Non pathogenic *Vibrio* environmental strains as a marine reservoir of virulence, fitness and antibiotic resistance genes and mobile genetic elements

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Introduction

Pathogenic *Vibrio* species are of major concern as they are responsible for infectious diseases in humans. However, other *Vibrio* species found in the aquatic environment and defined as non pathogenic for humans, such as *Vibrio alginolyticus, Vibrio harveyi, Vibrio anguillarum, Vibrio fluvialis* and others are also of interest in that, although rarely, they have been isolated in association with infections in humans. Although it has been largely considered that environmental bacterial isolates lack those virulence genes which usually are found in clinical strains, few recent studies indicate that virulence genes, or their homologous, could also be present in strains from environmental sources and that acquisition of such genes might have place in the aquatic environment. In this study we have screened a collection of marine *Vibrio* strains isolated in the area of the Venetian Lagoon for the presence of virulence and fitness genes usually present in *V. cholerae* and *V. parahaemolyticus* pathogenic strains. The collection has been also screened for the presence of antibiotic (AR) resistance traits. Particular interest has been dedicated to the detection and analysis of mobile genetic elements such as the V. *cholerae* pathogenicity island VPI-2 and the class 1 integron.

Materials and methods

A total of 152 *Vibrio spp* strains were isolated from water, plankton, sediment and fish samples obtained in the area of the Venetian Lagoon and the mouth of the Po river during the period 2006-2009. All the samples obtained from the marine environment were inoculated in a modified alkaline peptone water (m-APW) at pH 8.5 and added with 3.5% NaCl, incubated for 7 hours at 25°C and subsequently plated onto the selective media thiosulphate-citrate-bile salts agar (TCBS) to select green and yellow colonies presumptively identified as non pathogenic *Vibrio* strains.

DNA extracted from *Vibrio* species was subjected to PCR with a Manual MasterTaq Kit (Eppendorf) more adequate to amplify the DNA from environmental samples and using primer pairs designed on the nucleotide sequence of the genes considered in this study and following described. Amplicons were sequenced after DNA purification by BRM Genomics.

Results

A collection of environmental *Vibrio* strains was screened for a battery of genes usually found in *V.cholerae* and *V. parahaemolyticus*. Also the class 1 integron and the genes associated to quinolone resistance, recently isolated in some clinical gram-negative and *Vibrio* strains, were searched. Three of the genes, *yopP*, *tdh* and *trh* are involved in *V. parahaemolyticus* virulence while in *V. cholera* the gene *ctxa* for the choleric toxin and the *nanH* gene, encoding a neuraminidase, were considered. As regards genes involved in the survival and persistence of vibrios in the environment, also called fitness genes, three *V. cholera* genes were chosen: *flrA* is a gene involved in the regulation of *V. cholerae* flagella synthesis and response to environmental changes, *V. cholerae vpsR* is involved in biofilm formation and environmental persistence, while *V. cholerae luxA* gene is involved in bioluminescent expression. Of the total strains examined, 57 isolates (38%) resulted positive to PCR using primers selected on the virulence and fitness genes: 18 strains carried the gene *nanH* and 3 the *yopP* gene while only one strain carried the *trh* gene encoding the TDH-related haemolysin. No strains showing *tdh* or *ctxa* amplicons were isolated. Moreover, 26, 8 and 20 strains presented respectively the expected amplicons for *flrA*, *vpsR* and *luxA*.

As concern AR genes, 13 strains (8%) carried the class 1 integron and 13 strains showed one or more different quinolone resistance genes (studies concerning the presence of quinolone resistance genesin *Vibrios spp* were conducted in collaboration with G. Cornaglia, University of Verona). Interestingly, fifteen of the strains carried contemporary more than one class of virulence, fitness or AR genes and two isolates carried contemporary genes of virulence, AR and fitness. The PCR products obtained after amplification of the selected genes were confirmed as specific amplicons by sequencing. The identification of strains resulting positive to PCR indicated that most of the strains isolated (71%) belonged to the species *V. alginolyticus*.

The strains carrying the *nanH* gene, which is located within the *V. cholerae* pathogenicity island VPI-2 (Figure 1), have been further analyzed from the genetic point of view.

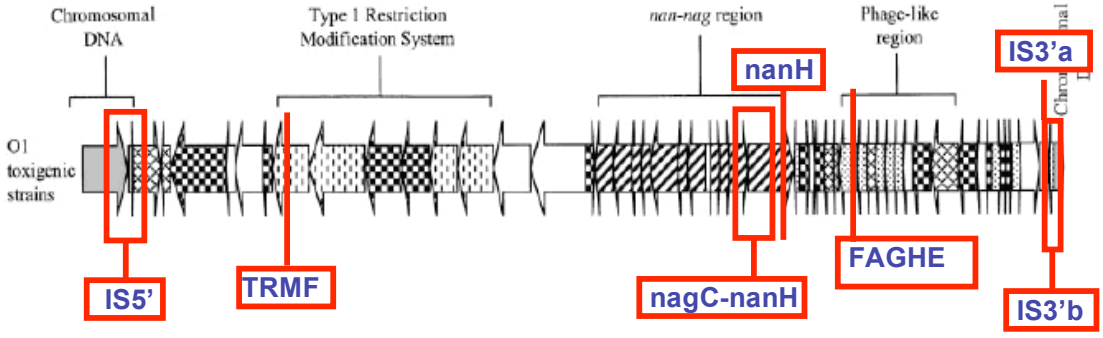

Figure 1.- *V. cholerae* VPI-2 and primers selected to analyze its structure in environmental *Vibrio* strains

To this end, a pair of primers was selected in the region *ir* which corresponds to the insertion site of the pathogenicity island into the chromosome (IS5'). PCR was also applied to search for the 3' insertion site of the *V. cholerae* VPI-2 into the chromosome (IS3'). PCR was also applied to detect the presence of the cluster *nan-nag* that codify for *nagC* and *nanH* genes and is homologous to an equivalent gene cluster in *Haemophilus influenza.* This cluster seems to be involved in the utilization of the sialic acid released by the enzymatic action of neuraminidase. Moreover a pair of primers was selected in the type 1 restriction modification system region (*TRMF*) and another one in the phage-like region *(FAGHE*).
As shown in table 1, of the 18 strains bearing the gene *nanH,* four (NPV3, NPV6, NPV7, NPV18) also contain both the 5' and 3' insertion sites of the pathogenicity island *VPI-2* into the chromosome. The strain NPV18 also carried the *nagC* gene. Strain NPV7 seems to present a modification in the 3' insertion site in that the DNA of this strain is amplified with the IS3'b primers but not with those corresponding to the IS3'a sequence. The TRMF and FAGHE clusters have not been detected in any of the strains. These four strains carried a version of the VPI-2 very similar to that described in *V. mimicus* by Jermyn & Boyd, 2005.
Moreover, we found some strains carrying only partial fragments of the island: two of the strains carried only the 5' insertion site and *nanH* but not the other extremity of the *VPI-2*, while four strains showed, on the contrary, the 3' insertion site plus *nanH* but not the 5' one. The strain NPV32 showed only the whole cluster *nagC-nanH* and lacks both the insertion sites. Finally, 7 *Vibrio* strains showed only the *nanH* amplicon. Ongoing inverse-PCR experiments will be used to investigate how these fragments of the island are inserted in the chromosome of the *Vibrio spp* environmental strains.

Conclusions

Only recently the vision of the marine bacteria belonging to non pathogenic species as innocuous microorganisms has began to change on the basis of few studies indicating that virulence genes, or their homologous, could be present in strains from environmental sources.

VPI-2 sequence detected	Environmental strain

IS5'+*nagC*+*nanH*+ IS3'a+ IS3'b IS5'+*nanH*+ IS3'a+ IS3'b IS5'+*nanH*+ IS3'b	*Vibrio spp* (NPV 18) *Vibrio spp* (NPV3*), V.alginolyticus* (NPV6) *V.alginolyticus* (NPV7)
IS5'+*nanH*	*V.alginolyticus* (NPV5), *V.alginolyticus* (NPV22)
nanH+ IS3'a	*Vibrio spp.* (NPV40), *V.alginolyticus* (33AD), *V.alginolyticus* (40AD), *V.alginolyticus* (48AD)
nagC +nanH	*Vibrio spp* (NPV32)
nanH	*V.alginolyticus* (NPV11), *V.alginolyticus* (NPV25), *V.anguillarum* (30AD), *V.anguillarum* (31AD), *V. parahaemolyticus* (34AD), *V.alginolyticus* (39AD), *V. alginolyticus* (52AD)

Although the incidence, in the isolated non pathogenic vibrios, of the classical virulence genes from human pathogenic *Vibrio, tdh, trh* and *ctxA* has resulted null or very low, the gene *nanH*, considered as a virulence associated gene in *V. cholera,* has been detected in 12% of our environmental strains. Some of these strains have acquired the gene *nanH* included in the pathogenicity island VPI-2 as, in fact, it is located in *V. cholera*. The possibility for a non pathogenic *Vibrio* strain of acquiring an entire or partial pathogenicity island is highly relevant being these island involved in bacterial virulence. VPI-2 is present in pathogenic *V. cholerae* isolates and is absent from nonpathogenic strains again supporting its role in pathogenicity. It is also here proposed a role for fitness genes in the bacteria pathogenicity in that those bacteria capable of persisting longer in the environment would have more chances of encountering an susceptible host and/or exchange genetic elements with the bacterial microflora present in the same ecological niche. To the best of our knowledge, these are the first quinolone resistant vibrios directly isolated from the marine environment: we conclude that the increased use of these antibiotics in fish farms is leading to selection of resistant bacteria which could constitute a concern in public health. We suggest that non pathogenic vibrios might represent a marine reservoir of virulence, fitness and AR genes and of genetic elements of medical interest and for this reason could constitute a risk for human health.

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