Gallium(III) Nitrate Inhibits Pathogenic *Vibrio splendidus* Vs by Interfering with the Iron Uptake Pathway

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Received: March 6, 2019

Revised: May 3, 2019

Accepted: June 6, 2019

First published online
June 10, 2019

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pISSN 1017-7825, eISSN 1738-8872

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It is well known that iron is critical for bacterial growth and pathogenic virulence. Due to chemical similarity, Ga^{3+} competes with Fe^{3+} for binding to compounds that usually bind Fe^{3+} , thereby interfering with various essential biological reactions. In our present study, gallium(III) nitrate [$\text{Ga}(\text{NO}_3)_3$] could repress the growth of *V. splendidus* Vs without complete inhibition. In the presence of $\text{Ga}(\text{NO}_3)_3$, the secretion of homogentisic acid-melanin (HGA-melanin) in *V. splendidus* Vs cells could be increased by 4.8-fold, compared to that in the absence of $\text{Ga}(\text{NO}_3)_3$. HGA-melanin possessed the ability to reduce Fe^{3+} to Fe^{2+} . In addition, HGA-melanin increased the mRNA levels of *feoA* and *feoB*, genes coding Fe^{2+} transport system proteins to 1.86- and 6.1-fold, respectively, and promoted bacterial growth to 139.2%. Similarly, the mRNA expression of *feoA* and *feoB* was upregulated 4.11-fold and 2.71-fold in the presence of 640 μM $\text{Ga}(\text{NO}_3)_3$, respectively. In conclusion, our study suggested that although $\text{Ga}(\text{NO}_3)_3$ could interfere with the growth of *V. splendidus* Vs, it could also stimulate both the production of Fe^{3+} -reducing HGA-melanin and the expression of *feoA* and *feoB*, which facilitate Fe^{2+} transport in *V. splendidus* Vs.

Keywords: *Vibrio splendidus* Vs, $\text{Ga}(\text{NO}_3)_3$ inhibition, iron uptake, HGA-melanin-mediated Fe^{2+} uptake

Introduction

In recent years, aquaculture of the sea cucumber, *Apostichopus japonicus* (Echinodermata: Holothuroidea), has developed rapidly and become an important part of the aquaculture industry in China [1]. However, with the rapid expansion of scale, a variety of diseases, for example, a typical skin ulcer syndrome (SUS), have emerged and hindered the healthy and sustainable aquaculture of *A. japonicus* [2]. *Vibrio splendidus* is one of the most important opportunistic pathogens that can infect *A. japonicus*, leading to outbreaks of SUS [3]. Thus, study of the pathogenic metabolic processes of *V. splendidus* is recommended to control this pathogen.

It is well known that iron is a critical element for bacterial growth [4], intracellular replication [5], and virulence [6]. However, the concentration of bio-available iron is usually lower than 10^{-18} M, a level far below what is needed for optimal bacterial growth [7]. Thus, bacteria have evolved

several strategies to compete for iron from extremely insoluble ferric oxyhydroxide polymers in the natural environment or from iron-protein complexes such as transferrin or lactoferrin in host tissues [8, 9]. The most common iron uptake pathways used by bacteria include those involving ferric complexes (e.g., ferri-siderophores, haem and haem-protein complexes, ferric-transferrin/lactoferrin complexes, and ferric-citrate). In addition to directly transporting Fe^{3+} , bacteria can also reduce Fe^{3+} to Fe^{2+} in the extracellular space and then transport Fe^{2+} into the intracellular space [10]. In *Legionella pneumophila*, homogentisic acid-melanin (HGA-melanin) was secreted to reduce Fe^{3+} to Fe^{2+} , and then Fe^{2+} was transferred into the intracellular space by the inner membrane protein FeoB [11, 12]. In previous studies, the iron uptake process had been shown to be associated with the pathogenesis of *V. splendidus* [13], and *V. splendidus* was able to acquire iron through the hydroxamate siderophore-IutA receptor iron acquisition pathway under conditions of low iron bio-

availability [14]. Thus, we wondered whether the infectivity of *V. splendidus* can be attenuated by interfering with its iron uptake process.

Ga³⁺ has an ionic radius and binding propensities very similar to those of Fe³⁺, and it can function as an iron analog [15]. Therefore, Ga³⁺ can bind to compounds that bind Fe³⁺, such as siderophore [16] and heme [17] transport systems for Fe³⁺ uptake, leading to the uptake of Ga³⁺ instead of Fe³⁺ [18]. This is called the 'Trojan horse' strategy, and in these cases, Ga-based compounds can be used as anti-infective agents. Simple gallium salts such as gallium nitrate [Ga(NO₃)₃] [19] and gallium maltolate [20] have been determined to exhibit antibacterial activity, and they represent a new class of anti-infective agents that are different from antibiotics [21].

In this study, the effects of Ga(NO₃)₃ on the growth and iron uptake pathways of *V. splendidus* Vs were determined. Since Ga(NO₃)₃ did not exhibit a strong inhibitory effect on *V. splendidus* Vs, we determined the effects of Ga(NO₃)₃ on the other iron uptake pathways in *V. splendidus* Vs. Based on the data obtained, the action mode of Ga(NO₃)₃ on the growth and the iron uptake of *V. splendidus* Vs was proposed.

Materials and Methods

Bacterial Strains, Culture Conditions, and Chemicals

The *V. splendidus* Vs used in this study was isolated from diseased *A. japonicus*, and its pathogenicity had been determined in our previous study [13]. *V. splendidus* Vs was cultured at 28°C in 2216E media consisting of 5 g/l tryptone, 1 g/l yeast extract and 0.01 g/l FePO₄ in aged seawater. When needed, 2,2'-dipyridyl (Aladdin, Shanghai, China), 8-hydroxyquinoline (Aladdin) or gallium(III) nitrate [Ga(NO₃)₃] was separately added into the media. Gallium(III) nitrate hydrate [Ga(NO₃)₃·xH₂O] was purchased from Sigma-Aldrich (Shanghai, China). The chemicals 2,2'-dipyridyl, iron (III) chloride hexahydrate, chromeazurol (CAS), hexadecyl trimethyl ammonium bromide (HTDMA), piperazine, sodium molybdate shuttle solution, ferric chloride, iron phosphate, vitamin C and hydroxylamine hydrochloride were all purchased from Aladdin China). Unless otherwise stated, all other chemicals used in this study were purchased from Sangon (China).

Inhibitory Effect of Ga(NO₃)₃ on the Growth of *V. splendidus* Vs

The ability of *V. splendidus* Vs to grow in 2216E media supplemented with different concentrations of Ga(NO₃)₃ was determined. *V. splendidus* Vs at an initial concentration of 6.0 × 10⁶ CFU/ml was inoculated into 2216E media supplemented with Ga(NO₃)₃ at concentrations of 20, 40, 80, 160, 320, 640, or 1,280 μM. *V. splendidus* Vs grown in 2216E media without Ga(NO₃)₃ was used as a control. The growth of *V. splendidus* Vs was monitored at

different time intervals, and 1 ml of each culture was taken to measure the OD₆₀₀ in a microplate reader (Molecular Devices, Shanghai, China) after culture for 0, 6, 12, 24, 36, 48, 60, 72, 84, or 96 h, respectively. The inhibitory activity of Ga(NO₃)₃ was determined using the following equation: inhibitory activity % = (C₀ - C)/C₀ × 100, where C₀ is the = OD₆₀₀ of *V. splendidus* Vs cultured in 2216E media without Ga(NO₃)₃, and C is the = OD₆₀₀ of *V. splendidus* Vs cultured in 2216E media with different concentrations of Ga(NO₃)₃. The inhibitory effect of Ga(NO₃)₃ on *V. splendidus* Vs was also tested in natural seawater. Cell-free seawater was obtained by filtration through a 0.22-μm polycarbonate membrane filter (Millipore). An overnight culture of *V. splendidus* Vs was washed three times with cell-free seawater, and the cell density was adjusted to 1.0 × 10⁸ CFU/mL based on the OD₆₀₀ determined using a UV-Vis spectrophotometer (Thermo Fisher, USA). The *V. splendidus* Vs suspension was then divided into eight parts, seven of which were separately supplemented with 20, 40, 80, 160, 320, 640, or 1,280 μM Ga(NO₃)₃, while the suspension without Ga(NO₃)₃ was used as a control. All eight groups were incubated at 28°C for 96 h, and then the cultures were diluted and spread on solid 2216E media. The bacterial colonies were counted after being incubated for 24 h. The inhibitory activity of Ga(NO₃)₃ was determined using the equation mentioned above.

Siderophore Quantification

The siderophores produced by *V. splendidus* Vs and determined in the presence of 0, 20, 40, 80, or 160 μM 2,2'-dipyridyl or Ga(NO₃)₃ were quantified using a method adapted from Schwyn and Neilands [22]. Briefly, *V. splendidus* Vs was incubated in 2216E media supplemented with 2, 2'-dipyridyl or Ga(NO₃)₃ for 48 h, and the supernatants were obtained by centrifuging the samples at 10,000 ×g for 10 min. A volume of 1 ml of cell-free supernatant from each culture was mixed with 1 ml of CAS bright blue dye and 20 μl of shuttle solution. Samples of 2216E media supplemented with various concentrations of 2,2'-dipyridyl or Ga(NO₃)₃ were also mixed with CAS and shuttle solution as the reference samples. The mixtures were kept at room temperature for 5 min, and then the OD₆₃₀ values were measured in a microplate reader. The relative quantities of siderophores were determined from the following equation: siderophore % = (Ar - As)/Ar × 100, where Ar is the = reference absorbance and As is the = sample absorbance [23].

Measurement of HGA-Melanin and Determination of HGA-Melanin Production by *Vibrio splendidus* Vs

To determine the maximum absorbance value of HGA-melanin, a method adapted from [24] was used. Briefly, supernatants were obtained and measured for their absorbance using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, USA). HGA-melanin made as described by Zheng *et al.* [12] was used as a positive control. Briefly, 200 μg/ml monomeric HGA in PBS was incubated at 37°C with agitation at 180 rpm for 72 h. To collect the natural HGA-melanin produced by *V. splendidus* Vs,

1.0×10^7 CFU/ml *V. splendidus* Vs was inoculated into 2216E media and grown for 72 h. The cell-free supernatant was then acidified to pH 2.0–3.0 using HCl. HGA-melanin was collected by centrifugation at $7,200 \times g$ for 20 min after precipitation for 30 min, and the precipitate was soaked in 6 M HCl for 4 h. Then, the precipitate was washed twice in ethyl alcohol and once in acetone. The final precipitate was dried at 60°C and dissolved with 1 M NaOH. Finally, the extracted HGA-melanin was adjusted to pH 7.0, and adjusted again to the same concentration as the synthetic HGA-melanin using a NanoDrop 2000 UV-Vis spectrophotometer.

To detect HGA-melanin production by *V. splendidus* Vs at different growth phases, an overnight culture of *V. splendidus* Vs was used to incubate 2216E media containing 1.0 g/ml L-tyrosine at an inoculation volume of 1% and aliquots were taken for measurement of the OD₆₀₀ values in a microplate reader at 12-h intervals for a total incubation period of 96 h. The HGA-melanin in the cell-free supernatant was monitored according to the maximum absorbance value obtained from spectroscopic analysis of the synthetic HGA-melanin. To detect HGA-melanin production by *V. splendidus* Vs in the presence of Ga(NO₃)₃, overnight cultures of *V. splendidus* Vs were inoculated at 1% volume into 2216E media supplemented with 320, 640, or 1,280 µM Ga(NO₃)₃. *V. splendidus* Vs grown in 2216E media without Ga(NO₃)₃ was used as a control. Then, the production of HGA-melanin was monitored as described above.

Fe³⁺ Reduction Assay

The reduction of Fe³⁺ by HGA-melanin was measured as described by Chatfield and Cianciotto [25]. Briefly, 400 µM ferrozine, 120 µM FeCl₃ and 120 µM FePO₄ in 250 µl Tris buffer (25 mM, pH 7.5) were mixed. Then, 10 µl of synthetic HGA-melanin, 10 µl of monomeric HGA (200 µg/ml), or 100 µl of bacterial HGA-melanin was added into each of the above mixtures, respectively. PBS solvent was used as a negative control for monomeric HGA and synthetic HGA-melanin, and 1 M NaOH (pH 7.0) was used as a negative control for the extracted HGA-melanin. Vitamin C and 1 mM hydroxylamine hydrochloride, which have defined Fe³⁺ reduction activity, were used as positive controls. The reactions were kept at room temperature for 12 h. The developed color was measured at 560 nm using a microplate reader (Molecular Devices, China).

Effects of HGA and HGA-Melanin on the Growth of *V. splendidus* Vs

V. splendidus Vs was precultured in 2216E media without 66.3 µM FePO₄ to the mid-logarithmic phase and then inoculated into 2216E media that was supplemented with 344.4 µM 8-hydroxyquinoline and grown to an OD₆₀₀ of 0.15. A total of 200 µl of the bacterial suspension was inoculated into the wells of a 96-well microtiter plate. Ten microliters of 2216E media with or without 66.3 µM FePO₄, 2216E with 344.4 µM 8-hydroxyquinoline, HGA, HGA-melanin or PBS solvent was also added into the wells. The inoculated plates were then incubated at 28°C for 60 h. Then, the

OD₆₀₀ of each well was recorded every 12 h. To prevent condensation, the lids of the microtiter plates were precoated with 10 ml of 0.05% Triton X-100 in 20% ethanol for 0.5 h and air dried [26].

Sample Collection for Real-Time Reverse Transcription PCR (RT-PCR)

To determine the effects of 2,2'-dipyridyl or Ga(NO₃)₃ on the expression of *iutA*, *V. splendidus* Vs was grown in 2216E media without 66.3 µM FePO₄ at 28°C to an OD₆₀₀ of approximately 0.2. Then, the culture was divided into three parts. Two of the parts were supplemented with 80 µM 2, 2'-dipyridyl or Ga(NO₃)₃, and the third part was left unsupplemented and used as a control. After incubation at 28°C for 6 h, 12 h, and 24 h, 2 ml of culture was taken, and the cell pellets were collected.

To determine the effects of HGA-melanin on the expression of *feoA* and *feoB*, *V. splendidus* Vs was grown in 2216E media without 66.3 µM FePO₄ until the mid-logarithmic phase. Then, the cells were washed three times with 2216E media supplemented with 344.4 µM 8-hydroxyquinoline and then resuspended to an OD₆₀₀ of 0.15. The resuspended *V. splendidus* Vs was divided into two parts. One part was supplemented with 100 µl of HGA-melanin, and the other part was supplemented with an equal volume of PBS as a control group. After incubation at 28°C for 10 min, 30 min, 60 min, 12 h, and 24 h, 2 ml of culture was taken and the cell pellets were collected.

To determine the effects of Ga(NO₃)₃ on the expression of *iutA*, *feoA* and *feoB*, *V. splendidus* Vs was grown in 2216E media without 66.3 µM FePO₄ at 28°C to an OD₆₀₀ of approximately 0.2. Then the culture was divided into two parts. One was supplemented with 640 µM Ga(NO₃)₃, and the other sample was left untreated and used as a control. After inoculation at 28°C for 10 min, 30 min, 60 min, 12 h, and 24 h, 2 ml of culture was taken and the cell pellets were collected.

Real-Time RT-PCR

RNA was extracted from *V. splendidus* Vs cells that were collected at different time points using a Bacterial RNA Isolation Kit (Omega). Then, 1 µg of RNA was reverse transcribed into cDNA using PrimeScript reverse transcriptase (Takara). Real-time RT-PCR was carried out using SYBR Premix Ex Taq (Takara) in an ABI 7500 real-time detection system (Applied Biosystems) as described previously [27]. Each assay was performed in triplicate using 16S rRNA as a reference control. Primers were designed according to the genomic sequence of *V. splendidus* Vs and their nucleotide sequences are shown in Table 1. Dissociation analysis of the amplification products was performed at the end of each PCR run to confirm that only one PCR product was amplified and detected. The comparative threshold cycle method (2^{-ΔΔCT} method) was used to analyze the mRNA levels.

Accession Numbers of the Bacterial Strains, Database Search and Statistical Analysis

The isolate of *V. splendidus* Vs has been deposited into the China

Table 1. Primers used in this study.

Primer name	Nucleotide sequence (5' → 3')
933F	GCACAAGCGGTGGAGCATGTGG
16SRTR1	CGTGTGTAGCCCTGGTCGTA
IutA ¹ F	GTACGATCTACCACCTCAC
IutAR	CGTAGTATTTTCGCAGGGTC
FeoA ² F	TCTCACAACCTAGAGCAAGGC
FeoAR	CGGCGAATCAACGTCACC
FeoB ³ F	GCACTAATCCCTTCTGTTG
FeoBR	TTGAATGCGAGGCTGAGGA

¹IutA is an abbreviation for the name of the protein of aerobactin siderophore receptor IutA.

²FeoA is an abbreviation for the name of the protein of the Ferrous iron transport A.

³FeoB is an abbreviation for the name of the protein of Ferrous iron transport B.

General Microbiological Culture Collection (CGMCC, China) with accession numbers 7.242. Statistical significance was determined by one-way analysis of variance (ANOVA). Statistical analyses were performed using two-tailed Student's *t* tests. In all cases, the significance levels were defined as **p* < 0.05, ***p* < 0.01 and ****p* < 0.001.

Results

Only Higher Concentrations of Ga(NO₃)₃ Could Inhibit Growth of *V. splendidus* Vs

Ga(NO₃)₃ at concentrations of 20, 40, 80, 160, 320, 640, or 1,280 μM Ga(NO₃)₃ was tested for its ability to inhibit the growth of *V. splendidus* Vs at different time points. The inhibitory effect of Ga(NO₃)₃ on the growth of *V. splendidus* Vs was dose-dependent and time dependent. Ga(NO₃)₃ at concentrations higher than 160 μM showed obvious inhibitory effects on the growth of *V. splendidus* Vs both at the exponential growth stage and with regard to the maximum biomass (OD₆₀₀). In the presence of 320, 640, or 1,280 μM Ga(NO₃)₃, the growth time needed to reach the maximum biomass was delayed for more than 12 h, and the maximum biomass was also reduced to 80%, 81%, or 66% of the control levels, respectively (Fig. 1A).

Compared with the inhibitory effect of Ga(NO₃)₃ in 2216E media, the inhibitory effect of Ga(NO₃)₃ in seawater was higher (Fig. 1B). After coincubation for 96 h, the percentage of viable cells of *V. splendidus* Vs had decreased to 43.5% in the samples supplemented with 160 μM Ga(NO₃)₃. The viable cells of *V. splendidus* Vs decreased to 11.14% in the presence of 320 μM Ga(NO₃)₃. When the concentration of Ga(NO₃)₃ was increased to 640 μM, the percentage of viable *V. splendidus* Vs cells decreased to 0.2% (Fig. 1B).

Fig. 1. (A) Growth curves of *Vibrio splendidus* Vs in the presence of 0, 20, 40, 80, 160, 320, 640, and 1,280 μM Ga(NO₃)₃. The bacteria were cultured at 28°C, and culture samples at different time points were taken for measurement of OD₆₀₀. (B) Inhibitory activity of Ga(NO₃)₃ on *V. splendidus* Vs in the presence of 0, 20, 40, 80, 160, 320, 640, and 1,280 μM Ga(NO₃)₃ in filter-sterilized seawater. After incubation at 28°C for 96 h, the cultures were diluted 10-fold, 100 μl was spread onto solid 2216E media, and the bacterial colonies were counted after 24 h of incubation. The data are the means for at least three independent experiments and are presented as the mean ± SE.

Effect of Ga(NO₃)₃ on Siderophore Production

The growth of *V. splendidus* Vs was not inhibited by 20 or 40 μM 2,2'-dipyridyl or Ga(NO₃)₃, but when the concentrations were higher, 2,2'-dipyridyl showed a stronger inhibitory effect than Ga(NO₃)₃ (Fig. 2A). In the presence of 160 μM 2,2'-dipyridyl, the growth of *V. splendidus* Vs was only approximately half that in the presence of 160 μM Ga(NO₃)₃. A concentration of 320 μM 2,2'-dipyridyl could nearly completely inhibit the growth of *V. splendidus* Vs, while 320 μM Ga(NO₃)₃ could only inhibit 10% of the growth. After measuring the growth, the siderophore production in the presence of 2,2'-dipyridyl or Ga(NO₃)₃ at different concentrations was determined. In the presence of 2,2'-

Fig. 2. (A) OD₆₀₀ of *V. splendidus* Vs grown with different concentrations of 2,2'-dipyridyl or Ga(NO₃)₃. *V. splendidus* Vs was grown in the presence of 0, 20, 40, 80, 160, and 320 μM 2,2'-dipyridyl or the same concentrations of Ga(NO₃)₃ at 28°C for 48 h, and then the OD₆₀₀ was measured using a UV-Vis spectrophotometer. (B) Quantitative determination of siderophore production by *V. splendidus* Vs in the presence of 0, 20, 40, 80, 160, and 320 μM 2,2'-dipyridyl or the same concentrations of Ga(NO₃)₃ at 28°C for 48 h. After incubation, the supernatants were collected for quantitative determination of siderophore production. The data are the means for at least three independent experiments and are presented as the mean ± SE. The relative quantities of siderophores produced by cells treated with Ga(NO₃)₃ were compared with those produced by cells treated with 2,2'-dipyridyl at each concentration. **p* < 0.05.

dipyridyl or Ga(NO₃)₃ at concentrations ranging from 20 to 160 μM, the relative siderophore production by *V. splendidus* Vs was significantly increased compared with that in the control group. However, the relative siderophore production in *V. splendidus* Vs supplemented with 2,2'-dipyridyl was higher than that in bacteria supplemented with the same concentration of Ga(NO₃)₃. The relative siderophore production was 57.6% in the presence of 80 μM 2,2'-dipyridyl, while in the presence of 80 μM Ga(NO₃)₃, the siderophore production was 31.6%, almost the same as in the control group. The relative siderophore production in

Fig. 3. The expression of the *iutA* gene in *V. splendidus* Vs at the mRNA level in the absence and presence of Ga(NO₃)₃ or 2,2'-dipyridyl.

The data are the means for at least three independent experiments and are presented as the mean ± SE. At each time point, the mRNA levels in cells treated with Ga(NO₃)₃ or 2,2'-dipyridyl were compared with those in cells without treatment. ****p* < 0.001, ***p* < 0.01, and **p* < 0.05.

the presence of 40 μM, 80 μM, 160 μM, or 320 μM Ga(NO₃)₃ was 75.9%, 51.2%, 41.3%, or 13.3% of that in the presence of the corresponding concentration of 2,2'-dipyridyl, respectively (Fig. 2B). Combining the OD₆₀₀ and siderophore production data, it could be concluded that there was less siderophore production in each individual cell in the presence of Ga(NO₃)₃ than in the presence of the same level of 2,2'-dipyridyl.

Effect of Ga(NO₃)₃ on Expression of *iutA*

The expression of *iutA* was upregulated 4.3- and 1.3-fold at the mRNA level in cells cultured with 80 μM Ga(NO₃)₃ for 12 h and 24 h, respectively, compared with control cells (Fig. 3). In cells treated with 80 μM 2,2'-dipyridyl, the expression of *iutA* was upregulated 1.1- or 13.2-fold at the mRNA levels after 12 h or 24 h of incubation, respectively (Fig. 3). These results suggested that the addition of either Ga(NO₃)₃ or 2,2'-dipyridyl could upregulate the expression of *iutA*; however, the effect of Ga(NO₃)₃ was not as strong as that of the same amount of 2,2'-dipyridyl.

V. splendidus Vs Produced HGA-Melanin That Promoted Growth at the Stationary Phase

The absorbance spectrum and absorbance peak of HGA-melanin produced by *V. splendidus* Vs showed a maximum absorbance at 329 nm (data not shown). The growth of *V. splendidus* Vs declined after incubation for 24 h; however, the HGA-melanin in the supernatant of *V. splendidus* Vs culture rapidly accumulated at this stage. After incubation

Fig. 4. Growth and HGA-melanin production of *V. splendidus* Vs. *V. splendidus* Vs in 2216E media containing 1.0 g/ml L-tyrosine was incubated at 28°C.

The OD₆₀₀ of *V. splendidus* Vs cultures and the OD₃₂₉ of their cell-free supernatants were measured at 12-h intervals for a total of 96 h. The data are the means for at least three independent experiments and are presented as the mean ± SE.

for 48 h, the growth of *V. splendidus* Vs reached a stationary phase, and at this phase, the accumulation of HGA-melanin also tended to be stable (Fig. 4).

HGA-melanin was synthesized and used as a positive control for the HGA-melanin secreted by *V. splendidus* Vs. Pure monomeric HGA was used to generate HGA-melanin and then tested for its ability to reduce Fe³⁺ as a control.

Both synthetic HGA-melanin and pure monomeric HGA showed Fe³⁺ reduction activity acting as reducing agents of vitamin C and hydroxylamine hydrochloride, compared with the negative control (Fig. 5A). HGA-melanin extracted from the culture supernatant of *V. splendidus* Vs also showed obvious Fe³⁺ reduction activity (Fig. 5B). The Fe³⁺ reduction activity of natural HGA-melanin was similar to that of synthetic HGA-melanin. Taken together, the results indicated that *V. splendidus* Vs could synthesize HGA-melanin that could directly mediate Fe³⁺ reduction.

To determine whether HGA-melanin with Fe³⁺ reduction activity could promote the growth of *V. splendidus* Vs under iron-limited conditions, we applied the monomeric HGA and polymerized HGA-melanin to 2216E media supplemented with 8-hydroxyquinoline. The results showed that *V. splendidus* Vs cultured in 2216E media with 8-hydroxyquinoline and HGA or with 8-hydroxyquinoline and HGA-melanin for 48 h exhibited 112.4% or 139.2% of the growth, respectively, of *V. splendidus* Vs cultured in 2216E media with 8-hydroxyquinoline alone (Fig. 6).

Ga(NO₃)₃ Stimulated HGA-Melanin Production and the Expression of *feoA* and *feoB* in *V. splendidus* Vs

HGA-melanin production by *V. splendidus* Vs significantly increased in the presence of Ga(NO₃)₃ and the increase occurred in a dose-dependent manner. The production of HGA-melanin in individual cells increased by 1.4-, 2.9-,

Fig. 5. (A) Fe³⁺ reduction by HGA or synthetic HGA-melanin. Vitamin C and hydroxylamine hydrochloride were used as positive controls, and PBS was used as a negative control. (B) Fe³⁺ reduction by extracted HGA-melanin. Acid-precipitated HGA-melanin obtained from the culture supernatants of *V. splendidus* Vs was mixed with FeCl₃. NaOH was used as a negative control for the extracted HGA-melanin. Vitamin C or hydroxylamine hydrochloride was used as a positive control. The reactions were allowed to proceed at room temperature for 12 h. The changes in color associated with ferrozine binding to Fe²⁺ were measured. The data are the means for at least three independent experiments and are presented as the mean ± SE. Comparisons were made between the OD₅₆₀ values of the reactions catalyzed by HGA, synthetic HGA-melanin, or extracted HGA-melanin and the corresponding negative control values. ****p* < 0.001, ***p* < 0.01, and **p* < 0.05.

Fig. 6. OD₆₀₀ of *V. splendidus* Vs in the presence of HGA or HGA-melanin at different time points.

PBS was used as a negative control for HGA and HGA-melanin. The data are the means for at least three independent experiments and are presented as the mean $\pm$ SE. At each time point, the OD₆₀₀ values of cultures with HGA or HGA-melanin were compared with those of cultures without treatment. ** $p < 0.01$, * $p < 0.05$.

and 4.8-fold, when *V. splendidus* Vs was grown in 2216E media in the presence of 320 μ M, 640 μ M, or 1,280 μ M Ga(NO₃)₃, respectively (Fig. 7).

It is clear from the above data that in the presence of Ga(NO₃)₃, *V. splendidus* Vs could stimulate the production of HGA-melanin, an Fe³⁺-reducing reagent, to promote its own growth. *FeoA* and *feoB* are two genes encoding proteins that facilitate the transportation of Fe²⁺ into cells. The expression of *feoA* was upregulated 1.9- and 1.4-fold at the mRNA level in cells treated with HGA-melanin for 1 h and 24 h respectively (Fig. 8A). Under the same conditions, the mRNA level of *feoB* was upregulated 2.1- and 6.1-fold

Fig. 7. OD₃₂₉ values of cell-free supernatants from *V. splendidus* Vs cultures in the presence of different concentrations of Ga(NO₃)₃.

The OD₃₂₉ of the cell-free supernatant from a *V. splendidus* Vs culture grown without Ga(NO₃)₃ was used as a control. The data are the means for at least three independent experiments and are presented as the mean $\pm$ SE. At each time point, the OD₃₂₉ of the culture with Ga(NO₃)₃ was compared with that of the culture without treatment. ** $p < 0.01$, * $p < 0.05$.

at 1 h and 24 h, respectively (Fig. 8B). The expression of *feoA* and *feoB* was also upregulated at the mRNA level in cells treated with 640 μ M Ga(NO₃)₃. The expression of *feoA* and *feoB* was upregulated 4.1- and 2.7-fold after treatment for 30 min, respectively (Fig. 9B).

Discussion

V. splendidus is an opportunistic pathogen that causes severe mortality and morbidity in its hosts [28]. In our previous study, the iron uptake process was determined to

Fig. 8. (A) Expression of *feoA* at the mRNA level in the presence of HGA-melanin. (B) Expression of *feoB* at the mRNA level in the presence of HGA-melanin. The data are the means for at least three independent experiments and are presented as the mean $\pm$ SE. At each time point, the mRNA levels in the cells treated with HGA-melanin were compared with those in the cells without treatment. ** $p < 0.01$, * $p < 0.05$.

Fig. 9. (A) The expression of *feoA* at the mRNA level in the presence of $\text{Ga}(\text{NO}_3)_3$. (B) The expression of *feoB* at the mRNA level in the presence of $\text{Ga}(\text{NO}_3)_3$. The data are the means for at least three independent experiments and are presented as the mean $\pm$ SE. At each time point, the mRNA levels in the cells treated with $\text{Ga}(\text{NO}_3)_3$ were compared with those in the cells without treatment. ** $p < 0.01$, * $p < 0.05$.

be associated with the pathogenesis of *V. splendidus* Vs [13]. One of the iron uptake pathways in *V. splendidus* Vs involves the use of Fe^{3+} via a hydroxamic siderophore-IutA-receptor, as proposed in our previous study [14]. Ga^{3+} has a similar radius as Fe^{3+} , and it could compete with Fe^{3+} for binding to biomacromolecules that need Fe^{3+} in biologic systems [29]. Thus, Ga^{3+} has been used as an anti-infective agent in *Pseudomonas aeruginosa* [30], *Staphylococcus aureus* [31], *Acinetobacter baumannii* [32] and *Rhodococcus equi* [33]. In our present study, the potential of $\text{Ga}(\text{NO}_3)_3$ to be used as an antibacterial substance to repress *V. splendidus* Vs was determined. $\text{Ga}(\text{NO}_3)_3$ inhibited the growth of *V. splendidus* Vs, especially in an oligotrophic environment under iron deficiency. However, the inhibitory effect of $\text{Ga}(\text{NO}_3)_3$ on *V. splendidus* Vs was not as strong as that on *A. baumannii* [32], *Mycobacterium avium* subsp. *paratuberculosis* (MAP) [34], *Mycobacterium tuberculosis* [35], *Klebsiella pneumonia* [36], *E. coli*, and *Enterococcus faecalis* [37]. Moreover, the inhibitory effect of $\text{Ga}(\text{NO}_3)_3$ on the growth of *V. splendidus* Vs decreased when *V. splendidus* Vs was cultured to the stationary phase. This led us to wonder if there were other iron uptake pathways in *V. splendidus* Vs that could not be suppressed by $\text{Ga}(\text{NO}_3)_3$.

Although $\text{Ga}(\text{NO}_3)_3$ or 2,2'-dipyridyl could increase the production of siderophores, the increase caused by $\text{Ga}(\text{NO}_3)_3$ was smaller than that caused by 2,2'-dipyridyl. In view of this, Ga^{3+} may compete with Fe^{3+} for binding to the siderophores produced by *V. splendidus* Vs given that siderophores could chelate Ga^{3+} instead of Fe^{3+} , as proposed previously [21]. The addition of $\text{Ga}(\text{NO}_3)_3$ or 2,2'-dipyridyl could upregulate the expression of *iutA*, but the upregulation caused by $\text{Ga}(\text{NO}_3)_3$ was smaller than that caused by the

same level of 2,2'-dipyridyl. This result suggested that Ga^{3+} might attenuate the Fe^{3+} absorption pathway mediated by the hydroxamic siderophore-IutA-receptor in *V. splendidus* Vs. Our result is consistent with previous reports that Ga^{3+} mimics Fe^{3+} and further affects the Fe^{3+} absorption pathway mediated by siderophores [38, 39].

HGA-melanin produced from the catabolism of tyrosine via *p*-hydroxyphenylpyruvate (HPP) and homogentisic acid (HGA) has been reported in *P. aeruginosa* [40, 41], *Shewanella colwelliana* [42], *Alcaligenes eutrophus* CH34 [43], *Burkholderia cepacia* [44] and *L. pneumophila* [45]. HGA-melanin is a multifunctional polymer that can facilitate bacterial growth, partly due to its ability to reduce Fe^{3+} . The Fe^{3+} reduction activity of HGA-melanin has been demonstrated in *L. pneumophila* [45] and *Shewanella algae* [46, 47]. Our results showed that *V. splendidus* Vs could produce HGA-melanin which possessed Fe^{3+} reduction activity, and that HGA-melanin could promote the growth of *V. splendidus* Vs under iron-limited conditions. These findings are consistent with findings regarding the HGA-melanin produced by *L. pneumophila* which mediated Fe^{3+} reduction, and thus increasing Fe^{2+} availability to bacterial cells with the help of FeoA and FeoB, members of Feo transporter system for specific acquisition of Fe^{2+} from the external environment [10, 12, 48, 49]. Our results are the first to reveal the pathway of Fe^{2+} uptake mediated by HGA-melanin in *V. splendidus* Vs.

Although $\text{Ga}(\text{NO}_3)_3$ could repress Fe^{3+} uptake [38, 39], $\text{Ga}(\text{NO}_3)_3$ also showed a stimulatory effect on the HGA-melanin-mediated Fe^{2+} uptake pathway in the current study. In the presence of $\text{Ga}(\text{NO}_3)_3$, both HGA-melanin production and the expression of *feoA* and *feoB*, were

upregulated. The increase in the expression of *feoA* and *feoB* in the presence of $\text{Ga}(\text{NO}_3)_3$ occurred earlier than that in the presence of exogenously added HGA-melanin. The difference in the response time may have been due to the different actions of the two chemicals: HGA-melanin, as an Fe^{2+} reducing reagent, may have functioned much later than $\text{Ga}(\text{NO}_3)_3$, an Fe^{3+} chelator. When $\text{Ga}(\text{NO}_3)_3$ was added, Ga^{3+} could enter the cells instead of Fe^{3+} , causing the cells to sense that they were under iron-limited conditions. Under these conditions, the ferric uptake regulator (Fur) could function and regulate the related expression [50]. In *Streptococcus suis* and *Vibrio cholera*, Fur has been reported to negatively regulate the *feo* system [51, 52]. Combined, all the data suggested that in the presence of $\text{Ga}(\text{NO}_3)_3$, the growth of *V. splendidus* Vs was inhibited due to the replacement of Fe^{3+} by Ga^{3+} , while the Fe^{2+} uptake pathway mediated by HGA-melanin was stimulated to compensate for growth. However, the stimulated Fe^{2+} uptake pathway could not completely compensate for the damaged Fe^{3+} uptake pathway, so the combined effect of $\text{Ga}(\text{NO}_3)_3$ involved a decrease in the maximum OD_{600} and a delay of the stationary phase. The inhibitory effect of $\text{Ga}(\text{NO}_3)_3$ on the growth of *V. splendidus* Vs was weaker in the 2216E media than in seawater. Therefore, $\text{Ga}(\text{NO}_3)_3$ shows promising potential for use in preventing disease in *A. japonicus* infected with *V. splendidus* Vs in aquaculture.

Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (41676141 and 41576139), the Ningbo Science and Technology Development Plan (2015C50057), and the K.C. Wong Magna Fund at Ningbo University.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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