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Research Article

# Screening for and Identification of an Anti-clam *Vibrio* Marine Bacterium from an Aquaculture Pond in the Yellow Sea

The identification and use of probiotic bacterial stains is a practical approach to protect clams grown in aquaculture farms from disease. The inhibition of the pathogenic bacterium *Vibrio alginolyticus* was used as a trait to select a candidate probiotic bacterial strain in this study. An ideal bacterial strain, SW-1, was isolated from seawater from a clam farm. The selected isolate SW-1 was identified based on its physiological, morphological, and biochemical characteristics and its 16S rDNA sequence. The experiments showed that strain SW-1 had a high similarity to *Pseudoalteromonas piscicida* and could inhibit the growth of *V. alginolyticus* (*V*.-MP-1). SW-1 also improved the survival of clams following challenge with the pathogenic *V*.-MP-1. The mortality of clams was 100% after infection with $10^8$ CFU/mL of *V. alginolyticus*, whereas mortality was only 11% when clams were infected with $10^8$ CFU/mL of *V*.-MP1 while simultaneously exposed to the same concentration of *Pseudoalteromonas* SW-1, indicating that *Pseudoalteromonas* SW-1 could be used as a probiotic to protect farmed clams, and thus reduce the effects of antibiotics on aquatic environment.

**Keywords:** Fish disease; *Meretrix meretrix*; Probiotic; 16S rDNA

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## 1 Introduction

Aquaculture production is valued at US$98.4 billion and accounts for 35.7% of aquatic production worldwide [1]. In the case of China, the total level of domestic aquatic production has been reported to be over 47.5 million tons, which accounts for 62% of global production in terms of quantity and 51% of global value in 2008. *Meretrix meretrix* (Linnaeus) is one of the main shellfish varieties that is grown along the southeast coast of China. In Jiangsu Province, for example, *M. meretrix* production in 2008 was approximately 0.06 million tons, with an estimated first-sale value of US$ 62.6 million, representing nearly a half of the country's total [2]. Disease outbreaks caused by pathogenic bacteria, commonly of the genus *Vibrio*, are a major cause of mortality in shellfish larvi-culture and can result in financial losses for commercial growers [3]. A variety of antibiotics have been used for the prevention of diseases, but the overuse of antibiotics can lead to the emergence of resistant bacteria and environmental problems [4–6], such as: the abuse of antibiotics may remain in high concentration in aquatic products and in surface water, resulting in the high risk of these chemical to human health and the environment [7, 8]. Therefore, alternatives to antibiotics, such as functional foodstuff or natural antimicrobial materials, are needed.

The term "probiotics" traditionally refers to live microbial feed supplements that beneficially affect the host animal by improving its intestinal microbial balance [9–11]. Recently, the interest in the use of probiotics in aquaculture has increased. In particular, a variety of in vivo and in vitro studies on the positive effect of using probiotics on fish, shrimp, and molluscs have been performed [12–14]. Currently, a number of preparations of probiotics, such as lactic acid bacteria, are commercially available. According to the claims of the producers, these products are effective in supporting the health of aquatic animals and are safe.

There are a few reports on the use of probiotic bacteria relevant to the prevention of shellfish diseases, both in China and abroad. The objective of this study was to screen for and identify anti-clam *Vibrio* bacteria and then to provide a basis for the exploitation of probiotic products for sustainably developing aquaculture [15–17].

## 2 Materials and methods

### 2.1 Isolates of *Pseudoalteromonas* SW-1 (*P.*-SW-1)

Water samples were collected from *M. meretrix* farms located in Qidong County, Jiangsu, China (lat. 30°45′36N and long. 120°21′4E). To isolate *P.*-SW-1, aliquots (0.1 mL) of diluted ($10^{-1}$) water samples were spread onto ZoBell's Marine Agar plates (composition: 1 g yeast extract, 5 g peptone, 0.1 g $FePO_4$, 20 g agar, 1000 mL seawater, pH 8.0). The plates were incubated at 30°C for 48 h, and then the colonies were purified by plate streak methods. Colonies

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**Abbreviations:** **CFU**, colony-forming unit; **MB**, marine broth; **P.-SW-1**, *Pseudoalteromonas* SW-1; **V.-MP-1**, *Vibrio* MP-1.

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causing inhibition of *Vibrio* MP-1 (*V.*-MP-1) were isolated [15] and pure cultured. Isolates were frozen at −70°C in marine broth (50%; MB diluted 1:1 with sterile seawater) containing 30% glycerol. The best activity strain was identified based on their morphological and physiological characteristics. The morphological, physiological, and biochemical characteristics were similar to the species of *Pseudoalteromonas*.

## 2.2 Pathogen isolate (*V.*-MP-1)

A pathogen causing clam disease (*Vibrio alginolyticus*) was isolated from diseased clams that had been collected at the Qidong Yellow Sea Breeding, in 2009. These clams were peeled off and ground, diluted tenfold with sterilized water, and then placed on ZoBell's Marine Agar plates. The plates were incubated at 28°C for three days. The colonies from the sample tissues were then incubated on TCBS agar plates. After two days, yellow and blue strains were removed and purified. The colonies were purified and identified based on their morphological and physiological characteristics and partial 16S rDNA gene sequence analyses. The results showed that the colonies were similar to *V. alginolyticus* [16] (GenBank accession no. JX046039). The virulence tests showed that the strain *V.*-MP-1 was the clam pathogenic bacteria. Isolates were frozen at −70°C in marine broth (50%; MB diluted 1:1 with sterile seawater) containing 30% glycerol. Working strains were stored on ZoBell's Marine Agar slants at 4°C.

## 2.3 Identification of strain SW-1 by phylogenetic analysis

DNA from bacterial isolates cultured in Luria–Bertani (LB) broth was extracted by 1 mL of culture by centrifugation. The total DNA was extracted using a DNA extraction kit according to the manufacturer's instructions (Genetech, China). 16S rDNA was amplified with the universal primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′). The PCR amplification was performed as follows: one cycle of 5 min at 95°C, 40 s at 94°C, 30 s at 58°C, and 1.5 min at 70°C, followed by 30 cycles of 8 min each at 72°C. The PCR products were purified and sequenced by Invitrogen Corporation in Shanghai. DNA sequence homology searches were performed using the online BLAST search engine in GenBank at the National Center for Biotechnology Information (NCBI). The phylogenetic tree for the data set was constructed using the neighbor-joining method with MEGA version 4.0.2.

## 2.4 Optimal temperature, pH and salinity for the growth of *P.*-SW-1

The optimal conditions for the growth of *P.*-SW-1 were determined by growing the bacterium in ZoBell's MB at various temperatures (25, 30, 35, and 37°C), pH (5.0, 6.0, 7.0, 8.0, and 9.0), and salinities (0.2, 0.5, 0.8, 1.0, and 1.5 mol/L) using a 1% inoculum (cultures concentration in ZoBell's MB medium to approximately $10^6$ coloniy-forming unit (CFU)/mL after inoculation). These cultures were incubated for 24 h in duplicate. The absorbance (a measure of growth) was measured with a 722 spectrophotometer at a wavelength of 600 nm.

## 2.5 Growth characteristics of *P.*-SW-1

Pure colonies of *P.*-SW-1 were transferred to a hard glass tube containing 5 mL of ZoBell's MB and incubated 24 h at 30°C. Then, these cultures were used to inoculate Erlenmeyer flasks containing ZoBell's MB, and the flasks were incubated on a rotatory shaker (180 rpm) at 30°C. The growth of *P.*-SW-1 was measured at 600 nm at regular intervals (0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, and 32 h). The absorbance measurement of the sterile ZoBell's MB was used as the blank measurement for each respective well.

## 2.6 Antibacterial testing

The antibacterial activity of *P.*-SW-1 was evaluated by the paper-disk agar diffusion method against different strains. *Vibrio alginolyticus* (GenBank accession no. JX046039) was isolated from the clam farming in China. *Achromobacter* sp. YZ (GenBank accession no. EF617310), and *Bacillus* J1 (GenBank accession no. FJ815201) were obtained from Nanjing Agricultural University and Jiangsu Key Laboratory of Marine Biology. Inocula were prepared by diluting overnight (24 h at 30°C) cultures in ZoBell's MB medium to approximately $10^6$ CFU/mL. Absorbent disks (Whatman, diameter 6 mm) were impregnated with *P.*-SW-1. The diameters of the growth inhibition zones were measured after incubation at 30°C for 24 h. The experiment was performed in quadruplicate.

## 2.7 Virulence tests

The possible probiotic *P.*-SW-1 and the pathogenic strain *V.*-MP-1 were tested in experimental clam infections. All inocula were prepared by diluting overnight (24 h at 30°C) cultures in ZoBell's MB medium to approximately $10^7$, $10^8$, or $10^9$ CFU/mL. Each group of 15 clams was randomly allocated in 25-L fiberglass tanks, and these inocula were poured into the tanks in triplicates. Survival was monitored for 15 days.

# 3 Results and discussions

## 3.1 Bacterial isolates

The strain with the best inhibitory activity, SW-1, grew on ZoBell's MB in the form of sub-round, straw yellow colonies. This bacterium was found to be a Gram-negative aerobic heterotrophic prokaryote that is widely distributed in the marine environment. The dimensions of the bacterium were 0.6–0.9 μm. The physiological and biochemical characteristics of strain SW-1 are shown in Table 1. Some characteristics of the SW-1 strain were identical to those previously reported for *P. piscicida* [17, 18].

## 3.2 Identification of the strain SW-1 by phylogenetic analysis

The 16S rDNA of strain SW-1 was amplified with primers 27f and 1492r. The genomic DNA is shown in Fig. 1. The almost-complete 16S rDNA gene sequence (1398 bp) of strain SW-1 was determined, and this sequence was submitted to GenBank (GenBank accession no. JX046040). The analysis of the 16S rDNA sequence indicated that the strain shared 99% identity with *Pseudoalteromonas*. In addition, a neighbor-joining tree (Fig. 2) clearly demonstrated that strain SW-1 was a member of *Pseudoalteromonas* at the 16S rDNA sequence homology level. It was therefore evident from the phylogenetic data that stain SW-1 represented a species of the genus *Pseudoalteromonas*.

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**Table 1.** The physiological and biochemical characteristics of strain SW-1 in comparison with those of *P. piscicida*

| Item | Strain SW-1 response | *P. piscicida*[a] response |
|---|---|---|
| Growth at | | |
| 4°C | + | + |
| 30°C | + | + |
| 35°C | + | + |
| 40°C | − | − |
| Gram stain | − | − |
| Straight rod | + | + |
| Motility | + | + |
| Polar flagella | + | + |
| Colony pigment | Yellow, insoluble in water | Yellow, insoluble in water |
| Oxidase | + | + |
| Gelatinase | + | − |
| Requires NaCl for growth | + | + |
| Utilization of | | |
| D-Glucose | + | + |
| Sucrose | − | − |
| Citrate | − | + |
| Lactose | − | − |
| M.R. reaction | − | − |
| Voges–Proskauer test | − | − |
| Indole production | − | − |
| $H_2S$ production | + | − |
| Amylase | − | − |

+, positive; −, negative.
a) Ivanova et al. [17] and Longeon et al. [18].

## 3.3 Optimal temperature, pH and salinity for the promotion of antagonistic action by *P.*-SW-1

According to the analysis of bacterial biomass, expressed as the absorbance at 600 nm ($OD_{600}$) (Fig. 3), strain SW-1 could grow at temperatures from 25 to 37°C (Fig. 3A). The highest biomass ($OD_{600} = 0.68$) was observed at 30°C. Within the temperature range of 25 to 30°C, the biomass increased with increasing temperature. When the temperature exceeded 30°C, the biomass decreased.

The effect of the NaCl concentration in the growth media is presented in Fig. 3B. ZoBell's MB containing 0.5 mol/L NaCl was optimum for growth, followed by ZoBell's MB containing 0.8 mol/L NaCl. Minimal growth was observed in ZoBell's MB containing 1.5 mol/L NaCl.

The pH range supporting the growth of strain SW-1 was 6.0–9.0. The highest biomass ($OD_{600} = 0.80$) was observed at pH 8.0, and at pH 5.0, there was no growth at all (Fig. 3C). At pH > 8.0, the biomass decreased.

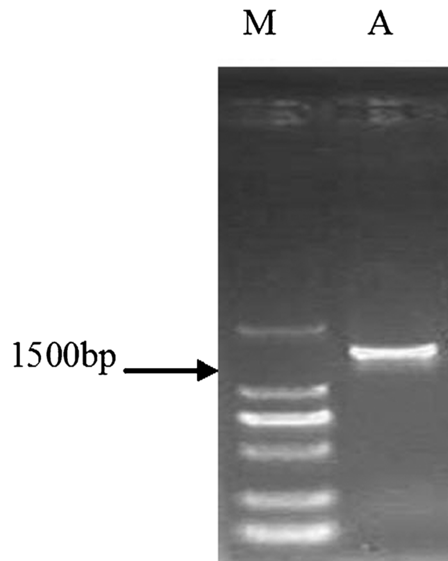


**Figure 1.** M is the marker, and A is the genomic DNA of strain SW-1.

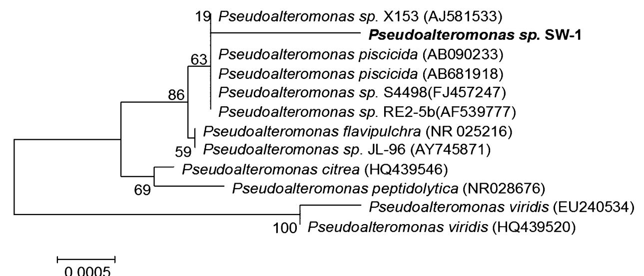


**Figure 2.** Phylogenetic tree based on comparison of the 16S rRNA gene sequences to determine the position of strain SW-1. The phylogenetic tree was generated using the neighbor-joining method. Bootstrap values, expressed as percentages of 1000 replications, are given at the branch points.

## 3.4 Growth characteristics of *P.*-SW-1

The resulting growth curves showed that in the range of 0–4 h, the absorbance at 600 nm of *P.*-SW-1 remained low (i.e., the bacterium was in the lag phase), and between 6 and 20 h, the biomass of *P.*-SW-1

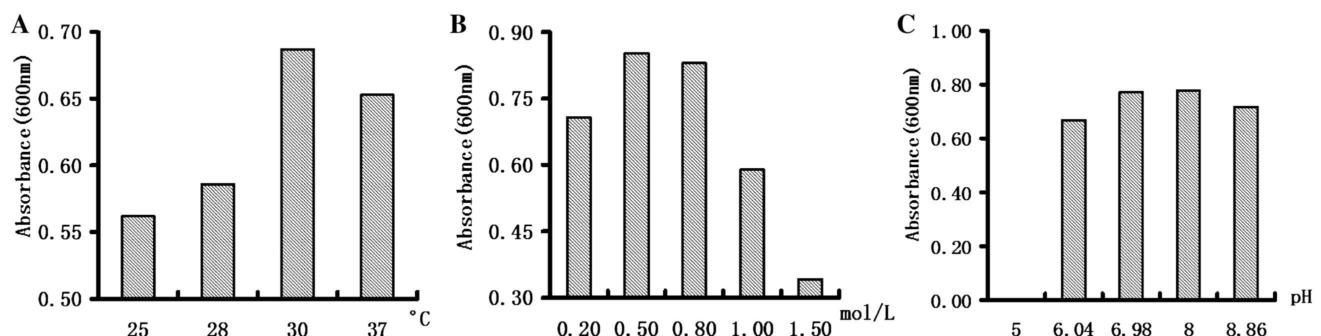


**Figure 3.** Effects of culture conditions (A, incubation temperature; B, incubation salinity; C, incubation pH) on the biomass of strain SW-1.

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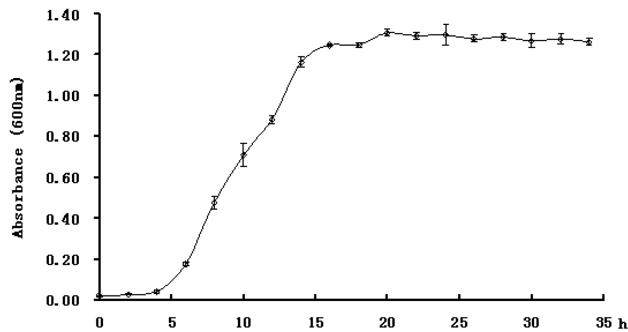


**Figure 4.** Growth curves for strain SW-1.

**Table 3.** Mortality of clams in virulence tests with different amounts of *P.*-SW-1 and *V.*-MP-1

| Bacterial species | Inoculating dose (CFU/mL) | Survivors/total (15 days) |
|---|---|---|
| Control | 0 | 13/15 |
| *V.*-MP-1 | $10^7$ | 0/15 |
| | $10^8$ | 0/15 |
| | $10^9$ | 0/15 |
| *P.*-SW-1 | $10^7$ | 15/15 |
| | $10^8$ | 15/15 |
| | $10^9$ | 15/15 |
| *V.*-MP-1 and *P.*-SW-1 | $10^8$ | 14/15 |
| | | 14/15 |
| | | 12/15 |

rapidly increased (logarithmic phase; Fig. 4). After 20 h, the biomass of the *P.*-SW-1 was stable.

## 3.5 Antibacterial testing

Strain *P.*-SW-1 showed good inhibition of *V. alginolyticus*, *Achromobacter* sp. YZ, and *Bacillus* J1 in paper-disk agar diffusion assay. The zone of inhibition was between 16 and 20 mm (Table 2).

## 3.6 Virulence tests

Virulence tests showed that the isolated strain *P.*-SW-1 was not pathogenic to clams. Over the 15-day test, the mortality of clams was zero, compared with 30% mortality in the control group (Table 3). Clams infected with $10^7$–$10^9$ CFU/mL of *V.*-MP-1 exhibited mortality of 100%. In contrast, clams infected simultaneously with $10^8$ CFU/mL of *V.*-MP-1 and *P.*-SW-1 exhibited an average mortality of only 11%.

Along the coast of the Yellow Sea in China, *M. meretrix* has become one of the most important commercial marine bivalves [2]. In the 1990s, mass mortality of *M. meretrix* due to unknown causes was reported [19]. Later, the *Vibrios* bacteria were identified as a major reason for the mass mortality of *M. meretrix*. However, the usages of antibiotics can cause water pollution, and indirectly affect human health [20]. So, Utilization of microorganisms is a very effective way to resist the pathogen hazards.

In this study, *P.*-SW-1 was isolated from seawater from a clam farm. The isolated *P.*-SW-1 strain was identified by morphological, physiological, and biochemical assays (Table 1), but these assays alone are never sufficient to confirm the taxonomic status. As suggested by Garrity et al. [21], 16S rDNA sequencing is the only alternative method for conclusive identification. Based on the partial 16S rDNA sequences and phylogenetic analysis using the neighbor-joining method, SW-1 is a member of the genus *Pseudoalteromonas* and is closely related to *P. piscicida*, *Pseudoalteromonas* sp. X153 and *Pseudoalteromonas* sp. S4498 (Fig. 2). This result clearly indicated that the SW-1 is very closely related to a strain *Pseudoalteromonas* sp. X153, which was isolated from a pebble collected at St. Anne du Portzic (France) and was found to be highly active against ichthyopathogenic *Vibrio* strains [18]. Therefore, strain SW-1 is a bacterial strain belonging to the species *P. piscicida*, a conclusion that is consistent with the results of the morphological and physiological assays.

**Table 2.** The zone of inhibition of *P.*-SW-1 for different strains

| Strain | Zone of inhibition (mm) | Average zone of inhibition (mm) |
|---|---|---|
| *Vibrio alginolyticus* | 17 | 16.7 |
| | 16 | |
| | 18 | |
| | 16 | |
| *Achromobacter* sp. YZ | 18 | 17.3 |
| | 17 | |
| | 18 | |
| | 16 | |
| *Bacillus* J1 | 19 | 19 |
| | 20 | |
| | 19 | |
| | 18 | |

The results presented in Table 2 indicate that *P.*-SW-1 could inhibit not only the *Vibrio* bacteria that are pathogenic to clams but also other strains, such as *Achromobacter* sp. YZ and *Bacillus* J1. Many studies have confirmed that species of *Pseudoalteromonas* display antagonistic activities against a variety of target organisms [18, 22, 23].

The antibacterial testing results showed that the *P.*-SW-1 isolate from the aquaculture farm caused a wide range of resistance to several strains tested. The data in Table 3 clearly show that *P.*-SW-1 was non-pathogenic to clams even at a very high level of $10^9$ cells/mL. After infection with *P.*-SW-1 for 15 days, clams exhibited improved survival following challenge with pathogenic bacteria (*V.*-MP-1). Robertson et al. [24] found that the administration of a single probiotic or a mixture of the three probiotics for 14 days resulted in increased levels of survival after challenge. The use of other strains as probiotics for protection against bacterial pathogens among fishes is well documented [25–28]. Thus, we believe that *P.*-SW-1 is a promising probiotic that can be used to treat farmed clams whenever *V.*-MP-1 counts rise to undesirable levels in aquaculture systems.

Additionally, virulence tests showed that the isolated strain *P.*-SW-1 was not pathogenic to clams, suggesting that *P.*-SW-1 could be safely used in aquatic farms to improve the survival of clams and the aquaculture environment.

# 4 Concluding remarks

It can be stated that the *P.*-SW-1 strain has the properties of a biocontrol agent for use in clam culture farms. There is also a potential to use this strain for the control of *Vibrio* spp. in aquaculture systems and maintain the clam aquaculture sustainable development in the future.

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