Phylogenetic relationships amongst the saltwater members of the genus *Bacteriovorax* using *rpoB* sequences and reclassification of *Bacteriovorax stolpii* as *Bacteriolyticum stolpii* gen. nov., comb. nov.

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Members of the saltwater genus *Bacteriovorax*, formerly known as the marine *Bdellovibrio*, are obligate predatory bacteria that prey selectively on other Gram-negative bacteria. Previous phylogenetic analysis based on the 16S rRNA genes of saltwater *Bacteriovorax* isolates from environmental samples revealed 11 distinct phylogenetic clusters based on ≥96.5% gene sequence similarity. In other micro-organisms, the gene coding for the β-subunit of RNA polymerase (*rpoB*) has been shown to be more discriminating than 16S rRNA genes. In this study, *rpoB* sequences from *Bacteriovorax* isolates were analysed to determine whether the results would be consistent with those based on 16S rRNA gene sequences. A 1242 bp region of the *rpoB* gene from 74 saltwater *Bacteriovorax* strains and two freshwater isolates, *Bacteriovorax stolpii* Uki2T and *Peredibacter starrii* A3.12T, was amplified by PCR and analysed. The sequences were aligned and phylogenetic trees were constructed using a neighbour-joining algorithm. The resulting tree showed that the *rpoB* sequences produced smaller subdivisions of isolates, but were nevertheless consistent with the clusters determined using 16S rRNA gene sequences. Thus, the highly conserved 16S rRNA gene sequences provided good phylogenetic information and the *rpoB* gene sequences permitted greater differentiation in order to further subdivide phylogenetically distinct groups within the genus *Bacteriovorax*. Also, on the basis of the extensive diversity and large distance between the saltwater members of the genus *Bacteriovorax* and the freshwater/soil *Bacteriovorax*, a reclassification of *Bacteriovorax stolpii* as *Bacteriolyticum stolpii* gen. nov., comb. nov. is proposed. A new family, *Peredibacteraceae* fam. nov., is also described.

INTRODUCTION

Members of the genera *Bdellovibrio* and *Bacteriovorax* and similar organisms are obligate predatory bacteria that prey on other Gram-negative bacteria and can be isolated from many environmental sources (Stolp, 1968). They have a unique life cycle in which they have an extracellular attack phase followed by penetration into the periplasm of a host cell. In the periplasm, they undergo filamentous growth and divide into several daughter cells that lyse the host cell to enter the extracellular attack phase (Ruby, 1991).

The genus *Bacteriovorax* was proposed with the reclassification of *Bdellovibrio stolpii* and *Bdellovibrio starrii* as members of the genus (Baer et al., 2000). Subsequently, the saltwater isolates *Bacteriovorax marinus* and *Bacteriovorax litoralis* were placed into the genus based on 16S rRNA gene sequence analysis, DNA G+C ratio, DNA–DNA hybridization and salinity experiments that differentiated them from *Bdellovibrio* spp. (Baer et al., 2004) and the freshwater *Bacteriovorax* strains, *Bacteriovorax stolpii* and *Bacteriovorax starrii* (subsequently renamed *Peredibacter starrii* by Davidov & Jurkevitch, 2004). Delineation of

Abbreviation: *rpoB*, β-subunit of RNA polymerase.

The GenBank/EMBL/DDBJ accession numbers for the *rpoB* gene sequences determined in this study are EF536750–EF536823 and EU240892–EU240893.
species within the saltwater members of the genus *Bacteriovorax* is not yet resolved. Identification and classification of these micro-organisms are difficult because of the general absence of morphological or physiological features that can be tested in the presence of the prey cells. A number of attempts to set up reliable identification schemes based on phenotypic characterization or prey susceptibility, as well as initial molecular characterization, have been reported in previous studies (Seidler *et al.*, 1969; Taylor *et al.*, 1974; Marbach *et al.*, 1975; Sutton & Besant, 1994; Píneiro *et al.*, 2004).

The *rpoB* gene, which encodes the β-subunit of RNA polymerase, has been shown to be a useful and more discriminating alternative to the 16S rRNA gene for inferring phylogenetic relationships (Case *et al.*, 2007). It has previously been demonstrated to be a suitable target on which to base species identification for the genera *Staphylococcus* (Drancourt & Raoult, 2002), *Mycoplasma* (Kim *et al.*, 2003), *Bacillus* (De Clerck & De Vos, 2004) and *Acinetobacter* (La Scola *et al.*, 2000).

In the case of the saltwater members of the family *Bacteriovoracaceae*, previous phylogenetic analysis based on 16S rRNA genes of isolates from various locations worldwide has revealed eight distinct phylogenetic clusters, along with three outlier isolates, based on ≥96.5% gene sequence similarity (Píneiro *et al.*, 2007). In order to better define the phylogeny of the genus *Bacteriovorax*, 74 saltwater isolates, representatives of the 16S rRNA gene clusters and three outlier isolates, were selected, their *rpoB* genes were sequenced and a phylogenetic tree was constructed. The *rpoB* gene sequences from two freshwater isolates, *Bacteriovorax stolpii* and *Peredibacter starrii* (Baer *et al.*, 2000), were included. The objective of this study was to demonstrate that phylogenetic analysis based on the *rpoB* gene from *Bacteriovorax* environmental isolates was consistent with and more discriminatory than the phylogenetic clustering obtained using 16S rRNA genes. Finally, the reclassification of *Bacteriovorax stolpii* as *Bacteriolyticum stolpii* gen. nov., comb. nov. is proposed based on the extensive diversity between the saltwater members of the genus *Bacteriovorax* and the isolates derived from freshwater and soil.

**METHODS**

**Bacterial strains and DNA processing.** Seventy-four saltwater *Bacteriovorax* isolates were obtained from environmental samples collected in various countries as previously described (Píneiro *et al.*, 2007). The freshwater/soil strains *Bacteriovorax stolpii* and *Peredibacter starrii*, donated by Dr E. Jurkevitch, were also included in the study. Lytic plaques of each isolate were obtained using a double overlay method with prey *Vibrio parahaemolyticus*, as described previously (Schoeffield & Williams, 1990). *Bacteriovorax stolpii* and *P. starrii* were maintained as described by Baer *et al.* (2000). Prey cells were removed by filtration and DNA was extracted from the *Bacteriovorax* cells using a Qiagen kit according to the manufacturer’s specifications. The DNA samples utilized were the same as those previously used for sequencing the 16S rRNA genes from these isolates (Píneiro *et al.*, 2007).

**RESULTS AND DISCUSSION**

The primers chosen for *rpoB* gene amplification from the *Bacteriovorax* isolates included a region that began and ended at bases 2073 and 3315, respectively, of the *Bacteriovorax marinus* SJT *rpoB* gene (http://www.sanger.ac.uk/Projects/B_marinus/). The *rpoB* gene sequences were obtained from 74 saltwater and one freshwater *Bacteriovorax* isolate, aligned and analysed using a neighbour-joining algorithm. The phylogenetic tree was constructed using *G. sulfurreducens*, another member of the class *Deltaproteobacteria*, as the outgroup as previously described for 16S rRNA gene analysis (Píneiro *et al.*, 2007). The phylogenetic tree analysis based on *rpoB* gene sequences showed 15 groups, labelled A to O, and numerous singletons (Fig. 1). The groups were defined by bootstrapping of 1000 replicates. If groups were defined as those isolates that grouped together with bootstrap value of ≥96%, then 15 groups were identified. Alternatively, if the groups were assigned by sequence similarity of ≥96% between members of the same group, then only 9 groups could be found. Groups C, D and E, G and H, and K and L coalesced when classified by similarity, since the isolates had
gene sequence similarities of \( \geq 96 \% \). Less stringent conditions will produce larger groups, but the groups need to be based on data from additional loci.

The \( rpoB \) gene sequences of the saltwater \emph{Bacteriovorax} isolates were compared with those of the freshwater isolates \emph{Bacteriovorax stolpii} and \emph{Peredibacter starrii}. The genetic distances to \emph{Bacteriovorax stolpii} ranged from 19 to 24 \% nucleotide differences and for \emph{P. starrii} from 24 to 27 \%. These distances were comparable to those within the saltwater \emph{Bacteriovorax} isolates (21 to 24 \%) and were less than to \emph{Bdellovibrio bacteriovorus} (37 to 41 \%) and to other bacteria (43 to 51 \%). While these distances clearly indicate that the various taxa are distinct, the distance metric is unlikely to be accurate because of the high likelihood of multiple substitutions at a single site.

A comparison of the \emph{rpoB} sequences of the saltwater \emph{Bacteriovorax} isolates with those from genera of other classes of the phylum \emph{Proteobacteria} revealed insertion/deletion mutations (Fig. 2). In this paper, insertion/deletions are referred to relative to \emph{Bacteriovorax marinus} SJ\( ^T \) and no inference is intended regarding the phylogeny of these events. When other bacterial species (belonging to various classes of the phylum \emph{Proteobacteria}) were examined, a 6 bp insertion was found starting at base 2363 of \emph{Bacteriovorax marinus} SJ\( ^T \) in the species \emph{G. sulfurreducens}, \emph{Salmonella enterica} and \emph{Y. pestis}, while a 3 base insertion was found in \emph{Silicibacter pomeroyi}, \emph{Bdellovibrio bacteriovorus} and \emph{D. vulgaris} (Fig. 2a). A second insertion/deletion occurrence started at nucleotide position 3052 of the \emph{Bacteriovorax marinus} SJ\( ^T \) sequence. \emph{Silicibacter pomeroyi} was found to have a 15 bp deletion in

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**Fig. 1.** Phylogenetic tree for \emph{Bacteriovorax} isolates based on \emph{rpoB} gene sequences. Percentages with cluster designations are \emph{rpoB} gene sequence similarity. The numbers on the branches correspond to bootstrap values based on 1000 replicates. Groups that bootstrap together \( \geq 96 \% \) of the time based on their \emph{rpoB} gene sequence are represented by letters. Clusters based on 16S rRNA gene sequence analysis are numbered consistently with previously published works (Davidov & Jurkevitch, 2004; Pineiro et al., 2007). GenBank accession numbers are given in parentheses. Sanger*: http://www.sanger.ac.uk/Projects/B_marinus/. Bar, 10 nucleotide changes.
this region and the two gammaproteobacteria had 54 bp deletions (Fig. 2b). A third region where insertion/deletion events were detected was around bases 3135 to 3239 of the *Bacteriovorax marinus* sequence. Through this region *Bacteriovorax marinus*, *Bdellovibrio bacteriovorus* and *D. vulgaris* had the same number of nucleotides, *G. sulfur-reducens* had 3 more nucleotides, while *Silicibacter pomeroyi*, *Salmonella enterica* and *Y. pestis* had 3 fewer nucleotides (Fig. 2c). It is worth noting that all of the insertions/deletion events were multiples of three, consistent with the conservation of the reading frame so that a proper enzyme can be formed. These insertions/deletions did not separate phylogenetic groups within the *Bacteriovorax* isolates. The first separated *Bacteriovorax* isolates from the other members of the class *Deltaproteobacteria*, the second separated the deltaproteobacteria from the other subdivisions and the third separated out *G. sulfurreducens* from the other members of the class *Deltaproteobacteria*.

The phylogenetic groups based on *rpoB* gene sequences were compared with the clusters based on 16S rRNA gene sequences (Fig. 1). The 16S rRNA gene clusters III, IV, V, IX, X, XII, XIII were identified previously (Pineiro et al., 2007) and were composed of isolates with ≥96.5% gene sequence similarity. In general, there was very good agreement between the groups identified by *rpoB* gene sequencing and the clusters identified by 16S rRNA gene sequencing. Groups C, D and E, based on *rpoB* gene sequences, and cluster V, based on 16S rRNA gene sequences, had identical sets of isolates and contained only isolates from estuarine environments. Similarly, groups K and L and cluster XI had an identical set of isolates and were from tropical locations (Miami, FL, USA, and Trinidad, Cuba). The groups A and B, G and H, and I, K and L, and M and N, were most closely related to the other groups from the same 16S rRNA gene cluster (X, III, XI and XII). These groups may well represent smaller phylogenetic taxa within the clusters defined by 16S rRNA gene sequence analysis as is the case for the genus *Prochlorococcus* which has six ecotypes with 16S rRNA gene sequences that diverge by less than 3% (Johnson et al., 2006). The genus *Salinispora* has three species with 99% or greater similarity at the 16S rRNA gene locus (Jensen & Mafnas, 2006) and *Yersinia pestis* has species defined both by biochemical tests and by multi-locus sequencing typing that cannot be distinguished by 16S rRNA gene sequences (Kotetishvili et al., 2005). These observations and the groups found within the identified clusters may indicate that the family *Bacteriovoracaceae* is even more diverse than shown in this study. However, before these taxonomic groups are elevated to recognized species, additional information will be required to meet the criteria set forth by the ad hoc Committee for the Re-evaluation of the Species Definition in Bacteriology (Stackebrandt et al., 2002) and others (Gurtler & Mayall, 2001; Rosselló-Mora & Amann, 2001).

Although insertion/deletion events are generally rare in 16S rRNA genes, there were four locations where these events have occurred in the 16S rRNA gene of the family...
Bacteriovoracaceae. Two events distinguish the predatory
deltaproteobacteria. One, a single base insertion relative to
the Escherichia coli sequence (AF233451) near E. coli
nucleotide position 1113 (Fig. 3a), was found in all isolates
from the families Bdellovibrionaceae and Bacteriovoracaceae.
A second single base insertion was found in the family
Bacteriovoracaceae near E. coli nucleotide position 436. In
contrast, members of the family Bdellovibrionaceae had a
23 base insertion at this location (Fig. 3b). This genetic
variance unambiguously separated these two families of
predatory bacteria. The other two events subdivided the
family Bacteriovoracaceae. The third location was near E. coli
nucleotide position 164 where most saltwater members of
the family Bacteriovoracaceae had a single base deletion, in
contrast to the freshwater Bacteriovorax/Peredibacter lineage
that had a 10 base deletion. The saltwater cluster XIII,
including strain MED2, also had the larger deletion,
consistent with the presence of strain MED2 as an outlier
at the base of the rpoB phylogenetic tree. The fourth location
was between nucleotide positions 181 and 199 in the E. coli
sequence. There were six distinct patterns within the family
Bacteriovoracaceae and two more within the family
Bdellovibrionaceae. The family Bdellovibrionaceae had three
or five more bases than E. coli (Fig. 3d), consistent with the
insertion in the 16S rRNA gene near nucleotide position 436
and the rpoB difference near to nucleotide position 2363 of
Bacteriovorax marinus SJ
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. These three observations all
supported the separation of these two families. There were
five distinct patterns within the family Bacteriovoracaceae
(Fig. 3d). Three patterns were found in the saltwater clusters.
16S rRNA gene clusters V, IX and X had 16 more bases than the
E. coli sequence, clusters III, IV, XI and XII had 17 bases
more than E. coli, and cluster XIII containing MED2 had
only four more bases. The freshwater isolates also showed
three patterns, cluster VII containing P. starrii had nine
more bases than the E. coli sequence, while cluster I
containing Bacteriovorax stolpii and strain PNEc1 had 14
and 15 more bases, respectively. These data confirm the
division of cluster XIII away from the other saltwater isolates
and the separation of the saltwater and freshwater isolates.
The partitions are also consistent with the groupings seen
using the rpoB gene sequence. Clusters V, IX and X
corresponded to rpoB groups A–F; clusters III, IV, XI and
XII to groups G–O; and cluster XIII, containing MED2, was
distinct. The distinctiveness of cluster XIII may explain why,
despite repeated attempts, we were unable to obtain an rpoB

Fig. 3. CLUSTAL _ X v1.81 multiple sequence alignment for 16S rRNA genes. All asterisks represent deleted bases and all plus
signs represent inserted bases relative to the E. coli 16S rRNA gene (GenBank accession no. AF233451). The underlined
nucleotides in the E. coli sequence are associated with the number underneath.
sequence from the other isolate in the cluster, GSL4B. The isolate GSL37 was an outlier located near the base of the rpoB gene tree and was ambiguously placed on the basis of 16S rRNA gene sequence alone. The pattern for insertions/deletions in the 16S rRNA gene for this isolate was the same as that of isolates in clusters V, IX and X. Finally, the outlier isolates previously revealed by 16S rRNA gene sequence analysis, strains IP and NZ7, were consistent with the phylogenetic analysis using the rpoB gene.

In conclusion, we found that the rpoB nucleotide sequences were more variable than the 16S rRNA gene sequences. The variation was consistent with that observed in sequence and insertions/deletions in the 16S rRNA locus. Some of the 16S rRNA gene clusters were subdivided by the greater nucleotide variation in the rpoB gene, possibly indicating that more narrowly delineated phylogenetic groups may be defined among the saltwater isolates of the genus Bacteriovorax. The formal establishment of defined taxonomic groups at the species level will require information from additional loci.

The wide dissimilarities in 16S rRNA gene sequence analysis between the family Bdellovibrionaceae and the newly described family Bacteriovoracaceae served as a basis for the construction of the genus Bacteriovorax (Baer et al., 2000) and, even more recently, the reclassification of Peredibacter starrii was proposed (Davidov & Jurkevitch, 2004). The differences in 16S rRNA and rpoB gene sequences between the genera Bdellovibrio, Bacteriovorax and Peredibacter are large enough to place each in its own family. Our additional data give rise to additional taxonomic categories. The reclassification of Bacteriovorax stolpii as Bacteriolyticum stolpii gen. nov., comb. nov. is proposed based on the extensive diversity between the saltwater members of the genus Bacteriovorax and the freshwater/soil isolates.

Description of Bacteriolyticum gen. nov.

Bacteriolyticum (Bac.teri.o.lyt.i.cum. Gr. n. bacterion staff, cane and in biology, a bacterium; Gr. adj. lutikos able to loosen, able to dissolve; N.L. neut. n. Bacteriolyticum a dissolver of bacteria).

This genus consists of Gram-negative bacteria that prey upon other Gram-negative bacteria to complete a biphasic life cycle. The morphological description of the genus is the same as that of the type and only described species, Bacteriolyticum stolpii.

Description of Bacteriolyticum stolpii comb. nov.

Bacteriolyticum stolpii (stol'pi.i. N.L. masc. gen. n. stolpii of Stolp, named after the American microbiologist Stolp).


Other homotypic synonym: Bacteriovorax stolpii Baer et al. 2000.

The description of the species is based on those of Baer et al. (2000) and Seidler et al. (1972). The species is separate from the genera Peredibacter and Bacteriovorax as Bacteriovorax stolpii has very low levels of DNA–DNA relatedness, 4% and 7.7% to P. starrii and Bacteriovorax marinus SJT, respectively (Baer et al., 2004). 16S rRNA gene sequence analysis shows the similarity between the freshwater/soil isolate Bacteriovorax stolpii and the saltwater species Bacteriovorax marinus SJT to be 89%. The maximum 16S rRNA gene sequence similarity to any saltwater member of the genus Bacteriovorax is 90.8% and ranges down to 81% (Pineiro et al., 2007). The type strain is Uki2T (=ATCC 27052T=DSM 12778T=DSM 50722T).

Description of Peredibacteraceae fam. nov.

Peredibacteraceae (Pe.re.di.bac.ter.a.ce ae. N.L. fem. n. Peredibacter the genus of Gram-negative, aerobic, predatory bacteria is the type genus of the family; suffix aceae denoting family; N.L. fem. pl. n. Peredibacteraceae the Peredibacter family).

The description of the family Peredibacteraceae is based on the description of the genus Peredibacter (Davidov & Jurkevitch, 2004) and the data from this study. This family is composed of Gram-negative, vibrioid shaped bacteria about 0.5 μm in length. Obligate predators of other Gram-negative bacteria. They exhibit a biphasic life cycle consisting of a motile attack phase and a phase that dwells in the periplasm of prey bacteria. They are a monophyletic offshoot of the family Bacteriovoracaceae. The only recognized species are found in freshwater and soil environments. The DNA G+C contents of the saltwater Bacteriovorax strains SJT, AQ and JS5 are in the range of 37.7–38.3 mol% (Baer et al., 2004) compared with the higher values of 43.5 mol% for Peredibacter starrii and 41.8 mol% for Bacteriolyticum stolpii [previously Bacteriovorax stolpii (Seidler et al., 1972)]. These species grow on prey in freshwater. The two species have distinctive numbers of nucleotides between E. coli bases 181 and 199 that differ from both the families Bacteriovoracaceae and Bdellovibrionaceae. The type genus is Peredibacter (Davidov & Jurkevitch, 2004 who revised Seidler et al., 1972).
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