Antonie van Leeuwenhoek (2015) 107:305–311
DOI 10.1007/s10482-014-0327-9

SHORT COMMUNICATION

454 Pyrosequencing reveals diversity of *Bdellovibrio* and like organisms in fresh and salt water

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Received: 11 July 2014 / Accepted: 31 October 2014 / Published online: 9 November 2014

Abstract *Bdellovibrio*-and-like organisms (BALOs) are Gram-negative, predatory bacteria that inhabit terrestrial, freshwater and saltwater environments. They have been detected primarily by culture-dependent methods which have limitations. In this study, diversity and community structure of BALOs in freshwater and saltwater samples were characterized by 16S rRNA gene pyrosequencing with specific BALO group primers. Novel *Bacteriovorax* 16S rDNA sequences were found both in saltwater enrichment cultures and in situ environmental samples, but no new operational taxonomic units were detected in the freshwater samples. The results revealed unexpected diversity of BALOs and advance understanding of the similarities and differences between *Bdellovibrio* and *Bacteriovorax* diversity and distribution in the environment.

Keywords Pyrosequencing · BALOs · 16S rRNA · Diversity · *Bacteriovorax* · *Bdellovibrio*

Electronic supplementary material The online version of this article (doi:10.1007/s10482-014-0327-9) contains supplementary material, which is available to authorized users.

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Introduction

Current knowledge about the distribution and diversity in nature of the bacteria predators, *Bdellovibrio* and like organisms (BALOs), (Chauhan and Williams 2006; Davidov and Jurkevitch 2004; Davidov and Jurkevitch 2009; Kelley and Williams 1992; Petrović-Gegić and Baloš; Schloss et al. 2009; Snyder et al. 2002; Taylor et al. 1974) has been derived by culture methods which likely detect only a small proportion of the total bacteria present. Various culture methods have yielded BALO isolates that fall into four known genera, *Bdellovibrio, Bacteriolyticum* and *Peredibacter* found in fresh waters and soils, and *Bacteriovorax* found in salt water (Baer et al. 2000; Davidov and Jurkevitch 2004; Pineiro et al. 2008). More complete information about the heterogeneity of these predators may be revealed by the application of culture independent, high-throughput DNA sequencing technologies. This approach may show greater diversity of BALOs than culture methods as previously uncultured strains will be detected. The degree of diversity of BALOs in any niche may be a factor in the ecology and role of predator predation against a community of native bacteria as the more diverse the predator population the more bacteria are preyed upon since not all bacteria are susceptible to the same BALO strains. The results should encourage further research to address questions on the ecological and evolutionary significance of diversity among the BALOs and the selective forces that drive it.

Materials and methods

Freshwater samples (10L, surface water, about 0.5 m depths) were collected from Lake Bradford (N 30°24.096′, W 84°20.248′) (site 248) and Lake Munson (N 30°21.968′, W 84°18.000′) (site 000) located in Tallahassee, FL (USA). Salt water samples (10L, surface water, about 0.5 m depths) were obtained from three sites (N30° 4.734′, W-84° 10.714′) (site 714); N 30°04.658′, W 84°10.970′;(site 970) N 30°04.466′, W 84°10.784′) (site 784) in the Saint Marks National Wildlife Refuge located in Tallahassee, FL (USA). During sampling, water temperature (YSI 30, Yellow Springs Instruments conductivity (YSI 30, Yellow Springs Instruments), salinity (YSI 30, Yellow Springs Instruments) and pH (AR15, Fisher Scientific) were measured (Supplementary Table 1). Following collection, samples were stored on ice for transport to the laboratory.

In the laboratory, the fresh and salt water samples, respectively, were mixed and filtered sequentially through 0.8 μm and 0.45 μm filters (Nalgene, Rochester, NY, USA) to remove debris and larger organisms. Five hundred mL of the 0.45 μm filtrate was passed through 0.1 μm filters (Whatman, Florham Park, NJ, USA) to capture the environmental BALOs on the filter surface. Filters were then stored at −20 °C for subsequent analysis by pyrosequencing.

BALO enrichment cultures were established by dispensing 500 mL of the 0.45 μm filtrate of water samples into 2L Erlenmeyer flasks and amending with suspensions of the bacterial prey, *Vibrio vulnificus* FLA042 (Vv) and *Vibrio parahaemolyticus* RIMD (Vp), to yield an optical density (OD) measurement of 0.7 at 600 nm. The enrichment microcosm flasks were shaken at room temperature for 120 h. Samples were removed and filtered through a 0.1 μm filter to capture BALOs and the filters held at −20 °C until being analyzed by pyrosequencing.

DNA extraction and pyrosequencing

Genomic DNA was extracted (Mo-Bio PowerWater® DNA Isolation Kit, Carlsbad, CA) from bacteria captured on the filters from both the environmental water samples and the BALO enrichment culture. DNA yield and purity were measured spectrophotometricly (NanoDrop Technologies, Delaware).

For pyrosequencing the 16S rRNA genes from the salt water BALOs were amplified with *Bacteriovorax*-specific primers Bac-676F and Bac-1442R (Davidov et al. 2006), and from the fresh water predators with *Bdellovibrio*-specific primers Bac-63F and Bac-842R (Jurkevitch et al. 2000). 2 μL of template DNA was aliquoted into illustra™ PuReTaq Ready-To-Go™ PCR Beads (GE Healthcare, Waukesha, WI) with 20 μL PCR grade molecular water and 1.5 μL of each primer for a total reaction mixture of 25 μL. PCR was conducted on a Biorad thermocycler (Hercules, CA) and conditions were as follows: 3 min initial denaturation at 95 °C, 35 cycles of 30 s denaturation at 95 °C, 1 min annealing at 55 °C, 2 min elongation at 72 °C, and a final extension for 7 min at 72 °C. The resulting amplified products were sequenced with a 454 GS FLX system (454 Life Sciences) at the Research and Testing Laboratory LLC. in Lubbock, TX (USA). Reads were deposited into the NCBI Short Read Archive (Accession no. PRJNA222495). Novel *Bacteriovorax* OTUs sequences were deposited in GenBank (KM107982-KM107999).

Sequencing data were analyzed using the single software platform, MOTHUR v.1.26.0 (Schloss et al. 2009). For the phylotype-independent approach, sequences were clustered into operational taxonomic units (OTUs) at a distance threshold of 0.03 (97 % similarity) via the average neighbor algorithm (Schloss and Westcott 2011). To minimize the effects of random sequencing error, low quality sequences were eliminated as described by Schloss and Westcott (2011). After removal of barcodes and primers, the remaining sequences were trimmed so that all started and ended at similar positions in their alignment to the SILVA database and underwent screening for chimeras through UCHIME (Edgar et al. 2011). After removal of chimeras, sequences were classified using the Ribosomal Database Project (RDP) Naïve Bayesian Classifier (minimum confidence of 50 %) (Lan et al. 2012). Contaminants such as *Vibrionaceae* and *Chromatiaceae* were removed.

The MOTHUR program was used to process sample data to generate indices of diversity, rarefaction curves and to perform the Fast UniFrac test to compare the phylogenetic structure of the libraries. A neighbor-joining tree was constructed with representative sequences of each OTU selected by MOTHUR. All sequences were then aligned using the muscle program (Edgar 2004). MEGA 5.0 (Tamura et al.

Table 1 Richness estimation, diversity indices and coverage in the 10 samples

Group[a]	Number of cleaned sequences	Goods coverage (%)	Number of OTUs	Diversity index		Richness estimators	
				Shannon[b]	Simpson[b]	Chao 1[c]	Ace[c]
Site 970	998	90.93	86	2.11	0.54	362	702
Site 970C	741	99.03	66	1.74	0.27	274	477
Site 784	1,424	93.01	97	1.97	0.52	474	1,056
Site 784C	949	92.11	73	1.68	0.31	320	957
Site 714	1,192	91.11	111	2.16	0.41	362	465
Site 714C	590	92.11	59	1.59	0.31	203	403
Site 248	1,251	98.65	63	0.48	0.92	94	100
Site 248C	1,312	99.06	67	0.23	0.83	48	51
Site 000	728	97.54	56	0.92	0.79	89	167
Site 000C	839	97.65	68	0.58	0.46	74	83

All parameters were done using the Mothur platform at a 3 % genetic distance

[a] C, enrichment samples

[b] Chao 1 and ACE are non-parametric richness estimators

[c] Invsimpson and Shannon are diversity indices

2011) and Dendroscope 3.0 (Huson and Scornavacca 2012) were used to generate and edit the neighbor-joining tree.

Results

Diversity of *Bacteriovorax* in saltwater

Using *Bacteriovorax* specific 16S rRNA gene primers to probe the three salt water samples and the enrichment cultures made from these yielded 5,896 high quality sequences with average lengths of 530 bps. The Good's coverage estimate (90.93–9.03 %) (Table 1), showed the majority of the bacterial community was covered. The diversity indices were consistently higher in the environmental samples than in the enrichment samples. The Chao1 and ACE richness estimate for site 784, the highest salinity site, was higher than other samples for both enrichment and environmental samples (Table 1).

Reference sequences of known *Bacteriovorax* clusters (Pineiro et al. 2007) from GenBank were used to constitute and run a local Blastn program (Tatusova and Madden 1999) to determine taxonomic similarities with sequences from the water samples. 91.9 % of total sequences were affiliated with the four known BALO phylotype clusters (Fig. 1a, Fig. S2, Table S2 and Table S3) with the most abundant of these being *Bacteriovorax* XIII present in all salt water samples. Cluster V was greater in the enrichment cultures than in environmental samples. Novel sequences with 91.7–96.06 % similarity to known *Bacteriovorax* sequences were found in all samples and were dominant at 47.79 % of the sequences in the environmental water sample from site 970. At the other sites the novel sequences ranged from 2.81 to 4.12 % of the total abundance(Fig. 1a). Based on similarity to the known reference sequences, the novel sequences were divided into 8 groups (Fig. 1a, Fig. S2, Table S2 and Table S3). Phylogentic analysis suggested these novel OTUs formed several different clusters (Fig. S1). For example, the most abundant, group II, which included 68 distinct OTUs, fell into cluster PI, cluster PII, cluster PIII and cluster PV.

Diversity of *Bdellovibrio* in freshwater

From freshwater samples 4,140 high quality sequences with average read lengths of 490 bps were obtained. Good's coverage estimate ranged from 97.54 to 99.06 % (Table 1). The Shannon diversity indices were higher in environmental samples than in the enrichment samples. The Simpson index for the environmental samples ranged from 0.79 to 0.92, and for enrichment samples from 0.46 to 0.83. The Chao1 richness values of the enrichment and environmental samples from Lake Bradford were lower than the Lake Munson samples (Table 1).

Reference sequences from nine freshwater *Bdellovibrio* sp. clusters (Davidov and Jurkevitch 2004) were downloaded from Genbank to run a local Blastn program (Tatusova and Madden 1999). A neighbor joining tree was constructed with the 90 represented OTUs from environmental and enrichment samples and the reference sequences. The results yielded three

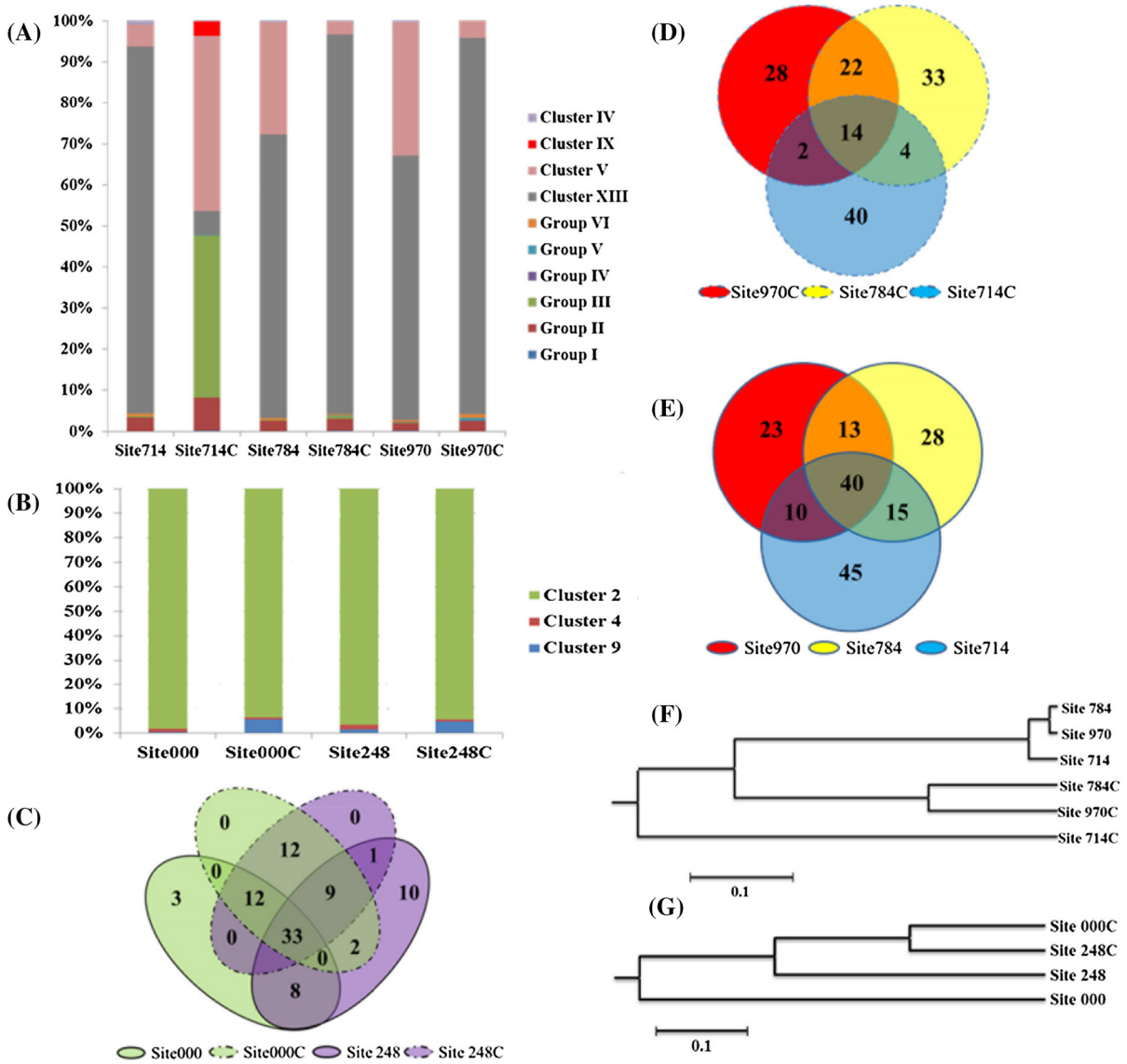

Fig. 1 Distribution and community similarities of BALOs in water samples. **a** Distribution of *Bacteriovorax* in salt water samples; **b** Distribution of *Bdellovibrio* in freshwater samples; **c** Venn diagram of OTUs in freshwater samples; **d** Venn diagram of OTUs in enrichment salt water samples; **e** Venn diagram of OTUs in environmental salt water samples; **f** ThetaYC neighbor joining tree based on a 0.03 genetic distance from salt water samples; **g** ThetaYC neighbor joining tree based on a 0.03 genetic distance from freshwater samples

large clades (Fig. 1 and Fig. S2). They were clustered with known cluster 2, cluster 4 and cluster 9 (Davidov and Jurkevitch 2004), respectively. The sequences of clade 3 were more abundant and nearly evenly distributed in environmental and enrichment samples from Lakes Munson and Bradford. No novel cluster of *Bdellovibrio sp*. were found in either of the lakes. Thirty three OTUs were shared among all samples (Fig. 1c, Table S4 and Table S5). Several OTUs were only detected in environmental samples (Fig. 1c, Table S4 and Table S5). Three OTUs belonging to clade 2 and clade 3, respectively were only found in Site000.

In assessing the similarity of the BALO community obtained directly from the environment with that from the enrichment cultures, significant differences

($P < 0.0001$) were observed. This was true for both the salt water (Fig. 1) and fresh water (Fig. 1) communities.

Discussion

High throughput sequencing strategy using pyrosequencing was applied to gain greater insight into the relative abundance and diversity of BALOs in the aquatic environment as previous studies using culture methods have limited capacity to detect all strains present (Staley and Konopka 1985). Studies that used, denaturing gradient gel electrophoresis (DGGE), culture-independent method, to detect BALOs in salt water (Chen et al. 2012) and soils (Davidov et al. 2006) showed greater diversity of the predators than was shown by culture. However, culture independent methods that are more sensitive than DGGE are currently available and will likely show even greater diversity of microorganisms in environmental samples.

In this study, we explored the diversity of BALOs in fresh and salt water environmental samples and in laboratory enrichment cultures by 454 pyrosequencing which provides deeper insights into microbial communities in ecological niches (Amend et al. 2010; Manter et al. 2010) than culture methods or DGGE. With the expectation that multiple preys may enhance recovery of more predator types than a single prey, two different *Vibrio* species were used to amplify the BALO community in enrichment cultures from environmental samples,

The results show that the *Bacteriovorax* are much more diverse than previously known. Phylogenetic analysis of pyrosequencing data showed that the eight known *Bacteriovorax* clusters all obtained previously by culture methods (Pineiro et al. 2007) made up a small branch in the neighbor joining tree (Fig. S1), whereas, novel *Bacteriovorax* sequences in the environmental samples were more abundant (Fig. 1a). To be consistent with the previous systems reported by Davidov (Davidov and Jurkevitch 2004) and Pineiro studies (Pineiro et al. 2007), we named saltwater *Bacteriovorax* clusters using Roman numerals, and fresh water *Bdellovibrio* clusters in Arabic numerals. All the *Bacteriovorax* novel clusters detected in this study carry the designation "P" to note they were identified based on analysis of 454 pyrosequencing data. All the novel clusters (from cluster PI to cluster PVII) were observed to be unrelated to previous taxa. OTUs from group II clustered with OTUs from other groups such as Group I and Group III, and they fell into different branches by phylogenetic analysis, which is inconsistent with Blastn results classifications. This suggests the arbitrary boundary of less than 97 % similarity in 16S rDNA gene sequence as the threshold to define species (Brenner et al. 2001) may not be met by BALO species. In this study, the novel sequences showed 91.7–96.06 % similarity to known *Bacteriovorax* sequences (Fig. S1). They fell into seven different groups by phylogenetic analysis, the minimum distance among them were 0.07. However, to define bacterial species more information is required than the 16S rDNA sequence only.

In comparison to the relatively large diversity of *Bacteriovorax* observed, the diversity of *Bdellovibrio* in freshwater was much smaller. There were no novel *Bdellovibrio* sp. 16S rDNA sequences detected in our pyrosequencing database. The sequences detected showed 97.08–100 % similarity to the known cluster 2, cluster 4 and cluster 9 (Davidov and Jurkevitch 2004) (Fig. S2).

Distinctly different BALO community structures were observed in the enrichment and environmental samples for both salt water and freshwater (Fig. 1a). There were more novel clusters detected in the environmental water samples than in the enrichment culture with the exception of samples from saltwater site 714. This was most evident in samples from site 970 where the novel clusters comprised 47 % of the relative population in the environmental sample compared to 5 % for the enrichment culture. This example shows that culture grossly underestimated the BALO diversity present.

The known *Bacteriovorax* clusters were fairly evenly distributed between the salt water samples and the enrichment culture. This is not surprising since the known isolates were recovered originally by culture methods on the same prey bacteria used in this study, which indicates their ability to grow under the culture conditions utilized. OTUs belonging to Cluster V were dominant in all freshwater samples, water and enrichment, but more so in enrichment samples. Cluster V has been reported to occur primarily in lower salt estuarine waters (Pineiro et al. 2013), which is consistent with the salt water body sampled in this study. To the contrary, Cluster XIII tended to be more abundant in environmental

water than enrichment cultures. The distribution of Cluster XIII in all salt water sites was surprising as it was only recovered previously from a site in Spain.

Some marked differences were observed in the *Bdellovibrio* community structures between the two freshwater sites, Lake Munson and Lake Bradford. The enrichment samples yielded a much different population structure with Clade 1 being a minor component (1.5 %) in Lake Bradford whereas in Lake Munson it was barely detectable (0.05 %). Clade 3 was relatively evenly distributed in both environmental and enrichment samples from Lakes Munson and Bradford.

Although pyrosequencing could produce errors in sequences (Hamady et al. 2008), the results from this study show a more diverse and complex BALO community in fresh and salt waters than previously known which leads to the matter of the significance of this diversity. For example, does a more diverse BALO community results in greater predation on a broader range of environmental bacteria than a homogeneous community? Other questions include: Is BALO diversity an indicator of the diversity of their bacterial prey; what impact does changes in prey population and community structure have on the diversity of the BALO community and is there an evolutionary basis for the diversity among the BALOs? The answers to such questions may have important implications in bacterial predation and ecology. The results and questions raised from this study should foster investigations at more sites covering larger geographic areas using methods as pyrosequencing to more fully show the diversity and distribution of BALOs in aquatic systems.

Acknowledgments This work was financially supported by the National Science Foundation Division of Education and Human Resources, HBCU-RISE Grant-0531523. The bacterial prey (*V. vulnificus* FLA042 (Vv) obtained from Dr. Paul Gulig, University of Florida and *V. parahaemolyticus* RIMD (Vp) from Dr. Gonzalez-Escalona, Narjol, US Food and Drug Administration).

Conflict of interest There is no conflict of interest in this study.

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