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ENVIRONMENTAL BIOTECHNOLOGY

Quorum sensing-disrupting coumarin suppressing virulence phenotypes in *Vibrio splendidus*

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Abstract In the present study, the effects of an environmental friendly natural reagent coumarin, on the growth and potential virulence factors, as well as its ability to interfere the infection of *Vibrio splendidus* (Vs), were determined. Coumarin showed no effects on the maximal growth of Vs, and biofilm formation of Vs, while it significantly decreased protease activity and hemolytic activity by 43 and 80%, respectively. Correspondingly, coumarin exhibited an obviously protective effect, with a relative percent survival of 60% upon *Apostichopus japonicus* from infection by Vs. To preliminarily investigate the mechanism underlining the inhibitory effects, regulation of genes *Vsm* and *Vsh* respectively related to protease activity and hemolytic activity by supernatant and supernatant extract containing acyl-homoserine lactones (AHLs) and coumarin was determined. Cell-free supernatant from higher density and its ethyl acetate extract containing AHL signal molecules could respectively upregulate the mRNA level of *Vsm* by 17.4- and 2.3-fold and *Vsh* by 7.2- and 5.0-fold, when Vs was at lower cell density. However, coumarin could reduce the stimulatory effects of both the supernatant and its ethyl acetate extract. Combining all the results in our study, it was suggested that coumarin could be considered as an alternative to be used for controlling infection of Vs, downregulating the expression of potential virulence factors through interfering the AHL-mediated pathways.

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Keywords Coumarin · *Vibrio splendidus* Vs · Protease · Hemolytic activity · Protective effect

Introduction

The invertebrate sea cucumber *Apostichopus japonicus* (Echinodermata, Holothuroidea) is an economically important species in Chinese marine culture (Liu et al. 2010a); however, with its rapid expansive and intensive farming, skin ulceration syndrome (SUS), manifesting swollen mouth, viscera ejection, skin ulceration, and massive mortality have resulted from pathogenic infection, leading to huge economical loss (Deng et al. 2008; Li et al. 2010). *Vibrio splendidus* is one of the most important opportunistic pathogens that could infect *A. japonicus*, leading to the outbreak of SUS (Zhang et al. 2006). Thus, ecological strategies with the merits of high efficiency and environmental friendly to protect *A. japonicus* from infection by *V. splendidus* have become urgently recommended and have become prior choices, compared with the traditional methods of using antibiotics and chemotherapeutics, which own the shortage of drug-resistant development, environmental pollution, and residues in the environment (Zhang et al. 2010; Li et al. 2015).

Coumarin is derived from plants such as Rutaceae, Umbelliferae, Asteraceae, Leguminosae, Thymelaeaceae, and Solanaceae, and its pharmacological safety has been established. Small dosage of coumarin (≤0.64 mg/kg BW/day) in food or cosmetics does not pose any risk to human health (Felter et al. 2006). Thus, coumarin has been suggested as a powerful alternative for disease control, as it is determined to be an antibacterial reagent and a universal quorum sensing inhibitor (QSI) (Gutiérrez-Barranquero et al. 2015). The antimicrobial activities of coumarin had been detected in the leaf extracts of *Petroselinum crispum* and *Ruta graveolens* (Ojala

et al. 2000). Later, a series of 45 coumarin derivatives and a parent coumarin showed more or less pronounced antibacterial potencies, affecting both Gram-positive and Gram-negative bacteria (de Souza et al. 2005). Apart from its antibacterial potential, the inhibitory effects of coumarins on the virulence phenotypes were also investigated. Coumarins could inhibit fimbriae production, swarming motility, and biofilm formation to prevent the infection by *Escherichia coli* O157:H7 (Lee et al. 2014). Recently, coumarin was reported to be an inhibitor of quorum sensing signal molecules acylhomoserine lactones (AHLs) in *Pseudomonas aeruginosa* and reduced expression of *rhlI* and *pqsA*, leading to the inhibition of biofilm formation and phenazine production (Gutiérrez-Barranquero et al. 2015). But no attempt has been carried out to investigate whether coumarin could be used as a protective reagent to deal with the problems posed by infection of *V. splendidus* in aquaculture of *A. japonicus*.

In this study, the effects of various concentrations of coumarin on the growth and potential virulence factors, including proteolytic and hemolytic activities of *V. splendidus* (Liang et al. 2016; Zhang et al. unpublished data), were determined. The messenger RNA (mRNA) levels of genes *Vsm* and *Vsh* related to metalloprotease and hemolytic activity after the addition of cell-free supernatant, its ethyl acetate extract, and coumarin were also investigated. Furthermore, in order to determine whether it could be potentially used in aquaculture, the ability of coumarin to protect *A. japonicus* from infection by *V. splendidus* was also explored.

Materials and methods

Bacterial strains, culture conditions, and chemicals

V. splendidus Vs was cultured in 2216E media consisting of 5 g L^{-1} tryptone, 1 g L^{-1} yeast extract, and 0.01 g L^{-1} $FePO_4$ in aged seawater at 28 °C. *Chromobacterium violaceum* CV026 was cultured in Luria–Bertani (LB) media (Sambrook et al. 1989) supplemented with 50 μg mL^{-1} kanamycin at 28 °C. Sheep blood medium was prepared using 2216E media supplemented with 5% sheep blood cells. Coumarin was purchased from Aladdin (China) and was dissolved in ethanol. Unless otherwise stated, all other chemicals used in this study were of the highest purity and were purchased from Sangon (Shanghai, China). To detect the effect of coumarin on the growth of Vs, cells grown overnight were inoculated into fresh 2216E media containing coumarin at concentrations of 0, 0.27, 1.35, and 6.75 mM at an initial OD_{600} of 0.05. Cells of Vs were continually cultured at 28 °C for 6, 12, 24, 36, 48, 60, and 72 h, and aliquots were taken out at selected time points for the measurement of OD_{600} nm by UV-1600PC (MAPADA, China).

AHL detection

The cross-streaking method with CV026 as an indicator strain was used to detect short-chain AHLs from C4 to C8 in the supernatant of Vs as descrbied previously (McClean et al. 1997; Han et al. 2010). Briefly, Vs was cross-streaked perpendicular to a horizontal streak of CV026 on LB agar plates and incubated at 28 °C for 24 h, and then a developed color was observed. AHLs were extracted and condensed as described by Viswanath et al. (2015). Vs was cultured to OD_{600} of approximately 1.2 as described previously, culture was centrifuged at 12,000 rpm for 15 min, and the spent supernatant was extracted twice with acidified ethyl acetate (0.1% glacial acetic acid). The organic phases were combined and concentrated using a rotary evaporator at 45 °C. The concentrated extracts were re-dissolved in acetonitrile and filtered through 0.22-μm membrane filters. Both the cell-free supernatant samples and ethyl acetate extract samples were detected using CV026 as an indicator strain. *Pseudomonas aeruginosa* PA1 was used as a positive control, while 2216E media was used as a negative control. Ten microliters of ethyl acetate extract was added to the hole near to the streak of CV026 on LB agar plates and incubated at 28 °C for 24 h, and then the developed color was observed.

Protease activity

The protease activity of Vs was determined as described previously (Zhang et al. 2009). Vs were grown in 2216E media at 28 °C supplemented with coumarin at concentrations of 0, 0.27, 1.35, and 6.75 mM, and cell-free supernatants from the culture were obtained at the time intervals of 9, 12, 24, and 50 h by centrifugation at 12,000 rpm, followed by filtration through 0.22-μm membrane filters. Fifty microliters of cell-free supernatants was added to 450 μL of 0.5% azocasein and incubated at 37 °C for 2 h, respectively. The reaction was stopped by the addition of 0.5 mL 10% trichloroacetic acid followed by centrifugation. The absorbance of transparent supernatant was measured at 350 nm by SpectraMax 190 (Molecular Devices, China).

Hemolytic activity assay

Hemolytic activity was determined according to Natrah et al. (2011a) with minor modifications. Briefly, cell-free supernatants of Vs culture were obtained as described above, 100 mL of each supernatant was added into an oxford cup on blood agar and incubated for 12 h at 28 °C, and then the developed transparent circle was observed. To quantitatively determine the hemolytic activities of the Vs supernatant, 100 mL of each supernatant was incubated with 1 mL of erythrocyte suspension at 28 °C for 2.5 h and then shocked to release

hemoglobin, followed by centrifugation. Then, OD_{450} nm was measured by SpectraMax 190 (Molecular Devices, China).

Biofilm development

The ability of Vs to form biofilm on a polystyrene microtiter dish was determined as described by Zhang et al. (2016). Briefly, the bacterial cells of Vs were cultured in a 96-well polystyrene microtiter plate for 12, 24, and 48 h, respectively. Unattached cells were washed away with PBS for five times. The attached cells were treated with Bouin's fixative for 1 h and stained with 1% crystal violet solution for 30 min. Plates were then rinsed with running water, and bound crystal violet was dissolved in ethanol for 30 min. The absorbance at 570 nm was measured by SpectraMax 190 (Molecular Devices, China).

Sample collection for real-time reverse transcriptase PCR (RT-PCR)

To detect the effect of coumarin on the expression of *Vsm* and *Vsh*, Vs was grown in 2216E media supplemented with coumarin at concentrations of 0 and 6.75 mM for 12 h. To detect the effects of the supernatant collected from higher cell density and coumarin on the expression of *Vsm* and *Vsh*, Vs were cultured in 2216E media to approximately OD_{600} of 1.2, and the cell-free supernatant was collected as described above. The supernatant was used to re-suspend the cells with OD_{600} of 0.35; meanwhile, the supernatant containing coumarin was also used to re-suspend another aliquots of cells with OD_{600} of 0.35. The cells with OD_{600} of 0.35 continuously grown in 2216E media were used as a control. All of the three aliquots were cultivated for another 10, 30, and 60 min. To determine the effects of the signal molecules in the supernatant and coumarin on the expression of *Vsm* and *Vsh*, Vs was grown in 2216E media to OD_{600} of approximately 0.3 and then was divided into four parts: one part was supplemented with ethyl acetate extract of supernatant collected from culture with an OD_{600} of 1.2; the second part was supplemented with ethyl acetate extract containing coumarin; the third part was supplemented with ethanol; and the fourth part was supplemented with ethyl acetate and ethanol. Then, each sample was cultured for another 30 min. After treatment under the above conditions, all of the cells were collected by centrifugation and used for real-time RT-PCR.

Real-time RT-PCR

Real-time RT-PCR was carried out as described by Zhang et al. (2013). Total RNA was extracted using the bacterial RNA kit (Omega). DNA contamination was eliminated by taking DNase treatment. cDNA was synthesized from 250 ng of total RNA using the Reverse Transcription System (Takara) and random hexamers according to Takara's instructions. Real-time RT-PCR was carried out in an ABI 7500 real-time detection system by using the SYBR ExScript RT-PCR kit (Takara, China). The primers for RT-PCR were VsmRTF3 (5′-CTCCAACAGAGCCTCGTCG-3′) and VsmRTR3 (5′-GTTCTCATCCAATCTCACCATCA-3′) for *Vsm* and VshRTF5 (5′-GCCAAGCACCGTTC AAAAGA-3′) and VshRTR5 (5′-GAAAAGCCATGCCA CACACC-3′) for *Vsh*. Each assay was performed in triplicate using 16S ribosomal RNA (rRNA) as a control. The primers used for 16S rRNA were 933F (5′-GCACAAGCGGTGGA GCATGTGG-3′) and 16SRTR1 (5′-CGTGTGTAGCCCTG GTCGTA-3′). The comparative threshold cycle method $2^{-\Delta\Delta CT}$ was used to analyze the relative mRNA level of *Vsm* and *Vsh*.

Infection of *A. japonicus*

Live healthy *A. japonicus* (weight 15 ± 3 g) were collected from Dalian Pacific Aquaculture Company (Dalian, China) and were allowed to acclimate for at least 3 days before use. Before artificial infection, 84 individuals of *A. japonicus* were randomly divided into seven groups and immersed with bacterial cells of Vs at a concentration of 4.0×10^6 CFU mL^{-1}. Each group was cultured with the addition of coumarin at concentrations of 0, 1.7, 8.5, 17, 85, and 170 μM, which were selected to be approximately analog with the ratio of bacterial cell number and concentration of coumarin in the above culture condition, according to the previous study of Li et al. (2015). Then, *A. japonicus* were monitored for mortality at 16 °C for 7 days. During this period, the death of *A. japonicus* was recorded daily. The relative percent of survival (RPS) was calculated according to the following formula: RPS = [1 − (% mortality in coumarin pretreated seawater / % mortality in control)] × 100 (Amend et al. 1981).

Database search and accession numbers for *V. splendidus*

Searches for nucleotide and amino acid sequence of *Vsm* and *Vsh* of *V. splendidus* LGP32 were conducted in the database NCBI. Isolate of *V. splendidus* Vs was deposited into the China General Microbiological Culture Collection (CGMCC, Beijing, China) with an accession number CGMCC No. 7.242.

Results

Coumarin suppressed the enzyme activity of Vs

The growth of Vs in 2216E media supplemented with coumarin at concentrations of 0, 0.27, 1.35, and 6.75 mM exhibited approximately the same trend, and the growth did not show a

significant difference at the late stationary phase. This result suggested that coumarin almost had no effect on the growth of Vs. All the growth of Vs under the tested coumarin concentrations increased rapidly from 6 to 24 h and approximately achieved stationary phases at 48 h (Fig. 1). Protease activity of supernatant collected from Vs culture supplemented with coumarin at the tested concentrations exhibited a similar and rapid increased trend from 9 to 24 h. After 24 h, the total protease activities were decreased with the increased concentration of coumarin. When the grown time reached 50 h, the protease activity of individual cell was decreased to 86, 77, and 43% in the presence of coumarin at concentrations of 0.27, 1.35, and 6.75 mM, respectively (Fig. 2). These results showed that coumarin could inhibit the protease activity of Vs.

The grass green hemolytic circle produced by the supernatant of Vs on the sheep blood agar indicated that Vs had apparent α-hemolytic activity. Hemolytic activity of Vs had a generally downward trend as the concentrations of coumarin increased. The hemolytic activity of individual cells decreased to 94, 81, and 80% in the presence of coumarin at concentrations of 0.27, 1.35, and 6.75 mM (Fig. 3). These results showed that coumarin possessed the ability to suppress hemolytic activity of Vs.

Also, Vs showed poor ability to develop biofilm on polystyrene microtiter. Furthermore, under the present tested conditions, it exhibited no effects on biofilm formation (data not shown).

Coumarin protected *A. japonicus* from infection by Vs

During the 7-day observation, a rapid increase in survival rate was observed, as the concentrations of coumarin increased from 8.5 to 170 μM. When *A. japonicus* was cultured in seawater that had been pretreated with 170 μM coumarin, the survival rate of *A. japonicus* was calculated to be 67% after they were co-immersed with Vs. This survival rate was significantly higher than the survival rate of 17%, which was obtained when *A. japonicus* was reared in seawater without coumarin co-immersed with the same cell density of Vs.

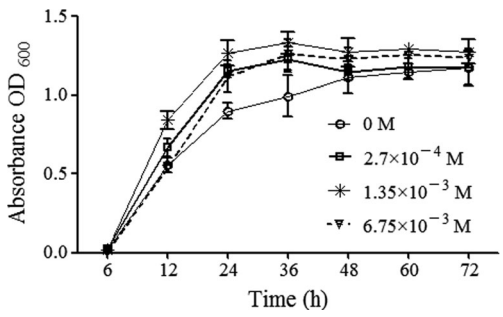

Fig. 1 Growth of Vs in 2216E media supplemented with various concentrations of coumarin. Aliquots were taken at different time points for measurements of absorbance at 600 nm. Data are the means for three independent experiments and are presented as the means ± SE

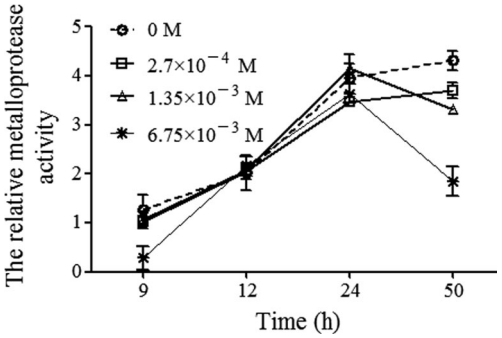

Fig. 2 The effect of coumarin on protease activity. Vs was grown in 2216E media supplemented with coumarin, and the supernatant was incubated with azocasein at 37 °C for 2 h. After being terminated with trichloroacetic acid, aliquots of supernatant were taken at different time points for measurements of absorbance at 350 nm, and the relative protease activity of individual cells was expressed as OD_{350}/OD_{600}. Data are the means for three independent experiments and are presented as the means ± SE

Kaplan–Meyer analysis showed that 170 μM coumarin exhibited an RPS of 60% upon *A. japonicus* when challenged with 4.0×10^6 CFU mL^{-1} Vs ($P = 0.050$), which suggested that coumarin showed an obvious protective effect on *A. japonicus* from infection by *V. splendidus* (Fig. 4).

Reduction of gene expression of potential virulence factors *Vsm* and *Vsh* by coumarin

To further explore the effect of coumarin on the mRNA level of *Vsm* and *Vsh*, Vs was grown in 2216E media supplemented with 0 and 6.75 mM coumarin at an initial OD_{600} of 0.05. After culture for 12 h, OD_{600} of Vs in the media containing different concentrations of coumarin was not significantly different; however, the mRNA levels of both *Vsm* and *Vsh* in the Vs cells supplemented with 6.75 mM coumarin obviously downregulated to respectively 23 and 11% compared with that in Vs cells grown in null 2216E media (Fig. 5), which indicated that coumarin could suppress the expression of potential virulence genes *Vsm* and *Vsh*.

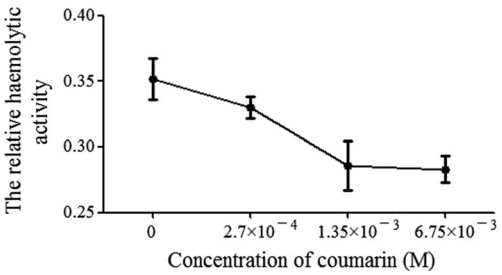

Fig. 3 Effect of coumarin on the hemolytic activity. Vs was grown in 2216E media supplemented with coumarin, and the supernatant was incubated with erythrocyte suspension for 2.5 h. Aliquots were taken for measurements of absorbance at 450 nm, and the relative hemolytic activity of individual cells was expressed as OD_{450}/OD_{600}. Data are the means for three independent experiments and are presented as the means ± SE

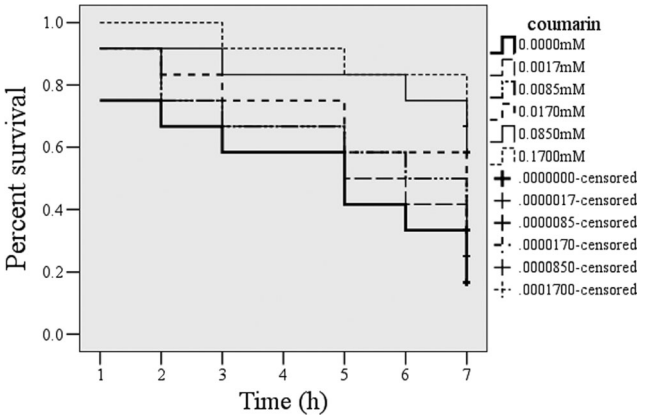

Fig. 4 Percent survival of *A. japonicus* in the presence of various concentrations of coumarin after being challenged with Vs during a period of 7 days. Percent survival was determined using the Kaplan–Meier analysis. Data are the means for three independent experiments and are presented as the means ± SE

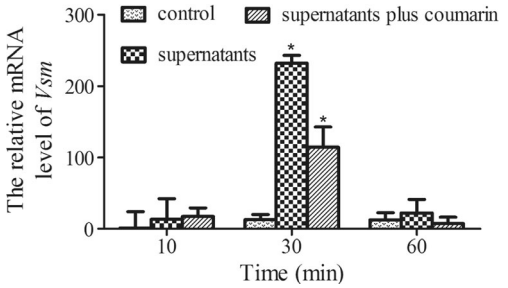

Fig. 6 Effect of supernatant and coumarin on the expression of *Vsm.* Vs was propagated at 28 °C to an OD_{600} of 0.35. Cells were resuspended in the supernatant of OD_{600} of 1.2 and the supernatant containing coumarin, and the cells continuously grown were used as a control. All of the cells were continuously cultured at 28 °C for another 10, 30, and 60 min. Total RNA was extracted from cells and used for real-time RT-PCR. The mRNA level of *Vsm* was normalized to that of 16S rRNA. Data are the means for three independent experiments and are presented as the means ± SE

The effect of supernatant and coumarin on the expression of *Vsm* and *Vsh*

As shown in Fig. 6, the mRNA levels of *Vsm* in Vs cells treated with supernatant were upregulated to 13.0-, 17.4-, and 6.6-fold at 10, 30, and 60 min, respectively, and the highest stimulatory effect was obtained in the cells collected at 30 min. However, the mRNA level of *Vsm* in Vs cells treated with supernatant containing coumarin were upregulated 1.29-fold at 10 min and downregulated to 49 and 32% at 30 and 60 min, compared with the expression of *Vsm* in Vs cells treated with supernatant. And the expression of *Vsm* was strongly downregulated particularly after Vs was cultured with coumarin for 60 min.

Similarly, after treatment with supernatant collected from OD_{600} of 1.2, the mRNA levels of *Vsh* were upregulated to 4.0-, 3.2-, and 7.2-fold, respectively, at 10, 30, and 60 min, and the highest stimulatory effect was observed in cells at 60 min (Fig. 7). However, the mRNA level of *Vsh* in cells treated with supernatant containing coumarin had a reduced trend, and it was downregulated to 97, 40, and 10% at 10, 30, and 60 min compared with the *Vsh* treated with the supernatant. And compared with controls, the mRNA levels of *Vsh* in cells treated with supernatant containing coumarin were upregulated to 3.8- and 1.3-fold at 10 and 30 min, respectively; later, it downregulated to 70% at 60 min. These results indicated that the supernatant obtained from the Vs culture with higher density could upregulate the expression of *Vsm* and *Vsh* in cells at lower cell density, while coumarin could inhibit the upregulation effect of the supernatant and downregulate the gene expression of *Vsm* and *Vsh*. What is more, the effect of coumarin on the expression of both *Vsm* and *Vsh* exhibited lagged effect on the time scale compared with that of the supernatant.

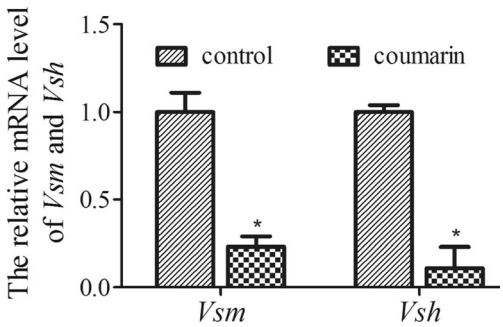

Fig. 5 Effect of coumarin on the expression of *Vsh* and *Vsm*. Vs was grown in 2216E media supplemented with coumarin at 28 °C to an OD_{600} of 1.2, and cells were collected by centrifugation. Total RNA was extracted from cells and used for real-time RT-PCR. The mRNA level of *Vsh* and *Vsm* was normalized to that of 16S rRNA. Data are the means for three independent experiments and are presented as the means ± SE

The effect of ethyl acetate extract and coumarin on the expression of *Vsm* and *Vsh*

It was shown that both Vs and PA1 induced the formation of a purple pigment with CV026, while CV026 mixed with 2216E

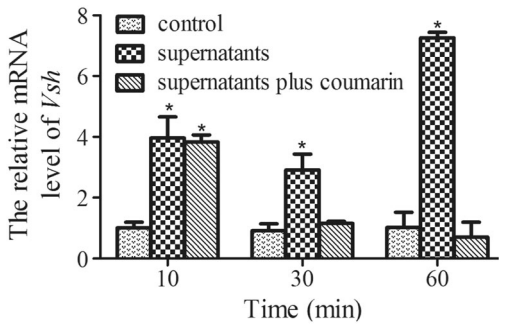

Fig. 7 Effect of supernatant and coumarin on the expression of *Vsh.* Vs was propagated at 28 °C to an OD_{600} of 0.35. Cells were resuspended in the supernatant of OD_{600} of 1.2 and the supernatant containing coumarin, and cells continuously grown were used as a control. All of the cells were continuously cultured at 28 °C for another 10, 30, and 60 min. Total RNA was extracted from cells and used for real-time RT-PCR. The mRNA level of *Vsh* was normalized to that of 16S rRNA. Data are the means for three independent experiments and are presented as the means ± SE

media exhibited unchanged bacterial color. When ethyl acetate extract of supernatant collected from the Vs culture with OD_{600} of 1.2 was added into culture of CV026, the purple color was also developed (Fig. 8a). These results indicated that Vs could produce relatively short AHLs signal molecules that could be extracted using ethyl acetate.

To investigate the effect of the AHLs on the expression of *Vsm* and *Vsh*, ethyl acetate extract was added into the Vs culture with an OD_{600} of 0.35. It showed that ethyl acetate extract could upregulate the expression of *Vsm* and *Vsh* to 2.3- and 5.0-fold, while in the presence of coumarin, expressions of *Vsm* and *Vsh* were downregulated to 33 and 25%, compared with the samples supplemented with ethyl acetate extract respectively (Fig. 8b). These results indicated that the ethyl acetate extract containing AHLs could upregulate the expression of *Vsm* and *Vsh*; however, coumarin could downregulate the stimulatory effect. Thus, it was postulated that AHLs might contribute to the upregulation of *Vsm* and *Vsh*, and coumarin might downregulate the gene expression through inhibiting AHLs.

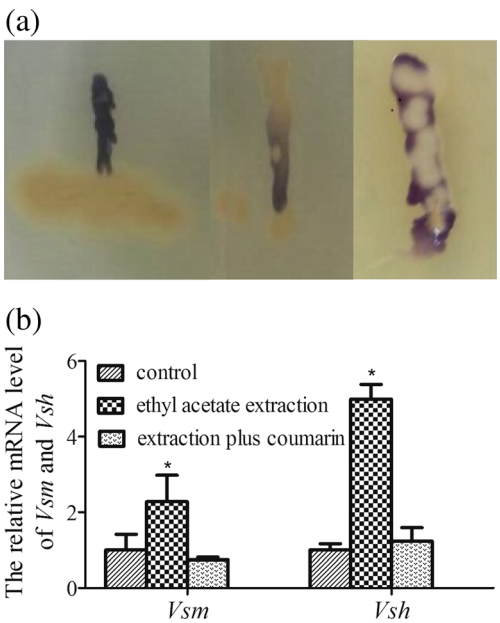

Fig. 8 **a** Detection of AHLs. In all of the three samples, *horizontal line* was tested strain and *vertical line* was CV026. The tested strains were *I* PA1 as positive control, *II* Vs, and *III* ethyl acetate extract. **b** Effect of ethyl acetate extract and coumarin on the expression of *Vsm* and *Vsh.* Vs was cultured at 28 °C to an OD_{600} of 0.3, then cells were divided into four parts: one part was supplemented with ethyl acetate extract; the second part was supplemented with ethyl acetate extract containing coumarin; the third part was supplemented with ethanol; and the fourth part was supplemented with ethyl acetate and ethanol. Then, each sample was cultivated for another 30 min. Total RNA was extracted from cells and used for real-time RT-PCR. The mRNA levels of *Vsm* and *Vsh* were normalized to that of 16S rRNA. Data are the means for three independent experiments and are presented as the means ± SE

Discussion

Quorum sensing (QS), which mediates cell-to-cell communication between bacteria, depends on the production, secretion, and detection of small diffusible autoinducers, such as AHL signaling molecules (Zhang and Li 2016). *V. splendidus* has been reported to produce various AHLs, including C4-HSL and 3-OH-C4-HSL (Bruhn et al. 2005; Decker et al. 2013; Purohit et al. 2013). In this study, the purple pigment formation induced by *V. splendidus* and the ethyl acetate extract of the supernatant suggested that *V. splendidus* could produce short-chain AHLs from C4 to C8 and secrete them into the extracellular media. Furthermore, it has been demonstrated that QS could regulate virulence factors including extracellular toxin (Manefield et al. 2000), siderophore (Lilley and Bassler 2000), protease (Mok et al. 2003), type III secretion system (Henke and Bassler 2004), phospholipase, caseinase, and gelatinase (Natrah et al. 2011a) in different aquatic pathogens, and in *V. splendidus* LGP32, QS could display both intra- and inter-specific effects on the expression of the two protease genes, *Vsm* and *Vam*, for *Vibrio aestuarianus* 02/041 (Decker et al. 2013). However, the function of AHLs in *V. splendidus* has never been described. In the present study, our results showed that the ethyl acetate extract containing AHLs could significantly upregulate the expression of *Vsm* and *Vsh*, which indicated that AHLs might take part in the regulation of virulence factors in *V. splendidus* through the QS system.

Coumarin was reported to inhibit biofilm formation of *E. coli* O157:H7 (Lee et al. 2014) and inhibit phenazine production and swarming motility of *P. aeruginosa* (Gutiérrez-Barranquero et al. 2015). In the present study, similar to the undisturbed effect of *Bacillus* sp. QSI-1 on the growth of *Aeromonas hydrophila* YJ-1 (Chu et al. 2014), coumarin also showed little effect on growth of *V. splendidus*, while it decreased the mRNA expression of two important virulence factors, *Vsh* and *Vsm*, even in the presence of the ethyl acetate extract of the supernatant (AHLs contained), as well as the infection of *V. splendidus*. Considering the fact that coumarin has been identified to be a universal QSI, with potent anti-virulence activity in a broad spectrum of pathogens and the quorum sensing system (Gutiérrez-Barranquero et al. 2015), coumarin reduced the enzyme activity of *V. splendidus*, through downregulating the mRNA level by interfering the AHL-mediated signaling pathways, similar to what occurred in *P. aeruginosa* PAO1 which was inhibited by phenylacetic acid as QSI (Musthafa et al. 2012). This suggested that coumarin might be an environmentally friendly reagent to reduce the virulence factors of *V. splendidus*, in the same way like chemicals which does not inhibit the growth of pathogens (Truchado et al. 2015), without destroying the environment of microbial communities and producing antibiotic-resistant bacteria (Natrah et al. 2011b; Gutiérrez-Barranquero et al. 2015).

In the study of Chu et al. (2014), in which a quorum quenching bacterium QSI-1 can protect zebrafish (*Danio rerio*) from *A. hydrophila* infection and significantly decreased the mortality of infected zebrafish, coumarin was applied to protect *A. japonicus* from *V. splendidus* as another QSI. Coumarin with a RPS of 60% was comparable to the other QSIs such as halogenated classical furanones (Zang et al. 2009), furanones F2 (Liu et al. 2010b), and alkyl maleican hydrides (Steenackers et al. 2010), with the merit of non-toxicity. Compared with Chu et al. (2014) using the complicated supernatants of *Bacillus* sp. as QSI, coumarin alone even at low concentration could significantly increase the survival of *A. japonicus*. Thus, coumarin exhibited the possibility to be used as a QSI to combat infection by *V. splendidus* in the future. Currently, inhibition of QS systems is primarily achieved by inhibiting the synthesis of autoinducers, degrading autoinducers, interfering with autoinducer receptors (Zhang and Li 2016), in light of this background, serial experiments could be performed in the future to identify which ways are occupied by coumarin to modulate *V. splendidus*.

Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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