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Inhibitory activity of *Phaeobacter* strains against aquaculture pathogenic bacteria

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Summary. A total of 523 bacterial strains were isolated during a 4-year period from mollusc hatcheries (flat oyster and clams) in Galicia (NW Spain). All of the strains were tested for their antibacterial activity against three larval pathogens (*Vibrio anguillarum* USC-72, *V. neptunius* PP-145.98, and *Vibrio* sp. PP-203). Of the isolates, 52 inhibited at least one of the target strains, and 11 inhibited all of them. The main source of active strains was oyster larvae, followed by water, tank surfaces, spat, and broodstock. Four similar strains, belonging to the genus *Phaeobacter*, showed the strongest activity. Strain PP-154, selected as representative of this group, displayed a wide spectrum of inhibitory activity against aquaculture pathogens, especially against members of the genus *Vibrio*, which is responsible for the most larval deaths. The inhibitory ability of such strain on solid medium was confirmed in seawater experiments, and the optimal conditions for antibacterial activity were established. These strains are promising probiotics for aquaculture facilities. Their potential benefit is based on the capacity to control the proliferation of a variety of aquaculture bacterial pathogens in mollusc larval cultures. [**Int Microbiol** 2009; 12(2):107-114]

Keywords: *Phaeobacter* · probiotics · mollusc larval pathogens · antibacterial activity

Introduction

The culture of bivalves is an important economic sector in Galicia (NW Spain), where environmental conditions allow different species to achieve sizes of commercial interest within a shorter period of time than in other areas [21]. In the cases of flat oysters (*Ostrea edulis*) and clams (*Ruditapes decussatus* and *Venerupis pullastra*), overexploitation, the decline of natural beds, and the failure of natural recruitment have led to uncontrolled imports, with the consequent introduction of pathogens to molluscs.

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Setting up hatcheries are the best way to guarantee a regular supply of autochthonous spat, but these facilities are often affected by outbreaks of lethal infections. Bacteria, mainly from the genus *Vibrio*, have been identified as the etiological agents responsible for disease outbreaks in larval cultures [23,24,32]. General control methods consist of chemotherapy and disinfection of seawater, but both have been proven to be inefficient and are associated with significant disadvantages [1,7,17], such as the development of antibiotic resistances, toxicity to larvae, and the release of chemical residues into the environment. Vaccines, a good solution in fish aquaculture, are not yet available for bivalves [3].

The most promising approach is the use of probiotics [36,37]. The term is most often applied to human and veterinary uses (homeotherms), especially in the context of the regulation of intestinal balance and, at present, also to the biocontrol of phytopathogenic bacteria [34]. But the application of probiotics in aquaculture implies a wider definition

because of the constant flow of microorganisms and the narrow interaction between the animal and the environment. According to Verschuere et al. [36], the term "probiotic" refers to those live microbial adjuncts which have a beneficial effect on the host.

The use of live bacteria with activity against pathogens is especially interesting in bivalve hatcheries [9,22,27,29], because the larvae are released and exposed to environmental microbiota during early ontogenic stages. The filter-feeding behavior of these animals increases the influence of the surrounding water. In this report, we present the results of a search for probiotically active strains in bivalve hatcheries in Galicia. Of a group of four isolates with the highest bacterial inhibitory activity, *Phaeobacter* strain PP-154 (deposited in the Spanish Type Culture Collection for patent purposes, CECT 5891) was found to be representative, and its potential use in aquaculture was further investigated. In addition, the taxonomic position of the four strains was studied.

Materials and methods

Isolation of strains. During a 4-year period, samples were taken from different compartments of three bivalve hatcheries located in Galicia (NW Spain) and processed as previously described [24]. Briefly, serial dilutions of the samples (seawater, larvae, phytoplankton, and tank surfaces) were plated onto Thiosulphate Citrate Bile Sucrose (TCBS, Oxoid, UK) and Marine Agar (MA, Pronadisa, Spain), and incubated at 23 ± 1°C for 24 h and for 7 days, respectively. Different colony types were isolated to obtain pure cultures on MA. Strains were stored frozen at –80°C in Marine Broth (MB, Pronadisa, Spain) containing glycerol (15% vol/vol).

Preliminary screening for inhibitory activity of several *Vibrio* strains. The antibacterial activity of the isolates was assayed on solid medium according to the spot method [18]. Two larval pathogens isolated in our laboratory from the same hatcheries, *Vibrio neptunius* PP-145.98 and *Vibrio* sp. PP-203 [24], and the marine pathogen *V. anguillarum* (USC-72) were used as target strains. Each was prepared as a 24-h culture in MA medium and resuspended in sterile seawater (SSW, aged natural seawater, filtered through a 1.0-mm filter and then autoclaved). The suspensions were adjusted to tube 1 of the MacFarland scale and spread on appropriate plates. A small amount of the isolates to be tested, cultured on MA, were deposited in spots (ca. 2–3 mm diameter) onto the surfaces of seeded plates. Plates were incubated for 24 h at 23 ± 1°C. Inhibition areas around the spot of at least 1 mm were considered positive for antibacterial activity. A representative strain from the group of four isolates with the strongest activity was selected and hereafter is referred to as strain PP-154.

Screening inhibitory activity of strain PP-154 against aquaculture bacterial pathogens. A modification of the spot method was used to evaluate the activity of strain PP-154 against different strains, including members of the genus *Vibrio*, as well as aquaculture pathogens belonging to *Aeromonas*, *Photobacterium*, *Pseudomonas*, *Streptococcus*, and *Tenacibaculum* (species detailed in Tables 2 and 3). Briefly, strain PP-154 was cultured in MB and incubated on an orbital shaker maintained at 23 ± 1°C. After 4 days, 1.5-ml samples were centrifuged (5000 ×*g*, 3 min). The supernatant was collected in a sterile tube and the pellet was resuspended in 40 µl of SSW. Both fractions were adsorbed to blank disks (20 µl/disk) and deposited on plates of the appropriate medium seeded with each of the target strains mentioned above. Blank disks with SSW were used as negative controls to exclude any non-specific activity. Inhibition areas of at least 1 mm around the edge of the disk were considered positive.

Antagonistic activity of strain PP-154 to pathogenic *Vibrio* under different culture conditions. Three types of interactions between PP-154 and the pathogens *V. neptunius* PP-145.98 and *Vibrio* sp. PP-203, simulating possible events in the hatchery, were analyzed: (i) Water conditioning. Strain PP-154 was added to SSW at the beginning of the experiment, and the pathogen was inoculated 24 h later, to evaluate whether previous colonization of strain PP-154 enhanced the inhibition of pathogen. (ii) Coculture. Probiotic and pathogen were inoculated simultaneously at the beginning of the experiment. (iii) Treatment. The pathogenic strain was inoculated 24 h before, with the aim of determining whether strain PP-154 was able to stop the growth of a pathogen previously established in the culture system.

Cultures were carried out in 250-ml flasks containing 100 ml of SSW. The strains, grown on MA plates, were resuspended in SSW and bacterial suspensions were inoculated to final concentrations of 10^4 CFU/ml for the pathogenic strains PP-145.98 and PP-203, and 10^6 CFU/ml for the probiotic strain PP-154. As a control, each strain was grown alone. For interaction assays, cultures were incubated on an orbital shaker at 100 rpm. Two different temperatures, 23 ± 1°C and 17 ± 1°C (the usual temperatures in Galician hatcheries during the year), were assayed to evaluate the influence of temperature on the interaction between strain PP-154 and the pathogens. Samples were taken at regular intervals after inoculation; appropriate dilutions were plated on MA and incubated at 23 ± 1°C. The number of CFU/ml was recorded after 24–72 h depending on the strain. In mixed cultures, the strains were clearly identified by colony morphology on MA. Strain PP-154 forms cream-brown colonies with a brown diffusible pigment, whereas the pathogenic vibrios form white-gray colonies.

Growth and antibacterial activity of strain PP-154. The growth of strain PP-154 was assayed on different culture media: MA/MB, TSA-1 (1% NaCl), MHA-1 (Mueller Hinton Agar with 1% NaCl; Oxoid, UK), BHA-1 (Brain-Heart Infusion Agar with 1% NaCl; Oxoid, UK), King A and King B (Cultimed, Spain), TCBS, TCBS-M (rehydrated with 50% SSW), LBSS (Luria-Bertani Agar, Pronadisa, Spain; with 1.4% Sea Salts, Sigma, Spain) [38] and 1/2 YTSS [10].

Antibacterial activity was assayed against strain PP-145.98, following the spot method explained above and using MB. At regular intervals, optical density (OD) was measured in a Lambda 3 spectrophotometer (Perkin Elmer, USA) at 540 nm, and samples were taken for antibacterial activity. In addition, the influence of pH on the antibacterial activity of strain PP-154 was studied in MB medium in the range of 4.5–10.5. Growth was determined by turbidity and confirmed by spreading on MA plates (10 ml, sterile inoculation loop). For both experiments, the activity of each fraction (pellet and supernatant) against the pathogenic strain *V. neptunius* PP-145.98 was assayed by the modified spot method described above for the screening of antibacterial activity.

Identification of strain PP-154. Strain PP-154 was examined for its phenotypic characteristics using classical procedures as previously described [2,24]. Additional phenotypic analyses were carried out with the API 20E, API 20NE, and API ZYM systems (bioMérieux, France), but using saline solution (NaCl 0.85 %) to prepare the inocula. Optimal conditions of salinity were determined in SSW diluted to 36, 25, 15, 10 and 5‰, measuring bacterial growth at OD_{540}. The temperature range of growth was determined by plating on MA and incubating at 8, 15, 25, 37, and 43ºC for up to 2 weeks. Sensitivity to the vibriostatic agent pteridine (O/129, 150 µg) was determined by the disc diffusion method. The inhibition zone on MA was determined after 24-48 h of incubation at 23 ± 1°C. The16S rRNA gene was sequenced following the procedures described in [24].

Results

Isolation of strains and antibacterial activity in solid medium. From the original MA and TCBS plates, 523 isolates were selected and subcultured to obtain pure cultures. Of these, 52 strains (9.4%) showed inhibitory effects against at least one of the three target strains (Table 1). The highest number of active strains was isolated from oyster larvae (18.6%), followed by culture seawater (10.6%), tank surfaces (8.6%), oyster spat (8.2%), and oyster broodstock (6.9%) (Table 1).

Eleven isolates displayed antagonistic effect against all target strains. Four similar strains (PP-154, PP-639, PP-694, and PP-847) showed the strongest inhibition. Strain PP-154 was isolated from spat (flat oyster), strain PP-639 from larvae (flat oyster), and strain PP-694 from gonads (flat oyster). Isolate PP-847 was obtained from phytoplankton culture (*Phaeodactylum* sp.). Strain PP-154 was selected as representative of this group and therefore subjected to further examination.

Antibacterial activity and antagonism assays of strain PP-154. Pellets of strain PP-154 showed a wide inhibitory activity against *Vibrio* species (Table 2) and several other of the aquaculture bacterial pathogens tested (Table 3). In all cases, the supernatant displayed none or only very weak activity.

In water conditioning experiments, pathogen growth was clearly inhibited by strain PP-154 compared with the control, with final reductions of viable counts of *V. neptunius* strain PP-145.98 (Fig. 1A,C,E) and *Vibrio* sp. PP-203 (Fig. 1B,D,F) of ca. 1–2 log-units. Similar results were obtained in the coculture experiments. The final numbers of pathogens in the treatment experiments were slightly lower than those recorded in controls. These results confirmed the activity displayed on solid media. In the conditioning and the coculture experiments, both of which included a control, high numbers of strain PP-154 were maintained (1.5×10^6 and 5.3×10^6 CFU/ml). Temperature did not influence the interaction between strain PP-154 and *Vibrio* pathogens (Fig. 1).

Table 1. Spectrum of the antibacterial activity of the isolates against the three target strains and the distribution of the isolates in relation to the different sources in mollusc hatcheries

		No. of target strains inhibited[a]		
Source	No. of isolates	Strain A	Strain B	Strain C
Oyster broodstock	131	9	6	4
Oyster larvae	129	24	15	4
Oyster spat	49	4	4	3
Clam broodstock	35	0	0	0
Clam larvae	21	0	0	0
Water	104	11	8	4
Phytoplankton	19	1	1	1
Tank surfaces	35	3	1	1
Total	523	52	35	17

[a]Target strains. Strain A: *Vibrio anguillarum* USC-72; Strain B: *V. neptunius* PP-145.98; Strain C: *Vibrio* sp. PP-203.

Table 2. Antagonistic activity of strain PP-154 against members of genus *Vibrio*

Species	Target strain[a]	Inhibition[b]
Vibrio aestuarianus	ATCC 35048	+
V. alginolyticus	CCM 2578	+
	MA-1	±
V. anguillarum	USC R-72	+
	TM 20.1(O11)	+
V. fischeri	NCIMB 1274	+
V. fluvialis	ATCC 33812	+
V. harveyi	177 PA	±
	PC 92.1	+
V. logei	ATCC 15382	±
V. mimicus	ATCC 33653	+
V. natriegens	ATCC 14048	+
V. neptunius	PP-145.98	+
V. parahaemolyticus	ATCC 27969	+
V. pelagius	ATCC 25916	+
	752.1	+
V. proteolyticus	ATCC 15338	+
V. splendidus	RPM 754.1	+
	RPM 753.1	+
	PC 399.1	+
V. tapetis	CECT 4600	+
	GR0705RD	+
	GR0202RD	+
	CMJ 10.7	+
V. tubiashii	ATCC 19106	+
V. vulnificus	ATCC 27562	+
	A1 AC	+
Vibrio sp.	PP-203	+
Vibrio sp.	PP-638	+

[a]The isolates used in the study belong to our laboratory collection, except those obtained from ATCC (American Type Culture Collection, USA), NCIMB (National Collection of Industrial, Marine and Food Bacteria, UK) and CECT (Spanish Type Culture Collection).
[b]Key: (+) inhibition zone ≥10 mm around the disk; (±) inhibition zone <1 mm.

Table 3. Antagonistic activity of strain PP-154 against fish pathogens other than *Vibrio*

Species	Target strain	Inhibition[a]
Aeromonas hydrophila	TR 401/02	+
	X 021104-01	+
A. salmonicida	PC 520.1	±
	RSP 43.1	–
	ATCC 14174	–
	ASF 1-1	–
Edwardsiella tarda	H 14.1	+
	ACC 36.1	+
Pseudomonas anguilliseptica	CECT 899	+
	AZ 196.1	+
	TW P1	+
P. fluorescens	CAN 228-1	–
	AZ 235.1	–
	AZ 239.1	–
Photobacterium damselae subsp. *piscicida*	Lgh 41/01	+
	PC 435.1	+
Tenacibaculum maritimum	PC 503.1	+
	RI 136.1	+
Lactococcus garviae	AR 1	–
	LG 3682	–
Streptococcus parauberis	ACC 2.1	+

[a]Key: (+) inhibition zone ≥10 mm around the disk; (±) inhibition zone <1 mm; (–) absence of inhibition.

Strain PP-154 grew well on MA, producing rounded cream-brown colonies with a brown diffusible pigment. Growth on MB was likewise evidenced by pigment formation, in both static and shaken cultures. Growth and pigment formation were also observed on LBSS and 0.5YTSS, with the cultures displaying antibacterial activity (data not shown). On the other media, no growth or only very weak growth was achieved.

The relation between bacterial growth and antibacterial activity against *V. neptunius* PP-145.98 was established for strain PP-154 in MB in shaking cultures at 23 ± 1ºC. The strongest inhibition was detected between days 4 and 8 (Fig. 2). The supernatant was only weakly active at days 6–8 (data not shown). Strain PP-154 grew at pH 6.0-10.0, but not at pH 5.8 or 10.3. Pigmentation in liquid medium was brown in cultures grown at pH 6.0–7.8, but at pH values higher than 8.8 the pigment was yellowish. The pellet was active against *V. neptunius* PP-145.98 at pH 6.0–10.0, with a maximum at pH 7.0–7.8. The supernatant showed weak activity at pH 7.7–7.8.

Identification of strain PP-154. Strain PP-154 is a motile short rod, gram-negative, oxidase and catalase-positive. It is oxidative in ZOF and OF medium with glucose. It is negative for the following tests: indol, nitrate reduction, Simmon's citrate, gas and acid production from glucose, H_2S production, degradation of starch, gelatinase, lipase and urease. It is positive for hydrolysis of esculine. The isolate grew at salinities between 15 and 36‰, and at a range of temperatures from 15 to 37°C. In API 20E, the only positive test was ONPG. In API 20NE, the tests for ESC and PNPG were positive. In API ZYM, strain PP-154 displayed alkaline phosphatase, leucine arylamidase, and acid phosphatase activities, and showed weak reactions for esterase lipase C8 and valine arylamidase. It was sensitive to the vibriostatic agent O/129.

Strain PP-154, based on its 16S rDNA sequence, is affiliated with the genus *Phaeobacter* (class Alphaproteobacteria, order Rhodobacterales). Isolates PP-639, PP-694, and PP-847 showed identical phenotypic and genetic characteristics (data not shown), with genetic similarity (16S rDNA) to strain PP-154 being ca. 99%. Clustering obtained by the Clustal method indicated that these strains are highly related to *Phaeobacter gallaeciensis* (99.2%) and to *Ph. inhibens* (99.6%) (Fig. 3). The 16S rDNA sequence of strain PP-154 is available from GenBank under accession no. AJ296158.

Discussion

Of the 523 bacterial isolates obtained from Galician bivalve hatcheries, 52 displayed antagonism against at least one of the three target strains, *Vibrio neptunius* PP-145.98, *Vibrio* sp. PP-203, and *V. anguillarum* USC-72. Flat oyster was the best source of antagonistic isolates. The higher percentages of isolates obtained from larvae (18.6%) than from spat (8.2%) and broodstock (6.9%) might be explained by the fact that adults are maintained in flow-through systems, in which a heavy bacterial colonization is difficult. Although closed systems could favor antibacterial activity, they could also promote the proliferation of opportunistic pathogens. Hence, the maintenance of a tight balance of appropriate strains in the hatchery ecosystem could facilitate the survival of larvae. There are few reports describing the search for isolates with antibacterial activity in bivalve hatcheries [22,26]. The isolate characterized in this work, *Phaeobacter* strain PP-154, showed a wide spectrum of antibacterial activity, as has been determined for other marine bacteria [6,9,16,19,27,29]. However, a comparison of the antibacterial activity of marine isolates is very difficult due to the high variability in number of bacterial isolates, target strains, media, and methods employed [13–15,25]. Ruiz-Ponte et al. [29] studied the anti-

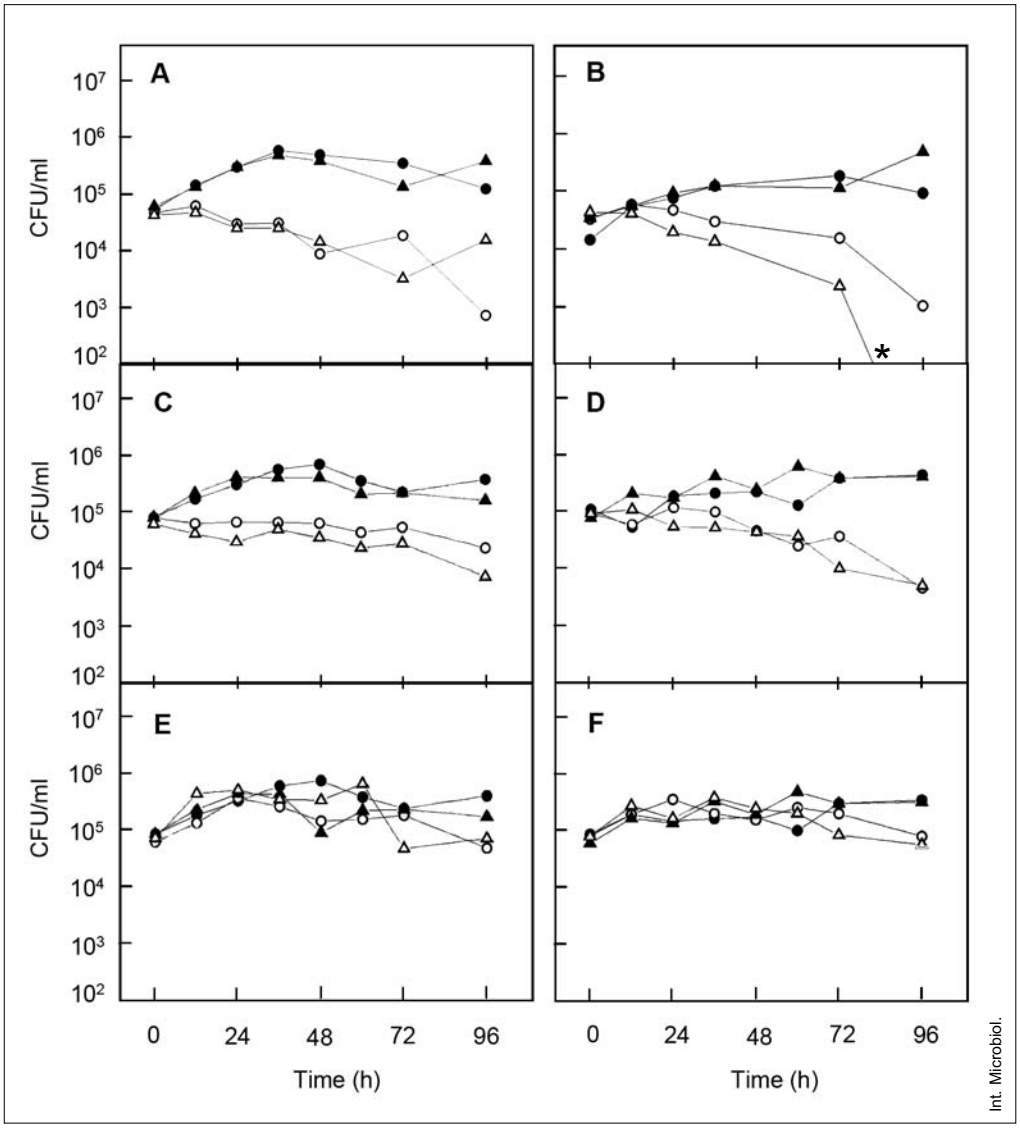

Fig. 1. Growth inhibition of pathogenic *Vibrio neptunius* PP-145.98 (**A,B,E**) and *Vibrio* sp. PP-203 (**B,D, F**) by the probiotic strain PP-154 in three different types of mixed cultures: "conditioning" (A,B), "coculture" (C,D) and "treatment" (E,F). Experiments were carried out at 17°C (circles) and at 23°C (triangles). For each graph, the growth of the pathogen alone (closed figures) and in the presence of strain PP-154 (open figures) is represented. The asterisk in the abscissa of panel B indicates the reduction of cell numbers below detection limits.

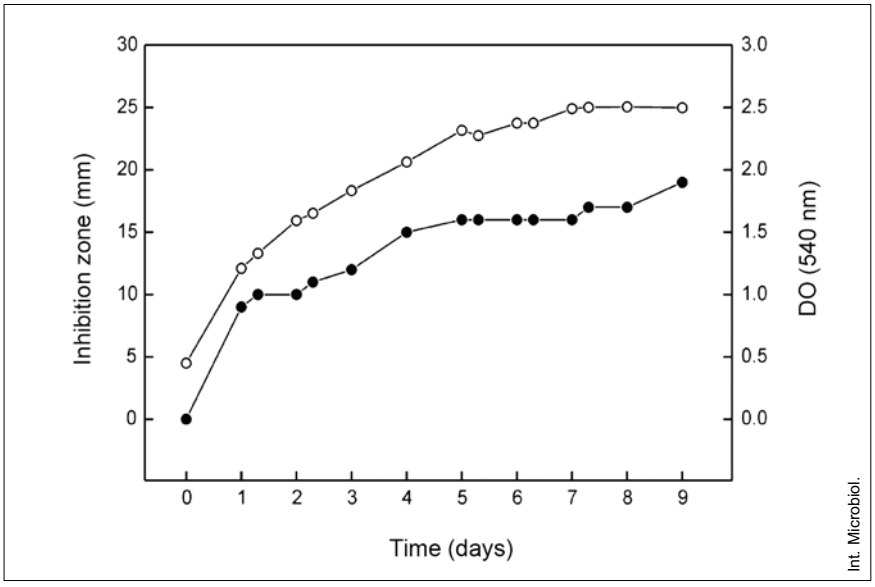

Fig. 2. Growth in MB of strain PP-154 (open circles) and antibacterial activity of the pellet against the pathogen *V. neptunius* PP-145.98 (closed circles).

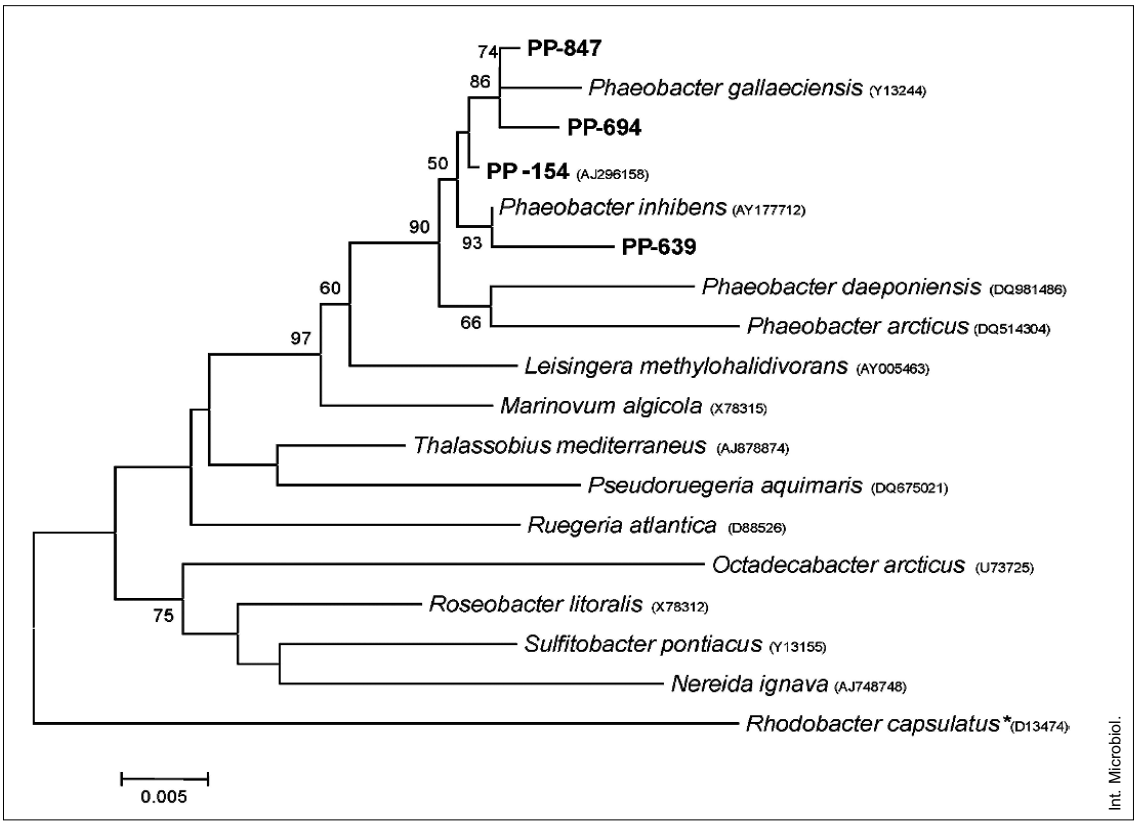

Fig. 3. Phylogenetic tree of 16S rRNA gene sequences for strains PP-154, PP-639, PP-694, PP-847, and the type strains of the closest Alphaproteobacteria, using the neighbor-joining method. *Rhodobacter capsulatus* (accession no. D13474) served as the outgroup. Horizontal branch lengths are proportional to evolutionary divergence. Significant bootstrap values of 1000 replicates appear next to the corresponding branch.

bacterial activity of *Phaeobacter gallaeciensis* (formerly *Roseobacter gallaeciensis*) BS107 using the spot method but not the double-layer method. Our strains were active in assays based on both methods (data not shown). The explanation for this divergence is that Ruiz-Ponte et al. [29] did not mix the target strain onto overlay agar and hence did not allow the contact between the active strain and the target one.

The antagonistic activity in solid medium does not necessarily imply effectiveness in liquid medium or in natural environments [11,33], and the assay media may influence the degree of in vitro inhibition [12,31]. For these reasons, assays in seawater were designed, using larval pathogens as target strains. The ability of strain PP-154 to inhibit vibrios in seawater confirmed the results obtained in solid medium.

It has been reported that high concentrations of inhibitory strain [35], or at least a difference of 2–3 log units between probiotic and pathogenic strains [5], may be needed to obtain antibacterial activity. However, even such differences between initial levels of probiotic and pathogen do not ensure successful inhibition [14]. In our experiments, good inhibitory activity was achieved with initial concentrations of about 10^6 and 10^4 CFU/ml for strain PP-154 and *Vibrio* pathogens, respectively, under identical experimental conditions. Strain PP-154 was able to inhibit vibrios when it was inoculated into the medium before or at the same time, but not after the pathogens were established, as reported for *Streptomyces tenjimariensis* [30]. However, there was no overgrowth of strain PP-154 in any assay. Therefore, our results indicate that strain PP-154 could be used as a preventive agent to inhibit the proliferation of vibrios in hatcheries, under pre-determined conditions.

The antibacterial compound secreted by strain PP-154 seems to be a secondary metabolite, produced in the stationary-phase, independently of the conditions of light/darkness (data not shown). The activity detected in the pellet indicates that the substance is attached to the bacterial cells, as was previously reported with other marine bacteria [18,27]. Hence, the slow release and cell lysis may explain the weak activity in the supernatant of old cultures. Bacterial cells of 4-day-old cultures in MB at pH 7.0–7.7 are optimal to obtain inhibitory activity, without significant release of the substance into the medium.

Our results are in disagreement with those obtained by other investigators with similar strains, in which the massive release of substances (inhibitors or effectors), or growth in MB and under static conditions were assumed to be essential for antibacterial activity [4,29]. According to the results of this work, the strains show inhibitory activity after culture in media other than MB, even in shake-cultures, and the inhibitory substance is not released into the supernatant.

The potential probiotic strain PP-154, as well as strains PP-639, PP-694 and PP-847, belong to the genus *Phaeobacter*, which was created by Martens et al. [19] to include *Phaeobacter gallaeciensis* (formerly *Roseobacter gallaeciensis* Ruiz-Ponte et al. [28]) and the new species *Ph. inhibens*. The phenotypic characteristics and 16S rDNA sequences of strain PP-154 isolate do not allow its allocation into one of these two species, especially since *Ph. inhibens* was described based only on a single isolate such that information on possible intraspecific variability is lacking. Further work is in progress to clarify the taxonomy of this group of bacteria.

In summary, the *Phaeobacter* strains characterized in this work are good candidates to be used as probiotics in aquaculture systems. They are marine bacteria with a wide spectrum of antibacterial activity, including against shellfish and fish pathogens, mainly *Vibrio* sp. They are able to develop inhibitory activity in seawater and do not proliferate to dangerous levels. Their biotechnological potential is based on the ability to control the proliferation of opportunistic pathogens in hatchery and farm environments.

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