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GENES AND GENOMES

Sequence Analyses of Type IV Pili from *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*

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Abstract Bacterial surface structures called pili have been studied extensively for their role as possible colonization factors. Most sequenced *Vibrio* genomes predict a variety of pili genes in these organisms, including several types of type IV pili. In particular, the mannose-sensitive hemagglutinin (MSHA) and the PilA pili, also known as the chitin-regulated pilus (ChiRP), are type IVa pili commonly found in *Vibrio* genomes and have been shown to play a role in the colonization of *Vibrio* species in the environment and/or host tissue. Here, we report sequence comparisons of two type IVa pilin subunit genes, *mshA* and *pilA*, and their corresponding amino acid sequences, for several strains from the three main human pathogenic *Vibrio* species, *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. We identified specific groupings of these two genes in *V. cholerae*, whereas *V. parahaemolyticus* and *V. vulnificus* strains had no apparent allelic clusters, and these genes were strikingly divergent. These results were compared with other genes from the MSHA and PilA operons as well as another *Vibrio* pili from the type IVb group, the toxin co-regulated pilus (TCP) from *V. cholerae.* Our data suggest that a selective pressure exists to cause these strains to vary their MSHA and PilA pilin subunits. Interestingly, *V. cholerae* strains possessing TCP have the same allele for both *mshA* and *pilA*. In contrast, *V. cholerae* isolates without TCP have polymorphisms in their *mshA* and *pilA* sequences similar to what was observed for both *V. parahaemolyticus* and *V. vulnificus*. This data suggests a possible linkage between host interactions and maintaining a highly conserved type IV pili sequence in *V. cholerae*. Although the mechanism underlying this intriguing diversity has yet to be elucidated, our analyses are an important first step towards gaining insights into the various aspects of *Vibrio* ecology.

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Introduction

Vibrio species are marine bacteria that naturally inhabit aquatic environments worldwide and are commonly associated with marine organisms. Some *Vibrio* species are pathogenic bacteria capable of producing life-threatening infections in humans typically following consumption of contaminated food, including seafood. Although the specific factors that contribute to the pathogenicity of vibrios in humans are well studied, little is known about the bacterial factors involved in the association of the bacteria with environmental organisms.

Bacteria display a variety of mechanisms that enable them to specifically interact with target cells. Many bacteria produce hair-like surface structures, called pili or fimbriae, which are often important for survival [1–3]. These adhesins have been clustered into groups based on amino acid sequence similarities among their pilin subunits [4]. One type of pili, the type IV group, are known to be involved in adhesion, immune escape, microcolony formation, transformation, and phage transduction [4] and are commonly found in Gram-negative bacteria, including numerous pathogens [4, 5]. Type IV pili are known to assist many bacterial species in survival in various environments, ranging from attachment to a variety of surfaces for biofilm formation [6–9] to colonizing the host [10–17]. These pili begin as prepilins possessing a hydrophilic leader peptide and are processed by a unique peptidase that cleaves the leader sequence to form a mature pilin protein [18]. After processing, mature pilin subunits assemble together to form pili through interactions between the conserved N-termini in the pilin cores, leaving the variable C-terminal regions to

interact with the environment [4]. Type IV pili are divided into two subclasses based on differences in amino acid sequence and length. Type IVa pili have both a shorter leader peptide and mature protein sequence, while type IVb pili have considerably longer leader sequences and overall length [4, 5, 18]. In addition to similarities in their amino acid composition, all type IV pili appear to have analogous architecture [4].

When examining the genomes of Gram-negative bacteria possessing type IV pili, type IVa pili biogenesis genes are scattered throughout the genome, but the genes or gene clusters are almost always flanked by the same genes, typically housekeeping genes. In addition, homologous gene sets for type IVa pili are found in virtually identical locations throughout more than 150 sequenced genomes. Considering these genes have not been found on any identifiable pathogenicity island, it suggests that these pili are ancient to many of the bacterial phyla possessing these genes [18]. In contrast, type IVb pili genes are fewer in number than type IVa genes and are typically found clustered within the genome. Moreover, the gene sequence order does not appear to be conserved amongst different organisms possessing the type IVb pili except for the universally conserved core proteins. In addition, when comparing N-terminal sequence homology, type IVa pilin subunits are more similar among themselves than to type IVb pilins or within the type IVb pili group. Furthermore, type IVa pili occur in bacteria with a broad host range, while type IVb pili have only been identified in colonizers of the human intestinal tract [4]. *Vibrio* species possess many type IV pili from both type IVa and b groups, but only a select few have been studied for their role in environmental and/or host survival. One thoroughly studied pili from the type IVb group is the toxin co-regulated pilus (TCP) from *Vibrio cholerae*, and it is known for its key role in virulence [19–21]. It is expressed by *V. cholerae* classical and El Tor biotypes from the O1 and O139 serogroups [22]. TCP is composed of TcpA subunits and appears as thick bundles on the electron microscope [4]. TcpA is processed by a TCP-specific signal peptidase, TcpJ, to form mature pilin subunits for assembly [22, 23]. The structure of TCP consists of the conserved N-terminal α-helices of TcpA buried in the core of the pilus, maximizing contact between subunits to provide overall strength. The structurally variable regions of the pilins interact to hold the core units together and coat the surface where interactions take place with the environment, i.e., the intestines [4]. In addition to colonization, TCP is the receptor for the CTXΦ phage [24, 25].

An additional well-studied *V. cholerae* type IV pilus is the mannose-sensitive hemagglutinin (MSHA), which belongs to the type IVa group. When examining operon composition, MSHA in *V. cholerae* consists of two operons where one operon encodes five prepilin subunits, including the major pilus subunit MshA, and the other contains genes involved in assembly and secretion [26]. In *V. cholerae*, the PilD peptidase has been shown to process the MshA subunits for assembly of the mature pilus structure [27, 28]. The MSHA pilus hemagglutinates red blood cells [29, 30] and is a receptor for filamentous phage [31–33]. It has been studied extensively in *V. cholerae* to identify any involvement in host colonization [19, 21, 34]. In *V. cholerae*, only the El Tor biotypes produce functional MSHA pili [29, 30], and during human colonization studies, the protein was repressed [35]. Expression of the MSHA pilus was tightly regulated so that when TCP was expressed, the MSHA protein was repressed; therefore, the MSHA pilus is considered an anticolonization factor in human disease [36]. When the MSHA pilus was constitutively expressed during colonization, it resulted in immune system recognition [35]. Thus, the MSHA pilus does not appear to be a virulence factor for *V. cholerae*, suggesting that expression of the gene product is for utilization in the environment. Studies have shown that the MSHA pilus is used to adhere to zooplankton exoskeletons as a survival strategy in the aquatic environment [37, 38], presumably by forming biofilms. *V. cholerae* and *Vibrio parahaemolyticus* are known to use the MSHA pilus to form biofilms on various surfaces [6, 8, 38], including chitin [39], which provides some supporting evidence for the role of the MSHA pilus in environmental survival.

Another pilus found in *Vibrio* spp. is the type IVa PilA pilus, also known as the chitin-regulated pilus (ChiRP). The PilA operon is composed of five open reading frames that constitute a single operon, consistent with other type IVa pili [28]. A mature PilA pilus is composed of PilA subunits that were processed by the PilD peptidase [28], the same peptidase that processes the MshA pilin subunits [27, 28]. The PilD peptidase is the fourth open reading frame in the PilA operon [28]. The PilA type IVa pilus is an integral player in the *V. cholerae* chitin utilization program [39]. Expression of the PilA protein has been shown to be induced by chitin in both *V. cholerae* [39] and *V. parahaemolyticus* [6]. PilA is involved in biofilm formation [6, 10], adherence to human epithelial cells [10], and colonization of oysters [11]. It has been implicated as a virulence factor for *V. vulnificus* [10], although direct evidence of its role in virulence has not been clearly described in other human pathogenic vibrios.

Taken together, the studies of the type IVa pili MSHA and PilA in various *Vibrio* spp. suggest that these proteins might be utilized by vibrios for environmental survival by attaching to chitinous substrates such as zooplankton. In contrast, the type IVb pilus, TCP, from *V. cholerae*, is critical for host colonization and has not be implicated in environmental survival, pointing out the possibility of two very distinct roles for the different subclasses of type IV pili.

During our efforts to investigate the roles of MSHA and PilA in *V. parahaemolyticus* colonization of the Pacific oyster, *Crassostrea gigas*, we noted sequence heterogeneities in these genes. This led us to examine these genes in other human pathogenic *Vibrio* species, such as *V. cholerae* and *V. vulnificus*.

Here, we present a comparative sequence analysis of the *mshA* and *pilA* pilin genes from several strains of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. These sequence analyses suggest that a selective environmental pressure has been applied to these genes, resulting in the observed sequence heterogeneities for all three *Vibrio* species examined.

Materials and Methods

Bacterial Strains

Thirteen of the *V. parahaemolyticus* bacterial strains sequenced were kindly provided by Dr. Yi-Cheng Su, Oregon State University Seafood Laboratory, Astoria, OR, USA. Genomic DNA for five tdh/trh negative strains of *V. parahaemolyticus* was obtained from Dr. Narjol-Gonzalez-Escalona, FDA, College Park, MD, USA. Genomic DNA for ten of the *V. vulnificus* strains sequenced were provided by Dr. Paul Gulig, University of Florida, Gainesville, FL, USA. Five of the *V. vulnificus* strains sequenced were provided by Dr. Kathy O'Reilly, Oregon State University, Corvallis, OR, USA. Bacterial strains were grown on Luria–Bertani agar supplemented with sodium chloride to a final concentration of 2%. All strains used in this study are listed in Table 1.

Sequencing

Genomic DNA from *V. parahaemolyticus* and *V. vulnificus* strains were isolated using the Qiagen DNeasy blood and tissue kit, following the protocol for DNA isolation included in the kit. Primers for sequencing each gene were designed for the region approximately 100 base pairs upstream from the start codon and 100 bp downstream of the stop codon for the gene of interest (Table 2). Polymerase chain reaction (PCR) was conducted using Invitrogen Platinum HiFi Supermix, following their standard protocol for PCR. PCR samples were quantified using the NanoDrop Spectrophotometer ND-1000. Sanger sequencing reactions for *V. parahaemolyticus* and *V. vulnificus* PCR products were performed at the Center for Genomic Research and Bioinformatics (CGRB), Oregon State University, Corvallis, OR, USA.

In Silico Analyses

The *in silico* sequence data for all the *V. cholerae* strains and additional *V. parahaemolyticus* and *V. vulnificus* strains were obtained from the Department of Energy Joint Genome Institute website: http://img.jgi.doe.gov/cgi-bin/pub/main.cgi. The *V. parahaemolyticus* and *V. vulnificus* sequenced DNA was translated into their predicted amino acid sequences using SeqTool and sequence alignments were created in ClustalW at the bioinformatics website for the CGRB: http://bioinfo.cgrb.oregonstate.edu/. Maximum likelihood phylogenetic trees were constructed using the MEGA 5 program: http://www.megasoftware.net/ using the Tamura–Nei model with nucleotide substitutions. Bootstrap values were calculated with 500 replicates. For the analysis of synonymous and nonsynonymous substitutions, calculations were made using the Synonymous Non-synonymous Analysis Program (SNAP): www.hiv.lanl.gov [40]. The program is based on the Nei and Gojobori [41] method for calculating synonymous and nonsynonymous rates of substitution with the incorporation of Ota and Nei [42] statistics. The package is described by Ganeshan et al. [43].

Results

Sequence Alignments

Overall, the sequence alignments for the DNA encoding the *mshA* and *pilA* genes from different strains of *V. cholerae, V. parahaemolyticus*, and *V. vulnificus* showed considerable sequence heterogeneity (Supplemental Figs. 1 and 2). Although the immediate 5′ regions are highly conserved in both genes, most of the gene sequences varied depending on the strain. Interestingly, *V. cholerae* exhibited distinct groupings for both genes, separating most clinical isolates from environmental isolates. In contrast, *V. parahaemolyticus* and *V. vulnificus* strains did not appear to group based on isolate origin or any other phenotype. Sequence alignments of the predicted amino acid sequences of MSHA and PilA from *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* are shown in Figs. 1 and 2. For *V. parahaemolyticus* and *V. vulnificus*, the predicted amino acids sequences for MSHA and PilA from both environmental and clinical isolates displayed notable sequence heterogeneity. With *V. cholerae* strains, most clinical isolates had conserved sequences for both MSHA and PilA. Most environmental isolates exhibited marked sequence heterogeneity, comparable to what was observed for the *V. parahaemolyticus* and *V. vulnificus* isolates.

Phylogenetic Trees

Maximum likelihood (ML) phylogenetic trees were constructed from the *mshA* (Fig. 3) and *pilA* (Fig. 4) sequences for the *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* isolates. Similar to the DNA and amino acid alignments, the *mshA* (Fig. 3a) and *pilA* (Fig. 4a) ML phylogenetic trees for *V. cholerae* clustered most clinical isolates into one branch, while environmental isolates exhibited various branching patterns. When ML phylogenetic trees were constructed for these two gene sequences from *V. parahaemolyticus* (Figs. 3b and 4b) and *V. vulnificus* (Figs. 3c and 4c), no discernable

Table 1 Strains used in this study

Vibrio cholerae

Strain	Serogroup	Biotype	Relevant genotype	Strain information	GenBank (PilA,MshA)
MAK757	O1, Ogawa	El Tor	TCP+, CTxA+	Pre-7th pandemic patient isolate, mildly toxigenic, from Celebes Islands in 1937	ZP_01954444, ZP_01953747
NCTC 8457	O1, Inaba	El Tor	TCP+, CT−	Saudi Arabia patient 1910, non-pandemic	ZP_01971084, ZP_01970913
B33	O1, Ogawa	Hybrid Classical/ El Tor	TCP+, CTxA+	Clinical isolates Beira, Mozambique 2004	ZP_04401766, ZP_01974939
O395	O1, Ogawa	Classical	TCP+, CT+	Clinical isolate, strain of the 6th pandemic in South Asia, isolated in 1965 from India	YP_001217923, YP_001218677
2470-80	O1, Inaba	El Tor	TCP+, CT−	Nontoxigenic environmental water isolate from the Gulf Coast, 1980, clonal with TCP+ CT+ isolates	ZP_01677376, ZP_01677345
N16961	O1, Inaba	El Tor	TCP+, CT+	Clinical isolate from Bangladesh, 1971	NP_232053, NP_230063
V51	O141		TCP+, CT−	Clinical Isolate from the United States, 1987	ZP_00748678, ZP_00749816
V52	O37		TCP+, CT+	Clinical isolate from Sudan, limited epidemic, not endemic	ZP_00746513, ZP_00747249
MO10	O139		TCP+, CT+	Clinical isolate from India and Bangladesh outbreak 1992, early isolate of O139 emergence	ZP_00758906, ZP_00758992
MZO-2	O14		TCP−, CT−	Clinical isolate from Bangladesh patients with diarrhea in 2001	ZP_01979911, ZP_01978309
1587	O12		TCP−, CT−	Clinical isolate from Lima, Peru, 1994, limited epidemic, not endemic, invasive	ZP_01949212, ZP_01949969
RC385	O135		TCP−, CT−	Persistent and luminescent environmental plankton isolate, Chesapeake Bay, 1998	ZP_00751854, ZP_00753463
623-39	non-O1/-O139		TCP−, CT−	Environmental water isolate from Bangladesh, 2002	ZP_01983325, ZP_01981981
AM-19226	O39		TCP−, CT−	Clinical isolate from Bangladesh, 2001	ZP_04962566, ZP_04962108
MJ-1236	O1, Inaba	El Tor "Matlab variant"	TCP+, CT+	Clinical isolate from patients with acute diarrhea, Matlab, Bangladesh 1994	YP_002877671, YP_002876957
VL426	non-O1/-O139	Albensis	TCP−, CT−	Diseased fish from Elbe River, Germany	ZP_04413813, ZP_04414508
TM 11079-80	O1, Ogawa	El Tor	TCP−, CT−	Environmental sewage isolate from Brazil, 1980	ZP_04410672, ZP_04409450
RC9	O1, Ogawa	El Tor	TCP+, CT+	Clinical isolate from Kenya 1985	ZP_04408866, ZP_04409227
TMA21	non-O1/-O139		TCP−, CT−	Environmental seawater isolate from Brazil, 1982	ZP_04403511, ZP_04402128
BX 330286	O1, Inaba	El Tor	TCP+, CT+	Water isolate from Australia, 1986	ZP_04397022, ZP_04396555
CIRS 101	O1, Inaba	El Tor, classical ctxB	TCP+, CTxA+	Clinical isolate from Dhaka, Bangladesh 2002	ZP_05420851, ZP_05417751
M66-2	O1		TCP+, CT−	1937 outbreak Indonesia, pre-7th pandemic isolate	YP_002811095, YP_002809171
CT 5369-93			TCP−, CT−	Sewage, Brazil 1993	ZP_06048448, ZP_06049688
RC27	O1	Classical	TCP+, CT−	Indonesia 1991	ZP_06036099, ZP_06035331
INDRE 91/1	O1, Inaba	El Tor	TCP+, CTxA+	Mexico 1991, first case of 7th pandemic in Mexico	ZP_06030661, ZP_06028721

Vibrio parahaemolyticus

Strain	Serotype	Relevant genotype	Strain information	GenBank (PilA,MshA)
RIMD 2210633	O3:K6	tdh+/trh-	Clinical strain from Osaka, Japan 1996	NP_798902, NP_799077
Peru-466		tdh+/trh+		ZP_05904882, ZP_05905780
K5030		tdh+/trh+		ZP_05776528, ZP_05778018
AN-5034	O4:K68	tdh+/trh+	Bangladesh 1998	ZP_05891366, ZP_05889900
SFL1009	O1:K2	tdh+/trh+	Isolated from sediment at Goose Point oyster farm, Willapa Bay, Washington, October 2002	JF923890, JF923914

Table 1 (continued)

SFL1027	O5	tdh+/trh+	Isolated from sediment at Oregon Oyster farm Yaquina Bay, Oregon, December 2002	JF923885, JF923903	
SFL1050	O1:K7	tdh+/trh+	Isolated from sediment at Goose Point oyster farm, Willapa Bay, Washington, July 2003	JF923892, JF923904	
SFL1079	O1:K4	tdh+/trh+	Isolated from seawater at Goose Point oyster farm, Willapa Bay, Washington, July 2003	JF923894, JF923905	
SFL1080	O5:K1	tdh+/trh−	Isolated from sediment at Goose Point oyster farm, Willapa Bay, Washington, July 2003	JF923891, JF923906	
10290	O4:K12	tdh+/trh+	1997 Washington outbreak strain	JF923888, JF923916	
10292	O6:K18	tdh+/trh+	1997 Washington outbreak strain	JF923893, JF923915	
BE98-2029	O3:K6	tdh+/trh−	1998 Texas outbreak strain	JF923886, JF923901	
O27-1C1	O5:K15	tdh+/trh+	1997 Oregon outbreak strain	JF923887, JF923908	
M25-0B	O4	tdh−/trh−	Environmental isolate from Washington, 1993	JF923900, JF923913	
UCM-V586	O8:K22	tdh−/trh−	Environmental isolate from Spain, 2003	JF923899, JF923912	
UCM-V441	O4	tdh−/trh−	Environmental isolate from Spain, 2002	JF923898, JF923911	
049-2A3	O4:K29	tdh−/trh−	Environmental isolate from Oregon, 1997	JF923896, JF923909	
357-99	O3	tdh−/trh−	Clinical isolate from Peru, 1999	JF923897, JF923910	
ATCC 17802		trh+	Shirasu food poisoning, Japan 1965	JF923889, JF923907	
Vibrio vulnificus					
Strain	Capsule	vcg	Virulence group	Strain information	GenBank (PilA,MshA)
98-783 DP-A1	1	E	2	Environmental isolate	JF923941, JF923921
99-520 DP-B8	2	E	3	Environmental isolate	JF923932, JF923920
99-581 DP-C7	2	E	3	Environmental isolate	JF923930, JF923917
99-584 DP-B12	2	E	2	Environmental isolate	JF923931, JF923922
99-736 DP-C7	2	E	2	Environmental isolate	JF923934, JF923924
99-738 DP-B5	2	E	5	Environmental isolate	JF923942, JF923923
S1-13	1	E	4	Environmental isolate	JF923933, JF923919
ATL-9580	1	C	5	Clinical isolate	JF923935, JF923918
CMCP6	1	C	4	Clinical isolate	NP_760518,NP_760356
YJ016	2	C	5	Clinical isolate, Taiwan	NP_935571, NP_935733
CP Mussel 10 PT					JF923939, JF923929
95-10-15 PT				isolated 10/18/05	JF923938, JF923928
960926 -1/4c PT				isolated 10/19/05	JF923937, JF923927
ATCC 27562				clinical isolate, Florida	JF923936, JF923926
OLOL-1				Katrina, isolated 10/18/05	JF923940, JF923925

grouping patterns appeared for either species, unlike the *V. cholerae* phylogenetic trees.

Substitution Analyses

We analyzed *mshA* and *pilA* for the rate of synonymous (silent) (d_S) and nonsynonymous (structural) (d_N) changes for the *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* isolates. For *mshA* from *V. cholerae*, the rate of synonymous (d_S) was 0.759, while the rate of nonsynonymous (d_N) was 0.471, with a d_N/d_S ratio of 0.621 (Table 3). The rate of synonymous changes for *V. parahaemolyticus* was 0.746 and that for *V. vulnificus* was 0.662. The rate of nonsynonymous changes for *V. parahaemolyticus* and *V. vulnificus* was 0.431 and 0.384, respectively. This resulted in a d_N/d_S

Table 2 Primers used in this study

Locus	Gene	Primer name	Primer sequence
pilA	VP2523	Shorter5′ VP2523 *Spe*I P1	5′-GATAATTGGGGGCATATCAACCTCTATAGTTTG-3′
		New3′VP2523 *Not*I P4	5′-ACCATGGGTGCATTCGTTGCAACCATCTGGATT-3′
		VP2523 sequence	5′-GCAACTTTCTACCAAAGAGTTTTACCTCACTCG-3′
	VV2278	VV pilA seq primer	5′-GTAAGTAACCAGATGTAAATAAAG-3′
		3′VV pilA seq P2	5′-GCCAAAAATCGCGCTTAGCTG-3′
mshA	VP2698	New5′VP2698 *Spe*I P1	5′-CGTAAACGCATTAAAGCCGCGATGCGCTATCCG-3′
		New3′VP2698 *Not*I P4	5′-CCATTAAGGTGAAACCACGAGTTTTCATTCAGT-3′
		5′VP2698 seq2	5′-TCGTCATTCTGCTCAAGCGGTAGA-3′
	VV2940	VV mshA seq primer	5′-CAAATGCTAAATGTACTTATATTC-3′
		VV 3′ mshA seq P2	5′-CTGCCAGTGCCAATATAGCGACTG-3′

of 0.577 for *V. parahaemolyticus* and 0.580 for *V. vulnificus* (Table 3). For *pilA*, the rate of synonymous changes was 1.109 for *V. cholerae*, 1.691 for *V. parahaemolyticus*, and 1.186 for *V. vulnificus*. The rate of nonsynonymous changes was 0.629 for *V. cholerae*, 0.642 for *V. parahaemolyticus* and 0.503 for *V. vulnificus*. This resulted in a d_N/d_S of 0.567, 0.380, and 0.424 for *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, respectively (Table 3).

Region Analyses

To compare the diversity of *mshA* and *pilA*, we examined neighboring genes from their respective operons, *mshC* and *pilB*, as well as the type IV pilin peptidase, *pilD*. The rate of synonymous and nonsynonymous changes for *mshC* was 0.135 and 0.039 for *V. cholerae*, 0.229 and .017 for *V. parahaemolyticus*, and 0.042 and 0.015 for *V. vulnificus*. This resulted in a d_N/d_S ratio of 0.290 for *V. cholerae*, 0.072 for *V. parahaemolyticus*, and 0.356 and *V. vulnificus* (Table 3). For *pilB*, the rates of synonymous and nonsynonymous for *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* was 0.176 and 0.008, 0.288 and 0.037, and 0.208 and 0.016 respectively. The d_N/d_S ratio for *pilB* was 0.047 for *V. cholerae*, 0.127 for *V. parahaemolyticus*, and 0.074 for *V. vulnificus*. For *pilD*, the synonymous and nonsynonymous rates calculated for *V. cholerae* were 0.122 and 0.005 with a d_N/d_S of 0.039. The *V. parahaemolyticus* strains used to calculate the synonymous and nonsynonymous rates of substitution for *pilD* had identical sequences; thus, the synonymous and nonsynonymous rates of substitution were zero, and the d_N/d_S ratio cannot be calculated. These rates are comparable with data from Chattopadhyay et al. [46], which calculated the rates of synonymous and nonsynonymous substitutions for *pilD* from *V. vulnificus* as 0.092 and 0.007 with a d_N/d_S ratio of 0.076.

TcpA and TcpJ

To compare the findings for *mshA* and *pilA* with another type IV pilin and its corresponding peptidase, we calculated the rates of synonymous and nonsynonymous substitutions for the toxin co-regulated pilus pilin subunit *tcpA* from *V. cholerae* and its processing leader peptidase *tcpJ* (Table 3). Only 13 *V. cholerae* strains out of the available 25 possess *tcpA* and *tcpJ*. The d_S and d_N for *tcpA* was 0.486 and 0.052 with a d_N/d_S ratio of 0.106. For *tcpJ*, the d_S and d_N was 0.003 and 0.000 with a d_N/d_S ratio of 0.000.

Discussion

The results from our sequence analyses of the *mshA* and *pilA* genes from several strains of three human pathogenic *Vibrio* species, *V. cholerae, V. parahaemolyticus*, and *V. vulnificus*, suggested that the various alleles observed were the result of selective pressure. When examining the *V. cholerae* predicted amino acid alignment (Fig. 1a) and phylogenetic tree (Fig. 3a) for the *mshA* gene, one distinct grouping emerged with highly conserved sequences for the MSHA pilin subunit. In fact, the isolates in this group, identifiable as one branch of the phylogenetic tree (Fig. 3a), were primarily from the O1 serogroup (13 out of 15) and clinical isolates (11 out of 15). This differs considerably from the remaining *V. cholerae* isolates examined, which were predominately environmental, non-O1/O139 strains (9 out of 10) with no apparent grouping pattern in the phylogenetic tree (Fig. 3a). When comparing the predicted amino acid alignments and phylogenetic trees for the *V. parahaemolyticus* (Figs. 1b and 3b) and *V. vulnificus* (Fig. 1c and 3c) strains sequenced, no grouping could be established based on either isolation source or phenotype, in contrast to what was observed for *V. cholerae*.

Figure 1 Amino acid sequence alignment of MshA from *Vibrio cholerae* (**a**), *Vibrio parahaemolyticus* (**b**), and *Vibrio vulnificus* (**c**). The predicted amino acid sequence alignments of MshA for *V. cholerae* (**a**), *V. parahaemolyticus* (**b**), and *V. vulnificus* (**c**) were constructed using the ClustalW program. *White* indicates normal residues. *Green* are similar residues. *Pink* are identical residues. *Black* indicates globally conserved residues

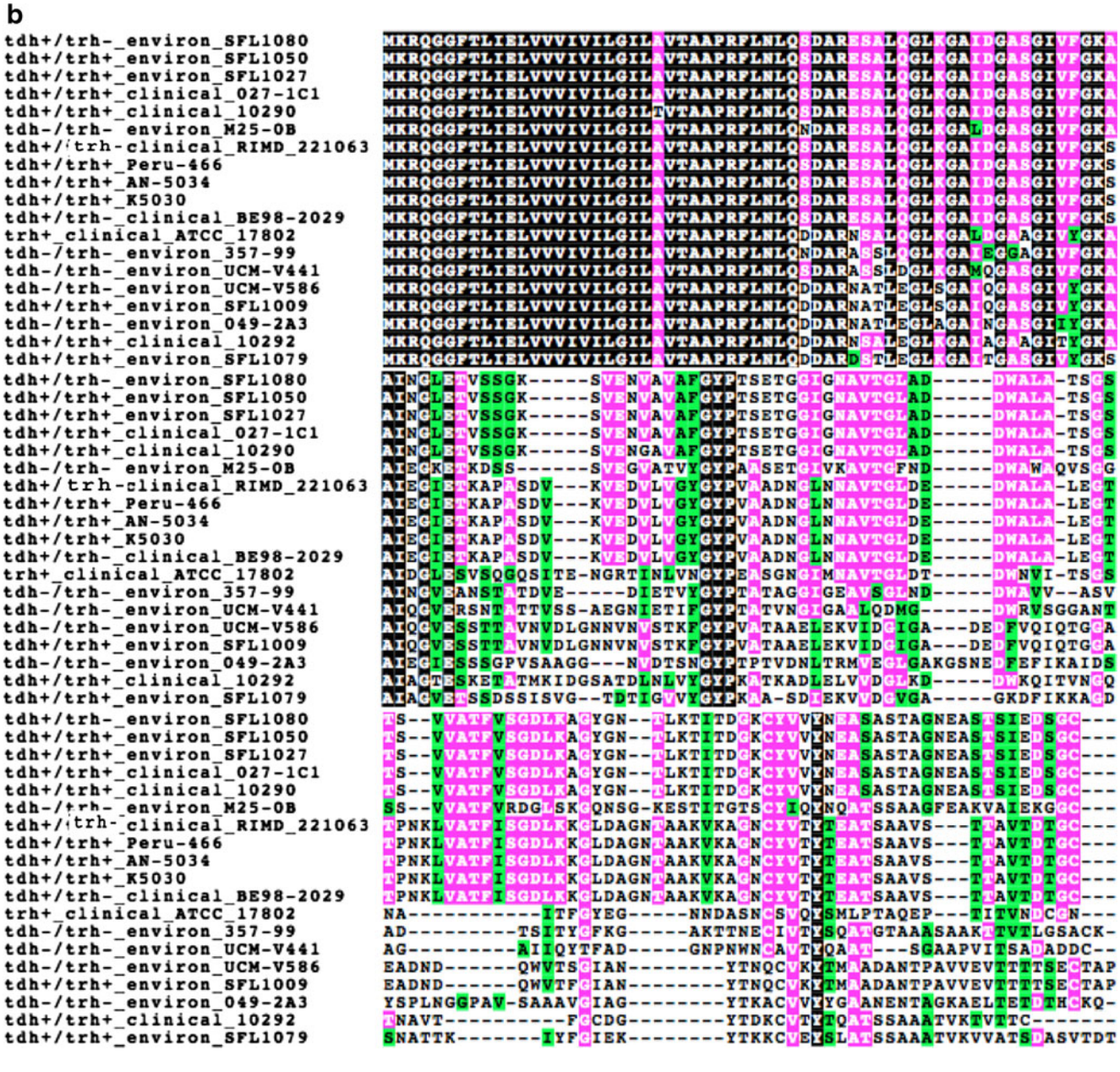

Figure 1 (continued)

Reviewing the sequence data for the PilA pilin subunit from *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, the *pilA* sequences exhibited a trend similar to what was observed for the MSHA pilin subunit. For *V. cholerae* strains, a group of highly conserved PilA sequences emerged and were primarily from the O1 serogroup (13 out of 14) and of clinical origin (11 out of 14). The remaining isolates were predominately non-O1/O139 (10 out of 11) and from an environmental source (6 out of 11). They did not have any clear pattern to their alignment (Fig. 2a) or tree branching (Fig. 4a). Consistent with the *mshA* findings, no apparent grouping pattern was observed for either the amino acid alignment or branching on the phylogenetic tree for any of the *V. parahaemolyticus* (Figs. 2b and 4b) and *V. vulnificus* (Figs. 2c and 4c) *pilA* genes sequenced. Taken together, our hypothesis is that a selective pressure has caused the differences observed in these two type IVa pili in *V. cholerae*, *V. parahemolyticus*, and *V. vulnificus*.

To test for selective pressure, the synonymous and non-synonymous nucleotide substitution rates were calculated to determine a d_N/d_S ratio [41]. In protein-coding sequences, synonymous substitutions (d_S) are structurally silent, while nonsynonymous substitutions (d_N) result in a change to the amino acid sequence. When a d_N/d_S ratio is calculated,

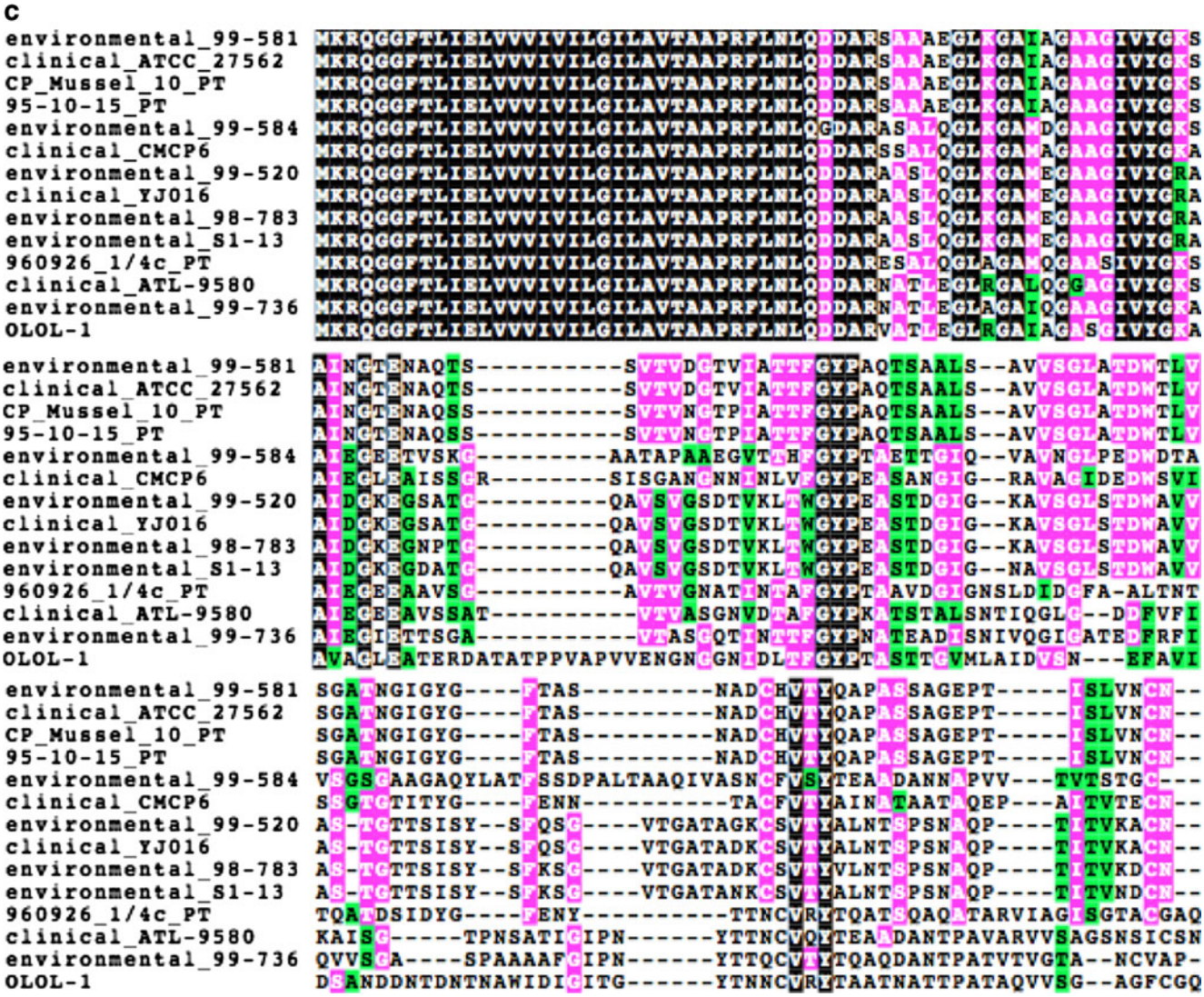

Figure 1 (continued)

typically the value suggests whether the substitutions are largely neutral ($d_N/d_S=1$), under a negative selection ($d_N/d_S<1$), or a positive selection ($d_N/d_S>1$) [44]. Table 3 shows the calculations for d_S, d_N, and d_N/d_S for the *mshA* and *pilA* genes from the different *Vibrio* strains analyzed, and the data suggest that a selective pressure has been applied to these two genes for all three *Vibrio* species. To further analyze the selective pressure applied to the type IV pili examined, we compared *mshA* and *pilA* with another gene in their corresponding operon, the neighboring genes *mshC* gene and *pilB*, respectively, to determine if a selective pressure has been applied strictly to the gene encoding the pilin subunit or to the entire operon. When comparing the d_N/d_S value for *mshA* with *mshC* and *pilA* with *pilB* for all three vibrios, the d_N/d_S values for the pilin subunits (*mshA* and *pilA*) are considerably larger than the neighboring gene in the operon (*mshC* and *pilB*) (Table 3). These results suggest that the neighboring genes (*mshC* and *pilB*) in both the MSHA and PilA operons are more conserved than their corresponding pilin subunits (*mshA* and *pilA*). Thus, it is possible that the pilin subunits are not under the same selective pressure as their neighboring genes.

Both *mshA* and *pilA* encoded pilins are processed by the same type IV prepilin peptidase, *pilD* [27, 28, 45]. When examining the d_N/d_S value for *pilD*, it was evident that the *pilD* gene maintained a highly conserved sequence. We calculated the *pilD* d_N/d_S for *V. cholerae* (0.039) but were unable to calculate it for *V. parahaemolyticus* because the sequences were identical for d_S (0.000) and d_N (0.000) so the d_N/d_S was 0:0 (Table 3). Despite the inability to calculate the d_N/d_S for *V. parahaemolyticus*, the results for *V. cholerae pilD* (0.039) were congruent with what was found for *V. vulnificus* (0.076) by Chattopadhyay et al. [46]. This suggests that a strong purifying selection has maintained the highly conserved *pilD* sequence in contrast to the general observation

Figure 2 Amino acid sequence alignment of PilA from *Vibrio cholerae* (**a**), *Vibrio parahaemolyticus* (**b**), and *Vibrio vulnificus* (**c**). The predicted amino acid sequences of PilA for *V. cholerae* (**a**), *V. parahaemolyticus* (**b**), and *V. vulnificus* (**c**) were aligned using the ClustalW program. *White* indicates normal residues. *Green* are similar residues. *Pink* are identical residues. *Black* indicates globally conserved residues

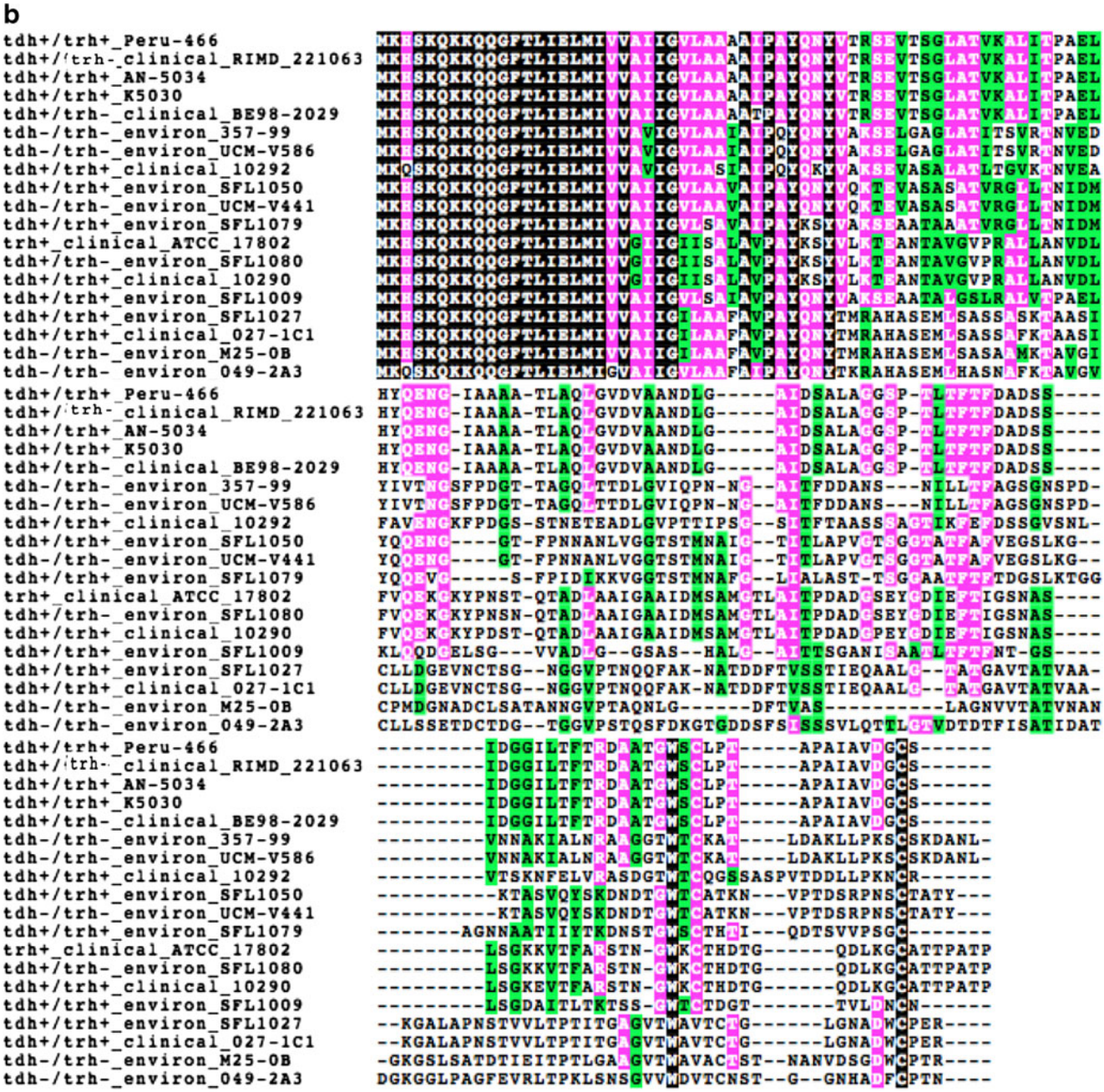

Figure 2 (continued)

for the *mshA* and *pilA* sequences. When examining the predicted amino acid sequences for both *mshA* (Fig. 1) and *pilA* (Fig. 2) for all three vibrios, it was clear that the N-termini remain highly conserved while the C-termini varied considerably. The N-termini region is recognized by the PilD peptidase for processing the protein into a mature pilin subunit [4]. If the N-terminal region of the type IVa pili proteins MSHA and PilA varied, it is possible that PilD would no longer process these proteins into mature subunits, while variations in the C-termini should still result in a mature pilin subunit. Thus, it appears that PilD has maintained a highly conserved sequence unlike the MSHA and PilA proteins it processes.

To further understand the variations observed in the MSHA and PilA pilins, the *V. cholerae mshA* and *pilA* sequences were compared to the type IVb pilin TCP from *V. cholerae*. The *tcpA* gene encodes the major pilin subunit of TCP and is processed by its own type IV pili peptidase TcpJ, encoded by *tcpJ* [23]. Contrary to *tcpA* that exhibit some variability in its sequences with mostly synonymous substitutions (d_S of 0.486) and few nonsynonymous

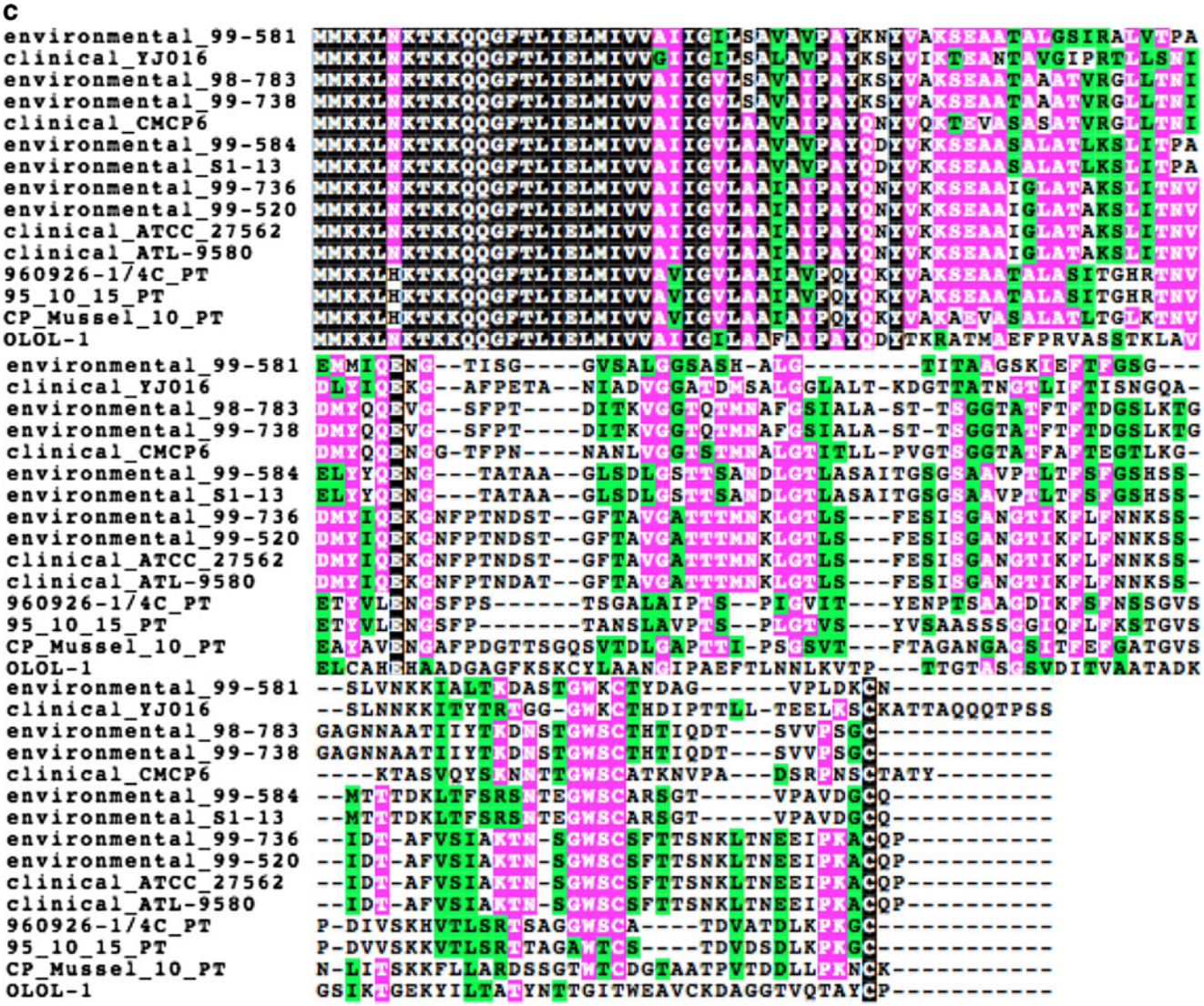

Figure 2 (continued)

substitutions (d_N of 0.052), *tcpJ* has relatively few substations overall (d_S of 0.003 and d_N of 0.000). The d_N/d_S for *tcpA* is 0.106 and that for *tcpJ* is 0.000, suggesting that these genes are under strong negative selection to maintain their sequences and structures. When examining the *V. cholerae* phylogenetic trees constructed for the *mshA* and *pilA* genes, the strains that possess TCP are all from the O1 serogroup and on a single branch (Figs. 3a and 4a). Looking at the amino acid alignment data, it was evident that the *V. cholerae* isolates containing all three type IV pili were highly conserved (Figs. 1a and 2a). To break it down further, the d_N/d_S ratio for *mshA* and *pilA* from the *V. cholerae* strains possessing TCP were also calculated, and the d_S and d_N for both genes were 0.000, resulting in an undefined d_N/d_S ratio (Table 3). Therefore, *V. cholerae* strains possessing all three type IV pili appear to be under a strong purifying selection. Even though some O1 *V. cholerae* isolates in this conserved branch were from environmental or unknown sources (3 out of 13), the fact that they possess TCP implies they could cause cholera. Taken together, the evidence suggests a connection between host interactions and highly conserved type IV pili in *V. cholerae*.

A previous study by Chattopadhyay et al. [46] analyzed *pilA* from 55 *V. vulnificus* strains of various origins and also determined that *pilA* is highly divergent. A total of 25 unique alleles were identified from the 55 analyzed strains, and the authors did not determine any relationship between the various alleles and pathogenicity of *V. vulnificus* [46]. They concluded that the genetic diversity of *pilA* in *V. vulnificus* was higher than neighboring genes (*pilBCD*) and thus was under strong positive, diversifying selection [46]. This conclusion was made despite the fact that the d_N/d_S ratio calculated for *pilA* was <1. The usefulness of the d_N/d_S ratio to detect positive selection is reduced when comparing gene polymorphisms within a single

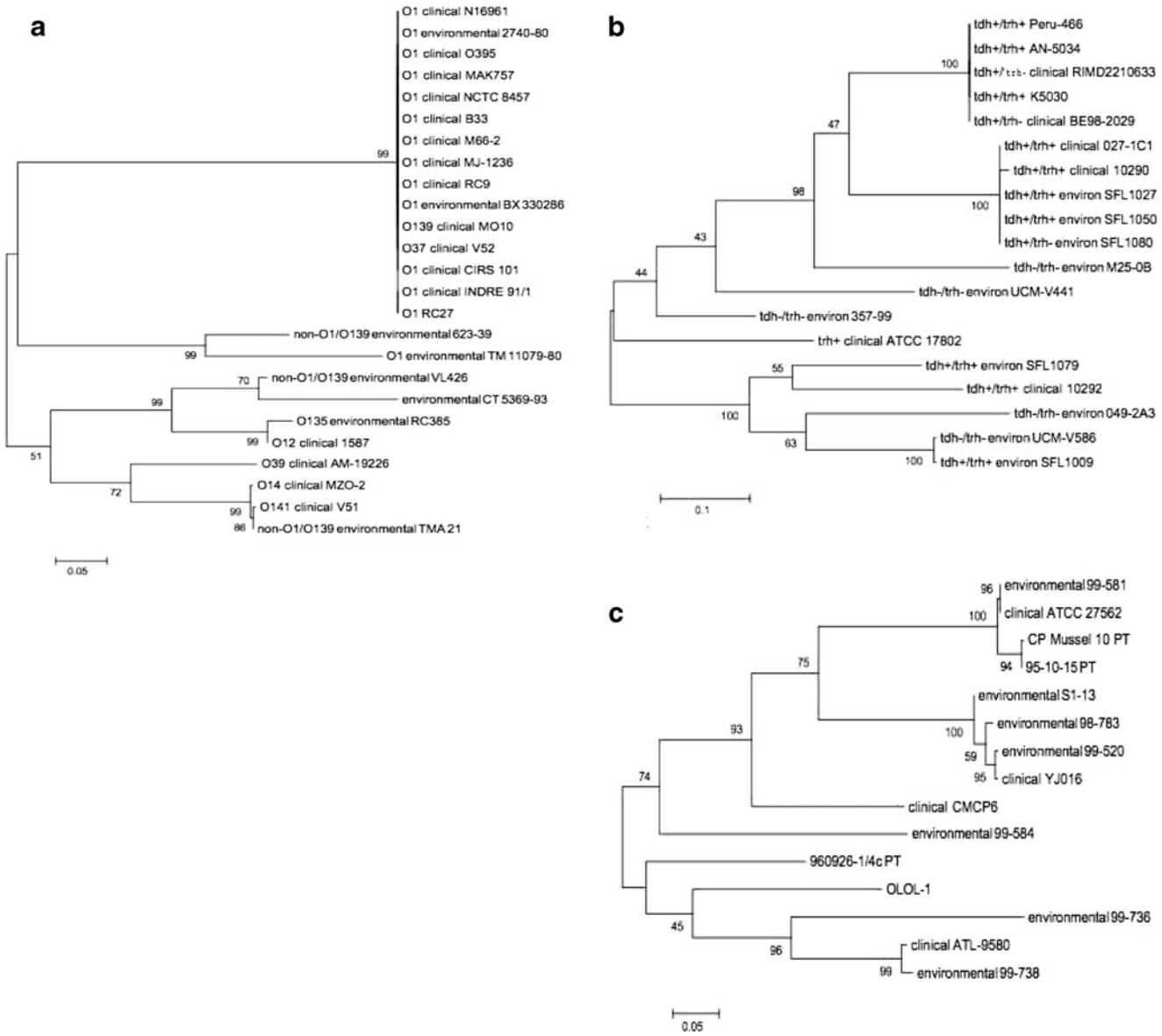

Figure 3 Bootstrap maximum likelihood phylogenetic trees for *mshA* from *Vibrio cholerae* (**a**), *Vibrio parahaemolyticus* (**b**), and *Vibrio vulnificus* (**c**). The bootstrap maximum likelihood phylogenetic trees for *mshA* from *V. cholerae* (**a**), *V. parahaemolyticus* (**b**), and *V. vulnificus* (**c**) were constructed using the gene sequences for *mshA* in the Molecular Evolutionary Genetics Analysis (MEGA) 5 software. All bootstrap values are listed

population compared to divergent populations [47]. Our results are consistent with their findings and also demonstrate that MSHA and PilA from *V. cholerae*, *V. parhaemolyticus*, and *V. vulnificus* exhibit higher genetic diversity than other genes in their corresponding operon (*mshC* and *pilB* and *pilD*).

Chattopadhyay et al. [46] suggested various ideas to explain their observation, including that the allelic variability in PilA for *V. vulnificus* could be the result of oyster innate immune system [46]. It was noted that since *V. vulnificus* commonly associate with shellfish in the environment and infections in humans are typically opportunistic, the selective pressure applied to this gene was probably not in response to an adaptive immune system [46]. Shellfish have an innate immune system that recognizes highly conserved motifs while lacking a well-developed adaptive immunity [48, 49]. Thus, the driving force behind the variations observed in the PilA protein could be the result of the innate immunity of shellfish, such as oysters, in part based on a previous study showing that PilA was involved in oyster colonization by *V. vulnificus* [11, 46]. Data from our laboratory also indicated that PilA and MSHA play a role in *V. parahaemolyticus* colonization of the Pacific oyster, *C. gigas* (Aagesen, A.M., and C.C. Häse, unpublished results), further supporting the idea that the shellfish immune system might be involved in applying pressure to

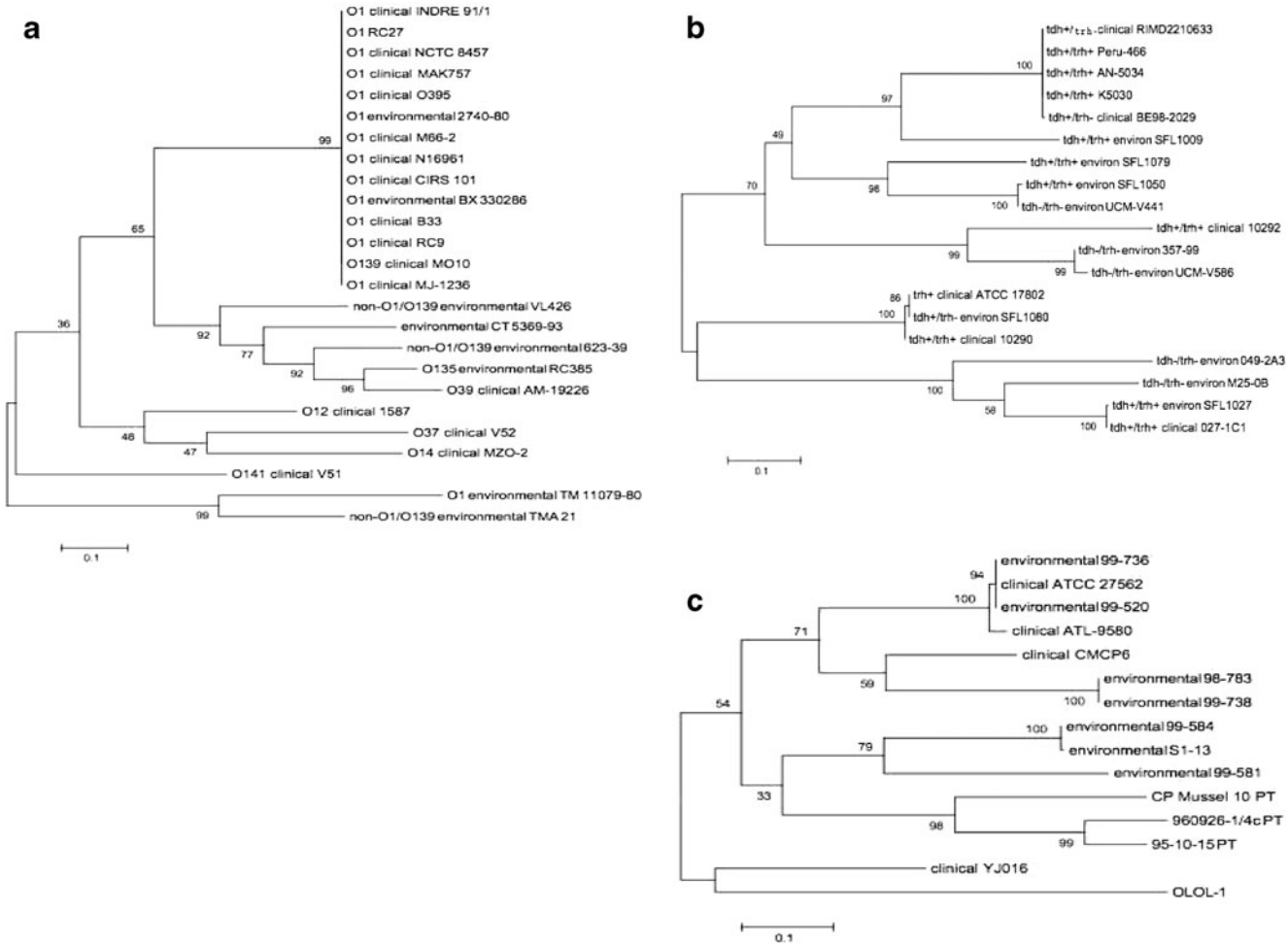

Figure 4 Bootstrap maximum likelihood phylogenetic trees for *pilA* from *Vibrio cholerae* (**a**), *Vibrio parahaemolyticus* (**b**), and *Vibrio vulnificus* (**c**). The bootstrap maximum likelihood trees for *pilA* from *V. cholerae* (**a**), *V. parahaemolyticus* (**b**), and *V. vulnificus* (**c**) were constructed using the gene sequences for *pilA* in the Molecular Evolutionary Genetics Analysis (MEGA) 5 software. All bootstrap values are listed

these pili proteins, thus causing variability. Studies using different strains expressing the various alleles for MSHA and PilA from *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* in shellfish interaction experiments are required to fully address this issue.

In addition to the shellfish immune system, other selective pressures in the environment could exist to cause the observed allelic diversity in MSHA and PilA, such as protozoan grazing, bacteriophages and DNA uptake [46]. Ideally, various alleles for MSHA and PilA from *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* would need to be examined to better understand the role of bacteriophages as a selective pressure causing the variations observed for these proteins. However, future studies using various alleles for MSHA and PilA are required to support these hypotheses.

In summary, this study illustrates significant diversity of the MSHA and PilA pilin subunits from *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. For all three vibrios examined in this study, *mshA* and *pilA* had considerably higher d_N/d_S ratios than any of the other genes examined, suggesting these genes are under a possible positive selection while the other genes examined are not. Another interesting finding was that *V. cholerae* strains that possess TCP also maintain highly conserved MSHA and PilA sequences, suggesting a connection with the host. Even though a selective pressure appears to exist causing the allelic variations observed for *mshA* and *pilA*, the mechanism(s) driving this diversification have yet to be determined. Several suggestions can be made, yet evidence to support these ideas awaits further experimental analyses. In addition, our observations raise an important point about the use of these genes in detection methods for these important human pathogens. In particular, some PCR-based detection methods utilize certain pathogen-associated genes as targets, including type IV pili genes [50, 51]. Realizing that the *Vibrio mshA* and *pilA* genes can be extremely variable at the 3′ ends of the genes is important to consider when designing primers

Table 3 Analysis of synonymous and nonsynonymous nucleotide substitutions for genes involved in type IV Pili function from *V. cholerae, V. parahaemolyticus*, and *V. vulnificus*

Gene locus	Organism	Sequence length (bp)	Number of strains	d_S	d_N	d_N/d_S
mshA	*V. cholerae*	438–537	25	0.759	0.471	0.621
	V. parahaemolyticus	456–504	19	0.746	0.431	0.577
	V. vulnificus	447–510	15	0.662	0.384	0.580
mshC	*V. cholerae*	489–513	25	0.135	0.039	0.290
	V. parahaemolyticus	131 (1–131 5′end)	15	0.229	0.017	0.072
	V. vulnificus	94 (1–94 5′end)	12	0.042	0.015	0.356
pilA	*V. cholerae*	420–504	25	1.109	0.629	0.567
	V. parahaemolyticus	405–486	19	1.691	0.642	0.380
	V. vulnificus	402–453	15	1.186	0.503	0.424
pilB	*V. cholerae*	1,689	24	0.176	0.008	0.047
	V. parahaemolyticus	248 (1–248 5′end)	19	0.288	0.037	0.127
	V. vulnificus	122 (1–122 5′ end)	14	0.208	0.016	0.074
pilD	*V. cholerae*	876	24	0.122	0.005	0.039
	V. parahaemolyticus	870	3	0.000	0.000	–
tcpA	*V. cholerae*	675	13	0.486	0.052	0.106
tcpJ	*V. cholerae*	762	13	0.003	0.000	0.000
mshA	*V. cholerae* with TCP		13	0.000	0.000	–
	V. cholerae without TCP		12	0.663	0.377	0.568
pilA	*V. cholerae* with TCP		13	0.000	0.000	–
	V. cholerae without TCP		12	1.270	0.615	0.484

to target these genes. Therefore, it is possible that a PCR protocol designed to amplify *mshA* and *pilA* from various *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* strains may not detect these genes simply due to the variations observed in this study. This is certainly something to consider when utilizing these genes in a PCR protocol.

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