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Nucleotide Sequence of the Thermostable Direct Hemolysin Gene of *Vibrio parahaemolyticus*

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The gene encoding the thermostable direct hemolysin of *Vibrio parahaemolyticus* was characterized. This gene (designated *tdh*) was subcloned into pBR322 in *Escherichia coli*, and the functional *tdh* gene was localized to a 1.3-kilobase *Hin*dIII fragment. This fragment was sequenced, and the structural gene was found to encode a mature protein of 165 amino acid residues. The mature protein sequence was preceded by a putative signal peptide sequence of 24 amino acids. A putative *tdh* promoter, determined by its similarity to concensus sequences, was not functional in *E. coli*. However, a promoter that was functional in *E. coli* was shown to exist further upstream by use of a promoter probe plasmid. A 5.7-kilobase *Sal*I fragment containing the structural gene and both potential promoters was cloned into a broad-host-range plasmid and mobilized into a Kanagawa phenomenon-negative *V. parahaemolyticus* strain. In contrast to *E. coli*, where the hemolysin was detected only in cell lysates, introduction of the cloned gene into *V. parahaemolyticus* resulted in the production of extracellular hemolysin.

Vibrio parahaemolyticus is an important agent of diarrhea associated with seafood consumption. It has been isolated from clinical sources as well as nonclinical sources including estuarine and marine environments in many parts of the world (4, 12). Experimental studies on the pathogenicity of this species have demonstrated the existence of a number of possible virulence factors including a hemolysin, a Chinese hamster ovary (CHO) cell elongation factor, and factors responsible for cytotoxicity, invasiveness, and adherence (4). Although there is insufficient evidence to support the direct relationship of any of these factors to the enteropathogenicity of *V. parahaemolyticus*, the thermostable direct hemolysin (TDH) (32), often called the Kanagawa phenomenon-associated hemolysin (28), has been considered a major virulence factor based on epidemiological evidence. TDH, which is usually detected by the Kanagawa phenomenon, i.e., characteristic beta-type hemolysis on special blood agars, is almost always produced by clinical isolates, but rarely by environmental strains (27, 31). For this reason, TDH has been the subject of extensive studies by many workers (reviewed in reference 35). The role of TDH in enteropathogenicity of *V. parahaemolyticus*, however, is still unclear, and a recent report has provided indirect evidence that fails to support the diarrheagenicity of TDH (16).

Because of this confusion, we are studying the molecular genetics of TDH to elucidate its involvement in the pathogenicity of *V. parahaemolyticus*. We have previously reported the cloning of the gene encoding TDH from a Kanagawa phenomenon-positive strain of *V. parahaemolyticus* into a phage lambda vector in *Escherichia coli* (19). We now report the DNA sequence and characterization of the gene encoding TDH, which we have designated the *tdh* gene. The position of the promoter of the *tdh* gene is discussed based on DNA sequence and on the results of subcloning and promoter cloning experiments. The cloned *tdh* gene was also introduced into a Kanagawa phenomenon-negative strain of *V. parahaemolyticus*, where the expression and transport of the TDH were examined.

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MATERIALS AND METHODS

Bacterial strains, media, and plasmids. The bacterial strains used in this study are listed in Table 1. *E. coli* strains HB101, Q359, and JM103 were used as hosts for propagating plasmids, bacteriophage λ, and bacteriophage M13 clones, respectively. LB medium (26) was used to grow all of the organisms except *E. coli* Q359 and JM103. When necessary, the medium was supplemented with the following antibiotics at the specified concentrations: ampicillin, 200 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 50 μg/ml; rifampin, 100 μg/ml; tetracycline, 30 μg/ml; or trimethoprim, 1 μg/ml. NZY broth (5) was employed for *E. coli* Q359, and YT medium (26) was used for strain JM103 to propagate phages as described below. The plasmids used are listed in Table 2. A broad-host-range vector, pCVD503, used to introduce the *tdh* gene into *V. parahaemolyticus* was constructed by modifying a wide-host-range cloning vector, pVK102, which is a derivative of pRK290 (9). pVK102 was first digested with *Bgl*II and religated to remove the *cos* site. This plasmid pJBK72 contained an *Hin*dIII site in the *kan* gene. A 3.3-kilobase (kb) DNA fragment consisting of the *cat* gene derived from pBR325 and *Hin*dIII cohesive ends was inserted into the *Hin*dIII site of pJBK72. The phenotype of the resulting plasmid pCVD503 was thus tetracycline resistance (Tc^r) and chloramphenicol resistance (Cm^r).

Chemicals, biochemicals, and enzymes. Restriction endonucleases and DNA modification enzymes were purchased from Bethesda Research Laboratories, New England Biolabs, and International Biotechnologies. Agarose (gel electrophoresis grade), cesium chloride (optical grade), bovine serum albumin (nucleic acid enzyme grade), replicative form DNA of M13 mp8 and M13 mp9, and M13 15-base primer were obtained from Bethesda Research Laboratories. Sodium dodecyl sulfate, *N,N,N',N'*-tetramethylphenylenediamine, and dithiothreitol were gel electrophoresis grade purchased from Bio-Rad Laboratories. ATP and deoxy- and dideoxytriphosphates were from P-L Biochemicals. [α-^{32}P]dATP was purchased from New England Nuclear Corp. All antibiotics, morpholinepropanesulfonic acid, and lysozyme (grade I) were obtained from Sigma Chemical Co. Acrylamide (electrophoresis grade), urea (Sequanal

TABLE 1. Bacterial strains employed

Strain	Genotype or relevant phenotype[a]	Source
Escherichia coli		
Q359	*hsdR hsdM*$^+$ *supE* $\phi 80^r$ P2	J. Karn
HB101	F$^-$ *hsdS20* (r_B, m_B) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ^-	S. Falkow
JM103	Δ(*lac-pro*) *thi rpsL supE endA sbcB15 hsdR4* F′ *traD36 proAB lacI*^{q} Δ*lacZM15*	J. Messing
Vibrio parahaemolyticus		
WP1	Wild type, Kanagawa phenomenon positive	Y. Takeda
S162-71	Wild type, Kanagawa phenomenon negative	M. J. Voll
S162-71-RIF	Spontaneous Rif^r mutant of S162-71	This study

[a] $\phi 80^r$, resistant to infection with bacteriophage $\phi 80$; P2, lysogenic for bacteriophage P2.

grade), and hippurate-Tris buffer (Hipp buffer) were from U.S. Biochemical, Pierce Chemical Co., and Gelman Sciences, Inc., respectively. Hipp buffer was employed at 0.06 M ionic strength, and phosphate-buffered saline was composed of 3.3 mM NaH_2PO_4–6.7 mM Na_2HPO_4–0.13 M NaCl (pH 7.2). Tryptone, yeast extract, and agar were from Difco Laboratories. NZ-amine (type A) was obtained from Sheffield Products. All the other chemicals used were of analytical or reagent grade. Purified TDH and anti-TDH rabbit serum were generous gifts from Y. Takeda.

Isolation of DNA. The recombinant lambda phage clone λJBK19 (19) was grown in *E. coli* Q359, and lysates were prepared as described by Blattner et al. (5). Phage was purified by centrifugation through CsCl block density gradients as described by Davis et al. (8), and phage DNA was then purified by extraction with phenol-chloroform followed by ethanol precipitation. Small amounts of plasmid DNA for rapid plasmid screening were isolated by the rapid alkaline extraction method of Birnboim and Doly (3) or the alkaline sodium dodecyl sulfate lysis procedure of Kado and Liu (18). Plasmid DNA used for restriction endonuclease mapping and cloning experiments was isolated by the Birnboim and Doly procedure (3) and purified by CsCl-ethidium bromide equilibrium density gradient centrifugation (23).

Restriction enzyme digestion and electrophoresis. *Bal*I enzyme was used according to the manufacturer's specifications. Digestion of DNA with all other enzymes was carried out as described previously (8), but modified to include bovine serum albumin (100 μg/ml) in the reaction mixture. Uncleaved plasmid DNA, restriction enzyme-digested DNA, and ligated DNA were analyzed by gel electrophoresis with 0.7 to 2.0% agarose gels or 5% polyacrylamide gels with Tris-borate-EDTA buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA). Gels were stained with ethidium bromide (1 μg/ml) and photographed with shortwave UV light. Restriction endonuclease maps of plasmids were constructed by analysis of single or double digests (or both) of plasmid DNA with appropriate enzymes.

Molecular cloning. In general, DNA fragments were separated by gel electrophoresis and isolated by electroelution (23) and purified by phenol-chloroform extraction. DNA to be inserted was ligated with a restriction enzyme-cleaved vector plasmid in a ligation buffer (10 mM Tris-hydrochloride, 10 mM $MgCl_2$, 3 mM dithiothreitol, 100 μg of bovine serum albumin per ml, 0.5 mM ATP, pH 7.5) at 15°C overnight. The ligation mixture was used to transform *E. coli* HB101 by the method of Mandel and Higa (22), except that the $CaCl_2$ solution was replaced by a transformation buffer (75 mM $CaCl_2$, 10 mM morpholinepropanesulfonic acid, 28 mM glucose, pH 6.5), and bacterial cells were incubated with shaking in LB broth for 1 h at 37°C after a heat shock at 42°C. Transformed cells were plated onto LB agar supplemented with the appropriate antibiotic(s). When necessary, the clones were screened for susceptibility to a pertinent antibiotic by replica plating. Plasmid DNA isolated from selected clones was analyzed for restriction enzyme digestion pattern.

Nucleotide sequence analysis. Various restriction endonuclease fragments isolated from fragment A of pCVD512 (Fig. 1) were cloned into appropriate restriction sites of vectors M13 mp8 and M13 mp9 (25), and phages were propagated in *E. coli* JM103. The nucleotide sequence was determined by the dideoxy chain termination method of Sanger et al. (33) with a 15-base primer as described by Messing (24). A length of nucleotide sequence in the fragment A was also obtained directly from pCVD512 as described by Wallace et al. (37). The DNA sequence was analyzed for palindromic sequences and for translation into amino acid residues with the aid of a Univac 1108 computer. The basic programs were adapted from those described by Gingeras et al. (13).

Introduction of the *tdh* gene into a Kanagawa phenomenon-negative *V. parahaemolyticus* strain. The cloned *tdh* gene was introduced into a Kanagawa phenomenon-negative strain of *V. parahaemolyticus* by using a broad-host-range vector, pCVD503, as described below. The 5.7-kb *Sal*I fragment of pCVD502 (Fig. 1) was cloned into the *Sal*I site present in the *tet* gene of pCVD503 by digesting both plasmids with *Sal*I followed by ligation. *E. coli* HB101 was transformed with the ligation mixture, and Cm^r Tc^s colonies were screened for intracellular TDH production. The resulting clone contained a recombinant plasmid designated pCVD504. Next, a helper plasmid, pRK2013, was introduced into HB101(pCVD504) by conjugation. The donor strain HB101(pRK2013) and the recipient strain HB101(pCVD504) were grown at 37°C to the midlog phase in LB broth containing kanamycin and chloramphenicol, respectively. These cultures were cross-streaked onto an LB agar plate containing kanamycin and chloramphenicol and incubated at 37°C. The presence of the plasmids pCVD504 and pRK2013 in the Km^r Cm^r transconjugants was confirmed by plasmid screening. In the final step, pCVD504 was introduced from the *E. coli* strain HB101(pCVD504, pRK2013) into a Kanagawa phenomenon-negative *V. parahaemolyticus* strain, S162-71, by conjugation as follows. A spontaneous rifampin-resistant (Rif^r) mutant, S162-71-RIF, was selected from strain S162-71 by plating S162-71 cells on LB agar containing rifampin and was used as the recipient strain. Donor and recipient strains were grown in LB broth (kanamycin and chloramphenicol supplemented for the donor strain) at 37°C to the midlog phase. A 0.5-ml portion of each culture was mixed, centrifuged in a microfuge, and suspended in 1 ml of LB broth. The bacterial cell suspension was collected on a membrane filter (0.4-μm pore size; Nuclepore Corp.), and the filter was incubated on LB agar at 37°C overnight. Cells were washed off the filter and spread onto LB agar containing rifampin and chloram-

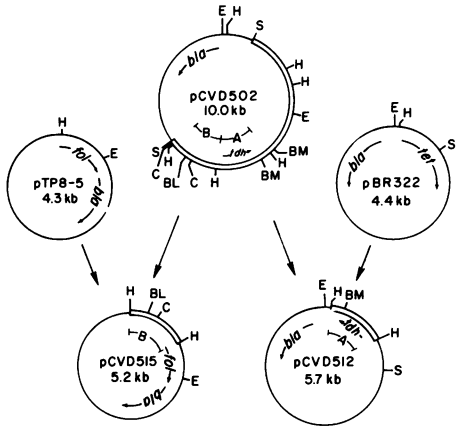

FIG. 1. Cloning of the 1.3-kb *Hin*dIII fragment (fragment A) and the 0.93-kb *Hin*dIII fragment (fragment B) of pCVD502 into plasmid vectors. Restriction sites are abbreviated as follows: BM, *Bam*HI; BL, *Bal*I; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I. Only *Bal*I and *Cla*I recognition sites found in fragment B are indicated. (□) DNA derived from *V. parahaemolyticus* WP1. The position and transcriptional direction of the *tdh* gene and the genes coding for ampicillin resistance (*bla*), tetracycline resistance (*tet*), and trimethoprim resistance (*fol*) are shown.

phenicol. The transconjugant was identified by colony morphology, positive cytochrome oxidase reaction, and the presence of pCVD504 as determined by plasmid screening.

Detection of intracellular TDH in *E. coli* clones. *E. coli* clones were grown in 30 ml of LB broth, supplemented with appropriate antibiotic(s) if necessary, with shaking (250 rpm) for 10 h at 37°C. After centrifugation (7,700 × *g*, 10 min, 4°C) the cell pellet was suspended in 0.5 ml of freshly prepared lysozyme-EDTA solution (2 mg of lysozyme per ml, 10 mM EDTA, 25 mM Tris-hydrochloride, 50 mM glucose, pH 8.0), and incubated on ice for 30 min. After the addition of 5 μl of 1 M $CaCl_2$, the sample was stored at −70°C. Before use, the frozen sample was thawed and sonicated on ice for 5 s to reduce viscosity. Hemolytic activity of TDH in the test sample was detected with a blood agar plate. The plate was prepared by mixing 15 ml of melted buffered agarose (0.01 M Tris-hydrochloride, 0.15 M NaCl, 5 mM $CaCl_2$, 1.5 mM NaN_3, 1% agarose, pH 7.3) and 5 ml of fresh rabbit erythrocytes washed and suspended to the original volume in phosphate-buffered saline in a 100- by 15-mm petri dish. A 50-μl sample of the sonicated cell lysate was placed in a well (6.5 mm in diameter) made in the blood agar plate, and the plate was incubated for 12 h at 37°C or for 24 h at 25°C to detect a cleared zone of hemolysis. Serological identification of TDH was performed by an Ouchterlony double immunodiffusion test. A 50-μl sample of the sonicated cell lysate was allowed to react with 100 μl of anti-TDH serum after diffusion through buffered agarose (0.8% agarose, 0.25 mM merthiolate in Hipp buffer) prepared in a 100- by 15-mm petri dish. A fused precipitation line formed by a test sample and purified TDH (100 μg) confirmed the identity of TDH in the test sample.

Assay for extracellular TDH from *V. parahaemolyticus*. The *V. parahaemolyticus* strain to be tested was grown in 100 ml of LB broth at 37°C with shaking (250 rpm) for 18 h. To grow strain S162-71-RIF(pCVD504), the medium was supplemented with 20 μg of chloramphenicol per ml. Culture supernatant fluid obtained by centrifugation (23,500 × *g*, 30 min) was mixed with 35.1 g of $(NH_4)_2SO_4$ and stirred on ice overnight. The precipitate was collected by centrifugation (16,300 × *g*, 10 min), dissolved in 1 ml of phosphate-buffered saline, and dialyzed against phosphate-buffered saline at 4°C. Any remaining precipitate was removed by centrifugation for 5 min in a microfuge. This 100× concentrate of

TABLE 2. Plasmids employed

Plasmid	Relevant genotype or phenotype	Comments	Source (reference)
pBR322	*bla*$^+$ *tet*$^+$		S. Falkow
pBR325	*bla*$^+$ *cat*$^+$ *tet*$^+$		S. Falkow
pTP8-5	*bla*$^+$ Tp^s	Contains the *fol* gene with an incomplete promoter	(17)
pVK102	*cos*$^+$ *tet*$^+$ *kan*$^+$	Broad host-range plasmid containing the *cos* site of phage λ	(20)
pRK2013	*kan*$^+$ *tra*$^+$		(11)
pJBK72	*tet*$^+$ *kan*$^+$	pVK102 derivative with *Bgl*II fragment containing the *cos* site removed	This study
pCVD502	*bla*$^+$ *tdh*$^+$	5.6-kb *Sal*I fragment of λJBK19[a] carrying the *tdh* gene inserted into the *Sal*I site of pBR322	This study
pCVD503	*tet*$^+$ *cat*$^+$	pJBK72 derivative containing the *cat* gene derived from pBR325	This study
pCVD504	*cat*$^+$ *tdh*$^+$	5.6-kb *Sal*I fragment of pCVD502 carrying the *tdh* gene inserted into the *Sal*I site of pCVD503	This study
pCVD511	*bla*$^+$ Tdh$^-$ Tc^s	1.3-kb *Hin*dIII fragment of pCVD502 carrying the *tdh* gene inserted into the *Hin*dIII site of pBR322	This study
pCVD512	*bla*$^+$ Tdh$^+$ Tc^s	1.3-kb *Hin*dIII fragment of pCVD502 carrying the *tdh* gene inserted into the *Hin*dIII site of pBR322 in opposite orientation to that of the insert in pCVD511	This study
pCVD515	*bla*$^+$ Tp^r	0.93-kb *Hin*dIII fragment of pCVD502 inserted into the *Hin*dIII site of pTP8-5	This study
pCVD516	*bla*$^+$ Tp^r	Two identical 0.93-kb *Hin*dIII fragments of pCVD502 inserted, in tandem, into the *Hin*dIII site of pTP8-5	This study

[a] λJBK19 is a recombinant phage carrying the *tdh* gene (19).

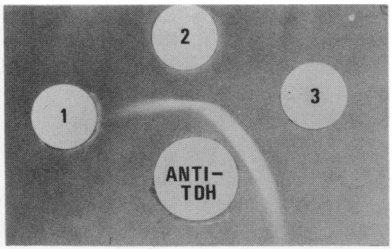

FIG. 2. Ouchterlony double immunodiffusion analysis of the TDH produced in *E. coli* HB101(pCVD512). Wells: 1, *E. coli* HB101(pBR322) lysate; 2, *E. coli* HB101(pCVD512) lysate; 3, purified TDH that was produced by *V. parahaemolyticus* WP1; anti-TDH, rabbit antiserum raised to the purified TDH.

culture supernatant fluid was diluted to 5 ml with distilled water, and the pH was adjusted to 4.2 with 0.1 M acetic acid. The acid precipitate was collected by centrifugation (12,000 × *g*, 15 min) and dissolved in 0.2 ml of phosphate-buffered saline, resulting in a 500× concentrate of culture supernatant fluid. Samples from strains WP1 and S162-71-RIF(pCVD504) were assayed for TDH by rocket immunoelectrophoresis in the following manner. A 4.6-ml volume of melted 0.8% agarose gel prepared in Hipp buffer was mixed with 50 μl of anti-TDH serum, poured onto a glass plate (50 by 50 by 2 mm), and allowed to solidify. Up to six wells (3 mm in diameter) were made in each plate, and a 5-μl sample was placed in each well and electrophoresed at 200 V for 2 h with Hipp buffer as a running buffer. The gel was incubated overnight at 4°C, and the results were recorded. There was a linear relationship between the concentration of purified TDH and the height of rocket precipitation lines ranging from 4 to 20 mm. Therefore, test samples were appropriately diluted so that the height of the rocket was within this range, and the concentration of TDH in test samples was determined from the height of the rockets using purified TDH as a standard.

RESULTS

Subcloning of the *tdh* gene. A 5.7-kb *Sal*I fragment was subcloned from λJBK19 into the plasmid vector pBR322 and transformed into *E. coli* HB101. The clones were screened for intracellular TDH production, resulting in the isolation of plasmid pCVD502. A restriction endonuclease map of pCVD502 was constructed (Fig. 1). Each of the four *Hin*dIII fragments within the *Sal*I insert was subcloned and tested for the ability to encode TDH. When a 1.3-kb *Hin*dIII fragment (fragment A, Fig. 1) was cloned into the *Hin*dIII site of pBR322 in both orientations, resulting in the recombinant plasmids pCVD511 and pCVD512, *E. coli* HB101 containing pCVD512 was found to produce TDH intracellularly. The TDH produced lysed erythrocytes and exhibited complete identity with purified TDH in an Ouchterlony double immunodiffusion plate (Fig. 2). However, no TDH was detected by either of these methods in *E. coli* containing pCVD511, which had fragment A inserted in the opposite orientation.

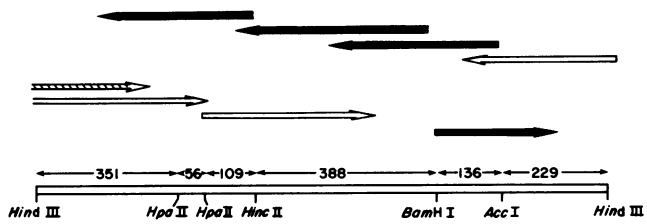

FIG. 3. Strategy employed to sequence fragment A of pCVD512 containing the *tdh* gene. Large arrows indicate the size and direction of sequences obtained in vectors M13 mp8 (⇨), and M13 mp9 (➡) by the method of Sanger et al. (33) and in pBR322 (⇨) by the method of Wallace et al. (37). Numerals represent the length of DNA fragments in base pairs.

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HindIII
1   AAGCTTACAGCTTGGTATGCCTAAAACTTGTCGTATTG
39  GCTGACAATTAATCTAAATAGGGCTGTTCGGCTTCGAAA
78  ATGATTTACGCAACAAAGCCATTCGGGCATTACTGTTAC
                          -35
117 TTATAGAGTCGTAAGTACCACCAAAGGTTTGAGAAAGCA
156 TTTTTCACAATTATTCAGTTTGCTTCTTTGGTTTTTTAG
        -10       +1
195 TTTTCATAACATCCGTCATTCTGGCAAAGTTATTAATCA
       P.B.
234 ATTCATGGGTTTTTT ATG AAA CAC CAA TAT TTT
       S.D.        MET LYS HIS GLN TYR PHE
267 GCA AAA AAA TCA TTT TTA TTT ATA TCC ATG
    ALA LYS LYS SER PHE LEU PHE ILE SER MET
297 TTG GCT GCA TTC AAA ACA TCT GCT TTT GAG
    LEU ALA ALA PHE LYS THR SER ALA PHE GLU
                                  HPAII
327 CTT CCA TCT GTC CCT TTT CCT GCC CCC GGT
    LEU PRO SER VAL PRO PHE PRO ALA PRO GLY
357 TCT GAT GAG ATA TTG TTT GTT GTT CGA GAT
    SER ASP GLU ILE LEU PHE VAL VAL ARG ASP
                              HPAII
387 ACA ACT TTT AAT ACC CAA GCT CCG GTC AAT
    THR THR PHE ASN THR GLN ALA PRO VAL ASN
417 GTA AAG GTC TCT GAC TTT TGG ACA AAC CGT
    VAL LYS VAL SER ASP PHE TRP THR ASN ARG
447 AAT GTA AAA AGA AAA CCG TAC GAA GAT GTT
    ASN VAL LYS ARG LYS PRO TYR GLU ASP VAL
477 TAT GGT CAA TCA GTA TTC ACA ACG TCA GGT
    TYR GLY GLN SER VAL PHE THR THR SER GLY
              HINC II
507 ACT AAA TGG TTG ACA TCC TAC ATG ACT GTG
    THR LYS TRP LEU THR SER TYR MET THR VAL
537 AAC ATT AAT GAT AAA GAC TAT ACA ATG GCA
    ASN ILE ASN ASP LYS ASP TYR THR MET ALA

567 GCG GTG TCT GGC TAT AAG AGC GGT CAT TCT
    ALA VAL SER GLY TYR LYS SER GLY HIS SER
597 GCT GTG TTC GTA AAA TCA GGT CAA GTA CAA
    ALA VAL PHE VAL LYS SER GLY GLN VAL GLN
627 CTT CAA CAT TCC TAT AAT TCT GTA GCT AAC
    LEU GLN HIS SER TYR ASN SER VAL ALA ASN
657 TTT GTT GGT GAA GAT GAA GGT TCT ATT CCA
    PHE VAL GLY GLU ASP GLU GLY SER ILE PRO
687 AGT AAA ATG TAT TTG GAT GAA ACT CCA GAA
    SER LYS MET TYR LEU ASP GLU THR PRO GLU
717 TAT TTT GTT AAT GTA GAA GCA TAT GAG AGT
    TYR PHE VAL ASN VAL GLU ALA TYR GLU SER
747 GGT AGT GGT AAT ATA TTG GTA ATG TGT ATA
    GLY SER GLY ASN ILE LEU VAL MET CYS ILE
777 TCC AAC AAA GAA TCG TTT TTT GAA TGT AAA
    SER ASN LYS GLU SER PHE PHE GLU CYS LYS
807 CAT CAA CAA TAAAAATAAAAAGCCACAGAATATTCT
    HIS GLN GLN TER                   PAL
843 GTGGCTTTGAAAATAATTAATAATAATTTTCTGATTTAT
                                BAMH I
882 GCATATCAAGGCTTTGTACTGAGGCATCCCCTCAGAAAT
921 CTGCCAGTGCATAGCCACCTTTTAATAGTTGCGTAGCTA
960 GAACCACCGCCGCAGCTAAGAGCTGTTGAGTTGTTATCT
999 CGTAGTCTGGTCGTCTGAGGACTTCCATTCCTAATCGAA
                ACC I
1038 TGGTAGACAGGACATTTCTATTTCTGACTGTATTCGCTT
1077 GAAAGTGCCTTTCCCATCCTTGGCTTTGCGCATGTATCC
1116 CTGCCAGCCAAAACATAAGTTGCAGCATTAGGGCTATCA
1155 GCAAAATAATATCGAACCTTTTGGAAAACTAGTGCGACT
1194 TTGACGTAGGCCTAATCCGTAAGCGGGACTCTTTAAGTC
1233 TCGGAAGGTTTCTTCTATCTCCATCAGCCTTTGCGTAAA
                HIND III
1272 GCTT
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FIG. 4. Nucleotide sequence of fragment A of pCVD512 containing the *tdh* gene. Only the coding strand is shown. Numerals indicate the positions of the bases starting at the 5′ end. Restriction endonuclease sites and amino acid sequences of TDH deduced from the nucleotide sequence are shown, respectively, above and below the nucleotide sequence. Broken underlines depict a possible signal peptide. The putative transcription initiation point is indicated as +1. Sequences showing homology with consensus sequences such as a Shine-Dalgarno sequence (S.D.), Pribnow box (P.B.), and −35 region are indicated by dots above the sequences. TER and PAL denote a termination codon and a palindromic sequence, respectively.

Nucleotide sequence analysis. The nucleotide sequence of the 1.3-kb *Hin*dIII fragment of pCVD512 was determined by the strategy summarized in Fig. 3. The DNA sequence data and predicted amino acid sequence of TDH are shown in Fig. 4. Fragment A contained 1,275 base pairs. The previously reported N-terminal amino acid sequence of TDH (39) allowed us to position the start of the mature TDH structural gene at nucleotide base position 321. The first termination codon in this reading frame was found at positions 816 through 818, indicating that the structural gene coding for mature protein is 495 nucleotides long, sufficient for 165 amino acids. Preceding the sequence for the mature protein, a sequence of DNA encoding a possible signal peptide was found. This 24-amino-acid peptide was similar to other known procaryotic signal peptides (10). No other significant open reading frame was observed on either strand. The region upstream of the putative signal peptide was examined for sequences similar to consensus promoter sequences (21). The −10 region or Pribnow box and the −35 region of a

putative promoter were assigned to positions 202 through 207 and 176 through 181, respectively. A possible transcription initiation point (+1) was assigned to the A at position 213 (Fig. 4). A putative Shine-Dalgarno sequence (positions 238 through 243) was found 6 bases before the ATG codon, and a 26-base-pair (bp) palindromic sequence (positions 825 through 851) was found immediately following the termination codon. The palindromic sequence consisted of 13-bp inverted repeats without a loop structure, and the axis of symmetry was located between positions 837 and 838. This region is presumably the site for transcription termination (30). The RNA transcript from this region will not be rich in G+C and will not contain a stretch of U residues and is therefore analogous to Rho-dependent termination signals (1).

Promoter cloning experiment. A 0.93-kb *Hin*dIII fragment of pCVD502 (fragment B, Fig. 1), was located immediately upstream of the *tdh* gene. This fragment was cloned into a promoter probe plasmid pTP8-5 to determine whether it contains a promoter functional in *E. coli* (Fig. 1). Fragment B was isolated and ligated with *Hin*dIII-digested pTP8-5 and transformed into *E. coli* HB101. Transformants were selected for trimethoprim resistance (Tp^r) and ampicillin resistance (Ap^r). A concentration of 1 μg/ml was chosen for trimethoprim because *E. coli* strain HB101(pTP8-5) was sensitive to this concentration of trimethoprim in the presence of ampicillin. Resistant clones were screened for plasmid content. Tp^r Ap^r clones harbored recombinant plasmid pCVD515 or pCVD516. pCVD515 (Fig. 1) had one fragment B, and pCVD516 (data not shown) had two fragments inserted in tandem in the *Hin*dIII site of pTP8-5. The results indicate that fragment B contains a promoter which is functional in *E. coli*. The clones HB101(pCVD515) and HB101(pCVD516) were both resistant to 2 μg of trimethoprim per ml, but susceptible to 5 μg of trimethoprim per ml, suggesting that the activity of the cloned promoter was relatively weak in *E. coli* as judged by the level of resistance to trimethoprim (17). The direction of transcription originating in the cloned promoter and reading into the *fol* gene was the same as the transcriptional direction of the *tdh* gene existing in fragment A of pCVD502 (Fig. 1).

Expression of the transferred *tdh* gene in *V. parahaemolyticus*. Expression of the transferred *tdh* gene in *V. parahaemolyticus* S162-71-RIF(pCVD504) was measured by the amount of TDH detected in the culture supernatant fluid. Under the incubation conditions specified above, this strain produced TDH at the level of 28 ng/ml, whereas strain WP1, the Kanagawa phenomenon-positive strain from which the *tdh* gene was originally cloned, produced TDH at 64 ng/ml.

DISCUSSION

The analysis of the DNA sequence of the *tdh* gene from *V. parahaemolyticus* reveals several interesting features. The amino acid sequence of TDH deduced from the DNA sequence predicts a mature protein of 165 amino acid residues with a calculated molecular weight of 18,496. In addition, a 24-amino-acid peptide preceding the mature protein was observed, suggesting the existence of a possible signal peptide. The calculated molecular weight of the preprotein is 21,140. Previous characterization of purified TDH (36) indicates that it consists of two subunits of identical molecular weight (21,000). It is evident that the *tdh* gene sequenced in this study encodes a single subunit of TDH. The *Hin*dIII fragment A of pCVD512 containing the *tdh* gene did not encode any additional protein. However, hemolysin expressed in *E. coli* HB101(pCVD512) was biologically active and serologically identical with purified TDH, indicating that it is bona fide TDH. Therefore our results suggest that the two subunits of TDH are identical and derived from the same gene copy. In addition, it can be assumed that the subunits expressed from the *tdh* gene cloned into *E. coli* are assembled into an active form (dimer) inside the cells, because active TDH was detected in sonicated cell lysates.

The amino acid sequence of TDH predicted from the DNA sequence closely agrees with information obtained from direct amino acid sequencing. The N-terminal amino acid sequence of TDH was reported by Zen Yoji et al. (39), and we find no discrepancies in the first 20 amino acids. After completion of the DNA sequencing, we learned of the complete amino acid sequence determined by Tsunazawa and colleagues (S. Tsunazawa, F. Sakiyama, and Y. Takeda, Annual Meeting of the Japanese Society for Biochemistry, October, 1983). As in our sequence, the complete amino acid sequence consisted of 165 residues. There was disagreement on seven of the residues, all of which were in regions where both DNA strands were sequenced.

The DNA sequence alone did not allow us to definitively position the promoter of the *tdh* gene. When a 5.7-kb *Sal*I fragment was inserted into pBR322 to yield pCVD502, the *tdh* gene was expressed in *E. coli*. However, when a 1.3-kb *Hin*dIII fragment of pCVD502 (fragment A, Fig. 1) was subcloned into pBR322, the *tdh* gene was expressed in one orientation (pCVD512), but not in the other (pCVD511). These results indicate that there is no functional promoter in fragment A that can be used to transcribe the *tdh* gene in *E. coli*, but that in clone HB101(pCVD512) the gene was transcribed from the antitetracycline promoter (38) or P1 promoter (34) of the vector plasmid pBR322.

The *tdh* gene in the larger *Sal*I fragment in pCVD502 is presumably transcribed from its own promoter, because the plasmid pBR322 does not have a promoter that can read through the *Sal*I site in a counterclockwise direction (34). One possibility is that there may be a promoter upstream of fragment A in the *Sal*I fragment which is functional in *E. coli* and transcribed in the same direction relative to the *tdh* gene. Weak promoter activity was detected by cloning fragment B of pCVD502 into a promoter probe plasmid. The nucleotide sequence of fragment A indicates, however, that the promoter existing in fragment B is removed from the putative Shine-Dalgarno sequence by at least 237 bp. Accordingly, it seems unlikely that this promoter is a natural promoter of the *tdh* gene. The nucleotide sequence data also suggested that there are sequences that resemble *E. coli* consensus sequences (−35 and −10 region) immediately upstream of the putative Shine-Dalgarno sequence in fragment A (Fig. 4), and we tentatively propose this region as a promoter of the *tdh* gene. Among bases that demonstrated homology with the consensus sequences of the −35 and −10 regions (indicated by dots), all three in the −35 region and three of four in the −10 region agreed with the strongly conserved bases of these regions of *E. coli* promoters (14, 21). Furthermore, the sequences upstream of the −35 region were rich in A+T, like other *E. coli* promoters (30). Interestingly, the putative −35 and −10 regions are separated by 20 bp. It has been reported that promoters with consensus sequences were active in *E. coli* when the −35 and −10 regions are separated by 16 to 18 bp, but not when the distance is expanded to 19 or 20 bp (2). This spacing could explain why the presumptive promoter in fragment A was not functional in *E. coli*. At the present time, we do not have evidence that the proposed promoter is functional in *V. parahaemolyticus*, and nucleotide sequences of possible

promoter regions reported from members of the genus *Vibrio* are too meager to be compared.

Although TDH was detected in cell lysates of HB101(pCVD502), no zones of hemolysis were seen when this clone was grown on a blood agar plate (data not shown). Moreover, polymyxin B treatment (6) of HB101(pCVD502) showed that active hemolysin was present mostly in the cytoplasm, but not in the periplasmic space (data not shown). These observations indicate that TDH was produced in, but not secreted by, *E. coli*. Although few genes from the genus *Vibrio* have been cloned in *E. coli*, at least one precedent can be cited. Cholera enterotoxin is normally secreted by *Vibrio cholerae*, but not by *E. coli* containing cloned cholera toxin genes (29). TDH is normally secreted by *V. parahaemolyticus*, and a reasonable amount of TDH (28 ng of TDH per ml) was secreted when the cloned *tdh* gene was introduced into a Kanagawa phenomenon-negative *V. parahaemolyticus* strain. The DNA sequence suggests that the cloned *tdh* gene encodes a singal peptide and this supports the hypothesis of TDH secretion by *V. parahaemolyticus*. The 24 amino acids preceding the known N-terminal amino acid, phenylalanine, must be removed to generate the mature protein. In the middle of the proposed signal peptide, however, three charged lysine residues were found, which is unusual for *E. coli* signal peptides and may account for the lack of TDH secretion by the *E. coli* clones. As with *Vibrio* promoter sequences, there is a paucity of published *Vibrio* signal peptide sequences, so comparison between these organisms is difficult.

As mentioned above, we designated the gene encoding TDH as the *tdh* gene. The term "Kanagawa phenomenon-associated hemolysin" historically precedes TDH and is occasionally substituted for TDH because this hemolysin was originally discovered in association with the Kanagawa phenomenon. The Kanagawa phenomenon, however, does not always correlate with TDH production. Routine hemolysis tests for the Kanagawa phenomenon may give false-positive results, depending on experimental conditions (7), and the Kanagawa phenomenon test is not as easily read as a serological method to detect TDH (15). Therefore, we propose to use Tdh, instead of the Kanagawa phenomenon, for the phenotype associated with the *tdh* gene; the positive Tdh phenotype should be defined on the basis of biological activity (hemolysis, but not necessarily the Kanagawa phenomenon test) as well as serological reactivity with anti-TDH.

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