International Journal of Systematic and Evolutionary Microbiology (2004), **54**, 1011–1016 **DOI** 10.1099/ijs.0.02458-0

Reclassification of salt-water *Bdellovibrio* sp. as *Bacteriovorax marinus* sp. nov. and *Bacteriovorax litoralis* sp. nov.

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Bdellovibrios are unique, predatory bacteria with an intraperiplasmic growth and multiplication phase within their prey, which consists of many Gram-negative bacteria. Until recently, all bacteria that exhibited these traits were included in the genus *Bdellovibrio*. However, analysis of 16S rDNA sequences and other studies have demonstrated substantial genotypic, phenotypic and ecotypic diversity among the organisms in this genus (Baer *et al.*, 2000; Snyder *et al.*, 2002). This has resulted in reclassification of *Bdellovibrio stolpii* and *Bdellovibrio starrii* into the newly constructed genus *Bacteriovorax* (Baer *et al.*, 2000). In this study, examination of marine isolates of *Bdellovibrio* (designated SJ^T, AQ and JS5^T) has revealed them to be related more closely to the newly designated genus *Bacteriovorax*. Phylogenetic analysis of 16S rRNA gene sequences revealed that marine isolates SJ^T, AQ and JS5^T clustered in a separate clade from *Bdellovibrio bacteriovorus* 100^T as part of the clade that contains *Bacteriovorax* spp., indicating a much closer taxonomic relationship to the latter. DNA–DNA hybridization experiments also demonstrated $<5\,\%$ similarity between *Bdellovibrio bacteriovorus* 100^T and the marine isolates. Distinct differences between the salt-water group and *Bdellovibrio* spp. were also observed by determination of DNA G+C content, salinity growth testing and antibiotic sensitivity analysis. On the basis of the results from the studies described above, it is proposed that marine isolates SJ^T (=ATCC BAA-682^T=DSM 15412^T) and JS5^T (=ATCC BAA-684^T=DSM 15409^T) should be classified within the genus *Bacteriovorax* as the type strains of *Bacteriovorax marinus* sp. nov. and *Bacteriovorax litoralis* sp. nov., respectively.

Bdellovibrio and *Bdellovibrio*-like organisms (BALO) are Gram-negative bacteria that prey uniquely upon a wide variety of susceptible, Gram-negative bacteria. They exhibit a biphasic life cycle that consists of an intraperiplasmic growth phase within their prey and a free-living, extracellular phase, in which the highly motile organisms 'hunt' for new cells to attack. Soon after their discovery in 1962 (Stolp & Petzold, 1962), two groups of *Bdellovibrio* were recognized. The freshwater/terrestrial group can only tolerate sodium chloride concentrations of $<0{\cdot}5\,\%$ and is found in soil and freshwater (Varon & Shilo, 1968). Marine or halophilic organisms require sodium chloride at concentrations of $>0{\cdot}5\,\%$ and are found in oceans, seas, estuaries and other salt or brackish waters (Taylor *et al.*, 1974; Marbach *et al.*, 1976; Williams, 1979, 1987; Williams & Falkler, 1984; Sutton & Besant, 1994). In addition, marine bdellovibrios have DNA G+C contents of <38 mol% (Schoeffield, 1990), whereas those of freshwater/terrestrial organisms range from 47 to 51 mol% (Seidler *et al.*, 1972; Marbach *et al.*, 1976). The DNA G+C contents of members of the genus *Bacteriovorax* (Baer *et al.*, 2000) that were classified previously as *Bdellovibrio* range from 41 to 44 mol% (Seidler *et al.*, 1972). Few studies have described the properties of the marine bdellovibrios (Taylor *et al.*, 1974; Marbach *et al.*, 1976; Sutton & Besant, 1994) and these

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Abbreviations: BALO, *Bdellovibrio* and *Bdellovibrio*-like organisms; PD, prey-dependent; PI, prey-independent.

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Bacteriovorax marinus* SJ^T, *Bacteriovorax marinus* AQ and *Bacteriovorax litoralis* JS5^T are AF084854, AF084855 and AF084859, respectively.

have typically included a limited number of characters, as traditional laboratory-based tests cannot be used to characterize the predators because they do not grow in pure culture. The most common properties that are used to distinguish the predators are those in which BALO can be grown with their prey and include salinity and temperature growth ranges and prey susceptibility patterns. Although marine and terrestrial predators are quite distinct in these properties and their DNA G + C content, they have continued to be assigned to the same genus, *Bdellovibrio*. This suggests, misleadingly, that they are closely related and share many similar properties. In this study, we have shown that marine and terrestrial *Bdellovibrio* sp. are genetically and phenotypically diverse, as shown by comparison of 16S rDNA sequences, DNA–DNA similarity studies, determination of DNA G + C content and culture-based methods. We report the first evaluation of the taxonomic classification for three marine isolates (SJ^{T}, AQ and JS5^{T}) of *Bdellovibrio*.

Strains AQ, SJ^{T} and JS5^{T} were isolated by Schoeffield (1990) from water samples from the National Aquarium in Baltimore, St John's Island in the Caribbean and the Chesapeake Bay estuary, respectively. Marine, prey-dependent (PD) strains were grown in prey/sea water (PS) medium with *Vibrio parahaemolyticus* P-5 (Williams, 1987; Schoeffield, 1990; Williams *et al.*, 1995), whilst freshwater/terrestrial PD strains were grown in dilute nutrient broth (DNB) medium (Starr & Seidler, 1971) with *Escherichia coli* ML35. Prey-independent (PI) mutants were isolated from wild-type PD cultures by using the methods described by Seidler & Starr (1969) for freshwater/terrestrial strains or by Schoeffield (1990) for marine strains. Suspensions of PD isolates were prepared by filtration through a 0·3 μm filter (Millipore) for marine strains or a 0·45 μm filter (Nucleopore) for freshwater/terrestrial strains. After centrifugation at 27 000 ***g*** for 60 min, predator cells were resuspended in sterile 70 % artificial sea water (ASW) or DNB for marine and freshwater/terrestrial strains, respectively. Predator concentrations (cells ml^{-1}) were determined by the acridine orange direct-count method (Hobbie *et al.*, 1977). *Bdellovibrio* genomic DNA was purified by CsCl density gradients that were prepared as described by Ausubel *et al.* (1987). 16S rRNA genes were amplified by using primers 8–27F (5′-AGAGTTTGATCCTGGCTCAG-3′, modified from FD1) (Weisburg *et al.*, 1991) and 1492R (5′-GGTTACCTTGTTACGACTT-3′; Weisburg *et al.*, 1991; Reysenbach *et al.*, 1992). Both strands of the resulting amplicon from each isolate were sequenced completely and aligned with sequences that were published previously for members of the genus *Bacteriovorax* and closely related micro-organisms (Baer *et al.*, 2000) by using the PHYDIT program (Chun, 1995). Evolutionary trees were inferred by using four treeing algorithms that are implemented in the PHYLIP package: Fitch–Margoliash (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1981; Olsen *et al.*, 1994), maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987). Evolutionary distance matrices for the neighbour-joining and Fitch–Margoliash methods were generated by the method of Jukes & Cantor (1969). The final unrooted tree (Fig. 1) was evaluated by neighbour-joining bootstrap analyses, based on 1000 reassembled datasets. 16S rRNA gene sequences for isolates SJ^{T} (GenBank accession no. AF084854), AQ (AF084855) and JS5^{T} (AF084859) were compared with the sequences for *Bdellovibrio bacteriovorus* 100^{T} (AF084850, a gift from J. Tudor), *Bacteriovorax stolpii* Uki2^{T} = ATCC 27052^{T} (M34125) and *Bacteriovorax starrii* A3.12^{T} = ATCC 15145^{T} (AF084852) in GenBank. Subsequently, a similarity analysis was performed to compare the BALO 16S rDNA sequences with those of related taxa in the δ-*Proteobacteria*. The level of similarity found between 1155 nucleotide sites of *Bdellovibrio bacteriovorus* 100^{T} and marine isolates SJ^{T},

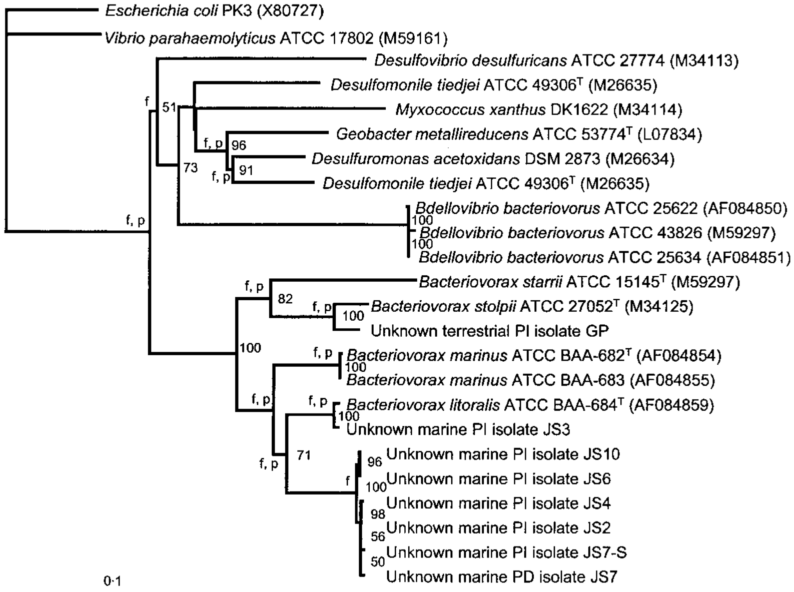

Fig. 1. Neighbour-joining phylogenetic tree, demonstrating the relationship between marine and terrestrial *Bdellovibrio* isolates. Values represent stability of branches (%), based on 1000 resamplings. Branches that are the same using the Fitch–Margoliash (f) and DNA parsimony (p) analyses are marked.

AQ and JS5^T was 81·7, 81·8 and 81·5 %, respectively. *Bdellovibrio bacteriovorus* 100^T had greater similarity to other genera in the *δ-Proteobacteria*, such as *Desulfovibrio desulfuricans*, 82·1 %; *Myxococcus xanthus*, 83·5 %; *Geobacter metallireducens*, 84·7 % and *Desulfomonile tiedjei*, 85·1 %. In contrast, 16S rDNA sequence similarity between the marine isolates (SJ^T and JS5^T) and *Bacteriovorax stolpii* Uki2^T was significantly higher (91·0 and 91·1 %, respectively). Similarity values between the marine isolates and *Bacteriovorax starrii* A3.12^T ranged from 88·6 (JS5^T) to 88·8 (SJ^T) %. Similarity between SJ^T and JS5^T was determined to be much higher (> 93 %). The distant relationship between the marine isolates and other *Bdellovibrio* spp. is apparent from the unrooted evolutionary tree (Fig. 1). The marine isolates did not cluster with the *Bdellovibrio bacteriovorus* clade, but within a larger group that is divided into two branches, one of which contains *Bacteriovorax* spp. and the second the marine strains. Data generated by the maximum-parsimony, maximum-likelihood and Fitch–Margoliash methods produced similar results.

Genomic relatedness of each isolate was investigated by DNA–DNA hybridization by using a direct binding assay (Johnson, 1988) with [α-^{32}P]dCTP-labelled probes on membranes (Denhardt, 1966). Intensity of hybridization was measured by using a STORM 840 PhosphoImager (Perkin-Elmer). Reciprocal experiments were performed for each pair of strains. DNA–DNA hybridization results revealed low similarity (< 3·5 %) between *Bdellovibrio bacteriovorus* 100^T [and also strains 109J (a gift from M. Thomashow) and 2484Se2 (= ATCC 25635)] and marine isolate SJ^T. *Bdellovibrio bacteriovorus* strains E (= ATCC 25634) and Ox9-2 (= ATCC 25635) showed a slightly higher degree of DNA–DNA similarity to SJ^T (5·7 and 5·2 %, respectively). Marine isolate JS5^T also demonstrated low similarity (< 3·6 %) to all *Bdellovibrio bacteriovorus* strains (100^T, 109J, Ox9-2, E and 2484Se2). A previous study (Baer *et al.*, 2000) demonstrated low similarity (< 4 %) between strains of *Bdellovibrio bacteriovorus* and the *Bacteriovorax* species (< 3–4 %). Isolate SJ^T demonstrated slightly higher similarity to *Bacteriovorax stolpii* Uki2^T and *Bacteriovorax starrii* A3.12^T (7·7 and 3·5 %, respectively). DNA–DNA similarity between JS5^T and *Bacteriovorax* species was slightly lower (4·9 and 3·1 %, respectively).

The DNA G + C content of PI isolates AQ and SJ^T was determined by thermal melting curves (Schoeffield, 1990). The HPLC method was used for PD isolate JS5^T (Mesbah *et al.*, 1989). The DNA G + C contents of SJ^T, AQ and JS5^T were 37·7, 38·3 and 37·8 mol%, respectively; these values are lower than the range of 47–51 mol% that has been reported for freshwater/terrestrial *Bdellovibrio* (Seidler *et al.*, 1972; Marbach *et al.*, 1976) and also than the range for the genus *Bacteriovorax* (41–44 mol%) (Seidler *et al.*, 1972).

Comparisons of the phenotypic properties of the marine and terrestrial *Bdellovibrio* strains confirmed the differences that were revealed by molecular methods. The enzymic reactions of each PD isolate and its PI derivative were examined by using the API ZYM test system (bioMérieux), according to the manufacturer's recommendations. The results reported in Table 1 were consistent for both PI and PD isolates, except where noted. Of the 19 enzyme substrates against which the isolates were tested, only four (valine and cystine aminopeptidases, trypsin and chymotrypsin) yielded differential reactions (Table 1). Reactions to valine and chymotrypsin differentiated salt-water from freshwater *Bdellovibrio*. A positive reaction for cystine differentiated isolates SJ^T and 109J (negative). Isolates AQ and JS5^T yielded variable results. Lack of trypsin activity distinguished the estuarine isolate, JS5^T, from all other isolates, which were positive.

Antibiogram profiles were generated for PD strains by using the double-agar overlay technique (Stolp & Starr, 1963) and, for PI strains, by the typical spread-plate method, as described by Guether & Williams (1993). Freshwater/terrestrial PD strains were tested on dilute (1/10) PYE (peptone/yeast extract) medium with *E. coli* ML35 as prey. For marine *Bdellovibrio*, dilute (1/10) SWYE (sea water/yeast extract/agar) medium was used with the prey, *V. parahaemolyticus* P-5. A second prey, *Pseudomonas fluorescens*, was used for testing both marine and terrestrial PD strains. The antibiotics tested are listed in Table 1. A resistant reaction was observed by growth of the predators up to the disc, as indicated by clearing or lysis of the prey lawn. In a sensitive reaction, the predators did not grow to the edge of the discs and there remained a turbid zone of prey cell growth. Control tests with only the prey lawn and without the predators were included. Tests were repeated three times for each isolate. The results reported in this study represent antibiotic susceptibilities that were consistent for both PD and PI isolates. The results revealed that susceptibility to methicillin yielded a clear distinction between marine (resistant) and freshwater (susceptible) BALO strains. Kanamycin was observed to distinguish between isolates SJ^T (susceptible) and JS5^T (resistant). No difference was found between the pattern susceptibilities of isolates SJ^T and AQ (Table 1).

The temperature growth range for each isolate was determined. For PD strains, double-agar overlay plates were incubated in humidified chambers for up to 2 weeks at 10, 15, 20, 25, 30, 35 and 40 °C. Plates incubated at 10 and 15 °C were incubated for up to 2 months. For these plates, the prey cell concentration in the top agar was increased to compensate for the slower growth rate of the prey. Mean plaque count and standard deviation (SD) were calculated from three replicate experiments. For testing PI strains, suspensions were prepared in sterile 70 % ASW for marine isolates and sterile distilled water for freshwater/terrestrial strains. Aliquots of the suspensions (0·1 ml) were spread-plated onto SWYE agar or PYE agar. Plates were incubated under the same conditions and mean counts were calculated, as for the PD predators. The results revealed that the temperature growth range was 15–35 °C for freshwater

Table 1. Differential characteristics of salt-water and freshwater *Bdellovibrio* strains

Unless otherwise noted, all results are for PD strains and their respective PI derivatives.

Characteristic	Salt-water isolates			Freshwater isolate 109J
	SJ^T	AQ	$JS5^T$	
Na^+ required for growth ($\geqslant 0{\cdot}5\,\%$)	+	+	+	−
DNA G+C content (mol%)	37·7*	38·3*	37·8	51·5†
Growth temperature (°C)	15–30	15–30	15–35	15–35
Enzyme activities tested:				
Alkaline phosphatase	+	+	+	+‡
Esterase (C4)	+	+	+	+‡
Esterase lipase (C8)	+	+	+	+
Lipase (C4)	v§	v§	−	−‡
Leucine aminopeptidase	+	+	+	+‡
Valine aminopeptidase	+	+	+	−‡
Cystine aminopeptidase	+	v§	v§	−‡
Trypsin	+	+	−	+‡
Chymotrypsin	−	−	−	+‡
Acid phosphatase	+	+	+	+‡
Phosphoamidase	+	+	+	+‡
α-Galactosidase	−	−	−	−‡
β-Galactosidase	−	−	−	−‡
β-Glucuronidase	−	−	−	−‡
α-Glucosidase	−	−	−	−‡
β-Glucosidase	−	−	−	−‡
N-Acetyl-β-glucosaminidase	v§	v§	v§	−‡
α-Mannosidase	−	−	−	−‡
α-Fucosidase	−	−	−	−‡
Antibiotic sensitivity:				
Methicillin (5 μg)	R‖	R	R	S
Ampicillin/sublactam (20 μg)	S¶	S	S	S
Carbenicillin (100 μg)	S	S	v§	S
Kanamycin (30 μg)	S	S	R	S
Gentamicin (10 μg)	S	S	v	S
Nalidixic acid (5 μg)	R	R	v§	R
Colistin sulphate (10 μg)	R	R	R	R
Polymyxin B (300 U)	S	S	v	R
Vancomycin (30 μg)	R	R	R	v§

*From Schoeffield (1990).

†From Seidler *et al.* (1969).

‡Only enzyme activities from PI isolates.

§v, Variable results observed for PD and PI strains.

‖R, Resistant to antibiotic.

¶S, Susceptible to antibiotic.

strains (with a few exceptions), 15–30 °C for marine strains AQ and SJ^T and 15–35 °C for isolate $JS5^T$ (Table 1).

Salinity growth range is not only a distinctive feature between marine and terrestrial BALO, but also among marine isolates. Those organisms that were isolated from the upper and mid-regions of the Chesapeake Bay estuary, where salinities range from 0·5 to 2 %, tended to grow at lower salinities than isolate SJ^T, which was recovered from ocean waters (salinity of approximately 3 %). Differences between ocean and estuarine isolates were also revealed by 16S rDNA sequence analysis. These differences warrant the separation of these organisms into different species within the same genus.

The results of this study provide conclusive evidence that the genus *Bdellovibrio* consists of molecularly diverse groups of micro-organisms that are not related closely to each other. The use of a predatory lifestyle as the sole criterion for classification of these organisms has resulted in the inclusion of phylogenetically diverse groups within the same genus. A change in the taxonomic status of marine *Bdellovibrio* is clearly warranted, based on the phylogenetic 16S rDNA sequence analysis, DNA–DNA similarity results, DNA G+C contents and phenotypic properties that are described in this investigation. Phylogenetic analysis of BALO 16S rRNA gene sequences revealed that marine isolates SJ^T, AQ and $JS5^T$ clustered in a separate clade from *Bdellovibrio bacteriovorus* 100^T, as part of the clade that contains *Bacteriovorax* spp., indicating a much closer taxonomic relationship to the latter. Results from other studies confirm that all marine *Bdellovibrio* isolates analysed to date fall into the same clade (Snyder *et al.*, 2002). It is appropriate, therefore, that salt-water *Bdellovibrio* should be reassigned to the genus *Bacteriovorax*. Here, we propose that marine isolates SJ^T and $JS5^T$ should be moved from the genus *Bdellovibrio* and assigned to the genus *Bacteriovorax*, as the type strains of *Bacteriovorax marinus* sp. nov. and *Bacteriovorax litoralis* sp. nov., respectively. Marine strain AQ should also be placed within *Bacteriovorax marinus*, due to its genetic similarities to isolate SJ^T. However, phenotypic and biochemical differences suggest that it may be a separate strain; this requires further study.

Description of *Bacteriovorax marinus* sp. nov.

Bacteriovorax marinus (ma′ri.nus. L. masc. adj. *marinus* of the sea, marine).

Cultural, biochemical and molecular characteristics of *Bacteriovorax marinus* are listed in Table 1. Optimal temperature range for growth is 15–30 °C. Salinity range is 10–60 parts per thousand (p.p.t.), with optimal growth between 20 and 30 p.p.t. Resistant to methicillin, nalidixic acid, colistin sulfate and vancomycin, but susceptible to kanamycin, carbenicillin, ampicillin/sublactam, gentamicin and polymyxin B (Guether & Williams, 1993). Enzyme activities for trypsin, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine aminopeptidase, valine aminopeptidase, acid phosphatase and phosphoamidase are detected. Closely related phylogenetically to both *Bacteriovorax stolpii* $Uki2^T$ and *Bacteriovorax starrii* $A3.12^T$, as determined by 16S rDNA sequence analysis. DNA G+C content is 37·7–38·3 mol%.

The type strain of *Bacteriovorax marinus* is SJ^T (=ATCC $BAA\text{-}682^T$ = DSM 15412^T). Isolated from the surrounding waters of St John's Island, US Virgin Islands. Reference strain is AQ.

Description of *Bacteriovorax litoralis* sp. nov.

Bacteriovorax litoralis (li.to.ra′lis. L. masc. adj. *litoralis* pertaining to the coast).

Cultural, biochemical and molecular characteristics of *Bacteriovorax marinus* are listed in Table 1. Optimal temperature range for growth is 15–35 °C. Salinity range is 0·25–30 p.p.t., with optimal growth at 5 p.p.t. Resistant to a range of antibiotics (methicillin, kanamycin, colistin sulfate and vancomycin), but susceptible to ampicillin/sublactam (Guether & Williams, 1993). No trypsin activity is detected (unlike *Bacteriovorax marinus* and *Bdellovibrio bacteriovorus* 109J), but enzyme activities are demonstrated for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine aminopeptidase, valine aminopeptidase, acid phosphatase and phosphoamidase. Closely related phylogenetically to both *Bacteriovorax stolpii* $Uki2^T$ and *Bacteriovorax starrii* $A3.12^T$, as determined by 16S rDNA sequence analysis. DNA G+C content is 37·8 mol%.

The type strain (and only strain to date) is $JS5^T$ (=ATCC $BAA\text{-}684^T$ = DSM 15409^T). Isolated from the gills of a crab captured on the Patuxent River, an estuary of the Chesapeake Bay.

Acknowledgements

This material is based on work that was supported by the National Science Foundation under grant no. OCE-9731055.

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