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Identification of a DNA Methyltransferase Gene Carried on a Pathogenicity Island-Like Element (VPAI) in *Vibrio parahaemolyticus* and Its Prevalence among Clinical and Environmental Isolates

Hui-zhen Wang, Minnie M. L. Wong, Desmond O'Toole, Mandy M. H. Mak, Rudolf S. S. Wu, and Richard Y. C. Kong*

Department of Biology and Chemistry and Research Centre for Coastal Pollution and Conservation, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon Tong, Hong Kong Special Administrative Region, People's Republic of China

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In this study we identified a putative virulence-associated DNA methyltransferase (MTase) gene carried on a novel 22.79-kb pathogenicity island-like element (VPAI) in *V. parahaemolyticus*. The *V. parahaemolyticus* MTase gene was shown by PCR to be prevalent (>98%) in pandemic thermostable direct hemolysin gene-positive isolates, which suggests that VPAI may confer unique virulence traits to pandemic strains of *V. parahaemolyticus*.

Vibrio parahaemolyticus, which is widely distributed in coastal and marine waters, has been the major cause of seafood-borne gastroenteritis in areas where raw or uncooked seafood is consumed (10). Although most *V. parahaemolyticus* strains are harmless to humans and animals, *V. parahaemolyticus* infections are often associated with strains that produce the thermostable direct hemolysin (TDH) and/or TDH-related hemolysin (TRH) (10, 22, 24). While sporadic cases of *V. parahaemolyticus* infections prior to 1996 were caused mainly by diverse serotypes, several major outbreaks of gastroenteritis that were reported since 1996 in many parts of the world, including the United States (2), Europe (19), Asia (5, 6, 22), and Africa (1), were associated with the emergence and pandemic spread of *V. parahaemolyticus* O3:K6 strains. Moreover, an increasing number of *V. parahaemolyticus* outbreaks have been linked to a number of clonal derivatives of O3:K6, including the O4:K68, O1:K25, and O1:KUT serovars (6, 8), which together constitute the so-called "pandemic group." These serovars can be distinguished from nonpandemic strains by the presence of the TDH gene (but not the TRH gene) (22) and by molecular techniques such as pulsed-field gel electrophoresis (7), arbitrarily primed PCR and group-specific PCR of the *toxRS* gene (20), *orf8*-PCR of the f237 filamentous phage (21), and multilocus sequence typing (8). Nevertheless, the occurrence of group-specific PCR-positive but *orf8*-negative pandemic isolates has recently been reported (4, 8, 14), and the group-specific *toxRS* marker has been detected in TDH gene-negative O3:K6 strains of *V. parahaemolyticus* (23).

To date, only a limited number of virulence-associated factors have been described for pandemic strains of *V. parahaemolyticus* (17, 25, 26). Comparison of genomic differences between O3:K6 and nonpathogenic strains by subtractive hybridization could reveal novel mechanisms relevant to the study of the enteropathogenicity of *V. parahaemolyticus*. Here we describe identification of a putative virulence-associated DNA methyltransferase (MTase) gene on a 22.79-kb pathogenicity island-like element on chromosome 1 of *V. parahaemolyticus*.

Identification of a unique genomic locus. The differential subtraction chain method of Luo et al. (16) was used to identify unique genomic sequences of a clinical *V. parahaemolyticus* O3:K6 isolate (strain QM98284) by subtractive hybridization with DNA from an environmental isolate (strain CECT611). Genomic DNAs of clinical strain QM98284 (tester) and environmental strain CECT611 (driver) were digested with SphI, and tester DNA was ligated to adaptors A and B (Table 1); this was followed by 10 PCR cycles consisting of 94°C for 30 s, 60°C for 1.5 min, and 72°C for 1.5 min. Driver fragments (100-fold excess) were added to adaptor-ligated tester fragments, and two rounds of subtractive hybridization were performed. Tester fragments were then amplified by PCR with adaptor-specific primers RDSCA and RDSCB (Table 1) for 40 cycles consisting of 94°C for 30 s, 65°C for 30 s, and 72°C for 1.5 min, and the products were cloned into the pCR2.1-TOPO vector (Invitrogen). Genomic Southern hybridization showed that clone 32 (containing a 254-bp insert) was present only in the tester strain (QM98284) and not in the driver strain (CECT611) (data not shown), and the clone was extended using a universal GenomeWalker kit (Clontech, United States) to produce a 5,195-bp fragment (designated CLONE-32) (Fig. 1). Comparison of CLONE-32 to the genome sequence of *V. parahaemolyticus* RIMD2210633 (17) showed that it exhibited >99% identity with a homologous region on chromosome 1 of this strain at positions 390994 to 396188 (accession no. NC_004603).

* Corresponding author. Mailing address: Department of Biology and Chemistry, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon Tong, Kowloon, Hong Kong SAR, China. Phone: 852-2788-7794. Fax: 852-2788-7406. E-mail: bhrkong@cityu.edu.hk.

TABLE 1. Gene-specific PCR primers and adaptor sequences used in this study

Target gene	Primer	Sequence (5′–3′)	Expected product size (bp)	Reference or source
TDH	Tdh-F	ATCTGTCCCTTTTCCTGCCC	457	26
	Tdh-R	TTCTTTGTTGGATATACACATTACC		
TRH	Trh-F	CTACTTTGCATTCAGTTTGC	355	12
	Trh-R	CTCTGATTTTGTGAAGACCG		
MTase	MTase-F	GTCTTGTCGAATAGAACTCTGA	683	This study
	MTase-R	TAAGCTCCAAAATCCATACG		
TTSS2	TTSS-F	GGAAGCCATTGCGAAAGATA	1,250	17
	TTSS-R	CACTCTCTTGTTGTTGCGTGA		
16S-23S rRNA gene intergenic spacer	Vpara-F	GCTGACAAAACAACAATTTATTGTT	174	13
	Vpara-R	GGAGTTTCGAGTTGATGAAC		
Putative ABC transporter (VP0379) and RpoD (VP0404)	VP0379-F	CTTGTTTTGCCGCTTCTTTC	900	17
	VP0404-R	CAACAGCAACAAGCCTGAA		
Differential subtraction chain fragment	RDSCA	GTCGCGGCCGCTAATACGACTC		
	RDSCB	GCCGGCAGATCGATACAGATG		
Adaptor A	Longer strand	GTCGCGGCCGCTAATACGACTCACTATAGG GACATG		
	Shorter strand	AGGGATATCACTCAGCATAATC		
Adaptor B	Longer strand	GCCGGCAGATCGATACAGATGTGGGACATG		
	Shorter strand	AGGGTGTAGACAGTAGCTAGA		

Characterization of the *V. parahaemolyticus* MTase gene. The *V. parahaemolyticus* MTase gene encodes a 233-amino-acid protein (Fig. 2), and a homology search showed that this protein exhibits a high level of amino acid identity (72%) with the mannose-fucose-resistant hemagglutinin (MFRHA) of *Vibrio cholerae* O1 (9). Analysis of the *V. parahaemolyticus* MTase and MFRHA deduced proteins using an HMM (hidden Markov models) search (at http://pfam.wustl.edu/hmmsearch.shtml) against the Pfam protein family database (3) showed that both proteins contain nine

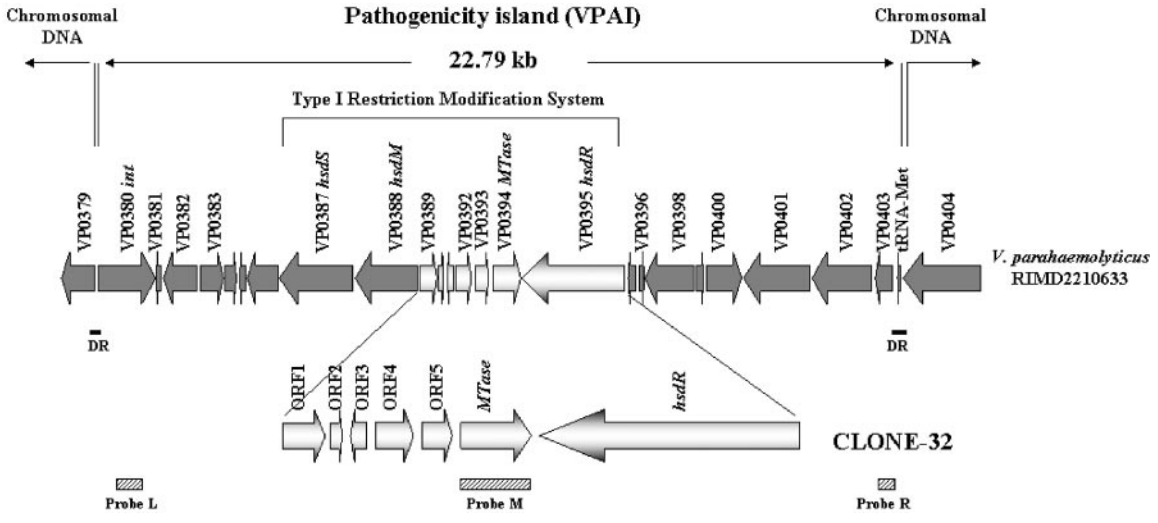

FIG. 1. Physical map of the pathogenicity island (VPAI) on chromosome 1 of *V. parahaemolyticus* encompassing the CLONE-32 sequence. Predicted ORFs are indicated by arrows, and the orientations show the directions of transcription. Gene designations assigned by the sequencing project of Makino et al. (17) are indicated above the arrows. Open arrows indicate ORFs in CLONE-32. DR indicates the 47-bp direct repeat sequences flanking VPAI. The positions of gene probes L, M, and R for Southern hybridization are indicated. Abbreviations: *MTase*, DNA methylase gene; *hsdM*, gene encoding type I restriction-modification system methylation subunit M; *hsdS*, gene encoding type I restriction-modification system specificity subunit S; *hsdR*, gene encoding type I restriction-modification system restriction subunit R.

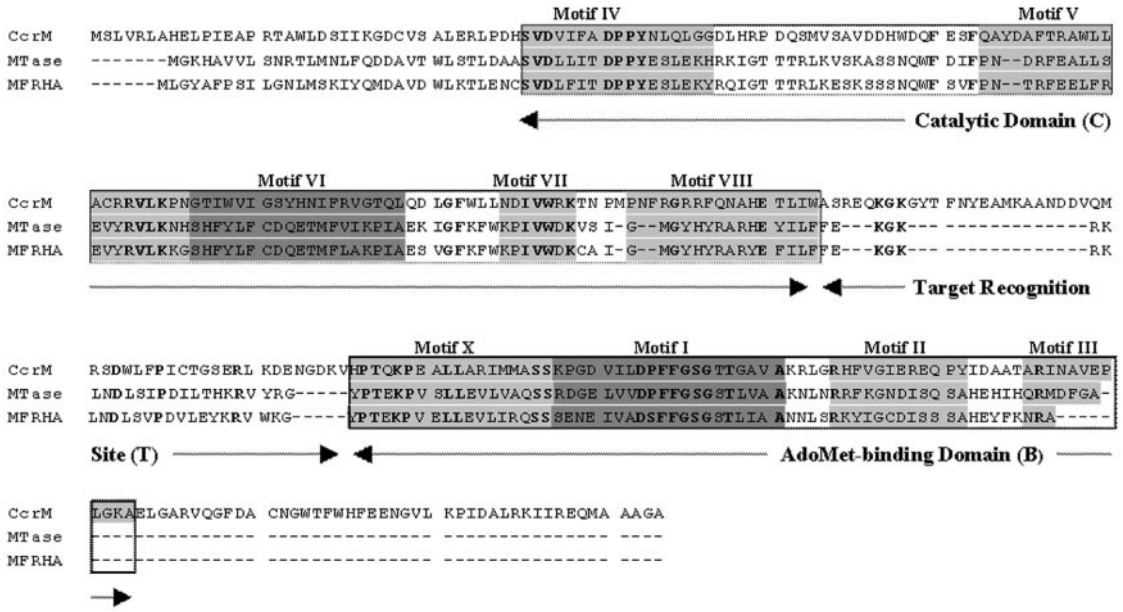

FIG. 2. Sequence alignment and domain organization of the *V. parahaemolyticus* MTase, MFRHA, and CcrM proteins. Conserved motifs are indicated by a gray background. The catalytic domain (C; consisting of motifs X, I, II, and II) and the AdoMet-binding domain (B; consisting of motifs IV, V, VI, VII, and VIII) are enclosed in boxes. The order of these domains is typical for β-class methyltransferases (18). Amino acids are designated by single-letter codes. Dashes indicate gaps inserted into the sequences for improved alignment. The proteins are highly conserved (72% identity, 90% similarity). The accession numbers for the CcrM (*Brucella abortus*), MTase (*V. parahaemolyticus*), and MFRHA (*V. cholerae*) protein sequences are AAB71351, VP0394, and X64097, respectively.

highly conserved sequence motifs (Fig. 2) typical of N4-N6 MTases (18). Specifically, motif I (DXFXGXG, where X is any amino acid residue) and motif IV [(D/N/S)PP(Y/F)] are the two most highly conserved motifs, and they are essential for *S*-adenosyl-L-methionine (AdoMet) binding and catalytic activity, respectively. Based on the arrangement of the nine conserved motifs, the linear order of the AdoMet-binding, catalytic, and target recognition domains, and alignment with the well-known cell cycle-regulated methyltransferase (CcrM; a group β MTase found so far only in α-proteobacteria [15]), it seems likely that *V. parahaemolyticus* MTase and MFRHA are members of the β class of m^6-*N*-adenine MTases (11, 18). The fact that a mutant of *V. cholerae* defective in MFRHA exhibited significant attenuation in virulence potential (9) suggests that the MTase gene has an important role in virulence, and by extrapolation, it is likely that the *V. parahaemolyticus* MTase gene also has a similar pathogenic role in *V. parahaemolyticus*.

Association of the *V. parahaemolyticus* MTase gene with a pathogenicity island. Inspection of the sequences flanking the CLONE-32 locus in the *V. parahaemolyticus* RIMD2210633 genome resulted in identification of a putative phage-like integrase (*int*) gene (VP0380) 9,936 bp upstream of *hsdS* (VP0387) and a methionine-specific tRNA ($tRNA^{Met}$) gene sequence 7,656 bp downstream of *hsdR* (VP0395). Further analysis of this 22.79-kb genomic region revealed that it is flanked by a 47-bp direct repeat sequence (Fig. 1). These features suggest that CLONE-32 is carried on a pathogenicity island-like element (designated VPAI) that contains 25 open reading frames (ORFs) (Table 2). Ten of these ORFs were homologous to genes having known functions, including the genes encoding the type I restriction-modification complex (VP0387, VP0388, and VP0395), *V. parahaemolyticus* MTase (VP0394), phage P4-like integrase (VP0380), and proteins involved in DNA replication (VP0400), transcription regulation (VP0399), signal transduction (VP0382), and general metabolism (VP0386 and VP0392). Further analysis showed that the mean G+C content of the 25 coding genes of VPAI (43.79% ± 3.93%) is significantly lower than (but within 1 standard deviation of) the mean G+C content of the ca. 5,000 coding genes of the complete genome (accession no. NC_004603 and NC_004605; 4,992 genes; G+C content, 45.8% ± 4.19%) of *V. parahaemolyticus* ($P < 0.05$, as determined by a one-tailed *t* test), which suggests that VPAI was recently acquired by lateral gene transfer.

To define the chromosomal insertion site of VPAI, primers VP0379-F and VP0404-R flanking VPAI (Fig. 1 and Table 1) were used in PCRs with a number of *V. parahaemolyticus* MTase gene-negative and -positive isolates of *V. parahaemolyticus*. As expected, no PCR product was detected for the *V. parahaemolyticus* MTase gene-positive isolates examined (since VPAI is too large to be amplified), while a 0.9-kb PCR product was obtained for 10 of the 15 *V. parahaemolyticus* MTase gene-negative isolates tested (Table 3). DNA sequencing showed that the 22.79-kb VPAI is precisely inserted into the 47-bp direct repeat sequence at the 3′ end of the methionine tRNA gene (Fig. 1), and this was corroborated by Southern blot analysis of *V. parahaemolyticus* MTase gene-positive clinical (pandemic and nonpandemic) isolates and *V. parahaemolyticus* MTase gene-negative clinical and environmental isolates (Table 3).

TABLE 2. Characteristics of the 25 ORFs on VPAI of *V. parahaemolyticus* O3:K6

ORF	Length of protein (amino acids)	Homology as determined by BLASTP search	Amino acid identity (%)	*E* value
VP0379	267	ABC-type metal ion transport system, periplasmic component/surface antigen (*Rhodospirillum rubrum*)	48	9e-51
VP0380	455	Phage integrase (*Polaromonas* sp. strain JS666)	25	3e-29
VP0381	33	No significant match		
VP0382	263	Metallophosphoesterase (*Shewanella amazonensis* SB2B)	25	2e-11
VP0383	179	Hypothetical protein plu3931 (*Photorhabdus luminescens* subsp. *laumondii* TTO1)	58	2e-47
VP0384	95	Hypothetical protein (*Dictyostelium discoideum*)	25	0.14
VP0385	39	No significant match		
VP0386	245	Putative inner membrane protein (*Salmonella enterica* serovar Typhimurium LT2)	60	3e-81
VP0387	583	Type I restriction specificity (S) subunit (*Vibrio vulnificus* CMCP6)	45	8e-98
VP0388	496	Type I restriction DNA methylase (M) subunit (*Vibrio vulnificus* CMCP6)	99	0.0
VP0389	132	Hypothetical protein VV12032 (*Vibrio vulnificus* CMCP6)	92	3e-49
VP0390	36	Hypothetical protein MG00497.4 (*Magnaporthe grisea* 70-15)	42	3.3
VP0391	49	Hypothetical protein VFA0532 (*Vibrio fischeri* ES114)	61	4e-08
VP0392	121	Translation elongation factor Ts (*Vibrio vulnificus* CMCP6)	94	3e-52
VP0393	94	ORF23 (*Vibrio cholerae*)	83	5e-40
VP0394	221	Putative DNA methyltransferase X64097 (*Vibrio cholerae*)[a]	72	2e-101
VP0395	814	Type I restriction enzyme (*Vibrio vulnificus* CMCP6)	97	0.0
VP0396	61	Hypothetical protein ENSANGP00000010383 (*Anopheles gambiae* strain PEST)	31	9.7
VP0397	33	No significant match		
VP0398	385	Hypothetical protein VV12042 (*Vibrio vulnificus* CMCP6)	24	3e-23
VP0399	54	Putative transcriptional regulator (*Vibrio vulnificus* CMCP6)	61	4e-09
VP0400	271	DNA topoisomerase I:restriction endonuclease (*Polaromonas* sp. strain JS666)	36	1e-37
VP0401	529	Conserved hypothetical protein (*Nitrosomonas eutropha* C71)	23	7e-17
VP0402	473	Hypothetical protein lpp1915 (*Legionella pneumophila* strain Paris)	38	2e-75
VP0403	111	Hypothetical protein lpg1935 (*Legionella pneumophila* subsp. *pneumophila* strain Philadelphia 1)	31	1e-06

[a] The identity of the putative X64097 protein is based on the analysis described in this study.

Distribution of the genes encoding *V. parahaemolyticus* MTase, TDH, TRH, and type III secretion system 2 (TTSS2). The clinical isolates of *V. parahaemolyticus* used in this study were kind gifts from W. C. Yam (University of Hong Kong, Hong Kong), H. C. Wong (Soochow University, Taiwan), and G. B. Nair (International Centre for Diarrheal Disease Research, Bangladesh). *V. parahaemolyticus* strains were confirmed by PCR using the species-specific primers Vpara-F and

TABLE 3. Distribution of VPAI in *V. parahaemolyticus* MTase gene-positive and *V. parahaemolyticus* MTase gene-negative strains of *V. parahaemolyticus*

Strain	Serotype	*V. parahaemolyticus* MTase gene PCR product (683 bp)	Presence of 0.9-kb PCR product obtained with VPAI-screening primers[a]	Southern hybridization result for[b]:		
				VP0380 (probe L)	*V. parahaemolyticus* MTase (probe M)	VP0403 (probe R)
QM98284	O3:K6	+[c]	−	+	+	+
QM115996	O3:K6	+	−	+	+	+
AN-2416	O3:K6	+	−	+	+	+
AO-97	O3:K6	+	−	+	+	+
AP-24659	O1:K56	−	+	−	−	−
AP-19569	O5:KUT	−	+	−	−	−
TW-271	K8	−	+	−	−	−
TW-288	K6	−	+	−	−	−
TW-421	K29	−	+	−	−	−
TW-491	K15	−	+	−	−	−
QM-113892	Unknown	−	+	−	−	−
QM-113890	Unknown	−	+	−	−	−
T8-9	Unknown	−	+	−	−	−
T1-41	Unknown	−	+	−	−	−
QM-112998	Unknown	−	−	−	−	−
AP-33593	O4:K55	−	−	−	−	−
TW-414	K29	−	−	−	−	−
TW-435	K60	−	−	−	−	−
T7-25	Unknown	−	−	−	−	−

[a] VPAI-screening primers (VP0379-F and VP0404-R) located outside VPAI (Fig. 1) are expected to amplify a 0.9-kb fragment from *V. parahaemolyticus* strains that lack VPAI.
[b] See Fig. 1 for the locations of the three VPAI hybridization probes.
[c] +, gene amplimer or Southern hybridization signal present; −, gene amplimer or Southern hybridization signal absent.

TABLE 4. Source and characteristics of the clinical and environmental *V. parahaemolyticus* isolates used in this study

Source or strain (no. of strains)	Origin	Serovar	Genotype			
			TDH gene	TRH gene	TTSS2 gene	*V. parahaemolyticus* MTase gene
Pandemic strains (55)						
Human (11)	Hong Kong	O3:K6	+	−	+	+
Human (22)	Bangladesh	O3:K6	+	−	+	+
Human	Bangladesh	O3:K6	+	−	+	−
Human	Hong Kong	O4:K68	+	−	+	+
Human (12)	Bangladesh	O4:K68	+	−	+	+
Human (5)	Bangladesh	O1:KUT	+	−	+	+
Human (3)	Bangladesh	O1:K25	+	−	+	+
Nonpandemic strains (53)						
Human (2)	Bangladesh	O5:KUT	+	−	+	+
Human	Bangladesh	O5:KUT	+	−	+	−
Human	Bangladesh	O5:KUT	−	−	−	−
Human (3)	Bangladesh	O1:K56	+	−	+	−
Human	Bangladesh	O1:K56	−	+	−	−
Human	Bangladesh	O4:K55	−	+	−	−
Human	Bangladesh	O4:K11	−	+	−	−
Human (2)	Hong Kong	ND[a]	−	+	−	−
Human	Hong Kong	ND	+	+	−	−
Human (8)	Hong Kong	ND	+	−	+	−
Human (8)	Hong Kong	ND	+	−	+	+
Human	Taiwan[b]	K8	+	−	+	−
Human (3)	Taiwan	K3	+	−	+	−
Human (5)	Taiwan	K29	+	−	+	−
Human (2)	Taiwan	K60	+	−	+	−
Human (5)	Taiwan	K15	+	−	+	−
Human	Taiwan	K10	+	−	+	−
Human	Taiwan	K4	+	−	+	−
Human	Bangladesh	O8:K22	+	−	+	+
Human	Bangladesh	O4:K22	+	−	+	+
Human	Bangladesh	O1:K38	+	−	+	+
Human	Bangladesh	O4:K10	+	−	+	+
Human	Bangladesh	O2:K3	+	−	+	+
Human	Bangladesh	O3:K29	+	−	+	+
Environmental strains (39)						
CECT611 (marine)	Spain	ND	−	−	−	−
CECT612 (marine)	Spain	ND	−	−	−	−
CECT613 (plankton)	Spain	ND	−	−	−	−
Seawater (35)	Hong Kong	ND	−	−	−	−
T4-1 (marine)	Hong Kong	ND	−	−	−	+

[a] ND, serotype unknown.
[b] All Taiwanese strains examined in this study were isolated before 1996.

Vpara-R (Table 1) (13). Serotyping of *V. parahaemolyticus* was performed using commercial antisera purchased from Denka Seiken Co., Ltd., Tokyo, Japan. PCRs were carried out in 100-μl mixtures containing 50 ng DNA, each primer at a concentration of 0.2 to 0.25 μM, each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP) at a concentration of 0.2 mM, 1.5 mM $MgCl_2$, and 5 U of *Taq* DNA polymerase. Each PCR consisted of an initial denaturation step at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min. The PCR products were analyzed by agarose gel electrophoresis.

A total of 108 clinical isolates of *V. parahaemolyticus* (55 pandemic strains consisting of 34 O3:K6, 13 O4:K68, 3 O1:K25, and 5 O1:KUT isolates and 53 nonpandemic strains having diverse serotypes) and 39 environmental isolates (Table 4) were tested by PCR for the presence of the *V. parahaemolyticus* MTase gene and the three known virulence genes, the genes encoding TDH, TRH, and TTSS2 of *V. parahaemolyticus* (17, 25). As reported previously (17), the TTSS2 gene was detected by PCR in all TDH gene-positive pandemic and nonpandemic strains. The *V. parahaemolyticus* MTase gene was detected in 98.2% (54/55) of TDH gene-positive pandemic strains and in 55.2% (16/29) of TDH gene-positive nonpandemic strains but was not detected in any of the TDH gene-negative clinical strains examined. Intriguingly, all TDH gene-positive "prepandemic" clinical isolates (collected before 1996) from Taiwan (H. C. Chung, personal communication) were PCR negative for the *V. parahaemolyticus* MTase gene (Table 4), and a few strains were confirmed to lack VPAI in the genome by PCR and Southern blot analysis (Table 3). The *V. parahaemolyticus* MTase gene was detected in only 2.56% (1/39) of the TDH gene-negative environmental isolates examined. Taken together, these results indicate that the *V. para-*

haemolyticus MTase gene (and/or VPAI) may confer unique virulence or fitness traits to the TDH gene-positive pandemic strains of *V. parahaemolyticus*, enabling them to cause disease more frequently than strains belonging to other serovars. However, it is possible that some other ORFs (which encode hypothetical proteins whose functions are not known) in VPAI may code for virulence-associated factors. Clearly, further experiments are needed to understand the relevance of VPAI (on chromosome 1) and its relationship to the TDH gene (on chromosome 2) for the pathogenesis and virulence potential of *V. parahaemolyticus*.

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