

New *Vibrio* spp. associated to the clam culture in Ria de Pontevedra (NW Spain)

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Introduction

It is well known that *Vibrio* species constitute an important percentage of the heterotrophic bacteria associated with marine bivalves, especially oysters, mussels and clams. Several *Vibrio* species can be pathogenic and are associated with disease outbreaks, therefore the accurate identification of these microorganisms is important.

Vibrio splendidus has been found to be a dominant *Vibrio* species in coastal marine sediments, seawater and bivalves in temperate climates (Farto *et al.*, 2003; Le Roux *et al.*, 2006; Sobecky *et al.*, 1998). Several reports have proved that other species are phylogenetically related and they comprise the Splendidus clade (Beaz-Hidalgo *et al.*, 2009). Some of these species have been associated with mortality in a wide range of marine animals, such as mollusks (Garnier *et al.*, 2007; Gómez-León *et al.*, 2005; Lacoste *et al.*, 2001; Nicolas *et al.*, 1996), fish (Jensen *et al.*, 2003), shrimps (Leano *et al.*, 1998) and octopus (Farto *et al.*, 2003).

Currently, twelve species, including *Vibrio lentus*, *V. tasmaniensis*, *V. kanaloae*, *V. pomeroyi*, *V. chagasii*, *V. cyclitrophicus*, *V. gigantis*, *V. crassostreae*, *V. gallaecicus*, *V. celticus*, *V. atlanticus* and *V. artabrorum*. (Beaz-Hidalgo *et al.*, 2009, Beaz-Hidalgo *et al.*, in press).

Here we present some strains isolated from carpet-shell clams (*Ruditapes decussatus*) that may constitute new species within the Splendidus clade.

Materials and methods

During a sampling program performed in the year 2008 a collection of marine bacterial strains were isolated from carpet-shell clams (*R. decussatus*). The samples were obtained from clam cultures in two intertidal beds of the Ria de Pontevedra (north-western coast of Spain). In order to obtain the isolates, the organs of the clams were removed and homogenated in saline solution (1:1 weight/volume), and the dilutions were spread on marine agar (MA) and thiosulfate citrate bilis sucrose media (TCBS). The mayor colony types were isolated as pure cultures on MA after forty eight hours of incubation.

A phenotypic and biochemical characterization of a group of facultative anaerobic bacteria with fermentative metabolism was performed, and the *Vibrio*-like bacteria was selected. These isolates were analysed using the dichotomous keys of Noguerola & Blanch (2008). The 16S rRNA gene of the representative isolates of the groups were sequenced to confirm the results. Multilocus Sequence Analysis (MLSA) is currently under development in order to assign these strains to their corresponding taxa. Preliminary results indicate that new *Vibrio* species will be described.

Results and discussion

The phenotypical characterization allowed the identification of the species *V. anguillarum*, *V. alginolyticus* and *V. parahaemolyticus*. In addition, a high prevalence of the Splendidus clade was shown. However, for isolates within this clade identification by the sequencing of the 16S rRNA gene did not match with that achieved by the biochemical characterization, and the subsequent phylogenetic analysis showed that some of these new isolates formed tight genomic groups different from any currently known *Vibrio* species in the clade (Figure 1A). The eight strains selected showed differential characters with the other species of the clade as well as among them. It's interesting to mention that 5 isolates grow with 0% NaCl, and this fact could be related to the situation of the clam beds at the bottom of the estuary with a high influence of fresh water.

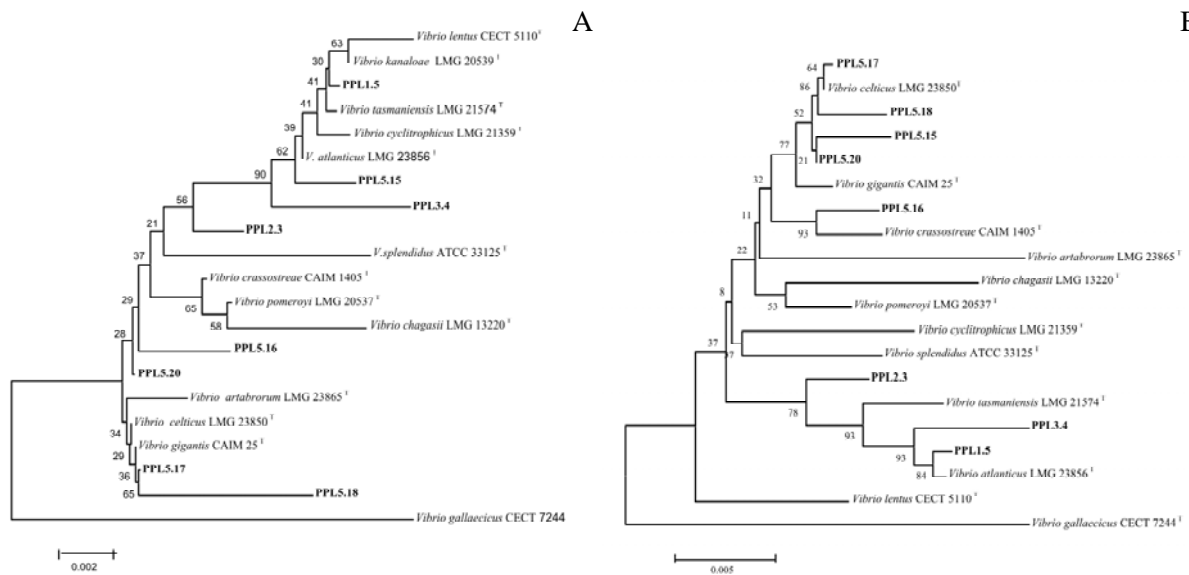


Figure 1: Phylogenetic trees based on the 16S rRNA gene sequence (A) and on concatenated sequences of the housekeeping genes *recA*, *pyrH*, *rpoA* and the 16S rRNA gene (B) obtained by the NJ method. Horizontal branch lengths are proportional to evolutionary divergence. Bootstrap percentages from 1000 replicates appear next to the corresponding branch.

The phylogenetic tree based on concatenated sequences of the 16S rRNA gene and three housekeeping genes (*recA*, *pyrH*, *rpoA*) clarify the position of the strains. (Figure 1B). Some strains like PPL5.17 or PPL 1.5 can be assigned to a previously described species (*Vibrio celticus* and *V. atlanticus*, respectively), while others cannot be assigned to any known species. This results were expected at some extent due to the known extreme conservation of the 16s rRNA gene among species belonging to this group and, therefore its low discriminatory value in order to define species. Again, our results reinforce the idea of the complexity of the Splendidus clade.

Conclusions

Although the *Vibrio* populations vary with the clam culture bed location, in both cases representatives of the Splendidus clade were the most predominant vibrios. This group of vibrios has been associated with mortalities in adult bivalve mollusks (Gay *et al.*, 2004). The classical tests in tubes and plates does not seem to match up with the sequencing of the 16S rRNA gene, and both methods seem to be unsuitable for the identification of species from the *Vibrionaceae* Family. Further studies including more housekeeping genes (currently in development) in MLSA, DNA-DNA hybridization and G+C content, are necessary to clarify the taxonomic position of the new isolates.

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