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Inhibition of marine *Vibrio* sp. by pyoverdine from *Pseudomonas aeruginosa* PA1

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HIGHLIGHTS

- Pyoverdine was purified and showed inhibitory effects on the growth of *Vibrio* sp.
- Pyoverdine affected the siderophore production and biofilm formation of *Vibrio* sp.
- The mechanism of inhibitory effect of pyoverdine was preliminary explored.
- Pyoverdine showed a RPS of 89% to *A. japonicas* when challenged with *V. splendidus*.

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ABSTRACT

Siderophores are low-molecular-weight chemicals that are secreted by many microorganisms to chelate iron from the external environment in order to facilitate their growth and diverse metabolisms. In this study, a fluorescent siderophore, pyoverdine, secreted by *Pseudomonas aeruginosa* PA1 was purified by affinity chromatography using Cu-sepharose. Pyoverdine was determined to have a molecular mass of 1333.54 Da, as determined by MALDI-TOF/TOF, and belong to type I pyoverdine, as determined by PCR analysis of its corresponding outer membrane ferri-pyoverdine receptor. Pyoverdine showed different degrees of inhibitory effects on the growth of marine *Vibrio* sp. strains. It was also shown that the biofilm developed by *Vibrio parahaemolyticus* WzW1 and Wz2121 and *Vibrio cyclitrophicus* HS12 was significantly reduced, alone with the repressed growth in the presence of pyoverdine. Siderophore production was determined in the strains of *Vibrio* sp. in response to the pyoverdine-induced iron-limited conditions. The siderophore production of most *Vibrio* sp. was up-regulated, with the exception of the bacteria that produced little siderophore. Furthermore, *Apostichopus japonicus* cultured in pyoverdine pretreated seawater showed a relative percent of survival of 89% when they were challenged by *Vibrio splendidus*. Our results demonstrated that pyoverdine may be a promising agent that could be potentially applied to treat vibriosis.

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1. Introduction

Vibrio sp. is a microbial genus that is ubiquitously found in aquatic ecosystems [1]. Isolates of *Vibrio alginolyticus*, *Vibrio harveyi*, *Vibrio parahaemolyticus* and *Vibrio splendidus* are important opportunistic pathogens that can infect a broad host range of cultured marine animals, including peneid shrimp, sea cucumber and fish of various species, leading to vibriosis in aquaculture [2–5]. For almost all bacteria, iron is an essential element and the cofactor of many important biological enzymes, including cytochrome reductase/oxidase, catalase, ferredoxin and iron-sulfur protein, which are involved in various vital biochemical cellular processes such as respiration, photosynthesis nitrogen fixation, DNA biosynthesis and resistance to reactive oxygen intermediates [6]. Despite the abundance of iron in the earth's crust, its normal concentrations of 10^{-17}–18^{-18} M in aerobic environments are not readily bioavailable for the rapid oxidization to ferric salts or precipitation under aerobic and neutral conditions, and these levels are far below the concentrations of 10^{-8}–18^{-6} M needed for the optimal growth of bacteria [7,8].

To quickly adapt to the ferric-limited environment, bacteria have evolved several strategies to scavenge iron from the external environment for their growth and colonization [9,10]. In most cases, heterotrophic bacteria synthesize and secrete

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ferric chelators, named siderophores, to scavenge iron and import it to maintain adequate intracellular levels of iron for bacterial metabolism [11,12]. Based on their chemical structures, siderophores are classified into four groups: catecholates (phenolates), hydroxamates, carboxylates (e.g., derivatives of citric acid) and mixed type [11,13]. After the ferri-siderophores complexes were formed, they are recognized by specific TonB-dependent outer membrane receptors (TBDRs), and then enter the intracellular space via an energy transduction complex, named the TonB system [14]. Among the more than 500 characterized siderophores, pyoverdines secreted by fluorescent *Pseudomonas* sp. with unique characteristics of fluorescence and high Fe^{3+} affinity are the most striking siderophores [14,15]. Pyoverdines are composed of a dihydroquinoline type chromophore responsible for the visualized blue-green fluorescence, a peptide of 6–12 aa connected to the carboxyl group of chromophore, and a side-chain bound to the N atom at C-3 of the chromophore [15]. The ferri-pyoverdines complex are recognized and transported into the intracellular space by specific TBDRs, the outer membrane ferri-pyoverdine receptors (Fpvs), whose specificity in binding and transporting cognate pyoverdines have been well established [16].

Consequently, the competition for iron between different microorganisms under iron-limited conditions and the importance of iron acquisition for bacterial pathogenesis in animals and plants led to the investigation of alternative efficient strategies to control and prevent disease [17,18]. The bacteria that produce siderophores with higher Fe^{3+} binding capacity have increasingly been used as biocontrol agents in both the aquaculture and agriculture. Previously, different strains of *Pseudomonas* sp. have been demonstrated to be used as biocontrol agents to inhibit pathogenic bacteria such as *Vibrio anguillarum*, *Erwinia carotovora*, *Alternaria canjani*, *Curvularia lunata*, *Fusarium* sp., *Bipolaris* sp., and *Helminthosporium* [19–21]. Pyoverdines use a combination of the catechol unit on the chromophore and hydroxamate- or hydroxy acid-containing amino acids on the peptide chain to chelate Fe^{3+}, with an association constant that is usually higher than10^{-20} M [22]. The high Fe^{3+} binding capacity of pyoverdine led us to wonder if pyoverdine can be used to inhibit the growth of marine bacteria, particularly the opportunistic *Vibrio* sp. pathogens, in aquaculture.

In our study, pyoverdine secreted by *Pseudomonas aeruginosa* PA1, an isolate from coastal seawater, was purified using Cu-agarose affinity chromatography. Its molecular mass and type classification were determined by MALDI-TOF/TOF and PCR amplification of *fpvs*, respectively. The effects of pyoverdine on the growth, biofilm formation and siderophore production of *Vibrio* sp. were determined and the inhibitory mechanism was preliminary explored. Furthermore, the possibility of pyoverdine to enhance the survival of *Apostichopus japonicus* after artificially challenged with *V. splendidus* was investigated.

2. Materials and methods

2.1. Bacterial strains, growth conditions, and chemicals

The bacterial strains used in this study and their respective isolation sources are listed in Table 1. All of these bacteria were identified by 16S rDNA, with PCR amplification using primers of 8F and 1492R, followed by nucleotide sequencing and BLAST searches in NCBI. The bacteria were cultured at 28 °C in 2216E medium consisting of 5 g/L tryptone, 1 g/L yeast extract and 0.01 g/L $FePO_4$ in aged seawater. Bacterial strains used to collect the cell-free supernatants (CFSs) were grown in Luria–Bertani (LB) medium [23] at 28 °C. To obtain a large amount of pyoverdine, *P. aeruginosa* PA1 was grown in synthetic succinic acid (SSA) medium consisting of 3.0 g/L KH_2PO_4, 6.0 g/L K_2HPO_4, 0.1 g/L $MgSO_4{\cdot}7H_2O$, 1.0 g/L $(NH_4)_2SO_4$ and 4.0 g/L succinic acid, with the pH adjusted to 7.0 using a 1 N NaOH solution before sterilization [24]. Unless otherwise stated, all chemicals used in this study were of the highest purity and were purchased from Sangon (Shanghai, China).

2.2. Preparation of CFSs, purification and MS characterization of pyoverdine

Acinetobacter cacolaceticus T32, *Pseudomonas putida* SP1, *Rhodococcus erythropolis* Re1, *Enterococcus faecalis* SW, *Endophytic bacillus* DJ61 and *P. aeruginosa* PA1 cells were separately cultured in LB medium for 24 h. The supernatants were obtained by centrifuging the culture at 10,000 × *g* for 5 min, and then filtered through 0.22-μm pore-size filter membranes to obtain the CFSs (Millipore). The CFSs were stored at −20 °C before testing their ability to inhibit the growth of *V. splendidus*, one of the representative opportunistic pathogens to cause skin ulceration syndrome (SUS) in the culture of *A. japonicus*.

The procedure used to purify pyoverdine was performed according to the method described by Yin et al. [25], with minor modification. Briefly, pyoverdine was bounded to the Cu-sepharose with high affinity and eluted with elution buffer consisting of 0.02 M sodium phosphate and 0.5 M NaCl, pH 3.5. The eluted pyoverdine was adjusted to pH 7.0 using NaOH. The molecular mass

Table 1
The strains used in this study.

Strains	Species	Sources
T32	*Acinetobacter cacolaceticus*	Isolated from coastal seawater
SP1	*Pseudomonas putida*	[27]
Re1	*Rhodococcus erythropolis*	Bought from CGMCC
SW	*Enterococcus faecalis*	Isolated from coastal seawater
DJ61	*Endophytic bacillus*	Isolated from coastal seawater
PA1	*Pseudomonas aeruginosa*	[25]
Wz2121	*Vibrio parahaemolyticus*	Isolated from diseased squid (This study)
WzW1	*Vibrio parahaemolyticus*	Isolated from diseased squid (This study)
Wz11	*Vibrio alginolyticus*	Isolated from diseased squid (This study)
Wz211	*Vibrio harveyi*	Isolated from diseased squid (This study)
Wz33	*Vibrio brasilliensis*	Isolated from diseased squid (This study)
Yt6	*Vibrio alginolyticus*	Isolated from coastal zone seawater (This study)
Yt8	*Vibrio cholerae*	Isolated from coastal zone seawater (This study)
HS0	*Vibrio splendidus*	Isolated from diseased sea cucumber (This study)
Vs	*Vibrio splendidus*	Isolated from diseased sea cucumber (This study)
HS311	Unidentified *Vibrio* sp.	Isolated from diseased sea cucumber (This study)
HS11	*Vibrio gigantis* sp. nov.	Isolated from diseased sea cucumber (This study)
HS12	*Vibrio cyclitrophicus*	Isolated from diseased sea cucumber (This study)

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