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Population dynamics of *Vibrio* spp. associated with marine sponge microcosms

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***Vibrio* is a diverse genus of marine-associated bacteria with at least 74 species and more expected as additional marine ecospheres are interrogated. This report describes a phylogenetic reconstruction of *Vibrio* isolates derived from one such unique ecosystem, marine sponges (Phylum Porifera) collected from depths of 150 to 1242 feet. 16S rRNA gene sequencing along with molecular typing of 16S–23S rRNA intergenic spacer regions clustered many sponge-associated *Vibrio* (*spp*) with current known species. That is, several benthic *Vibrio* species commensal with Porifera sponges seemed genetically linked to vibrios associated with coastal or shallow-water communities, signalling a panmictic population structure among seemingly ecologically disparate strains. Conversely, phylogenetic analysis provided evidence for at least two novel *Vibrio* speciation events within this specific sponge microcosm. Collectively, these findings earmark this still relatively unknown environment as a bastion of taxonomic and phylogenetic variability for the genus and probably other bacterial taxa.**

Subject Category: microbial ecology and functional diversity of natural habitats

Keywords: 16S rRNA gene sequencing; 16S–23S rRNA intergenic spacer region; Phylum Porifera; speciation; *Vibrio*

Introduction

The genus *Vibrio* comprises a diverse bacterial group consisting of at least 74 distinct species, many having been recently identified in the last several years. Vibrios belong to the class Gammaproteobacteria and are gram-negative, usually motile rods. Generally, the organisms are halophilic, mesophilic and chemoorganotrophic in nature and have a facultatively fermentative metabolism (Thompson *et al.*, 2004). Many of these species, such as *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, induce severe human disease, including gastroenteritis, after ingestion of contaminated raw or undercooked seafood, and wound sepsis (Gulig *et al.*, 2005; Saravanan *et al.*, 2007; Su and Liu, 2007). Vibrios retain remarkable biodiversity, persisting in a variety of geographic locales and eukaryotic hosts, including corals, molluscs,

sponges and zooplankton (Thompson *et al.*, 2004). Furthermore, environmental characteristics, such as water temperature and salinity, are also known to influence the diversity of *Vibrio* spp. in the environment (Beaz-Hidalgo *et al.*, 2010).

Given the multitude of aquatic environments harboring vibrios, it seemed reasonable to hypothesize that additional *Vibrio* species might exist in remote undersea environments. One such environment—marine sponges—consists of numerous sponge species inhabiting different depths and regions of benthic seas. Sponges maintain diverse symbiotic microbe populations that differ in composition from microbial populations of surrounding ambient waters (Hentschel *et al.*, 2006; Taylor *et al.*, 2007; Webster and Blackall, 2009). Moreover, sponge anatomy allows persistence of distinct microbiomes in the same individual. One such microbiome, for example, is the mesohyl (extracellular matrix), which consists of regions with varying O₂ concentrations (Hoffmann *et al.*, 2005). In this case, highly selective conditions may encourage speciation as microbial populations adapt from oxygen rich to anoxic regions. In this study, we examine the phylogenetic diversity of vibrios associated with commensal sponge hosts in the Phylum Porifera.

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Materials and methods

Vibrio isolates were collected at depths between 150 and 1242 feet from several geographic locales and different sponges (Figure 1) and are maintained in the Harbor Branch Marine Microbial Culture Collection. Isolation of *Vibrio* spp. from sponges was carried out as previously described (Sfanos *et al.*, 2005). Genomic DNA was isolated from pure *Vibrio* cultures using the ZR Fungal/Bacterial DNA kit (Zymo Research, Orange, CA, USA).

16S rRNA gene sequence analysis and 16S–23S rRNA intergenic spacer (IGS) analyses were performed, as described previously (Hoffmann *et al.*, 2010). PCR primers and reaction conditions used for 16S rRNA amplification and 16S–23S rRNA IGS analyses are listed in Supplementary Material. IGS PCR amplicons were resolved by capillary gel electrophoresis using the Agilent BioAnalyzer 2100 and the Agilent DNA 7500 Assay Protocol (Agilent Technologies, Santa Clara, CA, USA). DNA purification, 16S rRNA gene sequence and 16S–23S rRNA IGS analyses were repeated three times to assure accuracy.

16S rRNA amplicons were sequenced by Amplicon Express (Pullman, WA, USA) and aligned with 16S rRNA gene sequences from the US Food and Drug Administration *Vibrio* type strain collection and *Vibrio* 16S rRNA gene sequences available at Genbank (<http://www.ncbi.nlm.nih.gov>). DNA sequences were aligned in ARB (<http://www.arb-silva.de>) against the SSU ARB-Silva bacteria collection hosted by the Max Planck Institute for Marine Microbiology in Bremen, Germany (Ludwig *et al.*, 2004; Pruesse *et al.*, 2007). Phylogenetic analyses were performed using PAUP* version 4.0b10 (Swofford, 2003) and the Genetic Algorithm for Rapid Likelihood Inference (GARLI) software (Zwickl, 2006). New 16S rRNA gene sequences from 23 *Vibrio* isolates are available in GenBank under accession numbers GU223581–GU223603.

Results and discussion

Analysis of 16S rRNA gene sequences allowed construction of a maximum parsimony tree (Figure 1), as well as a maximum likelihood tree (Supplementary Material, Supplementary Figure S1), revealing similarities to known *Vibrio* species for which benthic *Vibrio* isolates were most closely related. It was noteworthy that both analytical methods produced identical tree topologies with respect to nearest neighbors of the feral sponge isolates. Furthermore, our 16S–23S rRNA intergenic sequence typing protocol produced excellent results for sponge-associated isolates and showed several interesting genotypic differences between these isolates and their shallow-water counterparts.

Phylogenetic analysis revealed several notable findings regarding the diversity of sponge-associated vibrios. It is noteworthy that *Vibrio*

isolates obtained from a *Scleritoderma* collected in Curacao (N376, N377, N382, N380, K350, K324 and K323) could not be differentiated by 16S rRNA gene sequencing indicating species homogeneity to *V. natriegens* and *V. alginolyticus*. IGS-patterns, however, suggested that these strains are more closely related to *V. alginolyticus*. In fact, these patterns were virtually identical, differing by a single 400-bp band absent in *V. alginolyticus* (Figure 2). Similarly, other *Vibrio* strains were either identical or nearly so to other known *Vibrio* species based on 16S rRNA gene sequence comparisons. For instance, strain J462 had a 16S rRNA gene sequence identical to *Aliivibrio fischeri*, while both strains J231 and J555 shared ~99.9% 16S rRNA gene sequence identity with *V. mediterranei*. In contrast to the Curacao strains, however, J462 generated an IGS-type pattern dissimilar to *A. fischeri* in actual number of bands generated. In addition, IGS-profiles generated for strains J231 and J555 differed substantially from patterns retained by their closest neighbors. Finally, strains N418 and N384 were identified as putatively novel *Vibrio* species—16S rRNA gene sequences showed significant divergence from known *Vibrio* species for both of these isolates (Figure 1). N384 was at least 19 steps (nucleotide substitutions) removed from *V. brasiliensis*, and N418, most closely related to *V. ichthyenteri* and *V. scophthalmi*, was separated by combined branch lengths of 15 and 13 steps, respectively. These distances far exceed the 1% 16S rRNA gene sequence divergence threshold as described by Acinas *et al.* (2004) and Harayama and Kasai, (2006) as the criterion for new and emerging species. As 16S rRNA gene sequence analysis represents a highly stable marker, divergence among sponge-associated vibrios and known reference sequences points to a distinct evolutionary separation explained, in part, by probable adaptation to a commensal existence in sponges.

16S rRNA gene sequences placed the majority of sponge-associated vibrios within the *Harveyi* clade (*Vibrio* core) (Sawabe *et al.*, 2007), which includes *V. parahaemolyticus*, *V. harveyii*, *V. alginolyticus*, *V. natriegens*, *V. rotiferanius* and *V. campbellii*, albeit precise species determinations were largely intangible. Follow-up inspection of IGS genotypes revealed that one group of Curacao strains could be differentiated from other members of the *Harveyi* clade differing by only one band from *V. alginolyticus*. Given the hyper-variable evolutionary structure of 16S–23S rRNA intergenic spacers, however, this subtle difference belies significant genetic diversity between strains. Rather, it is plausible that these vibrios, all from sponge environments, share a highly homogeneous (that is, population level) phylogenetic relationship with their shallow-water counterparts. Given the notable genetic homogeneity among these geographically disparate strains, it is reasonable to postulate a panmictic population structure (that is, members of the population move

freely across habitats, likely are able to exchange DNA and share a common gene pool), whereby members freely associate in spite of the physical distances separating these two groups of vibrios. Although further studies are essential to understanding this relationship, other *Vibrio* hosts, such as zooplankton, for example, may facilitate transport between these two microbiomes.

Near-shore vibrios often associate in commensal relationships with oysters and other commercial shellfish species. Although these microcosms remain distinct from deep-water environs, the relationship between these ecologically disparate vibrios is important because novel species could be 'incidental' sponge residents as a consequence of filtration. It is, however, important to note that more

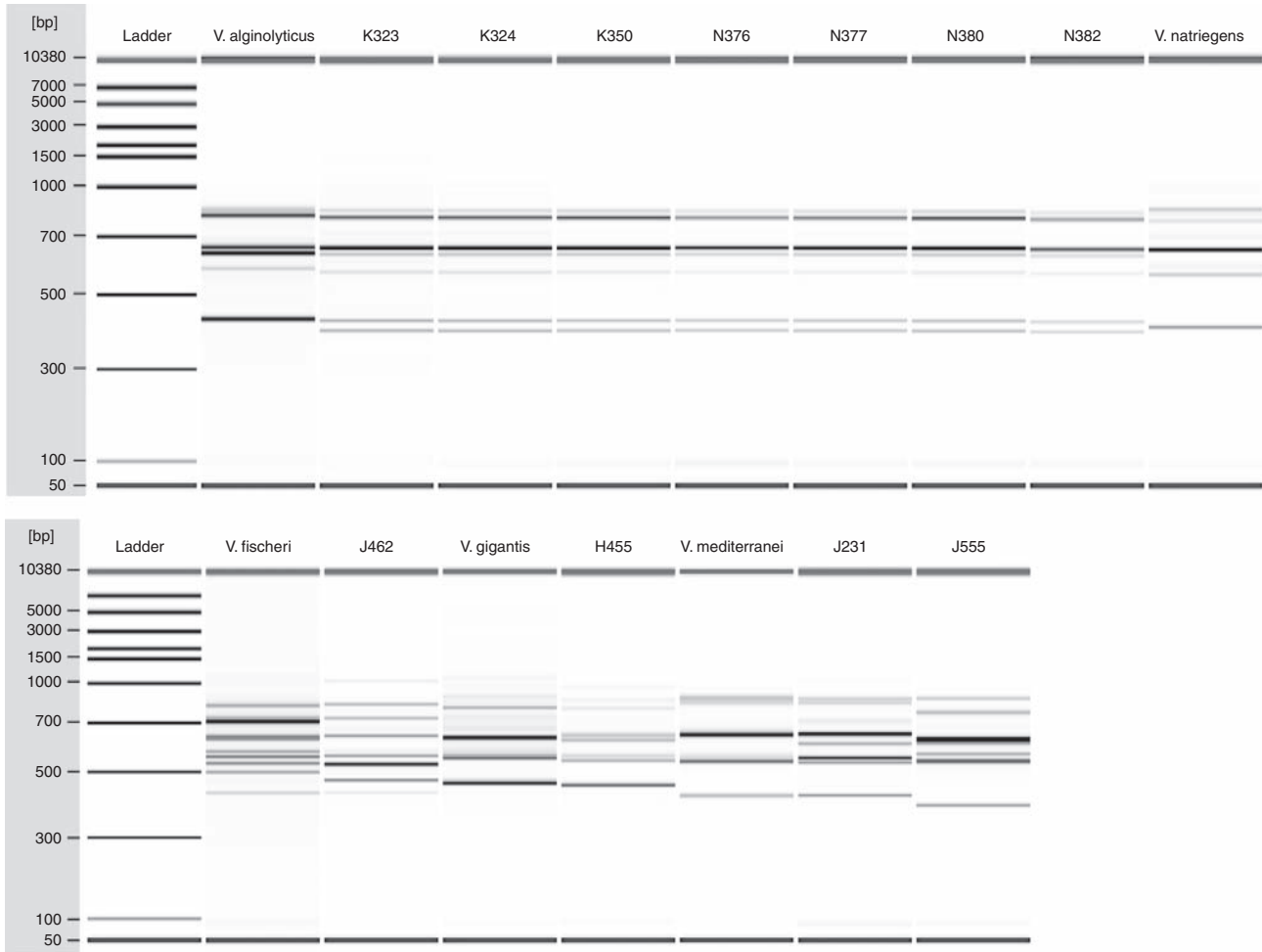


Figure 2 Representative virtual gel image, derived from the Agilent Bioanalyzer 2100, of 16S–23S rRNA IGS-type patterns of select *Vibrio* isolates and *Vibrio* reference species. Molecular weight standards are shown on the left of each gel image. Ln 1, Ladder; Ln 2, *V. alginolyticus* ATCC 17749; Ln 3, *Vibrio* K323; Ln 4, *Vibrio* K324; Ln 5, *Vibrio* K350; Ln 6, *Vibrio* N376; Ln 7, *Vibrio* N377; Ln 8, *Vibrio* N380; Ln 9, N382; Ln 10, *V. natriegens* ATCC 33898; Ln 11, Ladder; Ln 12, *Aliivibrio fisheri* ATCC 14546; Ln 13, *Vibrio* J462; Ln 14, *V. gigantis* LMG 22741; Ln 15, *Vibrio* H455; Ln 16, *V. mediterranei* ATCC 43341; Ln 17, *Vibrio* J231; Ln 18, *Vibrio* J555.

Figure 1 Maximum parsimony (MP) tree derived from 16S rRNA gene sequences from 80 reference species and 23 *Vibrio* isolates. MP analysis was performed using PAUP* v.4.0b10 under heuristic search methods using tree bisection-reconnection (TBR), and 1000 random, stepwise addition replicates to generate the MP phylogram shown here. Bootstrap analyses were performed with identical parameters as used for the maximum parsimony analyses with 1000 replicates being considered. Values shown without parenthesis represent branch lengths, while those shown in parenthesis represent Bootstrap values. Bootstrap values < 20% were not shown. The tree was rooted with *Escherichia coli*, *Erwinia amylovora*, and *Citrobacter freundii* (GenBank accession numbers A14565, AJ010485 and FJ768455). Isolates K875, K882, K878, K921, K873 and K872 were cultured from *Erylus* sp. collected at 470 feet in the Bahamas; N376, N377, N382, N380, K350, K324, K323, N384 and N418 were cultured from *Scleritoderma cyanea* collected at 795 feet in Curacao; L647 was cultured from *Corallistes* sp. at 670 feet from Gulf of Mexico, FL, USA; J036 was cultured from *Axinella* sp. at 150 feet, H455 was cultured from *Bubaris* sp. at 260 feet and J207 was cultured from *Higginsia strigilata* at 200 feet from Atlantic seaboard, FL, USA; J231 and J555 were cultured from Halichondrida at 472 and 735 feet, respectively, and J462 was cultured from Pachastrellidae at 1242 feet from Puerto Rico.

than 600 rRNA gene sequences taken from seawater showed little overlap with sequences derived from sponges (Hentschel *et al.*, 2002). This supports the notion that sponge environments impose strong selection on resident microflora, generally prohibiting habitation by planktonic strains. Furthermore, these observations suggest that N384 and N418 were established commensal strains. At a minimum, some of these strains may qualify as 'ecotypes' in the sense of Cohan (Cohan, 2002), because sponge microcosms offer structural and ecological diversity that could propel dynamic bacterial speciation. Whatever their final evolutionary significance, it is clear that sponges represent a largely unexplored frontier harboring a virtual pantheon of genetic diversity for this important group of bacteria.

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