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Sargasso Sea *Vibrio* bacteria: Underexplored potential pathovars in a perturbed habitat

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ABSTRACT

We fully sequenced the genomes of 16 *Vibrio* cultivars isolated from eel larvae, plastic marine debris (PMD), the pelagic brown macroalga *Sargassum*, and seawater samples collected from the Caribbean and Sargasso Seas of the North Atlantic Ocean. Annotation and mapping of these 16 bacterial genome sequences to a PMD-derived *Vibrio* metagenome-assembled genome created for this study showcased vertebrate pathogen genes closely-related to cholera and non-cholera pathovars. Phenotype testing of cultivars confirmed rapid biofilm formation, hemolytic, and lipophospholytic activities, consistent with pathogenic potential. Our study illustrates that open ocean vibrios represent a heretofore undescribed group of microbes, some representing potential new species, possessing an amalgam of pathogenic and low nutrient acquisition genes, reflecting their pelagic habitat and the substrates and hosts they colonize.

1. Introduction

Gammaproteobacteria of the genus *Vibrio* inhabit freshwater, coastal marine, open ocean, and abyssal habitats and can comprise a significant fraction of plant and animal microbiomes residing in these myriad aquatic environments, fulfilling crucial roles in biogeochemical cycling and ecosystem health (Hasan et al., 2015; Daniels and Shafaie, 2000; Michotey et al., 2020). Some vibrios are also known pathogens, such as *Vibrio cholerae*, the causative agent of cholera pandemics, notorious for its historic human toll (Baker-Austin et al., 2021). Of growing public health concern, non-cholera *Vibrio* spp. are now recognized as the dominant cause of human mortality from the marine environment (Baker-Austin et al., 2021). *Vibrio* spp. such as *V. alginolyticus, V. harveyi, V. parahaemolyticus, V. campbellii* and *V. vulnificus,* among others, are potent pathovars impacting both aquaculture-reared and wild bivalve and finfish stocks, causing life-threatening foodborne illnesses in humans via seafood consumption, as well as morbidity and mortality via wound infection (Baker-Austin et al., 2021; Daniels and Shafaie, 2000).

Plastic marine debris (PMD), first described in surface waters of the Sargasso Sea (Carpenter and Smith, 1972), has become a world-wide concern as it increasingly permeates habitats geographically distant from anthropogenic influence (Amaral-Zettler et al., 2020) and is known to persist decades longer than natural substrates in the marine environment (Amaral-Zettler et al., 2020; Zettler et al., 2013). Previous studies employing small-subunit rRNA gene amplicon surveys found that *Vibrio* spp. are common members of PMD microbial biofilms (also referred to collectively as the plastisphere) (Kirstein et al., 2019; Oberbeckmann et al., 2015; Pedrotti et al., 2022; Zettler et al., 2013). Additionally, non-quantitative, cultivation-independent rRNA gene amplicon surveys have implicated possible *Vibrio* pathovars consistently inhabiting the Great Atlantic *Sargassum* Belt (Michotey et al., 2020; Theirlynck et al., 2023), as well as wild fish stocks (Senderovich et al.,

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2010). Since 2011, rapidly expanding populations of these free-living brown macroalga *Sargassum* spp. in the Atlantic Ocean have been observed, including frequent and unprecedented seaweed accumulation events (Lapointe et al., 2021). This observation, in combination with anomalous rising ocean temperatures, spotlights *Vibrio* spp. for future ocean concerns (Vezzulli et al., 2016). To the best of our knowledge, little is known regarding the ecological relationship of vibrios with *Sargassum* spp. Additionally, genomic and metagenomic evidence has been lacking as to whether vibrios colonizing PMD and *Sargassum* indeed have pathogenic potential in humans (Wright et al., 2020).

In this study, we describe the isolation and genomic characterization of *Vibrio* spp. from the Sargasso and Caribbean Seas of the North Atlantic Ocean from samples of eel leptocephali (larvae), PMD, *Sargassum,* and seawater. From a total of 1188 *Vibrio* spp. isolates collected from two cruises in the North Atlantic Ocean, 16 cultivars were prioritized for full genome sequencing, annotation (Table 1) and comparative analyses with a metagenome-assembled genome (MAG) specifically generated for this study, from a Sargasso Sea PMD sample where *Vibrio* spp. were previously reported to be the dominant bacterial phylotype using small-subunit ribosomal gene sequencing (Zettler et al., 2013).

2. Methods

2.1. Collection of samples and isolation of Vibrio cultivars

Samples of various substrates were collected using either a rectangular neuston net (1 m x 0.5 m, 335 μm mesh) towed at the surface, or a 1-meter circular net (1-meter diameter, 335 μm mesh) at 10-m depth from the SSV *Corwith Cramer* as part of SEA Semester research cruises C-241 in May of 2012 and C-247 in May of 2013 (Fig S1, Table 1). Substrates including *Sargassum*, leptocephalus eel larvae, and PMD were rinsed twice in 0.2 μm filtered seawater then streaked onto CHROMagar™ *Vibrio* (CaV) agar plates (DRG, NJ, USA) and subsequently incubated at ambient temperature in the dark and examined every twelve hours for colony growth. Individual colonies were isolated by transferring to seawater tryptone plates (1 g Bacto tryptone, 20 gs BactoAgar (Becton Dickinson, NJ, USA) per 1 L natural seawater) to assure that single colonies were obtained. Cultivars were cryopreserved in a 1:9 solution of sterile glycerol:seawater tryptone broth at −80 °C.

2.2. Strain isolation and phylogenetic prioritization and gene copy number analyses

During two oceanographic cruises in the Sargasso Sea in May of 2012 (C241) and 2013 (C247) a focus was placed on *Vibrio* bacteria cultivars due to their presence on plastic debris and their underexplored nature in the open ocean. In all, 1188 total presumed *Vibrio* spp. cultures were obtained and cryopreserved from our CaV culture isolations (500 cultivars from C241; 688 cultivars from C247, Fig. S1). We prioritized strains using growth morphology on CaV agar based upon sucrose fermentation and pH indicator dye color ranging from pink to blue to colorless. Other prioritization parameters were: site obtained and substrates from which they were derived. We further prioritized our *Vibrio* cultivars employing heat shock protein 60 (*hsp*60) gene sequence phylogenetic analysis (Preheim et al., 2011) of over 100 cultivars, yielding 4 major clades with closest affiliations with *V. alginolyticus, V. campbellii, V. fortis*, and *V. parahaemolyticus* by average nucleotide identity (ANI) analysis in Fig. 1 in the main text and *hsp*60 phylogeny in Fig. S2.

2.3. Bacterial adhesion assay

Vibrio cultures revived from cryopreserved stocks in YT broth (10 g Bacto yeast extract, 5 g Bacto tryptone (Becton Dickinson, NJ, USA) in 1 L artificial seawater) (Mincer and Aicher, 2016) by overnight incubation with 100 RPM shaking at 23 °C in culture tubes filled with 10 ml of YT media were inoculated at a 1:1000 dilution and grown for 16–18 h. A microbial attachment assay was adapted (O'Toole and Kolter, 1998), for screening biofilm formation on plastic. Subsequent methods details can be found in the Supplemental Methods section. Results are reported in Table S1 and Fig. S3.

2.4. Microbial phenotype screening

Hemolysis assays were conducted using blood agar plates prepared from defibrinated sheep's blood (Fisher Scientific). Results were scored as alpha hemolysis (clear green or brown tinting to the blood, indicating the presence of methemoglobin), beta hemolysis (an entire clearing of the blood cells in the medium indicating full blood cell lysis), or gamma hemolysis (no noticeable change in the blood agar medium) as

Table 1
Data detailing the substrate sources, location and clade phylogeny of the isolates and MAG.

Strain	Source Substrate	Lat Lon	Cruise # (year)	Geographic Area	Clade
D417	Leptocephali	24.9519, −64.8520	C247–012–2-MN (2013)	Sargasso Sea	*V. alginolyticus*
D449	Leptocephali	20.6510, −64.6680	C247–006-NT (2013)	Sargasso Sea	*V. fortis*
B172	Polyethylene	18.6720, −64.5850	C241–002-NT (2012)	Tropical Atlantic	*V. parahaemolyticus*
B181	Polyethylene	18.6720, −64.5850	C241–002-NT (2012)	Tropical Atlantic	*V. parahaemolyticus*
B513	Polyethylene	27.3120, −63.5600	C241–017-NT (2012)	Sargasso Sea	*V. alginolyticus*
B516	Polyethylene	28.0870, −63.4820	C241–019-NT (2012)	Sargasso Sea	*V. alginolyticus*
D404	Polyethylene	20.6510, −64.6680	C247–006-NT (2013)	Sargasso Sea	*V. fortis*
D409	Polyethylene	20.0688, −64.6000	C247–008-NT (2013)	Sargasso Sea	*V. alginolyticus*
D420	Polyethylene	23.6686, −64.5186	C247–010-NT (2013)	Sargasso Sea	*V. fortis*
D421	Polyethylene	23.6686, −64.5186	C247–010-NT (2013)	Sargasso Sea	*V. alginolyticus*
D431	Polyethylene	26.6344, −64.6833	C247–014-MN (2013)	Sargasso Sea	*V. alginolyticus*
B511	Sargassum	24.8120, −64.5700	C241–013-NT (2012)	Sargasso Sea	*V. alginolyticus*
D401	Sargassum	20.6510, −64.6680	C247–006-NT (2013)	Sargasso Sea	*V. parahaemolyticus*
D406	Sargassum	20.0688, −64.6000	C247–008-NT (2013)	Sargasso Sea	*V. alginolyticus*
D415	Sargassum	24.9519, −64.8520	C247–012–2-MN (2013)	Sargasso Sea	*V. alginolyticus*
D173	Seawater	18.2174, −64.7180	C247-SS-001 (2013)	Carribean Sea	*V. parahaemolyticus*
MAG	Polypropylene	31.628, −41.415	C-230–01 (2010)	Sargasso Sea	*V. parahaemolyticus*

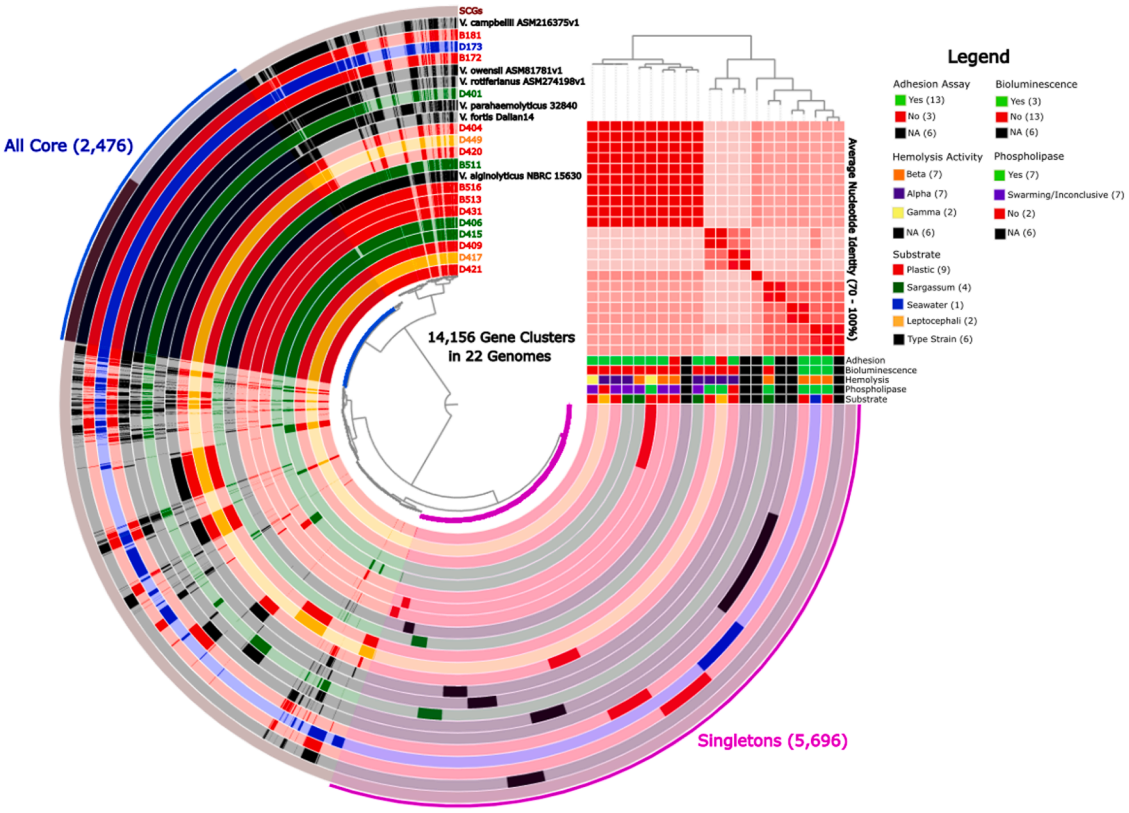

Fig. 1. *Vibrio* pangenome analysis of isolates analyzed in this study. The circular phylogram was created using Anvi'o 7.0 analyses of 16 environmental *Vibrio* isolates. The colors indicate the respective marine substrate from which each cultivar was isolated: eel leptocephalus (orange), plastic marine debris (red), *Sargassum* (green), seawater (blue), and type strains (black). All environmental cultivars were tested for biofilm formation, bioluminescence, hemolysis (see Section 2.4 for description), and phospholipase activity (see Supplemental Information). Results are color-coded in the legend. Average nucleotide identity (ANI) ranges between 100% (dark red) and 70% (pink) are also indicated, with the cladogram at the top right indicating ANI clustering. SCGs indicated by the outer circle denotes single copy genes. Strain names indicated at top continue through the circular dendrogram to the ANI and clade diagram.

previously described (Brown, 1919).

Extracellular phospholipase activity was screened on LB agar containing additional salt (LB 25 g, Milli-Q water 1 L, BactoAgar 15 g, NaCl 7 g, $CaCl_2$, 0.11 g) supplemented with 1% (v/v) egg yolk emulsion (Fisher Scientific, CA, USA) using a modified method (Kurioka and Liu, 1967). *Escherichia coli* MG1655 incubated in two separate conditions, 23 °C or 37 °C, was used as a negative control. Subsequent methods details can be found in the Supplemental Methods section.

2.5. Genomic sequencing and analyses

Vibrio spp. genomic DNAs were extracted using a modification of a previously published method (Zettler et al., 2013). See Supplemental Methods section for details.

We assembled *Vibrio* spp. reads into contigs using CLC genomics workbench v.7 (http://www.clcbio.com). The average coverage for each genome was between 40X and 117X. Genomes were not fully closed, and thus represent draft genomes. However, completeness of draft genome sequencing was assessed by single copy gene (SCG) representation, and all genomes were found to contain the full suite of universally conserved SCGs using BUSCO indicating that genomic coverage was near complete for each isolate.

SCGs were identified using Benchmarking sets of Universal Single-Copy Orthologs (BUSCO) (Simão et al., 2015) with the vibrionales_odb10 database as a reference. The percentage of SCGs from each isolate genome that were BUSCO notated as complete (C), duplicated (D), fragmented (F), and missing (M) are displayed in Fig. S4.

2.6. Metagenomic sequencing and recruitment analyses

DNA extraction, library preparation, quality control and sequencing of our metagenomic DNA library followed the same protocol as for the genomic sequencing but employed the DNA extraction protocol of Zettler et al., 2013. An entire lane of sequencing on an Ilumina HiSeq 1000 was dedicated to this sample, yielding approximately 243,882,048 raw reads (121,941,024 paired-end reads).

2.7. Pangenomic analysis

The *Vibrio* spp. pangenome was created using the freely available analysis and visualization platform Anvi'o (version 7 dev) (Eren et al., 2015) with modification of the pangenomic workflow (Delmont and Eren, 2018). Open reading frames were identified using Prodigal (V2.6.3) (Hyatt et al., 2010), and contig databases for all genomes were produced using the anvi-gen-contigs-database command. Hierarchical clustering of prodigal-designated genes was prepared using BLASTp, and putative homologous gene clusters were subsequently grouped using the Markov Cluster Algorithm (Hyatt et al., 2010) (minbit 0.5; inflation 2) and aligned with MUSCLE (V3.8.1551) using default parameters (Edgar, 2004). The average nucleotide identity of genomes was computed using the program pyANI (V0.2.10; ANIb) with a range of 70–100% similarity, and these data were used to construct a phylogenetic tree (distance: Euclidean; linkage: Ward) displayed in Fig. 1. Contig databases were annotated using COG20 categories, functions, and pathways (Galperin et al., 2021), as well as KOfams (Aramaki et al., 2020) and Pfams (Mistry et al., 2021). Additional query files containing the major and minor *Vibrio* virulence factors were obtained from the

Pathosystems Resource Integration Center (PATRIC) virulence factor database (Gillespie et al., 2011) and queried using Anvi'o, against all genomes using BLASTn (perc_identity 70; wordsize 18; evalue 1e-10) (Eren et al., 2015). Absence, or presence, as well as copy number of genes was verified by analyzing the aligned genome region (length) relative to the full query gene length (qlen), with a combination of BLASTn, TBLASTx (evalue 1e-10), exported amino acid sequences of target genes stored in Anvi'o, and COG20 functions, KOfams, and Pfams (all *Vibrio* spp. isolate annotation data included in Data S1 Table 1).

Anvi-get-enriched-functions-per-pan-group was used to calculate functional enrichment scores for annotation sources for each substrate category (Shaiber et al., 2020); eel leptocephali; plastic; *Sargassum*; seawater), and these scores were programmatically matched with unique identifiers of exported gene detection and coverage values for mapped metagenome reads (Supplemental Tables). Functions with a q-value less than 0.05 were considered enriched. Strains D404 and D449 displayed an ANIb within the cutoff to warrant a new species, as discussed in the Main Text, 86% in Anvi'o and 89% with GTDB-Tk, against the nearest fully-sequenced type strain *V. fortis* Dalian14 (Chaumeil et al., 2020; Ke et al., 2017).

2.8. Mutation analysis

Nucleotide sequences from gene clusters of interest were exported from Anvi'o and subsequently aligned with each clade's type strain using the local pair parameterization in MAFFT (v7.475) (Falda et al., 2012). The aligned sequences were imported into MEGA X for mutation analysis (Stecher et al., 2020) and the pairwise comparisons of the number of non-synonymous (dN) and synonymous (dS) mutations were calculated using the Nei-Gojobori method under the assumption that homologous recombination occurred at uniform rates (Nei and Gojobori, 1986). Full-length gene alignments with a ratio of dN/dS <1 were categorized as having experienced purifying selection, whereas genes with dN/dS >1 were classified as having underwent positive selection (Data S1 Table 2). Amino acid sequences were also exported for studying the syntenic region of the MSHA operon across isolates and type strains using the same parameters and tools as described above (Data S1 Table 3).

2.9. MEGAHIT approach for identifying Vibrio reads from the metagenome

We used trimmomatic v. 0.36 (Bolger et al., 2014) to quality trim and remove adapters from 121,941,024 raw reads, leaving 99.89% of the reads remaining. We used Kraken v. 1.0 (Wood and Salzberg, 2014) to assess the taxonomic composition of the trimmed reads and then assembled the reads using MEGAHIT v.1.1.3 (Li et al., 2015) with a k-min set to 21, k-max to 141 and a k-setp of 12. We used Quast v4.6.3 to evaluate the resulting assembly quality. The assembly coverage was performed by mapping-back the reads to the assembly using bwa-mem v. 0.7.17-r1188. The resultant .sam file was sorted and converted to a .bam file using SAMtools v. 1.8 (Li et al., 2015). We obtained the depth of coverage using the jgi_summarize_bam_contig_depths script available with Metabat2 (Kang et al., 2015). Metagenome-assembled genomes (MAGs) where computed using BinSanity v.0.2.6.4 (Graham et al., 2017) with the BinSanity-wf command and a minimum contig length of 500 bp resulting in four *Vibrio* bins of low completeness according to CheckM v. 1.0.11 (Parks et al., 2015) results (9.83, 6.54, 4.39, and 0).

Given that previous analyses showed a significant number of *Vibrio* reads, unbinned contigs were further identified in the assembly and coding sequences (CDS) detected with Prokka v1.14-dev (Seemann, 2014). Protein sequences were then annotated using diamond v0.9.22.123 (Buchfink et al., 2014) against the NCBI NR database downloaded on April 2019. Subsequent methods details can be found in the Supplemental Methods section.

2.10. Assessing MAG completeness

Mapped metagenomic reads to the different isolates were first dereplicated with a 100% identity using Vsearch v2.8.0 (Rognes et al., 2016) and mapped against the MEGAHIT assembly. In order to increase the confidence of the mapping, resultant .sam files were filtered with a minimum required quality of 30. Raw reads belonging to the mapped contigs and not present in our already selected contigs were also included in our new set of raw reads.

Due to the complex nature of the metagenomic sample, the assembly of metagenome assembled genomes MAGs was challenging, requiring a custom workflow (Fig. S5 and Data S1 Table 5). However, we were able to validate the completeness of a MAG belonging to the *Vibrio* clade in the subset of raw reads according to the following approach. First, we ran BUSCO v4.0.6 (Simão et al., 2015) to the assembled genome of *Vibrio* B172 (*Vibrio* sp. isolated from a piece of plastic which was determined closest to the MAG of interest) finding 1444 complete single-copy BUSCO genes out from 1445 from the vibrionales_odb10 BUSCO lineage. Next, using bwa mem, the selection of raw reads was mapped against the same reference genome, and subsequently using BEDTools v2.26.0 (Quinlan and Hall, 2010) with the coordinates of the identified SCGs by BUSCO and the mapping results, the coverage for each single-copy genes was computed, finding 1400 single-copy genes. Further results can be found in Data S1 Tables 5 and 6.

3. Results

3.1. Potential new species of Vibrio from the North Atlantic Ocean

Environmental *Vibrio* spp. cultivars segregated into 3 major clades by *hsp*60 phylogeny (Fig. S2) and Average Nucleotide Identity using BLAST (ANIb) with type strains *V. alginolyticus, V. campbellii* (known as a member of the "*Vibrio* Harveyi core" clade (Ke et al., 2017)), and *V. fortis* (Fig. 1). Strains D404, and D449 displayed an ANIb of 86% in Anvi'o and 89% with GTDB-Tk, against the nearest fully-sequenced type strain *V. fortis* Dalian14, suggesting they represent a new species within the *Vibrio* genus.

3.2. Pathogenic physiology and genes present in isolates and MAG

All 16 *Vibrio* spp. isolates were tested for substrate adhesion to the polymers PVC, PP, PS and glass, as well as the ability to oxidize or lyse red blood cells (alpha or beta hemolysis, respectively) and hydrolyse phospholipids. Interestingly, cell density appeared to introduce a factor of variability in substrate adhesion in our assays for strain D449, suggesting a quorum sensing mechanism could be influencing this phenotype (Fig. S3). Hemolysis and phospholipase assays were positive for 14 and seven of the 16 isolates, respectively (Fig. 1). The MAG mapped to about 48% of the B172 genome, the isolate that was most closely related by ANI (Data S1 Table 6).

Components of the accessory colonization factor (ACF) gene cluster, under control of the ToxR regulon in *V. cholerae* (Peterson and Mekalanos, 1988), were partially present in our isolates and the MAG, Fig. 2. The *acf*A gene, present in 12 of 16 isolates and the MAG, is required for full virulence and intestinal colonization in *V. cholerae* isolates in combination with the toxin co-regulated pilus gene cluster and the *acf*B, C, D accessory genes (Everiss et al., 1994), present to varying degrees in our isolates and more consistently in the MAG, which was missing only *acf*B (Fig. 2). The *ilp*A gene, found in all isolates and the MAG, encodes a lipoprotein known in *V. vulnificus* to enable full adhesion to human intestinal epithelial cells (K. J. Lee et al., 2010). Our MAG and all isolates except D404, D420, D449 possessed the multivalent adhesin molecule 7 (MAM7), a trans-membrane protein that is essential for lethality in mouse assays conducted with *V. parahaemolyticus* (Lim et al., 2014; Stones and Krachler, 2015). Genes encoding the mannose-sensitive haemagglutinin Type IV pili (MSHA) were well-represented in all

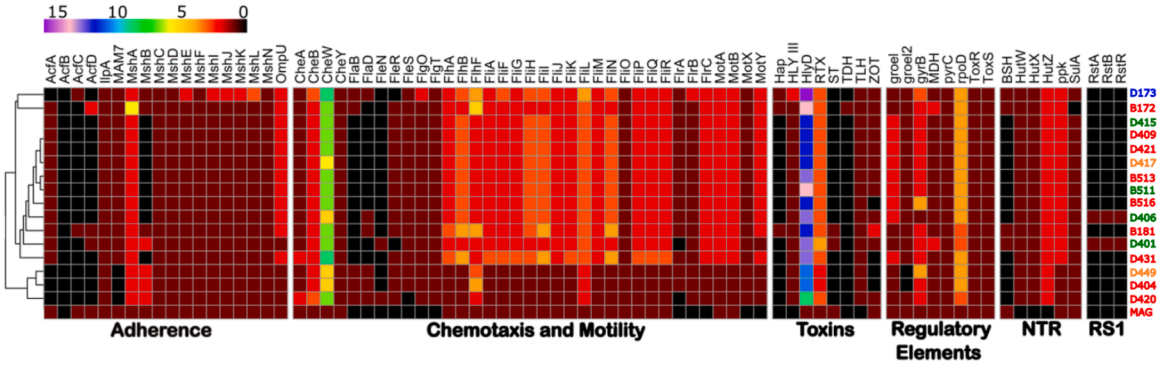

Fig. 2. Hallmark pathogenic genes present in our isolates and the PMD metagenome assembled genome (MAG) within the functional categories of adherence, chemotaxis and motility, toxins, regulatory elements, nutrition resistance factor (NTR) related genes, also known as persister genes, and the RS1 element of the cholera toxin phage. Heatmap colors indicate gene copy numbers from a range of 0 to 15.

isolates and the MAG, Fig. 2. In addition to MSHA type IV pili, Type II, III, and VI secretion system pathways were nearly complete for the MAG and all isolates that possessed genetic components associated with those pathways (Fig. 2 and Data S1 Table 7). All strains were capable of reproducible biofilm formation in our assays, on either multiple plastic polymer or glass substrates except for strains B516 and D449 (Table S2), emphasizing the importance of an attached lifestyle for these isolates.

Nutritional resistance (NTR) genes such as the full *hut*WXZ operon with multiple copies of the *hut*Z gene were present in all cultivars, but absent in the MAG (Fig. 2). HutZ, shown to be a crucial protein in *V. cholerae* for obtaining heme-bound iron once red blood cells are lysed (Wyckoff et al., 2004) and an important pathogenic determinant in the fish pathogen *Edwardsiella piscicida* (Shi et al., 2019) among other microbial fish pathovars (Lemos and Balado, 2020), was present in multiple copies in all *Vibrio* cultivars, but absent in the MAG. Bile salt hydrolase (BSH), an important gut colonization factor in vertebrate pathovars, was present in isolates B172, D173, B181, D401, and MAG. Other NTR genes polyphosphate kinase (*ppk*) and the SOS response regulated cell division inhibitor *sul*A were present in nearly all isolates and the MAG (Fig. 2). Repeats in toxin (RTX) which were hallmarks in the outbreaks of El Tor cholera epidemics, were present in all isolates and the MAG (Lin et al., 1999) as were *tox*R and *tox*S genes (crucial members of the ToxR regulon, involved in expressing cholera enterotoxin (CT) and TCP in epidemic *V. cholerae* strains (Bina et al., 2003) (Waldor and Mekalanos, 1996). Only *tcp*E was found in D404 and D449 (isolated from PMD and Leptocephalus, respectively) whereas the other 11 TCP pilus assembly genes were absent (Data S1 Table 1). Multiple full copies of various hemolysin genes were detected in all of our isolates and many presented this phenotype in our assay (Fig. 1, Fig. 2 and Supplemental Tables). Interestingly, isolates D401 and D406 were found to possess the *rs1*A, B, and R genes (Fig. 2), known to be part of a horizontally transferred lysogenic phage, RS1Φ using the same receptor as the CTXΦ phage (Lin et al., 1999). However, no presence of cholera toxin was observed in any of our isolates or MAG. Additionally, the gene encoding the Zonula occludens toxin, *zot*, required for the weakening of tight junctions in the vertebrate intestinal epithelial lining (Mauritzen et al., 2020) was present in a majority of our isolates and the MAG (Fig. 1, Fig. 2; Data S1 Table 7). Analysis of dN/dS also showed that the *zot* genes appear to be under strong positive selection ranging from 3.1 – 1.5 (Data S1 Table 3). Many of the isolates contained phosphate/-phosphonate ABC transport systems and strikingly isolates D404 and D449 possessed a near-complete phosphonate utilization pathway as characterized in *E. coli* (*phn*CDEFGHIJKLMNOP) missing only *phn*F (Data S1 Table1). However, a recent study showed through cloning studies in *E. coli* that *phn*GHIJKLM is only necessary for full phosphonate utilization (Jochimsen et al., 2011), suggesting isolates D404 and D449 possess a functioning carbon-phosphorous lyase pathway. Another interesting adaptation was the presence of proteorhodopsin in isolates B181 and D173, sharing near-identical amino acid similarity to *V. campbellii* (Data S1 Table 1).

4. Discussion

4.1. Vibrio ecology and pathology

The ecology and pathology of vibrios are well-studied in coastal regions with many cultivars physiologically characterized and fully-sequenced (1738 were represented in the IMG database at the time of analysis, adding confidence to our annotations) (Markowitz et al., 2012). With 147 species and 4 subspecies described, 24 *Vibrio* spp. are recognized as plant or animal pathogens (Grimes, 2020). Open ocean habitats present an extreme scenario, oligotrophic conditions force 'feast or famine' situations where key nutrients such as phosphorous are limiting, particularly in the Sargasso Sea (K. J. Lee et al., 2010). In the case of vibrios in these habitats it appears that pathogenic attachment, potential toxin production and hydrolytic enzymes targeted towards animal and plant tissues, likely provide an advantage.

Attachment is a key factor in pathogenesis and secretion-based attachment factors are well-represented in the *Vibrio* spp. isolates and MAG (Fig. 2 and Data S1 Table 7). Variability is common in secretion systems and function does not necessarily rely on a full complement of all known genes in a secretion system pathway (Izoré et al., 2011). The MSHA pilus is one of several biofilm and adhesion mechanisms in *V. cholerae*, which in concert with the toxin coregulated pilus TCP, is responsible in the formation of multilayered, complex biofilms on chitin (Yildiz and Visick, 2009). The MSHA system is also important in adhesion to diatom produced chitin, aiding persistence in the water column (Frischkorn et al., 2013) and has also been implicated in early colonization and adherence to plastic polymers (Bos et al., 2023). Multiple copies of the *msh*A genes are known to enhance adhesion, biofilm formation, DNA uptake, twitching motility, and virulence (Ellison et al., 2018). Of note, isolate B172 has seven copies of *msh*A, which is high compared to a calculated average of 2.1 (n = 1738) for other vibrios from our analysis (Fig. 2; Table S3; Data S1 Tables 1 and 2). An analysis of synonymous versus non-synonymous nucleotide substitutions showed that specific *msh*A alleles were under positive selection with dN/dS ratios as high as 2.8, suggestive of strong positive selection (Data S1 Table 3). Biofilm formation in many *Vibrio* spp. is commonly under flagellar influence (Yildiz and Visick, 2009), with motility being an important feature of the copiotrophic lifestyle of *Vibrio* spp. (Hunt et al., 2008) and pathovars (Daniels and Shafaie, 2000). Complementing motility, chemotaxis and lateral and polar (swarming) flagellar gene sets were well-represented in all isolates and the MAG (Fig. 2).

Additionally, synteny of the MSHA operon varied as two fundamental patterns (independent of strain phylogeny), and high diversity of amino acid compositions of *msh*A at the amino acid level (Figs. S6 and

S7, respectively). This *msh*A gene diversity could be an adaptation to the ecological pressures of vibrios in the open ocean and the many substrates that these isolates encounter, such as animal and plant tissues, in addition to plastic debris. Altogether, the adhesion and toxin genes present in the isolates and MAG display a genetic tool kit typical of *V. cholerae* and *V. parahaemolyticus* host colonization and biofilm formation genes, with multiple degeneracy and copy number, a theme similar to other *Vibrio* spp. pathovars (Yildiz and Visick, 2009). Positively selected pathogenic genes such as MSHA and other Type IV pilus mechanisms may also provide an advantage in competing on surfaces such as attachment to plastic and adhesion in the *Sargassum* phycosphere. More research is needed to understand the selective pressures on the MSHA operon and the role of *msh*A specifically in surface colonization in the wild. All of our isolates also possessed alginate lyase, some possessing multiple alleles (Data S1 Table 1), which could provide enhanced carbon utilization in the *Sargassum* phycosphere. Isolates B181 and D173 also possessed proteorhodopsin, nearly identical to the *V. campbellii* allele, previously determined to function as a proton pump and provide protection against starvation under oligotrophic conditions (Wang et al., 2012). Scavenging limited phosphorous through the carbon-phosphorous lyase pathway is an important adaptation in oligotrophic environments (Lee et al., 2017) and two of the *Vibrio* spp. isolates (D404 and D449) appear to have a complete pathway necessary for acquisition of phosphorous from phosphonate compounds (Jochimsen et al., 2011). Interestingly, these are also the two strains in our study that were the most divergent by ANI, possibly representing a new species within the *Vibrio* genus.

4.2. Potential for vertebrate host infection and phenotype expression

Hallmark pathogenic gene sets suggest that some of our *Vibrio* cultivars have the potential to infect vertebrate hosts, such as *acf*A, *ilp*A, bile salt hydrolase, *zot*, MSHA, ToxR and ToxS, with all these gene sets mirrored in the *Vibrio* MAG from PMD (Fig. 2, Data S1 Tables 5 and 6). It is interesting that this combination of pathogenic gene sets, particularly *acf*A, *ilp*A and MAM7 all appear to be separate key pathogenic determinants in *V. cholerae, V. vulnificus and V. parahaemolyticus*, respectively. The fact that the open ocean *Vibrio* spp. isolates and MAG possess these genes illustrates their unique mosaic nature and selective environmental pressures, compared to other well-characterized *Vibrio* spp. pathovars. The presence of the *zot* gene in these *Vibrio* spp. isolates and our MAG, and evidence for its strong positive selection in the cultivars, is intriguing and could have implications for the surrounding oligotrophic waters if it is causing vertebrate hosts to have a leaky gut and release nutrients to the environment which, in turn, could stimulate the growth of *Sargassum* and other surrounding organisms. The presence of the RS1Φ is intriguing as this is typical of only *V. cholerae* strains (Lin et al., 1999). Such similarities among our isolates and *V. cholerae* suggest some shared ancestry or gene transfer events between these clades. Additionally, the isolates all shared the presence of microbial collagenase, similar to other known *Vibrio* spp. pathovars (Data S1 Table 1). Collagen is a crucial component in animal tissue matrices, amounting to 25% of mammalian proteins, for example (Grimes, 2020).

Interestingly, cell density appeared to introduce a factor of variability in substrate adhesion in our assays, particularly for strain D449, suggesting a quorum sensing mechanism could be influencing this phenotype (Fig. S3). This mechanism has been found to be important in biofilm formation and virulence gene expression in *V. cholerae* where elevated cell density was shown to govern a committed point of no return, very similar to the biofilm formation phenomenon illustrated in Fig. S3 (Hammer and Bassler, 2004) Hemolysis and phospholipase assays were positive for 14 and seven of the 16 isolates, respectively (Fig. 1 and Table S2). Hemolysis and phospholipase phenotypes, known hallmarks of pathogenic vibrios, were mapped onto the clades of *V. alginolyticus, V. campbellii, V. fortis* and *V. parahaemolyticus*, according to their phylogenetic placement (Fig. 1).

4.3. Open ocean vibrios as opportunistic 'hunters'

In the vast open ocean, *Sargassum* represents an ancient and unique ecosystem for marine fauna and many of their larval and juvenile forms, including uniquely adapted species such as the *Sargassum* pipefish and seahorse (Coston-Clements et al., 1991). Predators have specifically adapted to these macroalgal oases of life in the nutrient depleted sea as well, notably, the endemic *Sargassum* fish (*Histrio histrio*) an aggressive piscivore exquisitely adapted to the *Sargassum* canopy via mimicry (Rogers et al., 2010). Similar adaptations taking advantage of the abundant sea life in these seaweed patches appear to have taken place at the microbial level. Members of the Vibrio Harveyi clade: *V. campbellii, V. owensii* and *V. rotiferianus* which includes relatives of our isolates, are recognized animal pathogens (Ke et al., 2017), while *V. fortis* has been linked to crustacean and seahorse disease (Wang et al., 2016). With the increase of *Sargassum* and PMD in the Sargasso Sea and their growing co-occurrence in other parts of the open ocean such as the Great Atlantic *Sargassum* Belt in the North Equatorial Recirculation Region, open ocean *Vibrio* spp. colonizing these substrates are expanding as well. *Sargassum* beaching events are now so commonplace that efforts to upcycle this seaweed biomass in the form of livestock feed and fertilizer are underway (Milledge and Harvey, 2016).

5. Conclusions

The MAG represents the first *Vibrio* spp. assembled from plastic debris, with its nearest relative to isolate B172 and possesses many pathogenic genes. Overall, given the complement of toxin, adherence, hydrolysis, and nutritional resistance gene sets in the open ocean *Vibrio* spp. isolates and MAG and additional positive functional screens for hemolysis and phospholipase phenotypes, it appears that some *Vibrio* spp. in this environment have an 'omnivorous' lifestyle targeting both plant and animal hosts in combination with an ability to persist in oligotrophic conditions. With increased human-*Sargassum*-PMD interactions, associated microbial flora of these substrates could harbor potent opportunistic pathogens. In particular, caution should also be exercised regarding the harvest and processing of *Sargassum* biomass until the risks are explored more thoroughly.

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Data and materials availability

All BioProject, MAG, BioSample and SRA numbers and GenBank accession numbers can be found in the Supplemental section Data S1 Tables.

CRediT authorship contribution statement

Tracy J. Mincer: Conceptualization, Investigation, Methodology, Visualization, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Ryan P. Bos:** Investigation, Methodology, Visualization, Writing – review & editing. **Erik R. Zettler:** Funding acquisition, Project administration, Writing – review & editing. **Shiye Zhao:** Methodology. **Alejandro A. Asbun:** Methodology, Visualization, Writing – review & editing. **William D. Orsi:** Visualization, Writing – review & editing. **Vincent S. Guzzetta:** Methodology, Writing – review & editing. **Linda A. Amaral-Zettler:**

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Declaration of Competing Interest

All authors declare that they have no competing interests.

Data availability

Data will be made available on request.

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Supplementary materials

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