Analysis of 16S rRNA genes in *Vibrio vulnificus*: type A, B, and more

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Introduction

Intragenomic heterogeneity among ribosomal operons in *Vibrio vulnificus*, a human pathogenic bacterium of marine origin, is purported as a probabilistic indicator of strain virulence and classifies *V. vulnificus* strains as 16S rDNA type A and B. The strong correlation between *V. vulnificus* 16S type B and clinical strains made the 16S rDNA a desiderable target for PCR-based detection methods for virulent strains. Interestingly, the quantitative real time PCR developed by Vickery et al. (2007) revealed the presence of both types in a single strain isolated from the northern Gulf of Mexico. This new variant, type AB, was present in up to 22% of the strains including clinical strains. Gordon *et al.* (2008) found a nearly equal proportion of type A/type B among clinical strains from Florida and suggested a geographical variation in the population structure of *V. vulnificus*. Both studies analyzed strains recovered along the same coast. In a previous work by our group, we reported a high intraspecies diversity among *V. vulnificus* strains from seaweater and bivalves sampled off of the eastern Coast of Spain (Arias et al., 1998), although those isolates were not ascribed to specific 16S rDNA types. 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 the Spanish Mediterranean Coast. Our results show an unexpected high degree of intragenomic heterogeneity in the 16S rDNA of the species *V. vulnificus*.

Material and Methods

Thirty-five *V. vulnificus* strains were initially used in this study. Strain nomenclature and origin are detailed in Table 1. DNA was extracted following standard protocols. 16S-RFLP typing was done according to Nilsson et al. (2003). Analysis of the restriction fragments was carried out by single strand conformation polymorphism analysis (SSCP) (Olivares-Fuster et al., 2006). Strains representing 16S types A and B as

well as atypical types were selected for 16S rDNA cloning and sequencing. Evolutionary distances were calculated using maximum parsimony and maximum likelihood. 16S rRNA secondary structure was calculated by free-energy minimization.

Results and Discussion

Out of 35 isolates, 28 strains were found to be type A, only two were type B and five could not be typed (Table 1).

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

Strain	Source	16S-*Hae*III type	16S-SSCP
ATTC 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[a]	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 4174	Diseased eel, Japan	A	3

[a] NT, non-typeable

To further analyze what appeared to be new 16S rDNA 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. Nearly complete 16S rDNA sequences were obtained from 400 clones from selected *V. vulnificus* strains. A total of 91 clones were sequenced from strain ATCC 27 562, 90 from C7184, 85 from C27, 58 from C30 and 76 from C34. A total of 65 unique sequences were identified among the 400 clones sequenced and deposited in GenBank (EF546244-EF546308). Sequence differed from two to 20 bp (99.93% to 97.87% sequence similarity, respectively). Polymorphisms were concentrated in the following areas along the 16S rDNA: zone 1 (175-220), zone 2 (435-485), and zone 3 (990-1035); numbers referred to *E. coli* numbering system. These regions correspond to helixes 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 one single nucleotide to a 15 bp insertion. Only environmental strains presented intervening sequences in helix 10. Eleven different types were found in helix 18 being all of them including compensatory mutations, which suggests ancient sequence divergence generated by lateral gene transfer. Up to 10 types were present in helix 37.

Based on the phylogenetic analysis, two main groups of sequences were observed: one containing all clinical isolates sequences plus a few sequences from environmental isolate C34 and the second grouping the rest of the sequences from Mediterranean Sea isolates. From this point of view, strain C34 would contain 16S rDNA variants typical from clinical as well as from environmental isolates. This instraspecific division based on 16S rDNA sequence could reflect an evolutionary trend driven by environmental factors. The mechanism by which *V. vulnificus* acquired so many 16S rDNA variants is unknown to us and needs further investigation.

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