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Characterization of a *Vibrio alginolyticus* Strain, Isolated from Alaskan Oysters, Carrying a Hemolysin Gene Similar to the Thermostable Direct Hemolysin-Related Hemolysin Gene (*trh*) of *Vibrio parahaemolyticus*▽

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A *Vibrio* strain isolated from Alaskan oysters and classified by its biochemical characteristics as *Vibrio alginolyticus* possessed a thermostable direct hemolysin-related hemolysin (*trh*) gene previously reported only in *Vibrio parahaemolyticus*. This *trh*-like gene was cloned and sequenced and was 98% identical to the *trh2* gene of *V. parahaemolyticus*. This gene seems to be functional since it was transcriptionally active in early-stationary-phase growing cells. To our knowledge, this is the first report of *V. alginolyticus* possessing a *trh* gene.

Vibrio parahaemolyticus is a gram-negative, estuarine bacterial species and is the leading cause of seafood-associated bacterial gastroenteritis worldwide (3, 4). During a *V. parahaemolyticus* outbreak associated with Alaskan oysters in 2004 (12), numerous *V. parahaemolyticus* strains were isolated from oysters. Many of these strains carried both thermostable direct hemolysin (*tdh*) and thermostable direct hemolysin-related hemolysin (*trh*) genes, which are associated with pathogenicity (1, 11, 13, 17). The *tdh* gene can be subdivided by sequence similarities into five subtypes (*tdh1* through *tdh5*), sharing 96 to 98% identity (14). In contrast, there exists among the *trh* genes a significant nucleotide sequence variation, and they can be clustered into two main subgroups, *trh1* and *trh2*, which share 84% identity (9).

During investigation of the Alaskan *V. parahaemolyticus* outbreak of 2004, two strains isolated from oysters tested positive for the presence of the *trh* gene but were negative for the presence of the *tlh* gene (thermolabile hemolysin), reportedly in all *V. parahaemolyticus* strains (2, 21, 22, 26). These strains were also urease positive, indicating the presence of the *ure* gene, which is genetically linked to the *trh* gene in *V. parahaemolyticus* (6, 7). The *trh* gene was detected using an alkaline phosphatase-labeled DNA probe designed specifically for the detection of *trh* (16). The presence of the *trh* but not the *tlh* gene in those isolates was very unusual, since the *trh* gene has been reported only in *V. parahaemolyticus*. These results suggest that vibrios other than *V. parahaemolyticus* could be a reservoir for *trh* in the environment.

In the present study, we described the characterization of a *Vibrio alginolyticus* strain isolated from Alaskan oysters that possesses and expresses a *trh* gene with 98% homology to the *trh2* gene of *V. parahaemolyticus*.

Bacterial strains and phenotypic characterization. *V. parahaemolyticus* strain 93A-5807 (clinical isolate) was obtained from the GCSL culture collection. This strain and those described below were originally isolated from thiosulfate citrate bile sucrose agar. Individual colonies were selected and streaked for purification on T1N1 agar as recommended in the FDA's Bacteriological Analytical Manual (BAM) (FDA/CFSAN; http://www.cfsan.fda.gov/~ebam/bam-9.html). Single-well-isolated colonies were selected for all phenotypic and genotypic assays. *V. parahaemolyticus* strains Vp 28 (AK 2228-1 0826), Vp 32 (AK 2162-1B 1054B), and Vp 33 (AK 2162-1A 1054A) were isolated from Alaskan oysters during the 2004 outbreak and were *tdh*, *trh*, *tlh*, and urease positive. The presence of *tdh* and *tlh* was determined using alkaline phosphatase-labeled DNA probe colony hybridization as described in the BAM. The presence of *trh* was determined using alkaline phosphatase-labeled DNA probe colony hybridization (16). This assay was conducted twice to confirm that there was no error in the detection of this gene. Urease production was determined as recommended in the BAM. *V. alginolyticus* ATCC 33787 was kindly provided by Marlene Janes of Louisiana State University. *V. alginolyticus* strains Va 29 (AK 1296-A2-1 1296) and Va 30 (AK 2208-1B 1073B) were also isolated from Alaskan oysters during the 2004 outbreak and were *trh* positive but *tlh* and *tdh* negative by the hybridization assay described in the BAM. They produced yellow colonies on thiosulfate citrate bile sucrose agar and grew in the presence of 10% NaCl, and biochemical characterization with API-20E (bioMérieux, Inc., Hazelwood, MO) (Table 1) indicated that these two isolates were *V. alginolyticus*. All strains were grown at 37°C in Luria-Bertani medium (LB).

DNA extraction, PCR amplification, cloning, and sequencing of the *trh*-like gene. In order to confirm the presence of structural *trh* genes in *V. alginolyticus* strains Va 29 and Va 30, we designed a pair of primers, trh_1F and trh_570R (Table 2), that amplified both *trh* subgroups (9) and hybridize at the 5′ and 3′ ends, respectively, of the *trh* genes reported in GenBank (http://www.ncbi.nlm.nih.gov). *V. parahaemolyticus* strains Vp 93A-5807, Vp 28, Vp 32, and Vp 33 were used as a positive

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TABLE 1. Biochemical properties of *V. alginolyticus* ATCC 33787, Va 29, and Va 30

Test	Result[a]
ONPG[b]	−
Arginine dihydrolase	−
Lysine decarboxylase	+
Ornithine decarboxylase	+
Citrate utilization	+
H_2S production	−
Indole production	+
Acetoin production	+ (−)
Gelatinase	+
Glucose fermentation	+
Mannitol fermentation	+
Inositol fermentation	−
Sorbitol fermentation	−
Rhamnose fermentation	−
Urease	+ (−) V
Sucrose fermentation	+ (−)
Melibiose fermentation	−
Amygdalin fermentation	−
Arabinose fermentation	− (+) V
Oxidase	+
Catalase	+
Growth in 1% tryptone plus NaCl	
0% NaCl	−
3, 6, and 8% NaCl	+
10% NaCl	+ (−)
Growth in alkaline peptone water at different temperatures	
4°C	−
20, 30, 35, and 40°C	+

[a] The results were the same for all strains, except that *V. alginolyticus* ATCC 33787 was negative for production of urease. Results in parentheses indicate expected *V. parahaemolyticus* results differing from *V. alginolyticus* results. V, variable.

[b] ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

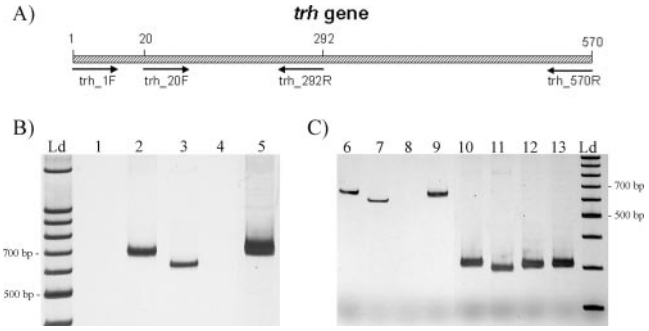

FIG. 1. Polyacrylamide gel electrophoresis of the products obtained after PCR amplification of the *trh* genes from the different strains used in this study. (A) Schematic representation of the *trh* gene, with the relative positions of the primers employed indicated by arrows. (B) PCR amplification products for the *trh* genes obtained with primers trh_1F and trh_570R. Lanes: 1, H_2O; 2, Vp 28; 3, Va 29; 4, Va 30; 5, Vp 32. (C) PCR amplification products for the partial *trh* genes obtained with primers trh_20F and trh_570R (6, Vp 28; 7, Va 29; 8, Va 30; and 9, Vp 32) and primers trh_1F and trh_292R (strains for lanes 10 to 13 are the same as those for lanes 6 to 9). Ld indicates a 100-bp size ladder.

control for the *trh* gene. Bacterial DNA was extracted using a DNeasy tissue kit (QIAGEN, Valencia, CA). Figure 1A shows the relative positions of the primers employed in this study. The PCR amplification and analysis of amplicons were performed as previously described (18).

For the two *V. alginolyticus* strains, only the Va 29 isolate yielded a positive reaction when these primers were employed (Fig. 1B), although the observed band was smaller than those obtained for Vp 28, Vp 31, Vp 32, and Vp 93A-5807. The *trh* PCR amplification was repeated five times with the same results, confirming the presence of the *trh* gene in Va 29 (results not shown). Additional primers (Table 2 and Fig. 1A) targeting other regions of reported *trh* sequences were also employed and indicated that strain Va 30 possesses a *trh*-like gene. The target sequence for the trh_570R primer located near the 3′ end of the *trh* gene appears to be missing or modified in this strain (Fig. 1C). As only Va 29 appeared to carry the entire *trh* gene, it was selected for further study. The sequence of the *trh*-like gene for this isolate was determined. The *trh* amplification products of strains Vp 28, Vp 29, and Vp 93A-5807 were used for cloning. PCR products were purified with a QIAquick PCR purification kit (QIAGEN) and later cloned into the TOPO TA-cloning vector, according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA). Clones were screened for the presence of plasmid with inserts by PCR as indicated above (Invitrogen), except that the primers used for PCR were trh_1F and trh_570R. Plasmids containing the fragment of interest were purified with a QIAprep Spin Miniprep kit (QIAGEN). Clones containing the *trh* genes from strains Vp 28, Va 29, and Vp 93A-5807 were selected and sequenced in both directions. The sequencing reactions were run by MCLAB (San Francisco, CA), employing primers M13F, M13R, trh_20F, and trh_292R (Table 2). DNA sequences were inspected individually and manually assembled. The alignments and sequence similarities were determined using BioEdit (5). Sequence analysis indicated that *V. parahaemolyticus* strains Vp 28 and Vp 93A-5807 carried a *trh1* gene, which is typical of most *V. parahaemolyticus* isolates that also possess

TABLE 2. Primers used in this study

Primer	Gene sequence (5′-3′)[a]	Target	Source or reference
trh_1F	ATGAAACTAARACTCTACTTTGC	*trh* gene	Present study
trh_570R	TTAAWTTTGTGAYWTACATTC	*trh* gene	Present study
trh_20F	TTGCTTTCAGTTTGCTATTGGCT	*trh* gene	24
trh_292R	TGTTTACCGTCATATAGGCGCTT	*trh* gene	24
Vp33	TGCGAATTCGATAGGGTGTTAACC	pR72H	10
Vp32	CGAATCCTTGAACATACGCAGC	pR72H	10
M13F	GTAAAACGACGGCCAGT	pCR2.1-TOPO	TOPO TA Cloning Manual (Invitrogen)
M13R	CAGGAAACAGCTATGAC	pCR2.1-TOPO	TOPO TA Cloning Manual (Invitrogen)

[a] Y, C/T; W, A/T; R, A/G.

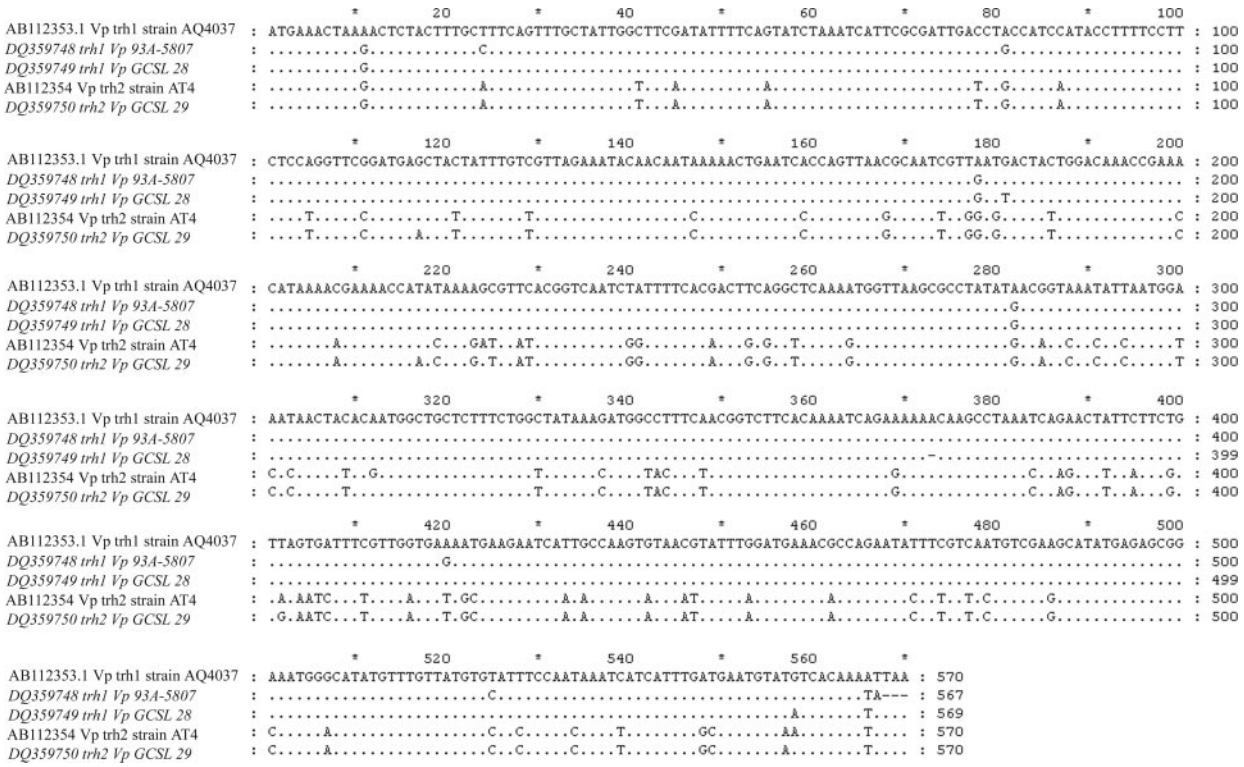

FIG. 2. Alignment of the *trh* gene sequences obtained in the present study along with two representatives of the *trh* gene sequences (*trh1* and *trh2*) retrieved from GenBank. All the sequences contain their respective GenBank accession numbers. The sequences generated from this study are in italics. Nucleotide positions conserved relative to those in the sequence located at the top are indicated by dots. Gaps included to preserve the alignments are indicated by dashes.

tdh (17). However, the *trh*-like gene of the Va 29 strain was 98% identical to a *trh2* gene of *V. parahaemolyticus* reported in GenBank (Fig. 2).

Determination of *V. parahaemolyticus* species DNA-specific marker pR72H. Previous results obtained by Ruimy et al. (20) showed that the 16S rRNA gene sequences of *V. alginolyticus* and *V. parahaemolyticus* are highly similar (99%) and consequently are unreliable phylogenetic markers for differentiating those species (20). To further exclude the possibility that this was an atypical *V. parahaemolyticus* strain, we examined Va 29 for an additional (other than *tlh*) *V. parahaemolyticus* species-specific DNA marker, pR72H (10). This test was previously validated using DNA-DNA hybridization between *V. alginolyticus* and *V. parahaemolyticus* (19). PCR was used to amplify a fragment (320 or 387 bp long) of an unknown function that is highly conserved in *V. parahaemolyticus* and absent in *V. alginolyticus*. Primers Vp33 and Vp32 (Table 2) were used under PCR conditions similar to those for the *trh* gene, except that 60°C was used as the annealing temperature. PCR products (10 μl each) were separated by agarose gel electrophoresis in a 2% agarose gel (Invitrogen) run at 75 V for 1.5 h in 1× Tris-acetate-EDTA. Amplification products were visualized by ethidium bromide staining. Figure 3 shows that only strains Vp 93A-5807, Vp 28, Vp 32, and Vp 33 possess this fragment, indicating that strains Va 29 and Va 30 were not *V. parahaemolyticus*.

Determination of the transcriptional activity of the *trh2* gene in Va 29. To determine whether the *trh2* gene of Va 29 was actively expressed, we isolated total RNA from early-stationary-phase (optical density at 600 nm = 1.0) cells of the Vp 28 and Va 29 strains. The presence of *trh* mRNA was determined by reverse transcription (RT)-PCR using primers trh_1F and trh_570R (Table 2) for the *trh* gene. Briefly, strains Vp 28 and

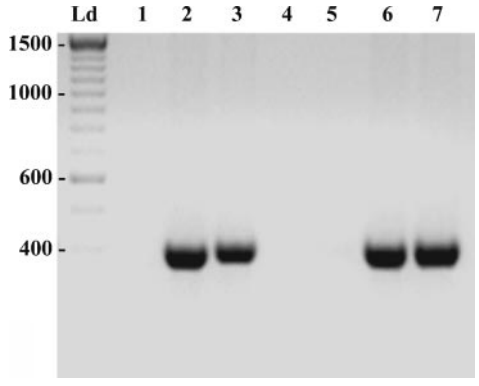

FIG. 3. Agarose gel electrophoresis of the products obtained after PCR amplification of the pR72H fragment. The PCR products were separated in a 2% agarose gel in 1× Tris-acetate-EDTA. Lanes: 1, H_2O; 2, Vp 93A-5807; 3, Vp 28; 4, Va 29; 5, Va 30; 6, Vp 32; 7, Vp 33. Ld indicates a 100-bp size ladder.

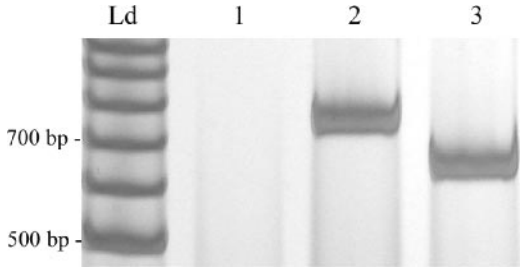

FIG. 4. Polyacrylamide gel electrophoresis of the products obtained after one-step RT-PCR amplification of the *trh* genes (with primers trh_1F and trh_570R) from the total RNA extracted from Vp 28 and Va 29 from early-stationary-phase growing cells. Lanes: 1, H_2O; 2, RNA Vp 28; 3, RNA Va 29. Ld indicates a 100-bp size ladder.

Va 29 were incubated at 37°C in LB until reaching an optical density at 600 nm of 1.0. One milliliter of each culture was centrifuged for 5 min at 5,000 × *g*. The pellets were resuspended in 100 μl of Tris-EDTA buffer. RNA extraction was carried out using an RNeasy mini kit (QIAGEN). The RNA was eluted with 50 μl of nuclease-free water. Prior to RT-PCR, 10-μl portions of each RNA solution were treated with DNase I (Invitrogen) for 15 min at room temperature, according to the manufacturer's instructions. DNase I was then inactivated by adding 1 μl of 25 mM EDTA solution to each reaction mixture and heating it for 10 min at 65°C. Portions (3 μl) of each reaction mixture were used as a template for RT-PCR. RT-PCR was carried out with a OneStep RT-PCR kit as indicated by the manufacturer (QIAGEN), using primers trh_1F and trh_570R. The amplification products were examined by electrophoresis in polyacrylamide gels and visualized by staining them with silver nitrate as previously described (18). After the RT-PCR, a single band equal in size to the band observed after *trh* DNA PCR amplification using the same primers was observed for each strain (Fig. 1A, lanes 2 and 3, and 4, lanes 2 and 3). This indicates that the *trh* gene in each strain was actively expressed in early-stationary-phase cells. DNA contamination was ruled out, as PCR amplification of the same RNA samples used in the RT-PCR did not give any product (data not shown). While transcriptional activity does not always result in full translational expression and function, it is likely that a functional protein is produced in this case, because the two *trh* genes have 98% identical DNA sequences and differ at only two amino acid residues. Additionally, *V. alginolyticus* is the closest relative of *V. parahaemolyticus*, further increasing the likelihood that a functional *trh2*-like toxin will be produced in *V. alginolyticus*.

Concluding remarks. Being an autochthonous marine bacterium, *V. alginolyticus* is probably subjected to a high level of recombination with the diverse, closely related bacterial strains populating marine environments. Marine environments provide a habitat where vibrios can be exposed to high levels of gene transfer by transduction (8), and consequently, putative transfers of virulence factor genes like *trh* can occur between marine bacteria.

This is the first report of *Vibrio* spp. other than *V. parahaemolyticus* possessing and expressing the pathogenicity marker *trh* of *V. parahaemolyticus*. The *tdh* gene has been reported in vibrios other than *V. parahaemolyticus* (26), and some authors (15, 23) have suggested that *tdh* and possibly *trh* were acquired by ancestral *V. parahaemolyticus* strains from another organism. The presence of a *trh2* gene actively expressed in a *V. alginolyticus* strain supports the hypothesis that this gene is transferred among vibrios. Xie et al. (25) showed that *V. alginolyticus* often possessed homologues of virulence genes (other than *trh*) of *V. parahaemolyticus* and *V. cholerae*, suggesting that *V. alginolyticus* can be a reservoir for these genes in the aquatic environment. The practical implications of these results are that detection of the *trh* gene in mixed cultures, such as broth enrichments or nucleic acid extracts of seafood or environmental samples, does not always imply that pathogenic *V. parahaemolyticus* is present.

Nucleotide sequence accession numbers. The *trh* gene sequences have been deposited in GenBank under accession numbers DQ359748 (*trh1* Vp 93A-5807), DQ359749 (*trh1* Vp GCSL28), and DQ359750 (*trh2* Va GCSL29).

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REFERENCES

1. **Ansaruzzaman, M., M. Lucas, J. L. Deen, N. A. Bhuiyan, X. Y. Wang, A. Safa, M. Sultana, A. Chowdhury, G. B. Nair, D. A. Sack, L. von Seidlein, M. K. Puri, M. Ali, C. L. Chaignat, J. D. Clemens, and A. Barreto.** 2005. Pandemic serovars (O3:K6 and O4:K68) of *Vibrio parahaemolyticus* associated with diarrhea in Mozambique: spread of the pandemic into the African continent. J. Clin. Microbiol. **43:**2559–2562.
2. **Bej, A. K., D. P. Patterson, C. W. Brasher, M. C. Vickery, D. D. Jones, and C. A. Kaysner.** 1999. Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tl*, *tdh* and *trh*. J. Microbiol. Methods **36:**215–225.
3. **DePaola, A., C. A. Kaysner, J. Bowers, and D. W. Cook.** 2000. Environmental investigations of *Vibrio parahaemolyticus* in oysters after outbreaks in Washington, Texas, and New York (1997 and 1998). Appl. Environ. Microbiol. **66:**4649–4654.
4. **Gonzalez-Escalona, N., V. Cachicas, C. Acevedo, M. L. Rioseco, J. A. Vergara, F. Cabello, J. Romero, and R. T. Espejo.** 2005. *Vibrio parahaemolyticus* diarrhea, Chile, 1998 and 2004. Emerg. Infect. Dis. **11:**129–131.
5. **Hall, T.** 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. **41:**95–98.
6. **Iida, T., K. S. Park, O. Suthienkul, J. Kozawa, Y. Yamaichi, K. Yamamoto, and T. Honda.** 1998. Close proximity of the *tdh*, *trh* and *ure* genes on the chromosome of *Vibrio parahaemolyticus*. Microbiology **144:**2517–2523.
7. **Iida, T., O. Suthienkul, K. S. Park, G. Q. Tang, R. K. Yamamoto, M. Ishibashi, K. Yamamoto, and T. Honda.** 1997. Evidence for genetic linkage between the *ure* and *trh* genes in *Vibrio parahaemolyticus*. J. Med. Microbiol. **46:**639–645.
8. **Jiang, S. C., and J. H. Paul.** 1998. Gene transfer by transduction in the marine environment. Appl. Environ. Microbiol. **64:**2780–2787.
9. **Kishishita, M., N. Matsuoka, K. Kumagai, S. Yamasaki, Y. Takeda, and M. Nishibuchi.** 1992. Sequence variation in the thermostable direct hemolysin-related hemolysin (*trh*) gene of *Vibrio parahaemolyticus*. Appl. Environ. Microbiol. **58:**2449–2457.
10. **Lee, C. Y., S. F. Pan, and C. H. Chen.** 1995. Sequence of a cloned pR72H fragment and its use for detection of *Vibrio parahaemolyticus* in shellfish with the PCR. Appl. Environ. Microbiol. **61:**1311–1317.
11. **Matsumoto, C., J. Okuda, M. Ishibashi, M. Iwanaga, P. Garg, T. Rammamurthy, H. C. Wong, A. DePaola, Y. B. Kim, M. J. Albert, and M. Nishibuchi.** 2000. Pandemic spread of an O3:K6 clone of *Vibrio parahaemolyticus* and emergence of related strains evidenced by arbitrarily primed PCR and *toxRS* sequence analyses. J. Clin. Microbiol. **38:**578–585.
12. **McLaughlin, J. B., A. DePaola, C. A. Bopp, K. A. Martinek, N. P. Napolilli, C. G. Allison, S. L. Murray, E. C. Thompson, M. M. Bird, and J. P. Middaugh.** 2005. Outbreak of *Vibrio parahaemolyticus* gastroenteritis associated with Alaskan oysters. N. Engl. J. Med. **353:**1463–1470.

13. **Nair, G. B., and J. C. Hormazabal.** 2005. The *Vibrio parahaemolyticus pandemic*. Rev. Chil. Infectol. **22:**125–130.
14. **Nishibuchi, M., and J. B. Kaper.** 1990. Duplication and variation of the thermostable direct haemolysin (*tdh*) gene in *Vibrio parahaemolyticus*. Mol. Microbiol. **4:**87–99.
15. **Nishibuchi, M., and J. B. Kaper.** 1995. Thermostable direct hemolysin gene of *Vibrio parahaemolyticus*: a virulence gene acquired by a marine bacterium. Infect. Immun. **63:**2093–2099.
16. **Nordstrom, J. L., R. Rangdale, M. C. L. Vickery, A. M. B. Phillips, S. L. Murray, and A. DePaola.** Evaluation of an alkaline phosphatase-labeled oligonucleotide probe for the detection and enumeration of the thermostable-related hemolysin (*trh*) gene of *Vibrio parahaemolyticus*. J. Food Prot., in press.
17. **Okuda, J., M. Ishibashi, E. Hayakawa, T. Nishino, Y. Takeda, A. K. Mukhopadhyay, S. Garg, S. K. Bhattacharya, G. B. Nair, and M. Nishibuchi.** 1997. Emergence of a unique O3:K6 clone of *Vibrio parahaemolyticus* in Calcutta, India, and isolation of strains from the same clonal group from Southeast Asian travelers arriving in Japan. J. Clin. Microbiol. **35:**3150–3155.
18. **Pizarro, J., E. Jedlicki, O. Orellana, J. Romero, and R. T. Espejo.** 1996. Bacterial populations in samples of bioleached copper ore as revealed by analysis of DNA obtained before and after cultivation. Appl. Environ. Microbiol. **62:**1323–1328.
19. **Robert-Pillot, A., A. Guenole, and J. M. Fournier.** 2002. Usefulness of R72H PCR assay for differentiation between *Vibrio parahaemolyticus* and *Vibrio alginolyticus* species: validation by DNA-DNA hybridization. FEMS Microbiol. Lett. **215:**1–6.
20. **Ruimy, R., V. Breittmayer, P. Elbaze, B. Lafay, O. Boussemart, M. Gauthier, and R. Christen.** 1994. Phylogenetic analysis and assessment of the genera *Vibrio*, *Photobacterium*, *Aeromonas*, and *Plesiomonas* deduced from small-subunit rRNA sequences. Int. J. Syst. Bacteriol. **44:**416–426.
21. **Taniguchi, H., H. Hirano, S. Kubomura, K. Higashi, and Y. Mizuguchi.** 1986. Comparison of the nucleotide sequences of the genes for the thermostable direct hemolysin and the thermolabile hemolysin from *Vibrio parahaemolyticus*. Microb. Pathog. **1:**425–432.
22. **Taniguchi, H., H. Ohta, M. Ogawa, and Y. Mizuguchi.** 1985. Cloning and expression in *Escherichia coli* of *Vibrio parahaemolyticus* thermostable direct hemolysin and thermolabile hemolysin genes. J. Bacteriol. **162:**510–515.
23. **Terai, A., K. Baba, H. Shirai, O. Yoshida, Y. Takeda, and M. Nishibuchi.** 1991. Evidence for insertion sequence-mediated spread of the thermostable direct hemolysin gene among *Vibrio* species. J. Bacteriol. **173:**5036–5046.
24. **Vickery, M. C. L., G. M. Blackstone, J. L. Nordstrom, and A. DePaola.** 2003. Detection and quantification of total and potentially virulent *Vibrio parahaemolyticus* using a 4-channel multiplex real-time PCR targeting the *tl*, *tdh*, and *trh* genes and a novel PCR internal control, abstr. Q-083, p. 529. Abstr. 103rd Gen. Meet. Am. Soc. Microbiol. American Society for Microbiology, Washington, D.C.
25. **Xie, Z. Y., C. Q. Hu, C. Chen, L. P. Zhang, and C. H. Ren.** 2005. Investigation of seven *Vibrio* virulence genes among *Vibrio alginolyticus* and *Vibrio parahaemolyticus* strains from the coastal mariculture systems in Guangdong, China. Lett. Appl. Microbiol. **41:**202–207.
26. **Zhang, X. H., and B. Austin.** 2005. Haemolysins in *Vibrio* species. J. Appl. Microbiol. **98:**1011–1019.