New Vibrio species associated to molluscan microbiota: a review

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The genus Vibrio consists of more than 100 species grouped in 14 clades that are widely distributed in aquatic environments such as estuarine, coastal waters, and sediments. A large number of species of this genus are associated with marine organisms like fish, molluscs and crustaceans, in commensal or pathogenic relations. In the last decade, more than 50 new species have been described in the genus Vibrio, due to the introduction of new molecular techniques in bacterial taxonomy, such as multilocus sequence analysis or fluorescent amplified fragment length polymorphism. On the other hand, the increasing number of environmental studies has contributed to improve the knowledge about the importance of vibrios as etiological agents of diseases is likely to increase over the coming years due to ocean warming.

INTRODUCTION
Coastal and estuarine environments are growing areas of bivalve molluscs, since they reduce the production and cause high economical losses. Some members of the genus Vibrio have been described as forming part of the molluscan microbiota. Some of them have been associated with mortalities of different molluscan species, seriously affecting their culture and causing high losses in hatcheries as well as in natural beds. For other species, ecological importance has been demonstrated being highly abundant in different marine habitats and geographical regions. The present work provides an updated overview of the recently characterized Vibrio species isolated from molluscs. In addition, their pathogenic potential and/or environmental importance is discussed.

Keywords: Vibriaceae, genus Vibrio, molluscan microbiota, new species, pathogenicity, ecology

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Vibrios spp. in cultured Manila clams (the subject of many studies (Morris, 2003; Su and Liu, 2007) and contaminated shellfish (mainly oysters), and therefore have been producing important outbreaks after the consumption of contaminated molluscs, and beside the well known species V. cholerae, V. parahaemolyticus, and V. nutrigenes. They recognized the genes mcrR (cholera toxin transcriptional activator) and rpoA (Sigma factor 070) as the most reliable for species identification, and proposed a scheme for species definition on the basis of the similarities of the concatenated sequences of the most resolving genes. In the last decade, more than 30 new species have been described in the genus Vibrio, many of them associated to marine environments and aquatic eukaryotic organisms. To mention some examples, among the new species described as free-living seawater bacteria are V. agarivorans (Macian et al., 2002b), V. ruber (Stich et al., 2003), V. aoteirua and V. quintilis (Lucena et al., 2012), V. azoreanus (Yoshitawa et al., 2009), or V. agamonensis (Yoshitawa et al., 2010). Associated to different marine organisms have been described, among others, V. caribbeanicus from sponges (Hoffmann et al., 2012), V. hemicentroti from sea urchin (Kim et al., 2013), V. corallifaciens, V. mariensis, V. shulin, V. stylophorae, and V. variabilis from corals (Kushmaro et al., 2001; Ben-Haim et al., 2005; Chimenti et al., 2011; Sheu et al., 2011), V. retiferiana from rotifers (Gomez-Gil et al., 2003a), V. comitans, V. gallus, V. insitu, V. neomae, V. parahaemolyticas, and V. venter from abalones (Hayashi et al., 2003; Sawabe et al., 2004a,b, 2007a), V. atypicus, V. hispanicus, V. owenni, V. pachini, V. asialimensis, and V. zhuhaiensis from crustaceans (Gomez-Gil et al., 2003b, 2004b; Cano-Gomez et al., 2010; Wang et al., 2010; Jin et al., 2012, 2013; Yoshitawa et al., 2012), V. hypoochampi from sea boxes (Bakalzer et al., 2010), and V. allicensius, V. simulans, and V. tanomansis from fish (Thompson et al., 2008d; Gomez-Gil et al., 2008, 2012).

Regarding the vibrios described as associated with bivalve molluscs, and beside the well known species V. alginolyticus, V. harveyi, V. mytali, V. parahaemolyticus, V. pectenicida, or V. vulniﬁcarus (Pujalte et al., 1993; Lambert et al., 1998; Arias et al., 1999; Pujalte et al., 1999), another example, in shellfish-growing areas of the US Northern Gulf Coast, the densities of V. vulniﬁcarus were high and almost constant at temperatures above 26°C and/or at salinity below 25 ppt, but decreased drastically below this temperature and/or above this salinity (Motes et al., 1998). The latter species together with Vibrio parahaemolyticus and V. cholerae are considered important human pathogens, producing important outbreaks after the consumption of contaminated shellfish (mainly oysters), and therefore have been the subject of many studies (Morris, 2003; Su and Liu, 2007; Jones and Oliver, 2009).

### NEW Vibrio SPECIES ASSOCIATED TO MOLLUSCS

The introduction of molecular techniques such as the fluorcent amplified fragment length polymorphism (FAFLP) and multilocus sequence analysis (MLSA) has allowed a more precise identiﬁcation of Vibrio species which were previously masked under other taxa (Thompson et al., 2001, 2005; Beaz-Hidalgo et al., 2008, 2010a; Pascual et al., 2010). In this sense, molecular studies have demonstrated the genetic diversity and the polyphyletic nature of V. splendidus (Thompson et al., 2001, 2005; Le Roux et al., 2002) and have enabled many new species to be described, such as Vibrio kanalai, V. pomeroyi, V. chaganti, or V. galliaecea (Thompson et al., 2003c; Beaz-Hidalgo et al., 2009b). Furthermore, polyphyletically identiﬁed V. harveyi strains were re-classiﬁed as V. campbellii by FAFLP, DNA–DNA hybridization (DDH), and MLSA (Gomez-Gil et al., 2004a; Thompson et al., 2007). Pascual et al. (2010) investigated the usefulness of an MLSA approach with six housekeeping genes to discriminate six tightly related species with DDH values close to 70%, namely V. harveyi, V. campbellii, V. retiferiana, V. parahaemolyticus, V. alginolyticus, and V. nutrigenes. They recognized the genes mcrR (cholera toxin transcriptional activator) and rpoA (Sigma factor 070) as the most reliable for species identiﬁcation, and proposed a scheme for species deﬁnition on the basis of the similarities of the concatenated sequences of the most resolving genes.

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French coasts (Gay et al., 2004b; Garnier et al., 2007). The syndrome is thought to be multifactorial involving physiological and environmental factors as well as pathogens. Labrenche et al. (2006a,b) demonstrated the pathogenic potential of *V. aestuari-
anus* in experimental oyster challenges. The characterization of a group of isolates obtained from diseased oysters in France led to the description of a new subspecies, named *V. aestuarianus* subsp. *francensis*, on the basis of DDH values close to the boundary limit for species definition (70%) and several phenotypic differences with the American isolates.

**Vibrio artabrensis** (Borges et al., 2011)
In a previous study (Beaz-Hidalgo et al., 2008), a collection of isolates obtained from Manila and carpet-shell clams and tentatively allocated to the genus *Vibrio* based on their phenotypic features were analyzed by FAFLP. One of the groups established, cluster 70, consisted of 8 isolates that could not be assigned to any of the known species of the genus *Vibrio*. Analysis of the 16S rRNA gene sequences allocated the isolates within the Splen-
didus clade forming a tight group. MLSA of five housekeeping genes, *atpA* (α-subunit of bacterial ATP synthase), *pyrH* [uridine monophosphate (UMP) kinase], *recA* (RecA protein), *rpoA* (RNA polymerase α-chain), and *rpoD*, supported their inclusion in that clade forming a well differentiated group with respect to the rest of species, being its closest neighbors *V. pomeroyi* and *V. kanaloae*. DNA–DNA reassociation values confirmed its sta-
tus of new species within the genus *Vibrio*. It is considered as an environmental species without pathogenic potential for clams.
**Vibrio atlanticus** (Díezquez et al., 2011)

From the same study of Beaz-Hidalgo et al. (2008) another group of five strains, designated as cluster 5, was likely to be also a new *Vibrio* species, being further characterized using the same approach employed for the description of *V. arthurbonum*. The phenotypic characterization, chemotaxonomy, MLSA, and DDH techniques confirmed the hypothesis that the clams isolates constituted a new species, related with *V. tasmanienis*, *V. kanalae*, and *V. cyclotrophicus* within the Splendidus clade. As *V. arthurbonum*, and since until now no pathogenic activity can be proved for *V. atlanticus*, it seems to be part of the normal environmental and clam microbiota.

**Vibrio brasilienis** (Thompson et al., 2003a)

Six isolates obtained from lion’s paw scallop (*Nodipecten nodosus*) larvae were identified as a tight group during a wide study on vibrios by FAFLP (Thompson et al., 2001). Further characterization of those isolates on the basis of phenotypic features, 16S rRNA gene sequencing, G + C content and DDH, allowed the description of the new species *V. brasilienis* within the Orientalis clade. Its pathogenic potential was demonstrated in experimental challenges using rainbow trout (*Oncorhynchus mykiss*) and Artemia *nauplii* as animal models (Austin et al., 2003). The extracellular products (ECP) of the strain tested were also harmful to the animals.

**Vibrio breoganii** (Beaz-Hidalgo et al., 2009a)

A group of seven non-motile, facultative anaerobic algolysic marine bacteria isolated from cultured Manila and carpet-shell clams in Galicia (NW Spain) were characterized employing a polyphasic approach, including the sequence analysis of the 16S rRNA gene and three housekeeping (atpA, recA, and rpoA) genes, FAFLP fingerprinting, G + C content, DDH, and phenotypic features. Phylogenetic analysis based on 16S rRNA gene sequences showed that the isolates were closely related to the species *V. omintum*, *V. raras*, and *V. inusitatus*, with sequence similarities of approximately 99%. However, phylogenies based on the sequences of the housekeeping genes grouped the isolates together and allocated them within the Halolcoli clade, although they can be differentiated from the other species in the clade by their FAFLP profiles. DDH experiments confirmed that they represented a new *Vibrio* species, that was named *V. breoganii*.

Some years later, this species was consistently detected in a meta-analysis of three separated studies aimed to identify the ecological population structure of *Vibrio* in the Plum Island Sound Estuary (Ipswich, MA, USA), mainly associated to large zooplankton and zooplankton. Interestingly, population of *V. breoganii* showed essentially identical results with respect to genetic breadth in all studies, regardless the season or the sampling method. This species constitutes a good example of how genotypic clusters established by MLSA can serve as a reasonable initial definition of cohesive unit from an ecological point of view, as well as of the ubiquity of *Vibrio* species in the marine environment.

In 2012, a strain of this species was included in one of the first studies examining the complete methylation pattern of a bacterial genome (Murray et al., 2012). The knowledge of the methylome could be of great interest due to the recognized importance of methylation for understanding fundamental microbiological processes, microbe adaptability, and disease pathogenicity.

**Vibrio celticus** (Beaz-Hidalgo et al., 2010b)

A group of four motile facultative anaerobic marine isolates obtained from cultured pullet carpet-shell (*Venerupis pullastra*) and Manila clams during 2004 and 2005 in Galicia (NW Spain) were studied using a polyphasic approach. It was found that they formed a tight phylogenetic group based on sequences of the 16S rRNA gene and four housekeeping (atpA, recA, rpoA, and rpoD) genes, indicating that the four isolates represented a novel species in the Splendidus clade of the genus *Vibrio*, for which the name *V. celticus* was proposed. In addition, the strains showed potential pathogenic activity for adult clams in virulence assays.

Recently, a study on the diversity of *Vibrio* spp. in the Eastern English Channel by means of sequencing of the housekeeping gene *pyrH* (Tall et al., 2013), revealed that *V. celticus* was the predominant species among other 20 *Vibrio* species isolated at ambient environmental temperature.

**Vibrio cartagadensis** (Lasa et al., 2013b)

It was described as a results of the polyphasic characterization of a group of four marine strains isolated from carpet-shell and Manila clams in Galicia (NW Spain). The study of the phenotypic characteristics, the analysis of chemotaxonomic features, the sequencing of the 16S rRNA and five housekeeping (atpA, pyrH, recA, rpoA, and rpoD) genes, as well as DDH, allowed the identification of the isolates within the genus *Vibrio*, being their closest neighbors *V. tapetis*, *V. pomeroyi*, and *V. crassostreae* (97.9%). The phylogenetic analysis of the five concatenated genes indicated the allocation of these strains in between the Splendidus and Anguillarum clades.

**Vibrio crassostreae** (Faury et al., 2004)

Described in 2004 on the basis of five strains obtained from oyster haemolymph, and originally identified as *V. splendidus*-like isolates. The authors employed a polyphasic approach including besides biochemical tests, fatty-acid methyl ester (FAME) analysis, 16S rRNA, and gyrB (DNA gyrase subunit B) genes sequencing, FAFLP fingerprinting, and DDH. Although all the genetic studies supported that the five strains constituted a novel *Vibrio* species within the Splendidus clade, their differentiation of the closest relatives was not possible on the basis of 17 phenotypic characters. However, the presence of fatty acids 16:0 iso and 14:0 iso allowed the differentiation of the new species from other *V. splendidus*-like species. It was described as a species with pathogenic potential for the oyster *C. gigas* (Gay et al., 2004a).

**Vibrio cortegadensis** (González-Castillo et al., 2014)

The description of this new *Vibrio* species was based on the study and characterization of one isolate obtained from cultured oyster (*C. gigas*) in Sonora (Mexico). The phenotypic characteristics and the 16S rRNA gene sequence of the isolate clearly placed it within...
the genus *Vibrio*, with *V. orientalis* and *V. rotiferans* as closest relatives. Curiously, these both species belong to different clades, as proposed by Sawabe et al. (2007b); the Orientalis clade and the Harveyi clade, respectively. MLSA technique clarified the definitive allocation of the isolate within the Orientalis clade, and the DNA relatedness measures by DDH experiments confirmed that it constituted a new *Vibrio* species. The proposed name, *Vibrio crossari*, was chosen to honor Prof. Dr. Jorge Crossa, microbiologist and specialist in vibrios.

**Vibrio fortiis** (Thompson et al., 2003b)
The species was defined on the basis of 10 isolates obtained between 1994 and 1999 from different hosts, including healthy and diseased lion’s paw scallop larvae, diseased *C. gigas* larvae, shrimp (*Litopenaeus vannamei*), Atlantic salmon (*Salmo salar*), as well as sea water. The geographical origins included different North and South American Countries, Tasmania, and UK. Genotypic analysis such as 16S rRNA gene sequencing and DDH confirmed the deliniation of this new species, differentiating it from the close species *V. splendidus* and *V. mytili*. Austin et al. (2005) confirmed the pathogenic potential of the species using rainbow trout and *A. nauplii* as animal models.

*Vibrio fortiis* was further isolated from spotted nose snapper (*Lutjanus guttatus*) in Mexico (Gomez-Gil et al., 2007) and from crown-of-thorns starfish (*Acanthaster planci*) in Australia and Guam (Rivera-Posada et al., 2011). It was also identified as one of the predominant *Vibrio* species in the Cariaco Basin, Venezuela (Garcia-Amado et al., 2011).

The extracellular polymeric substances from this biofilm forming *Vibrio* species were characterized (Kavita et al., 2013), showing potential for industrial applications.

**Vibrio gallesiacae** (Baca-Hidalgo et al., 2005b)
This species within the Splendidus clade was described on the basis of the characterization of three isolates obtained from Manila clams in Galicia (NW Spain). Phylogenetic analysis of the 16S rRNA gene and four housekeeping (*atpA, pyrH, rpoA*, and *rpoD*) genes, indicated that these strains were closely related to the Splendidus clade, being its closest relatives *V. splendidus*, *V. gigantis*, and *V. pomeroyi*. The FAFLP fingerprints and DDH values supported the MLSA results. It is considered as an environmental species without proved pathogenic potential.

**Vibrio gigantis** (Le Roux et al., 2005)
The polyphasic characterization of four isolates obtained from *C. gigas* haemolymph allowed the description of this new *Vibrio* species within the Splendidus clade. Although 16S rRNA gene sequence analysis did not permit a clear differentiation of *V. gigantis* from other phenotypically related species, other techniques including FAFLP, DDH, and sequencing of four housekeeping (*gyeB, rcrB* replication origin-binding protein), *rpoD*, and *rpoH* genes demonstrated that the isolates formed a tight genomic group, clearly differentiated from the neighboring species. The authors suggested, that as other *Vibrio* species present in the shellfish haemolymph, *V. gigantis* may play a role in the health of the host.

**Vibrio kanaloue** (Thompson et al., 2003a)
It was described on the basis of five isolates with different origins, including diseased oyster (*Ostrea edulis*) larvae from France, shrimp (*Penaeus chinensis*) from China and sea water from Hawaii (USA). Therefore, it has been described as an ubiquitous species in the aquatic environment. The five strains were originally detected in a wide FAFLP study (Thompson et al., 2001) as a separate cluster, showing a pattern clearly different from other *Vibrio* species, with which share the main phenotypic traits of the genus. Further DDH experiments confirmed that they were in fact a new species within the Splendidus clade.

Later studies on the virulence of other related strains were performed on the basis of experimental infections of *C. gigas*. After injection of strains, bacteria were localized at the periphery of the muscle and induced extensive lesions of the translucent part of the adductor muscle. Unfortunately, although using a polyphasic approach these strains were confirmed to be *V. splendidus*-related, no clear discrimination between *V. kanaloue* and *V. pomeroyi* was possible with the techniques employed. (Gay et al., 2004b).

**Vibrio lentus** (Macian et al., 2001a)
The study of 12 marine bacteria by means of cultural and physiological characterization, ribotyping, G+C content, DDH, and phylogenetic analysis on the 16S and 23S rRNA genes allowed the description of this new *Vibrio* species in 2001. All the strains had been isolated from Mediterranean oysters in Spain, and were phenotypically similar to *V. splendidus*. The name *V. lentus* was proposed since the strains showed a slow growth on Marine Agar. Thus, colonies of some of the isolates were not larger than 0.2 mm diameter after 3 days of incubation. Some years later *V. lentus* was isolated from diseased wild octopus (*Octopus vulgaris*) and from turbot (*Scophthalmus maximus*) also in Spain (Farto et al., 2005; Montes et al., 2005, 2006). In the case of octopus, experimental infections by bath challenge demonstrated that *V. lentus* was able to reproduce the skin lesions, colonize the internal organs, and induce mortality in healthy octopuses (Farto et al., 2003).

The presence of a lethal extracellular 39-kDa protease, similar to that of *Vibrio pelagius*, was detected in 15% of the ECP assayed belonging to strains of the *V. splendidus*–*V. lentus* related group by Farto et al. (2006), which suggested their potential risk for the health of reared aquatic organisms.

**Vibrio neptunius** (Thompson et al., 2003b)
Described during a polyphasic study of 21 isolates with diverse origins, like healthy and diseased lion’s paw scallop larvae, rotifers, and turbot larvae. The results clearly indicated that this group of strains constituted a new species within the Coralliibutyicus clade of the genus *Vibrio neptunius* was further identified as a zoological agent of a mortality episode of oyster (*O. edulis*) larvae occurred in a Galician hatchery (Prado et al., 2005).

Pathogenicity was confirmed in experimental tests where it shown to cause high mortalities (ranging from 98.5 to 100%) in 72 to 96 h after inoculation of larval cultures. The work of Prado and co-workers constituted the first description of *V. neptunius* as a molluscan pathogen. Later
studies with New Zealand Greenshell mussel (Perna canaliculus) larvae confirmed the pathogenetic potential for other molluscan species (Kesarcodi-Watson et al., 2009a,b).

Vibrio tapetis was also found in environmental studies as a predominant bacteria in the anoxic zone (García-Amado et al., 2011). In addition, during a study searching for novel antimicrobials in marine Vibrionaceae (Wetz et al., 2010), V. neptunius has been identified as a potential resource of antibacterial compounds with future applicability.

Vibrio astrovibrioides (Prado et al., 2014)
The species description relies on three strains isolated from a flat oyster (O. edulis) hatchery in Spain after episodes of high mortality (Prado et al., 2005). Pathogenicity was confirmed in experimental tests where the strains were able to cause high larval mortalities. The results of the phenotypic and genotypic analysis revealed that this group of strains constituted a new Vibrio species, closely related to V. pectenicida.

Vibrio pomeroyi (Thompson et al., 2003a)
As in the case of V. kanalase, V. pomeroyi was originally detected as a group of four isolates showing a characteristic FFAFLP pattern during a study on the genomic diversity amongst Vibrio isolates from different sources (Thompson et al., 2001). Two strains had been isolated from healthy bivalve larvae (N. nodosa) in Brazil and two from turbot in Spain. They were confirmed as a new Vibrio species within the Splendidus clade by means of DDH, phenotypic characterization, and FAME analysis. The studies of Gay et al. (2004a) and Austin et al. (2005) mentioned before demonstrated either non- or low virulence of V. pomeroyi in animal models.

Vibrio penticostis (Masian et al., 2004)
It has been described to accommodate four marine bacteria isolated from mussels, fish, and seawater at the Mediterranean coast of Spain. Phylogenetic analysis locate these strains in the vicinity of the Fluvialis–Furnissii clade, sharing with these species similarities slightly higher than 97% in their 16S rRNA gene sequences. Since one of the isolates were isolated after direct plating of a kidney sample from a diseased gilthead seabream (Sparus aurata), the pathogenic potential of the species cannot be discarded.

Vibrio tapetis subsp. britannicus (Balboa and Romalde, 2013)
Vibrio tapetis, described by Borrego et al. (1996), is the causative agent of an epizootic infection described in adult clams called Brown Ring Disease (BRD) constituting a major limiting factor for the culture of Manila clams in the Northwest of Spain. The techniques utilized included phylogenetic analysis based on sequences of 16S rRNA and MLSA of five housekeeping genes (atpA, recA, pyrH, rpoB, and rpoD), DDH, FAME analysis and more than 100 phenotypic traits. All the closest relatives were Vibrio species included in the Splendidus clade, such as V. kanalase, V. arctica, V. gigantis, or V. ceticus, from which it can be easily differentiated by several phenotypic characteristics. Current studies of some Chilean Vibrio strains isolated from fish seem to indicate that the geographical and host distribution of this species could be wider than expected.

Vibrio naff (Thompson et al., 2003a)
Three isolates obtained from bivalve and shrimp systems were identified as a tight group during a wide study on vibrios by FAFELP (Thompson et al., 2001). Further characterization of those isolates on the basis of phenotypic features, 16S RNA gene sequencing, G + C content, and DDH, allowed the description of the new species V. naffi within the Nereis clade. Considered as an environmental species, V. naffi demonstrated either non- or low virulence in the animal models (Austin et al., 2005).

MOLLUSC AND OTHER VIBRIONACEAE

Other Vibrionaceae described in the last years also associated to molluscan shellfish are representatives of the genera Alivibrio and Photobacterium.

The genus Alivibrio was established by Urbaneczyk et al. (2007) to accommodate the species V. fischeri, V. lages, V. salmonicida, and V. wodanis, on the basis of a study based on 16S rRNA gene sequencing and MLSA which results indicated that the four species represented a lineage within the Vibrionaceae distinct from other genera. Therefore, the authors proposed the reclassification of the species as Alivibrio fischeri (the type species), Lages, A. salmonicida and A. wodanis, respectively. The genus includes symbiotic (A. fischeri) and pathogenic (A. salmonicida) species for marine organisms (Urbaneczyk et al., 2007).

Photobacterium was one of the oldest described genus in the family Vibrionaceae. The type species is Photobacterium phosphoreum, which had been described by Cohn in 1878 as “Microacous phosphorum” (Gomez-Gil et al., 2011). At the time of writing, the genus Photobacterium contained 23 species (http://www.vibriobiology.net). Although most species have no described pathogenetic activity and are common inhabitants of marine environment, some species, i.e., both subspecies of Photobacterium damselae, are pathogenic for aquatic animals, mainly for fish.
Although classification of bacteria into a natural system has been
hindered by the lack of a generally applicable species concept, the
introduction of MLSA has provided much higher resolution
for microbial identification and taxonomy (Gevers et al., 2005).
The groups or species defined by means of MLSA are of partic-
lar interest for microbial ecology, since some theories predict
that they correspond to ecologically cohesive populations (Fraser
et al., 2009; Preheim et al., 2011). Some examples have been men-
tioned in this review, such as
V. breoganii
et al., 2009; Preheim et al., 2011). Some examples have been men-
tioned that these bacteria were closely related to
A. salmonicida

C. gigas
(Preheim et al., 2009). As indicated by Preheim et al.
(2009), future information on completed genomes, metagenomics, and meta-
transcriptomics will increase the understanding on the biology and
ecology of vibrios, providing new insights and solutions to prob-
lems with disease, nutrient cycling in the ocean, and opportunities
in marine biotechnology.

ACKNOWLEDGMENTS
The studies of the University of Santiago reviewed here were
supported in part by grants AGL2003-09307-C02-01, AGL2006-
13208-C02-01, and AGL2010-18438 from the Ministerio de
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 08 October 2013, paper pending published: 29 October 2013, accepted: 16 December 2013, published online: 02 January 2014.

Citation: Romalde JL, Diéguez AL, Lasa A and Balboa S (2014) New Vibrio species associated to molluscan microbiota: a review. Front. Microbiol. 4:413. doi: 10.3389/fmicb.2013.00413

This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology.

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