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Inhibition of *Vibrio* biofilm formation by a marine actinomycete strain A66

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Abstract China remains by far the largest aquaculture producer in the world. However, biofilms formed by pathogenic *Vibrio* strains pose serious problems to marine aquaculture. To provide a strategy for biofilm prevention, control, and eradication, extracts from 88 marine actinomycetes were screened. Thirty-five inhibited the biofilm formation of *Vibrio harveyi*, *Vibrio vulnificus*, and *Vibrio anguillarum* at a concentration of 2.5% (v/v). Thirty-three of the actinomycete extracts dispersed the mature biofilm. Six extracts inhibited the quorum-sensing system of *V. harveyi* by attenuating the signal molecules *N*-acylated homoserine lactones' activity. Strain A66, which was identified as *Steptomyces albus*, both attenuated the biofilms and inhibited their quorum-sensing system. It is suggested that strain A66 is a promising candidate to be used in future marine aquaculture.

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Introduction

China is by far the largest aquaculture producer, and it is growing rapidly (FAO 2005). Vibriosis is by far the most significant factor of aquaculture loss in China (Dong et al. 2003). *Vibrio harveyi* is a serious pathogen of marine fish and invertebrates, particularly penaeid shrimp (Austin and Zhang 2006). However, the pathogenicity mechanism remains elusive. Several studies have suggested that biofilms may be important for survival, virulence, and stress resistance of *Vibrio* spp. (Wai et al. 1998; Watnick and Kolter 1999; Watnick et al. 2001; Wang et al. 2003; Zhu and MeKalanos 2003; Faruque et al. 2006). The persistence and survival of *V. harveyi* in shrimp hatcheries has been attributed to its ability to form biofilms with resistance to disinfectants and antibiotics (Karunasagar et al. 1994).

Quorum sensing (QS) plays an important role in biofilm formation (Schembri et al. 2002). *Vibrio* spp. have been reported to utilize *N*-acylated homoserine lactones (AHLs) to coordinate expression of virulence in response to the density of the surrounding bacterial population (Zhu and MeKalanos 2003; Hammer and Bassler 2003). The AHL molecules are produced by LuxI homologues and constitute, in complex with LuxR homologues, transcriptional regulators (Waters and Bassler 2006). Fighting *Vibrio* infection by interfering with their command language and thereby disrupting virulence expression, instead of inhibiting growth, could serve as an alternative to conventional procedures (Thomas et al. 2005). Another strategy would be based on microorganisms or small molecules with variations in their chemical composition that would allow

them to block the AHL receptor site of the LuxR homologues, such as TraR, or, alternatively, to block the formation of active dimmers that are required for binding to and expression of target genes (Thomas et al. 2005). For example, furanones from alga *Delisea pulchra* was found to inhibit AI-2 signaling as well as homoserine lactone signaling (Ren et al. 2001), and furanone could protect the host from the pathogenic *V. harveyi* by disruption of AI-2 QS (Defoirdt et al. 2006; Tinh et al. 2006).

Marine actinomycetes are promising candidates for marine aquaculture (You et al. 2005; Zhang et al. 2005, 2007). In the present study, extracts from 88 marine actinomycete species revealed that strain A66 is a potential organism against biofilms produced by *Vibrio* spp.

Materials and methods

Bacterial strains and culture conditions

Marine actinomycetes were isolated from the marine sediments of the near-shore of South China Sea and grown in SSB medium (1% soybean extract [about 20 g of soybean powder was boiled for 20 min with 200 ml tap water], 3% sea salt, pH 7.5) or Gause 1 medium (HuanKai) agar at 28°C (You et al. 2005). The aquaculture pathogens *Vibrio vulnificus* V0105, *Vibrio anguillarum* AN0306, and *V. harveyi* H were isolated from infected fish and shrimp (Wu et al. 2001) and grown in a marine Luria–Bertani broth (mLB) medium (0.5% tryptone, 0.5% yeast extract, 3% sea salt, pH 6.0) with different carbon supplements (1% glucose, sucrose, lactose, maltose, xylose, fructose, glycerol, starch, or soybean extract) at 28°C. The total deoxyribonucleic acid (DNA) of marine actinomycete A66 was extracted using the EZNA kit (Omega). The 16s ribosomal DNA (rDNA) was amplified employing the combination of a conserved forward primer pA (5′-AGAGTTT GATCCTGGCTCAG-3′) and reverse primer pH (5′-AAG GAGGTGATCCAGCCGCA-3′). The polymerase chain reaction product is about 1.5 kb. The purified 16S rDNA was sequenced (ShangHai ShengGong Bio.). The sequence was aligned against corresponding sequences of representative strains from GenBank databases. The sequences were aligned manually using CLUSTAL X version 1.8 (Thompson et al. 1997) with available, almost complete, sequences of representative strains of the family Streptomycetaceae retrieved from the DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank databases. Phylogenetic trees were inferred by using the neighbor-joining (Saitou and Nei 1987) algorithms from the PHYLIP package version 3.5c (Felsenstein 1993). Evolutionary distance matrices were generated following the method of Kimura 1980.

Screening for biofilm inhibitors

Actinomycetes were incubated in 96 well plates containing 1 ml SSB medium per well at 28°C for 7 days and then centrifuged, and the supernatant was collected. The actinomycete supernatants were stored at 4°C within 1 week or stored at −20°C for months. *Vibrio* cultures were incubated in 96-well microtiter plates (polystyrene) containing 200 μl mLB medium with 1% glycerol per well at 28°C for 24 h. Planktonic cells and spent media were discarded, and adherent cells were gently rinsed twice with deionized water and allowed to air dry before being stained. The biofilms were stained by 200 μl 0.4% crystal violet solution (w/v) for 10 min, after which the dye was discarded and the wells were rinsed twice with deionized water. The wells were allowed to air dry before solubilization of the crystal violet with 200 μl of dimethyl sulfoxide. The optical density was determined at 570 nm in an enzyme-linked immunosorbent assay reader (Bio-Rad, Munich, Germany; Pratt and Kolter 1998). To screen the destroyer of mature biofilm, biofilms were developed in 96-well plates with mLB with glycerol (GmLB) at 28°C for 24 h. The mature biofilms were incubated with different dilutions of actinomycete supernatants, respectively, for 30 min at 37°C and assayed.

Developing the biofilm on cover glass

V. haveyi H was incubated with a cover glass in a 50-ml Erlenmeyer flask containing 1 ml GmLB medium at 28°C for 24 h. To study biofilm inhibition, the biofilm was developed with 0.5% actinomycete supernatants. To study the biofilm destruction, the mature biofilm was incubated with actinomycete supernatants for 30 min at 37°C. After dying with 0.4% crystal violet solution (w/v) for 10 min, the coverslips were washed with water and air dried and observed by microscope.

Bioassay of quorum-sensing inhibitors

The indicator strain *Agrobacterium tumefaciens*WCF47 (pCF372/pCF218) was obtained from Dr. Winans of Cornell University (Zhu et al. 1998) and was grown in AT minimal medium (Petit and Tempe 1978) at 28°C. The indicator strain WCF47(pCF372), which lacks its own *traI* gene and which has a plasmid-borne P*traI-lacZ* fusion could be induced to express β-galactosidase by *N*-AHSL. The bioassay was performed on an AT minimal medium plate with 0.04 μM 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (X-Gal) and 100 μM isopropyl-β-D-thiogalactoside (IPTG). Actinomycetes were incubated in 96 well plates with 0.1 ml Gause medium per well at 28°C for 7 days. Then, actinomycete strains were cocultured with *N*-

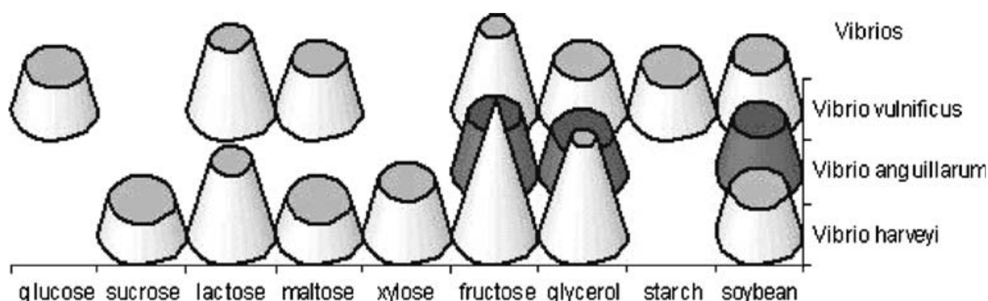


Fig. 1 Effect of carbon supplements on the formation of biofilms. The height of the cone reflects the thickness of the biofilms formed

AHSL in final concentrations of 5 mg/l at 28°C for 2 days. The *N*-AHSL were extracted from *A. tumefaciens* NT1/pTi C58ΔaccR (gift from Dr. Schweizer, Colorado State University, USA). After spotting the indicator strain WCF47 on the bioassay plate, the cocultures were spotted. After incubated at 28°C for 14 h, diffusion of *N*-AHLs was observed. Degradation of macromolecules was assayed by the standard method (Barcina et al. 1987).

Results

Isolation and identification of *Vibrio* pathogen from a marine aquaculture farm

V. harveyi H was isolated from infected white shrimp (*Litopenaeus vannamei*). *V. vulnificus* V0105 and *V. anguillarum* AN0306 were isolated from infected yellow croaker fish (*Pseudosciaena crocea*). Pathogenicity was confirmed by infecting the healthy shrimp or fish with monocultures (Liu, data not shown). The strains were stored at the Guangdong microbial culture collection center.

The effect of nutrient supplements on the formation of *Vibrio* biofilms

It has recently become apparent that nutrient cues play an important role in *Vibrio* biofilm formation (Davey and O'Toole 2000; Wong et al. 2002; Kierek and Watnick 2003). To develop a cheap, robust, and consistent biofilm assay, we first examined the effect of carbon supplements on the formation of pathogenic *Vibrio* biofilms. Nine carbon sources were tried in a standard quantitative 96-well format (O'Toole et al. 1999). Fructose, glycerol, and soybean extract were found to enhance the development of *Vibrio* biofilm as measured by the crystal violet-staining assay (Fig. 1). The mLB medium supplemented with 1% glycerol was designated as an effective medium for the biofilm assay.

Table 1 Inhibition of biofilm formation of pathogenic *Vibrio* spp. by marine actinomycete supernatants

	Vibrio harveyi	*Vibrio vulnificus*	*Vibrio anguillarum*
Inhibitors of biofilm formation	18	12	27
Inhibit biofilm formation but not inhibit the growth of *Vibrio* strains	15	3	16

Screening for biofilm inhibitors

The extracts of 88 marine actinomycetes, which were isolated from a marine aquaculture farm, were assayed by the screening system (You et al. 2005). At a concentration of 2.5% (v/v), 35 marine actinomycetes were found to inhibit at least one of the *Vibrio* species in biofilm formation (Inhibition rate >80°C). Eighteen, 12, and 27 of the marine actinomycetes could inhibit the biofilm formation of *V. harveyi*, *V. vulnificus*, and *V. anguillarum*, respectively (Table 1). Fifteen, 3, and 16 of them could inhibit the *Vibrio* biofilm but did not affect the growth of the *Vibrio* strains, respectively (Table 1), including the A66 extract. The data suggest that the mechanism of these marine actinomycetes against *V. harveyi* biofilm formation is different from that of the *V. anguillarum* biofilm.

At the top of the ranking lists of hits (Table 2), strain A66 not only attenuated the biofilm formation of *V. harveyi* but also dispersed its mature biofilm. At a concentration of 2.5% (v/v), the inhibition rate of A66 extract was 99.3%, and the degradation rate was 75.6%.

Table 2 Decrease in *N*-AHLs activity and inhibition of biofilm formation by marine actinomycetes

Marine actinomycete	Decrease in *N*-AHLs activity	Inhibition of biofilm formation		
		Vibrio harveyi	*Vibrio vulnificus*	*Vibrio anguillarum*
A66	+	+	+	−
A67	+	+	+	−
B15	+	−	+	+
86	+	+	−	−

Plus signs stand for relative intensity

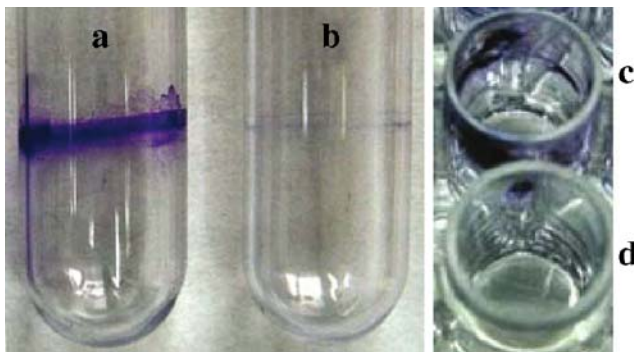
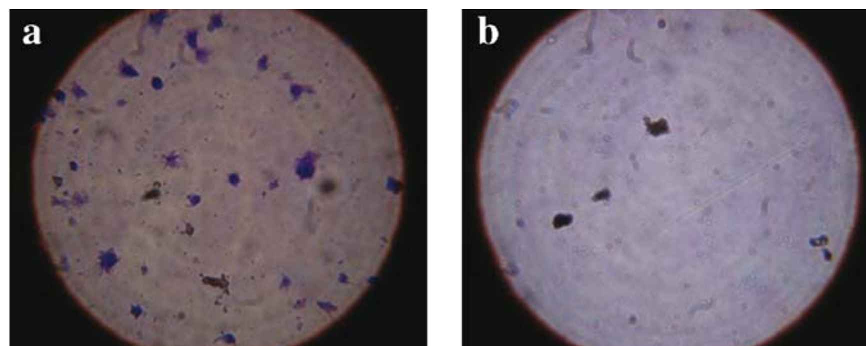


Fig. 2 Biofilm inhibition of *Vibrio harveyi* H by marine actinomycete A66 in borosilicate glass tubes and on polystyrene surface. **a** and **c** were controls. **b** and **d** were treated with A66 extracts

The above experiment was done on polystyrene microplates. We also tested the biofilm formation of *V. harveyi* in borosilicate glass tubes (Fig. 2) and on cover glasses (Fig. 3). For the control tube and well, a thick layer of biofilm was easily stained by crystal violet. However, when treated with A66 extracts at a concentration of 2.5% (v/v), biofilm reduction in *V. harveyi* was observed. Strain A66 not only reduced the quantity of microcolonies (Fig. 3b) but also attenuated the architecture of the biofilm (Fig. 3d). It is suggested that strain A66 can inhibited the *Vibrio* biofilm formation at both the initiation stage and the maturation stage. Because the QS system regulates biofilm maturation of *V. cholerae* (Zhu and MeKalanos 2003; Hammer and Bassler 2003), we further tested the effect of A66 on the QS system of *V. harveyi*.

An ultrasensitive bioassay system for the detection of AHLs was constructed in *A. tumefaciens* by using the T7 expression system to overproduce the AHL receptor TraR. This strain could detect many diverse AHLs, some at extremely low concentrations (Zhu et al. 2003). For the agar stripe assay we used, positive control could turn the second colony into blue, while all the colonies of the negative control remain white (Fig. 4). Strain A66 was found to attenuate the activity of *N*-AHLs and render all the colonies white. Marine actinomycetes strains A67, B15, and 86 also attenuate the activity of *N*-AHLs to some extent but not as dramatically as A66. They also inhibited biofilm formation of *Vibrio* spp. (Table 2). It thus suggests that the inhibition of biofilm formation may be associated with the blockage of its QS system.



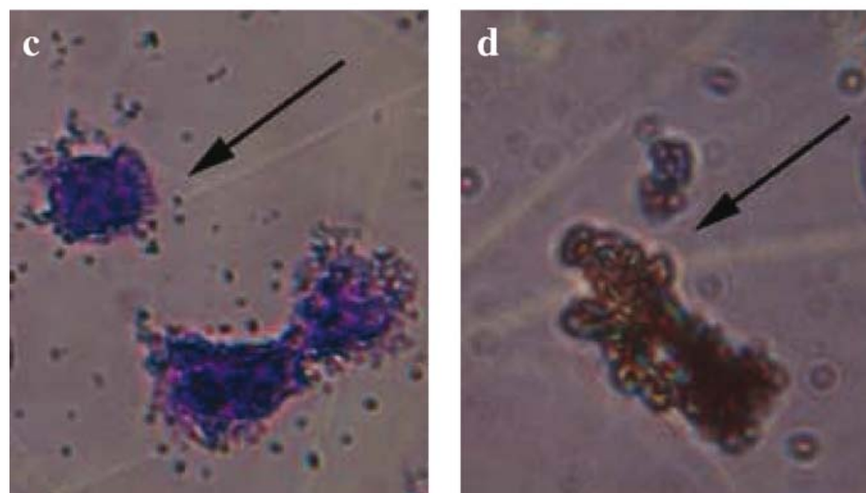


Fig. 3 Inhibition of formation of *Vibrio harveyi* H biofilm by marine actinomycete A66 on the cover glass. **a** and **c** were controls. A66 not only reduced the quantity of microcolonies **b** but also affected the architecture of biofilm **d**, making it looser

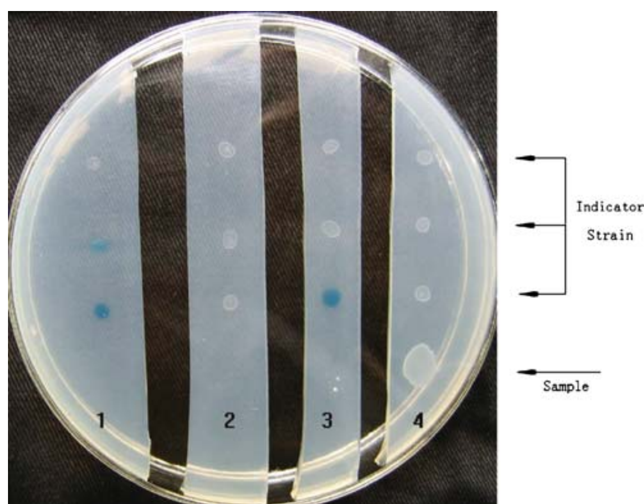


Fig. 4 Degradation of *N*-AHSL by marine actinomycete A66. *Lanes 1* and *4* were negative and positive control, respectively. The degradation activity of A66 (*lane 2*) is stronger than A67 (*lane 3*)

The *Vibrio* biofilm degradation activity of marine actionmycete A66 was further observed by the assay on cover glass (Fig. 5). The architecture of the microcolonies was totally destroyed.

Identification of marine actinomycete A66

The 16S rDNA sequence of strain A66 was sequenced. By comparison with other 16S rDNA sequences in GeneBank, the phylogenetic tree was constructed and shown in Fig. 6. Strain A66 and *Streptomyces albus* subsp. *albus* NBRC 3711, *S. albus* subsp. *albus* NBRC 3422, *Streptomyces saprophyticus* NBRC 13440, and *Streptomyces krainskii* NBRC 13053^{T} produced a consistent cluster with a high bootstrap value. The number of scored nucleotide identities and the overall percentage of similarity calculated by pairwise analysis indicates a sequence similarity of 99.9% between these organisms. All the other related *Streptomyces*

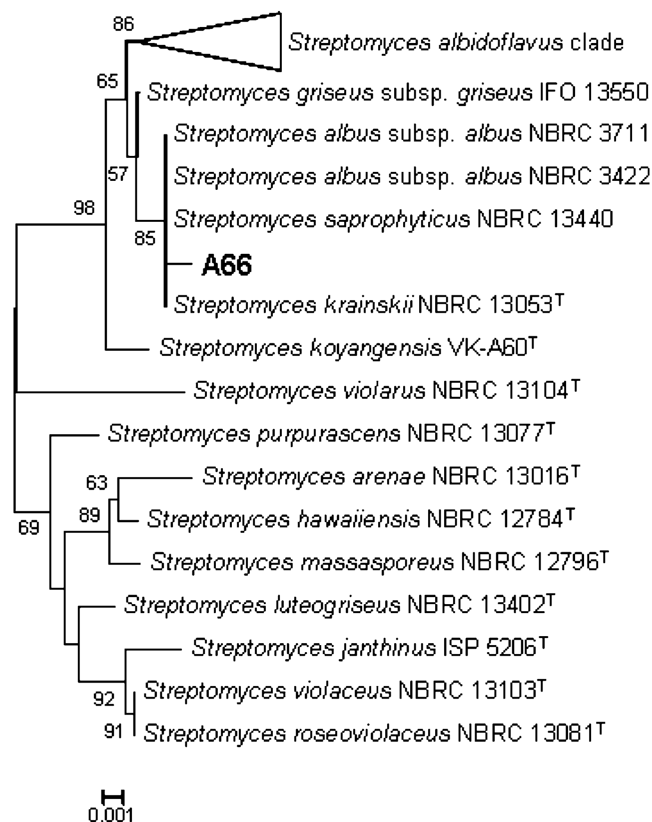


Fig. 6 Unrooted neighbor-joining tree (Saitou and Nei 1987) based on nearly complete 16S rDNA sequences, showing the position of strain A66 in the *Streptomyces* tree. *Numbers at nodes* are bootstrap values (%) based on 1,000 resampled datasets; only values greater than 50% are given. *Bar*, 0.001 nucleotide substitutions per nucleotide position

sequences shared relatively lower similarity with A66 and were recovered in distinct branches (Fig. 6).

Furthermore, comparison of the morphological features of strain A66 (Table 3) with those of the representative strains in the tree using Bergey's Manual of Systematic Bacteriology supposed a proposal that A66 is a strain of *S. albus*.

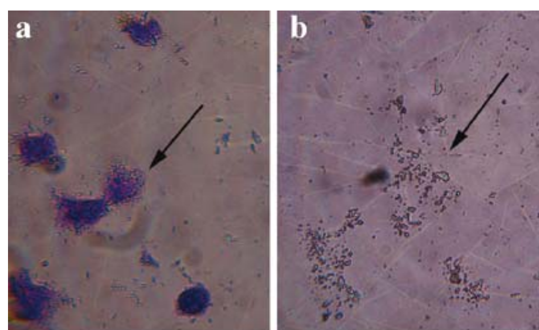


Fig. 5 *Vibrio harveyi* biofilm destroyed by marine actinomycete A66. **a** is the control. **b** contains A66

Discussion

In this study, marine actinomycete A66 was found to be one of the more promising candidates of an organism with potential use in marine aquaculture. The metabolites of actinomycete A66 reduced the number of microcolonies of *V. harveyi* by about tenfold (Fig. 3). This indicates that biofilm formation was possibly inhibited at the beginning of the attachment stage. Flagella and pili could facilitate initial adherence (Shi and Sun 2002). If they were enzymolysized or their expressions inhibited by the extracts from the actinomycete, the formation of biofilm could be inhibited.

Table 3 Morphological features of strain A66 cultured on Gause agar medium at 28°C for 14 days

Aerial mycelia present	Aerial mycelia color	Spores present	Spore color	Surface color	Reverse color	Exudate present	Exudate color	Diffusible pigment present	Diffusible pigment color
Yes, few	White	Yes, thick linear	White	White	Yellow	No	No	No	No

Another important step in biofilm development is the formation of the characteristic biofilm architecture. Figure 3 shows that the architecture of the *Vibrio* microcolonies that were treated with strain A66 extract was looser than that of the control. Exopolymeric substance (EPS) is essential for the development of biofilm architecture in *V. cholerae* (Watnick and Kolter 1999). Thus, it is possible that the natural product of marine actinomycetes in this study inhibits the expression of the exopolysaccharide synthesis gene, such as *vps*, and leads to the loose architecture of *V. harveyi* biofilm (Bomchil et al. 2003). It was reported that overproduction of EPS leads to alterations in biofilm architecture that correlate with an increased resistance of the cells to osmotic and oxidative stresses as well as killing by biocides such as chlorine (Wai et al. 1998; Yildiz and Schoolnik 1999). Thus, if marine actinomycete strain A66 loosens the architecture of the *Vibrio* microcolonies by inhibiting expression of EPS, it is possible that the resistance of the sessile cells to antibiotics would be reduced.

Another important role is played by the QS factor AHLs, which are required for the formation of a biofilm with a complex "wild-type" architecture. Givskov's group proved that the QS inhibitor (QSI) could promote the susceptibility of a *Pseudomonas aeruginosa* biofilm to a variety of antimicrobial compounds (Hentzer et al. 2003). Furthermore, biofilm formation and virulence were controlled by the QS system in parallel in *V. cholerae*. Thus, QS system inhibition is not only a means to control biofilm formation but also a way to reduce the virulence of a pathogen. Strain A66 could degrade the QS factor *N*-AHSL. It suggested that the loose architecture of the *V. harveyi* biofilm is probably related to the inhibition of the QS system by strain A66. However, Hammer and Bassler 2003 reported that in *V. cholerae* at low cell density, LuxO- and HapR-activated *vps* genes and the biofilm formation was enhanced. Furthermore, Zhu and MeKalanos 2003 reported that CqsA, one of two known autoinducer (AI) syntheses in *V. cholerae*, acts through HapR to repress *vps* gene expression. Waters and Bassler 2006 reported that AI addition represses GGDEF proteins in *V. harveyi*, which could be associated with repression of biofilm production in *Vibrio* spp. It means that the low concentration of AIs should enhance the formation of biofilm in *Vibrio* spp. These different results showed that the QS system may be not the most important factor of *Vibrio* biofilm formation, and a functional QS system is not an absolute requirement for *Vibrio* biofilm formation. There are three different *V. harveyi* AIs, including HAI-1, AI-2, and CAI-1 (Waters and Bassler 2006). In our study, strain A66 could degrade the *N*-AHSL, an acyl homoserine lactone, as HAI-1; The study of Zhu et al. (2003) showed that CAI-1 signaling is responsible for HapR-regulated biofilm formation but not AI-2; in Waters and Bassler's (2006) study, the GGDEF protein was repressed by AI-2 and HAI-1. Therefore, the different result may be due to the different functions of AIs.

The survival of pathogenic *Vibrio*, both in the natural environment and within the host organisms, is likely to be enhanced if the cells exist in biofilms (Zhu and MeKalanos 2003). It was reported that in vivo-formed biofilms are responsible for enhanced infectivity of *V. cholerae* shed in human stools (Faruque et al. 2006). This means that the biofilms shed from the host are more harmful, especially in the aquaculture environment. They could contaminate the whole farm and enhance the prevalence of vibriosis. Furthermore, biofilm bacteria can be up to 1,000-fold more resistant to antibiotic treatment than the same organism grown planktonically (Brooun et al. 2000). Thus, it is also important to destroy the mature biofilm in the environment. In this study, marine actinomycete strain A66 was able to destroy the biofilm of *V.harveyi*. Table 2 showed that strain A66 could also degrade the cell wall of bacteria and protein. The mechanism may involve an enzyme that could digest the compound within the biofilm, such as exopolysaccharide and protein, or the natural product could act by inducing biofilm self-destruction.

The biofilm, which is difficult to eradicate and is resistant to antibiotics, is the barrier of perpetuating infection in fish farms (Karunasagar et al. 1996). Thus, the biofilm inhibitor or destroyer could be combined with a conventional antibiotic to eradicate the biofilm. The multifunctional strain A66 is one of the most promising candidates to be used in marine aquaculture. It could control *Vibrio* biofilms in aquaculture farm by inhibiting their formation and dispersing the mature debris of the biofilm. Furthermore, it could inhibit the QS system, which relates to the infectivity, virulence, and biofilm formation of pathogens.

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