Identification and characterization of bacteria with antibacterial activities isolated from seahorses (*Hippocampus guttulatus*)

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Abstract

The aim of this study was to isolate bacteria from the intestinal content and cutaneous mucus of seahorses (*Hippocampus guttulatus*) that might produce antibacterial compounds against pathogenic *Vibrio* species. Comparative 16S rRNA gene sequence analysis indicated that antagonistic isolates were affiliated to the genera *Aquimarina*, *Aliivibrio*, *Brachybacterium*, *Jannaschia*, *Neptunomonas*, *Pseudoalteromonas*, *Pseudomonas*, *Ruegeria*, *Shewanella*, and *Vibrio*. The antibacterial activity of most of the isolates could be attributed to the production of organic acids or pH-dependent compounds. The only exceptions were *Aliivibrio fischeri* HG-12F, *Vibrio* sp. HG-3F, and *Vibrio* sp. HG-14F, which produced proteinaceous antibacterial compounds as demonstrated by the sensitivity to proteolytic enzymes. On the basis of these results, the potential as biological control agents of the isolates exhibiting inhibitory activities could be further studied in challenge experiments in fish.

Key words:

Seahorses, marine bacteria, *Vibrio* species, antagonism

Seahorse populations have declined in the last years, largely due to overfishing and habitat destruction.[1] Recent research efforts have, therefore, been focused to provide a better biological knowledge of these species. We have recently studied, as part of the *Hippocampus* project, wild populations of seahorse (*Hippocampus guttulatus*) in some areas of the Spanish coast and established breeding programs in captivity.[2]

With the development of intensive production methods, it has become apparent that diseases can be a significant limiting factor. *Vibrio* species are among the most important bacterial pathogens of marine fish. They are responsible for several diseases, and high mortalities due to vibriosis have been reported.[3,4] Thus, we have screened marine bacteria isolated from the intestinal content and cutaneous mucus of seahorses (*Hippocampus guttulatus*) for production of antibacterial compounds against pathogenic *Vibrio* species. This mechanism of competition offers the possibility of using these antagonistic microorganisms as biological control agents.[5-7]

Adult seahorses (n = 8) were collected from the coast of Galicia (NW Spain). The culturable microbiota was isolated from the intestinal content and cutaneous mucus as follows. Intestinal content from each seahorse was collected, weighed, homogenized using tissue grinders, and vortexed vigorously in sterile saline solution (8.5 g/l NaCl), while the cutaneous mucus was collected from the dorsal surface with a sterile cotton swab into a small amount of sterile saline solution. Ten-fold serial dilutions of samples were prepared and plated on marine agar (Difco, Detroit, MI), tryptic soy agar supplemented with 15 g/l NaCl (Cultimed), and *Cytophaga* agar prepared with 50% seawater [0.5 g/l tryptone, 0.5 g/l yeast extract, 0.2 g/l sodium acetate, 15 g/l agar, and adjusted to pH 7.2]. All plates were incubated for 3–7 days at 20ºC. Colonies with different morphological characteristics from each sample were selected, subcultured in suitable media and stored in sterile glycerol (15% v/v) at –80ºC.

In order to test the ability of the isolates to inhibit growth of pathogenic *Vibrio* strains (Table 1), all isolates were grown on suitable agar media at 20ºC for 2–3 days. After incubation, a loop of each isolate was spotted onto the surface of marine agar previously inoculated with overnight cultures of the indicator strain. Clear zones after overnight incubation at 20ºC indicated the presence of antibacterial substances.

Bacterial isolates showing antagonistic activity against pathogenic *Vibrio* strains were identified using the 16S rRNA gene, amplified from extracted genomic DNA with primers 27F and 907R and *Taq* DNA polymerase (Invitrogen).[8] PCR (95°C for 10 min; 30 cycles of 94°C for 30 s, 50°C for 1 min, and 72°C for 2 min; and 72°C for 10 min) yielded products of approximately 0.9 kb, which underwent sequencing. The sequences obtained were compared to those available in the GenBank, EMBL and DDBJ databases with the BLAST program.[9] Sequences were subsequently integrated within the ARB program package and analyzed with its alignment tools.[10] The phylogenetic tree was constructed by the neighbor-joining method with Jukes Cantor correction. The robustness of the tree topology was verified through calculating bootstrap values for the neighbor-joining tree and through comparison with the topology of a maximum likelihood tree, calculated by using the default settings in ARB.[10]

All antagonistic isolates were also studied for the characterization of antibacterial substances. Antagonistic isolates were grown in 100 ml of marine broth without agitation at 20ºC for 2 days. After incubation, the bacteria were removed by centrifugation (2000 *g*), and cell-free culture supernatants were recovered by passage through 0.22-μm-pore-size filters.

All cell-free culture supernatants were adjusted to pH 6.5 with 5M NaOH to eliminate the inhibitory effects produced by organic acids. Moreover, sensitivity of cell-free culture supernatants to trypsin and proteinase K (Sigma Chemical Co., St. Louis, MO)

at a final concentration of 1.0 mg/ml was also tested in buffers recommended by the supplier. Samples with and without enzymes were incubated at 37ºC for 2 h and residual activity was determined. To exclude potential inhibition by hydrogen peroxide, catalase (Sigma Chemical Co.) was added at a final concentration of 0.5 mg/ml and incubated at 37ºC for 30 min. All assays were independently repeated at least two times for reproducibility.

Indicator bacteria (*V. alginolyticus* N26-1, *V. harveyi* HT351, *V. ichthyoenteri* HT21, *V. parahaemolyticus* HT352 and *V. splendidus* HT29) were grown in 5 ml of tryptic soy broth supplemented with 1.5% NaCl at 20ºC for 24 h. The cells were harvested by centrifugation (2000 × *g*), washed twice with sterile saline solution, and resuspended in 5 ml of the same solution. The bacterial suspensions were transferred to marine agar plates. Four wells were made in each agar plate with a sterile Pasteur pipette, and cell-free culture supernatants (10 μl) from the isolates were placed into each well. The plates were incubated aerobically at 20ºC for 1-2 days and then examined for zones of inhibition.

In all, 250 bacterial isolates from the intestinal content and cutaneous mucus were analyzed. Only 13 of these produced zones of inhibition, ranging from 15 to 20 mm, against at least one of the indicator strains. Comparative 16S rRNA gene sequence analysis placed the antagonistic isolates in the *Gammaproteobacteria* (61.5%), *Alphaproteobacteria* (23.1%), in the CFB group of *Bacteroidetes* (7.7%) and *Actinobacteria* (7.7%). Bacterial isolates belonging to the *Gammaproteobacteria* were obtained exclusively from the intestinal content. In particular, the genera *Aliivibrio*, *Neptunomonas*, *Pseudoalteromonas*, *Pseudomonas*, *Shewanella*, and *Vibrio* were identified (Figure 1). The *Alphaproteobacteria* were represented by the genera *Ruegeria* and *Jannaschia*, which were isolated from the cutaneous mucus. Members of the CFB

126 group of *Bacteroidetes* and *Actinobacteria* were also identified from the cutaneous
127 mucus samples (Figure 1).
128 The cell-free culture supernatants from the 13 antagonistic isolates exhibited
129 antibacterial activitiy against at least one of the indicator strains (Table 1). Except for
130 *Vibrio* sp. HG-3F, *Aliivibrio fischeri* HG-12F, and *Vibrio* sp. HG-14F, the antibacterial
131 activities were completely lost when the cell-free culture supernatants were neutralized
132 to pH 6.5 (data is not shown), suggesting that the antibacterial activity of these isolates
133 could be attributed to the production of organic acids or pH-dependent compounds.
134 Inhibitory activity of the cell-free culture supernatants generally was not inactivated by
135 enzyme treatment, which indicates that the inhibitory compounds are not proteinaceous
136 (Table 1). The only exceptions were the cell-free culture supernatants from *Vibrio* sp.
137 HG-3F, *Aliivibrio fischeri* HG-12F and *Vibrio* sp. HG-14F, which were inactivated by
138 at least one proteolytic enzyme. These findings suggest that the inhibitory substances
139 produced by these three isolates are proteinaceous, which show similar biological
140 activities to bacteriocin or bacteriocin-like inhibitory substances (BLIS).
141 Bacteriocins are ribosomally synthesized peptides or proteins that are generally
142 effective against closely related species.[11] Evidence is abundant that bacteriocins are
143 important mediators of intra- and interspecies interactions and, consequently, a
144 significant factor in maintaining microbial biodiversity.[12] Although research efforts
145 have mainly focused on bacteriocins produced by Gram-positive bacteria, bacteriocin
146 production has also been reported in Gram-negative bacteria. Recent studies have
147 demonstrated BLIS production by *Vibrio harveyi*, *Vibrio mediterranei* and *Vibrio*
148 *vulnificus*.[13-15] In the present study, the isolate HG-3F, closely related to *Vibrio*
149 *parahaemolyticus* (97.6% similarity), showed inhibitory activity only against indicator
150 strains of *V. parahaemolyticus* and *V. splendidus*. Moreover, the isolate HG-12F,

identified as *Aliivibrio fischeri* (100% similarity), showed inhibitory activity only against indicator strains of *V. ichthyoenteri*, *V. parahaemolyticus* and *V. splendidus*. Finally, the isolate HG-14F, closely related to *Vibrio rotiferianus* (98.5% similarity), inhibited all indicator strains. The fact that BLIS can have similar or different immunity proteins could explain these results.[16]

In conclusion, in addition to showing that bacteria associated with seahorses are able to produce antibacterial compounds, we have demonstrated that some of these compounds are proteinaceous. The genus *Vibrio* includes several species pathogenic to fish and humans, some of which are resistant to chemotherapeutic treatment.[17] Therefore, the potential as biological control agents of isolates exhibiting inhibitory activities against such species could be further studied in challenge experiments in fish or other marine species.

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223 **Table 1.** Effect of proteolytic enzymes on the activity of the cell-free culture
224 supernatants
225
226 **Figure 1.** Phylogenetic tree of antagonistic isolates with the most closely related
227 bacterial species, based on 16S rRNA gene sequences and constructed by the
228 neighbour-joining method. Sequences determined in this study are shown in boldface
229 type. *Thermotoga maritima* DSM 3109^{T} was used as an outgroup. The scale bar
230 corresponds to 0.1 substitutions per nucleotide.
231
232
233
234
235

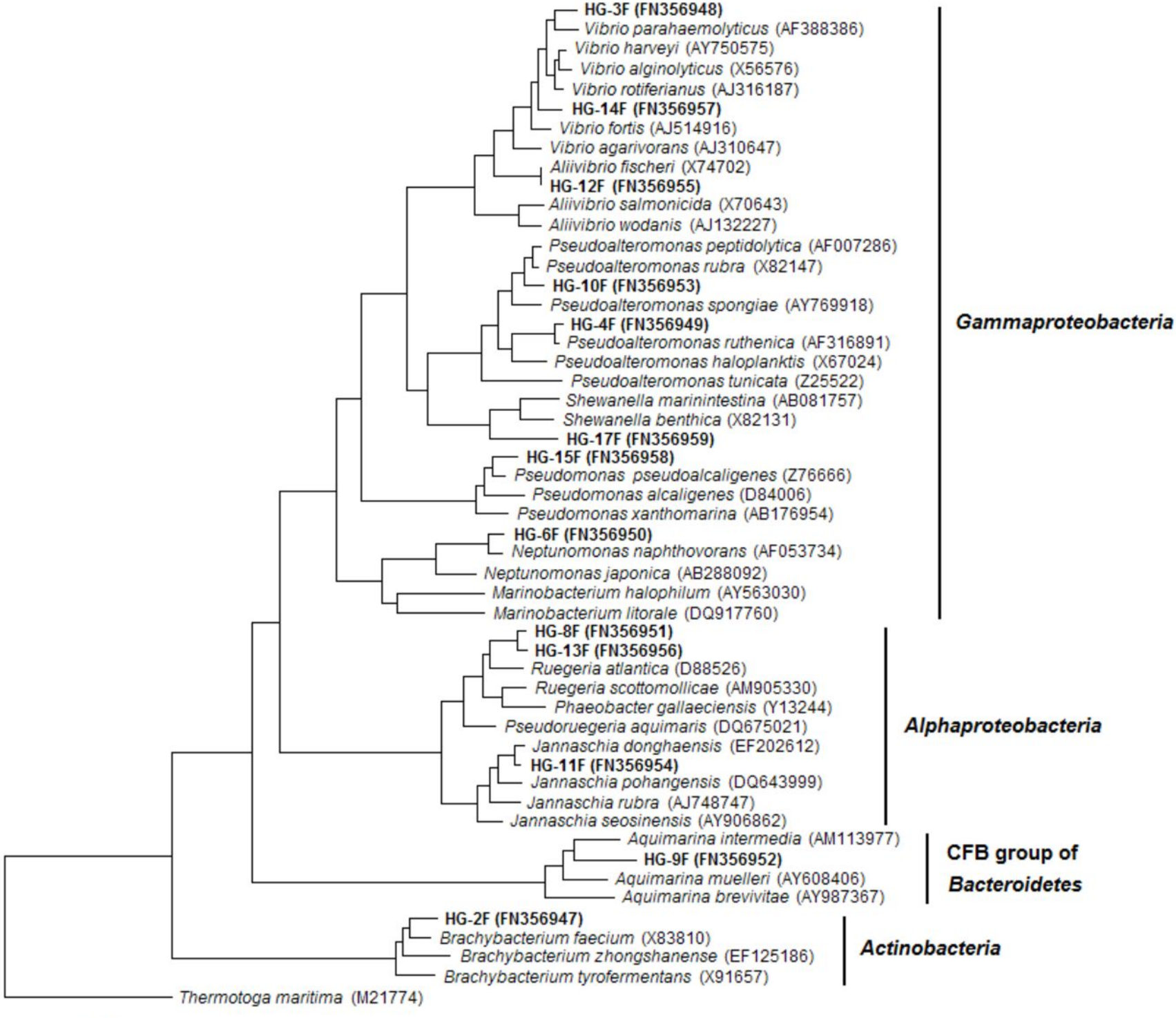
Table 1. Effect of proteolytic enzymes on the activity of the cell-free culture supernatants

Bacterial strain producers	Enzyme treatment	Indicator strains[a]				
		V. alginolyticus N26-1	*V. harveyi* HT351	*V. ichthyoenteri* HT21	*V. parahaemolyticus* HT352	*V. splendidus* HT29
Brachybacterium sp. strain HG-2F	Control[b]	+	+	+	+	+
	Proteinase K	+	+	+	+	+
	Trypsin	+	+	+	+	+
Vibrio sp. strain HG-3F	Control	–	–	–	++	++
	Proteinase K	–	–	–	–	–
	Trypsin	–	–	–	++	++
Pseudoalteromonas ruthenica strain HG-4F	Control	+	++	++	++	++
	Proteinase K	+	++	++	++	++
	Trypsin	+	++	++	++	++
Neptunomonas naphthovorans strain HG-6F	Control	+	+	+	+	++
	Proteinase K	+	+	+	+	++
	Trypsin	+	+	+	+	++
Ruegeria sp. strain HG-8F	Control	+	++	++	++	++
	Proteinase K	+	++	++	++	++
	Trypsin	+	++	++	++	++
Aquimarina sp. strain HG-9F	Control	+	+	+	+	++
	Proteinase K	+	+	+	+	++
	Trypsin	+	+	+	+	++
Pseudoalteromonas sp. strain HG-10F	Control	++	++	++	++	++
	Proteinase K	++	++	++	++	++
	Trypsin	++	++	++	++	++
Jannaschia donghaensis strain HG-11F	Control	+	+	+	+	+
	Proteinase K	+	+	+	+	+
	Trypsin	+	+	+	+	+
Aliivibrio fischeri strain HG-12F	Control	–	–	+	+	++
	Proteinase K	–	–	–	–	–
	Trypsin	–	–	–	–	–
Ruegeria sp. strain HG-13F	Control	+	++	++	++	++
	Proteinase K	+	++	++	++	++
	Trypsin	+	++	++	++	++
Vibrio sp. strain HG-14F	Control	+	+	+	+	+
	Proteinase K	–	–	–	–	–
	Trypsin	–	–	–	–	–
Pseudomonas sp. strain HG-15F	Control	+	+	+	+	+
	Proteinase K	+	+	+	+	+
	Trypsin	+	+	+	+	+
Shewanella sp. strain HG-17F	Control	+	+	+	+	+
	Proteinase K	+	+	+	+	+
	Trypsin	+	+	+	+	+

[a]*Vibrio* species isolated from disease processes in seahorses.

[b]Control samples consisting of cell-free supernatants without enzyme treatment.

Diameter of inhibition zone: +, 6 to 10 mm; ++, >10 mm; –, no inhibition zone.


HG-3F (FN356948)
Vibrio parahaemolyticus (AF388386)
Vibrio harveyi (AY750575)
Vibrio alginolyticus (X56576)
Vibrio rotiferianus (AJ316187)
HG-14F (FN356957)
Vibrio fortis (AJ514916)
Vibrio agarivorans (AJ310647)
Aliivibrio fischeri (X74702)
HG-12F (FN356955)
Aliivibrio salmonicida (X70643)
Aliivibrio wodanis (AJ132227)
Pseudoalteromonas peptidolytica (AF007286)
Pseudoalteromonas rubra (X82147)
HG-10F (FN356953)
Pseudoalteromonas spongiae (AY769918)
HG-4F (FN356949)
Pseudoalteromonas ruthenica (AF316891)
Pseudoalteromonas haloplanktis (X67024)
Pseudoalteromonas tunicata (Z25522)
Shewanella marinintestina (AB081757)
Shewanella benthica (X82131)
HG-17F (FN356959)
HG-15F (FN356958)
Pseudomonas pseudoalcaligenes (Z76666)
Pseudomonas alcaligenes (D84006)
Pseudomonas xanthomarina (AB176954)
HG-6F (FN356950)
Neptunomonas naphthovorans (AF053734)
Neptunomonas japonica (AB288092)
Marinobacterium halophilum (AY563030)
Marinobacterium litorale (DQ917760)
Gammaproteobacteria
HG-8F (FN356951)
HG-13F (FN356956)
Ruegeria atlantica (D88526)
Ruegeria scottomollicae (AM905330)
Phaeobacter gallaeciensis (Y13244)
Pseudoruegeria aquimaris (DQ675021)
Jannaschia donghaensis (EF202612)
HG-11F (FN356954)
Jannaschia pohangensis (DQ643999)
Jannaschia rubra (AJ748747)
Jannaschia seosinensis (AY906862)
Alphaproteobacteria
Aquimarina intermedia (AM113977)
HG-9F (FN356952)
Aquimarina muelleri (AY608406)
Aquimarina brevivitae (AY987367)
CFB group of
Bacteroidetes
HG-2F (FN356947)
Brachybacterium faecium (X83810)
Brachybacterium zhongshanense (EF125186)
Brachybacterium tyrofermentans (X91657)
Actinobacteria
Thermotoga maritima (M21774)
0.10