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Bdellovibrio exovorus sp. nov., a novel predator of *Caulobacter crescentus*

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The life cycle, prey range and taxonomic status of a *Bdellovibrio*-like organism, strain JSS^T, were studied. Strain JSS^T was isolated from sewage in London, Ontario, Canada, in enrichment culture with *Caulobacter crescentus* prey cells. During predation, this strain remained attached to the outside of a stalked *C. crescentus* cell. No periplasmic growth stage was observed and no bdelloplast was formed. The stalked cells of *C. crescentus* retained their shape and, after predation, were devoid of cytoplasmic content, as shown by transmission electron microscopy. A periplasmic growth stage has been a definitive character in the description of members of the genera *Bdellovibrio*, *Bacteriovorax*, *Bacteriolyticum* and *Peredibacter*. This is the first description of an epibiotic predator in this group of prokaryotic predators. The G+C content of the genomic DNA of strain JSS^T was 46.1 mol%. 16S rRNA gene sequence analysis showed that this strain was related to *Bdellovibrio bacteriovorus* strains HD100^T, 109J, 114 and 127 (90–93 % similarity). Phylogenetic analysis based on 16S rRNA gene sequences grouped strain JSS^T with the *Bdellovibrio* cluster, but at a distance from other *Bdellovibrio* isolates. On the basis of features of the life cycle and phylogenetic data, it was concluded that strain JSS^T merits classification as the type strain of a novel species, for which the name *Bdellovibrio exovorus* sp. nov. is proposed (type strain JSS^T =ATCC BAA-2330^T =DSM 25223^T).

Bdellovibrio bacteriovorus is a small, rapidly motile, Gram-negative bacterium that is an obligate predator of other Gram-negative bacteria. It has a life cycle characterized by a free-living, non-replicating attack phase and a reproductive phase within the periplasm of the prey cell. The first report of *Bdellovibrio bacteriovorus* described the isolates as predatory, ectoparasitic and bacteriolytic micro-organisms (Stolp & Starr, 1963). Subsequent studies by Starr & Baigent (1966) and Burnham *et al.* (1968) described prey cell penetration and the intraperiplasmic growth of the parasite. For many years, new isolates of predatory bacteria were assigned to the genus *Bdellovibrio*, as strains of *Bdellovibrio bacteriovorus*, based on their morphological characteristics (comma-shaped rods; motile by means of a single, polar, sheathed flagellum) and predatory life cycle. Seidler & Starr (1969) reported the DNA G+C contents of 11 isolates of prey-dependent bdellovibrios and noted that two isolates of uncertain nomenclature status had G+C contents of ~43 mol%, less than that of all other isolates, which had G+C contents of ~50 mol%. Two novel species were described by Seidler *et al.* (1972): *Bdellovibrio starrii* (the A3.12^T isolate from the original work of Stolp and Starr) and *Bdellovibrio stolpii* (the UKi2^T isolate from the Conti lab). These two species were subsequently transferred to a new genus, *Bacteriovorax* (Baer *et al.*, 2000), and then each to other new genera, as *Peredibacter starrii* (Davidov & Jurkevitch, 2004) and *Bacteriolyticum stolpii* (Piñeiro *et al.*, 2008). Collectively, *Bdellovibrio*, *Bacteriovorax*, *Bacteriolyticum* and *Peredibacter* species and strains and taxonomically uncharacterized isolates of intraperiplasmic, predatory prokaryotes are referred to as '*Bdellovibrio*-and-like organisms' (BALOs) (Snyder *et al.*, 2002). As predators, BALOs have been defined as having a cell cycle featuring an obligate, intraperiplasmic developmental stage inside the substrate bacterium (Jurkevitch, 2006).

This paper is dedicated to R. G. E. Murray, for his contributions to systematics.

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Abbreviation: BALO, *Bdellovibrio*-and-like organism.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JSS^T is EF687743.

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

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In a study on the protective function of paracrystalline protein surface layers (S-layers) against predation by *Bdellovibrio bacteriovorus* (Koval & Hynes, 1991), we isolated on *Caulobacter crescentus* CB2A, a predatory bacterium, strain JSS^T, which resembled *Bdellovibrio bacteriovorus* in many respects. This BALO is a small, motile, comma-shaped rod that possesses a single, polar, sheathed flagellum (29 nm wide) (Koval & Hynes, 1991). It is a prey-dependent predator that forms plaques on lawns of *C. crescentus*. In the present study, we report further characterization of strain JSS^T and provide evidence for the description of a novel species of *Bdellovibrio*.

Initial studies on the growth of strain JSS^T were done in co-cultures in 1/10-strength yeast extract-peptone medium supplemented with calcium (YP/10 plus Ca^{2+}) (Koval & Hynes, 1991). This was necessary because the *C. crescentus* strain with an S-layer (strain CB2NY66R) was unstable in the HEPES plus Ca^{2+} buffer used in other co-cultures in this study. However, the S-layer-negative strain of *C. crescentus* (strain CB2A) (=ATCC BAA-2331 =DSM 25117) is stable in HEPES buffer. HM buffer [3 mM HEPES (pH 7.6) with 1 mM $CaCl_2$ and 0.1 mM $MgSO_4$] was used for further studies, to correlate with other studies on *Bdellovibrio bacteriovorus* 109J (Thomashow & Rittenberg, 1978; Flannagan *et al.*, 2004). Dilute nutrient broth medium (Cotter & Thomashow, 1992) may also be used for co-cultures. For maintenance, co-cultures containing 1 ml strain JSS^T and 4 ml of a 24 h peptone-yeast extract (PYE) broth culture (Johnson & Ely, 1977; Koval & Hynes, 1991) of *C. crescentus* CB2A were incubated in 125 ml flasks with 20 ml HM buffer. The co-culture was incubated at 30 °C with shaking at 150 r.p.m. for 24–48 h. Cultures were transferred to sterile screw-capped tubes and kept at 4 °C for up to 1 month. Cell suspensions were plated for plaques using yeast extract-peptone-acetate medium (YPSC) (Koval & Hynes, 1991) or dilute nutrient broth medium. Plaques were small. For long-term storage, a 24–48 h culture of strain JSS^T cells was frozen in the presence of 25 % (w/v) glycerol at −80 °C. *Bdellovibrio bacteriovorus* strain 109J was grown on *Escherichia coli* ML35 (Flannagan *et al.*, 2004) and strain 6-5-S on *Aquaspirillum serpens* VHL (Koval & Hynes, 1991). *Bacteriolyticum stolpii* UKi2^T was obtained from J. Tudor (St. Joseph's University, Philadelphia, PA, USA); it was maintained on peptone-yeast extract medium (1 % peptone, 0.3 % yeast extract, 1 % agar, pH 6.8) (Seidler & Starr, 1969).

The *C. crescentus* strains used in this study are listed in Table 1 and were obtained from J. Smit (University of British Columbia, Vancouver, BC, Canada) and J. Poindexter (Barnard College, Columbia University, New York, NY, USA). All *C. crescentus* strains were maintained on PYE medium. Other Gram-negative bacteria used to test the prey range of strain JSS^T were from the culture collection of the Department of Microbiology and Immunology, University of Western Ontario.

Table 1. Predation of *Caulobacter crescentus* strains by strain JSS^T

Strain	S-layer	Predation	Source
CB2A	−	+	J. Smit
CB2NY66R	+	−	J. Smit
CB13bla	−	+	J. Smit
CB15A KSAC	−	+	J. Smit
Rmg1	−	+	J. Poindexter
A-19	+	−	J. Poindexter
CB15-BE	−	+	J. Poindexter

The age of prey cells affected the growth of strain JSS^T. When *C. crescentus* CB2A was grown in PYE medium, cells entered late exponential phase/early stationary phase in about 10–12 h. In co-cultures, strain JSS^T grew well on prey cells grown for 8, 14 and 24 h but, with prey cells grown for 48 h, predation was incomplete. There were always some residual, unattacked prey cells in the co-culture. Therefore, *C. crescentus* CB2A cells were routinely grown for 24 h before use.

Initial observations on the growth of strain JSS^T were made by phase-contrast light microscopy, at which time the lack of intraperiplasmic growth was noted (Koval & Hynes, 1991). Co-cultures were then examined by transmission electron microscopy. For thin sections, samples were fixed with acrolein (5 % v/v) and glutaraldehyde (0.25 %, v/v) in 50 mM cacodylate buffer, enrobed in Noble agar and post-fixed with 1 % osmium tetroxide and 1 % uranyl acetate. After dehydration in an ethanol series, samples were embedded in Spurr resin. Thin sections were post-stained with uranyl acetate and lead citrate. All preparations were examined in a Philips EM300 operating at 60 kV. Predators remained attached to the outside of the prey cell (Fig. 1) and did not enter the periplasmic space. At the attachment site (Fig. S1, available in IJSEM Online), a 'bulge' of prey outer membrane formed around the attached end of strain JSS^T. There was a localized degradation of peptidoglycan, but the electron-dense bilayer of the prey plasma membrane remained intact (Fig. S1b). No bdelloplast-like structures were ever seen in thin sections and the prey cells did not round up after attack by strain JSS^T. The stalked cells retained their original shape and, at the end of predation, were devoid of cytoplasmic content (Fig. 1b). Strain JSS^T is thus an example of an epibiotic predator (Davidov *et al.*, 2006).

How do strain JSS^T cells replicate? No long, aseptate filaments (analogous to those seen inside bdelloplasts) were ever seen on the outside of prey cells by light or electron microscopy. In thin sections (Fig. 1), cells of strain JSS^T appeared to be dividing by binary fission. For analysis by scanning electron microscopy (SEM), a 20 ml culture of strain JSS^T was centrifuged, resuspended in 5 ml YP/10 plus Ca^{2+} medium and added to 2 ml of a 24 h culture of *C. crescentus* CB2A in 18 ml YP/10 plus Ca^{2+} in a 125 ml side-arm flask. The co-culture was incubated at 30 °C with

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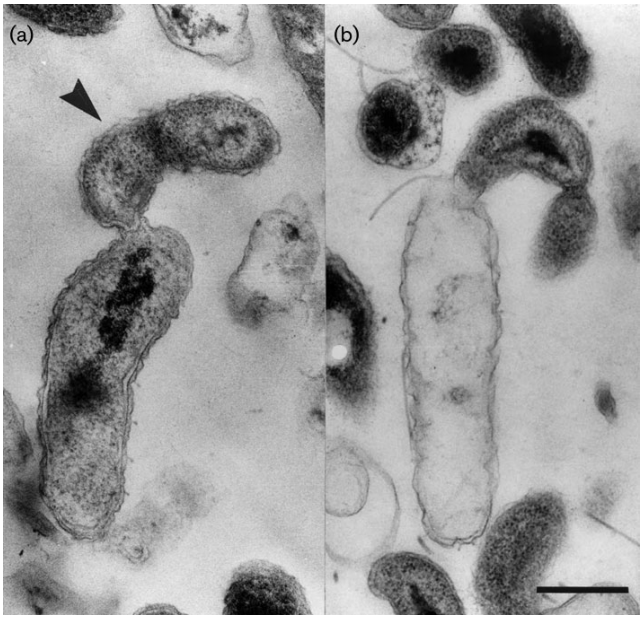

Fig. 1. Thin sections of cells of strain JSS^T attached to intact (a) and empty (b) stalked cells of *Caulobacter crescentus* CB2A. The arrowhead in (a) indicates the cell of strain JSS^T. Bar, 500 nm. The image in (a) is reprinted from the cover of *Journal of Bacteriology* vol. 183 issue 11 (2001) with permission from the American Society for Microbiology.

shaking until many JSS^T cells had attached to prey cells. Samples (0.5 ml) from co-cultures were filtered through a Nucleopore 0.2 μm polycarbonate filter (Millipore Corp.) in a 13 mm Swinney filter holder. Filters were fixed overnight in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), rinsed twice in buffer, post-fixed in 1 % osmium tetroxide for 1 h at 4 °C and then rinsed with buffer and distilled water before dehydration in a graded ethanol series. Samples were critical-point-dried with a SamDRI-PVT-3B model, gold coated and examined in a Hitachi model S-4500 field emission scanning electron microscope. Cells of strain JSS^T were seen attached to stalked prey cells (Fig. S2a), but no long filamentous extracellular forms were seen. It was concluded that strain JSS^T divides by binary fission. SEM preparations confirmed the evacuation and collapse of stalked prey cells during predation (Fig. S2b).

The prey range of strain JSS^T was tested on a panel of Gram-negative bacteria by an agar spot inoculation test (Schillinger & Lücke, 1989). An overnight culture (50 ml) of the test organism was centrifuged and resuspended in 1–3 ml YPSC medium, depending upon the size of the pellet. An aliquot (20 μl) of the cell suspension was added to 3.5 ml molten 0.6 % YPSC agar and poured over the surface of YPSC agar plates (1.5 %). After the semi-solid agar had solidified, 10 μl of a 24–48 h culture of strain JSS^T was placed on the surface of the semi-solid agar. Plates were incubated at 30 °C and observed for clearing of the lawn. None of the following Gram-negative bacteria were susceptible to predation by strain JSS^T: *Aeromonas salmonicida*, *Aquaspirillum serpens*, *Azospirillum brasilense*, *Enterobacter aerogenes*, *Escherichia coli*, *Erwinia herbicola*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas syringae*, *Rhizobium leguminosarum*, *Salmonella* sp., *Shigella boydii* and *Shigella flexneri*. Thus, strain JSS^T has a very limited prey range, compared with *Bdellovibrio bacteriovorus* 109J (Jurkevitch *et al.*, 2000) and other isolates (Stolp & Starr, 1963). All *C. crescentus* strains that did not possess an S-layer were susceptible to predation (Table 1). *C. crescentus* CB2A is not susceptible to predation by *Bdellovibrio bacteriovorus* strain 109J or 6-5-S (Koval & Hynes, 1991).

To determine the DNA base composition, late-stage co-cultures were filtered (0.45 μm) to remove residual prey cells. DNA isolation was performed using standard methods (Sambrook *et al.*, 1989). The genomic DNA G+C content was determined by thermal denaturation in 1× SSC. The G+C content was calculated using the equation of Mandel *et al.* (1970) [$G+C=1.99(T_m-66.0)$]. DNA from *Escherichia coli* K-12 and prey-independent *Bdellovibrio bacteriovorus* HI100 was used as reference DNA. The base composition of DNA from strain JSS^T was 46.1 mol% G+C. The base composition of DNA prepared from *Bdellovibrio bacteriovorus* HI100 was 49.7 mol% G+C. This value compares favourably with the published value of 50.4 mol% G+C (Burnham & Conti, 1984; Williams *et al.*, 2005). The thermal denaturation curve of the DNA preparation from strain JSS^T gave no indication of contaminating *C. crescentus* DNA (62–67 mol% G+C; data not shown).

Schwudke *et al.* (2001) proposed that the genetic locus *hit* (for host interaction) was restricted to *Bdellovibrio bacteriovorus* strains and could be used as a specific probe for this species. Therefore, we screened the genomic DNA of strain JSS^T for the presence of the *hit* locus by PCR amplification (according to Schwudke *et al.*, 2001) and Southern blotting (as described by Flannagan *et al.*, 2004) with a DIG-labelled *hit* locus probe. The *hit* locus was not identified in strain JSS^T by either method (data not shown). As positive controls, genomic DNA of *Bdellovibrio bacteriovorus* strains 109J and 6-5-S was screened. As a negative control, genomic DNA of *Bacteriolyticum stolpii* UKi2^T was screened. These results support the identification of strain JSS^T as a member of a novel species of *Bdellovibrio*.

For identification purposes, the 16S rRNA gene of strain JSS^T was amplified and sequenced in almost its entire length (1432 bp) as described by Jurkevitch *et al.* (2000). The 16S rRNA gene sequence (Genbank accession no. EF687743) had 93 % similarity to that of *Bdellovibrio bacteriovorus* HD100^T. Other *Bdellovibrio bacteriovorus* isolates (strains 109J, 114, 127) had similarity values of 90–93 % to strain JSS^T. Therefore, the closest relatives to strain JSS^T are members of the genus *Bdellovibrio*.

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A phylogenetic tree was reconstructed based on 16S rRNA gene sequences. Eleven good-quality, long (>1200 bp), representative cultured 16S rRNA gene sequences from *Bdellovibrio* strains were downloaded from GenBank on October 2011 and aligned by MUSCLE (Edgar, 2004). The alignment was trimmed at both ends in order for all the sequences to overlap each other fully, and a maximum-likelihood tree was reconstructed using MEGA5 (Tamura *et al.*, 2011), with *Bacteriovorax* as an outgroup. Our phylogenetic analysis confirmed that strain JSS^T is a member of the class *Deltaproteobacteria* and that it belongs to the *Bdellovibrio* cluster (Fig. 2). It is distantly related to *Bdellovibrio bacteriovorus* strains, though not as distant as isolate TRA2. A more extensive phylogenetic analysis (not shown) was undertaken using all 162 good-quality, long *Bdellovibrio* 16S rRNA gene sequences available at the RDP-II database, from both cultured and environmental samples. This phylogenetic tree confirmed the position of strain JSS^T in the *Bdellovibrio* lineage, and also revealed an interesting observation: the *Bdellovibrio* sequences split into two major clusters, one encompassing all the cultured strains alongside some sequences from uncultured organisms and another composed solely of sequences from uncultured bacteria. This suggests that many groups in this latter cluster may be very different from the 'classic' *Bdellovibrio*, thus providing ample opportunity for further study of the diversity of this genus.

Since the report of strain JSS^T by Koval & Hynes (1991), more BALOs have been isolated on lawns of *C. crescentus* CB2A, this time from another sewage treatment plant in London, Ontario, Canada, and from compost samples. These strains (KL1 to 8) had similar properties to strain JSS^T: extracellular attachment, no bdelloplast formation and no lysis of *Escherichia coli* ML35 (Shemesh *et al.*, 2003). The partial sequence of the 16S rRNA gene of strain KL8 (GenBank accession no. JQ080553) showed 99 % similarity to the 16S rRNA gene sequence of strain JSS^T.

A summary of the phenotypic characteristics of strain JSS^T and *Bdellovibrio bacteriovorus* HD100^T is provided in Table 2. Strain JSS^T lacks an intracellular, periplasmic growth phase, and no bdelloplast is formed during the life cycle. The prey range, as studied so far, is limited to *C. crescentus*. Additional chemotaxonomic data would be useful to support this proposal for a novel species of *Bdellovibrio*. However, these analyses need to be done with prey-independent mutants of predators, a rationale stated by Schwudke *et al.* (2001). The presence of remaining prey cells in a co-culture necessitates their separation from predators by filtration or differential centrifugation. This is not an easy task, especially on a large scale. Caution would have to be exercised in interpretation of chemotaxonomic data from prey-independent mutants, as the chemical composition of mutants may not be the same as that of the wild-type strain. Furthermore, analyses of properties such as fatty acid composition need to be conducted under standardized conditions, as the results vary with experimental conditions (temperature, growth stage, growth medium). For BALOs, the chemical composition of cells may also vary between the attack and growth phases.

Strain JSS^T differs from *Bdellovibrio bacteriovorus* as it does not demonstrate a periplasmic growth stage or form bdelloplasts. Strain JSS^T therefore represents a novel species, for which the name *Bdellovibrio exovorus* sp. nov. is proposed.

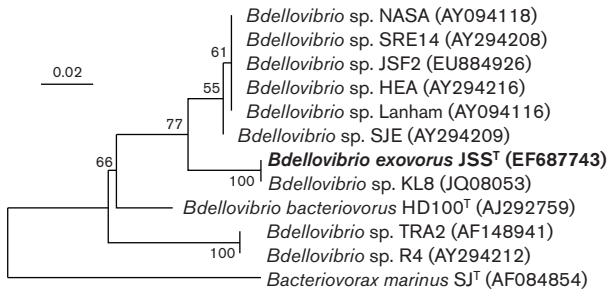

Fig. 2. Phylogenetic tree of the *Bdellovibrionales* based on 16S rRNA gene sequences, showing the position of strain JSS^T in the genus *Bdellovibrio*. Bootstrap values (>50 %) based on 1000 replicates are shown at branch nodes. Bar, 0.02 substitutions per nucleotide position.

Description of *Bdellovibrio exovorus* sp. nov.

Bdellovibrio exovorus (ex.o.vo′rus. Gr. pref. *exo-* outside; L. v. *vorare* to devour, to consume; L. suff. *-vorus* eating; N.L. masc. adj. *exovorus* eating outside, referring to the epibiotic life cycle).

Gram-negative, comma-shaped rods (0.5 μm wide, 0.5–1.4 μm long) with a single, polar, sheathed flagellum (29 nm wide). Obligate predator; the type strain was isolated from sewage in enrichment cultures with *Caulobacter crescentus* CB2A. Does not prey on *Escherichia coli*. Cells exhibit a biphasic life cycle: a free swimming attack phase and a growth phase whereby the predator attaches to and remains on the outside of the prey cell. There is no periplasmic stage within the prey cell. No bdelloplast is formed. Empty stalked *C. crescentus* cells remain after predation is complete. Small plaques are formed on lawns

Table 2. Phenotypic characteristics of strain JSS^T and *Bdellovibrio bacteriovorus* HD100^T

Both strains form vibroid cells with a single, polar, sheathed flagellum and both strains form plaques.

Characteristic	Strain JSS^T	*B. bacteriovorus* HD100^T
Width of flagellum (nm)	29	28
Intracellular growth	−	+
Bdelloplast formation	−	+
Prey range	*Caulobacter*	Wide

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of prey cells. Growth is by binary fission while attached to the prey cell. On the basis of 16S rRNA gene sequence similarity, belongs to the class *Deltaproteobacteria*, related to *Bdellovibrio bacteriovorus* (93 % similarity). Differs from *Bdellovibrio bacteriovorus* as it does not demonstrate a periplasmic growth stage or form bdelloplasts.

The type strain, JSS^T (=ATCC BAA-2330^T =DSM 25223^T), was isolated from sewage in enrichment cultures with *Caulobacter crescentus* CB2A. Similar isolates were obtained from compost and soil. The DNA G+C content of the type strain is 46.1 mol%.

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