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Evidence for Insertion Sequence-Mediated Spread of the Thermostable Direct Hemolysin Gene among *Vibrio* Species

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The *tdh* gene of *Vibrio parahaemolyticus* which encodes the thermostable direct hemolysin has been found in some strains of other *Vibrio* species. Analysis of seven *tdh* genes cloned from *V. parahaemolyticus*, *Vibrio mimicus*, and non-O1 *Vibrio cholerae* revealed that all *tdh* genes were flanked by insertion sequence-like elements (collectively named ISVs) or related sequences derived from genetic rearrangement of ISVs. The ISVs possessed 18-bp terminal inverted repeats highly homologous to those of IS*903* (2- to 4-bp mismatch) and were 881 to 1,058 bp long with <33.6% sequence divergence. These features and nucleotide sequence similarities among ISVs and IS*903* (overall homologies between ISVs and IS*903*, ca. 50%) strongly suggest that they were derived from a common ancestral sequence. A family of ISVs were widely distributed in *Vibrio* species, often regardless of the possession of the *tdh* genes, and one to several copies of the ISVs per organism were detected. A strain of *V. mimicus* possessed two copies of the ISVs flanking the *tdh* gene and three copies unrelated to the *tdh* gene. However, the transposition activity of the ISVs could not be demonstrated, probably because they had suffered from base changes and insertions and deletions within the transposase gene. The possible mode of ISV-mediated spread of the *tdh* gene is discussed from an evolutionary standpoint.

Vibrios are widely distributed in the marine environment, and some species can cause gastroenteritis in humans through seafood consumption (5, 18). The pathogenic mechanism of one such bacterium, *Vibrio parahaemolyticus*, is not clearly understood, but the thermostable direct hemolysin (TDH) produced by this organism is considered an important virulence factor (37). Recently, some strains belonging to other *Vibrio* species isolated from diarrheal patients were found to produce hemolysins similar to TDH. These species include non-O1 *Vibrio cholerae* (44, 45), *Vibrio mimicus* (15), and *Vibrio hollisae* (46).

We have been studying the gene (*tdh*) encoding TDH. Five *tdh* genes cloned from *V. parahaemolyticus* (designated *tdh*1 to *tdh*5) have been sequenced, and it was found that the coding regions had a sequence divergence of less than 3.3% (2, 29, 30). The genes encoding the TDH-like hemolysins of other *Vibrio* species have also been cloned (16, 31). The coding sequences of the genes cloned from non-O1 *V. cholerae*, *V. mimicus*, and *V. hollisae* had 96.7% or more, 97.0% or more, and 93.3% or more homologies, respectively, with genes *tdh*1 to *tdh*5. Therefore, these genes were included in the *tdh* gene family and were named NAG-*tdh*, Vm-*tdh*, and Vh-*tdh*, respectively (1, 39, 43).

While the majority of clinical strains of *V. parahaemolyticus* and *V. hollisae* possessed the *tdh* gene (28), only some clinical strains of non-O1 *V. cholerae* and *V. mimicus* had the *tdh* gene (16, 31). Evidence to support the hypothesis of plasmid-mediated transfer of the *tdh* gene between *Vibrio* species was obtained by comparative analysis of two plasmid-borne *tdh* genes (*tdh*4 and NAG-*tdh*) (1), and it could explain the distribution of the *tdh* gene in many *Vibrio* species. However, the *tdh* gene is usually present in the chromosome (30). In addition, we found that the *tdh* genes of *V. parahaemolyticus* were flanked by nucleotide sequences similar or identical to the terminal inverted repeat sequences of IS*102* (2, 27). Therefore, we put forward the hypothesis that the *tdh* gene may be associated with an insertion sequence (IS), possibly present on a transposon, and was transferred between chromosomes and plasmids through an IS-mediated mechanism. This hypothesis could also explain other observations, e.g., how the *tdh* gene was duplicated in Kanagawa phenomenon-positive (beta-hemolytic on Wagatsuma agar medium) strains of *V. parahaemolyticus* (30) and why some strains of *V. parahaemolyticus* spontaneously lost the *tdh* gene (29, 38).

In this article, we present evidence at a nucleotide sequence level to support the idea that IS-mediated transfer of the *tdh* genes and subsequent rearrangement of the ISs occurred during the evolution of the *tdh* genes. We first found that the Vm-*tdh* gene was flanked by IS-like sequences (collectively named ISVs) similar to IS*903*, which is related to IS*102*. We were then able to identify similar ISVs or related sequences in the regions flanking other *tdh* genes. We also found that a family of nucleotide sequences, which are similar to these ISVs but are not always associated with the *tdh* genes, are widely distributed in various *Vibrio* species. Transposition activity of the ISVs was not demonstrated, but, on the basis of nucleotide sequence analysis, we discuss a possible mode of IS-mediated *tdh* gene transfer from an evolutionary standpoint.

MATERIALS AND METHODS

Bacterial strains and plasmids. Wild-type *Vibrio* strains which were tested with three or four DNA probes by the DNA colony hybridization method (described below) were of clinical or environmental origin and were stock cultures in our laboratory. Of these strains, the following eight were used for the Southern hybridization and/or cloning experiments: *V. parahaemolyticus* WP1 and AQ3776 (30), *V. parahaemolyticus* AQ3860 (2), non-O1 *V. cholerae* 91 (16),

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TABLE 1. Plasmids used in this study

Plasmid	Description	Source or reference
pUC9		41
pUC118/pUC119		42
pACYC184		8
pCVD534	12.6-kb *Pst*I fragment containing the *tdh* gene copy of *V. parahaemolyticus* WP1 (Vp-*tdh*1) cloned into pBR322	28
pCVD538	8.1-kb *Sal*I fragment containing the *tdh* gene copy of *V. parahaemolyticus* WP1 (Vp-*tdh*2) cloned into pBR322	31
pKTN420	5.0-kb *Sac*I fragment containing the *tdh* gene copy of *V. parahaemolyticus* AQ3776 (Vp-*tdh*3) cloned into pUC118	This study
pCVD532	5.0-kb *Hin*dIII fragment containing the *tdh* gene copy of *V. parahaemolyticus* AQ3776 (Vp-*tdh*4) cloned into pBR322	28
pKTN201	6.4-kb *Sac*I fragment containing the *tdh* gene copy of *V. parahaemolyticus* AQ3860 (Vp-*tdh*5) cloned into pUC118	2
pCVD541	9-kb *Hin*dIII fragment containing the *tdh* gene copy of non-O1 *V. cholerae* 91 (NAG-*tdh*) cloned into pBR322	16
pCVD546	3.2-kb *Pst*I fragment containing the *tdh* gene copy of *V. mimicus* 6 (Vm-*tdh*) cloned into pBR322	31
pKTN419	9.8-kb *Ava*I fragment (blunt ended) containing the *tdh* gene copy of *V. mimicus* 6 (Vm-*tdh*) cloned into the *Sma*I site of pUC118	This study
pKTN412	0.67-kb *Bam*HI-*Hin*dIII fragment internal to ISV-ML cloned into the *Bam*HI-*Hin*dIII sites of pUC118	This study
pKTN403	0.4-kb *Alu*I-*Hae*III fragment internal to ORF1 cloned into the *Sma*I site of pUC9	This study
pKTN409	1.05-kb *Sac*I-*Hae*III fragment internal to ORF2 cloned into the *Sac*I-*Hin*cII sites of pUC118	This study
pKTN421	7.2-kb *Eco*RI fragment of *V. mimicus* 6 containing the sequence homologous to ISV-ML (ISV-M7.2) cloned into pUC118	This study
pKTN422	5.2-kb *Eco*RI fragment of *V. mimicus* 6 containing the sequence homologous to ISV-ML (ISV-M5.2) cloned into pUC118	This study
pKTN423	2.4-kb *Eco*RI fragment of *V. mimicus* 6 containing the sequence homologous to ISV-ML (ISV-M2.4) cloned into pUC118	This study
pGP704	Derivative of suicide vector pJM703.1	J. J. Mekalanos
pKTN430	1.5-kb *Hae*II fragment of pACYC184 carrying the chloramphenicol resistance gene (blunt ended) cloned into *Eco*RI site (blunt ended) of pGP704	This study
pKTN431	1.7-kb *Sph*I-*Xba*I fragment containing ISV-M5.2 cloned into the *Sph*I-*Xba*I sites of pKTN430	This study
pCHR71	Thermosensitive replication mutant of R388	33
pAT153	Nonmobilizable derivative of pBR322 lacking the transfer origin for conjugal transfer	40
pKTN435	1.7-kb *Sph*I-*Xba*I fragment containing ISV-M5.2 cloned into the *Sph*I-*Nhe*I sites of pAT153	This study

and *V. mimicus* 6 (31), all of which were isolated from clinical sources and possessed the *tdh* genes; non-O1 *V. cholerae* NRT-NAG71, a clinical strain lacking the *tdh* gene; O1 *V. cholerae* AQ1002, a clinical eltor strain possessing the cholera toxin (*ctx*) gene; and *V. parahaemolyticus* BG53, an environmental strain deficient in the *tdh* gene and which was used in the replicon fusion experiments. *Escherichia coli* HB101 (6), JM103 (23), and MC1061 (7) were used as hosts for construction and propagation of plasmids. *E. coli* MV1184 (42) was used as a host for preparation of single-stranded DNA. Other *E. coli* strains were used in the replicon fusion experiments: SY327 λ *pir* and SM10 λ *pir* (24) in the experiment with a suicide vector and DH1 (13) and C2110 *polA* Nal^r (34) in the experiment using a R388 derivative. The plasmids employed or constructed in this study are described in Table 1.

Reagents. Restriction enzymes and modification enzymes were purchased from Takara Shuzo Co. (Kyoto, Japan), Toyobo Co. (Osaka, Japan), and Boehringer Mannheim (Penzberg, Germany). Radioactive compounds used for nucleotide sequence determination and DNA probe labeling were obtained from Amersham Corp. (Buckinghamshire, England).

General genetic techniques and nucleotide sequence determination. Standard methods (21) and/or their modifications, described previously (29), were employed for DNA manipulations. For determining the nucleotide sequences of DNA fragments, the DNA fragments were cloned into pUC118/pUC119. Single-stranded DNA to be used for sequencing was then prepared by the method of Vieira and Messing (42), and the nucleotide sequence was determined by dideoxy chain-termination method as described by Mizusawa et al. (25).

DNA probes and hybridizations. Three DNA probes were constructed in this study. The probe specific to ISV-ML (described below) was constructed by cloning a 0.67-kb *Bam*HI-*Hin*dIII fragment internal to ISV-ML (Fig. 1) into pUC118, resulting in a recombinant plasmid, pKTN412. The probe DNA (*Bam*HI-*Hin*dIII fragment) was prepared from pKTN412. The probe specific to open reading frame 1 (ORF1) (Fig. 1) was constructed by cloning a 0.4-kb *Alu*I-*Hae*III fragment internal to ORF1 (Fig. 1) into the *Sma*I site of pUC9, resulting in a recombinant plasmid, pKTN403. In the *tdh* genes other than the Vm-*tdh* gene, the sequences homologous to ORF1 were interrupted by the terminal inverted repeats of ISVs or ISV-9TS at the site immediately upstream of the *Alu*I site (see Fig. 3). Therefore, the *Alu*I-*Hae*III fragment was considered specific to ORF1. pKTN403 was digested with *Bam*HI and *Eco*RI, which were immediately adjacent to the *Sma*I site in pUC9, and the 0.4-kb *Bam*HI-*Eco*RI fragment was isolated and used as the probe DNA specific to ORF1. The probe specific to ORF2 (Fig. 1) was constructed by cloning a 1.05-kb *Sac*I-*Hae*III fragment internal to ORF2 (*Hae*III site is located 0.28 kb

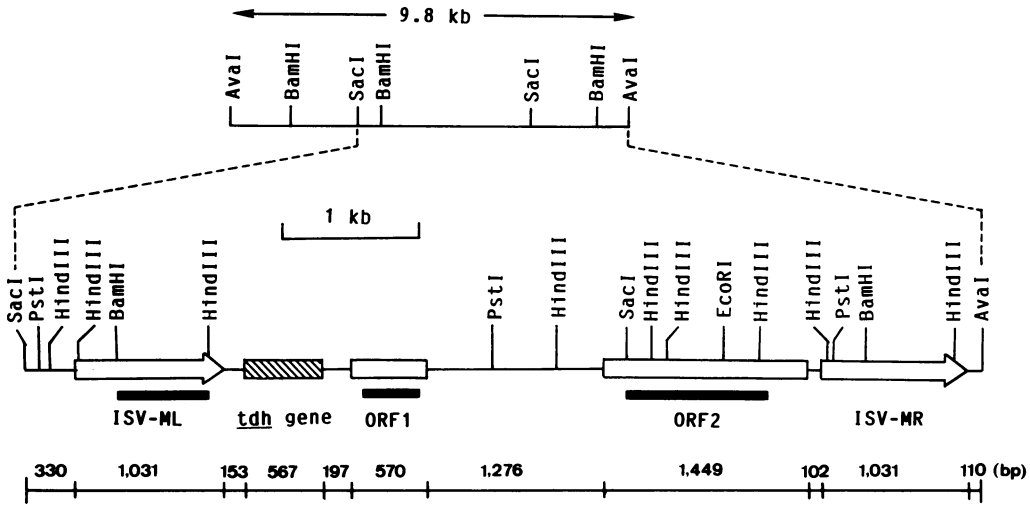

FIG. 1. Restriction map of the 9.8-kb *Ava*I fragment bearing the Vm-*tdh* gene and schematic representation of the genetic structures contained in the 6.8-kb *Sac*I-*Ava*I subfragment. The size (in base pairs) of each genetic structure is indicated at the bottom. The transcriptional directions of the *tdh* gene and two ORFs (ORF1 and ORF2) are from left to right. The orientations of two ISVs (ISV-ML and ISV-MR) are determined by homology with IS*903* (see text and Fig. 2 and 5). The approximate positions of three DNA probes constructed in this study (the probes specific to ISV-ML, ORF1, and ORF2) are indicated by solid bars under the respective genetic structures.

downstream of the *Eco*RI site shown in Fig. 1) into the *Sac*I- and *Hin*cII-cleaved pUC118, resulting in a recombinant plasmid, pKTN409. pKTN409 was digested with *Sac*I and *Pst*I (immediately flanking the *Hin*cII site), and the 1.05-kb *Sac*I-*Pst*I fragment obtained was used as the DNA probe specific to ORF2. The *tdh* gene probe, which was derived from a 406-bp fragment internal to the *tdh* gene (71% of the coding sequence), was described previously (28). The probe fragments were labeled with ^{32}P by the random priming method (9) and used for hybridization. The DNA colony hybridization test (28) and the Southern blot hybridization (30) were performed as described previously.

Replicon fusion experiments. Transposition activity of ISV-M5.2 (described below) was examined in two replicon fusion experiments. In the first experiment, a suicide vector system described by Miller and Mekalanos (24) was employed because we had already used this system successfully for constructing insertion mutations in *V. parahaemolyticus* (26). pGP704, a derivative of the suicide vector pJM703.1 (24) into which a polylinker was inserted, was constructed in J. J. Mekalanos' laboratory and obtained through J. B. Kaper. pGP704 was digested with *Eco*RI, blunt ended with a Klenow enzyme, and ligated with a blunt-ended 1.5-kb *Hae*II fragment of pACYC184 (8) carrying the chloramphenicol resistance gene. The resultant plasmid, pKTN430, was a suicide vector with two antibiotic markers (Ap^r Cm^r). The 1.7-kb *Sph*I-*Xba*I fragment bearing ISV-M5.2 was cloned into the *Sph*I- and *Xba*I-cleaved pKTN430 and transformed into *E. coli* SY327 λ *pir*, resulting in a recombinant plasmid, pKTN431 (Ap^r Cm^r). pKTN431 was purified and then transformed into *E. coli* SM10 λ *pir*, which can mobilize pGP704 derivatives. SM10 λ *pir*(pKTN431) was incubated with a recipient on a membrane filter (0.4-μm pore size; Nuclepore Corp.) for mating at 37°C for 12 h and then spread onto TCBS agar (Difco) containing chloramphenicol (20 μg/ml). The recipient strain used was *V. parahaemolyticus* BG53, which did not possess the sequences homologous to ISV-M5.2. Since pKTN431 cannot replicate without the function provided in trans by the *pir* gene, it is possible to detect a clone in which the whole plasmid pKTN431 was integrated into the host chromosome either by the transposition activity of ISV-M5.2 or by homologous recombination. Such a clone should grow on TCBS agar supplemented with chloramphenicol. The frequency of integration by homologous recombination is expected to be minimal in recipient strain BG53.

In the second experiment, we looked for the fusion of two plasmids mediated by ISV-M5.2. A 1.7-kb *Sph*I-*Xba*I fragment carrying ISV-M5.2 was cloned into the *Sph*I- and *Nhe*I-cleaved pAT153 (Ap^r), a nonmobilizable derivative of pBR322 in which the transfer origin for conjugal transfer was deleted (40). The resulting plasmid, pKTN435, was transformed into *E. coli* DH1 (*recA*). DH1(pKTN435) was then transformed with pCHR71, a thermosensitive replication mutant of R388 (Tp^r) compatible with ColE1 derivatives (33). The resultant donor strain, DH1(pKTN435, pCHR71), was mated with recipient strain C2110 *polA* Nal^r on the membrane filter at 30°C for 3 h and then spread onto Mueller-Hinton agar (Difco) containing trimethoprim, ampicillin, and nalidixic acid. Because pKTN435, if mobilized at all, cannot replicate in a *polA* host, there is a chance to detect a transconjugant which possesses the pKTN435-pCHR71 cointegrate.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this article have been assigned GenBank accession numbers as follows: M64120 for the 6,816-bp sequence containing Vm-*tdh* gene (Fig. 1); M64121 for ISV-3L (Fig. 2); M64122 for ISV-5L (Fig. 2); M64123 for ISV-5R (Fig. 2); M64124 for ISV-4R (Fig. 2); M64125 for ISV-NR (Fig. 2); M64126 for ISV-1L (Fig. 2); M64127 for ISV-ML (Fig. 2); M64128 for ISV-MR (Fig. 2); M64129 for ISV-M5.2 (Fig. 2); M64130 for ISV-M7.2 (Fig. 2); and M64131 for ISV-M2.4 (Fig. 2).

RESULTS

ISVs flanking the Vm-*tdh* gene. The nucleotide sequence of the coding region of the Vm-*tdh* gene cloned from *V. mimicus* 6 was determined previously (39). The complete nucleotide sequence of the 3.2-kb *Pst*I fragment of pCVD546 containing this Vm-*tdh* gene was determined in this study. A

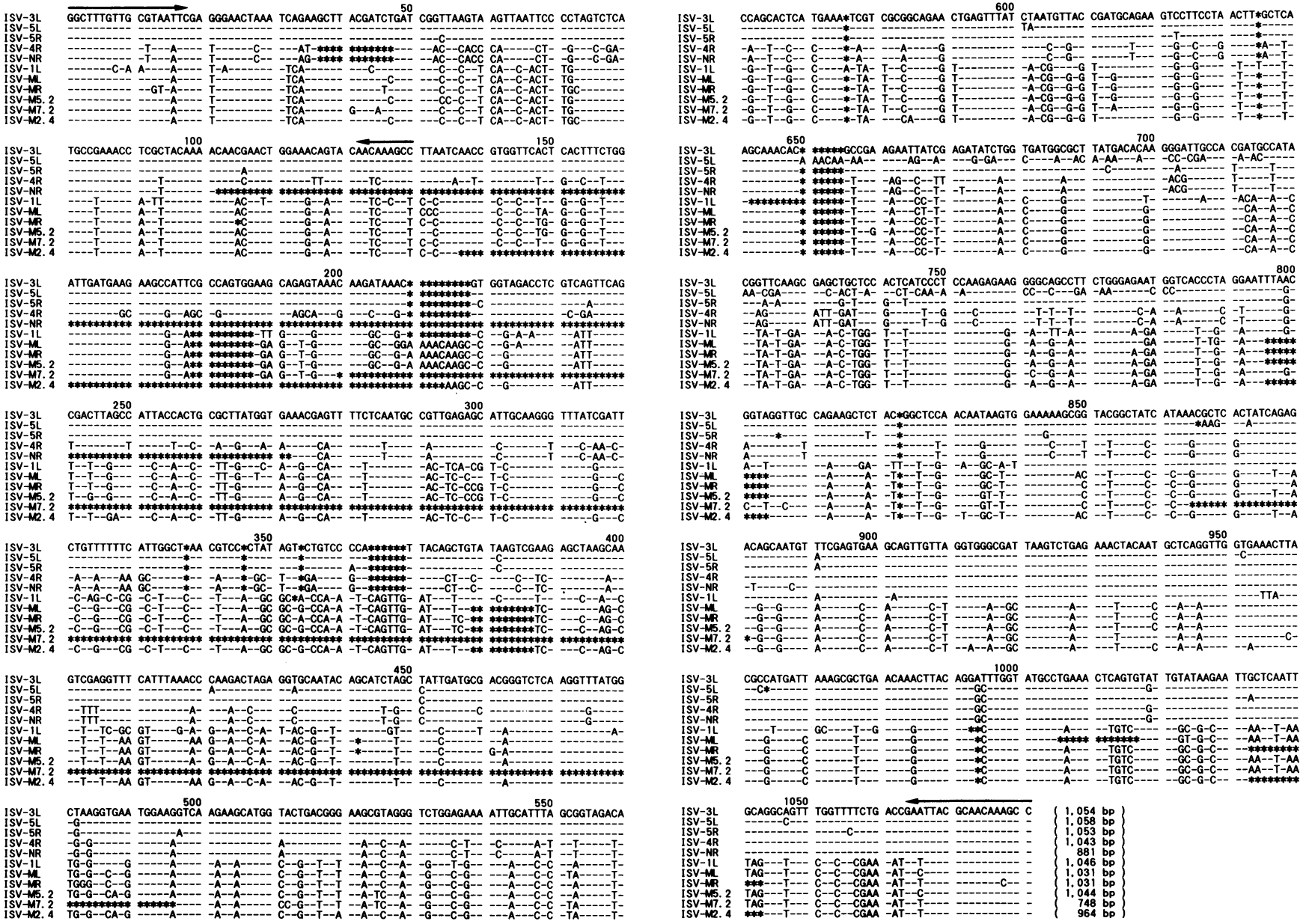

FIG. 2. Comparison of the nucleotide sequences of 11 ISVs aligned for maximum homology. Symbols used are the same as in Fig. 3. Since missing bases are included for alignment, the numbers do not necessarily indicate the actual base numbers of each ISV (indicated in parentheses at the end of the sequence). The long arrows show the 18-bp terminal inverted repeats of the ISVs. Nucleotides 121 to 130 in ISV-3L, ISV-5L, and ISV-5R (indicated by the short arrow) are identical to nucleotides 1,073 to 1,081 of these three ISVs, which are 9-bp terminal sequences of the terminal inverted repeats.

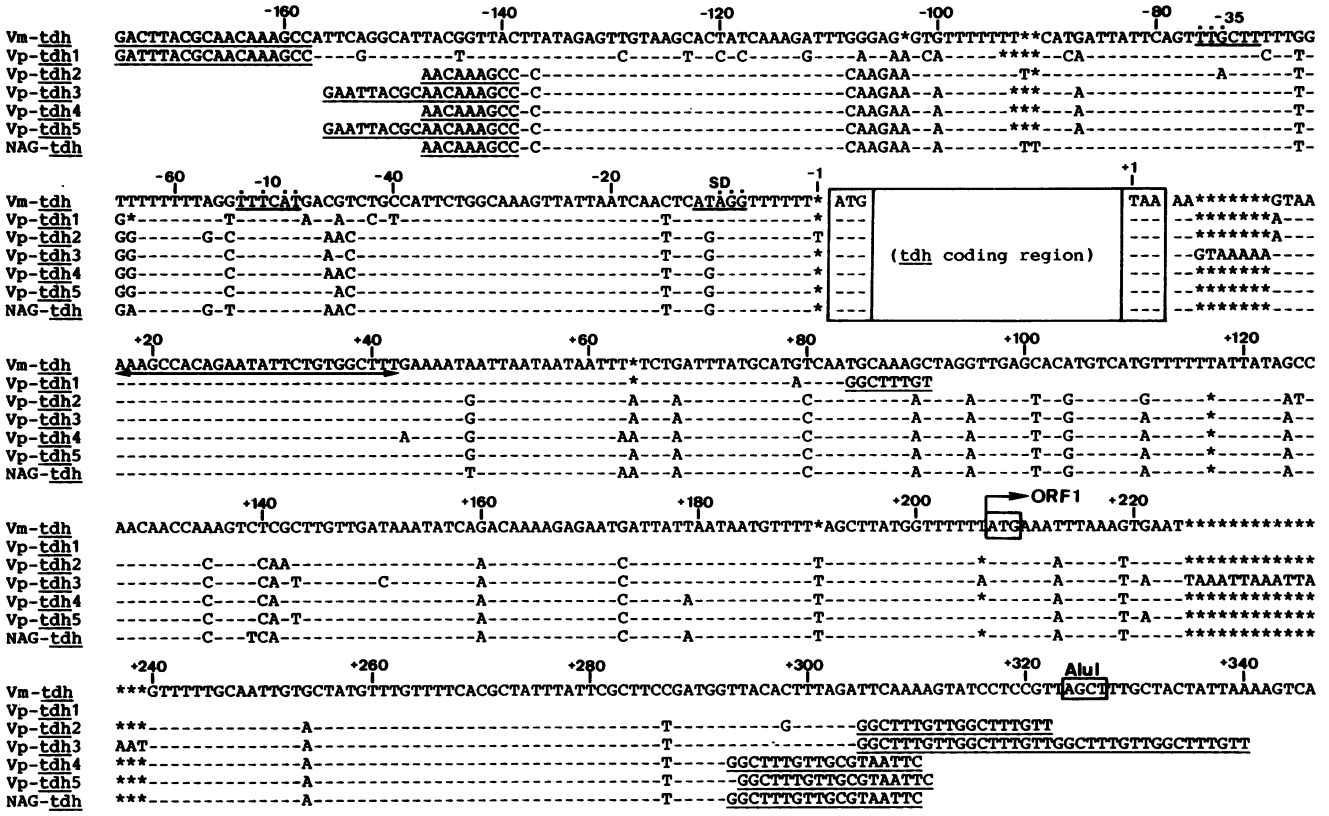

FIG. 3. Comparison of the nucleotide sequences immediately flanking the seven *tdh* coding sequences. The nucleotide sequences of these *tdh* coding sequences were reported previously (1, 2, 29, 30, 39). In this figure, the nucleotide sequences are aligned for maximum homology. Symbols: -, identical base to that of the Vm-*tdh*-bearing sequence; *, missing base. In the upstream regions, sequences showing homology with consensus sequences of *E. coli*, such as the Shine-Dalgarno sequences (SD) and −10 and −35 regions (−10 and −35) of the promoter, are indicated by dots above the underlined sequences. In the downstream regions, an inverted repeat sequence, a possible transcription terminator, is indicated (↔). The initiation codon of ORF1 downstream of the Vm-*tdh* gene (see Fig. 1) is also indicated (box). The underlined sequences at both upstream and downstream ends of the sequences were homologous to the terminal inverted repeat sequences of IS*102* and IS*903* and correspond to the terminal inverted repeats of the ISVs or related sequences (see Fig. 4).

1,031-bp ISV related to IS*903* (ca. 50% homology, shown below) was identified 153 bp upstream of the Vm-*tdh* coding region (ISV-ML in Fig. 1). Anticipating that there might be another ISV in the downstream region of the Vm-*tdh* coding region (thereby comprising a composite transposonlike structure), we cloned a larger 9.8-kb *Ava*I fragment containing the Vm-*tdh* gene from the chromosome of *V. mimicus* 6 into pUC118 (pKTN419) and determined the nucleotide sequence of the internal 6.8-kb *Sac*I-*Ava*I fragment (Fig. 1). An ISV was detected 3,594 bp downstream of the Vm-*tdh* coding region (ISV-MR in Fig. 1). Like ISV-ML, ISV-MR consisted of 1,031 bp and had 95.2% homology with ISV-ML, and these two ISVs were flanking the Vm-*tdh* gene in direct orientation. ISV-ML and ISV-MR possessed the terminal inverted repeats of 18 bp which were highly homologous but not identical to those of IS*903* (shown below). The G+C content of the 4,314-bp sequence existing between ISV-ML and ISV-MR was 32.6%, and it was in contrast to the G+C contents of ISV-ML (45.4%) and ISV-MR (46.6%). In addition to the Vm-*tdh* gene, there were two ORFs of 570 and 1,449 bp (ORF1 and ORF2, respectively, in Fig. 1) in the 4,314-bp region.

ISVs or related sequences flanking the other *tdh* genes. The findings mentioned above raised the possibility that the *tdh* gene might exist on a transposonlike element. We therefore analyzed the nucleotide sequences flanking the other *tdh* coding sequences which included five *tdh* genes of *V. parahaemolyticus* (previously named *tdh*1 to *tdh*5 and designated Vp-*tdh*1 to Vp-*tdh*5 in this study) and the NAG-*tdh* gene of non-O1 *V. cholerae*. These *tdh* coding sequences had high (96.7% or more) homologies with the Vm-*tdh* coding sequences and possessed sequences similar to the terminal inverted repeat sequences of IS*102* and IS*903* in the upstream and downstream regions (Fig. 3).

Various DNA fragments carrying the Vp-*tdh*1, -*tdh*2, -*tdh*3, -*tdh*4, and -*tdh*5 and NAG-*tdh* genes were subcloned from pCVD534, pCVD538, pKTN420, pCVD532, pKTN201, and pCVD541, respectively. The nucleotide sequences of the upstream and downstream regions of the six *tdh* genes were then determined. An ISV homologous to ISV-ML or ISV-MR or a related sequence was identified both upstream and downstream of all the six *tdh* coding sequences (schematically shown in Fig. 4). There existed ISVs similar to ISV-ML or ISV-MR upstream of the Vp-*tdh*1, Vp-*tdh*3, and Vp-*tdh*5 coding sequences and downstream of the Vp-*tdh*4, Vp-*tdh*5, and NAG-*tdh* coding sequences (designated ISV-1L, ISV-3L, ISV-5L, ISV-4R, ISV-5R, and ISV-NR, respectively; indicated in Fig. 4). The nucleotide sequences of these eight ISVs of vibrios, including ISV-ML and ISV-MR, were aligned for maximum homology and are presented in

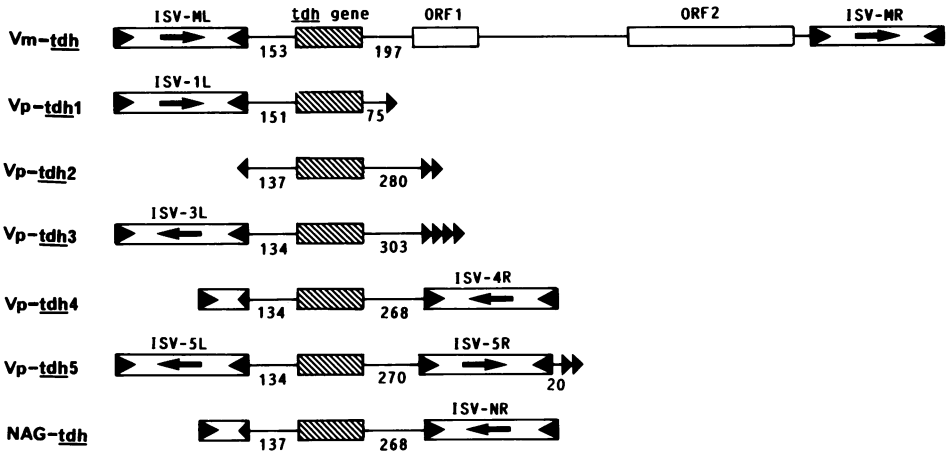

FIG. 4. Location of the ISVs and related sequences flanking seven *tdh* genes. ▶◀, ISV. The arrow denotes the orientation of the ISV determined from homology with IS*903* (see Fig. 2 and 5). ▶◀, truncated form of ISV-3L. The consensus sequences for the 18-bp terminal inverted repeats of the ISVs (▶, GGCTTTGTTGCGTAAATC; ◀, GATTTACGCAACAAAGCC) are included in the symbols for the ISVs. The 9-bp sequences, shorter versions of the 18-bp terminal inverted repeats of the ISVs, are indicated by ▸ (GGCTTTGTT) and ◂ (AACAAAGCC). Physical distances between the *tdh* coding sequences and ISVs or related sequences are indicated (in base pairs), except that the distance indicated downstream of the Vm-*tdh* gene is equal to that between the Vm-*tdh* coding sequence and ORF1. The nucleotide sequence downstream of ISV-5R (not shown in Fig. 3) was TCTGATGGTTACACTTTAGAGGCTTTGTTGGCTTTGTT.

Fig. 2. The length of the ISVs varied between 1,031 and 1,058 bp, with the exception of ISV-NR. ISV-NR suffered a 162-bp internal deletion, but the remaining nucleotide sequence was 99.3% homologous to that of ISV-4R.

A truncated form of ISV-3L (indicated in Fig. 4), a 130-bp sequence identical to bases 1 to 130 of ISV-3L, was present upstream of the Vp-*tdh*4 and NAG-*tdh* coding sequences in an orientation opposite to that of ISV-3L. As indicated in Fig. 2, the 9-bp terminal sequence of the truncated ISV (a sequence identical to bases 121 to 130 of ISV-3L) was identical to the 9-bp terminal sequence of the right terminal inverted repeat of ISV-3L (bases 1,073 to 1,081 in Fig. 2).

An ISV or an ISV truncate was absent, but the 9-bp sequence identical or homologous to the terminal inverted repeats of the ISVs (GGCTTTGTT or AACAAAGCC), a shorter version of the 18-bp terminal inverted repeat sequences, was present downstream of Vp-*tdh*1 and upstream of Vp-*tdh*2 (Fig. 3 and 4). These 9-bp sequences were collectively named ISV-9TS. ISV-9TS was tandemly repeated twice downstream of Vp-*tdh*2 and tandemly repeated four times downstream of Vp-*tdh*3 (Fig. 3 and 4). ISV-9TS tandemly repeated twice was also found 20 bp downstream of ISV-5R (Fig. 4).

To examine the presence or absence of ORF1 and ORF2 in vibrios, 368 strains of *Vibrio* species were tested under high-stringency conditions by DNA colony hybridization with DNA probes specific to ORF1, ORF2, and the *tdh* gene. The test strains consisted of 273 strains of *V. parahaemolyticus* (124 strains were *tdh* gene positive), 82 strains of non-O1 *V. cholerae* (1 strain was *tdh* gene positive), and 13 strains of *V. mimicus* (5 strains were *tdh* gene positive). The sequences homologous to ORF1 and ORF2 were present only in the five strains of *V. mimicus* which possessed the *tdh* gene.

Comparison of nucleotide sequences of ISVs and IS*903*. The nucleotide sequence homologies among the eight ISVs identified so far (Fig. 4) were calculated from the sequences shown in Fig. 2. The results indicated that these ISVs could be clustered into three homology groups. The first group consisted of ISV-3L, ISV-5L, and ISV-5R (92.2% or more homology), the second group consisted of ISV-4R and ISV-NR (99.3% homology), and the third group consisted of ISV-ML, ISV-MR, and ISV-1L (88.4% or more homology). Members of the first group had homologies of 77.7 to 84.2% with members of the second group and 66.4 to 72.1% with members of the third group. Members of the second group had homologies of 69.3 to 70.1% with members of the third group.

ISV-5R, ISV-4R, and ISV-ML were then chosen as representatives of the three groups, and these ISVs and IS*903* were aligned for maximum homology (Fig. 5). It is evident that they comprise a family of ISs. IS*903* contains an ORF (921 bp) that encodes a transposase composed of 307 amino acid residues (12). However, the ORFs identified in the ISVs were considerably shorter: 789 bp in ISV-5R, 564 bp in ISV-4R, and 453 bp in ISV-ML. The following observations could, however, be interpreted to mean that base substitutions, deletions, or insertions might have occurred in the ORFs which had originally encoded transposases. In ISV-5R, a single base deletion at the 235th codon might have caused a frameshift mutation. Otherwise, ISV-5R contains an ORF of 918 bp. Sequence comparison in Fig. 2 suggests that the missing nucleotide may be G (base 806). In ISV-4R, a stop codon (TAG) was identified at the 189th codon. Figure 2 suggests that it may be a point mutation from G to T (base 670). Substitution of T with G at this position will restore an ORF of 918 bp in ISV-4R. In ISV-ML, a single base deletion at the 116th codon may have resulted in a frameshift mutation. As suggested by Fig. 2, the missing base may be G (base 442). If the 442nd missing base is filled in with G, an ORF of 903 bp will be restored in ISV-ML. A 9-bp in-frame deletion is also seen in ISV-ML (codons 232 to 234).

In order to gain insight into the transposases which might have been originally encoded by the ISVs, the base deletions in ISV-5R and ISV-ML and the base substitution in ISV-4R were restored (as described above) and the amino acid sequences deduced from the nucleotide sequences were compared with that of IS*903* (Table 2). Conservation of the similar amino acid sequences in the putative transposases of

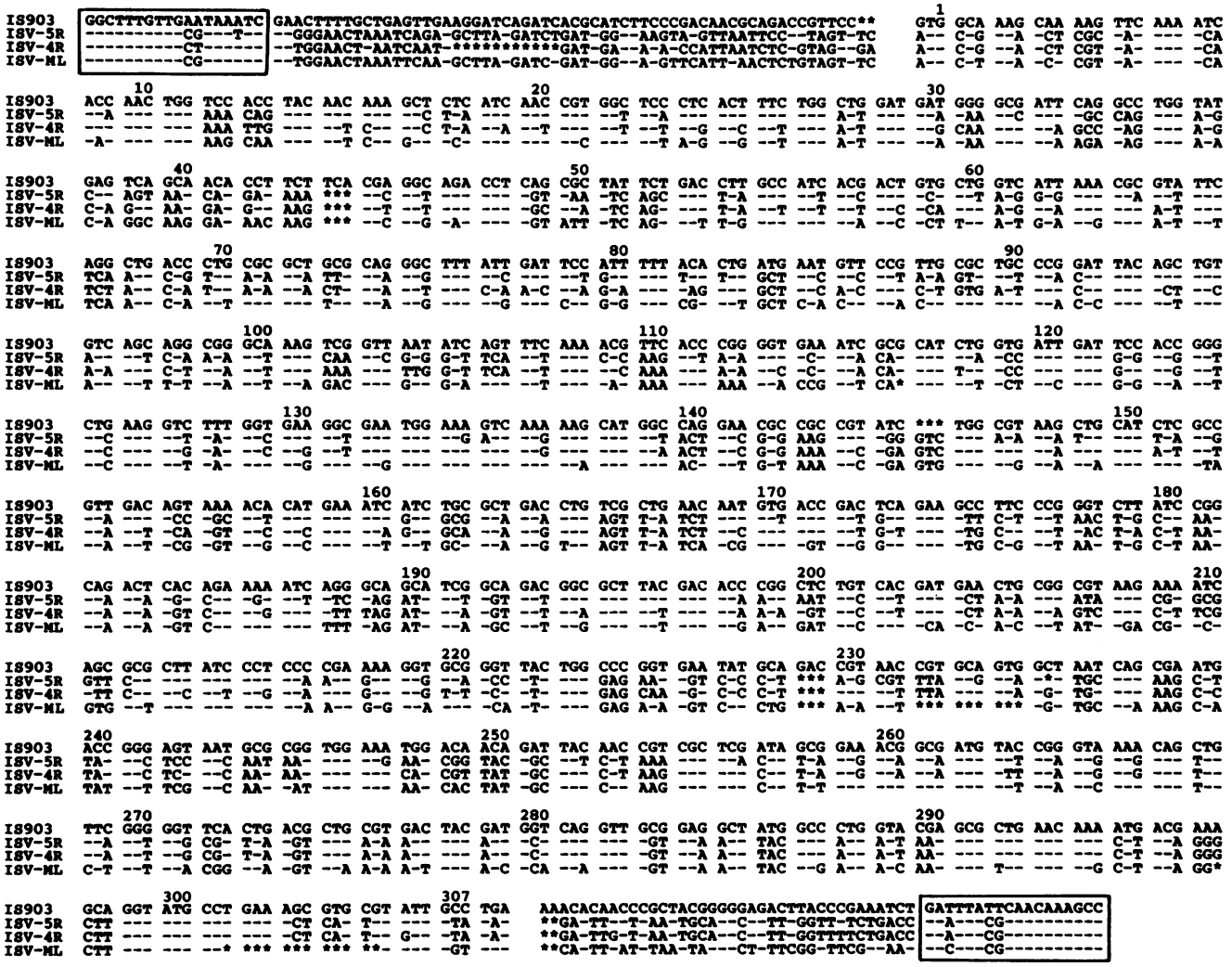

FIG. 5. Comparison of nucleotide sequences of IS*903*, ISV-5R, ISV-4R, and ISV-ML. The nucleotide sequences of IS*903* was reported previously (12). The sequences of ISV-5R, ISV-4R, and ISV-ML are aligned for maximum homology with IS*903*. Codons of the transposase encoded by IS*903* are numbered at the top. Symbols used are the same as in Fig. 3. The boxed sequences are the 18-bp terminal inverted repeats.

TABLE 2. Comparison of the aligned amino acid sequences of the putative transposases encoded by IS*903* and ISVs[a]

Sequence compared	% Identity	% Similarity[b]
IS*903* vs ISV-5R	60	79
IS*903* vs ISV-4R	59	78
IS*903* vs ISV-ML	54	76
ISV-5R vs ISV-4R	88	96
ISV-5R vs ISV-ML	82	92
ISV-4R vs ISV-ML	79	90

[a] The sequences of the putative transposases of ISVs were completed by the following base changes: insertion of G at base 806 in ISV-5R; substitution of G with T at base 670 in ISV-4R; insertion of G at base 442 in ISV-ML (see Fig. 2 and 5 and text).

[b] Values obtained when chemically similar amino acid groups are taken into account.

IS*903* and the ISVs (76 to 79% similarity) supports the notion that these proteins have similar functions. The degrees of identity between the putative transposases (IS*903* versus ISVs, 54 to 60%; between ISVs, 79 to 88%) indicate that these sequences have diverged from a common ancestral sequence but were probably modified in their individual genetic backgrounds.

Distribution of the ISV family in various *Vibrio* species. Strains (398) belonging to four *Vibrio* species were examined for the presence or absence of the DNA sequence homologous to ISV-ML and the *tdh* gene by the DNA colony hybridization test with specific DNA probes under high stringency. The results are summarized in Table 3. The *tdh* gene was detected in 143 strains belonging to *V. parahaemolyticus*, non-O1 *V. cholerae*, and *V. mimicus*. Of all the *tdh* gene-positive strains, 123 (86.0%) showed positive results and 14 (9.8%) showed weakly positive results with the ISV-ML-specific probe. In addition, of the *tdh* gene-negative strains belonging to *V. parahaemolyticus*, non-O1 *V. cholerae*, and O1 *V. cholerae*, 30.0 to 36.0% strains of each

TABLE 3. Results of the DNA colony hybridization test with DNA probes specific to the *tdh* gene and ISV-ML[a]

Species	No. of strains	DNA probe[b]	
		tdh gene	ISV-ML
V. parahaemolyticus	117	+	+
	14	+	+W
	6	+	−
	47	−	+
	36	−	+W
	65	−	−
V. cholerae (non-O1)	1	+	+
	32	−	+
	12	−	+W
	45	−	−
V. cholerae (O1)	3	−	+
	2	−	+W
	5	−	−
V. mimicus	5	+	+
	8	−	−

[a] Hybridization was performed under high stringency.
[b] +, positive; +W, weakly positive; −, negative.

species gave positive results and 13.5 to 24.3% strains of each species gave weakly positive results with the ISV-ML-specific probe. These results suggest that the nucleotide sequences homologous to ISV-ML are not only associated with the *tdh* gene but are also widely distributed among *Vibrio* species regardless of possession of the *tdh* gene.

Five strains representing the ISV-ML probe-positive strains were analyzed by the Southern blot hybridization with the ISV-ML-specific probe under high stringency (Fig. 6). The total DNA of *V. mimicus* 6 containing the Vm-*tdh* gene flanked by ISV-ML and ISV-MR appears to have several ISV-ML-like sequences, including these two ISVs (Fig. 6, panel 1). The total DNA of *V. parahaemolyticus* WP1, which possessed Vp-*tdh*1, Vp-*tdh*2, and ISV-1L, exhibited only one probe-positive band corresponding to ISV-1L (panel 2). Non-O1 *V. cholerae* 91 carries the plasmid-borne NAG-*tdh* and ISV-NR. The total DNA of this strain contained several probe-positive sequences, although such sequences were not identified in the plasmid DNA (panel 3). The results indicate that the ISV-ML-like sequences are present in the chromosome and that the homology of the plasmid-borne ISV-NR (69.3%) was not high enough to be detected with the ISV-ML probe. Non-O1 *V. cholerae* NRT-NAG71 did not carry the *tdh* gene, but several probe-positive sequences were detected in the total DNA extracted from this strain and the hybridization pattern was similar to that of non-O1 *V. cholerae* 91 (panel 4). O1 *V. cholerae* AQ1002 did not possess the *tdh* gene but had a sequence homologous to ISV-ML (panel 5). *V. parahaemolyticus* AQ3776, which carries the chromosomal Vp-*tdh*3 and ISV-3L and the plasmid-borne Vp-*tdh*4 and ISV-4R, was also examined. Two weakly positive bands, one in the chromosome and one on a plasmid, were observed only on extended autoradiography (data not shown). ISV-3L and ISV-4R (70.1% and 69.3% homologous to ISV-ML, respectively) were probably responsible for the weakly positive reactions. These results indicate that a family of ISVs with considerably diversified nucleotide sequences, thus not detectable by a single DNA probe, are distributed among various vibrios and that the copy number of the ISVs per organism may range from one to several.

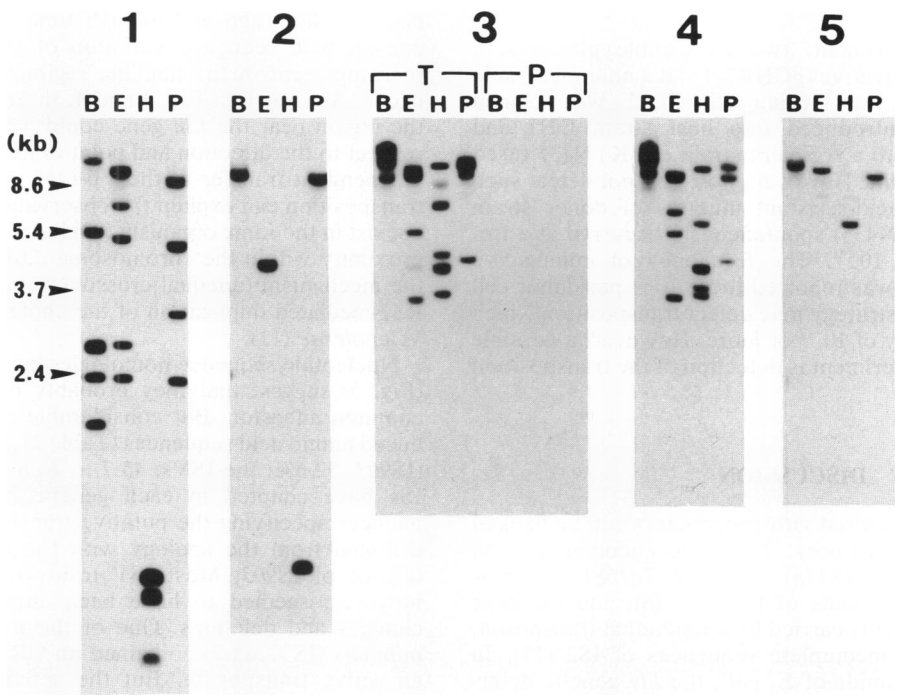

FIG. 6. Southern blot hybridization of the DNA isolated from representative strains of *Vibrio* species with ISV-ML-specific probe. Lanes are as follows: B, *Bam*HI digest; E, *Eco*RI digest; H, *Hin*dIII digest; P, *Pst*I digest. The DNAs examined are the total DNA of *V. mimicus* 6 (panel 1), total DNA of *V. parahaemolyticus* WP1 (panel 2), total DNA (T) and plasmid DNA (P) of non-O1 *V. cholerae* 91 (panel 3), total DNA of non-O1 *V. cholerae* NRT-NAG71 (panel 4), and total DNA of O1 *V. cholerae* AQ1002 (panel 5). Positions of the molecular mass markers (in kilobases) are indicated on the left.

Comparative analysis of five DNA sequences of *V. mimicus* homologous to ISV-ML. Of the ISV-ML-like sequences detected in the *V. mimicus* 6 chromosome, three sequences other than ISV-ML and ISV-MR (Fig. 6, panel 1) were cloned, and their sequence divergence was investigated. Because ISV-ML and ISV-MR did not possess an internal *Eco*RI site (Fig. 1), each probe-positive *Eco*RI fragment was expected to contain the complete ISV-ML-like sequence. Southern blot hybridization analysis with the ORF2-specific probe revealed that, among the probe-positive *Eco*RI fragments (11.0, 7.2, 5.2, 2.7, and 2.4 kb), the 11.0- and 2.7-kb fragments contained ISV-ML and ISV-MR, respectively (data not shown). The remaining *Eco*RI fragments (7.2, 5.2, and 2.4 kb) were cloned (resulting in pKTN421, pKTN422, and pKTN423, respectively). ISV-ML-like sequences were identified by subcloning and nucleotide sequence determination, and they were designated ISV-M7.2, ISV-M5.2, and ISV-M2.4, respectively. The nucleotide sequences of the three ISVs thus identified are aligned for maximum homology in Fig. 2, along with the other ISVs. Of the five ISVs of *V. mimicus*, only ISV-M5.2 contained a large ORF (915 bp, bases 80 to 1,003 in Fig. 2), which might encode a functional transposase.

In two experiments (see Materials and Methods for details), we examined whether the putative transposase of ISV-M5.2 is functional. In one experiment, we cloned ISV-M5.2 into a suicide vector which, although mobilizable by conjugation, cannot replicate in a recipient lacking the *pir* gene. Then, we sought a clone in which ISV-mediated integration of the whole suicide vector into the chromosome of the *pir* gene-deficient host occurred. This attempt was unsuccessful. Although this strategy may allow the putative transposase gene to be expressed in *Vibrio* species, the frequency of conjugative transfer from *E. coli* to *Vibrio* species and hence the limitation of the experiment cannot be determined.

In the other experiment, two compatible plasmids, a conjugative R388 derivative (pCHR71) and a nonmobilizable pBR322 derivative into which ISV-M5.2 was cloned (pKTN435), were introduced into host strain DH1 and conjugative transfer to a recipient strain of pKTN435 fused to pCHR71 was sought. However, we could not detect such a clone. Nalidixic acid-resistant mutants of donor strain DH1(pCHR71, pKTN435) spontaneously occurred at a frequency of 10^{-8} to 10^{-9}. The frequency of conjugative transfer of pCHR71 was reported to be 10^{-2} per donor cell (33). Therefore, this strategy may detect transposition which occurs at a frequency of 10^{-7} or more. However, a possible drawback of this experiment is detection of the transposition of an ISV in *E. coli*.

DISCUSSION

Genes encoding bacterial virulence factors can be flanked by ISs or related sequences. The gene encoding *E. coli* heat-stable enterotoxin (STIa) is part of Tn*1681*, which is flanked by inverted repeats of IS*1* (35, 36), and the gene specifying *E. coli* STII is carried by a functional transposon, Tn*4521*, flanked by incomplete sequences of IS*2* (17). In alpha-hemolysin plasmids of *E. coli*, the *hly* genetic determinant is flanked by IS*91*-like sequences which may be implicated in the spread of the *hly* genes (47). In *Vibrio* species, a 2.7-kb repeated sequence (RS1) of *V. cholerae* flanking the cholera toxin (*ctx*) gene (22) is the only IS-like sequence known so far. RS1 can itself transpose (4) and is involved in *recA*-dependent amplification of the *ctx* gene (11).

All the *tdh* genes examined in this study were shown to be flanked by the ISVs and/or related sequences. Association of the *tdh* genes with ISs was previously suggested by inverted repeat sequences flanking the *tdh* genes (2, 27). This study revealed that these inverted repeat sequences are actually the terminal inverted repeats of ISVs flanking the *tdh* genes. Although not all the *tdh* genes were flanked by complete ISVs, an ISV-related sequence (ISV truncate or ISV-9TS) was found in the region where a complete ISV was not detected.

One to several copies of ISVs or homologous sequences were widely distributed among various *Vibrio* species, but the sequences of the ISV family were appreciably diverse (33.6% maximum divergence among the sequenced ISVs). While the G+C contents of the ISVs (43.7 to 46.6%) are close to the average G+C contents of the vibrios (46 to 49%) (3), the figures are much higher than those for the G+C contents of the *tdh* genes and the intervening sequences existing between the ISVs or related sequences (31.5 to 33.7%). This finding suggests that the origin of the *tdh* gene is in some organism other than vibrios and raises the question of the mode of ISV-mediated acquisition of the *tdh* gene by these vibrios. Assuming that the ISVs were originally active as bacterial ISs (10), our hypothetical explanation is as follows. The insertion of the ISV in the region near the *tdh* gene followed by a second, nearby ISV insertion formed a *tdh* gene-bearing composite transposon. The *tdh* gene was transferred between different replicons by transposition, and plasmids could have been the vehicles for the *tdh* gene transfer between different organisms. The *tdh* gene was transferred first from the *tdh*-bearing nonvibrio organism to a *Vibrio* species and then among *Vibrio* species by this mechanism. Probably, ISV-mediated transfer of the *tdh* gene occurred long ago and enough time had elapsed to allow considerable sequence variation of the ISVs and genetic rearrangement in the flanking regions of the *tdh* genes to occur. As shown in Fig. 3 and 4, insertion of the ISVs into the region near the *tdh* gene could have been diverse with respect to the direction and point of insertion. Furthermore, independent transfer of the Vp-*tdh*1 and Vp-*tdh*2 genes by transposition can explain the observation that the two genes coexist in the same organism (30) but are not located in close proximity within the chromosome (26). This contrasts with the mechanism (unequal crossover event) proposed for the RS1-mediated duplication of the cholera toxin (*ctx*) gene in *V. cholerae* (11).

Nucleotide sequence homologies between IS*903* and ISVs (Fig. 5) suggest that they probably have diverged from a common ancestor. But considerable differences in the deduced amino acid sequences (Table 2) and the G+C contents (IS*903*, 52.6%; the ISVs, 43.7 to 46.6%) indicate that these ISs have adapted in each genetic background. The sequences specifying the putative transposases of ISVs were deduced from the analogy with the transposase gene sequence of IS*903*. Most ISV transposase gene sequences, however, seemed to have been altered because of base changes and deletions. One of the five ISVs found in *V. mimicus* (ISV-M5.2) contained an ORF which may encode an active transposase. But the activity of this presumed transposase was not demonstrated either because ISV-M5.2 did not have an active transposase or because the transposition was too infrequent to be detected by the systems we employed. These results suggest that the *tdh* genes introduced into vibrios had been fixed in their chromosomes and

that an absence of a selective pressure on active ISVs flanking the *tdh* genes allowed diversification and deletion of these ISVs, while the *tdh* coding sequences have been well conserved. Investigations on the expression of the four representative *tdh* genes (*tdh*1 to *tdh*4) of *V. parahaemolyticus* revealed that only the *tdh*2 gene was sufficiently expressed and was contributing to extracellular TDH production (30). However, the sequences immediately upstream of the initiation codon are highly homologous in the Vp-*tdh*2, -*tdh*3, and -*tdh*4 genes (Fig. 3) and thus could not explain the high-level expression of the Vp-*tdh*2 gene. One speculation is that as a result of genetic rearrangement involving the ISVs, the Vp-*tdh*2 gene became driven by a powerful promoter or an unknown activation system located upstream of ISV-9TS. Future studies analyzing the nucleotide sequences flanking variants of the *tdh* genes, e.g., the *tdh* gene of *V. hollisae* (43) and the *trh* gene of *V. parahaemolyticus* (32), which have less homology than that shared by the *tdh* genes examined in this study, may provide useful information on the evolution of the *tdh* gene and flanking ISVs.

An IS-mediated evolutionary process similar to our hypothesis has been proposed for the alpha-hemolysin determinant (*hly*) of *E. coli*. The *hly* determinant was considered to have originated from an organism other than *E. coli* because of low (40%) G+C content (19). In fact, similar hemolysin determinants were found in other organisms, including *Proteus vulgaris*, with 39% G+C content (20). The *hly* determinant is present on plasmids as well as in the chromosome in *E. coli* (14). Although systematic nucleotide sequence data are not available, the IS*91*-like elements flanking the *hly* determinant are considered to have played a role in spreading the *hly* determinant in an ancient time but have suffered considerable sequence changes during evolution (47). This type of gene transfer mechanism may be universally applied to explain the presence of a family of related bacterial virulence factors in various bacterial species.

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