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Analysis of 16S-23S rRNA Intergenic Spacer Regions of *Vibrio cholerae* and *Vibrio mimicus*

JONGSIK CHUN,† ANWARUL HUQ, AND RITA R. COLWELL*

Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, Maryland 21202

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***Vibrio cholerae* identification based on molecular sequence data has been hampered by a lack of sequence variation from the closely related *Vibrio mimicus*. The two species share many genes coding for proteins, such as *ctxAB*, and show almost identical 16S DNA coding for rRNA (rDNA) sequences. Primers targeting conserved sequences flanking the 3′ end of the 16S and the 5′ end of the 23S rDNAs were used to amplify the 16S-23S rRNA intergenic spacer regions of *V. cholerae* and *V. mimicus*. Two major (ca. 580 and 500 bp) and one minor (ca. 750 bp) amplicons were consistently generated for both species, and their sequences were determined. The largest fragment contains three tRNA genes (tDNAs) coding for $tRNA^{Glu}$, $tRNA^{Lys}$, and $tRNA^{Val}$, which has not previously been found in bacteria examined to date. The 580-bp amplicon contained $tDNA^{Ile}$ and $tDNA^{Ala}$, whereas the 500-bp fragment had single tDNA coding either $tRNA^{Glu}$ or $tRNA^{Ala}$. Little variation, i.e., 0 to 0.4%, was found among *V. cholerae* O1 classical, O1 El Tor, and O139 epidemic strains. Slightly more variation was found against the non-O1/non-O139 serotypes (ca. 1% difference) and *V. mimicus* (2 to 3% difference). A pair of oligonucleotide primers were designed, based on the region differentiating all of *V. cholerae* strains from *V. mimicus*. The PCR system developed was subsequently evaluated by using representatives of *V. cholerae* from environmental and clinical sources, and of other taxa, including *V. mimicus*. This study provides the first molecular tool for identifying the species *V. cholerae*.**

Vibrio cholerae is a noninvasive, gram-negative bacterium responsible for severe epidemics of cholera and endemic diarrhea in many parts of the world, especially developing countries (14, 33). On the basis of several genotypic and phenotypic characteristics, *V. cholerae* O1 strains can be subdivided into two biotypes, classical and El Tor. The current cholera pandemic, the seventh, which started in 1961, is caused by the El Tor biotype, whereas the classical O1 strains were responsible for previous pandemics (1881 to 1896 and 1899 to 1923). Non-O1 strains have not caused major cholera epidemics, until serotype O139, named Bengal, emerged in India in 1992 (1). Genetic and phenotypic evidence strongly suggests that the O139 strain arose from a *V. cholerae* O1 strain, probably an El Tor biotype, by horizontal gene transfer (3, 4, 15, 24, 49).

The species *Vibrio mimicus* is a biochemically atypical group of *V. cholerae* strains (17). *V. mimicus* produces a variety of toxins, including cholera toxin, and it causes sporadic diarrhea (8, 10, 37). It has been isolated from a number of environmental sources, including oysters, prawns, turtle eggs, rivers, and brackish waters, as well as clinical samples (8–10). On the basis of a full-length sequence comparison, *V. mimicus* has been determined to have genes coding for 16S rRNA (rDNA) nearly identical to those of *V. cholerae*, i.e., differing only in 6 of 1,456 nucleotides (41).

Genetic information derived from the rRNA (*rrn*) operon provides valuable taxonomic information. The rRNA-coding regions, notably 16S rDNA, have been used extensively to underpin phylogenetic structures at the species level or above (30, 51). Intergenic spacer regions (ISRs), especially those located between the 16S and 23S rDNAs, were thought to be under less evolutionary pressure and, therefore, to provide higher genetic variation than rRNA coding regions (20–23, 29, 31, 35, 44). In general, the ISR possesses a secondary structure and, frequently, tRNA genes (7). The number of *rrn* operons in bacteria varies from 1 to 11 and multiple operons often contain the same ISR. For example, *Escherichia coli* contains seven *rrn* operons, three of which comprise the ISR containing two tRNA genes for isoleucine and alanine; the remaining four have the ISR containing a single tRNA gene for glutamate (16). Therefore, genetic variations in ISR are not only interstrain but also intercistronic.

Nandi et al. (36) demonstrated that epidemic *V. cholerae* O1 and O139 strains have 9 *rrn* operons, whereas non-O1/non-O139 strains possess 10 operons. Using a pair of oligonucleotide primers flanking 16S and 23S rDNAs of *E. coli*, Coelho et al. (12) showed that ISR PCR amplification patterns from O1 classical, O1 El Tor, and O139 strains were different and, thereby, provide a potential tool for studying the epidemiology of *V. cholerae*. In the study reported here, the nucleotide sequences of ISR from *V. cholerae* O1 classical, O1 El Tor, O139, and non-O1/non-O139 strains, as well as those from *V. mimicus* strains, were obtained and analyzed to seek interspecies, interserotype, and intercistronic variations.

MATERIALS AND METHODS

Strains. Strains of *V. cholerae* included in this study, listed in Table 1, were grown on Luria-Bertani agar (LB; Difco Laboratories, Detroit, Mich.) at 37°C and maintained on LB slants at room temperature or as suspensions in 25% glycerol at −70°C.

PCR primers for ISR amplification. A pair of oligonucleotides were designed to amplify the ISR of *V. cholerae* and related taxa. The forward primer, pr16S-1541F-PstI (5′-TTTCTGCAGYGGNTGGATCACCTCCTT-3′ (the *Pst*I site is indicated by the underline), corresponding to 16S rDNA positions 1523 to 1541 of *E. coli* (5), was designed to match members of the domain bacteria, and the reverse primer, pr23S-25R-EcoRI (5′-ACGAATTCTGACTGCCMRGGCATCCA-3′ (the *Eco*RI site is indicated by the underline), corresponding to 23S rDNA

* Corresponding author. Mailing address: University of Maryland Biotechnology Institute, Center of Marine Biotechnology, 701 East Pratt St., Ste. 236, Baltimore, MD 21202. Phone: (410) 234-8885. Fax: (410) 234-8873. E-mail: colwell@umbi.umd.edu.

† Present address: Korean Collection for Type Cultures, Korean Research Institute of Bioscience and Biotechnology, Yusong, Taejon 305-600, Republic of Korea.

TABLE 1. Test strains used in this study

Strain no.	Species and serovar	Other designation(s) and information
RC2T	*V. cholerae* O1 classical	ATCC 14035
RC25	*V. cholerae* O1 El Tor	Clinical isolate; Mexico, 1991
RC4	*V. cholerae* O139	Clinical isolate; Bangladesh, J. Johnson AI1877
RC45	*V. cholerae* O22	J. Johnson Y334
RC48	*V. cholerae* O31	J. Johnson Y1, NRT36S
RC42	*V. cholerae* non-O1/non-O139	ATCC 14547, *V. albensis*; fish from the Elbe River
RC44	*V. cholerae* non-O1/non-O139	ATCC 25874, clinical isolate
RC47	*V. cholerae* non-O1/non-O139	ATCC 25872, clinical isolate
RC5T	*V. mimicus*	ATCC 33653; isolated from human ear; North Carolina
RC55	*V. mimicus*	Environmental isolate; Louisiana

positions 44 to 25 of *E. coli* (6), was designed based on sequences from *E. coli* (GenBank accession number V00331), *Pseudomonas aeruginosa* (Y00432), *V. cholerae* (U10956), and *Vibrio vulnificus* (U10951).

DNA isolation and PCR. Chromosomal DNA was isolated by using guanidine thiocyanate according to the method of Chun and Goodfellow (11). Approximately 50 ng of DNA was subjected to PCR amplification, in a total volume of 50 μl containing primers (each at a concentration of 0.4 mM), a mixture of deoxynucleoside triphosphates (each at a concentration of 200 mM), *Taq* polymerase, and buffer (Promega, Madison, Wis.). A DNA thermal cycler (PTC-200; MJ Research) used for thermal amplification was programmed for the following: (i) an initial extensive denaturation step, consisting of treatment at 94°C for 2 min; (ii) 30 reaction cycles, with each cycle consisting of treatment at 94°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min; and (iii) a final extension step, consisting of treatment at 72°C for 10 min. The PCR products were separated by 1% agarose gel electrophoresis, stained with ethidium bromide, and visualized with UV light.

Cloning. The ISR amplicons were purified with the Wizard PCR Mini-Prep Kit (Promega) according to the manufacturer's instructions. The preparations were digested in tubes containing *Eco*RI, *Pst*I, and buffer H (Promega) at 37°C for 1 h and then treated at 70°C for 15 min to inactivate the restriction enzymes. The digested ISR fragments were ligated into the predigested plasmids prepared as follows: pGEM-T Easy Vector (Promega) was recircularized by ligation, transformed into *E. coli* JM109, purified by using the Wizard Mini-Prep Kit, double-digested with *Eco*RI and *Pst*I, and purified from 1% agarose gels by using the GeneClean II Kit (Bio 101, Vista, Calif.). The ligation was achieved by using T4 DNA ligase (Promega). The resultant mixture was transformed into highly competent *E. coli* JM109, and the recombinants were selected according to the standard blue-white cloning procedure (43). The selected clones were grown in LB broth containing ampicillin (100 μg ml^{-1}), and the plasmids were purified with the Wizard Mini-Prep Kit. The size of the inserts was confirmed by 1% agarose gel electrophoresis after the *Eco*RI-*Pst*I treatment.

Sequencing of ISR. Nucleotide sequences of ISR inserts were determined by using the ABI PRISM Dye Terminator Cycle Sequencing Kit (Perkin-Elmer, Norwalk, Conn.) and an ABI 377 automated DNA sequencer. Two primers flanking the multiple cloning site of pGEM-T Easy Vector, prGTf (5′-TACGA CTCACTATAGGGCGA-3′) and prGTr (5′-CTCAAGCTATGCATCCAACG C-3′), were synthesized and used to sequence both DNA strands.

Data analysis. Nucleotide sequences were aligned by using the PILEUP program in the Genetics Computer Group package; the sequences were then adjusted manually. Evolutionary distances were calculated by using the model of Jukes and Cantor (25), and phylogenetic trees were inferred by the neighbor-joining method (42).

PCR for *V. cholerae* identification. A pair of primers, namely, prVC-F (5′-TT AAGCSTTTTCRCTGAGAATG-3′; positions 227 to 248 of *V. cholerae* RC2 ISR-2) and prVCM-R (5′-AGTCACTTAACCATACAACCCG-3′; positions 501 to 12 of 23S rDNA of *V. cholerae*), were synthesized and used for *V. cholerae*-specific PCR experiments. PCR conditions were identical to those for ISR amplification, except that annealing was done at 60°C for 1 min and extension was done at 72°C for 1 min. The results were confirmed by 1.5% agarose gel electrophoresis. For routine identification, cells were scraped from LB plates, boiled in distilled water, and used as PCR template DNAs. False-negative results due to PCR inhibition and insufficient template DNA were checked by performing PCR targeting of the universal region of 16S rDNA.

Nucleotide sequence accession numbers. Nucleotide sequences for ISRs determined in this study were deposited in GenBank under accession numbers AF114721 to AF114749.

RESULTS

PCR of ISR. PCR with the two primers, p16S-1541F-PstI and p23S-25R-EcoRI flanking 16S-23S rDNA, yielded a nearly identical band pattern for the *V. cholerae* and *V. mimicus* strains containing the two major bands (ca. 580 and 500 bp) and one minor band (ca. 750 bp) (Fig. 1). In addition, a faint band of ca. 700 bp was visible for both species.

Sequence analysis of ISR. Recombinant plasmids containing different ISR amplicons were screened by simultaneously digesting with *Eco*RI and *Pst*I, and those plasmids containing insert DNAs corresponding to three PCR amplicons of different sizes were identified and sequenced. The results of the sequence analyses are summarized in Fig. 2. In the type strain of *V. cholerae* (strain RC2T, O1 classical), the largest ISR (i.e., 750-bp amplicon; designated ISR-1) consisted of 686 nucleotides and contained three tRNA genes (tDNAs) coding for $tRNA^{Glu(UUC)}$, $tRNA^{Lys(UUU)}$, and $RNA^{Val(UAC)}$, respectively (anticodons are indicated in parentheses). Among the other *V. cholerae* strains, RC25 (O1 El Tor), RC47 (non-O1/non-O139), and RC48 (O31) showed an almost identical ISR-1 of the same length (Table 2) as that of RC2. In contrast, *V. mimicus* RC5T had a shorter version (i.e., 670 bp) and differed from the *V. cholerae* strains by 16 nucleotides. Such a length difference between two species is attributed to a 16-bp deletion in the *V. mimicus* strain, located between $tDNA^{Glu}$ and $tDNA^{Lys}$, where *V. cholerae* and *V. mimicus* strains had 19- and 3-bp spacers, respectively.

In all of the *V. cholerae* and *V. mimicus* strains examined in this study, the 580-bp amplicon, designated ISR-2, invariably contained $tDNA^{Ile(GAU)}$ and $tDNA^{Ala(UGC)}$ and had a total size of either 509 or 510 bp. Intraspecies ISR-2 sequence similarities among the *V. cholerae* and *V. mimicus* strains ranged from 99.0 to 100%, whereas the corresponding values for the interspecies comparisons were lower, ranging from 97.0 to 98.0% (Table 3).

The smallest ISR amplicon, designated ISR-3, corresponding to the ca. 500-bp PCR product, was 430 to 445 bp long and contained one tDNA coding for $tRNA^{Glu(UUC)}$. In contrast to ISR-1 and ISR-2, ISR-3 from some strains contained single

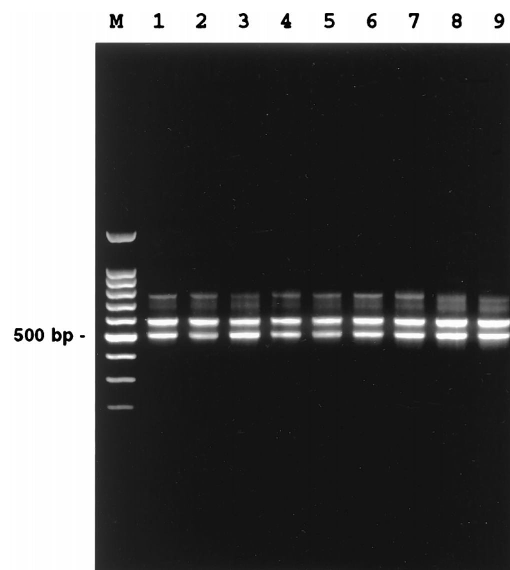

FIG. 1. Electrophoresis with a 1.5% agarose gel of PCR-amplified 16S-23S rRNA intergenic spacer regions of *V. cholerae* and *V. mimicus*. Lanes: M, molecular weight marker (100-bp ladder); 1, *V. cholerae* O1 classical RC2; 2, *V. cholerae* O1 El Tor RC25; 3, *V. cholerae* O139 RC4; 4, *V. cholerae* O22 RC45; 5, *V. cholerae* O31 RC48; 6, *V. cholerae* non-O1/non-O139 RC42; 7, *V. cholerae* non-O1/non-O139 RC44; 8, *V. mimicus* RC5; and 9, *V. mimicus* RC55.

FIG. 2. Schematic representation of 16S-23S rRNA intergenic spacer regions of *V. cholerae* and *V. mimicus*. An alignment of ISR sequences, corresponding to positions 224 to 276 of *V. cholerae* RC2 ISR-2, is presented in detail, and identities are indicated by a solid box. The positions of PCR primers, namely, prVC-F and prVCM-R, are indicated by arrows. For details, see the text.

tDNA^{Ala}, in addition to ISR-3 containing tRNA^{Glu} (Table 4). The additional ISR-3 containing one tRNA$^{Ala(GGC)}$ was recovered from *V. cholerae* RC2 and *V. mimicus* RC5 and RC55. Similarly, ISR-3 with tDNA$^{Ala(UGC)}$ was found in *V. cholerae* RC25 and RC44.

The corresponding clone containing a faint band of ca. 700 bp was not recovered. Therefore, it is not clear whether it resulted from nonspecific PCR or from amplification of authentic ISR.

Phylogenetic analysis based on ISR. Phylogenetic trees based on ISR-1, ISR-2, and ISR-3 (Fig. 3) showed consistent genealogical relationships among the *V. cholerae* and *V. mimicus* strains. In the case of ISR-3, the tDNA region was excluded from the neighbor-joining analysis, as it was not considered homologous. It was clear from the phylogenetic analyses and sequence comparisons that the ISRs from epidemic *V. cholerae* O1 classical, O1 El Tor, and O139 strains are very similar, if not identical. *V. cholerae* RC44 (non-O1/non-O139) was also found to be closely related to the epidemic *V. cholerae* strains. Other serotypes, including *V. cholerae* RC42, RC45, RC47, and RC48, showed significant sequence variation in the ISR. However, ISR-3 from RC42 was identical to the epidemic *V. cholerae* strains.

V. mimicus strains formed a separate monophyletic clade. The intraspecies ISR similarity values were 99.5 ± 0.4 and 99.1 ± 0.3 for *V. cholerae* and *V. mimicus*, respectively, whereas the corresponding value for interspecies comparisons was 97.3 ± 0.4. ISR-3 containing tDNA$^{Ala(UGC)}$ or tDNA$^{Ala(GGC)}$ formed a subclade within the *V. cholerae* clade. However, such a division was not observed for the *V. mimicus* strains (Fig. 3c).

Sequence analysis of tRNA genes found in ISRs. The primary structures of five tDNAs found in ISR-1 and ISR-2 were identical among the *V. cholerae* and *V. mimicus* strains. tDNA$^{Glu(UUC)}$ in ISR-3 was identical to tDNA$^{Glu(UUC)}$ in ISR-1. The results of a BLAST search of tDNAs are summarized in Table 5. None of the tDNAs from *V. cholerae* and *V. mimicus* was identical to published or deposited sequences in GenBank to date. tDNA$^{Glu(UUC)}$, which was found in both ISR-1 and ISR-3, showed the greatest similarity to *Aeromonas* tDNA$^{Glu(UUC)}$ found in ISR-3 (19). Similarly, two tDNAs coding for tRNA^{Ile} and tRNA^{Ala} located in ISR-2 showed the greatest similarity to those found in ISR-2 of *Aeromonas hydrophila*. tDNA^{Lys} and tDNA^{Val} found in ISR-1 were most similar to those found in *E. coli* and *Haemophilus influenzae*,

TABLE 2. ISR-1 sequence similarity values among *V. cholerae* and *V. mimicus* strains

Strain	Size (bp)	Sequence similarity values[a]				
		RC2	RC25	RC47	RC48	RC5
V. cholerae O1 classical RC2[T]	686		0/686	1/686	5/686	17/670
V. cholerae O1 El Tor RC25	686	100.0		1/686	5/686	17/670
V. cholerae NO[b] RC47	686	99.9	99.9		4/686	16/670
V. cholerae O31 RC48	686	99.3	99.3	99.4		16/670
V. mimicus RC5[T]	670	97.5	97.5	97.6	97.6	

[a] The values in the upper-right triangle indicate nucleotide differences/total number compared. The values in the lower-left triangle indicate the % similarity values.

[b] NO, non-O1/non-O139.

TABLE 3. ISR-2 sequence similarity values among *V. cholerae* and *V. mimicus* strains

Strain[a]	Size (bp)	Sequence similarity values[b]									
		RC2	RC25	RC4	RC42	RC44	RC45	RC47	RC48	RC5	RC55
V. cholerae O1 classical RC2^T	510		2/510	1/510	4/510	1/510	4/505	2/510	5/510	13/505	12/505
V. cholerae O1 El Tor RC25	510	99.6		1/510	4/510	1/510	4/505	2/510	5/510	13/505	12/505
V. cholerae O139 RC4	510	99.8	99.8		3/510	0/510	3/505	1/510	4/510	12/505	11/505
V. cholerae NO RC42	510	99.2	99.2	99.4		3/510	4/505	4/510	3/510	15/505	14/505
V. cholerae NO RC44	510	99.8	99.8	100.0	99.4		3/505	1/510	4/510	12/505	11/505
V. cholerae O22 RC45	509	99.2	99.2	99.4	99.2	99.4		4/505	3/505	13/509	12/509
V. cholerae NO RC47	510	99.6	99.6	99.8	99.2	99.8	99.2		5/510	11/505	10/505
V. cholerae O31 RC48	510	99.0	99.0	99.2	99.4	99.2	99.4	99.0		14/505	13/505
V. mimicus RC5^T	509	97.4	97.4	97.6	97.0	97.6	97.4	97.8	97.2		3/509
V. mimicus RC55	510	97.6	97.6	97.8	97.2	97.8	97.6	98.0	97.4	99.4	

[a] NO, non-O1/non-O139.
[b] See Table 2, footnote *a*.

respectively; the latter did not originate from ISR but from tRNA gene clusters. tDNA$^{Ala(GGC)}$ obtained from ISR-3 of strains RC2, RC5, and RC6 was nearly identical to the tDNA$^{Ala(GGC)}$ of *E. coli*, which was found in one of tRNA operons (26).

PCR specific for *V. cholerae*. Even though the ISR sequences for *V. cholerae* and *V. mimicus* were very similar, a region containing substantial genetic variation was identified next to the last tRNA coding genes, starting at position 224 of *V. cholerae* RC2^T ISR-2 (Fig. 2). A stretch of 17 nucleotides in ISR-2 and ISR-3 was consistently conserved within the species and different between species, from which the *V. cholerae* specific primer, named prVC-F, was designed (Fig. 2). The primer contains two degenerate nucleotides and five mismatches compared to *V. mimicus*. The reverse primer, prVCM-R, was derived from the sequence encompassing the 3′ end of ISR and the 5′ end of 23S rDNA and was complementary to all of the ISRs for *V. cholerae* and *V. mimicus*.

The results of PCR, based on the ISR sequence data, are shown in Fig. 4. The amplicon, the size of which was expected to be 295 to 310 bp, was apparent among more than 100 *V. cholerae* strains, including fresh clinical and environmental isolates from Bangladesh and the Chesapeake Bay, but not in *V. mimicus* strains. In addition, we confirmed negative results for *Listonella anguillarum* ATCC 19264^T, *Listonella pelagia* ATCC 25916^T, *Salinivibrio costicola* ATCC 33508^T, *Photobacterium damselae* subsp. *damselae* ATCC 33539^T, *V. aestuarianus* ATCC 35048^T, *V. alginolyticus* ATCC 17749^T, *V. campbellii* ATCC 25920^T, *V. carchariae* ATCC 35084^T, *V. diazotrophicus* ATCC 33466^T, *V. fischeri* ATCC 7744^T, *V. fluvialis* ATCC 33809^T, *V. furnissii* ATCC 35016^T, *V. hollisae* ATCC 33564^T, *V. natriegens* ATCC 14048^T, *V. nigripulchritudo* ATCC 27043^T, *V. orientalis* ATCC 33934^T, *V. proteolyticus* ATCC 15338^T, *V. salmonicida* ATCC 43839^T, *V. splendidus* ATCC 33125^T, *V. tubiashii* ATCC 19109^T, and *V. vulnificus* ATCC 27562^T.

DISCUSSION

Genetic information derived from the 16S-23S rRNA ISR can be used to differentiate closely related organisms (20, 22, 29, 39, 40, 44, 47, 52). One of the goals of this study was to underpin genealogical variation among *V. cholerae* species, especially those responsible for large-scale cholera epidemics.

TABLE 4. ISR-3 sequence similarity values among *V. cholerae* and *V. mimicus* strains

Strain[a]	Size (bp)	Sequence similarity values[b] with tDNA found in ISR-3:													
		tDNA$^{Glu(UUC)}$									tDNA$^{Ala(GGC)}$			tDNA$^{Ala(UGC)}$	
		RC2	RC25	RC4	RC42	RC44	RC45	RC47	RC48	RC5	RC2	RC5	RC55	RC25	RC44
tDNA$^{Glu(UUC)}$															
V. cholerae O1 classical RC2^T	431		0/431	0/431	0/431		6/429	1/431	2/431	14/426	46/424	56/419	57/419	42/424	42/424
V. cholerae O1 El Tor RC25	431	100.0		0/431	0/431	1/431	6/429	1/431	2/431	14/426	46/424	56/419	57/419	42/424	42/424
V. cholerae O139 RC4	431	100.0	100.0		0/431	1/431	6/429	1/431	2/431	14/426	46/424	56/419	57/419	42/424	42/424
V. cholerae NO RC42	431	100.0	100.0	100.0		1/431	6/429	1/431	2/431	14/426	46/424	56/419	57/419	42/424	42/424
V. cholerae NO RC44	431	99.8	99.8	99.8	99.8		7/429	2/431	3/431	15/426	47/424	57/419	58/419	43/424	43/424
V. cholerae O22 RC45	445	98.6	98.6	98.6	98.6	98.4		6/429	6/429	15/429	52/422	57/422	58/422	46/422	48/422
V. cholerae NO RC47	431	99.8	99.8	99.8	99.8	99.5	98.6		3/431	14/426	47/424	56/419	57/419	43/424	43/424
V. cholerae O31 RC48	431	99.5	99.5	99.5	99.5	99.3	98.6	99.3		12/426	48/424	56/419	57/419	42/424	44/424
V. mimicus RC5^T	430	96.7	96.7	96.7	96.7	96.5	96.5	96.7	97.2		58/419	44/423	49/423	52/419	54/419
tDNA$^{Ala(GGC)}$															
V. cholerae O1 classical RC2^T	424	89.2	89.2	89.2	89.2	88.9	87.7	88.9	88.7	86.2		14/420	15/420	8/425	4/425
V. mimicus RC5^T	424	86.6	86.6	86.6	86.6	86.4	86.5	86.6	86.6	89.6	96.7		5/424	18/420	16/420
V. mimicus RC55	424	86.4	86.4	86.4	86.4	86.2	86.3	86.4	86.4	88.4	96.4	98.8		19/420	17/420
tDNA$^{Ala(UGC)}$															
V. cholerae O1 El Tor RC25	425	90.1	90.1	90.1	90.1	89.9	89.1	89.9	90.1	87.6	98.1	95.7	95.5		4/425
V. cholerae NO RC44	425	90.1	90.1	90.1	90.1	89.9	88.6	89.9	89.6	87.1	99.1	96.2	96.0	99.1	

[a] NO, non-O1/non-O139.
[b] See Table 2, footnote *a*.

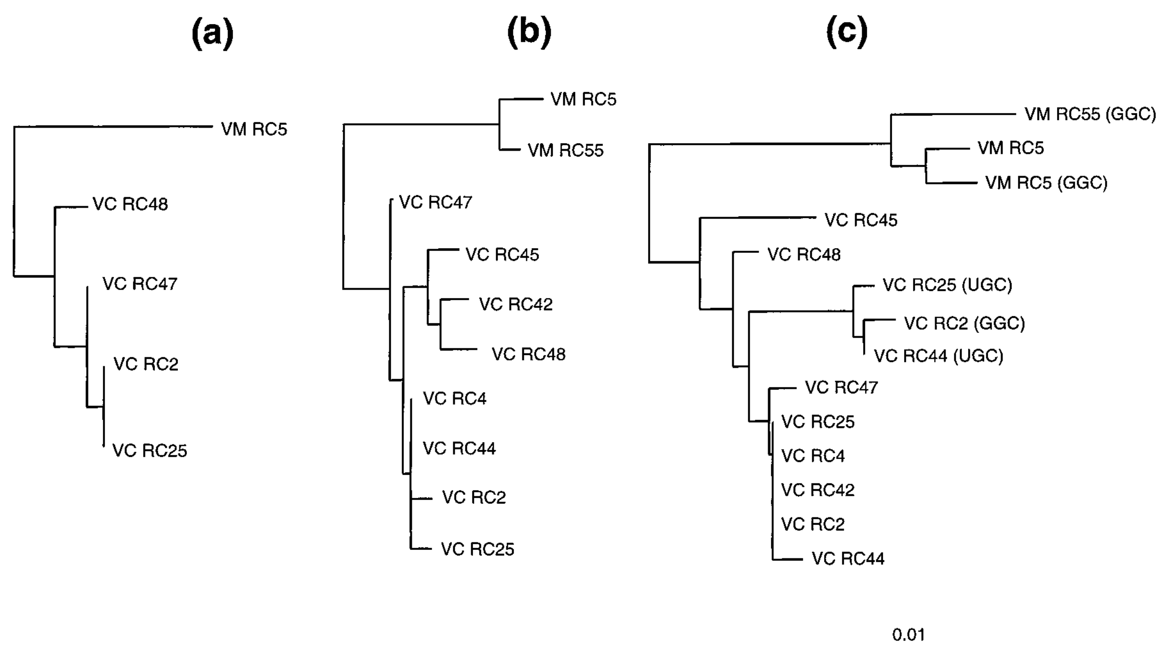

FIG. 3. Phylogenies of *V. cholerae* and *V. mimicus* strains based on ISR-1 (a), ISR-2 (b), and ISR-3 (c) sequences. A region coding for tRNA was excluded from analysis for ISR-3. Anticodons of tDNAs in ISR-3, other than $tDNA^{Glu}$, are indicated in parentheses. The unrooted evolutionary trees were inferred by using the neighbor-joining method. The scale bar represents 0.01-nucleotide substitutes per position. Abbreviations: VC, *V. cholerae*; VM, *V. mimicus*.

It was, therefore, disappointing to find so very little variation between the *V. cholerae* O1 and O139 strains. It has been pointed out by many investigators (3, 4, 15, 24, 48, 49) that serotype O139 may have arisen when a *V. cholerae* strain, probably an O1 El Tor, picked up genes responsible for its O antigen synthesis from other bacteria by lateral gene transfer. The close relationship between O1 and O139 strains, based on ISR sequence data, supports this hypothesis. Non-O1/non-O139 strains showed very little, albeit significant, variation from the O1 and O139 strains, except for the *V. cholerae* non-O1/non-O139 strain RC44.

The ISR PCR band patterns generated from *V. cholerae* and *V. mimicus* were almost identical (Fig. 1), which is in disagreement with the previous study of Coelho et al. (12), who reported that *V. cholerae* O1 classical, O1 El Tor, and O139 showed different ISR PCR patterns with low-stringency PCR conditions and primers based on *E. coli*. However, when their primer (NB-2) was compared with the recently available 23S rDNA sequence (GenBank accession number U10956) for *V. cholerae*, three mismatches, including two nucleotides at the 5′ end, were noted. It is therefore not clear that the PCR amplicons generated in the study of Coelho et al. originated from 16S-23S rRNA ISR. In contrast, our study was based on available 23S rDNA of *V. cholerae* and highly stringent PCR conditions.

The tRNA genes found in the bacterial 16S-23S rRNA ISR varied in number. *A. hydrophila* and *E. coli* contained two ISR types, one containing $tDNA^{Glu(UUC)}$ and the other $tDNA^{Ile(GAU)}$-$tDNA^{Ala(UGC)}$ (16, 19). In contrast, *Staphylococcus aureus* has three types, i.e., no tDNA, one $tDNA^{Ile(GAU)}$, and $tDNA^{Ile(GAU)}$-$tDNA^{Ala(UGC)}$ (21). The ISRs from mycobacteria have no tDNA (39, 47). It is interesting that the ISR containing $tDNA^{Ile(GAU)}$-$tDNA^{Ala(UGC)}$, which is equivalent to the ISR-2 found in *V. cholerae* and *V. mimicus*, was also present in a variety of bacterial taxa, including proteobacteria (16, 19, 23, 27, 29, 31, 34, 38), cyto-

TABLE 5. Sequence similarity of tDNAs found in 16S-23S rRNA intergenic spacer regions of *V. cholerae* and *V. mimicus*

ISR and tDNA	Matching organism(s)	% Similarity[a]
ISR-1		
$tDNA^{Glu(UUC)}$	*Aeromonas salmonicida*	93.4 (5/76)
	Aeromonas hydrophila	93.3 (5/75)
	Plesiomonas shigelloides, *Escherichia coli*, *Klebsiella pneumoniae*	92.1 (6/76)
$tDNA^{Lys(UUU)}$	*Escherichia coli*[b]	90.7 (7/75)
	Synechocystis sp.[b]	77.3 (14/75)
	Mycoplasma sp.[b]	76.3 (16/75)
$tDNA^{Val(UAC)}$	*Haemophilus influenzae*[b]	94.7 (4/76)
	Acholeplasma laidlawii,[b] *Escherichia coli*,[b] *Pseudomonas aeruginosa*[b]	85.5 (11/76)
ISR-2		
$tDNA^{Ile(GAU)}$	*Aeromonas hydrophila*	94.8 (4/77)
	Bacillus subtilis, *Haemophilus influenzae*, *Actinobacillus pleuropneumoniae*	93.5 (5/77)
$tDNA^{Ala(UGC)}$	*Aeromonas hydrophila*, *Escherichia coli*	96.1 (3/76)
	Haemophilus influenzae	94.7 (4/76)
ISR-3		
$tDNA^{Glu(UUC)}$	Identical to $tDNA^{Glu(UUC)}$ found in ISR-1	
$tDNA^{Ala(GGC)}$[c]	*Escherichia coli*[b]	97.4 (2/76)
	Aeromonas hydrophila,[e] *Escherichia coli*[e]	93.4 (5/76)
$tDNA^{Ala(UGC)}$[d]	*Vibrio cholerae*, *Vibrio mimicus*[e]	98.7 (1/76)
	Escherichia coli, *Aeromonas hydrophila*	97.4 (2/76)
$tDNA^{Ala(UGC)}$[f]	*Escherichia coli*, *Salmonella enterica*	96.1 (3/76)
	Vibrio cholerae[e]	94.7 (4/76)
	Haemophilus influenzae, *Pseudomonas syringae*	92.1 (6/76)

[a] Numbers of different nucleotides/total nucleotides compared are given in parentheses.
[b] tDNAs recovered from non-ISR.
[c] Obtained from strains RC2, RC5, and RC55.
[d] Obtained from strain RC25.
[e] $tDNA^{Ala(UGC)}$ found in ISR-2.
[f] Obtained from strain RC44.

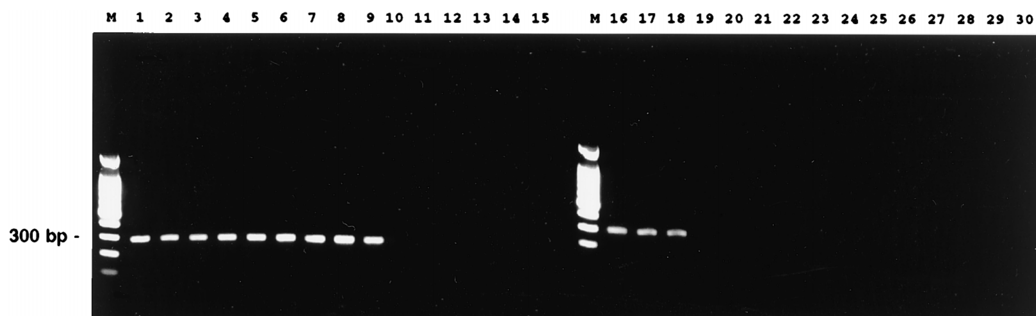

FIG. 4. Identification of *V. cholerae* by using PCR based on the 16S-23S rRNA ISR. Lanes: M, molecular weight marker (100-bp ladder): 1, *V. cholerae* O1 classical RC2; 2 to 5, *V. cholerae* O1 El Tor clinical isolates; 6 to 9, *V. cholerae* O139 clinical isolates; 10, *V. mimicus* RC5; 11 to 15, *V. mimicus* isolates; 16 to 18, *V. cholerae* non-O1/non-O139 isolates; 19, *V. aestuarianus* ATCC 35048^T; 20, *V. alginolyticus* ATCC 17749^T; 21, *V. campbellii* ATCC 25920^T; 22, *V. carchariae* ATCC 35084^T; 23, *V. diazotrophicus* ATCC 33466^T; 24, *V. fischeri* ATCC 7744^T; 25, *V. fluvialis* ATCC 33809^T; 26, *V. furnissii* ATCC 35016^T; 27, *V. hollisae* ATCC 33564^T; 28, *V. natriegens* ATCC 14048^T; 29, *V. salmonicida* ATCC 43839^T; and 30, *V. vulnificus* ATCC 27562^T.

phaga (2), gram-positive bacteria with a low G+C DNA (20, 28, 35, 45), and cyanobacteria and chloroplasts (46, 50). The recent finding of the same ISR type in *Aquifex aeolicus* (18), thought to represent the deepest evolutionary branch of bacteria, strongly suggests that this ISR type may be widespread among bacteria and present in the common ancestor of the domain *Bacteria*. Even though it is not possible to align ISR sequences between distantly related bacteria, two tDNAs found in ISR-2 can be compared that are likely homologous, i.e., originate from the same gene in an ancestral organism. The phylogenetic relationship based on $tDNA^{Ile(GAU)}$-$tDNA^{Ala(UGC)}$ sequences found in ISR-2 was readily comparable to current bacterial taxonomy based on 16S rDNA sequence data (data not shown). However, this type of ISR was not found among the archaea, actinomycetes, and mitochondria.

To date, the number of tDNAs found in the 16S-23S rRNA ISRs ranges from zero to two. It is, therefore, unexpected and surprising that the largest ISR amplicon of *V. cholerae* and *V. mimicus*, i.e., ISR-1, contained three tDNAs. In addition, the presence of $tDNA^{Lys}$ or $tDNA^{Val}$ in 16S-23S rRNA ISRs has not been reported before to occur in prokaryotes. We hypothesize that ISR-1 was generated from homologous recombination events between ISR-3 containing $tDNA^{Glu(UUC)}$ and other tRNA gene clusters containing $tDNA^{Lys}$ and $tDNA^{Val}$, since the region consisting of 144 bp of the 5′ end, including 76 bp coding for $tRNA^{Glu(UUC)}$, and of 235 bp of the 3′ end of ISR-1 was almost identical to ISR-3 among *V. cholerae* and *V. mimicus*. This event might have occurred in a common ancestor of *V. cholerae* and *V. mimicus*. At this stage of analysis, how far this event will date back is not certain, though additional 16S-23S rRNA ISR analyses with other vibrios and related taxa may provide an answer.

As in the case of the 16S rRNA sequence data, the 16S-23S rRNA ISR also provides a limited, albeit much higher, range of genetic variation. At the higher taxonomical level, it was not possible to compare the ISR with that of other bacteria examined to date except for the tDNAs. At the lower level, differentiation between the epidemic *V. cholerae* strains could not be achieved. However, information coded in the ISR was useful in separating *V. cholerae* from the closely related *V. mimicus*, a species category previously created for strains biochemically resembling *V. cholerae. V. mimicus* strains, like *V. cholerae*, are capable of producing many toxins, including cholera toxin, and share ecological niches with *V. cholerae*. It is therefore encouraging that *V. cholerae* can be identified by using the species-specific PCR method presented here. Currently available methods for identifying *V. cholerae* rely mainly on conventional biochemical tests that are time-consuming, are not always accurate, and require culturing. Techniques based on serology are critical, but they are useful only for detecting specific serogroups. In contrast, the PCR-based identification techniques are accurate, sensitive, and permit a large throughput. Furthermore, they allow direct detection without the necessity of culture. The latter is important, since it is now well documented that *V. cholerae* is present in the environment in the viable-but-nonculturable state. Monitoring for *V. cholerae*, at the species level, has significance because of the potential for conversion between *V. cholerae* serogroups; notably, conversion from non-O1 to O1 can occur in vitro and, probably, in vivo (13, 32). In conclusion, it is fair to say that the PCR-mediated identification system developed in the present study will provide an ecological and epidemiological tool for detecting *V. cholerae* in both the natural and clinical environments.

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