Polymorphism in repeated 16S rRNA genes is a common property of type strains and environmental isolates of the genus *Vibrio*

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Analysis of the 16S rDNAs obtained from cultures of single colonies of either type collection strains or environmental strains of the genus *Vibrio* revealed the presence of polymorphism in every one of the strains examined. Polymorphism was detected by visualization of heteroduplexes produced after 16S rDNA PCR amplification, a procedure that allows for the screening of a large number of isolates. Amplified 16S rDNAs obtained from both *Vibrio parahaemolyticus* and an environmental strain were cloned. Their nucleotide sequences revealed differences of up to 2% among 16S rDNAs from the same strain. Polymorphic sites were concentrated in a recognized variable stem–loop of bacterial 16S rRNA that contained in some cases up to 83% of the total mismatches observed. Most of the substitutions present in the stem–loop region showed compensating base covariation. The accumulation of so many compensating changes in the stem–loop region implies that the divergence of the different versions of this stem–loop is relatively ancient. This divergence could be the result of either a selection process or a lateral transfer of independently evolved genes.

**Keywords:** marine, 16S rDNA, *rrn*

**INTRODUCTION**

Acceptance of 16S rRNA as an appropriate tool for the reconstruction of evolutionary history and phylogenetic relationships (Amann *et al.*, 1995; Gray *et al.*, 1984) has been based on the fact that, among other assumptions, multiple copies of rRNA genes present in a single organism containing more than one *rrn* operon are either identical or nearly identical in nucleotide sequence. This assumption seems to be valid for some bacterial species, but not for others. For example, among some bacterial strains, for which the whole genome sequence is available, there are some strains with multiple *rrn* operons that nevertheless show no intra-16S rDNA polymorphism. Examples of these are *Treponema pallidum*, *Xylella fastidiosa*, *Synechocystis* PCC 6803 with two *rrn* operons; *Campylobacter jejuni* with three, *Neisseria meningitidis* with four and *Haemophilus influenzae* with six *rrn* operons. On the other hand, *Helicobacter pylori* and *Ureaplasma urealyticum* with two, *Escherichia coli* with seven, *Vibrio cholerae* with eight and *Bacillus subtilis* with ten *rrn* operons show differences between 16S rDNAs in different operons ranging from 0–6 up to 2% (www.ncbi.nlm.nih.gov and www.tigr.org). Polymorphism in repeated 16S rRNA genes had been initially suggested by the observation of nucleotide variations from 1 to 5% between pairs of 16S rRNA sequences from one bacterial strain or different bacterial strains of the same species deposited in GenBank (Felsenstein, 1985). 16S rRNA sequences from bacterial strains deposited in databases are usually obtained from a clone of a single operon or from the bulk of either 16S rDNA or 16S rRNA. Cloning of a single operon results in a sequence that may differ from those of the other operons, while direct sequencing of PCR or RT-PCR products produces a mean sequence in which differences between operons may be hidden.

More recently, Dahllof et al. (2000) showed 16S rDNA intraspecies heterogeneity in different bacterial isolates by denaturing gradient gel electrophoresis that was not observed in the gene for the RNA polymerase β subunit (rpoB). Altogether these data indicate that the existence of operons with different 16S rRNA in the same organism is a proven, though not general, phenomenon. It remains to be shown how common it is in nature.

The genus *Vibrio* contains a large number of closely related bacterial species with 16S rRNAs differing in nucleotide sequence from less than 1% up to 6% (Dorsch et al., 1992; Ruimy et al., 1994). The presence of several *rrn* operons has been shown in some *Vibrio* spp. and it may be common among members of this genus (Yamaichi et al., 1999). *Vibrio Fischeri* and *Vibrio harveyi* contain 8–11 operons (Lamfrom et al., 1978; Wolfe & Haywood, 1993; Fegatella et al., 1998). *Vibrio* spp. are indigenous to seawater where they can exchange genes, including RNA genes, by direct physical contact or indirectly through bacteriophages, known to be present in large amounts in seawater. This has been shown to be the case for *Vibrio cholerae* (for a review, see Wommack & Colwell, 2000).

In this study polymorphism was explored in type strains and environmental isolates of the genus *Vibrio* by a method which allows easy testing of large numbers of strains. It consists of the observation of the formation of heteroduplexes after PCR amplification of the 16S rDNAs (Espejo et al., 1998). When a sample containing several 16S rDNAs is PCR-amplified, products differing in sequence can anneal, forming hybrids (heteroduplexes) which migrate slower than the homoduplex during PAGE. We show that 16S rDNA polymorphism is a common phenomenon among type strains of validly described *Vibrio* species and of related strains isolated from oysters and coastal waters in Chile. Comparison of the sequences showed a high concentration of variable sites in particular regions of the molecule; 70–96 and 440–496 (*E. coli* numbering). The significance and origin of the observed polymorphism is discussed.

**METHODS**

**Strains and culture conditions.** Bacterial strains corresponded to *Vibrio paraaeromlyticus* ATCC 17802, *Vibrio vulnificus* ATCC 27562, *Vibrio algolyticus* ATCC 17749 and *Vibrio cholerae* (ISP NO 01) and were directly obtained from the respective culture collections. Environmental isolates were obtained from seawater samples collected from two points along the Chilean coast; Coloso and Horcon at latitudes 23° S and 32° S, respectively. The samples were filtered through Whatman No. 1 filter paper and the bacteria were subsequently collected in a 0.4 μm pore size membrane (Millipore). The bacteria in the membrane were resuspended by vortexing in 2 ml alkaline peptone water (APW). Thereafter, 100 μl of the suspension was streaked on thiosulfate citrate-bile-sucrose agar (TCBS) plates containing 2% NaCl. The plates were incubated overnight at 37 and 17 °C for 16–18 h. Strains isolated from oysters were obtained from oyster homogenates prepared as described previously (Romero & Espejo, 2002).

**DNA extraction and hybridization.** DNA for PCR was isolated from single bacterial colonies picked from marine agar plates, resuspended in 50 μl TE (0.01 M Tris, 0.001 M EDTA, pH 8.0) and lysed by boiling for 15 min. The lysate was then centrifuged at 5000 g for 15 s and 1.5 μl of the supernatants was directly used as template for amplification.

For Southern blotting, DNA of *V. parahaemolyticus* was purified as described by Sambrook et al. (1989). DNA (3.5 μg) was then digested with 9 units EcoRI (Gibco-BRL), resolved on a 1.5% agarose gel and the Southern blot was performed on Hybond-N+ membrane as described by the manufacturer (Boehringer Mannheim). A DNA probe of 1.4 kb, specific for 16S rDNA, was generated by PCR amplification of DNA extracted from *V. parahaemolyticus* and labelled by random priming with digoxigenin 11-dUTP using a DIG DNA labelling kit (Boehringer Mannheim) as recommended in the manufacturer’s instructions. Hybridization and immunological detection of DIG-labelled probe were performed using a DIG-luminescence detection kit, according to the manufacturer’s instructions (Boehringer Mannheim). Hybridization was performed using standard hybridization buffer at 68 °C with washing as recommended by the manufacturer (Boehringer Mannheim).

**PCR amplification of DNA, heteroduplex analysis, cloning and sequencing of the amplified 16S rDNA.** 16S rDNA PCR amplification and the heteroduplex mobility assay were performed as described by Espejo et al. (1998), except that electrophoresis was conducted at 150 V. PCR products were cloned into TOPO TA, according to the procedure indicated by the manufacturer (Invitrogen). Plasmid DNA was obtained by a rapid alkaline extraction miniprep (Birnboim, 1983). The DNA was diluted (1:500, v/v) in sterile distilled water and 15 μl was used for 16S rDNA amplification as described above. For sequencing, plasmids were purified with a Wizard Plus SV Miniprep rapid plasmid miniprep system (Promega). Cloned 16S rDNA genes were sequenced with an Applied Biosystems 310 automatic sequencer using ABI Prism dye terminator sequencing kits with M13 universal primers or primers annealing to conserved internal sequences. These primers were Eubac27F (5′-AGAGTTTGTACTCCTGGTCAG-3′), 357F (5′-CTCTTACGAGGCCGACCA-3′), 946F (5′-CCCGCACAAACCGTTGGA-3′), 1492R (5′-GGTTACCTTGTATTA CGACTT-3′), 1100R (5′-GGTTTGCCCCGCTGTGTT-3′) and 518R (5′-CGTATTACCGGGGCCGCTGCGG-3′). DNA sequences were inspected individually and manually assembled. The alignments and sequence similarities were performed with CLUSTAL W at the EMBL Outstation, European Bioinformatics Institute (EBI). Sequence similarities at specific sites were manually calculated. The secondary structure analysis was carried out with the mfold programs, using the sequence analysis software package (Genetics Computer Group, Madison, WI, USA).

**RESULTS**

16S polymorphism observed by the formation of heteroduplexes after amplification of the 16S rDNAs of different *Vibrio* species

When the 16S rDNAs in the DNA extracted from cultures of *V. algolyticus*, *V. parahaemolyticus* and *V. vulnificus*, obtained from single colonies, were PCR-amplified, additional bands with slower than expected electrophoretic migration rates were observed after electrophoresis in polyacrylamide gels (Fig. 1a). As previously shown (Espejo et al., 1998), these bands
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Correspond to heteroduplexes formed between single strands of amplified 16S rDNAs differing in nucleotide sequence. Single strands formed after the denaturation step of the amplification cycle can anneal during the temperature decrease to the annealing step. When the strands anneal are not 100% complementary, heteroduplexes are formed. Subjecting the product to an additional amplification cycle, after appropriate dilution to avoid annealing of the single strands (Delwart et al., 1993), can show their heteroduplex nature. In these conditions the heteroduplexes disappear. Furthermore, the presence of different sequences can be again revealed by the reappearance of the heteroduplexes if the latter product is subjected to denaturation and renaturation. Fig. 1(b) shows the results obtained after subjecting the heterogeneous amplification products of V. parahaemolyticus (shown in Fig. 1a) to this procedure. The same results were obtained with the amplification products of the other two species (not shown). These results suggest that some of the rrn operons in each of these species contained polymorphic 16S rDNAs. Based on previous results relating the relative electrophoretic migration rate of the heteroduplexes to that of the homoduplex with sequence dissimilarity (Espejo et al., 1998), the sequence differences may be estimated to be about 2–3%. If only the relative migration rate of the heteroduplexes formed by the Vibrio species is considered (Fig. 2), the equation can be restated as percentage dissimilarity = −12.96 × relative migration rate + 13.03. Using this equation, the intra-organism sequence dissimilarity observed in each of these strains can be estimated to be between 0.6 and 1.1%.

Southern blot analysis, and cloning and sequencing of the 16S rDNA in different rrn operons

The above observations prompted us to further characterize the rrn operons of these Vibrio species. Southern blot hybridization of EcoRI-restricted V. parahaemolyticus DNA with a 16S rDNA-specific probe yielded six bands, indicating the presence of at least six rrn operons in this genome (Fig. 3a). Hybridization between fragments and observation of heteroduplex formation indicated that fragments 5 and 6 contained 16S rDNA with different sequences (not shown). The amplification product of V. parahaemolyticus DNA was then cloned and the clones were distinguished according to the nature of products observed above the 1·45 kb band after PAGE. Lanes: P, amplification products of V. parahaemolyticus; R, products obtained after a single amplification cycle of samples in lane P diluted 1/10 (this dilution was performed to prevent annealing between single-stranded products (five times the volume applied for electrophoresis in lane P was used to compensate for the dilution performed, assuming that the product was doubled after the amplification cycle)); RR, products obtained after denaturation and renaturation of the samples applied in lane R. The position of molecular size markers is shown.
plexes, and the migration rate of the heteroduplex if formed. Hybridization between the 16S rDNA amplified from two clones, Vp23 and Vp16, resulted in the formation of three bands, producing a similar pattern to that observed after amplification of the whole genome (Fig. 3(a)). The lowest and most intense band corresponds to the homoduplex, while the other two, appearing above this one, correspond to the reciprocal hybrids formed by each plus and minus complementary strand (Espejo et al., 1998). Although the extent of dissimilarity in this hybrid pair is identical, non-paired regions may form distinct structural conformations in each hybrid, decreasing the mobility to different extents (Jensen & Straus, 1993). The potentiality of generating the three bands observed after amplification of the whole genome of V. parahaemolyticus with only two different 16S rDNAs suggested that the polymorphism in this strain might consist of only two different 16S rDNAs. The 16S rDNAs amplified from the above-mentioned clones and from clones Vp44 and Vp27, which hybridized like clones Vp23 and Vp16, respectively, were sequenced. The amplification product of fragment 6, eluted from a parallel gel to that used for the Southern blot shown in Fig. 3(a), was also sequenced. Hybridization of 16S rDNA amplified from this fragment rendered similar heteroduplexes to those obtained with the amplified products of clones 16 and 27. In every clone, the whole 16S rRNA region between primers Eubac 27f and 1492R was sequenced. The main differences in sequence observed are summarized in Table 1. From 4 up to 19 different nucleotide sites were found between the 16S rDNAs of the analysed clones. Interestingly, 63–83% of these sites were concentrated in a stem–loop of the variable region between positions 440 and 496 (E. coli numbering), called RV2 in Table 1.

### 16S rDNA polymorphism in environmental bacterial strains clustering among vibrios

Bacterial isolates from oysters or seawater with 94% or higher similarity in 16S rDNA to V. parahaemolyticus, V. cholerae and V. vulnificus were selected for this study. This value was chosen because the highest degree of dissimilarity between classical strains of the genus Vibrio is 7%. The similarity was estimated by the relative migration rate of the 16S rDNA heteroduplexes as shown in Fig. 2. 16S rDNA from the 20 strains selected by this criterion was amplified and examined for the formation of heteroduplexes, indicative of 16S rDNA polymorphism. The amplification products of every strain examined showed the presence of additional bands, migrating immediately above the amplified 16S rDNA, corresponding to heteroduplexes. At least three of these bands were observed in two strains, two bands in eight strains and one band in ten strains. Fig. 4 shows the heteroduplexes observed after amplification of 16S rDNA from some of these strains. A few of the strains rendered heteroduplexes with slower relative migration rates than those observed in V. parahaemolyticus (see lanes 2d and 3d, Fig. 4), suggesting the existence of up to 18% dissimilarity between 16S rRNAs genes in these strains. Fig. 4 also compares the heteroduplexes generated by hybridization of the amplified 16S rDNAs of these strains with those of V. parahaemolyticus. The heteroduplex nature of the bands observed in these isolates was confirmed by amplification after dilution.

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Table 1. Comparison of V. parahaemolyticus (VP) 16S rDNA amplified from different clones and from a restriction fragment containing 16S rDNA (F6)

H indicates the number of heteroduplexes observed between amplified 16S rDNA of the corresponding clones. T indicates the total number of differences found by comparison of 1514 sites. RV2 indicates the number of differences found in the 440–496 region (E. coli numbering). VP17802T corresponds to the sequence reported for V. parahaemolyticus ATCC 17802T. ND, Not done.
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Fig. 4. (a) PAGE of 16S rDNA amplification products obtained from some environmental vibrio isolates and of the hybridization products of these amplification products with *V. parahaemolyticus* amplified 16S rDNA. Lanes 1d/—, 2d/—, 3d/— and 4d/— contain the amplification product from the corresponding vibrio isolates. Lanes 1d/+ , 2d/+ , 3d/+ and 4d/+ show the product of the hybridization between 16S rDNA amplified from the corresponding strain with that from *V. parahaemolyticus*. (b) Heteroduplex nature of bands observed after amplification of DNA from strain 3d. Lanes: P, amplification products; R, products obtained after a single amplification cycle of samples in lane P; RR, products obtained after denaturation and renaturation of the samples applied in lane R. The procedure was performed as described in the legend to Fig. 1(b). The position of molecular size markers is shown.

Fig. 5. Gel electrophoresis of the amplification products of different clones of 16S rDNA of the environmental strain 3d and of the product obtained after hybridization of amplified 16S rDNA of the different clones. Lanes 3d, 2, 7 and 8 show the products obtained after amplification for 16S rDNA of strain 3d and clones 2, 7, 8 obtained with amplified 16S rDNA from strain 3d. Lanes 2/7, 2/8 and 7/8 show the hybridization products between amplified 16S rDNA from the corresponding clones. Lane Ld shows the 1–5 kb molecular size marker.

Table 2. Comparison of 16S rDNA amplified from different clones of *Vibrio* sp. 3d containing 16S rDNA

H indicates the number of heteroduplexes observed between amplified 16S rDNA of the corresponding clones. T indicates the total number of differences found by comparison of 1514 sites. RV1 and RV2 indicate the number of differences found in the 70–96 and 440–496 regions (*E. coli* numbering), respectively.

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Cloning and sequencing of the different 16S rDNAs in environmental isolate 3d

16S rDNA from isolate 3d was amplified and subsequently cloned. Clones containing different 16S rDNAs were subsequently clustered according to the capacity of their amplified 16S rDNA to form heteroduplexes. Fig. 5 shows the heteroduplexes formed between amplified 16S rDNAs of clones clustered in different groups. Clones 3d2 and 3d7 rendered a pattern with two putative heteroduplexes (lane 2/7), similar in migration rate to those observed after amplification of the whole genomic DNA (lane 3d), suggesting that there were only two different 16S rDNAs in this strain. However, hybridization of the product of each of these clones with that of a third clone, named 3d8, produced only one putative heteroduplex band (lanes 2/8 and 7/8, Fig. 5). Since clones 3d2, 3d7 and 3d8 contain different 16S rDNAs, it is likely that isolate 3d contains at least three different *rrn* operons. The nucleotide sequence was obtained from the amplified 16S rDNA of clones 3d2, 3d7 and 3d8, and also from clone 3d4, which behaved like 3d8 in heteroduplex analysis. A summary of the nucleotide differences is shown in Table 2. From 5 up to 31 different nucleotide sites were found. These sites were concentrated in the stem–loops containing nucleotides 70–96.
DISCUSSION

Detection of heteroduplexes after PCR amplification of DNA from purified bacterial strains proved to be a reliable indicator of the presence of polymorphism within particular organisms. This method allowed us to screen a large number of type strains and environmental isolates of the genus *Vibrio* and to show that polymorphism in 16S rDNAs *rrn* operons of the same organism is a common phenomenon among bacterial species of this genus. The sequence heterogeneity estimated by the relative migration of the heteroduplexes was confirmed by nucleotide sequencing of cloned 16S rDNAs from strain 3d and *V. parahaemolyticus*. The absence of polymorphism in the amplification product of cloned 16S rDNA, observed by either heteroduplex formation or sequencing, indicates that the reported polymorphism is not generated during PCR amplification.

The number of bands corresponding to heteroduplexes does not correspond to the number of genes differing in sequence. As shown earlier, reciprocal hybrids formed by each plus and minus complementary strand can display different migration rates; therefore two different 16S rDNAs can produce two heteroduplexes (Espejo et al., 1998). This is exemplified by the hybridization of the clones from strain 3d where only two clones rendered two heteroduplexes with different electrophoretic migration. Formation of heteroduplexes with slower electrophoretic migration rates than the homoduplex corresponded with the number of mismatches in the variable region. The retarded electrophoretic migration rate observed for the heteroduplexes might be due to interruptions of the double helix by either single-stranded regions or stem–loops in the region in which the mismatches are concentrated.

Comparative analysis of the sequences obtained from the different clones of *V. parahaemolyticus* and isolate 3d revealed details of the heterogeneity among *rrn* operons. Polymorphic nucleotide sites of the 16S rRNA were concentrated in two hypervariable regions in strain 3d and *V. parahaemolyticus*. The absence of polymorphism in the amplification product of cloned 16S rDNA, observed by either heteroduplex formation or sequencing, indicates that the reported polymorphism is not generated during PCR amplification.

ACKNOWLEDGEMENTS

It is a pleasure to acknowledge the support, help and advice of M. Garcia-Varela and J. P. Laclette for nucleotide sequencing in their laboratory at the Instituto de Investigaciones Biomédicas of the Universidad Nacional Autónoma de México. We would also like to thank Ilani Atwater for reviewing the manuscript and correcting the English grammar and style. This work was supported by grants FONDECYT 1990765 and FONDECYT 2000064 from the Comisión Nacional de Investigación Científica y Tecnológica, Chile. C. Moreno acknowledges a scholarship from Deutscher Akademischer Austausch Dienst (DAAD).
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Received 2 October 2001; revised 11 December 2001; accepted 17 December 2001.