The anti-quorum sensing activity and bioactive substance of a marine derived Streptomyces

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ABSTRACT
Since bacterial quorum sensing system has been verified to regulate many microbial physiological activities, such as virulence factor production and biofilm formation in pathogens, blocking quorum sensing has been recognized as a viable approach for the development of novel therapeutics in treating bacterial infections. In this study, quorum quenching activities of marine actinomycetes isolated from seawater sample were investigated. Out of 56 morphological different strains, 5 strains had been found exhibiting anti-quorum sensing activity against the violacein production of Chromobacterium violaceum 12472. One highly active strain HY026 was identified as Streptomyces parvulus based on the 16S rDNA sequence analysis and morphological features. Spent culture medium of HY026 could also significantly inhibit the biofilm formation of four bacteria. One major active compound named F1-4, accounting for 26.3% of crude extracts, was obtained from HY026 cultures and identified as actinomycin D based on the analysis of the NMR and MS. F1-4 could significantly inhibit the violacein production of C. violaceum with the inhibition rate of 64.9% but not affect the bacterial growth at the concentration of 12.5 \( \mu \)g/mL. F1-4 could also inhibit the prodigiosin production of Serratia proteamaculans 657 with the pigment inhibition zone of 13.5 mm at concentration of 25 \( \mu \)g/disc without affecting the bacterial growth. Another pure compound, cyclic (4-hydroxy-Pro-Phe) was also isolated and identified from this actinomycetes strain. The higher production and easier isolation of actinomycin D from S. parvulus HY026 indicated that this strain could be a potential source of quorum sensing inhibitors.

Introduction

Quorum sensing (QS), a communication system in bacteria which exists not only in the same species but also among different species, could regulate many microbial physiological activities, as well as the relationship among different bacteria. In response to the bacterial cell density, the production and sense of some small signal molecules, usually N-acylhomoserine lactones (AHL) in Gram-negative bacteria and oligopeptides in Gram-positive bacteria, the expression of many genes is regulated by bacterial quorum sensing system and in turn the bacterial behaviour [1,2].

Since many bacterial physiological activities regulated by quorum sensing system are detrimental, such as virulence factor production and biofilm formation in pathogens, possible function of quorum sensing inhibitors (QSIs) started to be notified [3,4]. It has been a new strategy to use QSIs in treating bacterial infections, because QSIs may block the QS and in turn prevent the virulence factor production as well as biofilm formation of pathogens. Additionally, QSIs do not directly target on the significant growth process of pathogen, and will not provide fatal selection pressure to the bacteria like common antibiotics, the generation of resistant mutants might be limited [5]. QSIs may have potential application in the control of bacterial diseases as well as the biofilm related continues infections and biofouling [6]. Besides, quorum sensing inhibitors are also useful research tools for understanding the functional roles of bacterial quorum sensing. Therefore, there have been more and more activities in developing quorum sensing inhibitors isolated from natural sources [7–9] or screened from synthesized compounds [5] in recent years. Many potent compounds and crude extracts have been found from plants [10] and microbes [11]. Some of them were AHL analogues which could mimic or compete with AHL signals, while some of them were AHL-lactonase or AHL-acylase which could degrade AHL signals [12,13].

The medicinal value of actinomycetes has already been recognized for several decades [14]. Hundreds of compounds with different structure or remarkable bioactivity have been isolated from actinomycetes, and about 70% of
the naturally derived compounds are currently in clinical use [15]. However, the anti-quorum sensing activity of actinomycetes has been rarely reported. Complex diversity of marine ecological environment gives marine actinomycetes the intense microbial competition for space and nutrient resources [16]. To mediate microbe–microbe interaction, marine actinomycetes might also exhibit ability in production of quorum sensing related metabolites.

In this study, 56 actinomycetes strains were isolated from seawater samples, and 5 strains were found exhibiting certain anti-quorum sensing activity against the violacein production of the reporter strain Chromobacterium violaceum by disc diffusion assay and pigment inhibition assay. Among them one highly active strain was identified as Streptomyces parvulus HY026. The active compound in metabolites of HY026 was also isolated and identified.

Materials and methods

Actinomycetes isolation and preservation

Seawater samples were collected from Lianyungang region (1 m depth) and brought to the lab. The samples were pretreated with water bath at 55 °C for half an hour to stimulate the germination of spores and to reduce the number of bacteria. Pretreated samples were then serial diluted to 10⁻³, 10⁻⁴ and 10⁻⁵. After that, 100 µL of each dilution was spread onto the Gauze No.1 agar medium plate which was prepared with artificial seawater and supplemented with 0.01% K₂Cr₂O₇. The agar plates were then incubated at 30 °C and checked once each day. Colonies grown on the plate were picked and further purified on fresh Gauze No.1 agar medium. The pure strains were stocked in 30% glycerol and kept at −80 °C.

Isolates cultivation and metabolites extraction

Pure isolates were subcultured in Gauze No. 1 liquid medium at 30 °C for 5 d. The cultures were extracted with equal volume of ethyl acetate (EtOAc) containing 5% acetone for two times. The organic layer was combined and evaporated using rotor vac (Shanghai Yarong, RES52CS) under reduced vacuum and at 35 °C. The residues were then dissolved in EtOAc or DMSO to the concentration of 50 mg/mL for bioassays.

Anti-quorum sensing activity screening

Disc diffusion assay and pigment inhibition assay were used to screen for the anti-quorum sensing active strains. In disc diffusion assay, reporter strain Chromobacterium violaceum 12472 was subcultured in LB liquid medium (containing 0.5% yeast extract, 1% tryptone, 1% NaCl, pH 7.0–7.2) overnight. Extracts were loaded onto the sterile paper disc (Φ 6 mm) to the test concentration. After the solvent was evaporated, the paper discs were placed onto the LB agar plates which were spread with 100 µL C. violaceum in advance. The pigment inhibition zones (in diameter) of the reporter strain created by extracts were observed after 24–48 h incubation at 30 °C. Serratia proteamaculans 657, which could produce red pigment prodigiosin (Miao et al. 2013), was also used as reporter strain to detect anti-quorum sensing activity of the isolated compounds using the same method. This strain was subcultured at 23 °C and the pigment inhibition zone (in diameter) was measured after 72 h incubation.

In pigment inhibition assay, overnight cultured C. violaceum 12472 was adjusted to OD₅₇₀ = 0.1 using fresh LB liquid medium and transferred to small test tube (2 mL/tube). The extracts of actinomycetes were added into the solution to the test concentration. Same volume of the solvent DMSO was served as negative control. All samples were cultured at 30 °C with rotation of 150 rpm for 24–48 h. The cultures were then centrifuged at 10 000 rpm for 30 min, bacterial cells were collected and added with 0.5 mL DMSO to dissolve the violet pigment from the bacterial cells. The cell debris in the resulted solutions was removed by centrifuge (10 000 rpm, 10 min). OD₅₇₀ of the solution was measured and the inhibition rate of violacein production was calculated based on the following formula:

\[
\text{Inhibition rate(\%)} = \frac{\text{OD}_{570\text{control}} - \text{OD}_{570\text{sample}}}{\text{OD}_{570\text{control}}} \times 100\%
\]

The strain with the highest activity was selected for further study.

Strain identification

The highly active strain was selected for identification based on the 16 s rDNA sequence analysis and morphological observation. Selected strain was cultured in Gauze No.1 liquid medium and the cells were collected by centrifugation (10 000 rpm, 10 min). Genomic DNA was extracted using the SK1201-UNIQ-10 DNA extraction kit (Sangon). The 16S rRNA genes (rDNA) in the genomic DNA were PCR amplified using the primers 7F (5’-CAGAGTTTGATCCTGGCT-3’) and 1540R (5’-AGGAGGTGATCCACGCGCA-3’). 16 s rDNA sequencing was performed by Shanghai Sangon Biotech Company. The strain was then identified by comparison with sequences available in Genbank databases. Similar sequences were aligned using multiple sequence alignment program MEGA (version 5.05). Gaps and positions with ambiguities were excluded from the phylogenetic analysis.
Phylogenetic analysis was performed using the neighbour-joining methods.

**Anti-quorum sensing confirmation assay**

To confirm that the violacein inhibition of reporter strain was due to the anti-quorum sensing activity of the positive extracts or active compounds, an additional assay was performed. Small pieces of agar within and outside the inhibition zone created by the extracts in disc diffusion assay were picked and added into fresh LB medium separately and shaken cultured at 30 °C for 24 h. The OD_{600} of the cultures were then measured and compared. Addition of agar in the fresh agar plate or agar within the penicillin created inhibition zone served as negative control. Cell growth of *C. violaceum* in liquid medium with or without the active compounds was also measured to confirm the anti-quorum sensing activity of the active compound. Reporter strain was inoculated into the fresh LB medium together with the active compounds at different concentration and then subcultured at 30 °C with rotation. The OD_{600} of the cultures at 0, 8, 16, 24 and 48 h were measured. The growth of reporter strain in each treatment was compared with negative control (solvent DMSO) to figure out whether the growth of reporter strains was affected by the active compounds.

**Biofilm inhibition assay**

The biofilm inhibitory activity of selected strain was measured by the following method. The selected active strain was subcultured in Gauze No. 1 liquid medium at 30 °C for 7 d. The cell free spent culture medium (spent medium in short) of the active strain was obtained by removing bacterial cells through centrifugation at 5000 rpm for 15 min and followed by filtration through 0.22 μm membrane. The biofilm inhibition assay was performed in 24-well plate. Four bacterial strains, *Pseudomonas aeruginosa* PAO1, *Staphylococcus aureus* 95005, *Micrococcus luteus* 95006 and *Ruegeria* sp. 01008, were selected as target bacteria. Target bacteria were inoculated into LB (for PAO1) or MB medium (1 mL in each well) and stationary cultured with or without spent medium for 10 h (24 h for PAO1) at 30 °C. Biofilms were stained with 200 μL 0.4% crystal violet and dissolved in 1 mL DMSO, and the OD_{570} of each treatment was measured by microplate reader to quantify the bacterial biofilm formation. Stationary bacterial growth in each well was also measured at OD_{600}. For each treatment, 4 replicates were performed.

**Microscopy observation**

The biofilms and the microbial cells were fixed overnight in a solution containing 2.5% glutaraldehyde. Samples were then washed with 0.1 mol/L PBS buffer (pH 7.4), dehydrated through a graded series of ethanol, critical point dried and gold sputtered, and examined by field emission scanning electron microscopy (FESEM, S-4800, Hitachi, Japan).

**Growth, metabolites production and activity profile of the selected strain during the cultivation process**

To study the growth, metabolites production and activity profile of the selected strain during the cultivation process, the strain was subcultured in 100 mL Gauze’s No.1 liquid medium at 30 °C with rotation, The cells in the 1–10 day cultures were collected by centrifugation at 10 000 rpm separately and the dry weight of the cells were measured after freezing dry. The supernatants were extracted by EtOAc and the extracts were weighed and 3–9 day extracts were tested for the anti-quorum sensing activity by disc diffusion assay.

**Isolation and identification of active compounds from selected strain**

The highly active strain was mass cultured and the cultures were extracted with EtOAc for three times. The organic fractions were combined and evaporated. The residue was suspended in MeOH/H$_2$O 9:1 and extracted with hexane for three times. Some H$_2$O was added to MeOH/H$_2$O layer to MeOH/H$_2$O 6.5:1 and extracted with dichloromethane (DCM) for three times. Hexane layer, DCM layer and MeOH/H$_2$O layer were concentrated and tested for their anti-quorum sensing activity. The active fraction was further isolated using column chromatography. The active components were identified using NMR (AVANCE 600, Bruker) and GC-MS (Trace DSQ, Thermo).

**Results and discussion**

**Strain isolation and anti-quorum sensing activity screen**

In total, 56 morphologically different actinomycetes strains were isolated and stocked. Each strain was subcultured in 100 mL Gauze No. 1 liquid medium and the extracts of the cultures were screened for anti-quorum sensing activity. The results of disc diffusion assay showed that 5 strains (HY004, HY012, HY026, HY072, HY086) had certain anti-quorum sensing activity with the violacein inhibition zone from 7.2 mm to 11 mm at a concentration of 250 μg/disc. Further screen showed
that 4 of the above active strains had some inhibition activity in pigment inhibition assay with the percentage of inhibition from 12.77% to 35.33% at a concentration of 250 \( \mu \text{g/mL} \) (Table 1). Strain HY026 showed the highest and remarkable activity in both assays; therefore, this strain was selected for further identification and active compounds isolation.

In recent years, more and more quorum sensing inhibitors related researches have been carried out. Actinomycetes, a rich and influential source of bioactive metabolites, however, received relatively less attention in the search for quorum quenching inhibitors. The primary screen results in this study indicated that marine actinomycetes should also be an important source of quorum sensing inhibitors and worthy of more attention in QSI development research.

### Identification of strain HY026

Genomic DNA of strain HY026 was extracted, the 16 s rDNA was PCR amplified and sequenced. The nearly complete 16S rRNA gene sequence of strain HY026 (1472 bp) was obtained and submitted to GenBank (Accession number: KJ200636.1). Comparative analysis of the 16S rRNA gene sequence with sequences deposited in GenBank using BLAST indicated that the strain belong to the genus *Streptomyces* and shared the highest sequence similarities with *Streptomyces parvulus* MARS-17 (GQ451836, 99.4%). Based on the 16S rDNA sequence analysis, the phylogenic tree of the strain HY026 and its related species were constructed as shown in Figure 1(a). HY026 grew faster on Gause’s No.1 agar plate and the colony was granular and rough, emossed in center, about 0.5 cm in diameter after five-day cultivation with white aerial haphae and brown substrate haphae (Figure 1(b)). Bright yellow pigment was produced after five-day cultivation and light purple spores could be observed under light microscopy. The filamentous bacterial cell structure was observed by FESEM (Figure 1(c)). Based on the sequence analysis and morphological observation, strain HY026 was identified as *S. parvulus*.

### Confirmation of the anti-quorum sensing activity of HY026

To confirm the anti-quorum sensing activity of *S. parvulus* HY026, small pieces of agar within and outside the inhibition zone created by HY026 extracts in disc diffusion assay, labeled as WI and OS independently, were picked and added into LB medium separately. After 24 h rotated cultivation, media of WI and OS treatments

| Strain No | Inhibition zone (mm) | Percentage of violacein inhibition |
|-----------|----------------------|-----------------------------------|
| HY004     | 7.2 ± 0.06           | 13.6 ± 0.56                       |
| HY012     | 7.45 ± 0.21          | 12.95 ± 1.54                      |
| HY026     | 10.9 ± 0.14          | 35.33 ± 1.07                      |
| HY072     | 6.85 ± 0.07          | 0                                 |
| HY086     | 7.6 ± 0.03           | 12.77 ± 1.39                      |

Figure 1. Phylogenetic tree (a), colony (b) and microscopic photo (c) of the strain HY026.
became turbid and the growth of bacteria were observed. OD$_{600}$ of the WI and OS treatments were 0.483 and 0.517 separately, while OD$_{600}$ of the negative control to which a piece of fresh agar was added was 0.048. No pigment production was observed in the treatment WI, while violacein production was observed in the treatment OS after 48 h cultivation. These results indicated that the violacein inhibition was mostly due to the interruption of quorum sensing system in *C. violaceum* rather than the inhibition of bacterial growth.

**Biofilm inhibitory activity of HY026**

The biofilm inhibitory activities of *S. parvulus* HY026 spent medium against four bacterial strains were investigated. The test concentration of the spent medium was 10%, 20% and 50% (v/v). The results showed that the biofilm formation of *Pseudomonas aeruginosa* PAO1, *Staphylococcus aureus* 95005, *Micrococcus luteus* 95006 and *Ruegeria* sp. 01008 were significantly inhibited by the spent medium at the lowest test concentration, and the inhibition rates were 37.12%, 53.72%, 22.20% and 45.98%, respectively (Figure 2). The biofilm inhibitory activity of the spent medium slightly increased along with the test concentration. This trend was verified by SEM observation. PAO1 formed dense and multi-layer biofilm in control, while PAO1 formed sporadic and single layer biofilm when treated with HY026 spent medium. The experiment results are shown in Figure 3.

![Figure 2](image1.png)

**Figure 2.** The biofilm formation inhibitive activity of spent culture medium of HY026 against four bacterial strains *Pseudomonas aeruginosa* PAO1, *Staphylococcus aureus* 95005, *Micrococcus luteus* 95006 and *Ruegeria* sp. 01008. The test concentration was 10%, 20% and 50% spent culture medium (v/v). Data plotted are mean ± SD of four replicates per treatment.

![Figure 3](image2.png)

**Figure 3.** The FESEM photographs of *Pseudomonas aeruginosa* PAO1 biofilms formed on glass cover slip. a: biofilm control, b–d: samples treated with HY026 spent medium (10%, 20% and 50%).
medium (Figure 3). The stationary bacterial growth was not affected at lower test concentration. However, slightly stationary bacterial growth inhibition was observed in the highest concentration treatment. The biofilm inhibition assay further verified the anti-quorum sensing activity of the strain S. parvulus HY026.

Lee et al. [17] reported the spent culture medium of a Streptomyces parvulus strain exhibited anti-biofilm and anti-hemolytic activities against S. aureus at a concentration of 1% (v/v). In comparison, the biofilm inhibitory activity of S. parvulus HY026 observed in this study was lower than that strain. This might due to the different sensitivity of the test bacterial species.

**Growth, metabolites production and activity profile of HY026 in the cultivation process**

The results of growth curve, metabolites production and activity profile of HY026 showed that the growth of the strain increased steadily in the first four days, then became faster from the fifth to the ninth day and dropped down in the tenth day. The metabolites production followed the similar trend of the growth (Figure 4(a)). Anti-quorum sensing activity was found in the third day extracts and the activity of the extracts reached a peak in the sixth day cultures and the violacein inhibition zone was over 16 mm in diameter. The activity was maintained to the eighth day cultures and slightly dropped from the ninth day (Figure 4(b)).

**Compound isolation of streptomyces parvulus HY026**

Streptomyces parvulus HY026 was mass cultured in 60 L at 30 °C for 9 d, and in total, 5.013 g of crude extracts was obtained from bacterial cultures by EtOAc extraction. The active DCM fraction was firstly separated using reduce pressure silica gel column chromatography eluted with DCM/MeOH from 30:1 (F1), 20:1 (F2), 15:1 (F3), 10:1 (F4), 5:1 (F5) to 0:1 (F6). The anti-quorum sensing activity of each fractions were tested by disc diffusion assay, and it was found that at a concentration of 250 μg/mL, subfraction F1 (3.95 g) and F2 (0.27 g) showed strong activity with a violacein inhibition zone of 16 mm, 14 mm, while very weak activity was detected in subfractions F3, F4, F5 and F6. Since the activity and amount of F1 was higher, this fraction was further separated using silica gel column chromatography eluted with DCM/MeOH from 1:0 (F1-1), 80:1 (F1-2), 60:1 (F1-3), 40:1 (F1-4), 30:1 (F1-5), 20:1 (F1-6), 15:1 (F1-7) to 0:1 (F1-8). The disc diffusion assay showed that the active substance is mainly located in F1-3 (1.2 g), F1-4 (1.32 g) and F1-5 (0.29 g) with a violacein inhibition zone of 17.9 mm, 19.2 mm and 14.1 mm, respectively.

The HPLC spectrum showed that F1-4 were relatively pure and then subjected to further identification. One pure compound was also isolated from the other active fraction, which was labeled as F1-4-3.

**Compound identification**

HRESI-MS spectrum showed that the molecular weight of F1-4 was m/z 1277.6180 [M+Na]+ (calcd. 1278.6255) and the suggested molecular formula of the compound was C62H86N12O16, which was the same as that of actinomycin D. The 13C NMR data of F1-4 was similar to the actinomycin D data reported by Chen et al. [18], and the slightly difference might be due to the different deuterated reagent used in NMR. The UV spectrum of this compound was also the same to standard actinomycin D (Sigma) (Figure 5). Therefore, F1-4 was identified as actinomycin D (Figure 6(a)). From the yield of F1-4, the production of actinomycin D in S. parvulus HY026 was accounting for 26.3% of crude extracts.

The pure compound F1-4-3 was a light yellow oily substance. Based on its 1H NMR data which was the
same as the data reported by Liu et al. [19], F1-4-3 was identified as cyclic (4-hydroxy-Pro-Phe) (Figure 6(b)). $^1$H NMR (600 MHz, DMSO-$d_6$) δ: 7.97 (1H, s, NH), 7.17-7.28 (m, 5H, H-2', 3', 4', 5',6'), 5.07 (1H, s, -OH), 4.40 (1H, t, $J = 5.0$ Hz, H-9), 4.30 (1H, dd, $J = 5.9, 11.0$ Hz, H-6), 4.19 (1H, s, H-4), 3.52 (1H, dd, $J = 4.6, 12.5$ Hz, H-3b), 3.16 (1H, d, $J = 12.5$ Hz, H-3a), 3.07 (2H, dd, $J = 5.0, 14.4$ Hz, H-10), 1.91-1.94 (1H, m, H-5), 1.53 (1H, m, H-5). The molecular formula of the compound was C$_{14}$H$_{16}$N$_2$O$_3$.

**Bioactivity of the isolated compound**

The anti-quorum sensing activity of F1-4 with different concentration was tested and compared with standard actinomycin D. It was found that the pigment inhibitory activity of F1-4 was similar to that of standard actinomycin D (Figure 7). The violacein production of *C. violaceum* was reduced up to 64.9% and 90.7% by F1-4 at a concentration of 12.5 and 50 µg/mL, while the pigment inhibition rate of positive control (25 µg/mL 3,4 dibromo-2(5H)-furanone, Sigma) was 55.6%. The cell growths of *C. violaceum* within 24 h in all the treatments were also measured. The results showed that the bacterial growth was not affected in the lower concentration treatment (6.25 µg/mL), and slightly affected in the medium concentration treatment (12.5 µg/mL), which was similar to the control (25 µg/mL 3,4 dibromo-2(5H)-furanone) (Figure 8). The violacein inhibitory activity of HY026 and compound F1-4 observed in this study was higher than or similar to some other natural substances reported in similar research [20,21], such as eugenol [22] and malyngolide [23]. Besides the remarkable violacein inhibitory activity, F1-4 and actinomycin D standard could also inhibit prodigiosin production in *Serratia proteamaculans* 657 with the inhibition zone of 13.5 and 14 mm at 25 µg/disc.

Actinomycin D is the most significant member of actinomycines, which are a class of polypeptide antitumour
antibiotics firstly isolated from soil Streptomyces in 1940 [24]. It is one of the well-known anticancer drugs, most commonly used in treatment of a variety of cancers, including: gestational trophoblastic neoplasia [25], rhabdomyosarcoma [26], malignant hydatidiform mole [27]. Actinomycin D also exhibited antimicrobial activity against some gram positive bacteria, such as Staphylococcus aureus by inhibiting the initiation of RNA synthesis in bacterial cells [28]. However, the anti-quorum sensing activity of actinomycin D has been ignored for a long time. The remarkable anti-quorum sensing activity of actinomycin D found in this study suggested that actinomycin D should have potential in anti-quorum sensing application. These findings also indicated that it might be valuable to pay more attention on the new bioactivity investigation of known antibiotics.

Cyclo-dipeptides were found from many microbial strains, which exhibited variety of bioactivities, including antibacterial and antitumour activity against many bacterial strains and tumour cell lines. Li et al. [29] reported that cyclic (4-hydroxy-Pro-Phe) exhibited antitumour activity against mouse breast cancer cell tsFT 210 at 5 μg/mL. However, cyclic (4-hydroxy-Pro-Phe) isolated in this study did not show any antitumour activity against HeLa cell at the concentration of 50 μg/mL. This might be due to its selectivity among different cell lines.

Conclusion

A marine derived actinobacterium HY026 was isolated from seawater sample and identified as Streptomyces parvulus in this study. The metabolites of this strain could significantly inhibit the violacein production of Chromobacterium violaceum 12472, biofilm formation of four bacterial strains including Pseudomonas aeruginosa PAO1, and prodigiosin production of Serratia proteam culans 657. The active component in the metabolites of HY026 was isolated and identified as actinomycin D. Since the noticeable anti-quorum sensing activity and high yield of actinomycin D, strain S. parvulus HY026 could have potential application in anti-quorum sensing compound development.

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Disclosure statement

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