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Research Article

Characterization of the Potential Probiotic *Vibrio* sp. V33 Antagonizing *Vibrio splendidus* Based on Iron Competition

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Background: *Vibrio splendidus* Vs is an important aquaculture pathogen that can infect a broad host of marine organisms. In our previous study, an antagonistic bacterium *Vibrio* sp. V33 that possessed inhibitory effects on the growth and virulence of a pathogenic isolate *V. splendidus* Vs was identified.
Objectives: Here, we further explored the antagonistic substances and antagonistic effects from the viewpoint of iron competition.
Materials and Methods: The main antagonistic substances in the supernatants from *Vibrio* sp. V33 were identified using the bioassay-guided method. The response of *V. splendidus* Vs under the challenge of cell-free supernatant from *Vibrio* sp. V33 was determined via sodium dodecyl sulfate-polyacrylamide gel electrophoresis and real-time reverse-transcription PCR.
Results: The main antagonistic substances produced by *Vibrio* sp. V33 have low molecular weights, are water soluble, and are heat-stable substances. Meanwhile, the iron uptake rate of *Vibrio* sp. V33 was higher than that of *V. splendidus* Vs. In the presence of cell-free supernatant from *Vibrio* sp. V33, expressions of two functional genes, *viuB* and *asbJ* related to ferric uptake processes in *V. splendidus* Vs, were up-regulated, whereas fur_{Vs} coding the ferric uptake repressor was suppressed below 0.5-fold. One gene coding phosphopyruvate hydratase does not change at mRNA level, but was up-regulated at protein level.
Conclusions: Our results suggested that antagonistic effect of *Vibrio* sp. V33 on the pathogenic isolate *V. splendidus* Vs was partially due to the stronger ability of *Vibrio* sp. V33 to seize iron. This cell-free supernatant from *Vibrio* sp. V33 created an iron-limited milieu for *V. splendidus* Vs, which led to the changed expression profiles of genes that were related to iron uptake in *V. splendidus* Vs.
Keywords: Bacterial Antagonist; Iron Uptake;*Vibrio* sp. V33; *Vibrio splendidus* Vs.

1. Background

Along with the rapid expansion of aquaculture, various bacterial infections have increasingly emerged and hindered healthy aquaculture development (1). Usually, antibiotics are used to treat bacterial disease, but more and more showed that probiotics, such as *Aeromonas* sp., *Agarivorans* sp., *Bacillus* sp. and *Vibrio* sp. have increasingly played their important roles in protection of cultured animals from pathogenic bacterial infection (2, 3).

Iron is essential for many key enzymes, particularly those involved in citric acid cycle, glycolysis, and oxidative phosphorylation (4, 5). Therefore, it is required by nearly all known organisms for survival and growth. Siderophores are specific ferric ion chelators that were composed of low-molecular-weight molecules, and have high affinity to iron (6). It was ubiquitously secreted by bacteria, including gram-negative *Escherichia coli, Salmonella typhimurium, Pseudomonas* sp. and *Vibrio* sp., and gram-positive *Staphylococcus* sp. and *Bacillus* sp. (6), leading to competition for iron between different kinds of bacteria (7). Antagonistic effects of siderophore-producing bacteria when they were exposed to iron-depleted conditions were reported previously (8, 9). *Bacillus cereus* inhibited the growth of a fish pathogen *Aeromonas hydrophila* partly by competing for iron through siderophore release (10). Similarly, seven strains of *Streptomyces* sp. isolated from the marine sediments of a shrimp farm were found to inhibit the growth of *Vibrio* sp. *in vitro* by producing siderophores (11).

Ferric uptake regulators are conserved iron-uptake-related proteins that regulate siderophore biosynthesis and corresponding receptors in most prokaryotic organisms, and they function as regulators when bound with Fe^{2+} (12). The expression of ferric-uptake-

related genes in bacteria was changed with varied iron levels. The mRNA expression of the gene coding Fur_{Vs} down-regulated in the presence of iron chelator 8-hydroxyquinoline (13). However, the expression of other iron-uptake-related genes in *V. splendidus* under iron-limited conditions remains largely unknown.

2. Objective

In our previous study, the antagonistic bacterium *Vibrio* sp. V33 was identified and found to inhibit the growth and virulence of a pathogenic bacterium *V. splendidus* (14). In the present study, the main antagonistic substances in the supernatant from *Vibrio* sp. V33 were characterized, and differential expressions of iron-uptake-related genes in *V. splendidus* Vs at both mRNA and protein levels in the presence of supernatant containing antagonistic substances were determined.

3. Materials and Methods

3.1. Bacteria, Culture Media

Vibrio sp. V33 was isolated from healthy cuttlefish, *Sepia pharaonis* in our previous study (14). *V. splendidus* Vs was isolated from *Apostichopus japonicus* suffering from skin ulceration syndrome in the indoor farms of the Jinzhou Hatchery, and its pathogenicity was determined in our previous studies (15). *V. splendidus* Vs and *Vibrio* sp. V33 were cultured at 28 °C in modified 2216E media consisting of 5 $g.L^{-1}$ of tryptone and 1 $g.L^{-1}$ of yeast extract in aged seawater. Unless otherwise stated, all chemicals used in this study were purchased from Sangon (Shanghai, China).

3.2. Growth Inhibition Assay

Vibrio sp. V33 was grown to OD_{600} of 0.7, 0.8, or 0.9, measured using a UV–Vis spectrophotometer (Beckman, USA). Then, supernatants were collected by centrifuged at 12000 × *g* and 4 °C for 5 min and they were filtered through 0.22-μm polycarbonate membrane filters (Millipore) to prepare the cell-free supernatant. The same volumes of different cell-free supernatants were separately added into cell pellet of *V. splendidus* Vs to make cell suspensions of the same concentrations. *V. splendidus* Vs suspensions in fresh media were used as a control. After incubated for 12 h, OD_{600} of each culture was measured using a UV–Vis spectrophotometer.

3.3. Characterization of Antagonistic Substances

Water solubility and organic solubility of the antagonistic substances were determined using the method developed by Jorquera *et al*. (16). Briefly, 500 mL cell-free supernatant of *Vibrio* sp. V33 was extracted with the same volumes of ethyl acetate and then evaporated at 42 °C to concentrate 100-fold. Then, 5 μL concentrated solution and the un-concentrated supernatant were separately added to the *V. splendidus* Vs suspensions. After incubated for 8 h, OD_{600} of each culture was measured using a UV–Vis spectrophotometer.

The molecular weight range of the antagonistic substances was estimated using the method of Chythanya *et al*. (17). Briefly, the cell-free supernatant from *Vibrio* sp. V33 was centrifuged using a 3-kDa molecular weight cut-off (MWCO) tubes at 6000 × *g* for 30 min at 4 °C. Two fractions (>3 kDa residue and <3 kDa filtrate) were collected. However, because the centrifuge tubes can only concentrate molecules with molecular mass >3 kDa, and the small molecular substances (<3 kDa) remained in the >3 kDa residue fraction. Moreover, the <3 kDa substances in both the residue filtrate fractions were the same. The same volume of the two fractions was added into *V. splendidus* Vs. Fresh media was also added into *V. splendidus* Vs and used as a control. After incubated for 8 h, OD_{600} of each culture was measured using a UV–Vis spectrophotometer. The inhibitory activity (I.A.) was measured as follows:

$$I.A.= 100-100\times\frac{OD_{600(a)}}{OD_{600(b)}}$$, where (a) is the treatment and (b) is the control.

3.4. Iron Uptake Rates Measurement

Ion uptake rates of *Vibrio* sp. V33 and *V. splendidus* Vs were measured using the method described by Lalloo *et al*. (18). Subsequently, 500 μL *Vibrio* sp. V33 or *V. splendidus* Vs were separately inoculated into 2216E media containing 0.01 $g.L^{-1}$ $FePO_4$ and each culture was grown at 28 °C. Samples were obtained every 12 h and centrifuged to collect supernatants for the remaining iron contents. Iron concentration was determined using a serum iron assay kit purchased from Nanjing Jiancheng Biochemistry (China).

3.5. Identification of Differentially expressed proteins by SDS-PAGE Combined with MALDI-TOF Mass Spectrometry

V. splendidus Vs was grown in 2216E media to an OD_{600} of approximately 0.2. Then, 1 mL cell-free supernatant of *Vibrio* sp. V33 was added to 3 mL of *V. splendidus* Vs culture. Fresh media was also added into *V. splendidus* Vs culture and was used as a control. After grown for another 2 h, the culture was centrifuged at 8000 × *g* for 10 min. The cell pellets were collected and suspended

in cell lysate. The distinct bands between the sample treated with and without cell-free supernatant of *Vibrio* sp. V33 on the gel were collected and subjected to MALDI-TOF/TOF MS for protein identification. MS was performed on an ABI 5800 MALDI-TOF/TOF Plus mass spectrometer (Applied Biosystems, Foster City, USA). Data were acquired through a positive MS reflector by using a CalMix5 standard for the calibration of the instrument (ABI5800 calibration mixture).

3.6. Real-Time Reverse-Transcription PCR (Real-Time RT-PCR)

V. splendidus Vs was grown in 2216E media to an OD_{600} of approximately 0.2. Then, 10 mL <3 kDa filtrate from *Vibrio* sp. V33 supernatant (described above) was added to 40 mL culture of *V. splendidus* Vs. The same volume of fresh media was added to another aliquot of *V. splendidus* Vs and was used as a control. After inoculated for 10, 20, and 30 min, 2 mL cell pellet was collected. RNA was extracted from cells collected at different time points using a Bacterial RNA isolation kit (Omega) and then treated with RNase-free DNase. cDNA generated from 1 μg of DNase-treated RNA with PrimeScript reverse transcriptase (Takara) was used for real-time RT-PCR. Each assay was performed in triplicate with the 16S rRNA as internal control. The primers shown in **Table 1** were designed on the basis of our genomic sequence of *V. splendidus* Vs. SYBR Premix Ex Taq (Takara) was used for the real-time RT-PCR reactions in an ABI 7500 real-time detection system (Applied Biosystems) as described previously (13). Dissociation analysis was performed on the amplification products at the end of each PCR to make sure that only one PCR product was amplified and detected. The comparative threshold cycle method ($2^{-\Delta\Delta CT}$ method) was used for the analysis of the mRNA levels. The expression of *fur* gene (the ferric uptake regulator, Supporting Information **Fig. S1**), *asbJ* gene (siderophore ABC transporter, Supporting Information **Fig. S2**) and *viuB* gene (one siderophore-interacting protein, Supporting Information **Fig. S3**) from *V. splendidus* Vs was compared with that of expression in the cells before treatment, which was used as 100% expression.

Table 1. Primers used in this study.

Primer Name	Nucleotide Sequence (5′→3′)	Products
RTF3	ACAACAACCAGACTGCCAACA	223 bp
RTR3	GATAACTTCACCGCAGTCTAAACAT	
ASBJF	GCGTTATTCATCGGGCAGGG	168 bp
ASBJR	GCCCAGGATGGATGGGGTTAG	
ViuBF	CAGAATGTGCTGGCGGCTAC	216 bp
ViuBR	CCACTTCTGCGTTCATTGCC	
ENOF	AAGAAGGTCTGCGTATCGGC	205 bp
ENOR	GCACAGTCCATAGCAAGAGTAAC	
933F	GCACAAGCGGTGGAGCATGTGG	300 bp
16SRTR1	CGTGTGTAGCCCTGGTCGTA	

3.7 Strain No. of Bacterial Strains

The isolates of *Vibrio* sp. V33 and *V. splendidus* Vs were deposited into the China General Microbiological Culture Collection (CGMCC, Beijing, China) http://www.cgmcc.net/english/ (19) with strain No. 12561 and 7.242, respectively.

3.8 Database Search and Statistical Analysis

Statistical analyses were performed by using the two-tailed *t*-test. Statistical significance was determined by one-way ANOVA (20). In all cases, the significance level was defined as * $P < 0.05$ and ** $P < 0.01$. The promoter prediction was conducted as the method described previously (21). The Fur binding DNA sequence used was 5′-GATAATGAT(A/T)ATCATTATC-3′ (22).

4. Results

4.1. Inhibitory Effect of Cell-Free Supernatant from Vibrio sp. V33

All the three supernatants collected from *Vibrio* sp. V33 at different densities showed inhibitory effects on the growth of *V. splendidus* Vs. OD_{600} was reduced to approximately 41.2%, 21.1% and 50.8% when the

added supernatants were collected from *Vibrio* sp. V33 at the cell densities of 7.0×10^8, 8.0×10^8, and 9.0×10^8 CFU.mL^{-1}, respectively (**Fig. 1**). This result suggests that the *Vibrio* sp. V33 secreted the antagonistic substances into its extracellular milieu, which showed a strong inhibitory effect on growth of *V. splendidus* Vs.

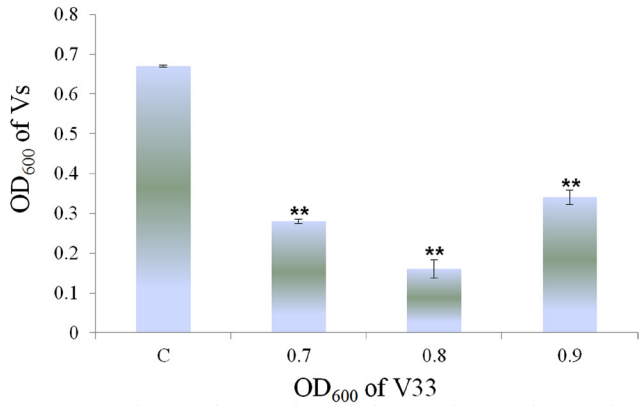


Fig 1. Antagonistic activity of the cell-free culture supernatants from *Vibrio* sp. V33 collected at different cell densities on the growth of *V. splendidus* Vs. The growth of *V. splendidus* Vs without inoculation of supernatant from *Vibrio* sp. V33 was used as control. Data are the means for at least three independent experiments and are presented as the means±SE.

4.2. Characterization of Antagonistic Substances

The relative I.A. of the antagonistic substances in the un-concentrated water phase and 100-fold concentrated organic phase were approximately 0.5- and 0.4-fold of that of the untreated cell-free supernatant (**Fig. 2A**). Thus, the substances in the water phase showed stronger antagonistic effect on growth of *V. splendidus* Vs than that in the 100-fold concentrated substance in the ethyl acetate extract, suggesting that the main antagonistic component was in the water-soluble phase. Similarly, when the cell-free supernatant collected from *Vibrio* sp. V33 was departed using the cut-off method at 3 kDa, the relative I.A. of the substances in the <3 kDa filtrate (un-concentrated) and >3 kDa (50-fold concentrated) residue were approximately 0.4 and 0.25-fold, respectively (**Fig. 2B**). The result suggested that the <3 kDa filtrate substances showed a much stronger antagonistic effect on *V. splendidus* Vs than the >3 kDa residue that contained the concentrated >3 kDa plus the unconcentrated <3 kDa substances. Thus, the main antagonistic substances in the supernatant from *Vibrio* sp. V33 were water soluble substances with molecular mass of less than 3 kDa.

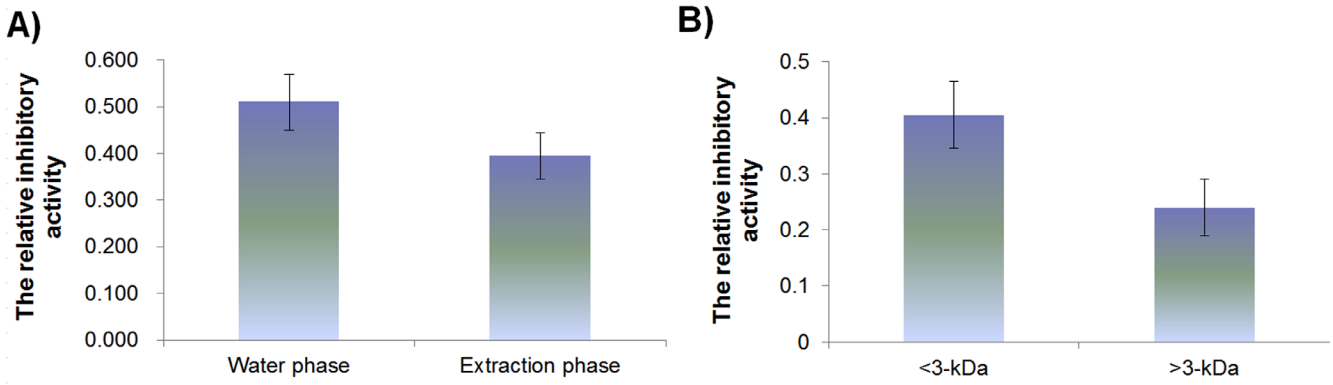


Fig 2. (A) I.A. ratio of the substances in the water phase and 100-fold concentrated organic phase. (B) I.A. ratio of the substances in the <3 kDa filtrate and >3 kDa residue.

4.3. Determination of Iron Uptake Rates of Vibrio sp. V33 and V. splendidus Vs

When *Vibrio* sp. V33 and *V. splendidus* Vs were grown for 12 h, the remaining iron concentrations in both cultures were around 2.09 mg.L^{-1} and no significant difference was observed (**Fig. 3**). Both the growth of *Vibrio* sp. V33 and *V. splendidus* Vs needed iron, because the remaining iron concentrations in the *Vibrio* sp. V33 and *V. splendidus* Vs cultures after cultured for 24 h were 1.79 and 1.91 mg.L^{-1}, respectively. The iron uptake rate of *V. splendidus* Vs was slower than that of *Vibrio* sp. V33 by approximately 0.01 mg.L^{-1}·h^{-1}. The significant difference in iron content appeared after cultured to 36 h, at this time point the iron uptake rates of *Vibrio* sp. V33 and *V. splendidus* Vs were 0.039 and 0.014 mg.L^{-1}·h^{-1}, respectively. *Vibrio* sp. V33 possessed a higher iron uptake rate of 0.025 mg.L^{-1}·h^{-1} than that of *V. splendidus* Vs. Our results suggested that *Vibrio* sp. V33 was stronger in the ability to compete for iron and caused an iron deprivation the environment for *V. splendidus* Vs.

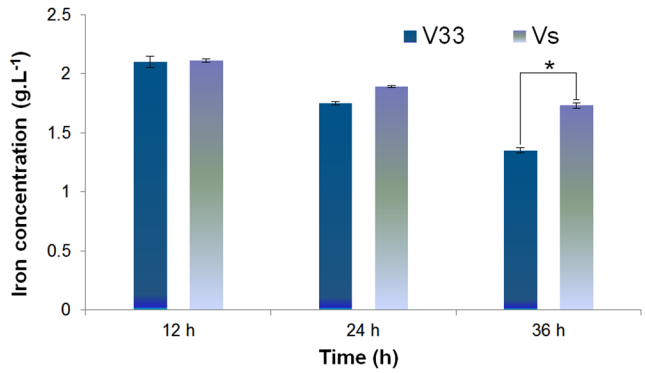


Fig 3. Iron concentrations remained in the culture after growth of *V. splendidus* Vs and *Vibrio* sp. V33 at different time points. Data are the means for at least three independent experiments and are presented as the means±SE.

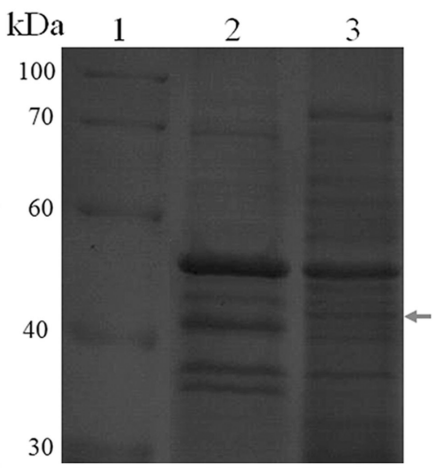
4.4. Analysis of Differentially Expressed Proteins

The differentially expressed proteins in *V. splendidus* Vs cell grown in the presence or absence of the cell-free supernatant of *Vibrio* sp. V33 were determined using SDS-PAGE. One distinctive band with an approximate molecular weight of 50 kDa was detected (**Fig. 4B**). The expression of this protein was up-regulated after treated for 2 h. The protein was identified based on the fragments obtained from MS and our genomic sequence of *V. splendidus* Vs, as well as its blast in NCBI. It showed a 99.8% sequence identity to a phosphopyruvate hydratase from *V. splendidus* (gi|490 873392|ref|WP_004735393.1|; E=0) (**Fig. 4A**), except that F422 was substituted by Y422 in phosphopyruvate hydratase from *V. splendidus* Vs. The nucleotide sequence of its coding gene corresponds to the *eno* gene (Supporting Information **Fig. S4**). However, no significant difference was noted at the mRNA level between the cells treated with cell-free supernatant of *Vibrio* sp. V33 and the control sample (**Fig. 4**C), implying that the cell-free supernatant of *Vibrio* sp. V33 influenced the expression of phosphopyruvate hydratase at the protein level rather than at the mRNA level under the tested conditions.

A)

>WP_004735393.1 MULTISPECIES: phosphopyruvate hydratase [Vibrio]
MSKIVKVLGREIIDSRGNPTVEAEVHLEGGFVGMAAAPSGASTGSREALELRDGDKSRFLGKGVLKAIEA
VNGPIADALVGKDAKAQADVDQVMLDLDGTENKSKFGANAILAVSLANAKAAAAAKGMPLYEHIAELNGT
AGQFSMPLPMMNIINGGEHADNNVDIQEFMIQPVGAKTLKEGLRIGAEVFHNLAKVLKSKGYSTAVGDEG
GFAPNLKSNAEALEVIAEAVAAAGYELGKDVTLAMDCAASEFFDKEAGIYNMKGEGKTFTSEEFNHYLAE
LANNFPIVSIEDGLDESDWDGFKHQTELLGDKLQIVGDDLFVTNTKILAEGIEKGVANSILIKFNQIGSL
TETLAAIKMAKDAGYTAVISHRSGETEDATIADLAVGTAAGQIKTGSMSRSDRVAKYNQLIRIEEALGSK
APFNGLKEVKGQ

B)



C)

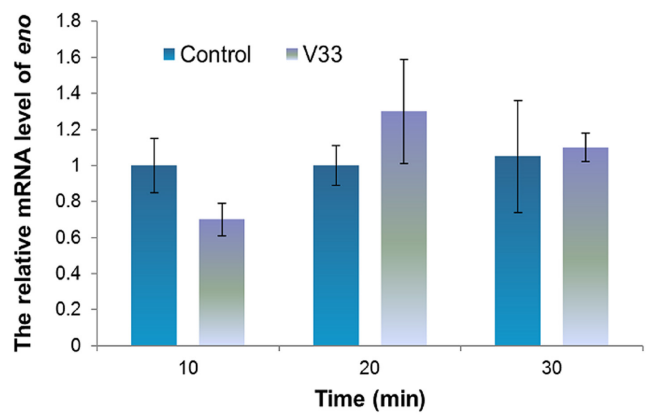


Fig 4. (A) Protein sequences of phosphopyruvate hydratase from *Vibrio* sp. **(B)** SDS-PAGE analysis of the differentially expressed protein in the cells treated with and without cell-free supernatant form *Vibrio* sp. V33. Lane 1, the protein marker; lane 2, the proteins from the *V. splendidus* Vs without supernatant treatment (control); lane 3, the proteins from the *V. splendidus* Vs treated with cell-free supernatant collected from *Vibrio* sp. V33. **(C)** Expression of the eno gene in the presence of the cell-free culture supernatants from *Vibrio* sp. V33. Data are the means for at least three independent experiments and are presented as the means±SE.

4.5. Expression of Iron-Uptake-Related Genes in V. splendidus Vs

Expression of fur_{Vs} was down-regulated to 0.06-, 0.48-, and 0.30-fold at the mRNA level after treatment with the cell-free supernatant from *Vibrio* sp. V33 for 10, 20 and 30 min, respectively (**Fig. 5A**). In *V. splendidus* Vs, there also existed an *asbJ* gene coding a protein with a 97% sequence identity to a siderophore ABC transporter from *V. splendidus* 12B01 (gi|490869579|ref|WP_004731594.1; E=0), and a *viuB* gene coding a protein with a 93% sequence identity to a siderophore-interacting protein ViuB from *Vibrio cyclitrophicus* (gi|498114642|ref|WP_010428798.1; E=1.2416E-180). The mRNA level of *asbJ* in the cells treated with the cell-free supernatant from *Vibrio* sp. V33 was down-regulated to 0.51- and 0.6-fold at 10 and 20 min, respectively, but up-regulated to 1.69-fold at 30 min (**Fig. 5B**). Similarly, the mRNA level of *viuB* in cells treated with the cell-free supernatant from *Vibrio* sp. V33 was down-regulated to 0.91- and 0.65-fold after 10 and 20 min, respectively, but up-regulated to 1.55-fold after 30 min (**Fig. 5C**). Apparently, the core regulator in the iron uptake process, Fur, was the most affected by iron level and was down-regulated earlier, but the expressions of functional genes had a time lag to be up-regulated. These results suggest that the expression profiles of the iron-uptake-related genes were affected by the cell-free supernatant from *Vibrio* sp. V33. This observation strengthened our speculation that the cell-free supernatant from *Vibrio* sp. V33 may inhibit the growth of *V. splendidus* Vs through creating an iron deficient environment.

4.6. Sequence Analysis of Up-Stream of the Functional Genes

The promoter regions and transcription factor binding sites of the functional genes, *viuB*, *asbJ*, and *eno* were analyzed. The 1 kb upstream regions from the start codon ATG of the three functional genes were used for analyses. BPROM prediction suggested that the upstream regions of the three functional genes contained the typical promoter regions that include the −35 and −10 domains (**Fig. S4**). The sequence of the Fur binding site was also searched adjacent to the −35 and −10 domains. Fur_{Vs} binding site was presented in both the promoters of P_{viuB} and P_{asbJ}, but not in the promoter of P_{eno}. This notion further indicated that the regulator Fur_{Vs} may control the expression of *viuB* gene and *asbJ* gene, however, the expression regulation of *eno* may not occur at mRNA level.

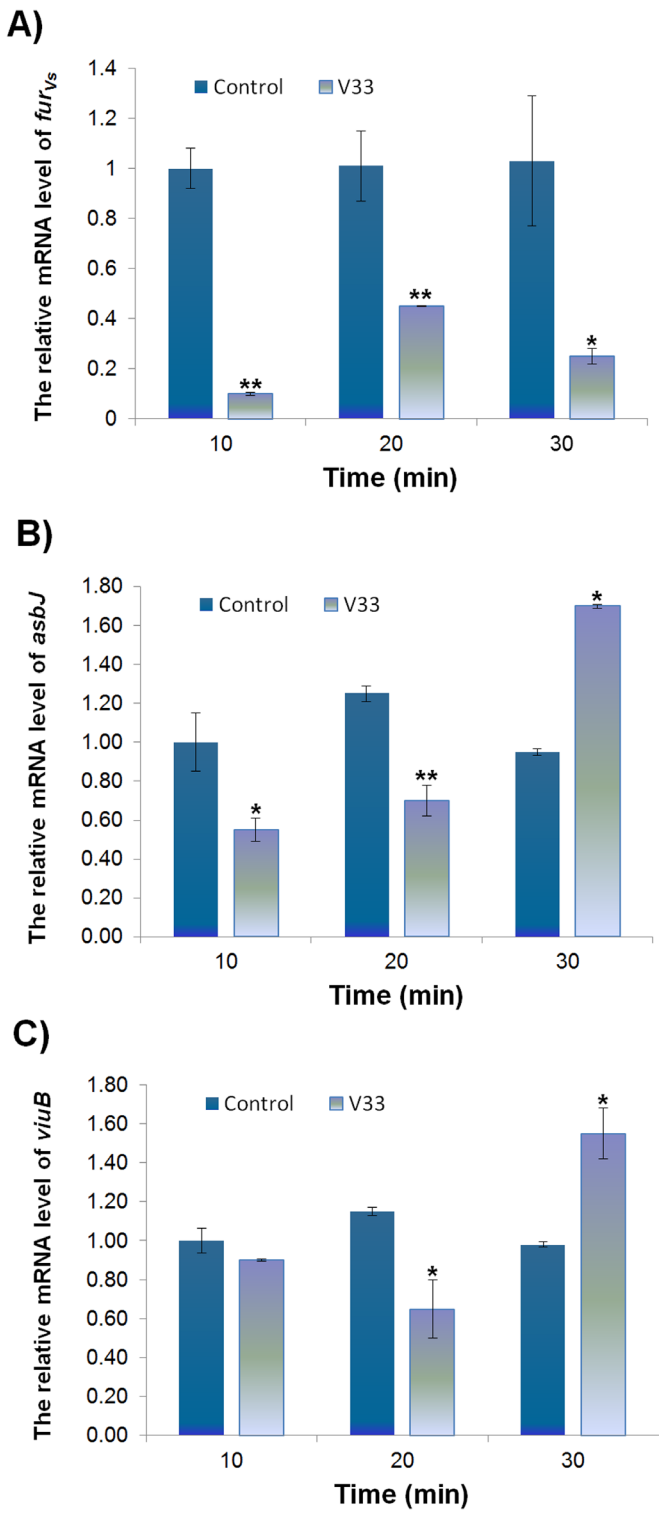


Fig 5. Expression of iron-homeostasis-related genes in the presence of the cell-free culture supernatants of *Vibrio* sp. V33. Data are the means for at least three independent experiments and are presented as the means±SE.

5. Discussion

Competition for iron via siderophore piracy is an important antagonistic mechanism that is utilized by potential probiotic bacteria to inhibit various pathogenic infections (7, 10, 23). In our previous study, we found that the antagonistic bacterium *Vibrio* sp. V33 produced more siderophores than that by *V. splendidus* Vs (14). In the present study, we further pointed out that the main antagonistic substances of *Vibrio* sp. V33 were thermostable and water soluble, and had low molecular weights. Combined with the higher iron uptake rate of *Vibrio* sp. V33, we speculated that the inhibitory effect of *Vibrio* sp. V33 was similar to that of *Vibrio* sp. E, which inhibited a non-siderophore producing strain of *V. splendidus*, vibrio P, also partly due to the high ability to compete for iron (24).

In the present study, the protein level of one phosphopyruvate hydratase (enolase) was up-regulated in *V. splendidus* Vs in the presence of the cell-free supernatant from *Vibrio* sp. V33. The involvement of phosphopyruvate hydratase in the iron uptake process was consistent with the up-regulation expression profiles of α-enolase in *Bacteroides fragilis* under iron-limited conditions (25). This finding is also supported by other reports that correlate dietary iron deficiency with the regulation of the glycolytic pathway (26, 27). The increased enolase expression in glycolysis suggests the necessity for sufficient ATP production under iron-limited conditions (28).

A previous study suggested that competition for iron via siderophore piracy can affect the gene expression patterns during bacterial interactions (29). Fur affects siderophore production in many bacterial species and controls the expression of most iron-uptake-related functional genes, such as those codes the siderophore ATP-binding cassette (ABC) transporter *asbJ* and the siderophore-receptor *viuB*, which were involved in ferric vibriobactin uptake or utilization (30-33). In the present study, the expressions of iron-related genes in *V. splendidus* were determined in the presence of antagonistic substrates. fur_{Vs} expression was reduced in the presence of the cell-free supernatant of *Vibrio* sp. V33, which was the same to the phenomena that observed under iron-limited conditions created by 8-hydroxyquinoline (13). This result further strengthened our speculation that *Vibrio* sp. V33 inhibited the pathogenic isolate *V. splendidus* Vs through creation of the iron-limited circumstance. Along with the reduced mRNA level of fur_{Vs}, the up-regulated mRNA levels of *viuB* and *asbj* and the presence of Fur_{Vs} binding sites in the two promoters suggested that the antagonistic effect of *Vibrio* sp. V33 may perform through iron competition. Such effect was similar to the probiotic influence of *Pseudomonas fluorescens* toward *Vibrio anguillarum* (34) and *Aeromonas salmonicida* (35,36) and that of *Pseudomonas* sp. and *Psychrobacter* sp. toward *Vibrio anguillarum* and *A. salmonicida* subsp. *Salmonicida* (36,37). In our present study, under iron-limited circumstance created by *Vibrio* sp. V33, the up-regulation of phosphopyruvate hydratase, a multifunctional protein contributing to glycolysis/gluconeogenesis and other biological pathophysiological processes, was consistent with that observed in *Cryptococcus gattii* (38). However, our study further highlighted that the up-regulation occurred at the protein level and not directly regulated at mRNA level by the most important iron uptake regulator Fur.

6. Conclusions

Vibrio sp. V33 has previously been identified to be an antagonistic bacterium of a pathogenic isolate *V. splendidus* Vs, but none of its antagonistic substances has been characterized. It was determined that the iron uptake rate of *Vibrio* sp. V33 was higher than that of *V. splendidus* Vs, which was also supported by the following points: on one hand, the active tracking method showed that the main antagonistic substances produced by *Vibrio* sp. V33 were of low molecular weights, water soluble, and heat-stable, which belonged to the characteristics of siderophores. On the other hand, the expressions of two functional genes, *viuB* and *asbJ* related to iron uptake processes in *V. splendidus* Vs were upregulated, which meant that the iron uptake pathway was involved in the interaction between *Vibrio* sp. V33 and *V. splendidus* Vs. All of the data indicated that competition for iron may be the main antagonistic process of the *Vibrio* sp. V33

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Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Lafferty KD, Harvell CD, Conrad JM, Friedman CS, Kent ML, Kuris AM, Powell EN, Rondeau D, Saksida SM. Infectious

diseases affect marine fisheries and aquaculture economics. *Ann Rev Mar Sci*. 2015;**7**:471-496. doi: 10.1146/annurev-marine-010814-015646
2. Newaj-Fyzul A, Al-Harbi AH, Austin B. Review: Developments in the use of probiotics for disease control in aquaculture. *Aquaculture*. 2014;**431**(2):1-11. doi: 10.1016/j.aquaculture.2013.08.026
3. Hai NV. The use of probiotics in aquaculture. *J Appl Microbiol*. 2015;**119**(4):917–935. doi: 10.1111/jam.12886
4. Konhauser KO, Kappler A, Roden EE. Iron in microbial metabolisms. *Elements.* 2011;**7**(27):89-93. doi: 10.2113/gselements.7.2.89
5. Fourquez M, Devez A, Schaumann A, Gueneugues A, Jouenne T, Obernosterer I, Blain S. Effects of iron limitation on growth and carbon metabolism in oceanic and coastal heterotrophic bacteria. *Limnol Oceanogr.* 2015;**59**(2):349-360. doi: 10.4319/lo.2014.59.2. 0349
6. Hider RC, Kong XL. Chemistry and biology of siderophores. *Nat Prod Rep.* 2010;**27**(5):637-657. doi: 10.1039/b906679a
7. Fgaier H, Eberl HJ. Antagonistic control of microbial pathogens under iron limitations by siderophore producing bacteria in a chemostat setup. *J Theor Biol*. 2011;**273**(1):103-114. doi: 10.1016/j.jtbi.2010.12.034
8. Gram L, Loevold T, Nielsen J, Melchiorsen J, Spanggaard B. *In vitro* antagonism of the probiont *Pseudomonas fluorescens* strain AH2 against *Aeromonas salmonicida* does not confer protection of salmon against furunculosis. *Aquaculture.* 2001;**199**(1):1-11. doi: 10.1016/s0044-8486(01)00565-8
9. Spanggaard B, Huber I, Nielsen J, Sick EB, Pipper CB, Martinussen T, Slierendrecht WJ, Gram L. The probiotic potential against vibriosis of the indigenous microflora of rainbow trout. *Environ Microbiol*. 2001;**3**(12):755-765. doi: 10.1046/j.1462-2920.2001.00240.x
10. Lalloo R, Moonsamy G, Ramchuran S, Görgens J, Gardiner N. Competitive exclusion as a mode of action of a novel *Bacillus cereus* aquaculture biological agent. *Lett Appl Microbiol*. 2010;**50**(6):563-570. doi: 10.1111/j.1472-765x.2010.02829.x
11. You JL, Cao LX, Liu GF, Zhou SN, Tan HM, Lin YC. Isolation and characterization of actinomycetes antagonistic to pathogenic *Vibrio* spp. from nearshore marine sediments. *World J Microbiol Biotechnol.* 2005;**21**(5):679-682. doi: 10.1007/s11274-004-3851-3
12. Troxell B, Hassan HM. Transcriptional regulation by ferric uptake regulator (Fur) in pathogenic bacteria. *Front Cell Infect Microbiol.* 2013;**3**:59. doi: 10.3389/fcimb.2013.00059
13. Liang WK, Zhang SS, Zhang WW, Li CH, Han QX. Characterization of functional fur_{Vs} as a biomarker for detection of aquatic pathogen *Vibrio splendidus*. *J World Aquacult Soc*. 2016;**47**(4):577-586. doi: 10.1111/jwas.12288
14. Liu NN, Zhang SS, Zhang WW, Li CH. *Vibrio* sp. 33 a potential bacterial antagonist of *Vibrio splendidus* pathogenic to sea cucumber (*Apostichopus japonicus*). *Aquaculture*. 2017;**470**:68-73. doi: 10.1016/j.aquaculture.2016.12.028
15. Zhang WW, Liang WK, Li CH. Inhibition of marine *Vibrio* sp. by pyoverdine from *Pseudomonas aeruginosa* PA1. *J Hazard Mater*. 2016;**302**:217-224. doi: 10.1016/j.jhazmat.20015.10.003
16. Jorquera MA, Riquelme CE, Loyola LA, Munoz LF. Production of bactericidal substances by a marine vibrio isolated from cultures of the scallop *Agropecten purpuratus*. *Aquacult Int.* 2000;**7**(6):433-448. doi:10.1023/A:1009246007208
17. Chythanya R, Karunasagar I, Karunasagar I. Inhibition of shrimp pathogenic vibrios by a marine *Pseudomonas* I-2 strain. *Aquaculture.* 2002;**208**(1):1-10. doi:10.1016/s0044-8486(01)00714-1
18. Lalloo R, Moonsamy G, Ramchuran S, Görgens J, Gardiner N. Competitive exclusion as a mode of action of a novel *Bacillus cereus* aquaculture biological agent. *Lett Appl Microbiol*. 2010;**50**(6):563-570. doi: 10.1111/j.1472-765x.2010.02829.x
19. Wu L, McCluskey K, Desmeth P, Liu S, Hideaki S, Yin Y, Moriya O, Itoh T, Kim CY, Lee JS, Zhou Y, Kawasaki H, Hazbón MH, Robert V, Boekhout T, Lima N, Evtushenko L, Boundy-Mills K, Bunk B, Moore ERB, Eurwilaichitr L, Ingsriswang S, Shah H, Yao S, Jin T, Huang J, Shi W, Sun Q, Fan G, Li W, Li X, Kurtböke I, Ma J. The global catalogue of microorganisms 10K type strain sequencing project: closing the genomic gaps for the validly published prokaryotic and fungi species. *Gigascience*. 2018;**7**(5). doi: 10.1093/gigascience/giy026
20. Mollah MM, Jamal R, Mokhtar NM, Harun R, Mollah MN. A hybrid one-way anova approach for the robust and efficient estimation of differential gene expression with multiple patterns. *PLoS One*. 2015;**10**(9):e0138810. doi: 10.1371/journal.pone.0138810
21. Umarov RK, Solovyev VV. Recognition of prokaryotic and eukaryotic promoters using convolutional deep learning neural networks. *PLoS One*. 2017;**12**(2): e0171410. doi: 10.1371/journal.pone.0171410
22. Escolar L, Pérez-Martín J, de Lorenzo V. Binding of the Fur (ferric uptake regulator) repressor of *Escherichia coli* to arrays of the GATAAT sequence. *J Mol Biol*. 1998;**283**(3):537-547. doi: 10.1006/jmbi.1998.2119
23. Lamb AL. Breaking a pathogen's iron will: Inhibiting siderophore production as an antimicrobial strategy. *Biochim Biophys Acta*. 2015;**1854**(8):1054-1070. doi: 10.1016/j.bbapap.2015.05.001
24. Gatesoupe FJ. Siderophore production and probiotic effect of *Vibrio* sp. associated with turbot larvae, *Scophthalmus maximus*. *Aquat Living Resour*. 1997;**10**(4):239-246. doi: 10.1051/alr:1997026
25. Sijbrandi R, Den Blaauwen T, Tame JR, Oudega B, Luirink J, Otto BR. Characterization of an iron-regulated alpha-enolase of *Bacteroides fragilis*. *Microbes Infect*. 2005;**7**(1):9-18. doi: 10.1016/j.micinf.2004.09.013
26. Kitamura M, Takayama Y, Kojima S, Kohno K, Ogata H, Higuchi Y, Inoue H. Cloning and expression of the enolase gene from *Desulfovibrio vulgaris* (Miyazaki F). *Biochim Biophys Acta.* 2004;**1676**(2):172–181. doi: 10.1016/j.bbaexp.2003.11.008
27. Tosco A, Siciliano RA, Cacace G, Mazzeo MF, Capone R, Malorni A, Leone A, Marzullo L. Dietary effects of copper and iron deficiency on rat intestine: a differential display proteome analysis. *J Proteome Res*. 2005;**4**(5):1781-1788. doi: 10.1021/pr0501012
28. Sun B, Wang Z, Zhu F. Molecular cloning of kuruma shrimp *Marsupenaeus japonicus* phosphopyruvate hydratase and its role in infection by white spot syndrome virus and *Vibrio alginolyticus*. *Aquaculture*. 2016;**455**:87-96. doi: 10.1016/j.aquaculture.2016.01.002
29. Traxler MF, Seyedsayamdost MR, Clardy J, Kolter R. Interspecies modulation of bacterial development through iron competition and siderophore piracy. *Mol Microbiol.* 2012;**86**(3):628-644. doi: 10.1111/mmi.12008
30. Butterton JR, Calderwood SB. Identification, cloning, and sequencing of a gene required for ferric vibriobactin utilization

by *Vibrio cholerae*. *J Bacteriol.* 1994;**176**(18):5631-5638. doi: 10.1128/jb.176.18.5631-5638

31. Köster W. ABC transporter-mediated uptake of iron, siderophores, heme and vitamin B_{12}. *Res Microbiol*. 2001;**152**(3-4):291-301. doi: 10.1016/s0923-2508(01)01200-1
32. Najimi M. Characterization of the siderophore transport protein AsbJ in *Aeromonas salmonicida* subsp. *salmonicida*. *Comp Clin Pathol*. 2012;**21**(6):1427-1431. doi: 10.1007/s00580-011-1292-6
33. Wyckoff EE, Mey AR, Leimbach A, Fisher CF, Payne SM. Characterization of ferric and ferrous iron transport systems in *Vibrio cholerae. J Bacteriol*. 2006;**188**(18):6515-6523. doi: 10.1128/jb.00626-06
34. Gram L, Melchiorsen J, Spanggaard B, Huber I, Nielsen TF. Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* AH2, a possible probiotic treatment of fish. *Appl Environ Microbiol.* 1999;**65**(3):969-973. doi: 10.1007/s11095-006-0251-4
35. Smith P, Davey S. Evidence for the competitive exclusion of *Aeromonas salmonicida* from fish with stress-inducible furunculosis by a fluorescent pseudomonad. *J Fish Dis*. 1993;**16**(5):521-524. doi: 10.1111/j.1365-2761.1993.tb00888.x
36. Lazado CC, Caipang CMA, Brinchmann MF, Kiron V. *In vitro* adherence of two candidate probiotics from Atlantic cod and their interference with the adhesion of two pathogenic bacteria. *Vet Microbiol.* 2011;**148**(2-4):252-259. doi: 10.1016/j.vetmic.2010.08.024
37. Lazado CC, Caipang CMA, Rajan B, Brinchmann MF, Kiron V. Characterization of GP21 and GP12: two potential probiotic bacteria isolated from the gastrointestinal tract of Atlantic cod. *Probiotics Antimicrob Proteins*. 2010;**2**(2):26-134. doi: 10.1007/s12602-010-9041-8
38. Crestani J, Carvalho PC, Han XM, Seixas A, Broetto L, Fischer JdeS, Staats CC, Schrank A, Yates JR, Vainstein MH. Proteomic profiling of the influence of iron availability on *Cryptococcus gattii*. *J Proteome Res.* 2012;**11**(1):189-205. doi:10.1021/pr2005296