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MEETING PRESENTATION

Evolution of a Vegetarian Vibrio: Metabolic Specialization of *Vibrio breoganii* to Macroalgal Substrates

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ABSTRACT While most *Vibrionaceae* are considered generalists that thrive on diverse substrates, including animal-derived material, we show that *Vibrio breoganii* has specialized for the consumption of marine macroalga-derived substrates. Genomic and physiological comparisons of *V. breoganii* with other *Vibrionaceae* isolates revealed the ability to degrade alginate, laminarin, and additional glycans present in algal cell walls. Moreover, the widely conserved ability to hydrolyze animal-derived polymers, including chitin and glycogen, was lost, along with the ability to efficiently grow on a variety of amino acids. Ecological data showing associations with particulate algal material but not zooplankton further support this shift in niche preference, and the loss of motility appears to reflect a sessile macroalga-associated lifestyle. Together, these findings indicate that algal polysaccharides have become a major source of carbon and energy in *V. breoganii*, and these ecophysiological adaptations may facilitate transient commensal associations with marine invertebrates that feed on algae.

IMPORTANCE Vibrios are often considered animal specialists or generalists. Here, we show that *Vibrio breoganii* has undergone massive genomic changes to become specialized on algal carbohydrates. Accompanying genomic changes include massive gene import and loss. These vibrios may help us better understand how algal biomass is degraded in the environment and may serve as a blueprint on how to optimize the conversion of algae to biofuels.

KEYWORDS macroalgal carbohydrates, horizontal gene transfer, metabolic specialization, *Vibrionaceae*, ecology, adaptation, macroalgae, seaweed, *Vibrio*, algae, degradation, polysaccharide

Competition for resources is a major driver in the diversification of organisms, affecting metabolic strategies as well as habitat and organismal associations (1–4). While marine bacteria of the family *Vibrionaceae* are typically considered specialized for animal associations, a remarkable variety of lifestyles have arisen along with the capacity to utilize distinct assortments of diverse substrates during environmental or host interactions (5–10), and these changes yield promising bioengineering insights and applications (11, 12). Accordingly, fine-scale mapping of differences in metabolic capabilities and habitat associations among closely related isolates has enabled the identification of distinct populations with high resolution, offering valuable insights into the selective forces and fitness trade-offs in natural environments (13–16).

Recently, we identified an adaptive radiation among marine vibrios that was mediated by horizontal gene transfers, leading to ecophysiological differentiation and

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fine-scale resource partitioning of alginate, a brown algal cell wall glycan (17). This differentiation was particularly dramatic in *V. breoganii*, where diverse carbohydrate-active enzymes (CAZymes) were repeatedly acquired from multiple sources leading to the multicopy presence of each type of enzyme and facilitating the rapid degradation of large alginate polymers (17). This high degree of differentiation led us to hypothesize that, rather than simply obtaining an additional metabolic capability, *V. breoganii* has evolved into a macroalgal specialist.

This hypothesis initially appeared to be at odds with much of the literature describing *V. breoganii*, which was first isolated from the guts of clams (18) and subsequently observed in a diverse array of marine invertebrates, including mussels, crabs, and octopi (19, 20). However, *Vibrio halioticoli*, a close relative of *V. breoganii*, has a well-characterized association within the abalone gut, where it facilitates host nutrient absorption by degrading macroalgal carbohydrates (21–24). These reports also raise the question of whether the presence of *V. breoganii* within the guts of diverse invertebrate hosts reflects stable long-term symbioses or merely transient associations due to ingested algal material. Accordingly, we sought to further characterize the metabolic capabilities differentiating *V. breoganii* from other *Vibrio* populations and the extent of ecophysiological specialization to a macroalga- or host-associated lifestyle.

Using a combination of substrate-specific growth assays together with an analysis of the CAZyme content in a diverse collection of *Vibrio* populations, we demonstrate the specialization of *V. breoganii* to macroalgal carbohydrates, including alginate and laminarin, along with a shift away from animal-derived chitin and glycogen polymers and amino acids. Comparative genomics further reveals changes associated with this metabolic specialization and adaptations to a sessile seaweed-associated lifestyle, including the loss of motility. Together, these findings illustrate how algal polysaccharides became a major source of carbon and energy for *V. breoganii*, likely driving distinct ecological associations with algal detritus and marine invertebrates.

RESULTS AND DISCUSSION

***V. breoganii* isolates are macroalgal carbohydrate specialists.** To investigate metabolic specialization within *V. breoganii*, representative sequenced isolates from diverse *Vibrionaceae* populations were subjected to growth assays encompassing a wide variety of carbon substrates, including an extensive panel of carbohydrates with environmental relevance. Genomes were also analyzed using the dbCAN database to identify the CAZyme repertoire of each strain, and the Carbohydrate-Active enZymes database was used to identify annotated substrates for predicted enzymes (25, 26). Together, these substrate utilization and CAZyme profiles differentiate *V. breoganii* from other *Vibrio* populations and reflect a specialization for macroalgal carbohydrates.

In addition to the previously characterized alginate pathway, *V. breoganii* and most other *Vibrio* populations assessed could utilize a wide variety of macroalga-associated substrates as a sole carbon source (Fig. 1A; see also Fig. S1 in the supplemental material). These commonly utilized algal substrates included mannitol, an important sugar alcohol used for carbon storage in brown algae, and galactose, a building block of carrageenans found in red macroalgae.

However, *V. breoganii* isolates differentiated themselves from most *Vibrio* populations in their ability to utilize a broader variety of beta-glucans, which are an important category of glucose-based polysaccharides composed of beta-glycosidic bonds found primarily in algal cell walls. While most *Vibrio* populations could grow on the beta-glucan cellobiose, a glucose disaccharide joined with a beta-1,4 linkage, only *V. breoganii* possessed the capacity to also utilize gentiobiose, the beta-1,6-linked glucose disaccharide. Furthermore, *V. breoganii* isolates were able to grow on longer beta-D-glucan polysaccharides from barley with a backbone of beta-1,3 linkages and exhibited limited growth on lichenan, a glucose polysaccharide consisting of beta-1,3- and beta-1,4-glucans. Perhaps most importantly, *V. breoganii* was the only *Vibrio* population consistently capable of growing on laminarin, the principal storage glycan in brown


3 2 1 0
10 8 6 4 2 0
3 2 1 0
V. breoganii
V. ordalii
V. sp F10
V. sp F6
V. kanaloae
V. sp F12
V. sp F13
V. tasmaniensis
V. splendidus
V. lentus
V. cyclitrophicus
V. rumoiensis
A. salmonicida-logei
A. fischeri
E. calviensis-like
E. norvegicus
C. Amino Acids
Glycine
Arginine
Tyrosine
Serine
Histidine
Alanine
Glutamine
Asparagine
Proline
B. Carbohydrate Active Enzymes
GT025
CBM69
AA06
CBM48
CBM50
GH023
CE16
CE04
AA10
CBM73
CBM14
CBM05
GH129
GH116
GH019
GH018
GT004
GH088
GH016
GH003
GH043
GH036
AA02
GH010
GH004
PL17
PL15
PL07
PL06
Misc.
Glycogen
Chitin
beta-Glucans
Cell wall
Alginate
A. Carbohydrates
Glycogen from oyster, Type II
N–Acetyl–D–glucosamine 99%
Laminarin from L. digitata
Laminarin
Lichenan (Icelandic Moss)
Gentiobiose
D (+) Cellobiose
beta–D–Glucan (Barley)
D–(+)–Galactose (99%)
D–Mannitol (98%)
D–Glucose
Alginic Acid from M. pyrifera
Alginic Acid
Tree scale: 0.05

algae. This abundant glycan is characterized by beta-1,3-glucans with occasional beta-1,6 linkages and branches (27).

Interestingly, the expanded capacity of *V. breoganii* to degrade beta-glucans appears to be shared with the more distantly related *Enterovibrio* populations of *E. norvegicus* and *E. calviensis*. These *Enterovibrio* populations could often degrade a similar suite of beta-glucans yet were incapable of degrading alginate polysaccharides. Similarly, although rare, a few non-*breoganii Vibrio* strains were capable of degrading additional beta-glucans, likely reflecting the independent and rapid horizontal acquisition of novel metabolic capacities.

To investigate the mechanistic basis of the expanded nutritional capacity in *V. breoganii*, we next analyzed the CAZyme content of each sequenced isolate. We found that *V. breoganii* isolates encode a CAZyme profile distinct from that of most *Vibrio* populations (Fig. 1B and S2), primarily defined by the alginate degradation pathway and an expanded repertoire of glycoside hydrolases implicated in the degradation of beta-glucans.

We previously discovered that *V. breoganii* repeatedly acquired and significantly expanded the alginate degradation pathway (17). Here, we extended our analysis to include additional strains across diverse *Vibrio* populations and assessed all CAZyme motifs to identify additional evidence of metabolic specialization. The expansion of the alginate degradation pathway was reaffirmed, in that every *V. breoganii* strain contained between 3 and 5 polysaccharide lyase 6 (PL6) domains, implicated in initiating extracellular polymer cleavage, whereas no other strain representative of other *Vibrio* populations encoded more than two of these alginate-specific lyases. Similarly, every *V. breoganii* strain contained more PL7 domains (between 8 and 12), which are important for cleaving mannuronate and guluronate linkages in alginate, than any other assessed *Vibrio* genome (between 0 and 7), further supporting an expanded capacity for extracellular degradation. The same was true when considering the oligoalginate lyase domains PL15 and PL17, responsible for intracellular degradation of alginate oligosaccharides into monomers, where *V. breoganii* had more of these alginate-related domains on average than did any other population.

We also found *V. breoganii* to be enriched in additional glycoside hydrolases (GHs), including a variety with activity relevant to macroalgal carbohydrates (28). These include GH36 ($P = 4.6e{-}5$), which have been implicated as galactosidases involved in breaking down carrageenans and carbohydrates found in red macroalgae, as well as others with wide-ranging substrate specificity, like GH4 ($P = 0.12$) and GH43 ($P = 1.1e{-}8$), which are often enriched in cell wall-degrading organisms (29). Additionally, some *V. breoganii* isolates encoded GH10 domains ($P = 0.03$) with xylan substrates often found in seaweeds (30), while every representative from this clade contained auxiliary activity 2 (AA2) motifs ($P = 0.01$), which can confer lignin peroxidase functions that can facilitate the breakdown of cell wall components (31).

FIG 1 Substrate utilization and CAZyme content of diverse *Vibrio* populations. (A) Differential growth of representative *Vibrio* populations on diverse carbohydrates. Each square of the heatmap reflects a growth score. Differences between initial and maximum OD_{600} values for each replicate were scored for growth according to threshold criteria, with average scores shown ($<$0.05, 0; 0.05 to 0.15, 1; 0.15 to 0.25, 2; $>$0.25, 3). Rows are ordered according to the phylogenetic species tree of the genomes based on concatenated ribosomal genes. Columns are arranged using hierarchical clustering, such that carbohydrate utilization scores with similar phylogenetic distribution are placed closer together. Only carbohydrates that displayed differential growth among strains are included here. The growth scores for all carbohydrates are shown in Fig. S1. (B) Hierarchical cluster analysis of all CAZymes in representative *Vibrio* genomes reveals the mosaic presence and absence of GH families as well as cohesive CAZyme repertoires indicating similar glycan catabolism and scavenging strategies within populations. All results are consolidated in a matrix, where rows represent species and columns represent CAZymes. Each cell depicts the absolute abundance of a CAZyme family in a strain. The CAZyme abundance matrix is presented as a heatmap, where rows are ordered according to the phylogenetic species tree of the genomes based on concatenated ribosomal genes. Columns are arranged using hierarchical clustering, such that CAZymes with similar phylogenetic distributions are placed closer together. Thus, absent or present blocks indicate loss or gain of a set of related genes in related species, respectively, e.g., the loss of chitin metabolism (GH18 and GH19) or the acquisition of laminarinases (i.e., GH16) in all *Vibrio breoganii* strains. The CAZyme contents shown here reflect differences between *V. breoganii* and other populations. Comprehensive CAZyme content is provided in Fig. S2. (C) Differential growth of representative *Vibrio* populations on amino acids. Each square of the heatmap reflects a growth score. Differences between initial and maximum OD_{600} values for each replicate were scored for growth according to threshold criteria, with average scores shown ($<$0.05, score 0; 0.05 to 0.15, score 1; 0.15 to 0.25, score 2; $>$0.25, score 3). Rows are ordered according to the phylogenetic species tree of the genomes based on concatenated ribosomal genes. Columns are arranged using hierarchical clustering, such that amino acid utilization scores with similar phylogenetic distribution are placed closer together. The amino acid substrates shown here reflect differences between *V. breoganii* and other populations. All amino acid substrate results are shown in Fig. S3.

Further reinforcing their distinct enzymatic repertoire, *V. breoganii* isolates encode more hydrolases implicated in beta-glucan (GH88, $P = 2.3e{-}13$) and laminarin degradation (GH3, $P = 5.0e{-}15$; GH16, $P = 1.1e{-}9$) than any other population. This abundance of laminarin-related CAZymes is consistent with *V. breoganii* growth on laminarin and beta-glucans in substrate utilization assays, and these CAZymes likely provide a mechanism of action for the breakdown of complex glucose-based polysaccharides (32). Furthermore, *V. breoganii* isolates are enriched in glycosyltransferase 4s (GT4s) ($P = 5.6e{-}4$), an enzyme family with activity on diverse substrates employed in the biosynthesis of oligosaccharides, polysaccharides, and glycoconjugates (33). This abundance in GT4 may reflect an increased capacity or specialization for utilizing distinct carbohydrates for anabolism.

***V. breoganii* cannot degrade chitin or glycogen.** While *V. breoganii* isolates displayed an expanded capacity to degrade macroalgal carbohydrates, substrate utilization growth assays reveal a diminished capacity to grow on a variety of substrates metabolized by the majority of *Vibrionaceae*. In particular, *V. breoganii* isolates were unable to utilize the animal-derived carbohydrates chitin and glycogen.

While most vibrios are widely recognized for their intimate associations with chitin (34–36), *V. breoganii* isolates are unable to metabolize this abundant marine polysaccharide. Although they have retained the ability to grow on the chitin monomer *N*-acetyl-D-glucosamine (Fig. 1A), *V. breoganii* isolates are unable to grow on chitin polymers in liquid culture or degrade them in plate-based chitinase assays (Table S2). Despite the presence of chitin metabolism pathways in more basal *Vibrio* populations, every *V. breoganii* strain lacked any GH18, GH19, GH116, or GH129 domain, suggesting a specific loss from this population. While they contain moderate numbers of GH23 domains, to which have been attributed chitinase activities, these enzymes also have annotated activities on lysozyme and peptidoglycan, so these likely reflect general cellular biosynthesis demands.

Carbohydrate binding motifs (CBM) and additional accessory CAZymes also reflect a shift away from chitin utilization. We found that *V. breoganii* lacked any CBM5, CBM14, or CBM73 motif, each implicated in binding chitin, which were observed in other *Vibrio* populations. Additional carbohydrate-modifying enzymes working in conjunction with CAZymes also indicate a shift away from chitin utilization. One enzyme with accessory activities implicated in chitin degradation (AA10) was absent in *V. breoganii* isolates yet widely distributed across most *Vibrio* populations. Similarly, the carbohydrate esterase 4 (CE4), responsible for the deacetylation of chitin sugars, was absent among *V. breoganii*, as was the acetylesterase CE16, which has activities on a variety of substrates, yet was widely distributed among other *Vibrio* populations.

In addition to a loss of chitin degradation ability, *V. breoganii* isolates were also incapable of using glycogen as a sole carbon source, unlike the majority of other vibrios. Glycogen is the primary energy storage polysaccharide in tissues of bivalves and other marine invertebrates and is composed of linear chains of glucose linked with alpha-1,4-glycosidic bonds with periodic branch points created by alpha-1,6-glycosidic linkages (37). Despite thriving on glucose monomers, *V. breoganii* isolates failed to degrade glycogen polysaccharides. While *V. breoganii* does encode a variety of GH13 domains which can include glycogen hydrolases, this family of hydrolases is multifunctional and not specific for glycogen degradation (38). Interestingly, the more basal *Enterovibrio* populations were also incapable of utilizing glycogen, suggesting that this metabolic capacity might have influenced evolutionary differentiation among the vibrios.

Similarly, most *V. breoganii* isolates assessed lacked a variety of additional CAZymes otherwise widely distributed throughout most *Vibrio* populations. *V. breoganii* isolates lacked any AA6s, which have been characterized as 1,4-benzoquinone reductases and are involved in the biodegradation of aromatic compounds, and they did not encode any CBM69 domains, which have been implicated in binding starch substrates and are also broadly distributed among populations. Most *V. breoganii* isolates also lacked

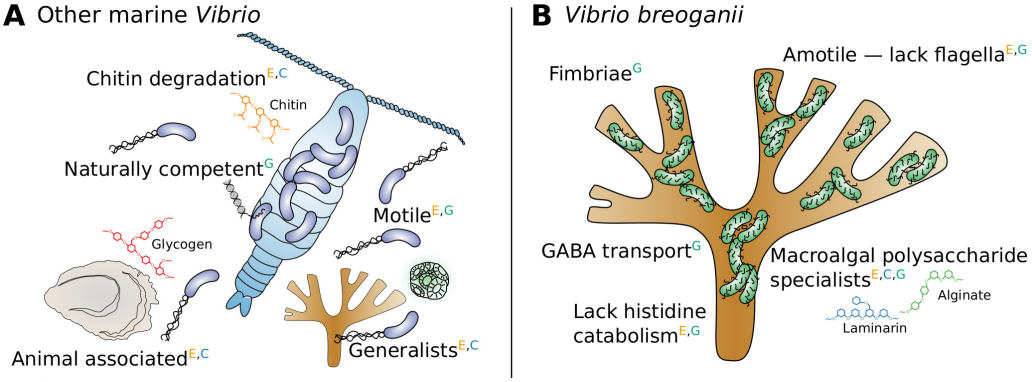

FIG 2 Features differentiating *V. breoganii* from other marine *Vibrio* species. Ecological associations and physiological traits distinguish *V. breoganii* from most other marine *Vibrio* species. Here, we illustrate properties common among other marine vibrios but absent in *V. breoganii* (A), and traits specific to *V. breoganii* (B). Superscript letters denote the type of supporting evidence for each feature: E, experimental evidence; C, CAZyme database analysis; G, gene cluster analysis.

GT25, which has been implicated as glucosyl- or galactosyltransferases, suggesting a metabolic shift away from substrates commonly utilized by other *Vibrio* populations.

While lacking the CAZymes required to break down chitin and glycogen, *V. breoganii* continue to encode binding motifs recognizing these abundant animal-associated marine polysaccharides. Two CBM50 domains with chitin-binding attributes remain in the *V. breoganii* genomes at an abundance similar to those all other vibrios assessed. Similarly, all *V. breoganii* genomes encoded CBM48 motifs with glycogen-binding functions, despite their inability to metabolize this carbohydrate. This glycogen-binding motif was less widely distributed and limited to the *V. breoganii*, *Vibrio logei*, *Vibrio fischeri*, and *Vibrio* sp. strain F-10 populations. The presence of these respective domains might indicate that although *V. breoganii* isolates have lost the ability to utilize these abundant animal-associated resources, they have maintained the ability to sense and respond to these important environmental cues.

***V. breoganii* isolates are poor scavengers of amino acids.** In addition to a diminished capacity to degrade and metabolize animal-derived carbohydrates, *V. breoganii* isolates were also inefficient at utilizing a variety of amino acids (Fig. 1C and S3). While every *Vibrio* strain grew to high density on media containing diverse amino acids, most *V. breoganii* strains were comparatively poor at metabolizing many specific amino acids (L-serine, L-tyrosine, L-arginine, L-glycine, L-alanine, L-glutamine, and L-proline) relative to most *Vibrio* populations. Furthermore, no *V. breoganii* strain tested was capable of growing on L-histidine as a sole carbon source. Indeed, genomic analysis revealed *V. breoganii* isolates have lost the ancestral enzymatic machinery required for histidine catabolism (IRP005923, IRP005920, IRP023636, IRP005921, and IRP023637), which is found almost universally among the other *Vibrio* genomes assessed (Fig. S4).

The diminished capacity of *V. breoganii* isolates to scavenge amino acids was surprising given how critical nitrogen acquisition can be under nitrogen-limited conditions, but it may be ecologically consistent with specialization for macroalgal carbohydrates. As the protein content in macroalgae biomass is relatively small (39), the impaired ability to scavenge amino acids might reflect their relatively scarce abundance on macroalgal surfaces, which would lead to relaxed selection on scavenging mechanisms (10).

Surprisingly, gene cluster analysis revealed that all *V. breoganii* isolates encode a transporter for gamma-aminobutyric acid (GABA), a nonprotein amino acid, as well as enzymes for its degradation (Fig. 2 and Table S3). The transporter is absent from all other isolates, and the enzyme was present in 50% of *Vibrio kanaloae* genomes and 33% of *Vibrio* sp. strain F12 genomes, yet it was absent from all other populations. GABA is found in the tissue of many macroalgae (40), so the enrichment of these genes could be consistent with macroalgal associations and reflect alternative scavenging mechanisms.

Ecophysiological adaptations distinguish *V. breoganii*. To identify additional distinguishing characteristics of *V. breoganii*, we expanded our genomic analysis beyond metabolism and assessed physiological traits relevant for habitat associations, summarized in Fig. 2.

Given the well-documented importance of chitin in *Vibrio* habitat associations and regulatory cues, along with the surprising loss of chitin utilization within *V. breoganii*, we investigated additional chitin-related functions within the representative genomes. We discovered that nearly all *V. breoganii* representatives have lost much of the genetic machinery associated with natural competence, transformation efficiency, and motility (Table S3). All *V. breoganii* isolates investigated lack the natural competence-associated genes *comP*, *pilB*, *pilM*, *pilN*, and *pilP*, yet retain some competence components (*comEA*, *comEC*, and *comF*) and pilin genes (*pilC*, *pilD*, *pilG*, *pilO*, *pilQ*, *pilT*, and *pilW*).

Flagellar proteins have also been lost, and agar stab assays confirmed that all *V. breoganii* strains were nonmotile, while nearly all representatives from other populations demonstrated motility (Table S4). This loss of motility might be indicative of trade-offs associated with different metabolic and dispersal strategies (15). Extensive environmental sampling has demonstrated that *V. breoganii* isolates are rarely observed in free-living fractions within the water column, instead favoring a particle-attached lifestyle (17, 19, 41). Metabolic specialization enabling higher growth rates on high-molecular-weight alginate and the capacity to degrade an expanded repertoire of laminarins and beta-glucans likely facilitate this stable association with insoluble macroalgal detritus (17, 19). Accordingly, this shift in nutritional resources enabling a predominantly surface-attached lifestyle might explain the loss of motility, as *V. breoganii* isolates could rely on passive dispersal via particles and ingestion by detritivore or scavenger hosts.

Conclusions. While most vibrios display little host preference and a dominance of generalist populations, we demonstrate how *V. breoganii* strains have become specialized for a macroalga-associated lifestyle, as indicated by a distinct substrate utilization profile, a unique CAZyme repertoire, and ecophysiological adaptations. We also describe a shift away from commonly utilized animal-derived carbohydrates and amino acids, suggesting a transition away from potentially saprophytic associations toward a transient commensal relationship with diverse marine invertebrate hosts.

It has been well documented that most bacteria in marine invertebrate guts are often only transiently associated with their hosts (42–45). However, some vibrios appear to be stable commensal residents that contribute directly to host physiology (46, 47). For example, *V. halioticoli* strains have a strong association with abalone gut, where they facilitate host nutrient absorption by degrading brown algae (21–24). Although *V. breoganii* is also highly specialized for macroalgal carbohydrates and falls within the *Halioticoli* clade, previous observations of *V. breoganii* within diverse hosts likely reflect merely transient commensalisms.

Unlike *V. halioticoli*, which has rarely been characterized outside its abalone hosts or aquaculture facilities (23, 48), *V. breoganii* has been found in the guts of diverse invertebrate hosts (18–20). Furthermore, extensive environmental sampling in Plum Island Estuary (MA) revealed distinct seasonal and habitat associations between *V. breoganii* and particles within the water column where they appear to be strictly associated with algae and algal detritus (17, 19, 41). Accordingly, invertebrate hosts of *V. breoganii* likely reflect a transient by-product of algal or detritivore feeding strategies rather than specific host associations.

Here, we demonstrate how *V. breoganii* isolates have become metabolically specialized for degrading macroalgal carbohydrates, facilitating surface-attached lifestyles on insoluble algal particles. These macroalgal associations likely explain their presence within diverse marine invertebrates following the ingestion of algal detritus. We also show *V. breoganii* has lost the ability to degrade animal-derived carbohydrates and impaired amino acid scavenging, likely contributing to more benign commensal relationships with diverse hosts, minimizing potentially disruptive impacts of colonization

on host physiology. In turn, these diverse hosts may provide stable habitats and useful waste products and facilitate dispersal. Accordingly, the metabolic specialization of *V. breoganii* to macroalgal carbohydrates appears to drive transient commensal presence within diverse marine invertebrates, illustrating selective pressures and fitness tradeoffs driving *Vibrio* evolution in natural environments.

MATERIALS AND METHODS

Isolates and culture conditions. The strains tested here originated from previous studies on the ecological population structure of *Vibrionaceae*. Briefly, isolates were obtained either from size-fractionated water samples, handpicked algal detritus particles and zooplankton, or different body parts of marine invertebrates by plating samples on *Vibrio*-selective marine thiosulfate-citrate-bile salts-sucrose (TCBS) medium (BD Difco TCBS with 1% NaCl added) (19, 34, 49). Individual colonies were picked and purified by restreaking three times, alternating between 1% tryptic soy broth (TSB) medium (BD Bacto with 2% NaCl added) and marine TCBS medium. Cultured isolates were grown in marine broth 2216 (MB2216, catalog no. 279110; Difco) at room temperature with shaking, unless otherwise specified. Substrate utilization assays were performed in minimal medium adapted from Tibbles-Rawlings medium (TRMM) (50, 51). The strains included in this study had been previously sequenced to enable genomic analyses, and all sequenced *V. breoganii* isolates were alga associated.

Substrate utilization growth assays. Frozen glycerol stocks of *Vibrio* strains were inoculated into 1.5 ml MB2216 in deep-well blocks and grown with shaking for 48 h. Strains were then inoculated with a pin replicator into optically clear 96-well trays containing 200 μl substrate minimal medium solution (1g/liter in TRMM) at an approximately 1:1,000 (vol/vol) final dilution. Substrate minimal medium solutions were made by solubilizing carbohydrates or amino acids in Milli-Q water, filter sterilizing, and adding sterilized Tibbles-Rawlings components. Some substrates were relatively insoluble in water and required additional filters for sterilization, contributing to a lower effective substrate concentration or treatment to improve solubility. A complete list of assessed substrates and their treatment is provided in Table S1 in the supplemental data. Strains were grown in triplicate for each carbohydrate, with plate layouts rearranged in each replicate to control for potential evaporative effects. Culture trays were incubated at room temperature without shaking, and the optical density at 600 nm (OD_{600}) was monitored twice daily for 12 days. Differences between the initial and maximum OD values for each replicate were scored for growth according to threshold criteria ($<$0.05, score 0; from 0.05 to 0.15, score 1; from 0.15 to 0.25, score 2; $>$0.25, score 3), and average scores are shown. When scoring growth or no growth among triplicates, any inconsistencies were resolved by removing the anomalous replicate.

CAZyme annotation and analysis. Hidden Markov models (HMMs) of carbohydrate-active enzyme (CAZyme) domains were obtained from the dbCAN database (25). These were searched against all open reading frames (ORFs) from each genome using hmmscan (https://www.ebi.ac.uk/Tools/hmmer/search/hmmscan). The best-scoring domains were then filtered by E value ($<10^{-23}$) and alignment coverage ($>$0.8), both of which are more stringent cutoffs than those recommended by dbCAN (http://csbl.bmb.uga.edu/dbCAN/), in an effort to minimize false positives. When multiple domains were observed within an ORF, only the best scores for each category of enzyme are reported. Statistical testing of population-specific CAZyme enrichment was assessed using a predicted binomial distribution model. Annotated CAZyme substrate activities and families are described in the Carbohydrate-Active enZymes database (http://www.cazy.org/) (26).

Chitinase activity. A plate-based chitin clearing assay was used to assess the ability of representative *Vibrio* strains to degrade chitin polymers. Dense cultures grown in MB2216 were spotted (10 μl) onto MB2216 agar plates with a top layer supplemented with 2.5% colloidal chitin stained with Remazol brilliant blue R (52). After 5 days of incubation at room temperature, plates were scored for the presence or absence of visible zones of clearing surrounding or below lawns, indicating the degradation of insoluble chitin polymers.

Gene cluster analysis. All ORFs were clustered at 50% amino acid identity using MMSeqs2. For each genome, the count of each cluster was tabulated, and these counts were compared between *V. breoganii* genomes and genomes from other populations. Gene clusters that appeared to be either almost exclusive to or almost entirely absent from *V. breoganii* were considered for further analysis. The ORFs for each genome were annotated with InterProScan version 5.17-56.0 using the iprlookup, goterms, and pathways options. InterProScan is a program from EMBL-EBI that uses the InterPro database for annotations. The InterPro database used contains 15 databases, with TMHMM for predicted transmembrane proteins and SignalP for predicted signal peptide cleavage sites, as well as the 13 default databases which listed at https://github.com/ebi-pf-team/interproscan/wiki/HowToRun#included-analyses.

Motility assay. A standard agar stab assay was used to assess the potential for motility among isolates. Motility test agar medium was prepared with MB2216, Bacto agar (catalog no. 214010, 0.25% [wt/vol]; BD), and 2,3,5-triphenyltetrazolium chloride solution (catalog no. 17779-10X10ML-F; Sigma-Aldrich) and autoclaved. Medium was aliquoted in autoclaved glass tubes (catalog no. 47729-576; VWR) with plastic closures (catalog no. EW-04500-01, 5 ml of medium per tube; Cole-Parmer) and allowed to cool to room temperature. Using an inoculating needle (catalog no. TL0000; Thomas), a stab inoculation was made from a single colony for each strain into a medium-filled glass tube. The tubes were incubated at room temperature for 7 days before cultures were analyzed. Evidence of motility was assessed visually. If growth occurred only along the stab line, strains were considered nonmotile under these conditions; otherwise, strains were deemed motile. All results were confirmed via microscopy with liquid cultures in MB2216.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00020-18.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 2, PDF file, 2.5 MB.

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