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Nontoxigenic *Vibrio cholerae* Non-O1/O139 Isolate from a Case of Human Gastroenteritis in the U.S. Gulf Coast

Nur A. Hasan,[a,b,c] Talayeh Rezayat,[d] Peter J. Blatz,[d] Seon Young Choi,[a,c] Kimberly J. Griffitt,[e*] Shah M. Rashed,[a] Anwar Huq,[a,f] Nicholas G. Conger,[d*] Rita R. Colwell,[a,b,c,f,g] D. Jay Grimes[e]

Maryland Pathogen Research Institute, University of Maryland, College Park, Maryland, USA[a]; University of Maryland Institute for Advanced Computer Studies, University of Maryland, College Park, Maryland, USA[b]; CosmosID, Inc., College Park, Maryland, USA[c]; Keesler Air Force Base, Biloxi, Mississippi, USA[d]; Gulf Coast Research Laboratory, The University of Southern Mississippi, Ocean Springs, Mississippi, USA[e]; Institute for Applied Environmental Health, University of Maryland, College Park, Maryland, USA[f]; Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland, USA[g]

An occurrence of *Vibrio cholerae* non-O1/O139 gastroenteritis in the U.S. Gulf Coast is reported here. Genomic analysis revealed that the isolate lacked known virulence factors associated with the clinical outcome of a *V. cholerae* infection but did contain putative genomic islands and other accessory virulence factors. Many of these factors are widespread among environmental strains of *V. cholerae*, suggesting that there might be additional virulence factors in non-O1/O139 *V. cholerae* yet to be determined. Phylogenetic analysis revealed that the isolate belonged to a phyletic lineage of environmental *V. cholerae* isolates associated with sporadic cases of gastroenteritis in the Western Hemisphere, suggesting a need to monitor non-O1/O139 *V. cholerae* in the interest of public health.

Vibrio spp. are natural inhabitants of marine and estuarine environments, and they cause human infections that most commonly present as gastroenteritis or wound infections and/or septicemia (1, 2). The infection is generally acquired through the consumption of contaminated food or water or by the direct invasion of wounds (3). *Vibrio cholerae* is the causative agent of cholera, the severe watery diarrheal disease that has the potential to become pandemic. Based on variable somatic (outer membrane) O-antigen composition, more than 200 serogroups of *V. cholerae* have been recognized to date (4). Of them, toxigenic strains of *V. cholerae* typically belong to serogroups O1 or O139 and are rare in the United States (5). All other serogroups, notably the non-O1/O139 serogroup, are frequently isolated from environmental sources and have been associated with sporadic cases of gastroenteritis or extraintestinal infections. Although none has caused a pandemic yet (6), these serogroups have reportedly caused epidemics of cholera through the acquisition of genes carried on mobile elements (e.g., O antigens, vibrio pathogenicity island 1 [VPI-1], VPI-2, cholera toxin phage [CTXϕ], and heat-stable enterotoxin [NAG-ST]). Human illnesses caused by environmental *V. cholerae* non-O1/O139 serogroups are reported regularly (7). Over the past few decades, environmental studies have shown that nontoxigenic *V. cholerae* strains inhabit estuarine waters along the Atlantic and Gulf coasts (8–14). They are most commonly isolated from environmental sources, such as brackish water, oysters, and sewage, and have been reported in many countries, such as Bangladesh, Brazil, Guam, Great Britain, and the United States, even when cholera outbreaks had not been recorded for decades in those countries (2).

In the United States, the consumption of raw or undercooked seafood is the leading cause of non-O1/O139 *V. cholerae*-associated gastroenteritis, with isolated cases reported (8, 15). Outbreaks of intestinal illness caused by non-O1/O139 *V. cholerae* have been reported more commonly than would be expected (16–18). Since 2000, an average of 44 cases has been reported each year (http://www.cdc.gov/nationalsurveillance/cholera-vibrio-surveillance.html). The majority of infections to date have originated from the Gulf of Mexico, where the surface water is warm, often reaching 34°C in late summer (19), thereby providing optimal conditions for the growth of *Vibrio* spp. (8, 15, 20–24).

In this report, we describe the isolation of a nontoxigenic *V. cholerae* non-O1/O139 strain from a 30-year-old male with a history of hypertension who presented with a complaint of >30 episodes of watery nonbloody stools in a 48-h period. Using whole-genome sequencing technology, we sequenced the isolate to determine the presence of virulence factors and phylogenetic relatedness.

CASE REPORT

A 30-year-old male with a history of hypertension presented with a complaint of >30 episodes of watery nonbloody stools in a 48-h period. He had visited Lake Charles, LA, 2 days prior to the onset of symptoms and had eaten a seafood platter consisting of battered and fried catfish, shrimp, stuffed crab, and crawfish étouffée. The physical examination was unremarkable. The laboratory evaluation revealed a normal white blood cell count and normal renal function without electrolyte abnormalities. A stool culture was

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Address correspondence to D. Jay Grimes, jay.grimes@usm.edu.
* Present address: Kimberly J. Griffitt, Keesler Air Force Base, Biloxi, Mississippi, USA; Nicholas G. Conger, Wright Patterson Air Force Base, Ohio, USA.

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sent to the Keesler Air Force Base (AFB) laboratory, and only a *V. cholerae* isolate was obtained on blood and thiosulfate-citrate-bile salts-sucrose (TCBS) agars; it was identified as *V. cholerae* by the Keesler AFB laboratory using the Vitek 2 (bioMérieux, France). The patient was treated successfully with a one-time 300-mg dose of doxycycline, with complete resolution of symptoms.

MATERIALS AND METHODS

The Keesler AFB submitted a TCBS agar culture to the Mississippi Public Health Laboratory (MPHL) for confirmatory culture-based identification and to the University of Southern Mississippi Gulf Coast Research Laboratory (GCRL) for molecular identification. The isolate was also tested for motility, protease activity, hemolytic activity, and biofilm formation. The motility test was performed in motility indole urea (MIU), a semisolid medium in which a pure colony was inoculated through a stab with a sterile straight wire followed by incubation at 37°C overnight. Motility was shown by the spreading turbidity from the stab line or by turbidity throughout the medium. Protease activity was tested using a marine agar-skim milk agar double-layer plate onto which a layer of marine agar was overlaid on skim milk agar (25). The hemolytic activity was tested on commercially available blood agar (tryptic soy agar [TSA] plus 5% sheep blood; Hardy Diagnostic). Biofilm production was determined by a semiquantitative adherence assay on 96-well microtiter plates, as described previously (26). *V. cholerae* El Tor reference strain N16961 (ATCC 39315) was used as a positive control for the biofilm formation assay. Biofilm formation was quantified by the addition of 400 μl of 95% ethanol to each crystal violet-stained well of the microtiter plate followed by the determination of absorbance at 570 nm using a spectrophotometer (GENESYS 10S Bio UV/Vid spectrophotometer; Thermo Scientific).

Genomic DNA from the isolate was sent to the University of Maryland, where it was subjected to next-generation whole-genome Illumina and hybrid Ilumina/454 pyrosequencing; the libraries were constructed with target insert sizes of 3 kb and 100-bp paired-end reads. Hybrid and Illumina sequences were assembled using the Celera and Velvet assemblers, as described elsewhere (27). The combination of next-generation whole-genome Illumina and 454 pyrosequencing yielded good-quality draft assemblies (311 contigs) of the *V. cholerae* BJG-01 genome (PRJNA64001). The RAST subsystem annotation identified 3,467 predicted coding sequences and 97 noncoding RNAs. Approximately 23% of the predicted coding sequences were annotated as hypothetical proteins, including proteins conserved in other bacteria.

RESULTS AND DISCUSSION

The isolate was identified as non-O1/O139 *V. cholerae* by the Keesler AFB lab and the MPHL and was assigned the isolate designation BJG-01. Multiple yellow sucrose-fermenting colonies were subjected to confirmatory PCR at the GCRL with the *V. cholerae* transmembrane regulatory protein-producing gene (*toxR*) and the outer membrane protein W gene (*ompW*) (28). Subsequent molecular tests showed that the strain lacked the O biosynthetic genes *wbe* and *wbf* specific for serogroups O1 and O139, respectively, and the cholera toxin gene *ctxA* and toxin-coregulated pili *tcpA* gene, which confirmed that it was a nontoxigenic *V. cholerae* non-O1/O139 strain. The isolate BJG-01 exhibited motility on MIU medium, a typical trait of *V. cholerae*, and demonstrated protease activity, as evident by a zone of clear lysis around the bacterial growth on a double-layer plate containing marine agar overlay on skim milk agar. The isolate also showed beta hemolysis on blood agar (TSA plus 5% sheep blood; Hardy Diagnostic) and showed biofilm formation, a well-known survival strategy for *V. cholerae* under adverse conditions. The biofilm formation assay revealed that the isolate BJG-01 was strongly positive for biofilm formation (optical density at 570 nm [OD_{570}], 1.07 ± 0.13, 1.9× higher than that of reference strain N16961 [ATCC 39315] [0.57 ± 0.17]).

Although *V. cholerae* non-O1/O139 strains typically lack the two major virulence factors, cholera toxin (CTX) and toxin-coregulated pili (TCP) (6), they often contain a battery of genes coding for extracellular products that collectively play an important role in pathogenesis. The *V. cholerae* isolate BJG-01 contained several putative virulence factors associated with hemolysis and proteolysis. The genome of *V. cholerae* BJG-01 encodes a predicted heat-labile El Tor hemolysin (polyethylene glycol [PEG] 3222) known to induce fluid accumulation in the infant mouse (29); a predicted heat-stable hemolysin (PEG 2480) similar to the thermostable direct hemolysin of *Vibrio parahaemolyticus* (30); cytolysin and hemolysin; a pore-forming toxin (PEG 2791) associated with lethality, developmental delay, and intestinal vacuolation in *Caenorhabditis elegans* (31); and hemagglutinin/protease (PEG 2851). The genome of *V. cholerae* BJG-01 also encodes other putative virulence-related factors found in toxigenic and nontoxigenic *V. cholerae*, including *toxR-toxS* virulence regulators (PEG 1593 and 1594), the integron integrase IntI4 (PEG 841), RTX toxin (PEG 2870), multiple lipases, the outer membrane protein OmpU, and the type VI secretion system (T6SS). The widespread occurrence of many of these putative virulence factors in toxigenic and nontoxigenic *V. cholerae* isolates suggests that all *V. cholerae* strains have the potential to be virulent, but it is likely that these factors play an important but undetermined role in the ecology of *V. cholerae*. Hemolysin is an accessory virulence factor in *V. cholerae* believed to contribute to disease in humans; recently, hemolysin reportedly caused infection leading to death and developmental delay in a study using *Caenorhabditis elegans* (31). However, the presence of hemolysin genes in the genomes of pathogenic and nonpathogenic, as well as clinical and environmental, *Vibrio* spp. suggests that these genes may play a role in environmental fitness. Smith and Oliver (32) suggested that these hemolysins may have a function in the cold shock response. T6SS codes for a newly described mechanism for protein transport across the cell envelope of Gram-negative bacteria, and although first identified in *V. cholerae* V52 (33), T6SS gene clusters have been reported in *Pseudomonas aeruginosa*, *Salmonella enterica*, *Yersinia pestis*, and *Escherichia coli* O157:H7 (33). T6SS gene clusters are required for virulence and/or survival of a bacterium in the presence of a eukaryotic host (34–36) or other bacterium. Weber et al. (37) reported that T6SS in *Vibrio anguillarum* regulates the stress response, suggesting an ecological fitness function in addition to pathological consequences for the host. Thus, hemolysins and T6SS may serve a more basic purpose for *V. cholerae* in the environment, but they act as virulence factors when a human host is encountered.

Interestingly, we identified the osmoregulatory choline-glycine betaine locus, *betABIT*, in the genome of BJG-01; this trait was not previously reported in *V. cholerae* but has been described in both *V. parahaemolyticus* and *Vibrio vulnificus* (38, 39). Typically, the ectoine (*ectABC*) synthesis system is found in *V. cholerae*, and the glycine betaine system is found in *V. vulnificus*, whereas *V. parahaemolyticus* possesses both of these synthesis systems (38, 39). A BLAST search showed that the *betABIT* locus of *V. cholerae* shares a greater similarity with the *betABIT* locus of *Pseudomonas* spp. than with that of *V. parahaemolyticus*. The *betABIT* locus was interrogated among the available sequenced genomes of *V. cholerae* (>200) and was identified in three other *V. cholerae* strains,

GI Number	Insert Site	Chromosome	Size (bp)
VSP-1	VC0174-VC0186	1	14,038
VSP-2	VC0489-VC0517	1	26,866
VPI-1	VC0809-VC0848	1	48,875
VPI-2	VC1757-VC1810	1	57,573
SXT	VC0659	1	104,986
CTX	VC1451-VC1465	1	6,315
	VCA0569-VCA0570	2	6,318
TLC	VC1464~VC1479	1	10,612
GI-02	VC1582-VC1588	1	3,584
GI-03	VC1747-VC1753	1	4,866
GI-04	VC1819-VC1828	1	9,486
GI-10	VCA0877-VCA0885	2	4,901
GI-19	VCA0569-VCA0570	2	9,497
GI-47	VC0809-VC0848	1	15824, 51529(c)
GI-58	VCA0235-VCA0237	2	6,533
GI-61	VCA1067-VCA1064	2	6,421
GI-64	VC1560-VC1563	1	5,541
GI-67	VCA0735-VCA0734	2	37,871
GI-79	VCA0990-VCA0991	2	10,459
GI-85	VC1835-VC1854	1	15,489
GI-86	VC1234-VC1235	1	*5,574*
GI-87	VC1451-VC1479	1	*5,347*
GI-88	VC1328-VC1329	1	3,431
GI-89	VC2041-VC2042	1	*2,899*
GI-90	VCA0249-VCA0250	2	3,164

FIG 1 Reciprocal BLASTN analysis of major virulence factors and other genomic islands in *V. cholerae* BJG-01. Colors represent the relatedness of the virulence factors and the genomic islands in terms of their gene contents.

namely, *V. cholerae* bv. albensis VL426, *V. cholerae* RC385, and *V. cholerae* PS15.

Genomic islands, notably pathogenicity islands, contribute to the evolution and diversification of microbial life (40). They are clusters of genes with a common history distinct and divergent from the histories of the organisms in which they reside. Furthermore, genomic islands encode multiple complex and potentially advantageous traits frequently acquired by lateral transfer and may be major contributors to the evolution and diversification of *V. cholerae*. Using a comparative genomic approach, described previously by Chun et al. (41), we observed that *V. cholerae* BJG-01 does not contain the pathogenicity islands frequently found in toxigenic *V. cholerae* O1, namely, *Vibrio* seventh pandemic islands (VSP-I and VSP-II), *Vibrio* pathogenicity islands (VPI-1 and VPI-2), a cholera toxin phage (CTXϕ), an SXT element, and a toxin-linked cryptic plasmid (TLC) (Fig. 1). However, *V. cholerae* BJG-01 contains several factors necessary for the complete CTXϕ life cycle and for CTX production and translocation, including TolQRA, inner membrane proteins involved in CTXϕ attachment to the cell, XerCD tyrosine recombinases (which catalyze recombination between CTXϕ and the host genome), and LexA (involved in CTXϕ expression). In addition, the isolate contains 4 (GI-02 to GI-04 and GI-10) of the 10 genomic islands (GI-01 to GI-10) with various nucleotide similarities that are frequently found in toxigenic *V. cholerae* O1 isolates (Fig. 1 and Fig. 2). Further, *V. cholerae* BJG-01 encodes 12 other genomic islands, 6 (GI-47, GI-64, GI-86, GI-87, GI-88, and GI-89) in chromosome I (C-I) (Fig. 1) and 6 (GI-19, GI-58, GI-61, GI-67, GI-79, and GI-90) in chromosome II (C-II). The relative locations of these genomic islands with reference to the *V. cholerae* N16961 genome are depicted in Fig. 2. Our comparative genomic analysis also showed that *V. cholerae* BJG-01 contains GI-85, frequently found in most toxigenic and nontoxigenic *V. cholerae*, and encodes part of the Tol-Pal protein system, which are reported to be critical cell envelope components and affect cell morphology and virulence (42). Functional characteristics of the genomic islands are illustrated in Table S1 of the supplemental material.

Multilocus variable-number tandem-repeat (VNTR) analysis (MLVA), a discriminating tool used to trace human *V. cholerae* infections (43) and resolve distinct populations of clinical isolates from different geographic regions (44), showed that *V. cholerae* BJG-01 is genotype 12.12.5.10, genetically distinct (see Table S2 in the supplemental material) from *V. cholerae* non-O1/O139 isolates of clinical and environmental origin, including representative 2010 Haitian *V. cholerae* isolates (27).

The core oligosaccharide (OS) and O antigens are major regions of the *V. cholerae* lipopolysaccharide (LPS) synthesized by the *wav* and *wb** gene clusters, respectively. Analysis of the *wav* gene cluster (Fig. 3) revealed that the core OS of *V. cholerae* BJG-01 belongs to the type 3b core OS, identified previously in *V. cholerae* bv. albensis VL426 (41). However, the genetic organization of the O antigen appeared to be distinct, as the *wb** gene cluster of BJG-01 showed that the cassettes comprised several smaller gene sets specific to the *V. cholerae* BJG-01 O antigen (Fig. 3), such as those under GenBank accession numbers AFOU01000268.1 (three genes), AFOU01000125.1 (three genes), and AFOU01000130.1 (four genes) and gene sets identified in other *V. cholerae* strains, such as VCA_002417 of *V. cholerae* VL426, VchoM_03542 to VchoM_03544 of *V. cholerae* MO10, and *wbeW* of *V. cholerae* N16961. Similar extensive diversity in the

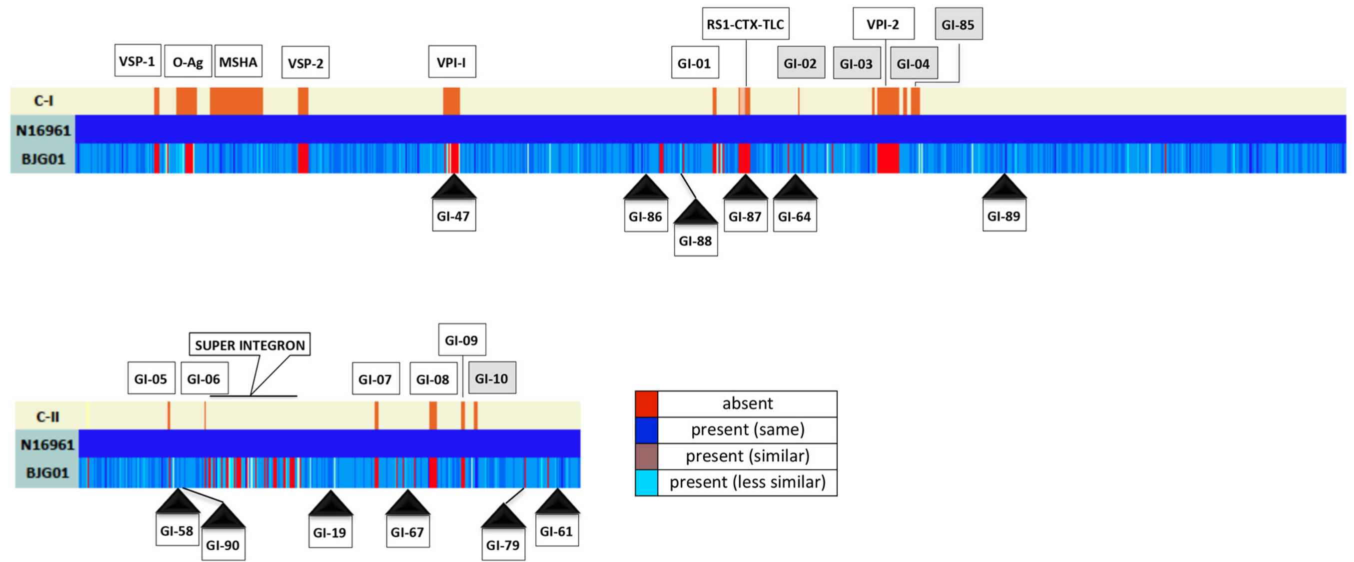

FIG 2 Chromosomal distribution of genomic islands in BJG-01 in reference to *V. cholerae* N16961. C-I, chromosome 1; C-II, chromosome 2. Rectangular boxes with solid black triangles represent the GIs and their relative positions (in reference to the N16961 genome) in the BJG-01 genome. The rectangular shaded boxes indicate the GIs that were present in N16961 and BJG-01. Colors represent the relatedness of the GIs in terms of gene content.

O-antigen gene clusters (*wb**) was reported previously by Chun et al. (41) and Nesper et al. (45) and indicates that the O-antigen gene clusters are horizontally transferred, since they comprise several smaller gene sets of origin.

The phylogeny of *V. cholerae* BJG-01 was inferred by constructing a genome-relatedness neighbor-joining tree, which employed homologous alignment of 1,163 highly conserved orthologous protein-coding genes (~1,182,674 bp) of 32 *V. cholerae* genomes. The deduced phylogenic tree (Fig. 4) shows that *V. cholerae* BJG-01occupies a non-O1/O139 phyletic lineage and forms a monophyletic clade with three *V. cholerae* strains (RC385, CT 5369–93, and V51) isolated from the Americas. *V. cholerae* RC385, an environmental strain belonging to serogroup O135, was isolated from the Chesapeake Bay. *V. cholerae* CT 5369–93 serotype O1 was isolated from Brazil in 1993, and V51 serotype O141 is a 1987 clinical isolate from the United States. Our conclusion is that the phyletic lineage of these *V. cholerae* non-O1/O139 strains represents a lineage resident in the Western Hemisphere.

In conclusion, *V. Cholerae* non-O1/O139 isolates can be pathogenic for humans and are autochthonous to aquatic environments throughout the world. The mechanisms by which non-O1/O139 *V. cholerae* functions successfully in two very distinct habitats (i.e., as a human pathogen but native to the aquatic environment) have yet to be fully described. Toxigenic epidemic strains of *V. cholerae* O1 that carry major virulence factors conferring pathogenicity have been described, but in nontoxigenic *V. cholerae* strains, the underlying mechanism(s) causing diarrhea in humans is not clearly understood. Here, we investigated a non-

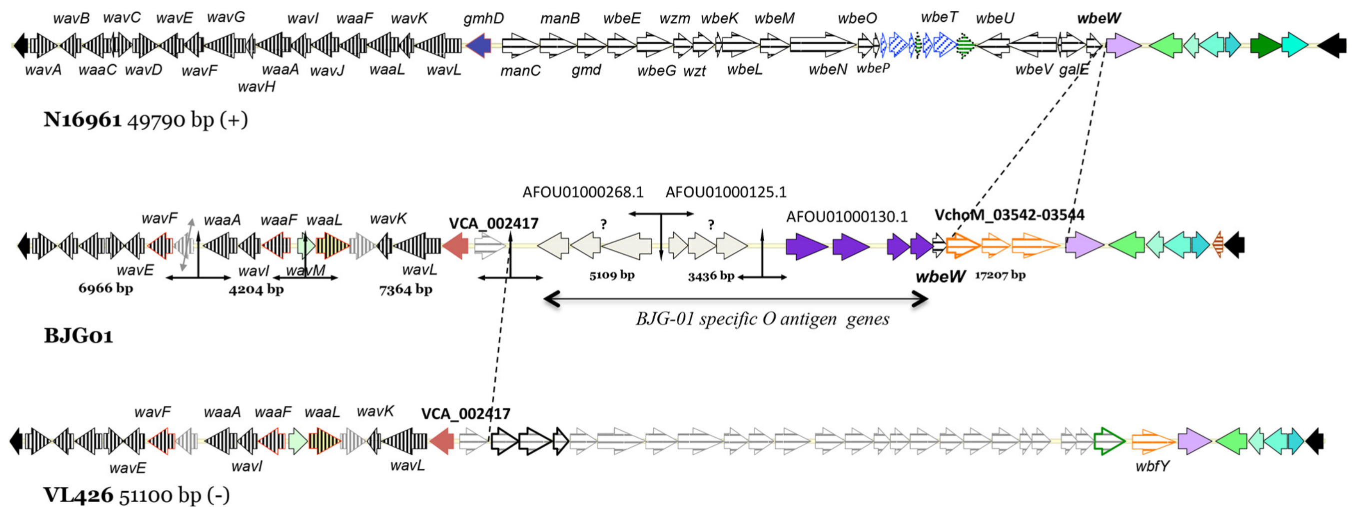

FIG 3 Genetic organization of *wav* and *wb** gene clusters in *V. cholerae* BJG-01 compared to that of *V. cholerae* bv. EIT or N16961 and *V. cholerae* bv. albensis VL426. Homologous aspects are indicated by the same colors. BJG-01 has a variant of the type 3b core oligosaccharide similar to that of VL426, whereas the O-antigen (*wb**) gene cluster appeared to be very distinct, with a set of BJG-01-specific gene sets.

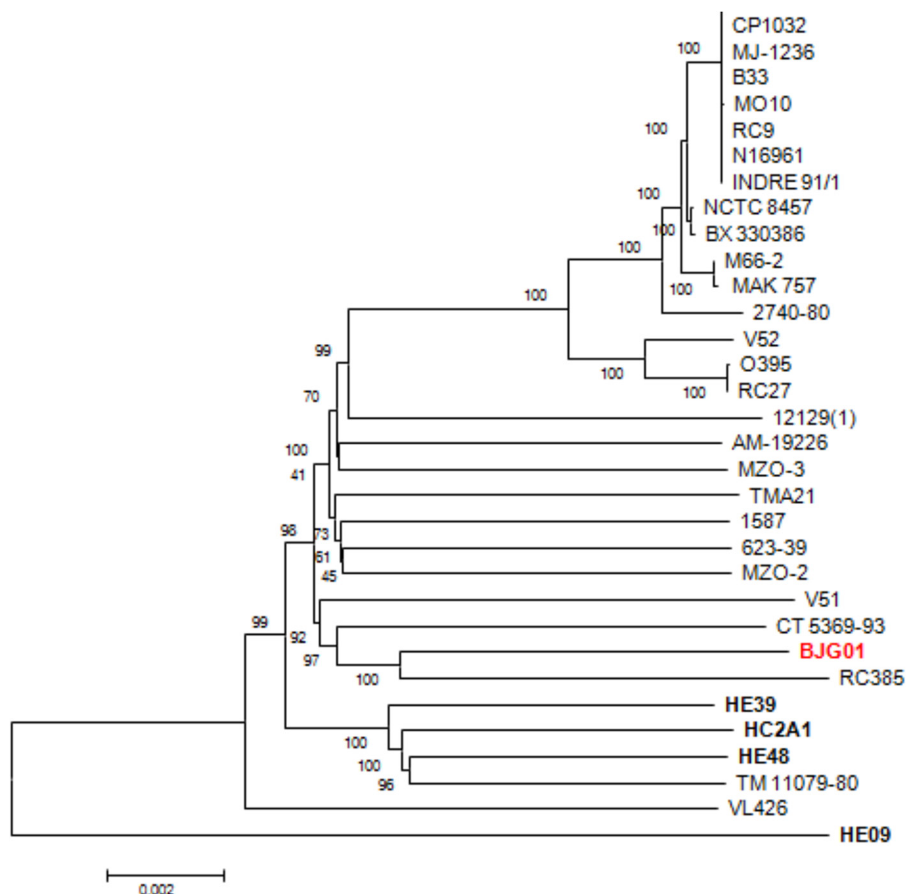

FIG 4 Phylogeny of *V. cholerae* BJG-01. The neighbor-joining tree was constructed based on 1,163 highly conserved orthologs representing 1,182,674 bp of the BJG-01 genome. The nucleotide substitution is the Kimura 2-parameter model. Numbers at the nodes indicate the bootstrap supports, and the bar indicates the number of substitutions per nucleotide position.

toxigenic strain of *V. cholerae* isolated from a clinical case and found it to lack most of the major virulence-encoding regions of toxigenic *V. cholerae* and an important virulence factor of non-O1/O139 *V. cholerae*, the NAG-specific enterotoxin gene (*stn*), reported to be essential for pathogenesis in a human volunteer study (46). Therefore, it appears that additional virulence factors yet to be determined are present in nontoxigenic *V. cholerae* associated with human disease. In this study, *V. cholerae* BJG-01 was found to encode a large number of genomic islands and other putative virulence genes. However, the functionality of these genomic islands remains obscure, as the majority have been annotated as either hypothetical proteins or proteins of unknown function. Some of the accessory genes detected in *V. cholerae* BJG-01 have previously been identified in other environmental *V. cholerae* non-O1/O139 isolates. VNTR genotyping indicated that *V. cholerae* BJG-01 is a distinct genotype, whereas phylogenetic analysis shows that it belongs to a phyletic lineage of non-O1/O139 *V. cholerae* associated with sporadic cases of gastroenteritis in the Western Hemisphere.

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