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High intragenomic heterogeneity of 16S rRNA genes in a subset of *Vibrio vulnificus* strains from the western Mediterranean coast

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Summary. Heterogeneity among ribosomal operons in *Vibrio vulnificus* is purported as a probabilistic indicator of strain virulence and classifies *V. vulnificus* strains as 16S rRNA genes type A and B. In this study, 16S rRNA genes typing of *V. vulnificus* strains isolated from the Valencia city coast, in the western Mediterranean, showed that 24 out of 30 isolates were type A, one was type B and five could not be typed. Single strand conformation polymorphism (SSCP) analysis of this gene region revealed complex patterns indicative of intragenomic ribosomal operon sequence heterogeneity. The 16S rRNA genes of three untypeable isolates C27, C30, and C34, along with type A (ATCC 27562) and B (C7184) reference strains, were amplified, cloned and sequenced. The number of unique 16S rRNA gene sequences was 4, 3, and 4 for the environmental isolates. The type strain of the species (ATCC 27562) presented only two 16S rRNA gene types, while the reference isolate C7184 of clinical origin had only one 16S rRNA gene type. Sequences differed from five to 35 bp (99.6% to 97.6% sequence similarity). Areas of variability concentrated in helices 10, 18, and 37 and included variants with short intervening sequences in helix 10. Most of the substitutions showed compensatory mutations suggesting ancient sequence divergence generated by lateral gene transfer. [**Int Microbiol** 2010; 13(4):179-188]

Keywords: *Vibrio vulnificus* · 16S rRNA genes · intraspecies heterogeneity · waterborne bacterial pathogens

Introduction

Vibrio vulnificus is an autochthonous marine and estuarine bacterium that can be found in association with both vertebrate and invertebrate marine animals. Considered one of the most dangerous waterborne bacterial pathogens, it has a fatality rate that can reach 50% for cases of primary septicemia. The majority of reported *V. vulnificus* infections are associated with the consumption of raw oysters (*Crassostrea virginica*) from the Gulf of Mexico. This bacterium species also includes fish-pathogenic strains that cause severe outbreaks in cultured eels [7].

Vibrio vulnificus harbors a high level of intrinsic phenotypic and genetic diversity [4,6–8,16]. Within the species, biotypes, serovars and genotypes have been described. Ribotyping, amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), and pulsed field gel electrophoresis (PFGE), among other methods, have been used to type *V. vulnificus* isolates [4,5,18]. However, all these fingerprinting methods failed to provide a clear association between genotypes and biotypes or serotypes and, even more relevant, they were unable to correlate a specific genotype with strain virulence.

In 2003, Nilsson et al. [27] proposed the use of a polymorphism present in the 16S rRNA genes of *V. vulnificus* as

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indicator for strain virulence. In their study, they reported 94% of the environmental *V. vulnificus* isolates as 16S rRNA genes type A while 76% of the clinical isolates were 16S rRNA genes type B (percent of type B strains was up to 94% when clinical fatalities were considered). This polymorphism had been first described by Aznar et al. [6], who found 17 nucleotide differences (out of 1526 bases) between the type strain of *V. vulnificus* ATCC 27562 and a clinical strain C7184. Most of those polymorphisms occur near helix 10 of the secondary structure for bacterial 16S rRNA, a variable region within the prokaryotic 16S rRNA genes [10]. Under experimental conditions, both type A and type B have been shown to be virulent for mice [20]; however, the majority of strains recovered from clinical samples (at least in the USA) belong to type B. The strong correlation between *V. vulnificus* 16S type B and clinical strains have made the 16S rRNA genes a desirable target for PCR-based detection methods for virulent strains [35]. The quantitative real time PCR developed by Vickery et al. [35] revealed the presence of both types in a single strain isolated from the northern Gulf of Mexico. This new variant, referred to as type AB since it contains both haplotypes, was present in up to 22% of the strains including clinical strains. Gordon et al. [13] have found a nearly equal proportion of type A/type B among clinical strains from Florida and have suggested a geographical variation in the population structure of *V. vulnificus*. Both studies analyzed strains recovered along the same coast.

Few studies have analyzed the variability of *V. vulnificus* 16S rRNA genes in isolates from outside the Gulf of Mexico. Kim and Jeong [21] have suggested that type B strains are present in equal numbers as type A strains in oysters and seawater from South Korea. A low incidence of *V. vulnificus* infections along the Mediterranean coast suggests a high prevalence of type A and type AB among *V. vulnificus* isolates from the region. Sanjuán et al. [31] have confirmed a higher percent of *V. vulnificus* type A and AB isolated in Spain and Israel. All the clinical strains from Israel described in their study are type AB, however, most of these Mediterranean isolates were recovered from diseased eels, not from environmental samples.

In a previous study our group reported a high intraspecies diversity among *V. vulnificus* strains from seawater and bivalves sampled off of the eastern coast of Spain, near Valencia, although those isolates were not ascribed to specific 16S rRNA genes types at that time [3]. The objective of the present study was to investigate the distribution of *V. vulnificus* types A, B, and AB in isolates from seawater and bivalves from Valencia in the Spanish Mediterranean coast. Our results show an unexpected high degree of intragenomic heterogeneity in the 16S rRNA genes of a subset of species of *V. vulnificus*.

Materials and methods

Strains, culture conditions, and DNA extraction. Thirty *V. vulnificus* strains previously isolated and characterized from Mediterranean samples collected from surface water, 100 m off-shore, at a beach 20 km north of Valencia city (39° 36′ 14.14″ N, 0° 15′ 36.06″ W) [2], were used in this study along with reference strains. Strain nomenclature and origin are detailed in Table 1. Bacteria were kept as glycerol stocks at –80°C in our collection and subsequently cultured in marine agar (Difco, Detroit, MI, USA) at 30°C. DNA extraction for all strains was accomplished from an overnight culture according to Pitcher et al. [30]. DNA was quantified using a Nanodrop spectrophotometer (Nanodrop, Wilmington, DE, USA) and properly diluted at 20 ng/µl.

16S-RFLP (restriction fragment length polymorphism) and 16S-SSCP (single-strand conformation polymorphism) analysis. Strains were ascribed to 16S type A or B according to Nilsson et al. [27]. Amplification of 16S rRNA genes was carried out with primers UFUL (5′-GCCTAACACATGCAAGTCGA-3′) and UFUR (5′-CGTATTA CCGCGGCTGCTGG-3′). These two primers target two highly conserved regions of the prokaryotic 16S rRNA gene that flank a region known to be variable among bacterial species corresponding to position 46 to 537 in the *Escherichia coli* numbering system [34]. PCR amplification and restriction analysis with *Hae*III (New England Biolabs, Beverly, MA, USA) were performed as described by Nilsson et al. [27]. Following digestion, restriction fragments were electrophoresed in a 3% TAE agarose-1000 (Invitrogen) gel for 90 min at 80V, stained with ethidium bromide, and photographed under UV light.

Single-strand conformation polymorphism (SSCP) analysis of the restricted products was carried out according to Olivares-Fuster et al. [28]. One µl of the restriction product was mixed with 5 µl of the denaturing-loading solution (95% formamide, 0.025% bromophenol blue), heated for 5 min at 98ºC and immediately cooled on ice. All 6 µl of the denatured products were separated by electrophoresis on GeneGel SSCP non-denaturing polyacrylamide gels (Amersham Biosciences, Piscataway, NJ, USA) hydrated with SSCP buffer A, pH 9.0 (Amersham Biosciences). A GenePhor electrophoresis unit (Amerham Biosciences) was used with the following running conditions: 5°C constant temperature, 25 min at 90 V, 6 mA, 5 W, plus 60 min at 500 V, 14 mA, 10 W. Gels were silver stained with the DNA Silver Staining Kit (Amersham Biosciences) and digitally photographed.

Full-length 16S rRNA genes were amplified using the universal primers 63V (5′-CAGGCCTAACACATGCAAGTC-3′) and 1387R (5′-GGG-CGG(A/T)GTGTACAAGGC-3′) . Fifty µl PCR amplifications were used containing 200 ng of template DNA, 20 pmol of each primer, 15 µl of 5X GoTaq PCR buffer (Promega, Madison, WI, USA), and 3 U of GoTaq DNA polymerase (Promega). Thermocycling conditions were: 5 min at 94ºC; 30 cycles of 30 s at 94ºC, 30 s at 55ºC, and 90 s at 72ºC; and a final extension step of 7 min at 72ºC. In order to ascertain whether PCR conditions had an effect on 16S-RFLP pattern complexity, we reduced the amount of template DNA to 10 ng as well as the number of amplification cycles (25, 20, and 15 cycles were assayed). Double restriction of the amplified product for RFLP analysis was achieved with enzymes *Afl*III and *Cac*8I (New England Biolabs). Total 16S-SSCP analysis was carried out according to [28], as described above.

To display the similarity between the 16S-SSCP profiles obtained, they were subjected to cluster analysis using the BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). Following conversion, normalization, and background subtraction with mathematical algorithms, levels of similarity between fingerprints were calculated with the Dice coefficient. Cluster analysis was performed using the unweighted pair-group method using average linkages (UPGMA).

Cloning, sequencing and sequence analysis of the 16S rRNA genes. Five strains were selected for 16S rRNA gene sequence analysis: ATCC 27562, C7184, C27, C30 and C34. Nearly full-length 16S rRNA gene sequences were amplified as described above (using 200 ng template DNA and 30 amplification cycles). PCR products were resolved in a 1% TAE agarose gel. Bands were excised and DNA purified using the GeneClean III kit (Qbiogene, Morgan Irvine, CA, USA), cloned into pCRII-Seq vector (Invitrogen) and transformed into *Escherichia coli* TOP10 chemically competent cells (Invitrogen) following manufacturer's instructions. After selection on *E. coli* FastMedia containing ampicillin and X-Gal (InvivoGen, San Diego, CA, USA), 96 clones containing the insert were isolated. Clones were automatically sequenced using an ABI 3730xl sequencer at Lucigen Corp. (Madison, WI, USA).

Sequence quality was enhanced by applying ABI long trace software [http://www.nucleics.com/longtrace-sequencing/] and subsequently automatically assembled using the Batch Sequence Assembly plugin in BioNumerics. Multiple alignment and cluster analysis of sequences was done using the Alignment & Mutation Analysis tool according to the BioNumerics proprietary algorithm. We used the online software Bellerophon [http://foo.maths.uq.edu.au/~huber/bellerophon.pl] to identify putative chimeras in our clones [17]. All putative chimeras were removed from the analysis. Prediction of *V. vulnificus* 16S rRNA secondary structure was completed by free-energy minimization using online software at [http://www.bioinfo.rpi.edu/applications/mfold/old/RNA]. BLASTn searches were performed to identify similar sequences in GenBank with search parameters adjusted for short input sequences. Similarly, the Ribosomal Database Project was used to search for similar intervening sequences.

Results

Analysis of 16S rRNA genes by RFLP and RFLP-SSCP. Thirty-five isolates were ascribed to 16S rRNA genes type A or B according to Nilsson et al. [27]. Out of these, 28 strains were found to be type A, only two type B and five could not be ascribed to either type nor were they type AB (Table 1). Figure 1A shows the typical 16S-RFLP patterns for types A and B and the unusual restriction bands displayed by the untypeable isolates. Strains C1, C27, C28, C30 and C34 presented several additional bands suggesting the presence of more than one sequence in the 16S rRNA gene amplified product. To increase the resolution power of the restriction analysis, 16S *Hae*III-restricted fragments were resolved by SSCP analysis. Partial 16S-SSCP profiles generated an average of nine bands of 16S types A and B while the untypeable strains showed up to 17 different bands by partial 16S-SSCP (Fig. 1B). It needs to be noted that in a SSCP profile each denatured double-stranded fragment is represented in the gel by several bands because as the single-strands migrate through the gel, the same strand of DNA may adopt several conformations that are not necessarily of equal intensity [32].

Figure 2 shows the similarities between the 16S-SSCP profiles. Using a position tolerance of 1% for band class definition in the BioNumerics software, only two of 18 band positions were found not to be polymorphic. At 85% similarity, six distinct 16S-SSCP types were defined (Table 1). We used 85% as cut-off based on previous studies that took into account the intrinsic variability associated to the analysis of these profiles under our laboratory conditions [28]. 16S-SSCP type 1 clustered two of the untypeable strains (C27 and C28) that shared identical profiles and were highly similar to strain C30. Fingerprints of strains C1 and C34 displayed the highest number of bands among all 16S-SSCP patterns and formed a separated 16S-SSCP cluster (16S-SSCP type 2).

Table 1. *Vibrio vulnificus* strains used in the study

Strain	Source	16S-*Hae*III type	16S-SSCP[a]
ATTC[b] 27562	Human wound infection, USA	A	3
C7184	Human blood, USA	B	4
Vv3, Vv4	Oyster, Alabama, USA	A	3
C26, C32, C35, C36, C37, C38, C39, C61, C66	Seawater, Valencia, Spain	A	3
C42, C60	Seawater, Valencia, Spain	A	6
C27, C28, C30	Seawater, Valencia, Spain	NT[c]	1
C1,C34	Seawater, Valencia, Spain	NT	2
C31	Seawater, Valencia, Spain	B	5
C4, C6, C7, C8, C9, C10, C13, C15, C16, C18, C19, C20, C22	*Donax* spp., Valencia, Spain	A	3
CECT[d] 4174	Diseased eel, Japan	A	3

[a]Genotype based on 16S-SSCP profiles.
[b]ATCC, American Type Culture Collection.
[c]NT, non-typeable.
[d]CECT, Spanish Type Culture Collection.

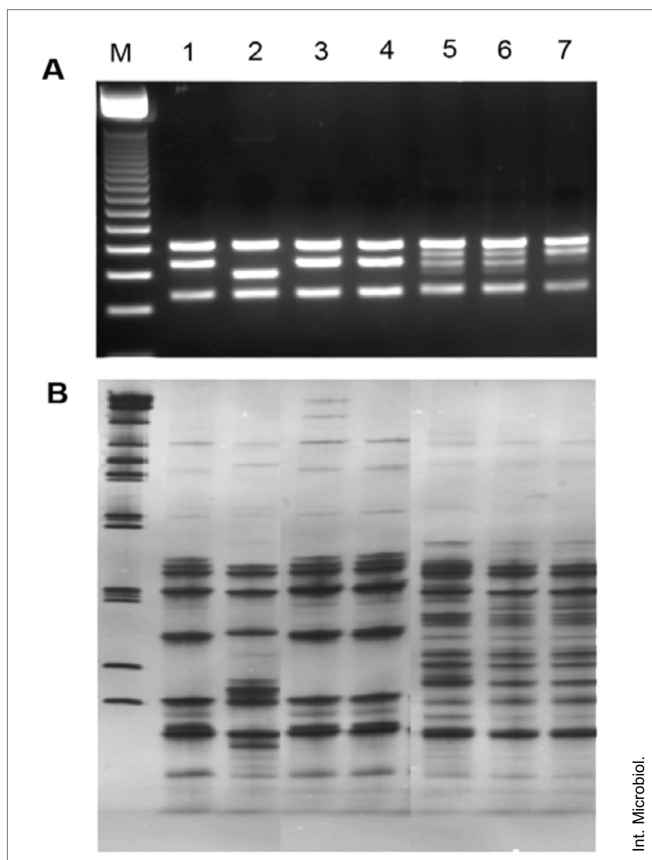

Fig. 1. RFLP analysis of 16S rRNA genes digested with *Hae*III. Panel A shows the fragments resolved through agarose gel. Panel B shows single-strand conformation polymorphism of the same restriction fragments. Lane 1, ATCC 27562 (type A); lane 2, C7184 (type B); lane 3, C4 (type A); lane 4, C26 (type A); lane 5, C27 (non-typeable); lane 6, C28 (non-typeable); lane 7, C34 (non-typeable); M, molecular weight marker.

Twenty-six isolates (all type A) formed a tight cluster at 85% similarity (16S-SSCP type 3). The only two type B isolates shared a similar, but not identical, profile and joined the main cluster at 65% similarity (16S-SSCP types 4 and 5). Interestingly, two Mediterranean Sea isolates ascribed to 16S type A showed the highest dissimilar profiles (16S-SSCP type 6) joining the rest of the groups at 55% similarity. Analogous results were obtained when the complete 16S rRNA genes were amplified and double restricted with *Afl*III/*Cac*8I (data not shown).

To rule out a possible contamination, all strains were subjected to 20 consecutive plate passes, each one starting from a single colony. DNA extraction was performed again after the 20th pass and typing was carried out as above. Identical results, untypeability for 16S A/B but the same complex 16S-SSCP fingerprints, were obtained for strains C1, C27, C28, C30 and C34. In addition, and to confirm that the unexpected high number of bands in strains C1, C27, C28, C30 and C34 were bona fide and not the result of amplification artifacts, PCR conditions were modified using low template DNA and fewer amplification cycles. 16S-SSCP profiles were identical regardless of the initial amount of template or the number of cycles (data not shown). To further analyze what appeared to be new 16S rRNA gene polymorphisms, strains C27, C30 and C34 were selected for cloning and sequencing along with ATCC 27562 and C7184 as reference strains for 16S type A and B, respectively.

Intragenomic heterogeneity revealed by sequencing of the 16S rRNA genes. Nearly complete 16S rRNA gene sequences (>1350 bp) were obtained from 400 clones from selected *V. vulnificus* strains. A total of 91 clones were sequenced from strain ATCC 27562, 90 from C7184, 85 from C27, 58 from C30, and 76 from C34. Contigs were created after automated assembly of trace files in BioNumerics. The number of chimeras present in each cloning event was determined by the Bellerophon software. Only 6% (5 chimeras out of 91 contigs) were found in the type strain ATCC 27562 and no chimeras were detected in C7184. However, 35% of the contigs from strain C27, 37% from C30 and 34% from C34 were identified as chimeras. All putative chimeras were removed from the analysis. The number of haplotypes recovered from each strain was determined after multiple sequence alignment and corresponding cluster analysis. Unique sequences were defined at <99% similarity but only when two or more contigs presented identical variable nucleotides in identical positions were those sequences considered to be individual haplotypes.

A total of 14 unique haplotypes were identified among the 400 clones sequenced and deposited in GenBank (HM996960–HM996973). Sequence differed from five to 35 bp (99.6% to 97.6% sequence similarity, respectively). Polymorphisms were concentrated in the following regions along the 16S rRNA genes: zone 1 (175–220), zone 2 (435–485), and zone 3 (990–1035); numbers referred to *E. coli* numbering system. These regions correspond to helices 10, 18, and 37, respectively. Figure 3 displays all variants found along with the consensus sequence for each zone. Up to seven different sequence types were found in helix 10 ranging from two nucleotides to a 15 bp insertion. Seven different types were found in helix 18 (all of them including compensatory mutations) while 5 types were present in helix 37.

Table 2 summarizes all the sequence types found per strain with the number of clones displaying each combined profile (helix 10: helix 18: helix 37) and their corresponding accession numbers. *Vibrio vulnificus* type strain ATCC 27562

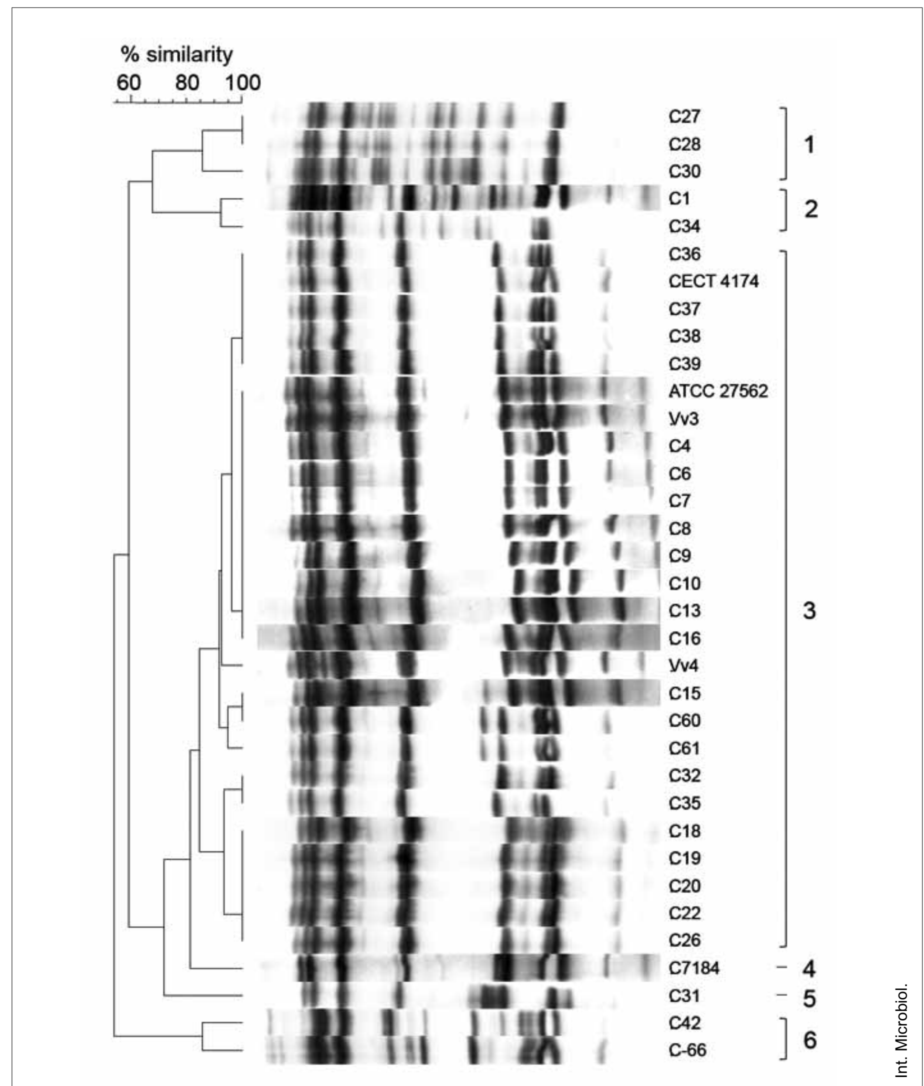

Fig. 2. Dendrogram based on the 16S-SSCP profile analysis. The tree was obtained by UPGMA cluster analysis of the 16S-SSCP profiles of 35 *Vibrio vulnificus* strains. Levels of linkage are expressed as percent of similarity based on the Dice coefficient. Defined 16S-SSCP types are indicated.

showed two 16S rRNA gene types. Profile H10-1: H18-1: H37-1 represented the majority of the clones (88.3%) and corresponded to previously described 16S type A. C7184 only showed one unique 16S rRNA gene sequence that matched 16S type B sequence. In particular, 16S type A and B are defined by a polymorphism present in helix 10. According to our classification system, H10-1 corresponded to 16S type A and H10-2 to type B. As was expected, based on 16S-SSCP analysis, environmental strains C27, C30, and C34 had more distinct 16S rRNA gene sequences than reference strains. The number of unique 16S rRNA gene sequences was 4 for C27, 3 for C30 and 4 for C34. Within each strain there was a predominant haplotype that constituted more than 40% of the total clones. Some helix types such as H10-1, H18-3 and H-37-6 were found in more than one strain while other helix variants seemed to be strain-specific.

Analysis of helix 10 intervening sequence. Three helix 10 variants presented an insertion of 14 bp (H10-4) or 15 bp (H10-5 and H10-6). Types H10-4, H10-5, and H10-6 were represented by 37, 15, and 8 clones, respectively. Insertions in the *rrn* are typically referred to as intervening sequences (IVS). Only environmental strains presented IVS in helix 10. The secondary structure of helix 10 was calculated for all types (Fig. 4). Types H10-1, H10-2, H10-3 and H10-7 conserved the typical secondary structure and the mutations found in those sequences were either compensatory mutations located in the stem without affecting its structure or were located in the loop. Types H10-4, H10-5 and H10-6 contain IVS and present an elongated stem of identical length (seven extra base-pair alignments in the stem). In addition, types H10-5 and H10-6 present an additional base pair in the loop region (five bp instead of the four present in

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Helix 10

                      .180      .190      .200      .210

Consensus    TGATGCCCTNGNNNNTAANNNNTANGGGCCAAAGAGGGGGACCTTCGG
H10-1        ||||AG|---------------|TC||CT|||||||||||||||||||
H10-2        |||||||---------------||C|||||||||||||||||||||||
H10-3        |A|||||---------------||C|||||||||||||||||||||||
H10-4        ||||AG||-C|GTTC|TCGGAA|TG|TCT||||||||||||||G|AA|
H10-5        |||||||||T|TTTA|||TGAAC|G|||||||||||||||||||||||
H10-6        |||||||||T|TTTA|||TGAAC|G|A|||||||||||||||||||||
H10-7        |A|---|||C|----|||--------|AG||||CA|||||||||||||

Helix 18

                  .440      .450      .460      .470      .480

Consensus    TNGTGAGGAAGGTGGTAGTGTTAATAGCACTATCATTTGACGTTAGCNA
H18-1        |C|||||||||||||||||||||||||||||||||||||||||||||G|
H18-2        |C|||||||||||||||||||||||||||||||||C|||||||||||G|
H18-3        |C||||||||||A||GTGT|||||||ACGC||T||||||||||||||G|
H18-4        |T|||||||||||||GTC|||||||||GGC|||||||||||||||||A|
H18-5        |T|||||||||||||GT||G||||||||GC|||||||||||||||||A|
H18-6        |T|||||||||||||GT|||||||||||GC|||||||||||||||||A|
H18-7        |A|G||||||||||||||||||||||||G||||||||||||||||C|T|

Helix 37

                       .1000     .1010     .1020     .1030

Consensus    CCAGAGAANCCAGCGGAGACGCNGGTGTGCCTTCGGGAACTCTGA
H37-1        ||||||||T|T||||||||||T||A||||||||||||||||||||
H37-2        ||||||||T||TA|||||||TG||A|||||||||||||G||||||
H37-3        ||||||||T||TA|||||||TG||A||||||||||||||||||||
H37-4        ||||||||G||||||||||||A|||||||||||||||||||||||
H37-5        ||||||||G||||||||||||A||||||||||||||||G||||||
H37-5        |T|C||||G||||||||||||A|||||||||||||||||||G|A|
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Fig. 3. Alignment of all the different *rrn* alleles found in the study. Vertical lines denote the same base pair; only different nucleotides are shown. Bold nucleotides denote conserved sequences. Compensatory mutations are underlined.

the rest of helix 10 types). Secondary structures of helices with IVS are predicted to be thermodynamically more stable than those without IVS. In this respect, H10-5 showed the lowest free energy. Only one identical sequence to IVS from *V. vulnificus* was found in the databases. Sequence type H10-5 was identical to a 16S rRNA gene sequence from *V. pommerensis* (GenBank accession no. AJ580582). In addition, two highly similar sequences to H10-4 and H10-6 respectively obtained from an uncultured bacterium from parrotfish (*Chlorurus sordidus*) (GenBank accession no. FJ654007) and from a *V. vulnificus* strain recovered from seawater in Indonesia (GenBank accession no. FJ404765) were found.

Discussion

Vibrio is one of several genera in which several *rrn* operons are present, and intragenomic heterogeneity is common [1,6,12,19,26]. In fact, among the *Vibrio* species whose genomes have been sequenced, the instraspecies diversity

Table 2. Summary of the 16S rRNA genes variants based on polymorphisms present in three areas for all strains included in the study

	Helix 10	Helix 18	Helix 37	No. clones (%)	Accession no.
ATCC 27562	H10-1	H18-1	H37-1	76 (88.3)	HM996963
	H10-1	H18-2	H37-1	10 (11.7)	HM996960
Total				86	
C7184	H10-2	H18-4	H37-4	100 (100)	HM996961
Total				91	
C27	H10-3	H18-5	H37-6	9 (16.3)	HM996966
	H10-4	H18-5	H37-6	24 (43.6)	HM996967
	H10-3	H18-3	H37-6	12 (21.8)	HM996968
	H10-5	H18-3	H37-6	10 (18.1)	HM996970
Total				55	
C30	H10-4	H18-5	H37-5	13 (46.4)	HM996969
	H10-6	H18-3	H37-6	8 (28.6)	HM699972
	H10-7	H18-5	H37-5	7 (25.0)	HM996973
Total				39	
C34	H10-1	H18-6	H37-2	22 (44.0)	HM996962
	H10-1	H18-6	H37-3	11 (22.0)	HM996964
	H10-5	H18-7	H37-2	5 (10.0)	HM996965
	H10-1	H18-7	H37-2	12 (24.0)	HM996971
Total				50	

of their 16S rRNA genes is relatively high (0.6 % diversity) when compared to the average diversity found in proteobacteria (0.2%) [29]. In the present study, we identified a higher degree of 16S rRNA gene sequence variability in the species *V. vulnificus* than what had been previously reported. According to the two *V. vulnificus* sequenced genomes (AE016795 and BA000037), intragenomic 16S rRNA gene diversity in the species is only 0.4 %; nevertheless, we found up to 2.4 % sequence diversity within one strain. In fact, the intragenomic heterogeneity found in the *V. vulnificus* analyzed in this study was above the common threshold (1 to 1.3%) used for distinguishing species.

Our data showed that no strain presented more than four different 16S rRNA gene sequences. This species reportedly contains up to nine *rrn* operons (eight in chromosome I and one in chromosome II) [9] thus the number of expected operons within the species might explain the different haplotypes found within a strain. All strains analyzed in this study have been previously typed by ribotyping with profiles showing 9 bands, indicating the expected number of *rrn* operons for this species [3]. Our sequence analysis revealed a higher percentage of chimera formation than expected. Literature reports estimate formation of recombinant molecules around 32% after 30 PCR cycles on 7 distinct haplotypes. Even though there were only three or four haplotypes in the environmental strains we analyzed, up to 37% of the chimeras were identified during our sequence analysis. Frequency of chimera formation in PCR is in function of the length and the sequence similarity of the amplified molecule [36]. In our study, the amplified sequences were highly similar (the least similarity within a strain was 97.6%) and long (ca. 1350 bp), which may explain why our percentage of recombinant molecules was high. Logically, as diversity in the original sample increases so does chimera formation [23]. Hence, the three environmental strains with three or four haplotypes produced a higher percentage of chimera formation than the type strain with only two haplotypes. Studies suggest that chimera formation by PCR can be reduced by decreasing the amount of template and using fewer amplification cycles. We compared several PCR protocols with the one we followed to test whether the number of bands observed by 16S-SSCP was due to chimera formation and therefore could be reduced, but we did not find any difference between profiles obtained by different protocols. We conclude that 16S-SSCP observed profiles showed predominant sequences generated during amplification and did not reflect PCR artifacts that were detected by sequencing.

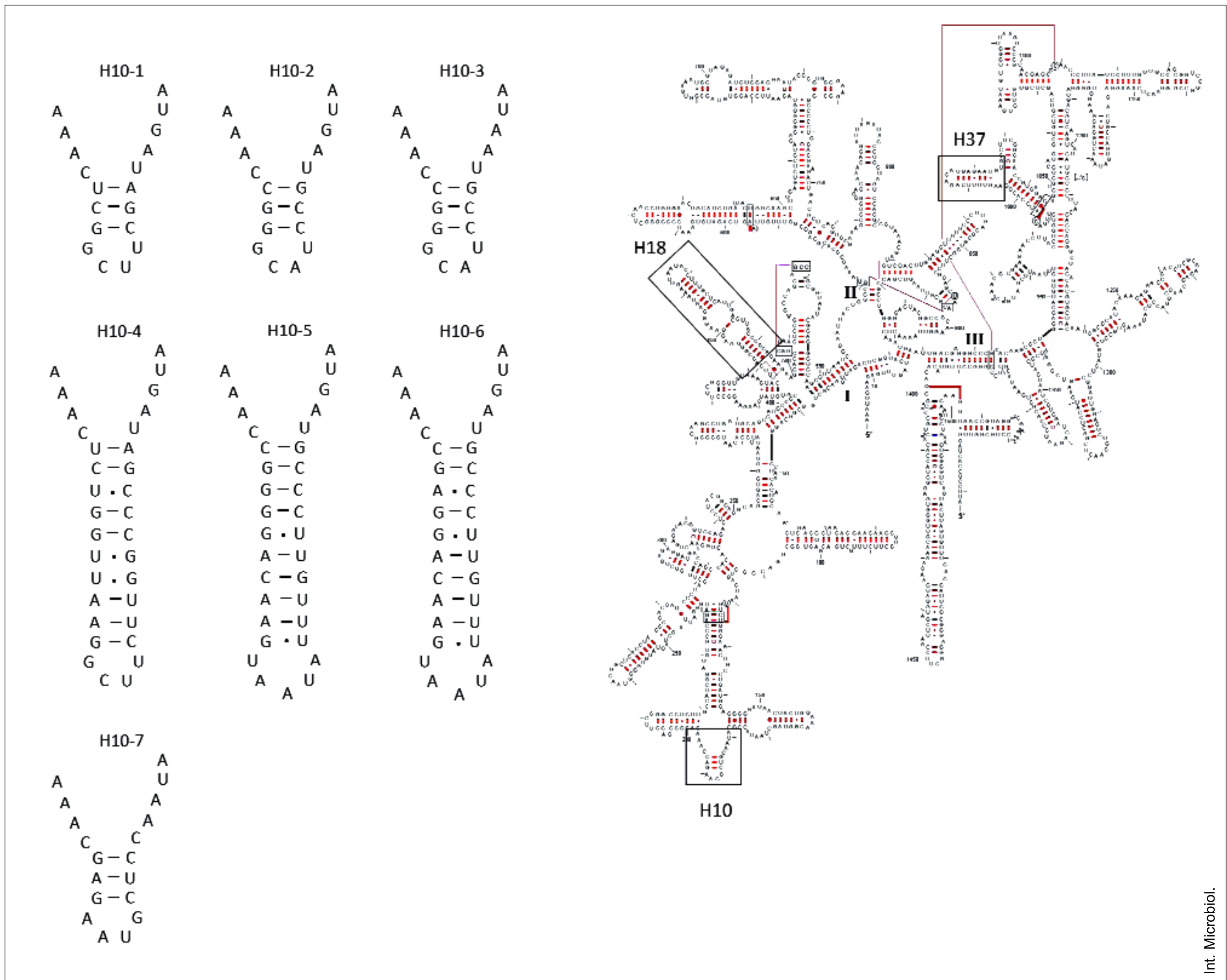

Fig. 4. Variants of helix H10 in 16S rRNA genes from *Vibrio vulnificus*. The helixes were drawn from the secondary structure model of *Escherichia coli* 16S rRNA [13]. Position of variable regions helix H18 and H37 are also indicated. Bars and dots indicate Watson-Crick pairing and non-Watson-Crick pairing, respectively.

Our data show three areas of variability in helix 10, helix 18 and helix 37. Helix 10 and helix 18 have been previously identified as variable regions within the genus *Vibrio* [11]. Note that some *V. vulnificus* 16S rRNA gene variants presented an insertion in helix 10 that extended the stem from three to 10 paired bases. This has been observed in other bacteria including piezophiles, and it has been postulated that longer stems could contribute to 16S secondary structure stabilization under high pressures [24]. The advantage of a longer stem at helix 10 for *V. vulnificus*, if any, is unknown and requires further investigation. Sequence polymorphisms in helix 18 may occur in *V. parahaemolyticus* and *V. splendidus* although at a much lower frequency than in *V. vulnificus.* Only four variants were identified in *V. splendidus*, with up to three present in the same strain [19]. Similar numbers have been reported for *V. parahaemolyticus* [15].

The higher number of different sequences recovered from environmental *V. vulnificus* isolates is in agreement with the idea that different rRNA variants function under different conditions. Bacteria with higher numbers of *rrn* are hypothesized to adapt quicker to new conditions than bacteria harboring fewer *rrn* copies [22]. Recently, it has been shown that different rRNA variants can be differentially expressed in prokaryotes [25]. However, whether *V. vulnificus* strains with high levels of *rrn* heterogeneity can express different 16S rRNA genes under different environmental conditions needs to be evaluated in future studies. Our data showed many more 16S rRNA gene variants in this species than pre-

viously reported and that sequence divergence between 16S rRNA gene variants found in some strains of *V. vulnificus* was larger than that between some *Vibrio* species. This should be taken into account when performing microbial diversity studies with samples in which this bacterium is abundant. The mechanism by which some *V. vulnificus* strains acquired such different 16S rRNA gene variants is unknown; however, the accumulation of numerous substitutions with compensatory mutations in all three helices implies ancient divergence of these *rrn* segments and suggests lateral gene transfer as source of variation [33]. Further studies are needed to evaluate the role these 16S rRNA gene variants play in the biology of *V. vulnificus*.

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