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Genes Similar to the Vibrio parahaemolyticus Virulence-Related Genes tdh, tlh, and vscC2 Occur in Other Vibrionaceae Species Isolated from a Pristine Estuary

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Detection of the human pathogen Vibrio parahaemolyticus often relies on molecular biological analysis of species-specific virulence factor genes. These genes have been employed in determinations of V. parahaemolyticus population numbers and the prevalence of pathogenic V. parahaemolyticus strains. Strains of the Vibrionaceae species Photobacterium damselae, Vibrio diabolicus, Vibrio harveyi, and Vibrio natriegens, as well as strains similar to Vibrio tubiashii, were isolated from a pristine salt marsh estuary. These strains were examined for the V. parahaemolyticus hemolysin genes tdh, trh, and tlh and for the V. parahaemolyticus type III secretion system 2α gene vscC2 using established PCR primers and protocols. Virulence-related genes occurred at high frequencies in non-V. parahaemolyticus Vibrionaceae species. V. diabolicus was of particular interest, as several strains were recovered, and the large majority (>83%) contained virulence-related genes. It is clear that detection of these genes does not ensure correct identification of virulent V. parahaemolyticus. Further, the occurrence of V. parahaemolyticus-like virulence factors in other vibrios potentially complicates tracking of outbreaks of V. parahaemolyticus infections.

Vibrio parahaemolyticus is the leading cause of seafood-associated gastroenteritis in the United States and the world (1). The U.S. Food and Drug Administration (1) estimates that 4,500 reported cases of V. parahaemolyticus gastroenteritis occur every year, and outbreaks of V. parahaemolyticus infections are increasing in frequency and expanding in geographic range (2, 3). This organism is ubiquitous in nearshore marine waters, and cell numbers are typically highest in surficial sediments (4) and in turbid waters bearing high loads of resuspended sediment (5, 6). Filter-feeding bivalve mollusks, such as oysters and mussels, can concentrate V. parahaemolyticus and other pathogenic vibrios (for examples, see references 7 and 8), resulting in levels in the mollusks capable of producing infection in a person that ingests them (9). Virulent V. parahaemolyticus strains are clearly a concern for seafood safety, and their detection is important anywhere that elevated levels of this organism are found.

Detection of V. parahaemolyticus in shellfish and environmental samples is typically based on molecular biological analysis of specific genes, particularly genes exclusive to this species and those strongly correlated with pathogenicity. The gene encoding the thermolabile hemolysin (TLH), designated tlh, encodes a phospholipase A2 (10). While its contribution to pathogenicity is unknown, expression of this gene is upregulated under conditions mimicking the human intestine (11, 12). tlh is considered to be a species-specific marker for V. parahaemolyticus (13, 14) and is frequently employed to identify this species (1, 13, 15–18). Genes encoding the thermostable direct hemolysin (TDH) and the homologous thermostable direct hemolysin-related hemolysin (TRH), tdh and trh, respectively, have been implicated in V. parahaemolyticus virulence (19, 20, 21). TDH and TRH are tetrameric proteins that act as porins and facilitate efflux of divalent cations and other solutes from and influx of water molecules into intestinal cells (11, 22, 23). Occurrence of tdh is correlated with the Kanagawa phenomenon (KP), a beta-hemolytic reaction on saline blood agar (Wagatsuma agar) (19). Numerous studies indicated that tdh and trh are found almost exclusively in clinical strains isolated from patients suffering from V. parahaemolyticus gastroenteritis (19, 21). Only about 1 to 2% of screened V. parahaemolyticus strains not derived from infected humans (i.e., environmental strains) were reported to carry these genes (24). These results were due, at least in part, to inadequate methodology, and recent studies have shown that high levels of environmental V. parahaemolyticus, 52% of strains from an area supporting intensive shrimp aquaculture (25) and 48% of strains from water and sediment in a pristine estuarine ecosystem (26), carry these genes. In addition, V. parahaemolyticus strains can carry two type three secretion systems, T3SS1 on chromosome 1 and T3SS2 on chromosome 2 (27). T3SS1 has been found in all tested strains of V. parahaemolyticus, while T3SS2 has been reported in virulent strains (27, 28). T3SSs are composed of 20 to 30 proteins and are responsible for translocating effector proteins directly into host cell cytoplasm. There are more than 100 described effector proteins having effects ranging from autophagy to cytotoxicity (29, 30). Additionally, there are two distinct types of T3SS2 (31). T3SS2α has been found in strains that carry tdh, while T3SS2β is correlated with trh (31, 32). Pathogenesis of V. parahaemolyticus does not appear to rely solely on a given virulence function; rather, virulence is complex and different strains may employ somewhat different strategies.

The utility of any of these genes as a molecular marker for V. parahaemolyticus or for evaluation of the potential pathogenicity of V. parahaemolyticus strains relies upon their specificity for V. parahaemolyticus.
parahaemolyticus. Sporadic reports of *V. parahaemolyticus*-like virulence genes in other species have appeared in the literature (33–39), but little is known regarding the distributions of these genes among *Vibrionaceae*. In particular, the occurrence of these genes in environmental strains has received very little attention. We screened a collection of *Vibrionaceae* strains isolated from a pristine estuary for *tlh*, *tdh*, and *trh* and for a gene encoding a T3SS2/H9251 outer membrane protein, *vscC2* (VPA1339), in order to evaluate the specificity of these genes for *V. parahaemolyticus*. Our results show that *tlh*, *tdh*, and *vscC2* are not found exclusively in *V. parahaemolyticus*.

**MATERIALS AND METHODS**

Sample site and strain isolation. Strains were isolated from the pristine North Inlet estuary near Georgetown, SC. The North Inlet-Winyah Bay National Estuarine Research Reserve protects the third largest watershed on the east coast of the United States, and 90% of the 18,916 acres are in their natural state. The keystone macrophyte *Spartina alterniflora* dominates the intertidal marsh, except at lower-salinity, higher elevations, where the subdominant *Juncus roemerianus* thrives. Fiddler crabs of the genus *Uca* are the biomass dominant fauna within the marsh. Their burrows are found throughout the intertidal marsh and have been shown to contain high levels of *V. parahaemolyticus* (40).

Sampling trips were made in August 2011 and again in September 2011 to coincide with periods of elevated *Vibrio* numbers (4, 40). Samples were collected at low tide from bulk sediment, *Uca* burrow lining sediment, *Uca* burrow water, sediment pore water, and creek water as described previously (4, 40). All samples were diluted with phosphate-buffered saline (400 mM NaCl, 1.75 mM NaPO₄, pH 7.4) and plated on thiosulfate citrate bile salts sucrose (TCBS) agar (BD, NJ) by following the FDA protocol (41) without the use of enrichment media. The TCBS plates were then incubated at 37°C for 48 h. Well-isolated colonies were streaked onto fresh TCBS plates for further characterization. Green colonies (typical appearance of *V. parahaemolyticus*) were collected and routinely cultured on saline Luria agar (SLA; per liter, 10 g tryptone, 5 g yeast extract, 27 g NaCl, 15 g Bacto agar).

Physiological characterization. Strains were tested for *β*-galactosidase activity, sucrose fermentation, and the ability to grow at 42°C (26). *β*-Galactosidase activity was determined (Oxoid, Hampshire, England) using cultures grown in 1 ml saline *Luria* broth (SLB) supplemented with 0.1% lactose and incubated at 37°C with shaking. Sucrose fermentation was determined in 1-ml broth cultures (per liter, 10 g casein digest peptone, 0.018 g phenol red, 5 g sucrose, 27 g NaCl, 15 g Bacto peptone), the month and year it was isolated (8 to 11 for August 2011 and 9 to 11 for September 2011), and the isolate number. For example, JS-8-11-4 refers to isolate number 4 from *Juncus* zone sediment (JS) collected in August 2011 (8–11).

*Wagatsuma* agar. Preparation of *Wagatsuma* agar followed the FDA bacteriological analytical manual and employed rabbit erythrocytes (41). After curing overnight, each plate was inoculated with one *Vibrionaceae* strain. Inoculated plates were incubated for up to 48 h at 37°C, and isolates...
producing beta-hemolysis were scored Kanagawa phenomenon (KP) positive.

**MLSA.** The identities of the North Inlet *Vibronaceae* isolates were determined by multilocus sequence analysis (MLSA) of concatenated 16S rRNA gene, recombinase A (*recA*), RNA polymerase alpha subunit (*rpoA*), and gyrB (*gyrB*) gene sequences. Bacterial genomic DNA was extracted using the Wizard genomic DNA purification kit (Promega, Madison, WI) and diluted to 25 ng µl⁻¹. The PCR program used to amplify *recA*, *rpoA*, and *gyrB* consisted of an initial denaturation at 95°C for 5 min, three cycles of 95°C for 1 min, 55°C for 2 min 15 s, and 72°C for 1 min 15 s, and then 30 cycles of 95°C for 35 s, 55°C for 75 s, and 72°C for 1 min, and a final elongation at 72°C for 2 min. Three cycles of 95°C for 1 min, 55°C for 2 min 15 s, and 72°C for 1 min, and a final elongation at 72°C for 7 min.

PCR products were sequenced, the resulting gene sequences were edited, and Neighbor-joining trees were constructed using the Kimura 2-parameter model with Mega version 5.05 (46).

**Nucleotide sequence accession numbers.** Sequence data obtained from this work were submitted to the NCBI GenBank and assigned the following accession numbers: JX453017 to JX453108, JX257004 to JX257006, and JX262990 to JX262992 (46).

**RESULTS**

Fifty-five strains of vibrios produced green (non-sucrose-fermenting) colonies on TCBS agar. MLSA demonstrated that 18 of these strains were not *V. parahaemolyticus* (Fig. 1). Thirteen of the 18 strains from the North Inlet estuary yielded negative results for β-galactosidase activity and sucrose fermentation and grew at 42°C, as would be expected for *V. parahaemolyticus* (42). The other five strains produced green colonies on TCBS agar, fermented sucrose to acid end products in broth, lacked β-galactosidase activity, and grew at 42°C.

Twelve of the North Inlet strains were identified as *Vibrio diabolicus*; these strains grouped with the *V. diabolicus* type strain HE800* in the MLSA tree. Three strains grouped with the *Vibrio tubiashii* type strain LMG 10936 but were not similar enough to the type strain to be considered true *V. tubiashii* strains. Three strains (JPW-8-11-4, JPW-9-11-6, and JPW-9-11-11) most likely represent a new species of *Vibrio* that is related to *V. tubiashii* and are referred to in the present study as *V. tubiashii*-like. The other *Vibronaceae* recovered from the North Inlet were strains of *Vibrio harveyi* (TBS-9-11-8), *Vibrio natriegens* (TBB-8-11-5), and *Photobacterium damselae* subsp. *damselae* (JPW-8-11-6).

All 18 strains were screened for the three hemolysin genes (Table 1). Six *thl* amplicons were detected. Two strains produced faint amplicons that, after multiple attempts, did not yield sequence

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**TABLE 1** Occurrence of genes and phenotypic characteristics considered diagnostic for virulence in *V. parahaemolyticus* and also found in non-*V. parahaemolyticus* environmental *Vibronaceae* strains

<table>
<thead>
<tr>
<th>Strain designation</th>
<th>Species</th>
<th>Presence of <em>a</em></th>
<th>Swarming motility</th>
<th>Colony appearance on TCBS agar</th>
<th>β-Galactosidase activity</th>
<th>Sucrose fermentation</th>
<th>Growth at 42°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>JBW-8-11-1</td>
<td><em>V. diabolicus</em></td>
<td>* + + + +</td>
<td>Green</td>
<td>− − + +</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>JPW-9-11-11</td>
<td><em>V. tubiashii-like</em></td>
<td>+ + + +</td>
<td>Green</td>
<td>− − + +</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>JPW-9-11-8</td>
<td><em>V. diabolicus</em></td>
<td>+ − − − −</td>
<td>Green</td>
<td>− − + +</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>JPW-8-11-8</td>
<td><em>V. diabolicus</em></td>
<td>+ + + + +</td>
<td>Green</td>
<td>− − + +</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>JPW-8-11-6</td>
<td><em>P. damsela</em></td>
<td>+ + + + +</td>
<td>Green</td>
<td>− − + +</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>JPW-9-11-6</td>
<td><em>V. tubiashii-like</em></td>
<td>+ + + +</td>
<td>Green</td>
<td>− − + +</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>JBW-8-11-3</td>
<td><em>V. diabolicus</em></td>
<td>− + + + +</td>
<td>Green</td>
<td>− − + +</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>CW-9-11-1</td>
<td><em>V. diabolicus</em></td>
<td>− + + + +</td>
<td>Green</td>
<td>− − + +</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>JS-8-11-4</td>
<td><em>V. diabolicus</em></td>
<td>+ + + + +</td>
<td>Green</td>
<td>− − + +</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>TPS-9-11-4</td>
<td><em>V. diabolicus</em></td>
<td>+ + + + +</td>
<td>Green</td>
<td>− − + +</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>TBBW-9-11-5</td>
<td><em>V. natriegens</em></td>
<td>+ + + + +</td>
<td>Green</td>
<td>− − + +</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>JBBW-8-11-4</td>
<td><em>V. diabolicus</em></td>
<td>+ + + + +</td>
<td>Green</td>
<td>− − + +</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>JBPW-8-11-4</td>
<td><em>V. tubiashii-like</em></td>
<td>+ + + +</td>
<td>Green</td>
<td>− − + +</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>JBW-9-11-8</td>
<td><em>V. diabolicus</em></td>
<td>+ + + + +</td>
<td>Green</td>
<td>− − + +</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>TS-9-11-7</td>
<td><em>V. diabolicus</em></td>
<td>+ + + + +</td>
<td>Green</td>
<td>− − + +</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>TBS-9-11-8</td>
<td><em>V. harveyi</em></td>
<td>+ + + + +</td>
<td>Green</td>
<td>− − + +</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>JBS-8-11-1</td>
<td><em>V. diabolicus</em></td>
<td>− + + + +</td>
<td>Green</td>
<td>− − + +</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>TBS-8-11-1</td>
<td><em>V. diabolicus</em></td>
<td>− + + + +</td>
<td>Green</td>
<td>− − + +</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

*a* An asterisk designates a faint amplicon detected but no sequence recovered.
data. This has been observed previously for some authentic *V. parahaemolyticus* strains (26). *tlh* gene sequences were recovered from the other four strains (Fig. 2). The *V. diabolicus* strains JPW-9-11-8 and JPW-8-11-8 contained *tlh* sequences that were each 99.3% similar to *tlh* gene sequence from the *V. parahaemolyticus* type strain ATCC 17802T. *tlh* was also detected in JPW-9-11-11 (*V. tubiashii*-like) and JPW-8-11-6 (*P. damselae*). *tlh* gene sequences from these species were 99.3 and 99.1% similar, respectively, to those from *V. parahaemolyticus* ATCC 17802T.

*tdh* amplicons were detected in 5 *V. diabolicus* strains and 1 *V. tubiashii*-like strain (Fig. 3). Two of the strains produced faint amplicons that after multiple attempts did not yield sequence data, indicating that these strains contain a variant of *tdh*. Four amplicons, all from *V. diabolicus*, yielded sequence data. Three of the *tdh* gene sequences had high similarities (97.6 to 99.2%) to the *tdh* sequence of the *V. parahaemolyticus* reference strain ATCC 33846, which was employed because the *V. parahaemolyticus* type strain ATCC 17802T does not contain *tdh*. The *tdh* sequence from CW-9-11-1 appeared to be divergent, having only 83.2% similarity to the ATCC 33846 *tdh* sequence. The translated peptide encoded by the CW-9-11-1 *tdh* gene sequence was 75.9% similar to the *tdh* sequence from *V. parahaemolyticus* strain ATCC 33846.

The hemolysin gene *trh* was not recovered from any of the non-*V. parahaemolyticus* strains examined in this study. This gene was also less...
common in North Inlet *V. parahaemolyticus* strains than *tlh* or *tdh* (26).

The T3SS2α gene *vscC2* was detected at the highest frequency of the genes examined. Twelve of the 18 non-*V. parahaemolyticus* strains (67%) contained *vscC2*. *vscC2* was detected in 7 *V. diabolicus* strains, 2 *V. tubiashii*-like strains, a *P. damselae* strain, a *V. natriegens* strain, and a *V. harveyi* strain (Fig. 4). All of the *vscC2* amplicons yielded sequences that were highly similar (92.9 to 98.8%) to the *vscC2* sequence from the *V. parahaemolyticus* clinical strain RIMD 2210633, in which *V. parahaemolyticus* T3SS2 was first described (27). In total, at least one *V. parahaemolyticus*-like virulence gene was detected in 16 of the 18 non-*V. parahaemolyticus* strains (89%) from North Inlet. None of the genes of interest were detected in two of the *V. diabolicus* strains (see below). These two additional strains (*V. diabolicus* strains JBS-8-11-1 and TBS-8-11-1) did not contain virulence factor genes but were included because they had virulence-related phenotypic characters (Table 1).

Seven of the 18 non-*V. parahaemolyticus* strains (38%) were KP positive. Three of these KP-positive strains contained *tdh*. The four strains that did not produce a *tdh* amplicon but were KP positive were J PW-9-11-6, J BW-9-11-8, TBS-9-11-8, and JBS-8-11-1, and three of these strains contained other virulence-related genes. *vscC2* was detected in J BW-9-11-8 (*V. diabolicus*) and TBS-9-11-8 (*V. harveyi*). *tlh* was detected in J PW-9-11-6 (*V. tubiashii*-like), but none of the four marker genes was detected in J BS-8-11-1 (*V. diabolicus*). Additionally, three strains that contained *tdh* did not produce a KP-positive phenotype.

**DISCUSSION**

All of the non-*V. parahaemolyticus* environmental *Vibrionaceae* strains examined in this study were identical in appearance to *V. parahaemolyticus* on TCBS agar, lacked β-galactosidase activity, and were able to grow at 42°C. Five strains fermented sucrose to acid end products in broth but were phenotypically identical to *V. parahaemolyticus* for all other tests. TCBS agar is a medium commonly used in the isolation of clinical and environmental vibrios; however, only 67% of the presumptive *V. parahaemolyticus* colonies were confirmed by *recA* phylogenetic analysis (26) to be *V. parahaemolyticus*. Clearly, results of TCBS agar plating and confirming physiological testing of the kind used here must be supported by further analysis to determine the identities of environmental strains. It should be noted that ≥38% of the non-*V. parahaemolyticus* strains examined demonstrated the Kanagawa phenomenon, which is also inadequate for precise identification of this species.

The thermolabile hemolysin gene *tlh* has provided a high-throughput and convenient means to determine *V. parahaemolyticus* numbers in samples, but this analysis is clearly subject to false positives arising from other *Vibrio* species. Unlike previous studies (34), the *tlh* sequence data recovered from non-*V. parahaemolyticus* vibrios indicate that *tlh* gene sequences in *V. diabolicus*, *V. tubiashii*-like, and *P. damselae* are highly similar to those in *V. parahaemolyticus*. This makes unambiguous species discrimination, even employing gene sequences, much more difficult. The mol% G+C content of *tlh* (47.6%) (13) corresponds to the mol% G+C contents of these four *Vibrionaceae* species (43 to 49%) (42), which indicates that any horizontal transfer of *tlh* within this family occurred far enough in the past for genetic drift to eliminate clear-cut evidence of this process. Some previous reports of *tlh* in non-*V. parahaemolyticus* vibrios attributed its detection to false-positive results (47). False-positive PCR amplifi-
cation arising from sample contamination is certainly possible, but true tlh amplicons may have been recovered instead. Additionally, tlh is highly similar to homologous hemolysin genes found in other Vibrio species, including V. harveyi (vhh), V. tubiashii (vth), and V. vulnificus (vvh). Wang et al. (36) determined that DNA probes for tlh and vhh could be used interchangeably, demonstrating that tlh probing for V. parahaemolyticus lacks the necessary specificity. Using tlh as the sole marker for V. parahaemolyticus densities may lead to overestimation of total V. parahaemolyticus counts by including other Vibrio species carrying tlh or similar hemolysins (V. harveyi, V. tubiashii, and V. vulnificus). The extent of this overestimation may be variable among different systems and sample types. It appears that no virulence factor gene sequence can be used to detect V. parahaemolyticus quantitatively without additional, supporting analyses.

The thermostable direct hemolysin gene (tdh) and a T3SS2α gene, both typical of virulent V. parahaemolyticus strains, are also not restricted to this species. In fact, virulence features generally attributed to V. parahaemolyticus were common in environmental Vibrionaceae strains that were isolated from an estuary having negligible human impacts. The apparent rarity of thermostable hemolysin genes in environmental non-V. parahaemolyticus are also quite high. The Vibrionaceae species in which these toxin genes were detected also expand the list of species that carry genes correlated with pathogenicity. tdh sequences have been recovered from clinical strains of Vibrio cholerae non-O1, Vibrio mimicus, and Vibrio hollisae (33), but environmental strains also carry this gene. Non-V. parahaemolyticus tdh sequences recovered in this study were similar to those of V. parahaemolyticus tdh, with the exception of tdh from V. diabolicus CW-9-11-1, which was clearly divergent from the other sequences and might represent a variant of tdh. It seems likely that these genes were transferred from an origin taxon to other Vibrio species, including V. parahaemolyticus and perhaps others. Phenotypic manifestation of tdh has frequently been demonstrated with Wagatsuma agar via the KP, but there was a poor correlation in these strains between tdh detection and KP. Three strains contained tdh yet were KP negative, and three strains were KP positive yet yielded negative results for canonical hemolysin gene detection. These results indicate that either detection of these virulence-related genes was faulty or other virulence mechanisms are responsible for the KP-positive result from JBS-8-11-1.

Recent studies have established a correlation between T3SS2 and hemolysin genes in V. parahaemolyticus, with T3SS2α distribution correlated with the presence of tdh in KP-positive stains and T3SS2β with thr (31, 32). We found no correlation between

![Neighbor-joining tree (Kimura 2-parameter model) of vscC2 gene sequences recovered from environmental Vibrionaceae isolates. Bootstrap values represent 1,000 replications; values less than 50 are not shown. The vscC2 gene sequence of Yersinia pestis served as the outgroup, and reference sequences were obtained from the NCBI GenBank.](attachment:figure4.png)
the TSS2α marker gene vscC2 and the presence of tdh, vscC2 was detected at high frequencies in environmental Vibrionaceae. The vscC2 gene sequences from V. parahaemolyticus strains are highly similar to those from non-V. parahaemolyticus strains, indicating a recent transfer of this gene. This gene has been reported in other vibrios, including V. cholerae non-O1 and V. mimicus strains (31, 37). Evidence of vscC2 in V. diabolicus, P. damsela, V. natriegens, V. harveyi, and V. tubiashii-like strains greatly expands the known distribution of the TSS2α gene vscC2 in Vibrionaceae.

V. parahaemolyticus hemolysin genes and TSS2 genes have been detected previously in the highly pathogenic V. cholerae non-O1 strain but also in vibrios that rarely, if ever, cause illness in humans, including V. alginolyticus, V. mimicus, and V. hollisae (33–35, 37–39, 48). None of the non-O1 V. parahaemolyticus strains we isolated belong to a species implicated in human infections, being either pathogenic to marine fauna (V. tubiashii, V. harveyi, and P. damsela) or considered to be nonpathogenic (V. diabolicus and V. natriegens). The V. diabolicus strains are of particular interest, because this species has seldom been reported since its description by Raguenes et al. (49). The frequent recovery of V. diabolicus strains from the pristine North Inlet estuary suggests that this species is more broadly distributed than previously thought, and the finding of V. parahaemolyticus-like virulence factor genes in this species implicates it as a reservoir for these genes. Additionally, the high similarity of the V. diabolicus and V. parahaemolyticus tdh and vscC2 sequences indicates that these genes are readily transferred among these and other Vibrionaceae species.

Maintenance of superfluous virulence factor genes in the absence of positive selection is unlikely, indicating other potential roles of these virulence factors in the environment. The pristine North Inlet estuary is a strongly nutrient-limited environment (26, 50). Virulence factors implicated in diseases of humans could also be used to acquire nutrients through damage to cells of estuarine organisms, and several Vibrio species may be able to acquire nutrients in this manner. If these species are ingested and expression of these genes and function of the gene products in human hosts occur, the emergence of pathogenic potential in these vibrios due to V. parahaemolyticus-related virulence factors would be confirmed. Shellfish-harvesting practices are based on the established seasonal population dynamics of environmental V. parahaemolyticus, which grows optimally in the warmer months. Transfer of V. parahaemolyticus virulence factors to other vibrios with different optimal growth conditions or geographic distributions potentially changes the dynamics of virulent Vibrio populations. Most research on the well-known pathogenic vibrios, V. cholerae, V. vulnificus, and V. parahaemolyticus, has focused on clinical and epidemiological impacts of the organisms, but if the addition of virulence factors to the genomes of Vibrio species that are not generally considered pathogenic is increasing their fitness in the environment, additional virulent Vibrio species, perhaps with different environmental preferences and different host ranges, could emerge.

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