bacterium during predatory growth. Mutagenesis showed that the *B. bacteriovorus tatA2* and *tatC* gene products are essential for both HI and HD growth, despite the fact that they partially complement (in SDS resistance assays) the corresponding mutations in *Escherichia coli* where neither TatA nor TatC are essential for life. The essentiality of *B. bacteriovorus* TatA2 was surprising given that the *B. bacteriovorus* genome encodes a second *tatA* homologue, *tatA1*. Transcription of *tatA1* was found to be induced upon entry to the bdelloplast, and insertional inactivation of *tatA1* showed that it significantly slowed the rates of both HI and HD growth. *B. bacteriovorus* is one of a few bacterial species that are reliant on a functional Tat system and where deletion of a single *tatA1* gene causes a significant growth defect(s), despite the presence of its *tatA2* homologue.

INTRODUCTION

The twin-arginine translocation (Tat) system transports folded proteins across the cytoplasmic membrane and is found in a wide variety of bacteria, whilst homologous systems are found in both archaea and eukaryotes (Sargent, 2007a; Yuan *et al.*, 2010). Proteins transported by the Tat system in bacteria have a consensus twin arginine motif S/T-R-R-X-F-L-K (where X is any polar amino acid), a hydrophobic region and an A-x-A cleavage recognition motif in their N-terminal leading sequence (Berks, 1996; Stanley *et al.*, 2000). Tat-transported proteins are typically involved in metabolism, redox reactions, metal acquisition and cell envelope maintenance (Berks *et al.*, 2005; Palmer *et al.*, 2005) and are involved in pathogenesis, symbiosis, quorum sensing and motility (Sargent, 2007a; Stevenson *et al.*, 2007).

In *Escherichia coli*, a functional Tat system consists of TatABC (the genes for which are encoded in an operon) and the TatA homologue TatE (Sargent *et al.*, 1998; Weiner *et al.*, 1998). TatB and TatC form a complex in *E. coli* (Bolhuis *et al.*, 2001) which recognizes the signal motif of a

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Abbreviations: eGFP, enhanced GFP; HD, host-dependent; HI, host-independent.

Two supplementary tables and two supplementary figures are available with the online version of this paper.

Tat substrate, whereupon the TatA proteins associate to form a channel complex of 12–35 monomers, associated with TatBC (Alami *et al.*, 2003; Dabney-Smith *et al.*, 2006; Gohlke *et al.*, 2005; Holzapfel *et al.*, 2007). The Tat substrate is transported across the membrane via the TatA channel and released by proteolytic cleavage at the C-terminal end of the signal peptide. TatA then disassociates from the TatBC complex to reset the process (Berks *et al.*, 2005; Lee *et al.*, 2006; Sargent, 2007a). Un- or mis-folded proteins are rejected then targeted for either degradation or refolding (DeLisa *et al.*, 2002; Lee *et al.*, 2006; Sargent, 2007a).

Deletion of *tatC* in *E. coli* abolishes Tat system function (Bogsch *et al.*, 1998) and whilst TatA and TatB share 25 % sequence identity, each is essential for full Tat function, suggesting that they play different roles (Sargent *et al.*, 2001). Alterations in the *E. coli* TatA protein sequence allow it to function as both TatA and TatB in a TatBE deletion strain (Blaudeck *et al.*, 2005). TatE has 60 % identity with TatA (Lee *et al.*, 2006) and in aerobically grown *E. coli*, *tatE* is expressed at very low levels compared with *tatA* and the deletion of *tatE* has no noticeable effect on Tat substrate transport or viability of planktonically grown cells (Jack *et al.*, 2001). However, in *E. coli* biofilms, *tatE* transcription is induced up to 2.12 times more than in planktonic cultures (Beloin *et al.*, 2004). Only in an *E. coli tatA tatE* double mutant was the Tat pathway blocked in all conditions (Sargent *et al.*, 1998); but TatE in *Pseudomonas stutzeri* is specifically required to transport proteins for denitrification (Heikkilä *et al.*, 2001). This and other studies suggest that second TatA homologues (TatEs), in diverse bacteria, play an important role in transportation under certain growth conditions (Brüser, 2007).

Bdellovibrio bacteriovorus (Stolp & Starr, 1963) is a small Gram-negative bacterium which preys upon other Gram-negative bacteria. *B. bacteriovorus* attaches to, penetrates through the outer layers of and grows within the periplasm of prey bacteria. *B. bacteriovorus* modifies the prey cell wall forming an osmotically stable structure called the bdelloplast, which is bounded by an intact prey outer membrane. Inside this, *B. bacteriovorus* delivers enzymes both to the prey periplasm and (at early stages of predation) via the (still intact) prey cell inner membrane to the cytoplasm to degrade the cytoplasmic contents of the prey cell. Prey-derived nutrients facilitate *B. bacteriovorus* growth and reproduction and *B. bacteriovorus* elongates into a filamentous cell. After consuming the bdelloplast interior, the filament divides into multiple progeny *B. bacteriovorus* which become flagellate and escape through the bdelloplast outer membrane to carry out further predation (Sockett, 2009; Thomashow & Rittenberg, 1979).

The *B. bacteriovorus* HD100 genome encodes approximately 290 hydrolytic enzymes, many of which will be transported into prey cells for degrading macromolecular constituents (Rendulic *et al.*, 2004). The percentage of the *B. bacteriovorus* HD100 genome encoding transport systems is higher than for other free-living bacteria. A systematic review by Barabote *et al.* (2007) predicted at least four types of cytoplasmic membrane transportation systems and five types of outer membrane secretion systems. Genes encoding the Tat system have been predicted in the *B. bacteriovorus* genome (Barabote *et al.*, 2007; Rendulic *et al.*, 2004), including *tatB* and *tatC* as well as two *tatA* homologues. In this study we show that *B. bacteriovorus* expresses a functional Tat system required for both predatory [prey/host-dependent (HD)] and axenic [prey/host-independent (HI)] growth and that at least one protein containing a Tat signal motif is exported from *B. bacteriovorus* into the cytoplasm of the prey bdelloplast during predatory growth. We show that deletion of *tatA1* slows both predatory and axenic growth, delays gene expression of some potential Tat substrates and stops the export of Tat substrate protein Bd1802 into the prey during predation. This shows that *B. bacteriovorus* TatA1 and TatA2 are not interchangeable and are responsible for the transport of different substrates, and that Tat-transported substrates can be further exported beyond the *B. bacteriovorus* outer membrane into the surrounding bdelloplast, possibly contributing to predatory processes.

METHODS

Bacterial strains, plasmids and culture conditions. All bacterial strains and plasmids used and created in this study are listed in Supplementary Table S1 (available with the online version of this paper). *E. coli* strains were grown in yeast extract and tryptone (YT) broth (Lambert & Sockett, 2008) at 29 °C with shaking at 200 r.p.m. for 16 h to give late-exponential-phase cultures for use as prey. *E. coli tat* mutants containing expression plasmids were grown in the presence of 50 μl ampicillin ml^{-1} and 100 nM IPTG. *E. coli* S17-1 (Simon *et al.*, 1983) was routinely used as prey for growth of *B. bacteriovorus* in predatory cultures at 29 °C with shaking at 200 r.p.m. (Hobley *et al.*, 2006), which typically contained 50 ml Ca/HEPES buffer (2 mM $CaCl_2$, 25 mM HEPES pH 7.6), 3 ml of a late-exponential-phase *E. coli* culture and 1 ml of a *B. bacteriovorus* predatory culture (which had completed prey lysis and contained typically 2.5×10^8 p.f.u. ml^{-1}). Antibiotic-resistant insertion-mutant *B. bacteriovorus* were grown with *E. coli* S17-1 carrying an appropriate plasmid as prey with the antibiotics at the following concentrations: kanamycin sulphate, 50 μg ml^{-1}, S17-1 pZMR100 (Rogers *et al.*, 1986); apramycin sulphate, 50 μg ml^{-1}, S17-1 pUC19:apraR; both antibiotics, S17-1 pLH003. HI derivatives of *B. bacteriovorus* strains were isolated and grown at 29 °C on peptone and yeast extract (PY) plates or in PY broth with shaking at 200 r.p.m. as described previously (Evans *et al.*, 2007; Shilo & Bruff, 1965).

***B. bacteriovorus* predation assays using *E. coli* MC4100 and B1LK0-P (Δ*tatC*) as prey.** *E. coli* strains MC4100 (Casadaban & Cohen, 1979) and B1LK0-P (Buchanan *et al.*, 2002) were grown as described above to late exponential phase, then diluted (in cell-free supernatant from the same cultures) and matched to OD_{600} 1.5. The same prey cell c.f.u. was not used to match the cultures due to the reported larger filamentous appearance of the B1LK0-P cells, meaning that more prey cell volume would be available for *B. bacteriovorus* replication in the B1LK0-P cells than the wild-type if matched c.f.u. were used (Stanley *et al.*, 2001). Three independent biological repeats of this experiment were carried out. A 150 μl volume of diluted *E. coli* prey was added to 1 ml of an overnight predatory culture of

B. bacteriovorus HD100 (grown as described above) and made to a final volume of 5 ml using Ca/HEPES buffer (*B. bacteriovorus* was replaced with 1 ml Ca/HEPES buffer in *E. coli*-only control cultures). These starting ratios of predator to prey allowed predation to be monitored over 4 h. Experimental cultures were incubated at 29 °C with shaking at 200 r.p.m., whilst enumerations were taken every hour (0–4 h) using standard serial dilutions on YT plates for prey enumerations and double-layer yeast extract peptone, sodium acetate, calcium chloride (YPSC) overlay plates for *B. bacteriovorus* p.f.u. counts as described previously (Hobley *et al.*, 2006).

RT-PCR detection of gene expression. RT-PCR was used to determine which of the *tat* system and substrate genes were expressed in the predatory life cycle and whether expression patterns differed in the *tatA1* mutant. RNA was isolated as described previously (Evans *et al.*, 2007; Lambert *et al.*, 2006) from both wild-type *B. bacteriovorus* HD100 and the *tatA1* mutant strain and from HID2, an HI derivative of *B. bacteriovorus* HD100 (Evans *et al.*, 2007). RT-PCR with 25 amplification cycles (allowing a semiquantitative view of expression levels) was carried out as described previously (Evans *et al.*, 2007) using the primers listed in Supplementary Table S2 (available with the online version of this paper).

Cross-species complementation tests for Tat function. *B. bacteriovorus* HD100 *tatA2* (Bd2572; $tatA2_{Bd}$), *tatB* (Bd3866; $tatB_{Bd}$), *tatC* (Bd3865, $tatC_{Bd}$) and *tatA1* (Bd2196; $tatA1_{Bd}$), amplified by PCR using Phusion High-Fidelity DNA (New England Biolabs) with primers listed in Supplementary Table S2, were cloned into the expression vector pTRC99a (Amann *et al.*, 1988) using the restriction sites engineered into the primers. The resulting plasmids pTRC-$tatA2_{Bd}$, pTRC-$tatB_{Bd}$, pTRC-$tatC_{Bd}$ and pTRC-$tatA1_{Bd}$ were sequenced by using pTRCFor and pTRCRev (Supplementary Tables S1 and S2) to ensure that orientation was correct and that no mutation had been introduced. Each of these plasmids, along with an empty vector control, was transformed into the cognate *E. coli tat* deletion mutant and the wild-type MC4100 strain to be tested for the SDS-resistance–Tat transport-functionality phenotype using 2 % (w/v) SDS Luria–Bertani (LB) agar plates as described previously (Buchanan *et al.*, 2002).

Insertional inactivation of *B. bacteriovorus* genes encoding Tat substrates and Tat transporter components. Two genes, Bd1802 and Bd3906, encoding potential Tat substrates were knocked out by silent deletion using modifications of the techniques used by Steyert & Pineiro (2007) and Roschanski *et al.* (2011). Approximately 450 bp of flanking DNA from either side of the gene was amplified using primers listed in Supplementary Table S2 and Phusion High-Fidelity DNA polymerase (New England Biolabs) and joined together by sequential cloning into vector pUC19 (Vieira & Messing, 1982), giving a deletion construct that still retained the Tat signal peptide and part of the C-terminal peptide coding regions (approximately the last 10 codons of each protein); these constructs were then transferred into the kanamycin-resistant suicide vector pK18*mobsacB* (Roschanski *et al.*, 2011; Schäfer *et al.*, 1994) and transformed into *E. coli* S17-1. Kanamycin resistance cassette insertion constructs were created for each *B. bacteriovorus tatA1*, *tatA2* and *tatC* gene. Each was amplified including flanking DNA, from genomic DNA of *B. bacteriovorus* HD100 using primers listed in Supplementary Table S2 and Phusion High-Fidelity DNA polymerase (New England Biolabs), and cloned into the *Eco*RI site of the cloning vectors pGEM7 (Promega; *tatA1* and *tatA2*) or pUC19 (Vieira & Messing, 1982; *tatC*). The 1.3 kb kanamycin resistance cassette from pUC4K (Yanisch-Perron *et al.*, 1985) was then cloned into a unique restriction site in each *tat* gene (*tatA2*, *Pst*I; *tatC*, *Bgl*II; *tatA1*, *Mfe*I); the constructs were then transferred into the conjugative vector pSET151 (Bierman *et al.*, 1992) and transformed into *E. coli* S17-1. The plasmids were conjugated into *B. bacteriovorus* HD100 as described previously (Evans *et al.*, 2007; Lambert *et al.*, 2006; Lambert & Sockett, 2008). Subsequent HD and HI *B. bacteriovorus* strains were tested by culture and colony PCR for evidence of a secondary crossover event (Evans *et al.*, 2007); putative deletion mutants were confirmed by Southern blotting (Southern, 1975) and sequencing (MWG Biotech) to confirm for Bd1802 the deletion of the gene and the loss of the suicide vector, and for *tatA1* the insertion of the kanamycin cassette at the correct site, the lack of a wild-type gene and also for the loss of the suicide vector.

***B. bacteriovorus* Tat substrate–GFP fusions.** The enhanced GFP (eGFP) gene was amplified from the vector pEGFP-C2 (Clontech) and cloned in-frame into the Bd1802 and Bd3906 genes, such that the predicted N-terminal Tat signal peptide and a further eight amino acids beyond the predicted cleavage site (APA–AG for 1802 and ALA–SL for 3906, where the (–) represents the predicted cleavage site) were fused in-frame to eGFP then a C-terminal peptide sequence of nine amino acids from each gene led from the eGFP in-frame to the natural stop codon of each gene.

These eGFP-tagged constructs were then cloned into the vector pK18:apraR (Supplementary Table S1) and transformed into *E. coli* S17-1. Plasmids were integrated (by a single crossover event) into the *B. bacteriovorus* HD100 genome by conjugation between the *E. coli* S17-1 donor strains with *B. bacteriovorus* HD100 as described previously (Evans *et al.*, 2007), creating apramycin-resistant *B. bacteriovorus* that were able to express an eGFP fused to both a putative Tat signal peptide and the original gene, both under the control of the native promoter.

Phase-contrast, fluorescence and electron microscopy. We used a Nikon Eclipse E600 epifluorescence microscope with a 100 × phase-contrast objective and images were captured using a Hamamatsu Orca camera with the SimplePCI software (version 5.3.1 from Digital Pixel) and enhanced overall by using the 'sharpen' and 'smooth' tools in the SimplePCI software for additional clarity. For fluorescence images, additional filter blocks were used: BV-2A (excitation 400–440 nm; emission 470+ nm) for eGFP detection, and hcRED (excitation 550–600 nm; emission 610–665 nm) for mCherry detection. Electron microscopy samples were stained with 1 % phosphotungstic acid (PTA) pH 7.0 and imaged with a JEOL 1200EX transmission electron microscope (Iida *et al.*, 2009).

Predation efficiency of HD *B. bacteriovorus*. The predation efficiency of the HD *B. bacteriovorus tatA1* mutant was compared with that of the wild-type strain HD100 over a single round of predation by light microscopy. Synchronous infections were set up as described previously (Morehouse *et al.*, 2011) giving a starting ratio of at least five *B. bacteriovorus* cells to each *E. coli* prey cell.

Growth rate comparisons of HI *B. bacteriovorus*. HI *B. bacteriovorus* growth rates in PY broth were measured by recording OD_{600} in 96-well plates in a Fluostar Optima plate reader (BMG Labtech) as described previously (Morehouse *et al.*, 2011).

RESULTS AND DISCUSSION

Identification and expression of genes encoding the Tat system in *B. bacteriovorus*

The Tat transport system is important in many bacteria as it transports folded proteins across the cytoplasmic membrane, including many proteins involved in redox reactions, metabolism and metal acquisition (Berks *et al.*,

2005; Palmer *et al.*, 2005). *B. bacteriovorus* can grow in a predatory manner as HD cells, seeking prey during a non-replicative motile 'attack-phase'. They invade prey, forming an infected structure called a bdelloplast. Inside this, *B. bacteriovorus* resides first in the prey periplasm; later they degrade the prey inner membrane, growing and replicating in the bdelloplast and finally lysing the prey outer membrane to release new progeny *B. bacteriovorus*. In the lab on peptone-rich media, 1 in 4.5×10^7 HD *B. bacteriovorus* can convert to a long-celled axenically growing form called HI. Such HI *B. bacteriovorus* replicate slowly but do take up nutrients and septate outside prey. The growing HD *B. bacteriovorus* carries out extensive transport of proteins required for the predatory hydrolysis of the prey contents and the HI *B. bacteriovorus* metabolizes complex peptone-based media; thus we hypothesized that the Tat transport system may play a role in predation and/or axenic growth.

Four genes encoding TatB (Bd3866), TatC (Bd3865), TatA2 (Bd2572) and TatA1 (Bd2196) have been identified in the *B. bacteriovorus* HD100 genome (Barabote *et al.*, 2007; Rendulic *et al.*, 2004). Unlike the *tatABC* operon in *E. coli*, *tatA2* in *B. bacteriovorus* is separated from *tatBC*, whilst the *tatA1* locus is separate again on the opposite DNA strand to *tatA2*, *tatB* and *tatC*.

B. bacteriovorus TatA2 shares 54 % amino acid sequence identity with $TatA1_{Bd}$. CLUSTALW alignment with *E. coli* TatA, TatE and *P. aeruginosa* TatA (Supplementary Fig. S1, available with the online version of this paper; http://www.ebi.ac.uk/Tools/clustalw/) showed $TatA2_{Bd}$ and $TatA1_{Bd}$ have high levels of identity in their N-terminal regions which are predicted to form a transmembrane helix by TransMembrane prediction using Hidden Markov Models (TMHMM) (http://www.cbs.dtu.dk/services/TMHMM/). A highly homologous transmembrane motif in the N terminus has also been found in the TatB protein sequences in *B. bacteriovorus*, *E. coli* and *P. aeruginosa* (Supplementary Fig. S1), but they have divergent C-terminal regions. Six transmembrane motifs are predicted within the $TatC_{Bd}$ protein which shares 35 % and 38 % sequence identity with TatC in *E. coli* and *P. aeruginosa*, respectively (Supplementary Fig. S1). Thus, bioinformatic analysis suggested that $TatA2_{Bd}$, $TatB_{Bd}$, $TatC_{Bd}$ and $TatA1_{Bd}$ are transmembrane proteins which are likely to function as a Tat system in *B. bacteriovorus*.

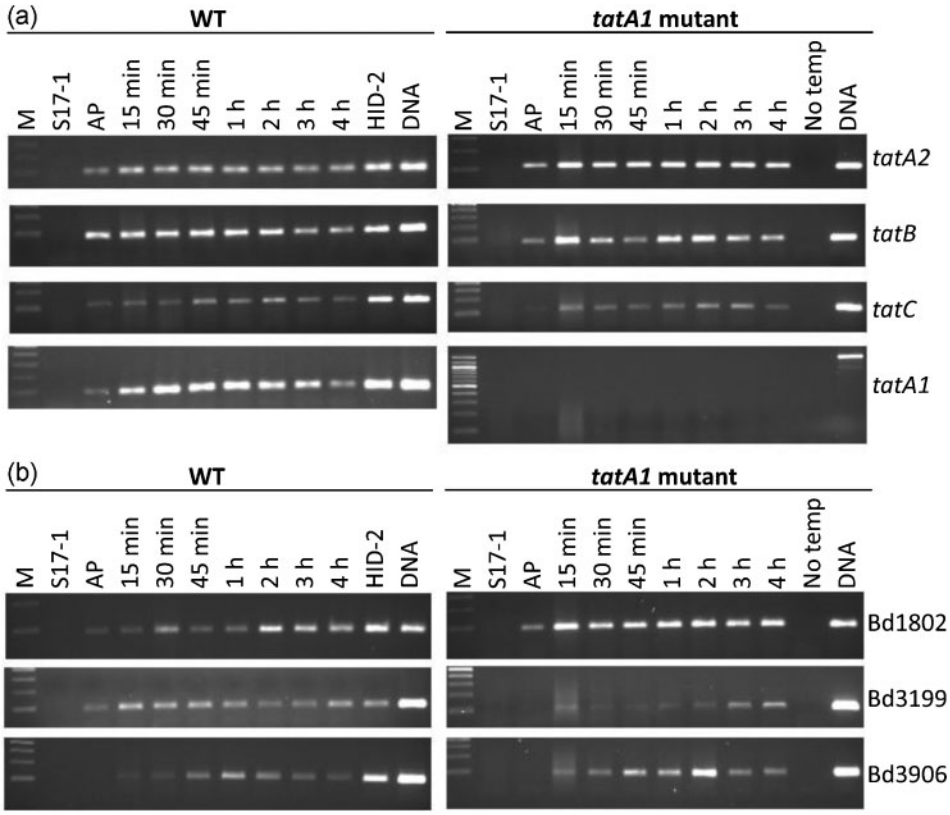

Fig. 1. RT-PCR on RNA isolated from wild-type and *tatA1* mutant *B. bacteriovorus* at different time points (15 min–4 h) during the HD predation cycle (on *E. coli* S17-1) and HI growth using primers designed to amplify internal fragments of the Tat system (a) and predicted Tat substrate (b) genes. Lanes: M, markers, NEB 100 bp ladder; AP, attack phase (free-swimming HD *B. bacteriovorus*); HID-2, wild-type HI strain; DNA, genomic DNA template. WT, Wild type.

RT-PCR (Fig. 1a) showed that the expression of *B. bacteriovorus tatA2* was constitutive throughout the predatory cycle, whilst both the *tatB* and *tatC* genes were expressed steadily during the first hour of predation and then expression gradually dropped. The expression of *tatA1* was highest during the first hour of predation; microarray analysis (Lambert *et al.*, 2010a) showed that the expression of $tatA1_{Bd}$ was increased 3.2-fold after 30 min of initial predatory growth, compared with expression in attack phase *B. bacteriovorus* without prey. Thus we hypothesized that in *B. bacteriovorus*, TatA1 may be an important constituent of a predatory Tat system that transports proteins that contribute to predation, possibly replacing the 'housekeeping' TatA2 in some Tat complexes with TatBC to specialize them for a burst of predatory substrate transport while TatBCA2 complexes continue constitutive housekeeping Tat substrate transport.

Complementation analysis of *B. bacteriovorus tat* gene product function in *E. coli*

E. coli tat mutants lose the ability to grow on LB agar plates containing 2 % SDS (Buchanan *et al.*, 2002; Ize *et al.*, 2003) due to the failure to transport Tat substrates, including AmiAC, which are required for maintenance of the cell envelope (Bernhardt & de Boer, 2003). *E. coli tat* mutants expressing similarly functional *tat* genes would show complementation by a restoration of growth on plates containing SDS. Each *B. bacteriovorus tat* gene ($tatA2_{Bd}$, $tatB_{Bd}$, $tatC_{Bd}$ and $tatA1_{Bd}$) was expressed from pTrc99A (a standard expression vector used routinely for complementation studies in *E. coli*, on which the gene of interest is under the control of an IPTG-inducible promoter) in both a wild-type *E. coli* MC4100 and the corresponding *E. coli* deletion strain [JARV16-P $\Delta tatAE_{Ec}$; BØD-P $\Delta tatB_{Ec}$; B1LK0-P $\Delta tatC_{Ec}$ (Bogsch *et al.*, 1998; Buchanan *et al.*, 2002; Sargent *et al.*, 1999)] and strains were tested for resistance/sensitivity to 2 % SDS with and without IPTG induction (Fig. 2).

The wild-type *E. coli* MC4100 resisted 2 % SDS both with and without the $tatA2_{Bd}$ or $tatA1_{Bd}$ genes (Fig. 2a), but the JARV16-P (Δ*tatAE*) strain did not resist 2 % SDS when transformed with either the empty plasmid or pTrc-$tatA1_{Bd}$ (even when IPTG concentrations were increased from 100 to 500 nM). Complementation by $tatA2_{Bd}$ was optimal when expression was induced by IPTG (similar levels of complementation were seen with IPTG concentrations between 100 and 500 nM) from the pTrc99A plasmid *in trans* in *E. coli* (Fig. 2a), suggesting that $TatA2_{Bd}$ was required in large amounts to form a Tat-specific pore that could dock functionally with the $TatBC_{Ec}$ machinery. Despite the extensive similarity between $TatA1_{Bd}$ and $TatA2_{Bd}$ when compared with the similarity between the two *E. coli* TatA homologues (Supplementary Fig. S1) and even when expression of $TatA1_{Bd}$ was induced by high levels of IPTG, the $TatA1_{Bd}$ protein was seemingly not sufficiently homologous to either $TatE_{Ec}$ or $TatA_{Ec}$ for *in trans* complementation to occur. We did not investigate whether low transcription of $tatA1_{Bd}$ was occurring for some reason in the *E. coli*, so this remains to be tested.

The $\Delta tatB_{Ec}$ strain, BØD-P, was sensitive to 2 % SDS and showed very poor growth when transformed with the vector pTrc99A (Fig. 2b) or vector containing $tatB_{Bd}$. Interestingly, IPTG-induced expression of $tatB_{Bd}$ repressed the growth of the wild-type MC4100. This effect was not Tat-specific as it was observed whether the *E. coli* was grown on SDS assay plates or on plain nutrient agar, thus probably overexpressed *B. bacteriovorus* TatB forms toxic aggregates in *E. coli*, possibly because it could not form productive interactions with the other *E. coli* Tat proteins due to sequence differences.

A related, but slightly different, effect was seen when $tatC_{Bd}$ was overexpressed in an *E. coli tatC* strain, B1LK0-P (Fig. 2c); complementation was seen but only when no IPTG induction from the plasmid pTrc99A promoter was used; if induction was used then growth of the *E. coli tatC* mutant and wild-type *E. coli* was inhibited on both 2 % SDS plates and nutrient media alone. These data suggested that low levels of $TatC_{Bd}$ restored the function of the Tat system in *E. coli* but that high levels of $TatC_{Bd}$ repressed growth.

To summarize, $TatA2_{Bd}$ and $TatC_{Bd}$ can replace the function of $TatAE_{Ec}$ and $TatC_{Ec}$, respectively; overexpression of $TatB_{Bd}$ or $TatC_{Bd}$ repressed wild-type *E. coli* growth and $TatB_{Bd}$ and $TatA1_{Bd}$ (even with high levels of IPTG induction of expression) did not complement the *E. coli tat* mutants, suggesting that they may have specialized functions in *B. bacteriovorus* or that $tatA1_{Bd}$ expression levels are for some reason insufficient in *E. coli*.

Predatory killing efficiency of *B. bacteriovorus* on an *E. coli tatC* mutant

Predatory *B. bacteriovorus* grows within the periplasm of prey, a part of the cell where proteins transported by the prey cells own Tat transport system reside. *E. coli* prey are viable and transcriptionally active for 15 min after *B. bacteriovorus* attachment (Lambert *et al.*, 2010b) and, at this time, *E. coli* upregulates the expression of *hybA*, a hydrogenase 2 4Fe–4S ferrodoxin-type component required for H_2 oxidation (Menon *et al.*, 1994), and *sufI*/*ftsP*, required for stability of the divisome under conditions of stress (Samaluru *et al.*, 2007). Both of these periplasmic proteins are known *E. coli* Tat substrates (Dubini *et al.*, 2002; Tarry *et al.*, 2009). To see if *E. coli* Tat substrates affected predation we tested the efficiency with which *B. bacteriovorus* HD100 could prey upon the *E. coli* MC4100 *tatC* derivative B1LK0-P (Bogsch *et al.*, 1998) compared with wild-type *E. coli* MC4100.

The starting inocula of *E. coli* cells were matched by OD_{600} to give approximately the same prey cell volume in each, instead of matching prey c.f.u., which, due to the larger cell size of the B1LK0-P strain, would have resulted in a greater prey cell volume available for *B. bacteriovorus* replication [and thus more *B. bacteriovorus* progeny (Kessel & Shilo, 1976)]. After 1 h co-incubation, the viable MC4100 prey

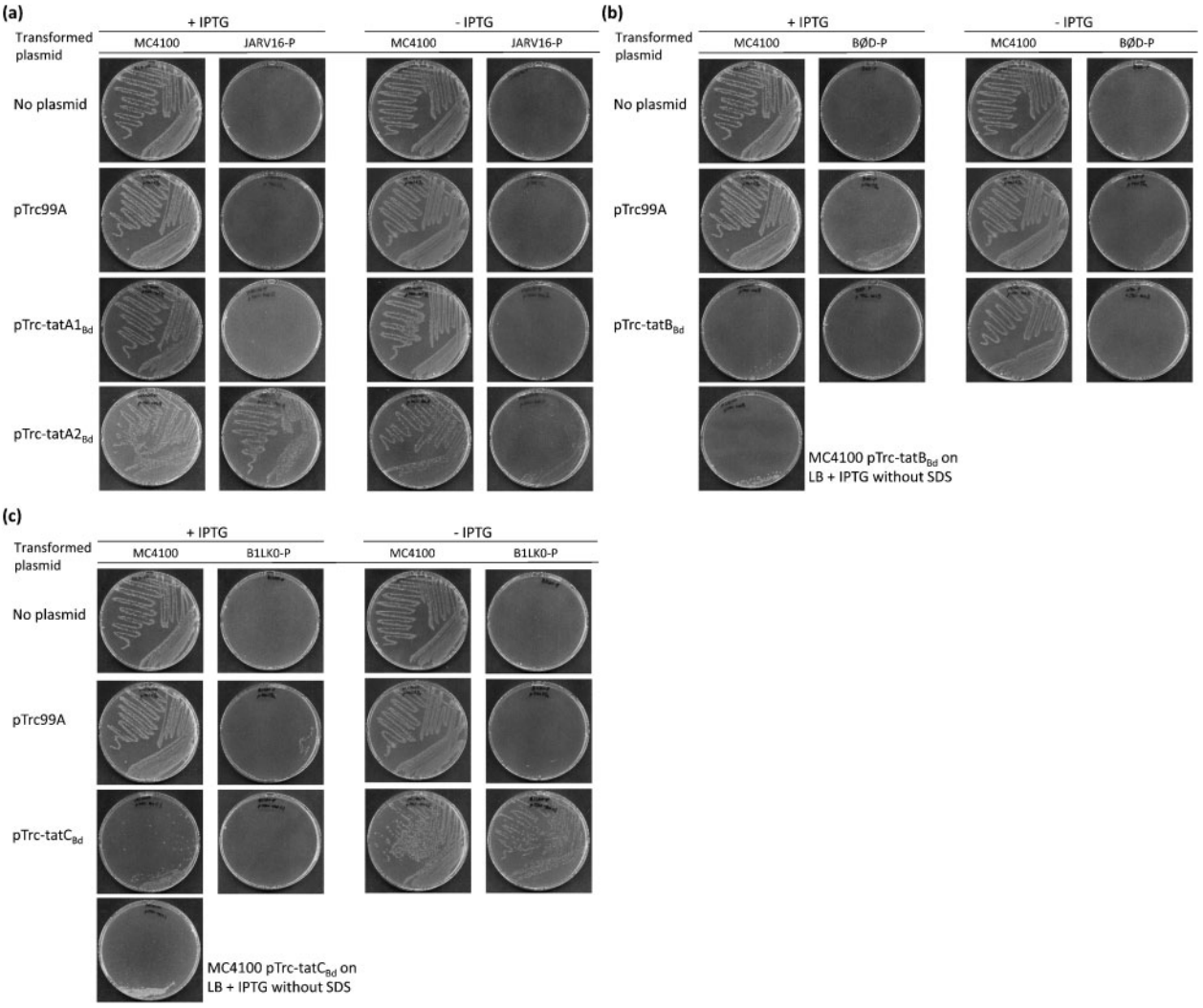

Fig. 2. Cross-species complementation tests of *E. coli* Tat mutants with *B. bacteriovorus* HD100 Tat genes. (a) *E. coli* MC4100 and JARV16-P (Δ*tatAE*$_{Ec}$) were transformed with empty pTrc99A, pTrc-tatA1$_{Bd}$ and pTrc-tatA2$_{Bd}$ and grown on 2 % SDS LB plates with or without 100 nM IPTG. (b) *E. coli* MC4100 and BØD-P (Δ*tatB*$_{Ec}$) were transformed with empty pTrc99A and pTrc-tatB$_{Bd}$ and grown on 2 % SDS LB plates with or without 100 nM IPTG. The separated plate (bottom left) shows the wild-type MC4100 strain transformed with pTrc-tatB$_{Bd}$ grown with 100 nM IPTG on an LB plate without SDS. (c) *E. coli* MC4100 and BILK0-P (Δ*tatC*$_{Ec}$) were transformed with empty pTrc99A and pTrc-tatC$_{Bd}$ and grown on 2 % SDS LB plates with or without 100 nM IPTG. The separated plate (bottom left) shows the wild-type MC4100 strain transformed with pTrc-tatC$_{Bd}$ grown with 100 nM IPTG on an LB plate without SDS.

population had been reduced to 11 % of its starting c.f.u. value, whilst the B1LK0-P prey had been reduced to 7 % (Fig. 3a); after 3 h, both prey populations had been reduced to 0.4 % of their initial c.f.u. values and the predation curves for both prey overlapped throughout. A corresponding rise in *B. bacteriovorus* viable counts was seen for both predatory cultures, with the *B. bacteriovorus* population preying upon wild-type MC4100 growing fourfold over 4 h and the *B. bacteriovorus* preying on the B1LK0-P increasing threefold over the same time period (Fig. 3b). Thus the yield of *B. bacteriovorus* from the B1LK0-P *tatC* mutant strain was slightly lower than for wild-type prey but not statistically significantly so, especially when we consider that the chance of double entry of *B. bacteriovorus* into a single prey was greater for the larger *tatC* mutant prey cells. No significant differences in the predation rates of *B. bacteriovorus* HD100 were seen when preying upon either wild-type MC4100 or B1LK0-P *tatC* mutant *E. coli* prey, other than those expected due to the cell division defects of the Δ*tatC E. coli* giving larger prey cells than the wild-type (Stanley *et al.*, 2001) and some chaining (which we observed).

We conclude that Tat-deficient and wild-type *E. coli* prey cells are both susceptible to predation and that

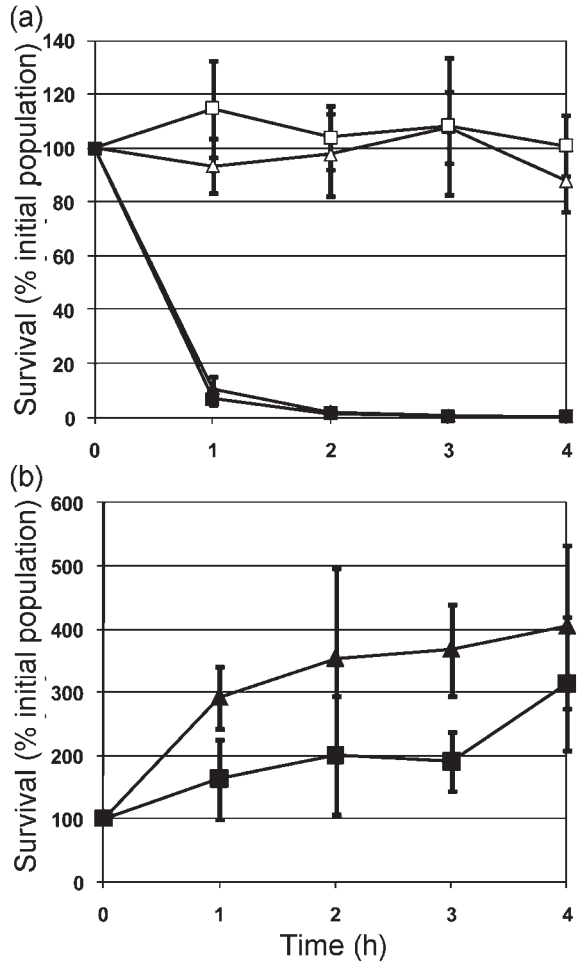

Fig. 3. Predation by *B. bacteriovorus* HD100 upon *E. coli* MC4100 and the BlLK0-P Δ*tatC* mutant. Prey were matched by initial OD_{600} readings for predation experiments. (a) *E. coli* prey populations (c.f.u.) as a percentage of starting population (c.f.u.). Data are *E. coli* MC4100 incubated alone (△), *E. coli* MC4100 with *B. bacteriovorus* HD100 (▲), *E. coli* B1LK0-P ($\Delta tatC_{Ec}$) incubated alone (□) and *E. coli* B1LK0-P with *B. bacteriovorus* HD100 (■). (b) *B. bacteriovorus* HD100 predator populations (p.f.u.) as a percentage of starting population (p.f.u.). Data are *B. bacteriovorus* HD100 with *E. coli* MC4100 (▲) and *B. bacteriovorus* HD100 with *E. coli* B1LK0-P (■). Error bars show SD.

Tat-exported substrates of prey do not significantly aid the survival or replication of predatory *B. bacteriovorus* in the periplasm of prey cells, or the defence of prey against predation, as only slight differences in *B. bacteriovorus* yield are seen (from a single round of infection, Fig. 3b).

Prediction and expression of potential Tat-transported substrates in *B. bacteriovorus*

Ten potential Tat substrates of *B. bacteriovorus* were predicted by the TatP programme (http://www.cbs.dtu.dk/services/TatP/; Bendtsen *et al.*, 2005; Table 1). All of them have twin-arginine motifs and potential cleavable Tat signals. Bd0673, Bd1160, Bd3199 and Bd3240 have unknown function, Bd2646 may be involved in membrane degradation, and Bd1802, Bd2540 and Bd2779 may be involved in redox reactions. Use of the recently published new prediction algorithm PRED-TAT (http://www.compgen.org/tools/PRED-TAT/; Bagos *et al.*, 2010) identified a further eight potential Tat substrates (Table 1), four of unknown function (Bd0882, Bd1908, Bd2530 and Bd3288), three more that may be involved in redox reactions (Bd1608, Bd1969 and Bd2010) and a phosphodiesterase (Bd3539). Because we anticipated significant redox challenges to *B. bacteriovorus* growing inside a dying bacterium, we reasoned that redox-active Tat substrates might modify the redox state of the prey cell contents allowing subsequent waves of predatory enzyme activities to be carried out, such as the scavenging of metals from macromolecules. Thus we wanted to test the contribution of some of these Tat substrates to predation.

Bd1802 is predicted to encode a high-redox-potential ferredoxin or high-potential iron–sulfur protein (HiPIP), containing all the known conserved residues essential to this type of protein, including the four cysteine residues that bind the Fe_4S_4 cluster to the protein backbone (González *et al.*, 2003). Thus Bd1802 represented a suitable redox-active protein to test. Interestingly, in contrast, the predicted products of two potential housekeeping genes of *B. bacteriovorus* had Tat secretion signals, indicating that they may be secreted into prey in the predatory process. They are Bd3112 which encodes a protein with two Pfam ribosomal protein motifs, S4/S4S9, indicative of RNA-binding capacity and Bd3906 coding for a protein with 31 % identity to a ParA DNA (chromosomal and plasmid) and protein partitioning protein from *E. coli*. We selected Bd1802 and Bd3906 as contrasting potential Tat substrates for further study but proceeded to test the expression of a large sample of predicted Tat substrate genes by RT-PCR.

Transcription of each of the TatP predicted genes was assayed throughout the predatory life cycle of *B. bacteriovorus* (and in HI cells); most had constitutive expression throughout the life cycle whilst three (Bd1802, 3199 and 3906, shown in Fig. 1b) had varying expression levels at different points in the developmental cycle. In wild-type *B. bacteriovorus*, the expression of Bd1802 increased (Fig. 1b) from attack phase to 30 min into predation, then briefly dropped at the 45 min time point and then returned to higher levels of expression at 2 h; expression then stayed steady until the end of the predatory cycle (Fig. 1b) at which time the *B. bacteriovorus* is growing as a coencytic filament using the hydrolysed contents of the prey cell cytoplasm. It also remained steady later when the cytoplasmic membrane itself of the prey cell is being degraded, exposing the *B. bacteriovorus* cell to the periplasmic contents of the prey as well as any remaining cytoplasmic contents, which may potentially include toxic compounds such as free radicals. The expression of Bd3199 peaked after 15 min of predation and then dropped slightly, whilst Bd3906 reached a peak in

Table 1. Predicted Tat substrates of *B. bacteriovorus* using the prediction algorithms TatP (Bendtsen *et al.*, 2005), PRED-TAT (Bagos *et al.*, 2010) and TAT-FIND (Rose *et al.*, 2002)

The N-terminal sequence of each gene is shown. The twin-arginine motif RR residues are highlighted in bold type and the FLK consensus sequences are underlined.

Gene	Description	Tat signal prediction program			N-terminal sequence
		TatP	PRED-TAT*	TATFIND	
Bd0673†	Hypothetical protein	Yes	Yes	Yes	MSMYYCKKT**RR**QFLVGSGKTLLALPLLPSLMPVEAFA
Bd0882	Hypothetical protein	No	Yes	No	MSDDSFKNFLKENAAPVPDAPLGESSRIWRHIEDRKH **RR**QRAWWVLVPAMAATLALVIAVKTQKP
Bd1160†	Conserved hypothetical protein	Yes	Yes	Yes	MGDGKNLT**RR**GFIAGGAVAGAAALVATPYERLLNAL
Bd1408	Outer-membrane protein	Yes‡	No	No	MAIHLSGFISEGFMKVFAALLLAALVTSSAAFAAPKTTAQN NATVKINPDTL**RR**LFLDRNVGIAISLNNVHQAKERVNVA
Bd1608	*hmoA*; molybdopterin oxidoreductase, iron–sulfur binding subunit	No	Yes	No	MRESDDEGGWA**RR**EFLKLMGASLAMASAGCIRRPVQKIV
Bd1802†	Putative high-potential iron–sulfur protein	Yes	Yes	Yes	MN**RR**GFFTTLAKITGVAVVAPTALNAVFSS
Bd1908	Hypothetical protein	No	Yes	No	MKEAMSVAPAS**RR**LPLTALLGIFGGAALGYYAYRKYKPS
Bd1969	*fabG*; oxidoreductase	No	Yes	No	MPAGTPASSAWPGLKMAEKGPEDQKTDKEELYSD**RR**R FAHGHNFSSAPTFATISPVQIHLRS
Bd2010	*fabF*; 3-oxoacyl-[acyl-carrier-protein] synthase II	No	Yes	No	MNSRFERPSKPQ**RR**VVVTGVGAVTPLGNTIEDSWAAAIRG
Bd2236	*tgt*; queuine tRNA-ribosyltransferase	No	No	Yes	MDGHNSTATDATMKLGEFKVHATDGHA**RR**ATLMTAHG PVQTPVFMAVGTKATVKA
Bd2530	Hypothetical protein	No	Yes	No	MKNDKLQKVLDEKTDLSSLKLS**RR**ELLETGVLSFAARVAV PGAVTSILTM
Bd2540†	Amine oxidase, flavin-containing	Yes	Yes	Yes	MLPFYAGEGLMAKSSFT**RR**EFLKISALGSSALA WGGCASAERFFM
Bd2646†	*choD*; putative cholesterol reductase	Yes	Yes	No	MKSYFS**RR**SFLKSSMAAGALGLLKSEDASAAPEI
Bd2779†	Putative amine oxidase	Yes	Yes	Yes	MQ**RR**DFLKTSLSVAALSSLPASSLAKGSEW
Bd3112†	Probable 30S ribosomal protein S4	Yes	No	No	MNRQGKTPRFKRQ**RR**LLTELPGMGKAGALERRPYPPG QHGL
Bd3199†	Hypothetical protein	Yes	Yes	Yes	MSINIARRAPVS**RR**LLFALSTSALILSGCASGSFKARQEQ
Bd3240†	Hypothetical protein	Yes	No	No	MRQSFDF**R**RLLFAYKSGRNLALMPSAYWSANMMKL
Bd3288	*ygiD*; hypothetical protein	No	Yes	No	MQCDRSALFHPCNSDLHFRGLAKQSKLADNQSMKLPSR PFS**RR**SLLFGAGSLAAVAGLSQLTSAKELLM
Bd3539	Phosphodiesterase/ Alkaline phosphatase D	No	Yes	Yes	MMSTS**RR**EFLENATKSMLIYTSLPAGLALAGNE
Bd3824	Hypothetical protein	Yes§	No	No	M**RR**TDLTMKNAKLIFTFAMMLTSLGAQAN
Bd3906†	*parA*; partition protein, ParA-like protein	Yes	Yes	No	MNVHAK**RR**TFLPQGGKMAKTICIANQKGGVGKTT

*PRED-TAT was published in 2010, after the majority of this study was completed.
†Genes initially identified by TatP and transcriptionally profiled.
‡Tat motif found after potential cleavage site.
§Potential Tat signal peptide but no Tat motif.

expression after 1 h of predation before dropping slightly; this differential expression of these genes throughout the predation cycle suggested that each may play a role in predatory growth, something that we tested later by mutagenesis and by GFP tagging of the Tat signal peptides of the two predicted substrates Bd1802 and Bd3906.

B. bacteriovorus Tat signal peptides were not recognized by the *E. coli* Tat system

Two predicted Tat signal sequences from *B. bacteriovorus* (from genes Bd1802 and Bd3906) were each translationally fused with a green fluorescent protein (eGFP) and expressed in *E. coli* using the accompanying native upstream *B. bacteriovorus* promoter. The *E. coli* also contained, as a periplasmic control, the vector pMal-p2-mCherry (Fenton *et al.*, 2010), which constitutively expresses mCherry fused to a *malE* signal sequence, allowing SecB-dependent transport into the periplasm. Each GFP fusion was cloned into *E. coli* MC4100 and its Δ*tatC* derivative B1LK0-P, revealing that whilst the Bd1802–eGFP fusion was expressed in both *E. coli* strains it was not exported to the periplasm (see Supplementary Fig. S2, available with the online version of this paper). As we later show (see Fig. 4) that the tagged Bd1802 protein is exported by *B. bacteriovorus*, this shows that the predicted (and indeed functional) Tat signal sequence from this *B. bacteriovorus* protein was not recognized by the *E. coli* Tat system. However, the Bd3906–eGFP fusion was not fluorescent (Supplementary Fig. S2), possibly due to the native Bd3906 promoter from *B. bacteriovorus* not being recognized by the *E. coli* transcription factors. (With hindsight we acknowledge that the signal motif predicted for Bd3906 contains three charged lysine residues in the typically hydrophobic region, so might not have been an ideal choice as a Tat substrate, but its expression profile, at a time of *B. bacteriovorus* secretion during predation encouraged us to test its function.)

Bd1802 a predicted *B. bacteriovorus* Tat substrate was exported into prey, yet was not essential for predatory or axenic growth

The predicted Tat signal-peptide–GFP fusions from the two *B. bacteriovorus* genes (Bd1802 and Bd3906) were integrated via a single crossover into the *B. bacteriovorus* genome, leaving them under the expression of their native promoter, as well as leaving a functional copy of the gene available (also expressed from a second copy of its native promoter). For the predicted Bd1802 Tat signal sequence–eGFP fusion protein during *B. bacteriovorus* predation, fluorescence was seen after 3 and 4 h of co-incubation with prey and eGFP was seen to be exported out of the growing *B. bacteriovorus* into the bdelloplast (Fig. 4). The appearance of eGFP later in bdelloplast development corresponded with the expression of the Bd1802 gene which was seen to be highest between 2 and 4 h after infection (Fig. 1b). No fluorescence for the Bd1802 Tat signal sequence eGFP fusion was seen in the attack-phase *B. bacteriovorus* (outside prey) or early in predation, as confirmed by Western blots of whole-cell protein from attack-phase *B. bacteriovorus* cells and late-stage bdelloplasts using an anti-GFP antibody (data not shown).

To test whether secreted Bd1802 high-potential iron–sulfur protein alone was performing a key function in the bdelloplast, a Bd1802 gene deletion strain was made; however, this retained predatory ability as well as the ability to grow axenically at normal rates (data not shown). We had hypothesized that during enzymic hydrolysis of the prey cell contents by *B. bacteriovorus*, Bd1802 may have acted to adjust the redox potential either of the prey cytoplasm to allow enzyme activity such as metal scavenging within the prey periplasm and/or to protect the growing and septating *B. bacteriovorus*. Clearly, from these data, the action of Bd1802 alone is not essential for this, and other redox-active proteins, some of which may be Tat substrates, must act also if bdelloplast redox balancing is an issue.

When the eGFP reporter fused with the predicted Bd3906 Tat signal peptide eGFP fusion (Bd3906 is a ParA homologue, predicted to be involved in DNA partitioning) was recombined into the *B. bacteriovorus* genome, fluorescence was observed throughout the growing *B. bacteriovorus* cell inside the bdelloplast between 2 and 4 h of predation (Fig. 4), after the peak of Bd3906 transcription seen at 1 h into predation by wild-type cells, although some attack-phase cells and some early bdelloplasts showed fluorescence as well. There was no sign of export of the Bd3906 Tat signal peptide eGFP fusion into the prey cell, the growing filamentous *B. bacteriovorus* were clearly seen to be fluorescent, whilst the rest of the prey bdelloplast remained dark (Fig. 4). Western blots with an anti-GFP antibody again confirmed the presence of the eGFP in both attack-phase *B. bacteriovorus* cells and in the bdelloplasts (data not shown). From this we concluded that Bd3906 has an intracellular role in *B. bacteriovorus*, probably as a main ParA protein for chromosome partitioning. As there are other ParA homologues in *B. bacteriovorus*, we had originally considered that Bd3906 might have had an exported role in prey DNA binding but multiple attempts to delete the Bd3906 gene in *B. bacteriovorus* (in both predatory and axenically growing cells) were unsuccessful, probably due to a need for this protein in *B. bacteriovorus* DNA partitioning. It remains of interest that DNA encoding a Tat-signal-like-sequence was found at the 5′ end of a *parA* gene but degenerate Tat signal sequences are reported to remain in genomes (Ize *et al.*, 2009; Sargent, 2007b; Turner *et al.*, 2004). These have often lost the second arginine in the canonical RRxFLK motif as well as having a scattering of acidic and polar residues in the hydrophobic region of the degenerate signal peptide(three K residues are present in that of Bd3906) and some bind chaperone-like proteins (Vergnes *et al.*, 2006). It may be that chaperone binding aids septal location of ParA in growing *B. bacteriovorus* filaments; also Tat chaperones may be involved in regulation of expression of gene products with a real or degenerate Tat signal motif, but testing this is beyond the scope of this study.

In *B. bacteriovorus*, *tatA2* and *tatC* are likely to be essential for cell viability, whilst deletion of *tatA1* significantly slows both predatory and axenic growth

To broadly characterize the roles of Tat substrates in *B. bacteriovorus*, we turned to the genes for the Tat system

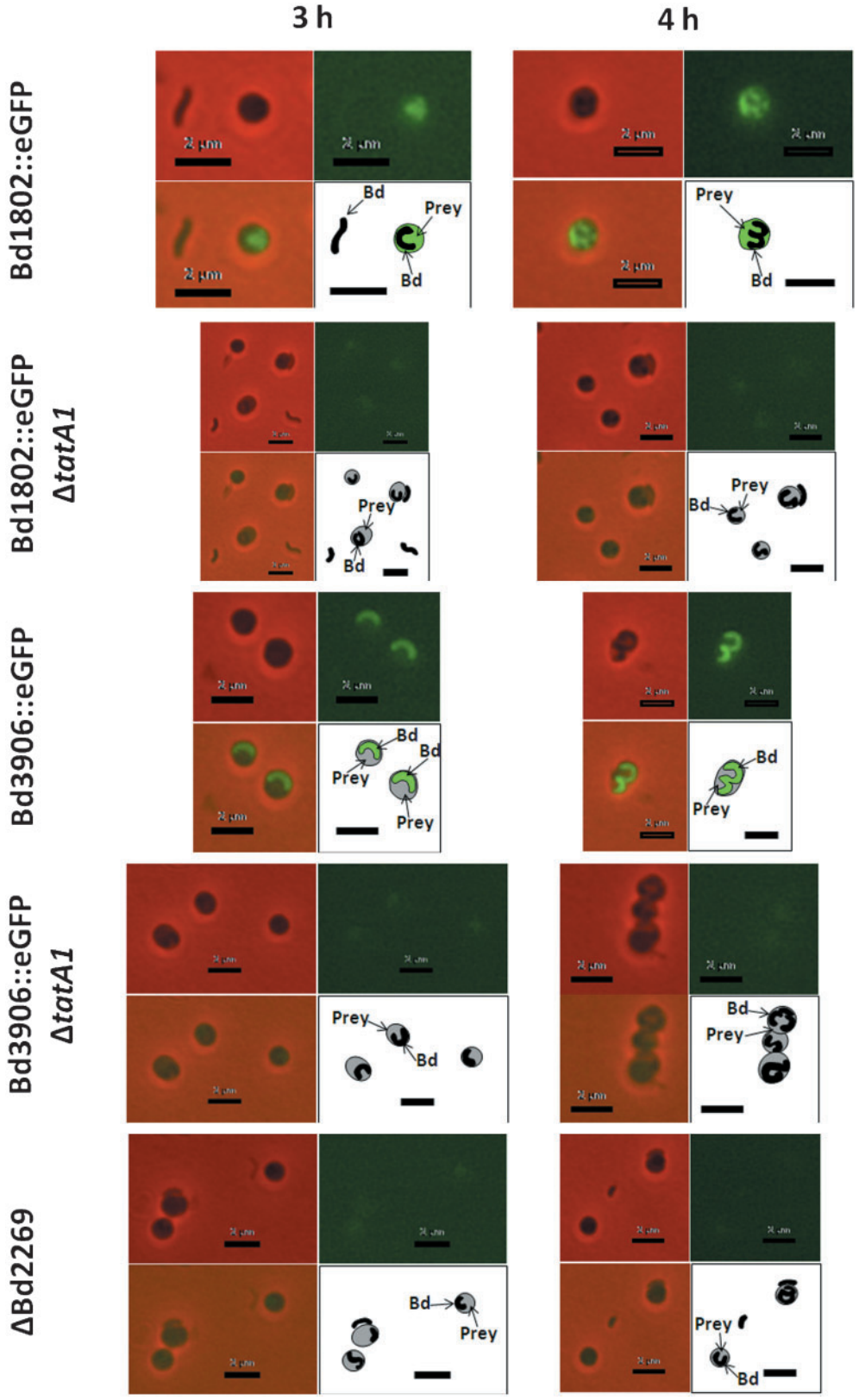

Fig. 4. Predatory growth by *B. bacteriovorus* strains with Tat signal sequence–eGFP fusion constructs integrated into the genome. The prey used was non-fluorescent *E. coli* S17-1. Images were taken after 3 and 4 h of predation, when *B. bacteriovorus* was in mid–late developmental stages, prior to release from the bdelloplast. Fluorescence was rarely seen before the 3 h time point for wild-type *B. bacteriovorus* and not at all for the $tatA1_{Bd}$ mutant. The *B. bacteriovorus* Bd2269 mutant was used as a wild-type, non-fluorescent, apramycin-resistant *B. bacteriovorus* control.

itself. Constructs containing each of the $tatA1_{Bd}$, $tatA2_{Bd}$ and $tatC_{Bd}$ genes disrupted with a kanamycin-resistance cassette were conjugated into wild-type *B. bacteriovorus* HD100 and resulting cultures (both of HD and HI *B. bacteriovorus*) were screened for recombinant mutant strains. Of these, only the *tatA1* gene could be successfully disrupted, as, for example, screening only 91 HD cultures for recombinant mutant strains yielded two *tatA1* mutants but 868 HD cultures and 480 HI cultures were screened unsuccessfully for recombinants after attempted construction of a *tatC* mutant, and similar numbers were unsuccessfully screened for a *tatA2* mutant. Thus, we concluded that both $tatA2_{Bd}$ and $tatC_{Bd}$ are likely to be essential for *B. bacteriovorus* cell viability, for both predatory and axenic growth, whilst $tatA1_{Bd}$ is not.

In liquid culture conditions, the $tatA1_{Bd}$ mutant attacked *E. coli* prey and completed the predatory cycle, yielding the same number of viable *B. bacteriovorus* progeny as the wild type ($2.5 \times 10^8 \pm 0.5 \times 10^8$ p.f.u. ml^{-1} in a typical predatory culture) after 24 h incubation with matched starting prey. However, when plated onto YPSC double-layer agar overlay plates with *E. coli* prey, the *tatA1* mutant produced very small plaques, over a longer period of time, when compared with the wild-type. It took 5–6 days for the plaques to appear on the prey overlay plates compared with 1–2 days for the wild-type, suggesting that inactivation of *tatA1* did slow *B. bacteriovorus* predation under these conditions.

Predatory cultures of both wild-type *B. bacteriovorus* HD100 and the $tatA1_{Bd}$ mutant with *E. coli* S17-1 prey (Fig. 5a) were seen, by microscopy, to attach to and invade prey and form bdelloplasts at the same rate; a finding confirmed by the use of the luminescent predation assay (Lambert *et al.*, 2003, 2006) which showed that the initial prey death was equal for both the wild-type and $tatA1_{Bd}$ mutant *B. bacteriovorus* strains (data not shown). The wild-type *B. bacteriovorus* lysed the bdelloplasts after 4 h in synchronous cultures (Fig. 5a) (seen as a reduction in the density of bdelloplasts and an increase in the number of free-swimming *B. bacteriovorus*), whilst in the *tatA1* mutant cultures, there remained a large number of bdelloplasts even after 6 h of incubation. This was probably caused by either slow development of the *tatA1* mutant within the bdelloplast or delayed release of progeny. Septation was seen in bdelloplasts made by the wild-type or *tatA1* mutant *B. bacteriovorus* (Fig. 5c), but the *tatA1* mutant cells septated later than the wild-type and the *B. bacteriovorus* were wider (Fig. 5b). Morphological differences have been reported for *tat* mutants in other bacterial species (Ize *et al.*, 2003; Kimura *et al.*, 2006; Stanley *et al.*, 2001).

HI axenically growing derivatives were isolated from the predatory *tatA1* mutant and their growth rate was found to be 3.2-fold slower (Fig. 5d), compared with a kanamycin-resistant HI control which has wild-type growth rate (*fliC1* merodiploid; Morehouse *et al.*, 2011). There was a large 44 h lag time before HI growth initiated for the *tatA1* mutant, compared with only a 2 h lag for the control. These data (Fig. 5d) show the growth of four separate HI isolates for each strain, thus it is not an HI-isolate-specific effect [we checked this because there is published diversity in HI growth rates for wild-type *B. bacteriovorus* (Barel & Jurkevitch, 2001)]. Thus, deletion of the *tatA1* gene in *B. bacteriovorus* causes significant reduction in growth rates of both predatory and especially axenic HI cells.

That growth of the *tatA1* mutant (both predatory and axenically) is slowed, but not abolished, and that deletion of either $tatC_{Bd}$ or $tatA2_{Bd}$ is lethal, suggests that *B. bacteriovorus* requires both TatBCA1 and TatBCA2 complexes for efficient growth, although *B. bacteriovorus* with just TatBCA2 complexes can survive in both growth modes. Recent *Bacillus subtilis* studies show that the loss of a TatA homologue can be compensated for by overexpression of the second homologue (van der Ploeg *et al.*, 2011) and early *E. coli* studies showed that overexpression of *tatA* homologue *tatE* (above native expression levels) can compensate for the loss of *tatA* (Sargent *et al.*, 1999) but without regulated overexpression tools for *B. bacteriovorus*, such experiments are not yet possible. With this in mind, we still hypothesize that Tat_{Bd} substrates may be either TatBCA1- or TatBCA2-optimized, and that some normally TatBCA1-specific substrates may be transported slowly, or in small amounts, by the TatBCA2 complexes in the $tatA1_{Bd}$ mutant. Sequence analysis of the potential *tat*-substrates alone is unlikely to ever reveal which are TatBCA1- or TatBCA2-specific, as the signal peptide binds to the TatBC complex, causing a conformational change which allows either TatA1 or TatA2 to dock, creating the full transport apparatus (Sargent, 2007a).

Altered expression of tat_{Bd} system and substrate genes in the *B. bacteriovorus tatA1* mutant

Practically, it was too challenging to study all the Tat substrates and TatABC proteins by GFP tagging in wild-type and *tatA1 B. bacteriovorus*, so RT-PCR using the *B. bacteriovorus tatA1* mutant and wild-type total RNAs (Fig. 1) was used to measure any regulatory effects of the *tatA1* mutation on potential substrate gene expression and genes expressing other components of the Tat transport system. In both the wild-type and the $tatA1_{Bd}$ mutant, the expression of $tatA2_{Bd}$ was steady throughout the cycle, whilst the expression profile of both $tatB_{Bd}$ and $tatC_{Bd}$ altered when $tatA1_{Bd}$ was deleted. In the absence of $tatA1_{Bd}$, both $tatB_{Bd}$ and $tatC_{Bd}$ had two peaks in expression, one at 15 min and one at 2 h into predation, compared with in the wild-type where their expression remained steady, slightly dropping towards the end of the life cycle (Fig. 1a).

Of the ten potential Tat_{Bd} substrates identified by TatP above, three had altered expression patterns in the $tatA1_{Bd}$ mutant compared with the wild-type strain. One of these, Bd1802, the potential high-redox-potential ferredoxin, shown previously to have a recognized Tat signal sequence

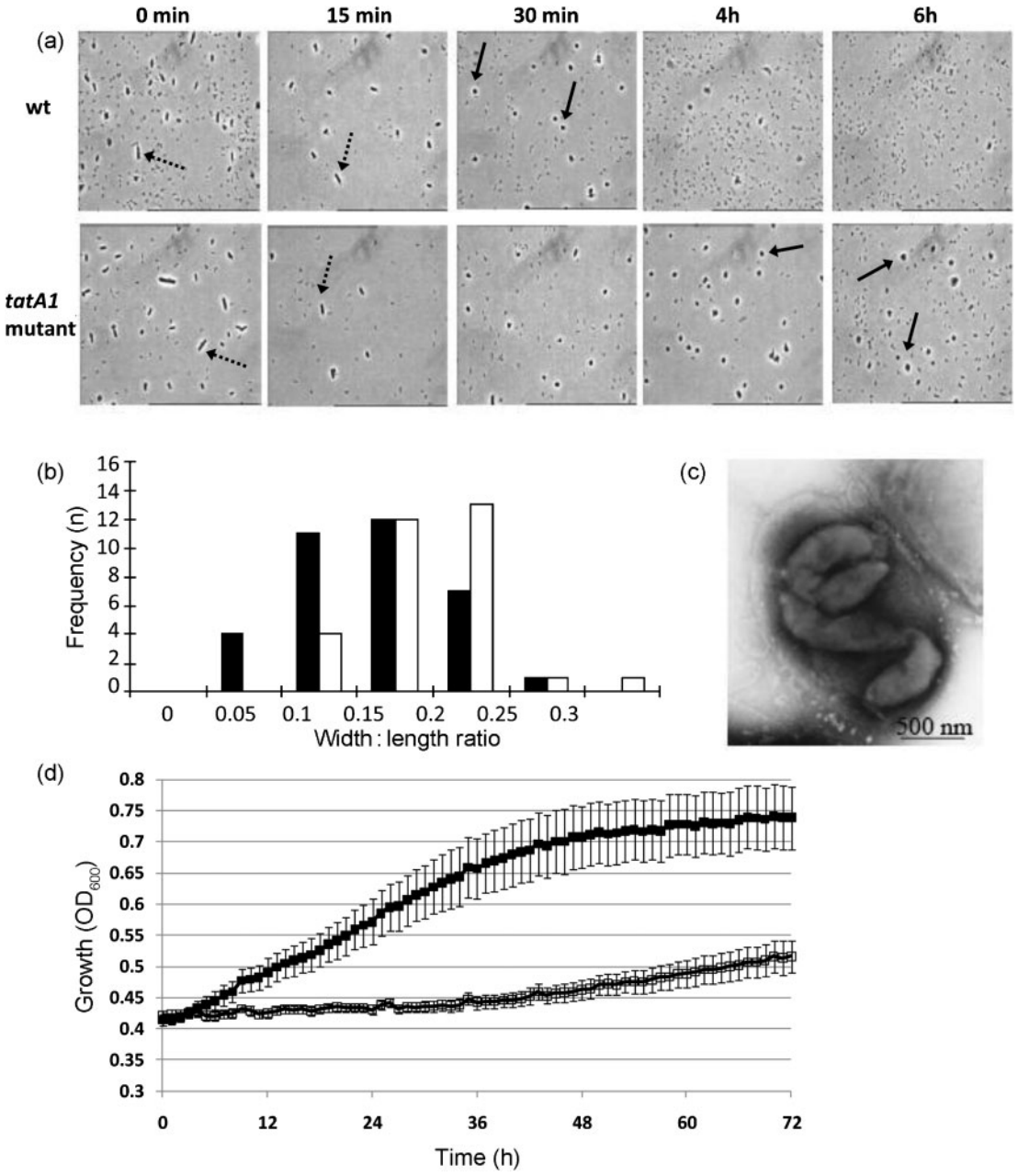

Fig. 5. Growth and morphology of the *B. bacteriovorus tatA1* mutant. (a) Microscopic analysis of bdelloplast persistence during predation of *E. coli* S17-1 (indicated by dashed arrows) by *B. bacteriovorus* HD100 (wt) and the *tatA1* mutant. By 6 h the wild-type had lysed all the bdelloplasts (indicated by solid arrows); this was accompanied by an increase in small *B. bacteriovorus* cells, whilst the *tatA1* mutant culture still contained many bdelloplasts. (b) The distribution of width : length ratios of progeny *B. bacteriovorus* cells recently released from bdelloplasts of wild-type HD100 (black bars) and the *tatA1* mutant (white bars). (c) Typical electron microscope image of the *B. bacteriovorus tatA1* mutant septating within a bdelloplast. (d) Growth of HI *B. bacteriovorus* strains. Each point is mean of six OD_{600} measurements from individual cultures of four separate HI isolates of each strain. The *fliC1* merodiploid HI strains (■) were used as a wild-type kanamycin-resistant control (Morehouse *et al.*, 2011); error bars show the 95 % confidence interval around the mean at each point. □, *B. bacteriovorus tatA1* mutant.

that caused an eGFP fusion protein to be exported from *B. bacteriovorus* into the prey (Fig. 4), was abnormally highly expressed throughout the predatory cycle in the *tatA1* mutant, whereas in the wild-type it had varying expression levels, being most highly expressed at 2 h into predation (Fig. 1b). Expression of the Bd1802 Tat signal sequence–eGFP fusion protein in the $tatA1_{Bd}$ mutant strain resulted in no visible fluorescence (Fig. 4) and eGFP could not be

detected in Western blots (data not shown). This suggests that the lack of a TatBCA1 system in the $tatA1_{Bd}$ mutant causes a paucity of Bd1802 protein in *B. bacteriovorus* which may be sensed, feeding back to induce higher expression of the Bd1802 gene. In the absence of a suitable Tat transport system to export it, any Bd1802 protein made must be targeted for degradation – thus we did not detect eGFP fluorescence in the *tatA1* mutant.

Bd3199, a conserved hypothetical, predicted Tat substrate, had a peak of expression at 15 min in wild-type cells, but in the *tatA1* mutant its expression gradually increased, peaking after 4 h of predation. A delay in the expression of Bd3906, a ParA homologue, was seen in the *tatA1* mutant, where its expression peaked after 2 h of predation, whereas in the wild type it peaked after 1 h (Fig. 1b). The Tat signal sequence from Bd3906 fused to the eGFP reporter protein (as described above) was also integrated into the wild-type and the *B. bacteriovorus tatA1* mutant strain. This reporter construct was strongly fluorescent within the wild-type *B. bacteriovorus* cell within the prey bdelloplast but did not show any fluorescence in the *tatA1* mutant strain (Fig. 4) and no eGFP could be detected in Western blots of whole-cell protein from both *B. bacteriovorus tatA1* mutant attack-phase cells and late-stage bdelloplasts (data not shown). So, although the Bd3906 signal sequence does not appear to be recognized by the Tat export apparatus, alterations to the Tat system by deletion of the *tatA1* gene did have an effect on gene expression and protein production. This is slightly puzzling but may suggest that regulatory feedback from defects in the Tat transporter still signal to regulate expression of even those genes with ancient degenerate Tat signals, such as those on Bd3906, even though those substrates are no longer themselves Tat-transported.

Conclusions

The Tat transport system has been shown to be essential for growth of several halophilic archaea (including *Halobacterium* sp. NRC1 and *Haloferax volcanii*; Dilks *et al.*, 2005; Rose *et al.*, 2002), important in virulence of multiple bacterial species (De Buck *et al.*, 2008), but until now identified as only being essential for the survival of one bacterial species, *Sinorhizobium meliloti* (Pickering & Oresnik, 2010) where genomic *tat* gene deletion was only possible when the cells contained a plasmid containing a supportive copy of the same *tat* gene. Few bacteria have two *tatA* (or *tatE*) homologues; the best characterized is *E. coli*, where deletion of *tatE* has no significant phenotype and, at native expression levels, TatE can only partially complement the loss of TatA (Sargent *et al.*, 1999). Interestingly our cross-complementation studies show that *B. bacteriovorus tatA2* can complement the loss of *E. coli tatA* and *tatE*, but that *B. bacteriovorus tatA1* cannot. We have shown that a functional TatBCA2 system is likely to be required for *B. bacteriovorus* survival and that although deletion of *tatA1* can be tolerated, it has a significant detrimental effect on predatory and especially axenic growth rates and that TatA1 is required for transport of at least one Tat substrate (Bd1802) into the prey cell during predation. That the *tatA1* mutant is attenuated in growth but the Bd1802 deletion mutant is not, shows that the other TatBCA1 substrate(s) must contribute to the wild-type growth rate of axenic and predatory *B. bacteriovorus*. Furthermore, it is likely that there are some TatBCA2-specific substrates that are essential for *B. bacteriovorus* viability in both growth modes. The very slow axenic growth phenotype of a single *tatA/E* homologue is unprecedented, even more so given the high levels of gene redundancy seen in other dual orthologous gene systems in *B. bacteriovorus* (Lambert *et al.*, 2006; Rendulic *et al.*, 2004).

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