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# A Novel Retron of *Vibrio parahaemolyticus* Is Closely Related to Retron-Vc95 of *Vibrio cholerae*

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**Some bacteria produce a satellite RNA-DNA complex termed msDNA, multicopy single-stranded DNA. In this report, msDNA from *Vibrio parahaemolyticus*, a cause of acute gastroenteritis, was identified and named msDNA-Vp96. The retron element containing the *ret* gene, encoding the reverse transcriptase (RT) that is responsible for msDNA production, was cloned and characterized. Comparison of msDNA-Vp96 and msDNA-Vc95, from *Vibrio cholerae*, showed a high level of sequence similarity. We exchanged the two *ret* genes to examine whether msDNA was produced by the RT from different sources. We found that RT-Vp96 of *V. parahaemolyticus* was able to synthesize msDNA-Vc95 of *V. cholerae* and vice versa. To the best of our knowledge, this is the first report that RT from different bacterial species can synthesize msDNA.**

***Keywords***: reverse transcriptase, msDNA, pathogenic bacteria, vibrios

## Introduction

Bacterial reverse transcriptase (RT) is an essential enzyme for the production of an unusual satellite RNA-DNA complex, multicopy single-stranded DNA (msDNA) (Yamanaka *et al*., 2002). msDNA was originally discovered in Gram-negative soil myxobacteria, such as *Myxococcus xanthus* and *Stigmatella aurantiaca*, and then later found in *Escherichia coli* and other bacteria. The genetic element that is responsible for the msDNA synthesis is called a retron and consists of *msr*, a coding region of the RNA part of msDNA, *msd*, a coding region of the DNA part of msDNA, and *ret*, the gene for RT.

Retrons in *E. coli* are highly diverse, while retrons in myxobacteria are more closely related to each other. To date, seven different msDNAs from *E. coli* have been characterized. msDNAs have been found in enteropathogenic *E. coli* (EPEC), which causes severe diarrhea, especially in infants and children, and enteroaggregative *E. coli* (EAEC), which causes persistent diarrhea in children (Lim *et al*., 1990). DNA sequences of msDNAs from one EPEC and one EAEC strain have been determined and named msDNA-Ec78 and msDNA-Ec83, respectively (Lim, 1992; Lima and Lim, 1997). msDNAs have also been found in some strains of *Salmonella* and *Klebsiella pneumoniae*, which are human pathogens (Rice *et al*., 1993). A retron has been characterized in *Salmonella enterica* serovar Enteritidis (Rychlik *et al*., 2001), while another retron was also characterized in *S. enterica* serovar Typhimurium, and was named retron-St85 (Ahmed and Shimamoto, 2003). In multidrug-resistant strains (DT104) of *S.* Typhimurium, retron-St85 is linked to the *Salmonella* genomic island (SGI1), which contains several antibiotic resistance genes (Boyd *et al*., 2000).

*Vibrio cholerae* is the cause of epidemic and pandemic cholera. Only the O1 and O139 serogroups of *V. cholerae* are believed to cause epidemic cholera outbreaks (Faruque *et al*., 1998). We previously identified an msDNA from *V. cholerae* strain O139 and named it msDNA-Vc95 (Shimamoto *et al*., 1999). We then searched for the msDNA-Vc95 sequence in the published complete *V. cholerae* O1 El Tor genome (Heidelberg *et al*., 2000), and found a retron containing *msr-msd*, the *ret* gene, and two additional open reading frames (ORFs) of unknown function (Shimamoto *et al*., 1999).

*Vibrio parahaemolyticus* is another *Vibrio* species that is recognized as a major cause of acute gastroenteritis related to seafood consumption. In this report, we identified a novel msDNA from *V. parahaemolyticus* and cloned and characterized the retron elements from *V. parahaemolyticus* and *V. cholerae* O139.

## Materials and Methods

### Bacterial strains and plasmids

*Vibrio parahaemolyticus* clinical isolate AQ3354 and *V. cholerae* O139 clinical isolate MDO-6 were used in this study. *E. coli* TG1 (*supE*, *hsd*Δ5, *thi*, Δ[*lac-proAB*]/F′[*traD*36, *proAB*$^+$, *lacI*$^q$, *lacZ*ΔM15]) was used as a host for all other genetic manipulations. Luria-Bertani (LB) medium (Lennox, 1955) was used for the growth of *E. coli* cells. *V. parahaemolyticus* and *V. cholerae* cells were grown in *Vibrio* LB medium (1% polypeptone, 0.5% yeast extract, 1.5% NaCl, pH 7.5) at 37°C under aerobic conditions. All plasmids used in this study are listed in Table 1.

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**Table 1.** List of the plasmids used in this study

| Name of plasmid | Characteristics of plasmids | References |
|---|---|---|
| pVc-RET1 | pBlueSTAR carrying retron-Vc95 of *V. cholerae* O139 MDO-6 | This study |
| pVp-RET1 | pBlueSTAR carrying retron-Vp96 of *V. parahaemolyticus* AQ3354 | This study |
| pINIIIlpp$^{p-5}$ | Expression vector; ampicillin resistance, *lpp* gene promoter and *lac* promoter-operator | Inouye and Inouye (1985) |
| pGB2 | General cloning vector ; spectinomycin resistance | Churchward *et al.* (1984) |
| pBluescript II SK(-) | General cloning vector ; ampicillin resistance | Alting-Mees and Short (1989) |
| pGB-VcRT1 | pGB2 carrying *ret* of *V. cholerae* : start codon ATG | This study |
| pGB-VcRT2 | pGB2 carrying *ret* of *V. cholerae* : start codon TTG | This study |
| pGB-VpRT1 | pGB2 carrying *ret* of *V. parahaaemolyticus* | This study |
| pBS-VcMS1 | pBluescript II SK(-) carrying *msr-msd* region of *V. cholerae* | This study |
| pBS-VpMS1 | pBluescript II SK(-) carrying *msr-msd* region of *V. parahaemolyticus* | This study |

### Isolation of msDNA and DNA sequence determination

*Vibrio parahaemolyticus* AQ3354 cells were grown in 200 ml of *Vibrio* LB medium, and the msDNA was isolated by alkaline lysis, as described previously (Shimamoto *et al.*, 1993). The sequence of the msDNA was determined by the chemical sequencing method of Maxam and Gilbert (Maxam and Gilbert, 1980). msDNA was isolated from *V. cholerae* MDO-6, and the sequence was determined as described previously (Shimamoto *et al.*, 1999). msDNAs were prepared from *E. coli* transformants harboring plasmids carrying the cloned retron fragments by the same method as used for *V. parahaemolyticus* and *V. cholerae*.

### Cloning and sequencing of the retron elements from *V. parahaemolyticus* and *V. cholerae*

Chromosomal DNA was isolated from *V. parahaemolyticus* AQ3354 and *V. cholerae* MDO-6 using a previously described miniprep method (Ausubel *et al.*, 1995). Chromosomal DNA was partially digested with *Sau*3AI and separated by agarose gel electrophoresis. The digested DNA fragments, approximately 7–20 kb in length, were eluted from the gel and DNA libraries of the *V. parahaemolyticus* AQ3354 and

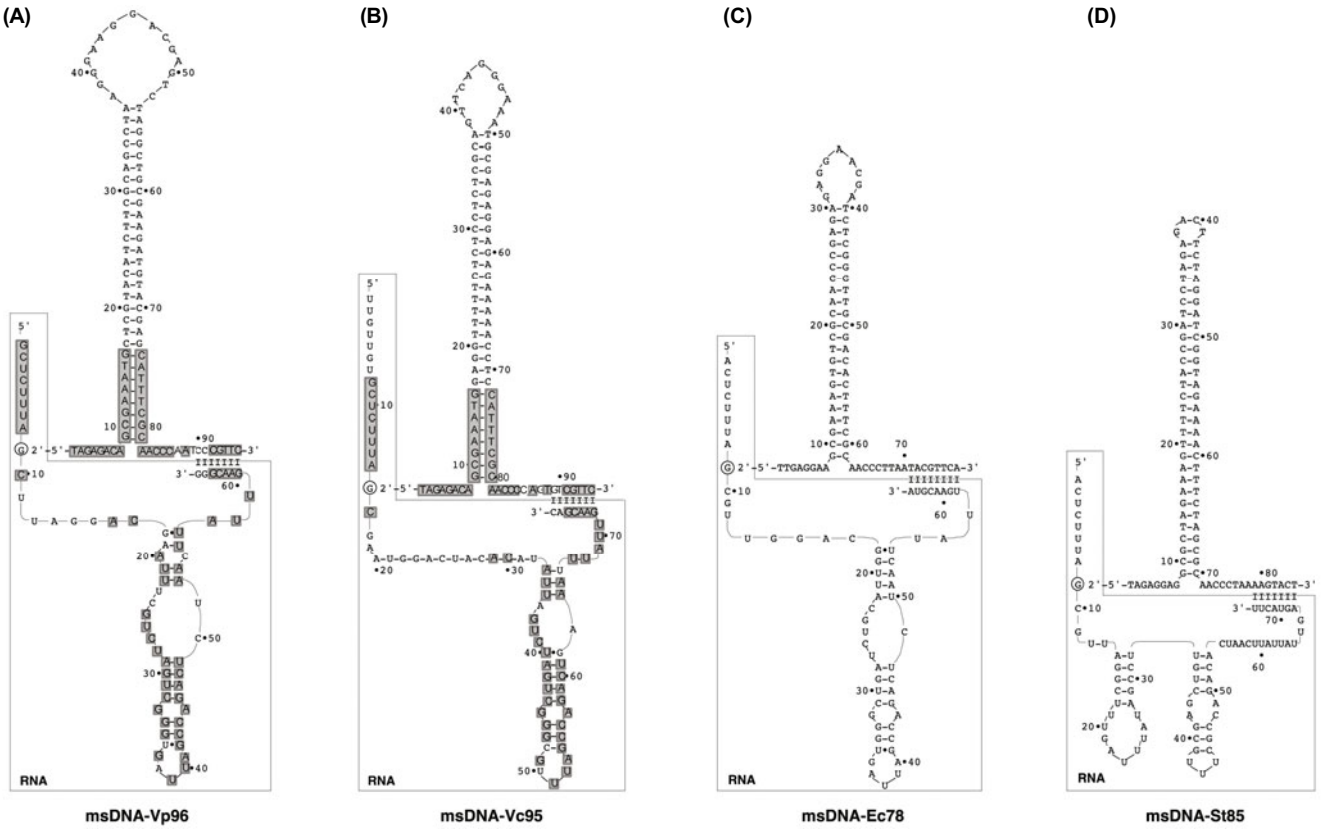


**Fig. 1. Predicted secondary structures of msDNAs of some pathogenic bacterial species.** RNA portions are boxed and the branching G residue that forms the 2′, 5′-phosphodiester linkage is circled. Both RNA and DNA are numbered from their 5′ ends. msDNA-Vp96 (A) was isolated from *V. parahaemolyticus* AQ3354 in this study. msDNA-Vc95 (B) is from *V. cholerae* O1/O139 (Shimamoto *et al.*, 1999), msDNA-Ec78 (C) is from EPEC 110 (Lima and Lim, 1997), and msDNA-St85 (D) is from *S.* Typhimurium (Ahmed and Shimamoto, 2003). The nucleotides conserved between msDNA-Vp96 and msDNA-Vc95 are shaded.

*V. cholerae* MDO-6 genomes were constructed using a λ phage vector, λBlueSTAR (Novagen, USA), as described previously (Shimamoto *et al*., 2001). According to the msDNA sequences determined as described above, two sets of oligonucleotide primers were used for PCR: msd-VpF (5′-CC TAAGGGAAGAACGAGTC-3′) and msd-VpR (5′-CGGG ATTGGGTTGCGAA-3′) were used for amplification of a part of the *msd* region of *V. parahaemolyticus* AQ3354, and msd-VcF (5′-TTCAGGGAAATGCGAAAGGA-3′) and msd-VcR (5′-GAACGACACTGGGGTTGCGA-3′) were used to amplify the *msd* region of *V. cholerae* MDO-6. *msd* fragments of 60 bp and 56 bp were amplified by PCR using the chromosomal DNA of *V. parahaemolyticus* and *V. cholerae* as templates, respectively. As the *msr-msd* region and the *ret* gene form an operon in most of the retrons found to date, the whole retron can be cloned by using the *msd* DNA fragment as a probe. Each amplified *msd* DNA fragment was labeled with alkaline phosphatase using an AlkPhos Direct Labeling and Detection System (GE Healthcare, Japan) and used as a probe for plaque hybridization assays, as described previously (Shimamoto *et al*., 2001). A positive phage clone was selected from each genomic library, then *E. coli* BM25.8 cells (Novagen, USA) (Palazzolo *et al*., 1990) were infected with the selected phages to subclone the genomic DNA fragment from the λBlueSTAR vector into the pBlueSTAR plasmid vector (Novagen) by Cre recombinase-mediated excision. pVc-RET1 and pVp-RET1, containing the retron elements, were then cloned from *V. cholerae* and *V. parahaemolyticus*, respectively.

The nucleotide sequences of the retrons from *V. cholerae* and *V. parahaemolyticus* were determined from both the sense and antisense strands by the dideoxynucleotide chain termination method (Sanger *et al*., 1977). The sequence data of the retrons of *V. cholerae* and *V. parahaemolyticus* reported in this paper have been submitted to the DDBJ/GenBank/EMBL nucleotide sequence databases with the accession numbers AB191408 and AB079134, respectively.

### Construction of plasmids

The *V. parahaemolyticus ret* gene was amplified by PCR using primers Vp-RT1ND (5′-TCCATATGAACTTAGTG AAACGAC-3′) and Vp-RT4BH (5′-TCGGATCCTTATTC ATGACCCGCCTC-3′), and cloned into pBluescriptII SK(−) (Alting-Mees and Short, 1989). Following sequence confirmation, the *Nde*I-*Bam*HI fragment containing the *ret* gene was inserted into pINIIIlpp$^{p-5}$ (Inouye and Inouye, 1985). The *Xba*I-*Bam*HI fragment containing the *lpp-lac* promoter and the *ret* gene was then ligated into pGB2 (Churchward *et al*., 1984). As the *ret* gene of *V. cholerae* has two *Nde*I sites, one site was deleted by site-directed mutagenesis using two-step PCR (Higuchi *et al*., 1988), without changing the amino acid sequence of the RT. The resulting PCR fragment was cloned in pBluescript SK(−). Subsequent steps of the cloning procedure were the same as those described for the *ret* gene of *V. parahaemolyticus*. Each DNA fragment of the *msr-msd* regions from *V. cholerae* or *V. parahaemolyticus* was cloned into the *Eco*RV site of pBluescript II SK(−) after blunting.

### DNA and amino acid sequence analyses

Multiple alignment of bacterial RT amino acid sequences was performed using the Clustal W program (Thompson *et al*., 1994) available at the GenomeNet server (http://www.genome.ad.jp/). All other genetic analyses, including the promoter search and secondary structure prediction of RNA and DNA, were carried out with the GENETYX-MAC program (GENETYX, Japan).

## Results and Discussion

### Structure of msDNA-Vp96 from *V. parahaemolyticus*

A novel msDNA from *V. parahaemolyticus* AQ3354 was detected and isolated. DNA sequencing showed that the DNA portion of the msDNA was 96 bases in length. Thus the msDNA, the retron, and the RT of *V. parahaemolyticus* were named msDNA-Vp96, retron-Vp96, and RT-Vp96, respectively. The predicted secondary structure of msDNA-Vp96 is depicted in Fig. 1A. Interestingly, there are no mismatched base pairs in the DNA stem structure, a property

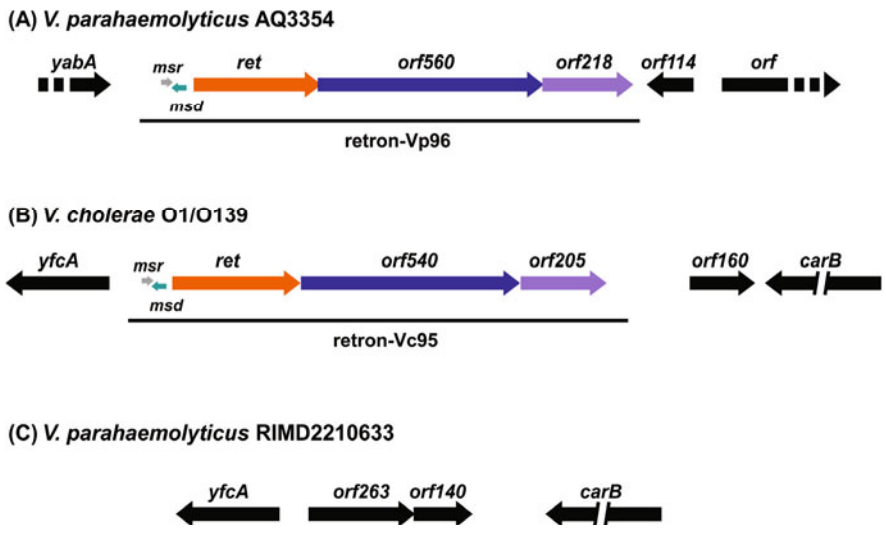


**Fig. 2. Gene organization of retrons and their flanking regions.** (A) retron-Vp96 and its flanking region, from *V. parahaemolyticus* AQ3354; (B) retron-Vc95 and its flanking region, from *V. cholerae* O1, O139; (C) gene organization of the genomic region between *yfcA* and *carB* of *V. parahaemolyticus* RIMD2210633.

also observed in msDNA-Vc95 from *V. cholerae* (Fig. 1B), msDNA-Ec78 from EPEC (Fig. 1C), and msDNA-St85 from *S.* Typhimurium (Fig. 1D). msDNA-Vp96 appears to have no mutagenic effect, unlike some msDNAs from other bacteria (Maas *et al.*, 1994; Mao *et al.*, 1996). msDNAs with no mismatched base pairs in their DNA stems may have some specific function(s) in pathogenic bacteria. In addition to the common DNA stem structure, these four msDNAs have significant identities in both their DNA and RNA regions, as described below.

The secondary structure of the RNA portion of msDNA-Vp96 was also predicted, as depicted in Fig. 1A. The RNA stem region of msDNA-Vp96 was found to have significant similarity to that of msDNA-Vc95 (Fig. 1B). These regions are important for recognition by bacterial RTs and synthesis of msDNAs (Shimamoto *et al.*, 1993; Inouye *et al.*, 1999). This led us to investigate whether RTs from retron-Vp96 and retron-Vc95 could synthesize msDNAs from different sources.

### Retron elements of *V. parahaemolyticus* and *V. cholerae*

Cloning and sequencing of retron-Vp96 and retron-Vc95 showed that gene organization of the two retrons was the same (see Figs. 2A and 2B). The nucleotide sequence of retron-Vc95 from *V. cholerae* O139 was identical to that of *V. cholerae* O1, as reported previously (Shimamoto *et al.*, 1999). Each retron consists of *msr-msd*, a coding region of msDNA, and three ORFs. The first ORF of retron-Vp96 encodes a retron-type RT of approximately 313 residues. The amino acid sequence of RT-Vp96 is similar to RT-Vc95 (49% identity). The second and third ORFs encode hypothetical proteins with predicted sizes of 560 and 218 residues, respectively. The amino acid sequence of the second ORF has similarity with that of retron-Vc95 (34% identity). Although the functions of both ORFs are unknown, the second ORF (ORF560) contains nucleotide-binding motifs similar to the second ORF (ORF540) of retron-Vc95. The second and third ORFs may have some specific functions in bacterial cells.

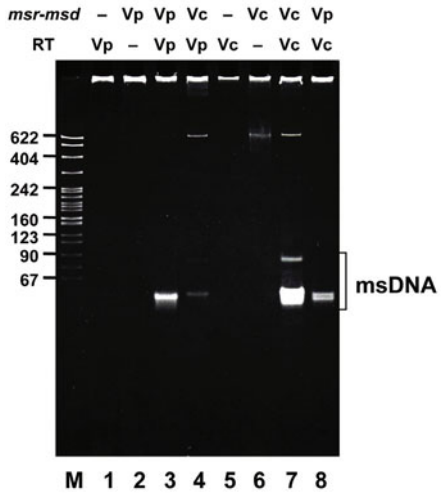


**Fig. 3. msDNA production in different *E. coli* transformants.** msDNAs were subjected to polyacrylamide gel electrophoresis following RNase A treatment. Lanes: M, pBR322 digested with *Msp*I used as a molecular weight marker; 1, *E. coli* TG1/pGB-VpRT1; 2, TG1/pBS-VpMS1; 3, TG1/pGB-VpRT1/pBS-VpMS1; 4, TG1/pGB-VpRT1/pBS-VcMS1; 5, TG1/pGB-VcRT2; 6, TG1/pBS-VcMS1; 7, TG1/pGB-VcRT2/pBS-VcMS1; 8, TG1/pGB-VcRT2/pBS-VpMS1.

Retron-Vc95 was inserted directly between the *yfcA* gene and the *carB* gene in the *V. cholerae* chromosome, as described previously (Inouye *et al.*, 2011; Fig. 2B). In contrast, the *yabA* gene and an unknown ORF were found on either side of retron-Vp96 (Fig. 2A). We searched for both the *yfcA* gene and the *carB* gene in the complete *V. parahaemolyticus* RIMD2210633 genome (Makino *et al.*, 2003) and found that two ORFs, *orf263* (VP0473) and *orf140* (VP0472), were inserted between *yfcA* and *carB* in the *V. parahaemolyticus* genome (Fig. 2C). This clearly indicated that retron-Vp96 is located in a different chromosomal region from retron-Vc95. Previous work has shown that retron-Vc95 is located in a region without any other mobile genetic elements (Inouye *et al.*, 2011). The insertion mechanism and mobility of retron-Vp96 could be different from that of retron-Vc95. Retron-Vp96 may be linked to other mobile elements, such as transposons or phages. In addition, retron-Vp96 of *V. parahaemolyticus* does not appear to be linked to pathogenicity (as described below), and therefore retron-Vp96 might come from other sources. In fact, a 29-base pair inverted repeat was found in the upstream region of retron-Vp96 (Fig. 4A). This inverted repeat may be related to the insertion or mobility of retron-Vp96. Investigation of retron-Vp96 mobility may provide important insight into new mechanisms of DNA transposition. In addition, a genomic fragment including retron-Vp96 was recently found to be circularized and excised from the genome (Ishida *et al.*, unpublished data).

### Translation initiation sites of the *ret* genes

It was previously predicted that RT-Vc95 was 298 amino acids in length (Shimamoto *et al.*, 1999). However, the amino terminal region of the expected RT-Vc95 protein was about 10 amino acid residues shorter than those of similar bacterial RTs (Shimamoto *et al.*, 1999). In addition, it has been predicted that a minor initiation codon, TTG, located 75 bp upstream of the ATG codon, is the initiation codon for the *ret* gene, based on *V. cholerae* O1 El Tor genome sequence data (Heidelberg *et al.*, 2000; Ahmed and Shimamoto, 2003; see Fig. 4B). Because the consensus sequence of the ribosome binding site does not exist in the upstream region of either the ATG or TTG codon, we predicted another TTG codon between these two was the true initiation codon of the *ret* gene for RT-Vc95 (Fig. 4B). To investigate this, RT-expression plasmids carrying the *ret* gene with transcription starting from each initiation codon were constructed. *E. coli* was transformed with a plasmid carrying the *msr-msd* region of *V. cholerae* as well as each RT expression plasmid, and msDNA synthesis was examined. msDNA was detected from TG1/pGB-VcRT2/pBS-VcMS1 (Fig. 3, lane 7), but not from TG1/pGB-VcRT1/pBS-VcMS1 (see Table 1 for characteristics of the plasmids; data not shown).

In the case of *V. parahaemolyticus*, the ATG codon shown in Fig. 4A is most likely to be the initiation codon for the *ret* gene, considering the ribosome binding site and distance between the *msr-msd* region and the *ret* gene. An RT expre-

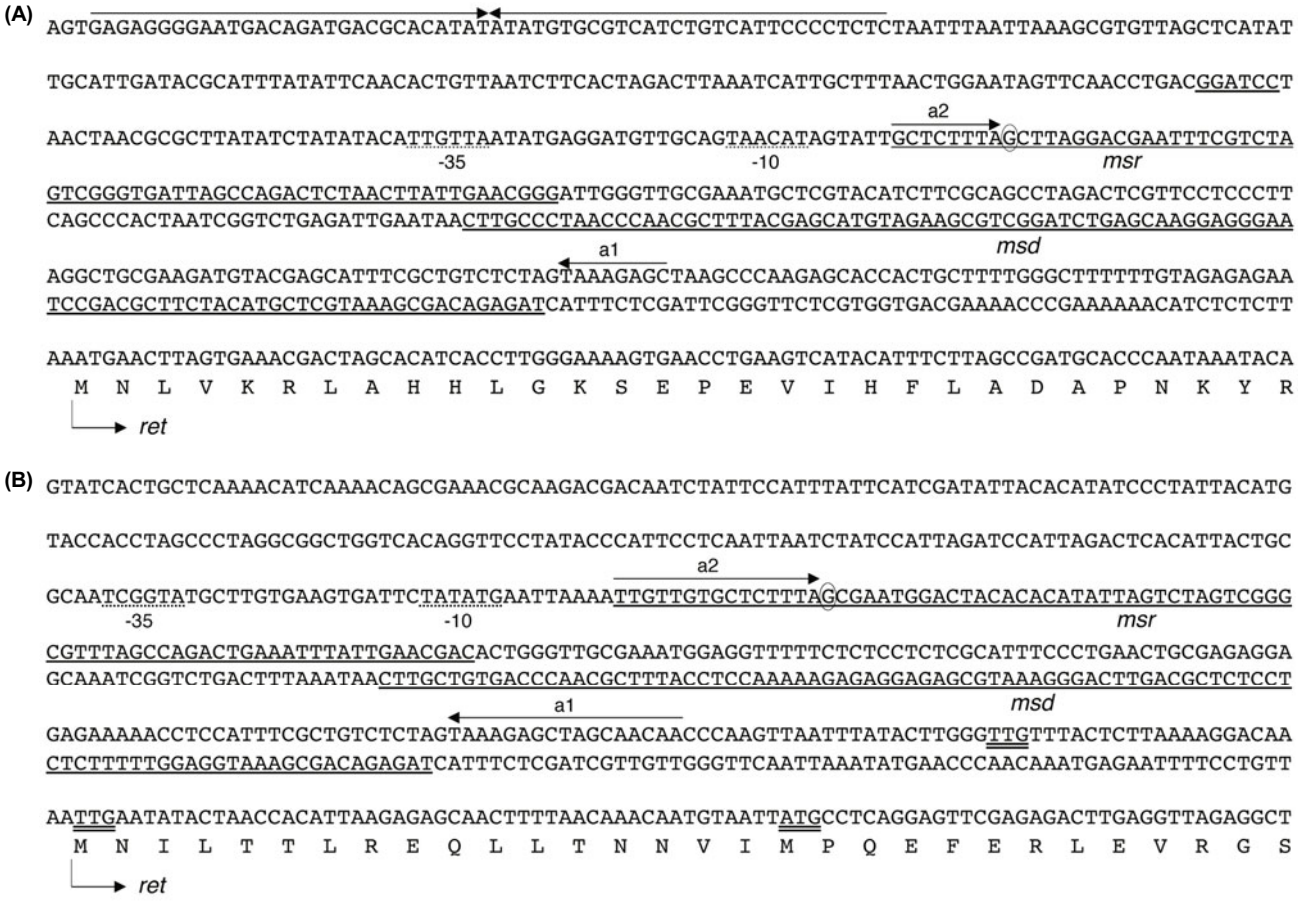


**Fig. 4. Nucleotide sequences of the upstream and promoter region, the *msr-msd* region, and a part of the *ret* gene of retron-Vp96 (A) and retron-Vc95 (B).** Sequences depicted in panels (A) and (B) correspond to nucleotide regions 451–990 (GenBank/EMBL/DDBJ accession no. AB079134) and 729–1,268 (GenBank/EMBL/DDBJ accession no. AB191408), respectively. The fourth and fifth lines in panels (A) and (B) are shown as double-stranded DNA. Inverted repeats are indicated by arrows. The predicted promoter sequences (-35 and -10) are marked by dotted lines. The branching guanosines in the *msr* regions are circled. The amino acid sequences are the amino-terminal regions of both RTs. Possible initiation codons for the *ret* gene are marked by double underlines in panel (B).

ssion plasmid (pGB-VpRT1) carrying the *ret* gene starting from the ATG codon was also constructed. *E. coli* TG1 was transformed with the plasmid carrying the *msr-msd* region of *V. parahaemolyticus* (pBS-VpMS1) and pGB-VpRT1, and msDNA synthesis was examined. As shown in Fig. 3, lane 3, msDNA was synthesized in the transformant, indicating that functional RT-Vp96 was produced from the predicted ATG codon.

### Reverse transcriptases of *V. parahaemolyticus* and *V. cholerae* are interchangeable between retron-Vp96 and retron-Vc95 for the production of each msDNA

The amino acid sequences of bacterial RTs and nucleotide sequences of msDNAs are diverse, and previous work has shown that RTs from different retrons could not synthesize alternate msDNA (Shimamoto *et al.*, 1993). The msDNA-Vp96 of *V. parahaemolyticus* is similar to msDNA-Vc95 of *V. cholerae*, especially in the region important for recognition by RTs (stem-loop structures of the RNA portions) (Inouye *et al.*, 1999; see Figs. 1A and 1B). We hypothesized that RT-Vc95 may be able to synthesize msDNA-Vp96, and vice versa. To investigate this possibility, *E. coli* TG1 was transformed with pGB2 carrying either the RT-Vp96 or RT-Vc95 gene, and pBluescript carrying *msr-msd* of either retron-Vp96 or retron-Vc95, and msDNA production was examined. As shown in Fig. 3, msDNA-Vp96 was produced not only by its own RT-Vp96 (lane 3), but also by RT-Vc95 (lane 8). Likewise, msDNA-Vc95 was produced by both its own RT-Vc95 (lane 7), and by RT-Vp96 (lane 4). A previous study showed that RT-Ec78 from *E. coli* could synthesize msDNA-Ec83 from *E. coli* and vice versa (Lima and Lim, 1997). To the best of our knowledge, this is the first report of RTs that are capable of synthesizing the msDNA of another species.

### Distribution of retron in *V. parahaemolyticus*

Although the whole genome sequence of *V. parahaemolyticus* RIMD2210633 has been reported (Makino *et al.*, 2003), we could not find retron-Vp96 in the published sequence. Thus, we investigated the distribution of retrons in different *V. parahaemolyticus* strains by means of msDNA isolation. We attempted to isolate msDNAs from *V. parahaemolyticus*

AQ3354 and 28 other strains and found that the msDNA-Vp96 migration band was not detected in any *V. parahaemolyticus* strains (data not shown). However, an msDNA-like band with a different migration pattern from msDNA-Vp96 was detected in *V. parahaemolyticus* OKA80-223 (an environmental isolate), suggesting that this strain may have another novel retron (data not shown). These data indicate that retrons may not be as common in *V. parahaemolyticus* as they are in *V. cholerae*. In comparison, all investigated strains of *V. cholerae* O1/O139, which can cause epidemic cholera, have msDNA-Vc95, whereas *V. cholerae* non-O1/non-O139 strains rarely have this retron (Inouye *et al.*, 2011). This may indicate that retron-Vc95 is related to pathogenicity of *V. cholerae*. As retron-Vp96 is not common in *V. parahaemolyticus*, it may indicate that retron-Vp96 has some specific function(s) and further analysis of the possible function of retron-Vp96 is warranted.

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