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Rapid Detection and Identification of *Vibrio anguillarum* by Using a Specific Oligonucleotide Probe Complementary to 16S rRNA

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Partial 16S rDNA from *Vibrio* collection type strains and recent isolates of *Vibrio*-related strains were sequenced and compared with previously published sequences. A 24-base DNA oligonucleotide (VaV3) was designed and used as a specific probe for detection and identification of *Vibrio anguillarum*. Its specificity was tested against collection type strains and environmental isolates and no cross-reaction was found. The probe detected 8 of the 10 *V. anguillarum* serovars. It was applied to screen different *Vibrio*-related strains isolated from marine hatcheries and fish farms. The detection limit in DNA-DNA slot blot hybridization was 150 pg.

Vibriosis is a systemic infection, primarily of marine and estuarine fish, which produces either skin ulcers or a septicemia characterized by erythema, hemorrhages, and anemia, causing significant mortalities especially in summer when the temperature of the water is above 15°C (31). This disease has been the main pathological problem since the initiation of marine fish culture (3, 13, 34). *Vibrio anguillarum* has been described as the main species causing vibriosis (5). It is a gram-negative motile rod with fermentative and respiratory metabolism. Its taxonomy has a history of controversy (25). Neither biochemical (14, 16, 21) nor immunological (6, 8) techniques provide an absolutely clear identification because strains vary considerably in their phenotypic properties (14). Moreover, the actual procedures do not allow *V. anguillarum* to be distinguished from other *Vibrio* species when it is not the dominant type, as happens in water, sediments, in the gut of carriers, etc.

Methods based on genomic analysis are fast and are usually more accurate than those based on phenotypic features (20). Among them, oligonucleotide probes based on 16S rRNA studies (37) are a powerful diagnostic tool in bacterial identification (26). The primary structure of rRNA includes both conserved and variable nucleotide sequences characteristic of different taxonomic groups, reliable even at the species-specific level (36). The aim of this study was to develop a nucleic acid probe that could be used to distinguish *V. anguillarum* from other *Vibrio* and bacterial species frequently isolated in marine hatcheries and fish farms. To that end, the V3 region (10) (positions 338 to 536, according to the *Escherichia coli* numbering system [4]) of the 16S rDNA of many strains was sequenced. After alignment and definition of a *V. anguillarum*-specific probe, its specificity and sensitivity were tested.

The bacterial strains used in the sequence comparison are listed in Table 1. In addition, 40 *Vibrio*-related recent isolates were used in the hybridization screening. They were grown either on marine broth (Difco) or Trypticase soy broth (ADSA) with 2% NaCl at 22°C. For solid media 15 g of agar per liter was added. Chromosomal DNA was isolated according to Ausubel et al. (2) but without using cetyltrimethylammonium bromide (CTAB). RNA was eliminated by digestion with 10 μg of DNAse-free RNAse per ml. DNA amplification components were as described by Saiki (29) but with 0.5 μM each universal eubacterial primer, whose sequences were ACTCCTACGGGAGGCAGC (positions 338 to 355) and GTATTACCGCGGCTGCTG (positions 536 to 519) (24). The thermal cycling was one cycle at 95°C for 2 min., 50°C for 30 s, and 72°C for 45 s followed by 35 cycles at 95°C for 30 s, 50°C for 30 s, and 72°C for 45 s. Later, 1 μl of the first amplification yield was added to a new fresh PCR reaction mixture in which the relative primer ratio was 40:1 (120 ng of the primer of the strand to be amplified and 3 ng for the other) in order to obtain single-stranded DNA (ssDNA) fragments by asymmetric amplification (17) using the same cycling protocol as that indicated above. Purification of amplified DNA was performed by chloroform extraction, ultrafiltration with 30,000-molecular-weight-cutoff units (Ultrafree MC; Millipore), washing, resuspension in TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), and precipitation with ethanol. The nucleotide sequence of PCR-amplified ssDNA was determined by using the dideoxynucleotide chain terminator method (33). Seven nanograms of the primer complementary to the strand to be sequenced was used to promote annealing. The sequencing reaction was performed with 0.75 U of T7 DNA polymerase and 10 μCi of [γ-^{35}S]ATP. From the study of the sequence alignment, one oligonucleotide (VaV3) was designed, and it was then chemically synthesized by Medprobe (Oslo, Norway). The oligonucleotide was labeled with [γ-^{32}P]ATP by 5′ end labeling (32) with T4 polynucleotide kinase. Unincorporated label was removed by gel filtration on NAP-10 columns (Pharmacia) equilibrated with TE buffer. Purified chromosomal DNA was blotted on Hybond-N membranes (Amersham) in order to check the specificity and the sensitivity. After cross-linking was done with UV light, membranes were incubated in hybridization solution (10× Denhardt's solution, 4× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% [wt/vol] sodium dodecyl sulfate [SDS], 2 mM EDTA, 50 μg of salmon sperm DNA per ml) at 55°C for 30 min. Then, 1.0×10^7 cpm of ^{32}P-labelled VaV3 was added to the solution and hybridized overnight at 55°C. Two washing steps performed at 55°C with 5× SSC plus 0.1% (wt/vol) SDS of 15 and 30 min were followed by two other washings at room temperature with 2× SSC. After that, membranes were exposed to X-ray films. In order to perform RNA-DNA colony hybridization, bacteria were patched on nylon membranes deposited onto marine agar plates and grown overnight at 22°C. Filters were processed for hybridization (19) with VaV3 as described above. For rehybridization after exposure to the

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TABLE 1. Bacterial strains used in the sequencing study

Species	Strain	EMBL accession no.
Aeromonas hydrophila	ATCC 7966	M59148
Escherichia coli	#[a]	J01859
Pasteurella multocida	NCTC 10322	M35018
Plesiomonas shigelloides	#	M59159
Proteus vulgaris	Monteil	J01874
Pseudomonas aeruginosa	ATCC 25330	M34133
Pseudomonas aeruginosa	DSM 50071	X06684
Pseudomonas aeruginosa	ATCC 15442	Z22989
Renibacterium salmoninarum	ATCC 33209	X51601
Vibrio alginolyticus	ATCC 17749	X56576
Vibrio anguillarum	ATCC 19264	X16895
Vibrio anguillarum O1[b]	ATCC 43305	Z23021
Vibrio anguillarum O2	ATCC 43306	Z23020
Vibrio campbellii	ATCC 25920	X56575
Vibrio diazotrophicus	ATCC 33466	X56577
Vibrio fluvialis	ATCC 33812	Z22990
Vibrio harveyi	#	M58172
Vibrio harveyi	ATCC 14126	X56578
Vibrio hollisae	ATCC 33564	X56583
Vibrio natriegens	ATCC 14048	X56581
Vibrio parahaemolyticus	ATCC 17802	X56580
Vibrio pelagius	ATCC 25916	Z22991
Vibrio proteolyticus	ATCC 15338	X56579
Vibrio vulnificus	ATCC 27562	X56582
Vibrio vulnificus	ATCC 33147	Z22992
Vibrio anguillarum O3	11008	Z23015
Vibrio anguillarum O1	A018	Z23016
Vibrio sp.	A032	Z22972
Vibrio sp.	A049	Z22983
Vibrio sp.	A050	Z22993
Vibrio sp.	A052	Z22994
Vibrio sp.	A053	Z22995
Vibrio anguillarum O1	A055	Z23017
Vibrio anguillarum O1	A056	Z23018
Vibrio sp.	A060	Z22996
Vibrio sp.	A061	Z22997
Vibrio sp.	A063	Z22998
Vibrio sp.	A065	Z22999
Vibrio sp.	A068	Z22973
Vibrio sp.	A070	Z22974
Vibrio sp.	A071	Z22975
Vibrio sp.	A073	Z22976
Vibrio sp.	A074	Z22977
Vibrio sp.	A075	Z22978
Vibrio sp.	A076	Z22979
Vibrio anguillarum O1	A077	Z23019
Vibrio sp.	A081	Z22980
Vibrio sp.	A082	Z22981
Vibrio sp.	A093	Z22982
Vibrio sp.	A094	Z22984
Vibrio sp.	A095	Z22985
Vibrio sp.	A096	Z22986
Vibrio sp.	B009	Z22987

[a] #, source not specified by the European Molecular Biology Laboratory.
[b] *V. anguillarum* serovar.

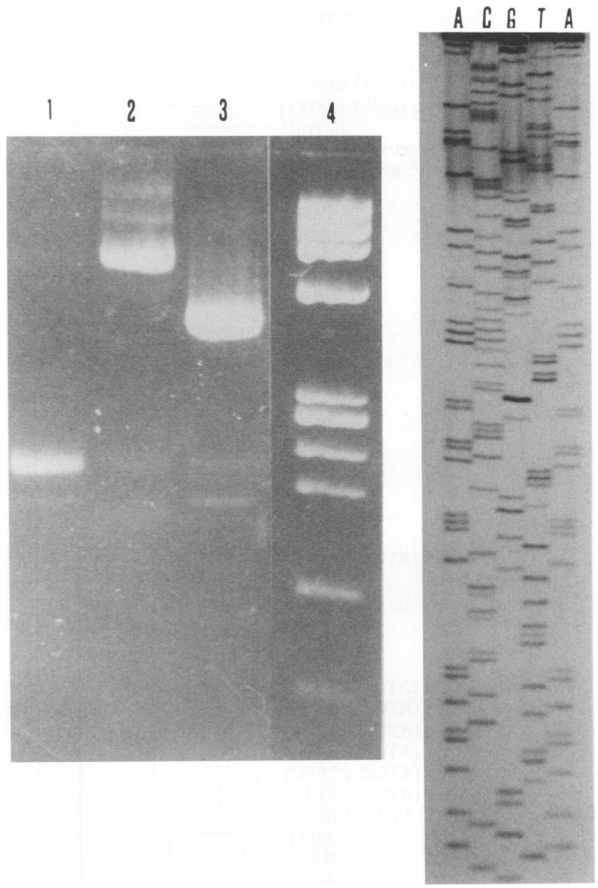

FIG. 1. (Left) Agarose electrophoresis (1% plus 3% NuSieve) of PCR-amplified V3 domain of 16S rDNA. Lanes: 1, double-stranded DNA (dsDNA) after symmetric amplification (199 bp); 2, 5′ ssDNA after asymmetric amplification of dsDNA of lane 1; 3, 3′ ssDNA after asymmetric amplification of dsDNA of lane 1; 4, standard molecular weight DNA marker (ϕX174 DNA, *Hae*III digested). (Right) Sequence from 5′ to 3′ from a 5′ ssDNA asymmetrically amplified V3 domain of 16S rDNA of a *Vibrio* sp. strain.

first film, membranes were washed from the VaV3 probe in 0.1% (wt/vol) SDS at 100°C and allowed to cool to room temperature. Then, rehybridization was performed with a universal eubacterial probe (positions 536 to 519) (24) which was used as a positive control for the method.

To identify species-specific probes, partial 16S rDNA sequences were obtained by direct sequencing from PCR products of 34 strains belonging to a wide group of marine bacteria. They included both type strains and *Vibrio*-related strains isolated from coastal waters, hatcheries, and fish in the Atlantic Ocean and Mediterranean Sea. The symmetric and asymmetric PCR provided abundant and homogeneous ssDNA fragments (Fig. 1). After gel electrophoresis, between 100 and 160 nucleotides were clearly determined for each strand (Fig. 1). The overlaps of the two complementary strand sequences allowed determination of the complete sequence of the V3 domain.

Partial 16S rDNA sequences (positions 338 to 536) were aligned with previously published sequences, using two different methods of pairwise alignment (9, 11) and the stationary Marcov model (28). Dendrograms of similarity were displayed using the neighbor-joining method (30), distant matrix method (15), and the weighted average clustering method by Sastaxan (D. Jacobs, University of Maryland). The three different methods used for aligning the sequences of the V3 region gave similar results. The dendrograms of similarity obtained were also very similar and clearly defined some clusters (Fig. 2). A clear separation of *Vibrio* from other genera, even taxonomically related genera, was observed by use of the V3 domain of 16S rRNA. All *V. anguillarum* strains constituted a homogeneous group which presents a similarity of over 99% for the studied sequence (Fig. 2). A 24-mer oligonucleotide (VaV3:

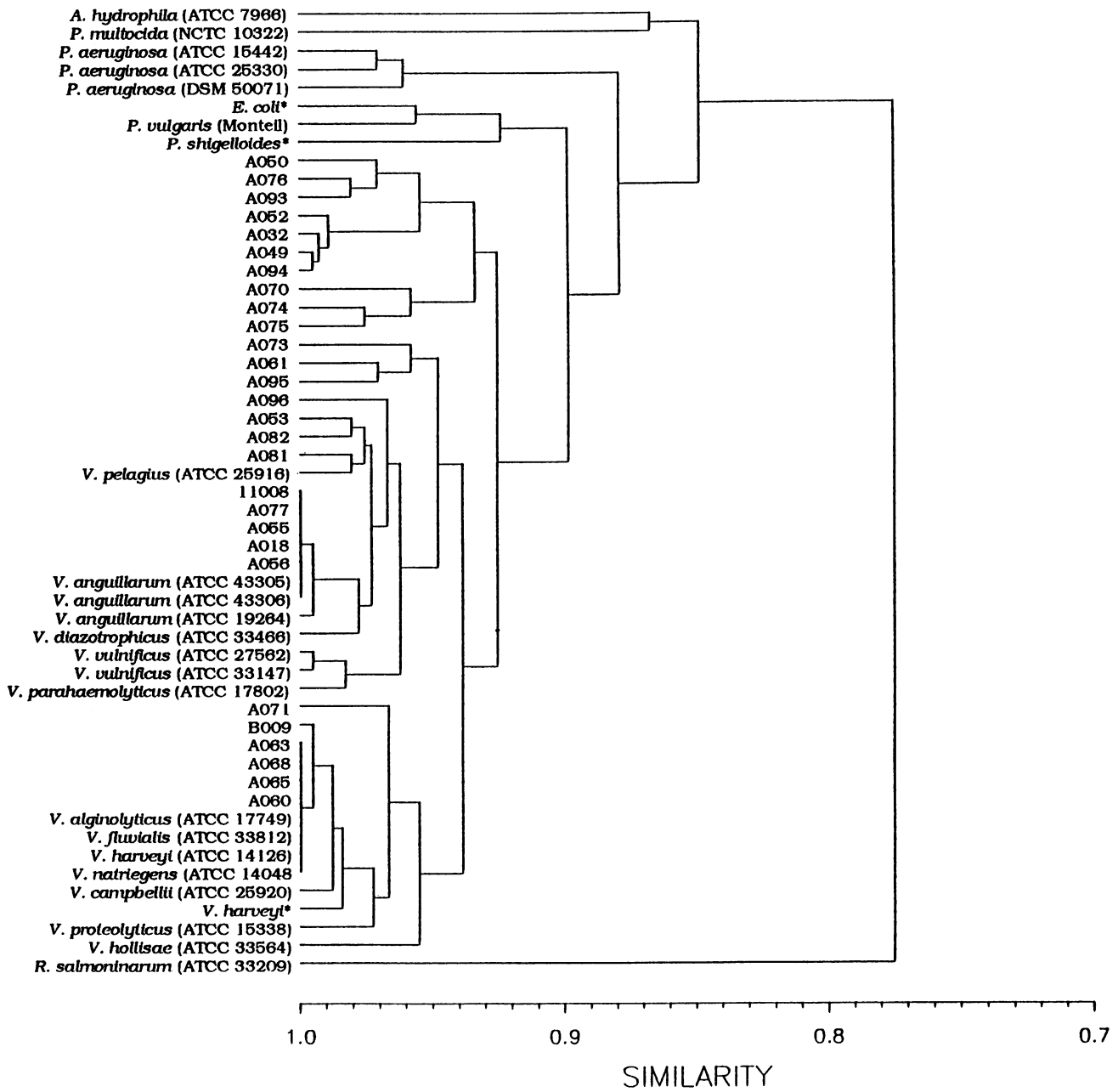

FIG. 2. Dendrogram based on partial 16S rRNA sequence (positions 338 to 536) showing the relationships among the 53 reference strains which were used in this study. The Sastaxan program (D. Jacobs, University of Maryland) was used. For organism names, see Table 1. *, source not specified by European Molecular Biology Laboratory. Numbers after *V. anguillarum* refer to serovar.

5′-TGATGCTGCTATTAACAACACCAC-3′, positions 477 to 454) was selected from the alignment as a *V. anguillarum* species-specific probe (Table 2). Computer-assisted analysis reveled optimal primary and secondary structures for probing (11). Except for *V. diazotrophicus*, which was 95.8% similar to *V. anguillarum*, the rest of the *Vibrio* type strains tested were at least 16.7% divergent. This oligonucleotide belongs to the V3 domain sequence, which is a highly variable region of the 16S rRNA, recently used by other investigators in phylogenetic studies of the genus *Vibrio* (12, 23). This region allows successful differentiation of *Vibrio* species from other genera, but is not sufficient to separate all *Vibrio* species from each other. However, it is shown here that it is sufficient for differentiation of *V. anguillarum* from other *Vibrio* species usually found mostly in fish farms and hatcheries. The DNA-DNA slot blot hybridization confirmed that VaV3 recognized *V. anguillarum* chromosomal DNA. These results were confirmed with RNA-DNA colony hybridization experiments (Table 3). No cross-reaction with other *Vibrio* species was detected. On the other hand, serovars O1 to O8 of *V. anguillarum* (35) hybridized strongly with VaV3 while serovars O9 and O10 never did hybridize. The three serovars (O1, O2, and O3) most

TABLE 2. Partial 16S rDNA sequences aligned for comparison

Species	Identity with VaV3 probe[a]: 3′ CACCA 5′ GTGGT	CAACA GTTGT	ATTAT TAATA	CGTCG GCAGC	TAGT 5′ ATCA 3′
V. anguillarum O1[b] (ATCC 43305)	*****	*****	*****	*****	****
V. anguillarum O2 (ATCC 19264)	*****	*****	*****	*****	****
V. anguillarum O2 (ATCC 43306)	*****	*****	*****	*****	****
V. anguillarum O3 (ATCC 43307)[c]	*****	*****	*****	*****	**N*
V. anguillarum O4 (ATCC 43308)[c]	*****	*****	*****	*****	**N*
V. anguillarum O5 (ATCC 43309)[c]	*****	*****	*****	*****	**N*
V. anguillarum O6 (ATCC 43310)[c]	*****	*****	*****	*****	**N*
V. anguillarum O7 (ATCC 43311)[c]	*****	*****	*****	*****	****
V. anguillarum O8 (ATCC 43312)	*****	K****	*****	****N	NN**
V. anguillarum O1 (A018)	*****	*****	*****	*****	****
V. anguillarum O1 (A055)	*****	*****	*****	*****	****
V. anguillarum O1 (A056)	*****	*****	*****	*****	****
V. anguillarum O3 (11008)	*****	*****	*****	*****	****
V. anguillarum O1 (A077)	*****	*****	*****	*****	****
V. diazotroficus (ATCC 33466)	*****	N****	*****	*****	****
Vibrio sp. (A096)	*Y**Y	*KN**	*****	***S*	****
V. pelagius (ATCC 25916)	**T**	**C**	*****	**G**	T*G*
V. campbellii (ATCC 25920)	**A**	**A**	*****	**T**	**T*
V. alginolyticus (ATCC 17749)	**A**	**A**	*****	**T**	**T*
V. fluvialis (ATCC 33812)	**A**	**A**	*****	**T**	**T*
V. harveyi (ATCC 14126)	**A**	**A**	*****	**T**	**T*
V. natriegens (ATCC 14048)	**A**	**A**	*****	**T**	**T*
Vibrio sp. (A060)	**A**	**A**	*****	**T**	**T*
Vibrio sp. (A065)	**A**	**A**	*****	**T**	**T*
Vibrio sp. (A068)	**A**	**A**	*****	**T**	**T*
Vibrio sp. (A063)	**A**	**A**	*****	**T**	**T*
Vibrio sp. (A071)	**A**	**C**	*****	**T**	**T*
Vibrio sp. (B009)	**R**	**A**	*****	**T**	**T*
V. harveyi (M58172)[d]	**A**	*NA**	*****	**T**	**T*
Vibrio sp. (A053)	*Y***	**A**	*****	*****	**T*
Vibrio sp. (A082)	*****	**W**	*****	***S*	**Y*
Vibrio sp. (A081)	*****	**C**	*****	**T**	****
V. proteolyticys (ATCC 15338)	**A*C	**A**	*****	*AT**	**T*
Vibrio sp. (A095)	*C**N	TAC**	*****	**G**	****
Vibrio sp. (A061)	**A**	TAC**	*****	**GTA	**T*
V. parahaemolyticus (ATCC 17802)	****C	AG***	*****	***C*	****
V. vulnificus (ATCC 27562)	*****	AG***	*****	***CT	****
V. vulnificus (ATCC 33147)	*****	AG***	*****	***CT	****
V. hollisae (ATCC 33564)	**A*C	**A**	*****	C*T**	G*T*
Vibrio sp. (A073)	**TTA	TGC**	*****	**GTA	TAG*
Vibrio sp. (A093)	*G***	A****	*****	**T**	***T
Vibrio sp. (A076)	*GT**	**A**	*****	**T**	**AT
Vibrio sp. (A050)	*GT**	**A**	*****	**T**	GCAT
Vibrio sp. (A094)	*G***	AGC**	*****	**GCT	***T
Vibrio sp. (A052)	*G***	AAC**	*****	**GCT	***T
Vibrio sp. (A032)	*G***	AGY**	*****	**GCT	***T
Vibrio sp. (A049)	*G***	AGC**	*****	**GCT	***T
Vibrio sp. (A070)	****N	TRC**	*****	**GTA	C**R
Vibrio sp. (A075)	*C**A	**C**	*****	**G**	T**G
Vibrio sp. (A074)	*G***	**C**	*****	**G**	***T
V. anguillarum O9 (ATCC 43313)[c]	**TTT	TGC**	*****	**GCA	GGG*
V. anguillarum O10 (ATCC 43314)[c]	**NT*	TGN**	*****	**GCA	GGG*
E. coli (J01859)[d]	*GA**	AAA**	*****	C*TTT	GCTC
Proteus vulgaris (Monteil)	***A*	AAA**	*****	C*TTT	G***
Plesiomonas shigelloides (M59159)[d]	*GYYA	C*A**	*****	C*TAG	TGGC
Aeromonas hydrophila (ATCC 7966)	*GTTG	A*GCC	*****	CGTAT	CAAC
Pseudomonas aeruginosa (ATCC 25330)	*GCAG	TAA**	*****	C*TTG	C*GT
Pseudomonas aeruginosa (DSM 50071)	*GCAG	TAA**	*****	C*TTG	C*GT
Pseudomonas aeruginosa (ATCC 15442)	*GCAG	TAA**	*****	C*TTG	C*GT
Pasteurella multocida (NCTC 10322)	*GATG	T*GT*	A****	*ATAG	CATC
Renibacterium salmoninarum (ATCC 33209)	ACATC	A**T*	*----[e]	----G	TGGT

[a] *, nucleotide identical to the *V. anguillarum*-specific probe sequence.
[b] *V. anguillarum* serovar.
[c] Sequenced by Rehnstam et al. (27).
[d] Source not specified by the European Molecular Biology Laboratory.
[e] -, gap position.

TABLE 3. RNA-DNA colony hybridization results for different collection type strains against VaV3 probe

Species	Strain	Hybridization
V. alginolyticus	ATCC 17749	–
V. anguillarum	ATCC 43305	+
V. campbellii	ATCC 25920	–
V. costicola	CCM 2811	–
V. damsella	ATCC 33539	–
V. fluvialis	ATCC 33812	–
V. harveyi	ATCC 14126	–
V. ordalii	ATCC 33509	–
V. parahaemolyticus	ATCC 17802	–
V. pelagius	ATCC 25916	–
V. proteolyticus	ATCC 15338	–
V. splendidus	ATCC 33125	–
V. vulnificus	ATCC 33147	–
Aeromonas hydrophila	CECT 398	–
Pseudomonas aeruginosa	ATCC 15442	–

frequently reported to be pathogenic for fish (18, 22, 35) are clearly recognized. Serovars O9 and O10, which are not recognized by our probe, have sequences in the V3 region clearly different from those of the remaining serovars. More extensive studies are required to establish whether they belong to the species *V. anguillarum*. Recent environmental isolates were also tested by both DNA-DNA slot blot hybridization and RNA-DNA colony hybridization. The identical results confirmed that 59% (40/68) of the strains tested, all those biochemically and serologically classified as *V. anguillarum*, were positive. Because RNA-DNA colony hybridization was as reliable as DNA-DNA slot blot hybridization, we suggest the use of RNA-DNA colony hybridization when working with environmental samples, since this method provides a higher number of potential targets and a more stable template-probe union than DNA-DNA hybridization. When the sensitivity of the VaV3 was evaluated by slot blot hybridization, the probe was able to detect as little as 150 pg of purified *V. anguillarum* chromosomal DNA.

Two DNA probes have already been designed for identification of *V. anguillarum* isolated from aquatic environments and organisms. A 526-bp cloned probe (1) was proposed for rapid identification of *V. anguillarum*, but it hybridized only with Japanese serotypes A and H. On the other hand the minimum amount of chromosomal DNA detectable by dot blot was 4 orders of magnitude greater than that detectable with the VaV3 probe. Another probe based on the 16S rRNA *V. anguillarum* sequence had also been established by studying only *V. anguillarum* collection strains and *E. coli* (27). It could hybridize with *Vibrio* species other than *V. anguillarum* because of its sequence. Although no correlation has been established between the sequence of VaV3 and the virulence of *V. anguillarum*, the availability of fast sensitive methods such as the one described here may help to improve the monitoring of *V. anguillarum* in fish farms and hatcheries.

Nucleotide sequence accession numbers. The partial 16S rDNA sequences reported in this paper have been deposited in the EMBL, GenBank, and DDBJ Nucleotide Sequence data bases under the accession numbers indicated in Table 1.

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