Research Article

Phylogenetic Analysis and Screening of Antimicrobial and Antiproliferative Activities of Culturable Bacteria Associated with the Ascidian *Styela clava* from the Yellow Sea, China

Lei Chen, Xue-Ning Wang, Chang-Ming Fu, and Guang-Yu Wang

Department of Bioengineering, School of Marine Science and Technology, Harbin Institute of Technology, Weihai 264209, China

Correspondence should be addressed to Lei Chen; chenleihit@163.com and Guang-Yu Wang; wanggy18_2007@163.com

Received 24 April 2019; Revised 4 July 2019; Accepted 28 July 2019; Published 28 August 2019

Academic Editor: Stefano Pascarella

Copyright © 2019 Lei Chen et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Over 1,000 compounds, including eceatinascidin-743 and didemnin B, have been isolated from ascidians, with most having bioactive properties such as antimicrobial, antitumor, and enzyme-inhibiting activities. In recent years, direct and indirect evidence has shown that some bioactive compounds isolated from ascidians are not produced by ascidians themselves but by their symbiotic microorganisms. Isolated culturable bacteria associated with ascidians and investigating their potential bioactivity are an important approach for discovering novel compounds. In this study, a total of 269 bacteria were isolated from the ascidian *Styela clava* collected from the coast of Weihai in the north of the Yellow Sea, China. Phylogenetic relationships among 183 isolates were determined using their 16S rRNA gene sequences. Isolates were tested for antimicrobial activity against seven indicator strains, and an antiproliferative activity assay was performed to test for inhibition of human hepatocellular carcinoma Bel 7402 and human cervical carcinoma HeLa cell proliferation. Our results showed that the isolates belonged to 26 genera from 18 families in four phyla (*Firmicutes*, *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes*). *Bacillus* and *Streptomyces* were the most dominant genera; 146 strains had potent antimicrobial activities and inhibited at least one of the indicator strains. Crude extracts from 29 strains showed antiproliferative activity against Bel 7402 cells with IC$_{50}$ values below 500 μg mL$^{-1}$, and 53 strains showed antiproliferative activity against HeLa cells, with IC$_{50}$ values less than 500 μg mL$^{-1}$. Our results suggest that culturable bacteria associated with the ascidian *Styela clava* may be a promising source of novel bioactive compounds.

1. Introduction

Ascidians (sea squirts) are sessile marine filter-feeding invertebrates belonging to the phylum *Chordata*. Studies on ascidians can be traced back to 1847 when their blood was observed to change color following exposure to air. Investigation of this unusual phenomenon led to the isolation of a series of hydroquinoid compounds called tunichromes from the blood of several species of ascidians [1]. It was not until 1974 that the first ascidian-derived bioactive metabolite, geraaryl hydroquinone, was isolated from an *Aplidium* species. This compound showed chemopreventive activity against leukemia, *Rous sarcoma* virus, and mammary carcinoma in test animals [2]. Over 1,000 compounds have been isolated from ascidians, the majority of which have bioactive properties that include antimicrobial, antitumor, and antimalarial activities [3]. Some of these compounds have been used as clinical drugs and preclinical leads [4].

Some ascidian-derived compounds, such as alkaloids, cyclic peroxides, peptides, and macrolides, have cytotoxic activities [5]. Trabectedin (also known as eceatinascidin-743 or ET-743), a milestone in the development of marine-derived drugs, was initially isolated from the Caribbean ascidian *Ecteinascidia turbinata* [6] and is of bacterial origin [7]. It was approved for the treatment of advanced soft tissue sarcoma in Europe, Russia, and South Korea and has also completed phase III randomized multicenter clinical trials for the treatment of advanced liposarcoma, leiomyosarcoma, and leiomyosarcoma [4, 8, 9].

Ascidians, together with marine sponges, corals, and other marine invertebrates, are also promising sources of novel bioactive compounds against bacterial and fungal
pathogens of both humans and fish [10]. As invertebrates, ascidians rely only on innate immunity that lacks somatic recombination and long-term immune memory and has a limited array of effector responses. The efficiency of the immune system can prevent the risk of infections and select appropriate mutualistic bacterial strains for gut colonization [11]. Forazoline A, a complex and novel marine polyketide from Actinomadura sp., isolated from the ascidian Ecteinascidia turbinata, showed antifungal activity against Candida albicans and demonstrated in vivo efficacy in a disseminated candidiasis model in mice with no toxicity and was also a candidate for human trials [12, 13].

The ascidian Styela clava is native to the Pacific coast of Asia, ranging from the Sea of Okhotsk to Japan, Korea, and northeastern China [14]. In China, it is mainly distributed in the Bohai Sea and the Yellow Sea. Recently, however, it has invaded different parts of the world’s oceans, being first found in British waters in 1953, then spreading up the North Sea coast as far as Denmark and south along the Atlantic coast to Portugal [15]. S. clava is viewed as an aggressive invader, which causes huge losses for local inshore shellfish farming [16]. However, it is beneficial to scientific research because of its rapid growth and low cost [17]. Several compounds have been isolated from S. clava, many of which have bioactive properties, including antihypertensive, anti-inflammatory, and antimicrobial effects [18, 19]. Two families of antimicrobial polypeptides, styelins and clavanins, were isolated from S. clava and identified, respectively. Styelin D showed activity against methicillin-resistant and susceptible strains of Staphylococcus aureus and Pseudomonas aeruginosa [20]. Clavatin A, which showed specific inhibition against Escherichia coli and S. aureus, is as effective as human cathelicidin LL-37 but is less toxic to humans [21].

However, collection and aquaculture of ascidians is sometimes difficult and may not be environmentally friendly, which severely limits the supply of these compounds [22]. The compounds isolated from marine invertebrates are often similar to those isolated from bacteria, which led to speculation that many of these compounds were synthesized by symbiotic bacteria rather than the animals themselves [7]. Studies on bacteria isolated from ascidians and their biological activities are relatively limited. In this study, the biodiversity of bacteria associated with S. clava in the Yellow Sea, China, was studied based on 16S rRNA gene sequence. Bacterial isolates were screened for potential antimicrobial and antiproliferative activities.

2. Materials and Methods

2.1. Sample Collection and Preparation. Ascidian samples (Styela clava) were collected from two locations of the coast of Weihai in the north of the Yellow Sea, China. One location was the intertidal zone of Xiaoshi Island, which is a sea cucumber national nature preserve (37°31’49”N, 122°0’5”E). The other was the surface of scallop farming net cages in Puyi Town (37°25’8”N, 122°17’59”E). After collection, the fresh samples were immediately placed into sterile plastic containers in a cooler and transported to the laboratory within 2–3 h. S. clava is a solitary ascidian with a leathery, bumpy, and often wrinkled outer skin. The ascidian samples were rinsed 3–5 times with filtered sterile seawater under sterile conditions to remove loosely attached microorganisms and other foreign matters from their surfaces. Following this, the S. clava gut was sampled using a sterile scalpel and tweezers and then homogenized.

2.2. Isolation of Bacterial Strains

2.2.1. Isolation of Common Bacteria. Homogenates (0.5 mL) were added to sterile flasks containing 4.5 mL of sterile seawater and glass beads, shaken on a rotary shaker (180 rpm) at 28°C for 30 min, and mixed thoroughly. Following this, ten-fold serial dilutions were performed with sterile seawater, ranging from 1:100 to 1:100,000. These dilutions (100 μL) were then plated on Petri dishes containing one of the 12 kinds of culture media described in Table 1. Inoculated plates were incubated at 28°C for at least 3 weeks.

2.2.2. Isolation of Actinobacteria. The homogenates were heated to 55°C in a water bath for 5 min [23]. The samples were serially diluted and plated on Petri dishes containing one of the 12 kinds of culture media described in Table 1. All media were supplemented with a final concentration of 20 μg nalidixic acid mL⁻¹ and 100 μg cycloheximide mL⁻¹. The inoculated plates were incubated at 28°C for at least 3 weeks.

2.3. Selection and Preservation of Isolated Strains. Single colonies growing on media were selected based on colony morphology, including growth rate, shape, size, pigmentation, and margin characteristics. Following subculturing and confirmation of strain purity, the isolated bacteria were preserved in 15% glycerin at -80°C.

2.4. 16S rRNA Gene Sequencing and Phylogenetic Analysis. Strains were selected based on their colony morphologies on 2216E solid culture media and the results of Gram staining, and their 16S rRNA genes were sequenced to determine their phylogenetic positions. Bacteria were cultured in 2216E liquid medium, and Actinobacteria were cultured in MI liquid media. Cells were harvested at stationary-phase by centrifugation (10,000 xg for 1 min). Genomic DNA was extracted by using a Bacterial Genome DNA Extraction Kit (Sangon Biotech, China). The universal primers 27F (5’-AGAGTTTGTATCCTGCTAG-3’) and 1492R (5’-GGTTACCTTGTAGACTT-3’) were used in polymerase chain reaction (PCR) amplification of the 16S rRNA gene. Extracted genomic DNA was used as a PCR template. Genomic DNA from the bacterial strain Escherichia coli was used as a positive control, and sterile distilled water was used as a negative control. DNA was denatured at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 90 s, with a final 10 min extension at 72°C. PCR products (~1,500 bp) were sequenced in Sangon Biotech, China.

Sequence similarity searches and the calculation of pairwise similarity values between isolated strains and their closely related types were performed using the
Pseudomonas aeruginosa 25922) and including the Gram-negative bacteria. Sizes against seven indicator bacterial or fungal strains, all 269 strains were tested by measuring inhibition zone activity was determined by observing the growth inhibition of the NCBI GenBank Database under the accession numbers KT758342–KT758375, KT758393–KT758412, KT758414–KT758461, KT758463–KT758477, KT758540–KT758561, KT758563–KT758575, and KT758600–KT758630.

Table 1: Compositions of the media for the isolation of bacteria associated with ascidian Styela clava.

| Media   | Composition                                                                 |
|---------|-----------------------------------------------------------------------------|
| 2216E   | Peptone 5 g; yeast extract 1 g; FePO₄ 0.01 g; agar 18 g; natural seawater 1 L; pH 7.6. |
| M1      | Soluble starch 10 g; yeast extract 4 g; peptone 2 g; agar 18 g; natural seawater 750 mL; deionized water 250 mL. |
| M2      | Glycerol 6 mL; arginine 1 g; K₂HPO₄ 3H₂O 1 g; MgSO₄ 7H₂O 0.5 g; agar 18 g; natural seawater 1 L. |
| M3      | L-asparagine 0.1 g; casein peptone 2 g; K₂HPO₄ 3H₂O 0.05 g; MgSO₄ 7H₂O 0.1 g; FeSO₄ 7H₂O 0.01 g; agar 18 g; nature seawater 1 L; pH 7.0. |
| M4      | Yeast extract 5 g; L- asparagine 1 g; glycerol 10 mL; K₂HPO₄ 3H₂O 1 g; KNO₃ 5 g; agar 18 g; nature seawater 1 L; pH 7.5-8.5. |
| M5      | Agar 18 g; natural seawater 1 L. |
| M6      | Aspartic acid 0.1 g; casein peptone 2 g; sodium propionate 4 g; K₂HPO₄ 3H₂O 0.05 g; MgSO₄ 7H₂O 0.1 g; FeSO₄ 7H₂O 0.01 g; agar 18 g; natural seawater 1 L; pH 7.0. |
| M7      | Soluble starch 10 g; casein 0.5 g; K₂HPO₄ 3H₂O 0.5 g; NaCl 20 g; deionized water 1 L; agar 18 g; pH 7.0. |
| M8      | Yeast extract 4 g; soluble starch 15 g; K₂HPO₄ 3H₂O 1 g; MgSO₄ 7H₂O 0.5 g; agar 18 g; natural seawater 1 L; pH 7.0. |
| M9      | Yeast extract 4 g; soluble starch 15 g; K₂HPO₄ 3H₂O 1 g; MgSO₄ 7H₂O 0.5 g; agar 18 g; natural seawater 1 L; pH 7.0. |
| M10     | Glucose 10 g; asparagine 1 g; K₂HPO₄ 3H₂O 1 g; FeSO₄ 7H₂O 0.001 g; ZnSO₄ 7H₂O 0.01 g; agar 18 g; natural seawater 750 mL; deionized water 250 mL; pH 7.0. |
| M11     | Glucose 10 g; acid hydrolyzed casein 0.3 g; KNO₃ 2 g; NaCl 2 g; K₂HPO₄ 3H₂O 0.2 g; MgSO₄ 7H₂O 0.05 g; FeSO₄ 7H₂O 0.01 g; CaCO₃ 0.2 g; agar 18 g; natural seawater 750 mL; deionized water 250 mL; pH 7.0. |
| M12     | Beef extract 5 g; peptone 10.0 g; NaCl 5.0 g; agar 18 g; nature seawater 1 L; pH 7.4-7.6. |

EzBioCloud Database [24]. The 16S rRNA gene sequences of related strains were downloaded from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). Sequences were further analyzed by performing sequence alignments using Clustal X [25], with manual modification. A phylogenetic tree was constructed using the neighbor-joining method, which was implemented in the software package Molecular Evolutionary Genetics Analysis (MEGA) (version 6.0) [26]. The 16S rRNA gene sequences of the isolated bacteria were submitted to the NCBI GenBank Database under the accession numbers KT758342–KT758375, KT758393–KT758412, KT758414–KT758461, KT758463–KT758477, KT758540–KT758561, KT758563–KT758575, and KT758600–KT758630.

2.5. Antimicrobial Activity Screening. Antimicrobial activity was determined by observing the growth inhibition of bacteria or fungi according to the method described by Chen et al. [27]. In this study, antimicrobial activities of all 269 strains were tested by measuring inhibition zone sizes against seven indicator bacterial or fungal strains, including the Gram-negative bacteria Escherichia coli (ATCC 25922) and Pseudomonas aeruginosa (ATCC 27853), the Gram-positive bacteria Bacillus subtilis (ATCC 6633) and Staphylococcus aureus (ATCC 6538), the human pathogenic fungi Candida albicans (ATCC 10231), and the aquatic animal pathogenic bacteria Vibrio parahaemolyticus (ATCC 17802) and Vibrio anguillarum. Most of the indicator strains were purchased from the ATCC, except for Vibrio anguillarum, which was donated by Associate Professor Yuxia Zou, Institute of Oceanology, Chinese Academy of Sciences. Two Vibrio strains were inoculated into 2216E liquid medium and incubated at 28°C for 12 h, while the other four indicator bacteria were inoculated into peptone-beef extract liquid medium and incubated at 37°C for 12 h. The indicator fungi were inoculated into Sabouraud liquid medium (Peptone 10 g; glucose 40 g; nature seawater 1 L, pH 5.6) and incubated at 28°C for 2–3 days. The isolated bacterial strains were inoculated into 2216E solid medium at 28°C for 3–5 days, and actinobacteria were inoculated in MI solid medium 28°C for 5–7 days. The indicator strain medium was added into 9 cm diameter Petri dishes and mixed with 0.1 mL of stationary phase cultures of the corresponding indicator bacterial suspension (or indicator fungal spore) to prepare antimicrobial assay plates. Plugs measuring 11 mm in diameter from isolated bacterial solid cultures were excised with a cork borer and placed on the surface of the corresponding indicator strain plates. Ampicillin, chloramphenicol, and norfloxacin (Sigma, St Louis, MO, USA) were used as positive controls. Disks of the same size were excised from 2216E and MI solid medium and used in antimicrobial assays as negative controls. Then the antimicrobial assay plates were cultivated at 37°C (or 28°C) for 2–3 days for indicator bacteria or at 28°C for 5–7 days for indicator fungi. The inhibition zones around the agar plugs were measured to gauge the antimicrobial activity of the isolated bacteria. Three or more biological repeats were performed for each isolate to establish average inhibition zone sizes.
2.6. Preparation of Crude Extracts. All of the isolated strains were inoculated from a frozen stock into 25 mL of either 2216E liquid medium (for bacteria) or M1 liquid medium (for actinobacteria) in an Erlenmeyer flask. The cultures were incubated at 28°C for 5–7 days on a rotary shaker at 180 rpm. The culture supernatants were subsequently extracted three times with 25 mL ethyl acetate. The organic layers were separated, combined, dried over anhydrous sodium sulfate, decanted, and dried under vacuum to obtain a crude extract. The crude extract from each strain was dissolved in 1 mg dimethyl sulfoxide (DMSO) mL⁻¹. The unoinoculated 2216E liquid medium (for bacteria) or uninoculated M1 liquid medium (actinobacteria) was extracted and used as negative controls in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay.

2.7. Antiproliferative Activity Screening. The antiproliferative activity of the crude extracts of all of the isolated strains was determined using the MTT assay as described by Chen et al. [27]. Briefly, human hepatocellular carcinoma Bel 7402 cells and human cervical carcinoma HeLa cells were purchased from the Cell Bank of the Chinese Academy of Science, China. Bel 7402 and HeLa were cultured in RPMI 1640 medium (HyClone, Thermo Fisher Scientific Inc., Logan, UT, USA) and Dulbecco's Modified Eagle Medium (DMEM) high-glucose medium (HyClone), respectively, supplemented with 10% fetal calf serum (FCS, Gibco, Grand Island, NY, USA) in 96-well microtiter plates (Corning, NY, USA). 5-Fluorouracil (5-FU, 10 ng·mL⁻¹, Sigma-Aldrich, St Louis, MO, USA) was used as a positive control. The IC₅₀ (50% inhibition concentration) values were defined as the concentration of an extract, which resulted in 50% growth inhibition of the tumor cells. Data are expressed as mean ± SE of three or more experiments.

3. Results

3.1. Phylogenetic Analysis of Isolated Strains. In this study, a total of 269 bacterial strains were isolated. A total of 125 strains were isolated from S. clava collected from Xiaoshi Island, and another 144 strains were isolated from S. clava collected from Poyu Town. Based on their colony morphologies and the results of Gram staining, a total of 183 strains were isolated from both locations were associated with the phylum Actinobacteria. These belonged to seven genera (Citrobacter, Kocuria, Micromonospora, Rhodococcus, Nocardiosis, Saccharomonospora, and Streptomyces) in six families (Micrococaceae, Micromonosporaceae, Nocardiaceae, Nocardiopsaceae, Pseudonocardaceae, and Streptomycetaceae). Among these isolates were Streptomyces. Eight strains were from the phylum Proteobacteria, and all of these were γ-Proteobacteria, belonging to four genera (Citrobacter, Halomonas, Sheinanella, and Vibrio) in four families (Enterobacteriaceae, Halomonadaceae, Shewanellaceae, and Vibrionaceae). Among these, Vibrio was the dominant genus. Further 31 strains were from the phylum Actinobacteria. These belonged to seven genera (Citroccus, Kocuria, Micromonospora, Rhodococcus, Nocardiosis, Saccharomonospora, and Streptomyces) in six families (Micrococaceae, Micromonosporaceae, Nocardiaceae, Nocardiopsaceae, Pseudonocardaceae, and Streptomycetaceae). Most of these isolates were Streptomyces. Eight strains were from the phylum Proteobacteria, and all of these were γ-Proteobacteria, belonging to four genera (Citrobacter, Halomonas, Sheinanella, and Vibrio) in four families (Enterobacteriaceae, Halomonadaceae, Shewanellaceae, and Vibrionaceae). Among these, Vibrio was the dominant genus. A single strain, HQB628, was from the phylum Bacteroidetes and belonged to the genus Tenacibaculum of the family Flavobacteriaceae.

In this study, the bacterial diversity associated with the ascidian species S. clava collected from different locations exhibited similar high-abundance bacteria and also exhibited differences in low-abundance bacteria. Among a total of 26 genera isolated during our study, nine genera (Kocuria, Micromonospora, Rhodococcus, Streptomyces, Bacillus, Fictibacillus, Halomonas, Sheinanella, and Vibrio) were shared by two ascidian samples from different locations (Table 2). The S. clava collected from both locations was associated with a larger proportion of bacteria of the genera Bacillus and Streptomyces. A total of 27 species represented the genus Bacillus, and 14 species represented the genus Streptomyces, with 16S rRNA gene sequence similarities in the range of 96.69%–100.00% (data listed in the Supplementary Material Tables S1 and S2). Ten genera were only isolated from ascidian samples collected at Xiaoshi Island, including three genera in the Actinobacteria, three genera in the Firmicutes, three genera in the α-Proteobacteria, and one genus in the γ-Proteobacteria. Seven genera were only isolated from ascidian samples collected at Poyu Town, including three genera from the Actinobacteria, one genus from the Bacteroidetes, two genera from the Firmicutes, and one genus from the γ-Proteobacteria.
Table 2: Identification of the 183 bacterial strains isolated from S. clava collected from two different locations (Xiaoshi Island and Poyu Town) based on the similarities of the 16S rRNA gene sequences.

| Phylum          | Genus           | Number of isolates from Xiaoshi Island | Number of isolates from Poyu Town |
|-----------------|-----------------|----------------------------------------|-----------------------------------|
| Actinobacteria  | Citricoccus     | 0                                      | 1                                 |
| Micrococcus     |                 | 4                                      | 0                                 |
| Kocuria         |                 | 1                                      | 1                                 |
| Micromonospora  |                 | 2                                      | 1                                 |
| Mycobacterium   |                 | 1                                      | 0                                 |
| Rhodococcus     |                 | 2                                      | 1                                 |
| Nocardiopsis    |                 | 0                                      | 2                                 |
| Actinoalloteichus|                | 1                                      | 0                                 |
| Saccharomonospora|               | 0                                      | 1                                 |
| Streptomyces    |                 | 21                                     | 24                                |
| Bacteroidetes   | Tenacibaculum   | 0                                      | 1                                 |
| Firmicutes      | Bacillus        | 34                                     | 52                                |
|                 | Fictibacillus   | 1                                      | 2                                 |
|                 | Halobacillus    | 2                                      | 0                                 |
|                 | Oceanobacillus  | 1                                      | 0                                 |
|                 | Virgibacillus   | 1                                      | 0                                 |
|                 | Paenibacillus   | 0                                      | 1                                 |
|                 | Salinicoccus    | 0                                      | 1                                 |
| Proteobacteria (the class α-Proteobacteria) | Altererythrobacter | 1                                      | 0                                 |
|                 | Phaeobacter     | 1                                      | 0                                 |
|                 | Ruegeria        | 4                                      | 0                                 |
| Proteobacteria (the class γ-Proteobacteria) | Citrobacter     | 0                                      | 1                                 |
|                 | Halomonas       | 2                                      | 2                                 |
|                 | Microbulbifer   | 2                                      | 0                                 |
|                 | Shewanella      | 1                                      | 1                                 |
|                 | Vibrio          | 5                                      | 4                                 |
| Total number    |                 | 87                                     | 96                                |

Three strains, HQB252, HQB272, and HQB233, had a sequence similarity of only 96.85% to Bacillus thermotolerans, 96.54% to Kocuria rosea, and 96.90% to Bacillus cereus, respectively. They did not cluster with any validly named species, suggesting that they are potentially novel species of the genus Bacillus and Kocuria.

3.2. Antimicrobial Activity of Isolated Strains. Antimicrobial activity screening tests showed that of the 269 bacterial strains tested 146 strains (54.28% of all strains) exhibited antimicrobial activities against at least one of the indicator strains used. A total of 27 strains (10.04% of all strains) inhibited at least three of the indicator strains (Table 3). One isolate, HQA032 (Streptomyces sp.), displayed inhibitory activity against six indicator bacterial strains.

Isolated strains exhibited higher antimicrobial activity against Gram-positive bacteria (B. subtilis and S. aureus) than against Gram-negative bacteria (E. coli, P. aeruginosa, V. parahaemolyticus, and V. anguillarum), with 117 strains (43.49%) inhibiting Gram-positive bacteria, while there were 40 strains (14.87%) inhibiting Gram-negative bacteria. A total of 98 inhibited B. subtilis. Among them, 25 strains had inhibition zones of more than 16 mm. Two strains HQA032 and HQA046 (both are Streptomyces sp.) had the strongest inhibitory effects against B. subtilis, with inhibition zone diameters of 22 mm and 27 mm, respectively. Activity against S. aureus was found in 67 strains (24.91% strains), especially strain HQA046 (Streptomyces sp.), which had an inhibition zone of 26 mm. However, low antimicrobial activity was found against Gram-negative bacteria. Only 12 strains inhibited the growth of E. coli, and
Figure 1: A neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships of bacterial strains associated with the ascidian *S. clava* and related taxa. A total of 26 genera were isolated from the ascidian *S. clava*, and we chose one species from each genus. Bootstrap values (>50%) based on 1,000 replicates are shown at branch nodes. *Pyrococcus abyssi* was used as an outgroup. Bars represent 0.05 substitutions per nucleotide position.

Only four strains showed activity against *P. aeruginosa*. As for antimicrobial activity against marine animal bacterial pathogens, 11 strains displayed activities against *V. Para-haemolyticus*, and 25 strains against *V. anguillarum*. In addition, 45 strains (16.73% strains) displayed activities against the human pathogenic fungus *C. albicans*, with two strains, HQA030 (*Actinoalloteichus* sp.) and HQA819 (*Streptomyces* sp.), both having inhibition zone diameters of more than 22 mm. These results suggested that some culturable strains associated with *S. clava*, especially actinobacterial strains,
Table 3: Antimicrobial activities of the isolated strains associated with ascidian *Styela clava*.

| Test strains | Ec | Pa | Bs | Sa | Ca | Va | Vp |
|--------------|----|----|----|----|----|----|----|
| HQA013       | -  | +  | +  | -  | -  | +++| ++ |
| HQA014       | -  | -  | +++| -  | -  | +++| ++ |
| HQA022       | -  | -  | ++ | +  | +  | -  | -  |
| HQA029       | ++ | -  | ++ | +  | -  | -  | -  |
| HQA030       | -  | -  | ++ | +  | -  | -  | -  |
| HQA032       | +  | +  | +++| +  | -  | +++| ++ |
| HQA034       | -  | -  | +++| +  | +  | -  | -  |
| HQA046       | -  | -  | +++| +++| -  | +++| ++ |
| HQA051       | -  | -  | ++ | +  | -  | -  | -  |
| HQA053       | -  | -  | +++| +  | -  | -  | -  |
| HQA056       | -  | -  | +++| ++ | -  | -  | -  |
| HQA057       | -  | -  | ++ | +  | -  | -  | -  |
| HQA058       | -  | -  | +++| ++ | +  | -  | -  |
| HQA802       | -  | -  | +++| +  | +  | -  | -  |
| HQA809       | -  | -  | +++| +++| +  | -  | -  |
| HQA811       | -  | -  | ++ | +  | -  | -  | -  |
| HQA819       | -  | -  | ++ | +  | +++| -  | -  |
| HQB224       | +  | -  | -  | +  | -  | -  | +++|
| HQB255       | -  | -  | +  | +  | -  | -  | ++ |
| HQB268       | +  | +  | +  | +  | -  | -  | -  |
| HQB288       | -  | -  | +  | +  | +  | -  | -  |
| HQB296       | -  | -  | +  | ++ | +  | -  | -  |
| HQB603       | -  | -  | ++ | +  | -  | -  | -  |
| HQB610       | +  | -  | +  | +  | -  | -  | -  |
| HQB613       | ++ | -  | ++ | +  | -  | -  | -  |
| HQB636       | ++ | -  | +++| +  | -  | +++| -  |
| HQB641       | -  | -  | +  | +  | +  | +  | -  |

Only the bacterial strains with antimicrobial activity against at least three indicator strains were listed. Antimicrobial activities were tested against *Escherichia coli* (Ec), *Pseudomonas aeruginosa* (Pa), *Bacillus subtilis* (Bs), *Staphylococcus aureus* (Sa), *Candida albicans* (Ca), *Vibrio anguillarum* (Va), and *Vibrio parahaemolyticus* (Vp).

Symbols of inhibition degree against indicator strains: (-), no inhibition; (+), 11 < inhibition zone < 13 mm; (++), 13 ≤ inhibition zone < 16 mm; (+++), 16 ≤ inhibition zone < 22 mm; (++++) inhibition zone ≥ 22 mm.

could be promising sources for the treatment of infected diseases.

### 3.3. Antiproliferative Activity of Isolated Strains

The MTT assay was used to evaluate the ability of crude extracts of the isolated strains to inhibit the proliferation of human hepatocellular carcinoma Bel 7402 cells and human cervical carcinoma HeLa cells. Our results showed that, among the 269 strains, the extracts of 187 strains (69.52% strains) showed antiproliferative activity against Bel 7402 cells, with 29 extracts (10.78%) showing high antiproliferative activity, with IC$_{50}$ below 500 𝜇g·mL$^{-1}$ (Table 4). In particular, the extract of HQB237 (*Bacillus* sp.) displayed strong antiproliferative activity against Bel 7402, with an IC$_{50}$ of 0.44 𝜇g·mL$^{-1}$.

The extracts from 208 strains (77.32% strains) showed antiproliferative activity against HeLa cells, with 53 strains (19.70% strains) having an IC$_{50}$ lower than 500 𝜇g·mL$^{-1}$ (Table 5). Five extracts significantly suppressed the proliferation of HeLa cells with IC$_{50}$ values less than 100 𝜇g·mL$^{-1}$.

### 4. Discussion

Because of their high biodiversity and chemodiversity, marine bacteria are considered a promising source of novel drugs. In recent years, the richness and diversity of microbial communities associated with ascidians have surprised many investigators. The research methods employed in their study have included both culture-dependent and culture-independent techniques, with the latter, including DNA fingerprinting techniques, metagenomic libraries, and the latest next-generation sequencing techniques [28].
To date, bacterial strains representing at least 34 genera belonging to 23 families in 5 phyla (Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria, and Verrucomicrobia) have been isolated from ascidians [10]. These include strains of Actinobacteria, Bacillus, Halomonas, Kocuria, Microbulbifer, Micrococcus, Ruegeria, Shewanella, and Streptomyces, among others. Some strains isolated from ascidians represent novel genera or novel species, including Ascidiaceibacter salegens, Halomonas halocynthiae, Labilibacter aurantiacus, Ruegeria halocynthiae, Streptomyces hyaluromycin, and Tenacibaculum halocynthiae [29–34]. The culture-dependent approach applied in this study resulted in the isolation of 269 strains distributed across 26 genera from 18 families in 4 phyla Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (α-Proteobacteria and γ-Proteobacteria). Bacillus and Streptomyces were the dominant genera. Of the genera found in our study, 15 genera have been previously isolated from both S. clava and other kinds of ascidians. These genera are Bacillus, Halobacillus, Halomonas, Kocuria, Microbulbifer, Micrococcus, Micromonospora, Nocardiospsis, Paenibacillus, Ruegeria, Shewanella, Streptomyces, Tenacibaculum, Vibrio, and Virgibacillus [10, 29–32]. A total of 11 genera, Actinoalloteichus, Altererythrobacter, Citricoccus, Citrobacter, Fictibacillus, Mycobacterium, Oceanobacillus, Phaeobacter, Rhodococcus, Saccharomonospora, and Saliniococcus, have not yet been isolated from ascidians using culture-dependent methods. The genera Actinoalloteichus, Citricoccus, Mycobacterium, Rhodococcus, and Saccharomonospora are rare Actinobacteria but have been isolated from marine environments previously [35–37]. Actinoalloteichus species were previously isolated from the soil in the cold desert [38], the rhizosphere of fig trees [39], and marine sponges [40]. Members of the Citricoccus genus were previously isolated from marine sponges [41], marine macroalgae [42], and marine sediments [43]. Some species of this genus such as Citricoccus nitrophenolicus use aromatic compounds as the only source of carbon and energy [44], and C. nitrophenolicus may show lipolytic activity at low temperatures [45]. The genus Altererythrobacter, belonging to the phylum Proteobacteria, has been isolated from various marine and terrestrial environments such as deep-sea water [46], tidal flats [47], marine sediments [48], forests, desert soil [49, 50], hot springs [51], and the air [52]. The genus Fictibacillus, belonging to the phylum Firmicutes, has been previously isolated from different environments, including hot springs [53], industrial wastes [54], metal ores [55], and marine sediments [56]. Our study increased the knowledge of the diversity of available ascidian-derived microorganisms, providing new resources useful for the screening of novel strains and bioactive compounds.

Our S. clava ascidian samples were collected from two locations, Xiaoshi Island and Poyu Town. The bacterial diversity associated with the ascidian species S. clava collected from different locations had similarities in high-abundance bacteria but also had differences in low-abundance bacteria. On the one hand, nine genera were shared by ascidian samples from both locations, and there were 69 strains (79.31% of 87 sequenced isolates from Xiaoshi Island), and 88 strains (91.67% of 96 sequenced isolates from Poyu Town) belonged to these nine genera, respectively. Among them, there are a larger proportion of bacteria belonged to two genera (Bacillus and Streptomyces). Strains belonging to the genus Bacillus were the greatest in number, with 39.08% (34 strains) and 54.17% (52 strains) of all the sequenced isolates from the two locations, respectively, and then followed by the genus Streptomyces, which accounted for 24.14% (21 strains) and 25.00% (24 strains) of the sequenced strains isolated from the two locations, respectively. These two genera are common in the marine environment and have been frequently isolated from marine animals [57]. On the other hand, the ascidian-derived strains from low-abundance
microorganisms were highly diverse, and this diversity of the tropical Pacific Ocean showed that ascidian-derived analysis of 32 different ascidians from a broad expanse highly variable environments [58]. Bacterial and chemical OTUs between disparate populations mask the effect of their abundant operational taxonomic unit (OTUs). These shared isolated populations revealed a striking similarity in the results showed that samples from three geographically abundant bacteria. In another study that investigated stable symbiotic relationship between ascidians and these genera collected at the two locations were different. Ten Data is expressed as mean ± SE of three or more experiments. genera, including 18 strains (20.69%), were only isolated from ascidian samples collected from Xiaoshi Island, and seven genera (nearly half of the 16 genera), including eight strains (8.33%), were only isolated from ascidian samples collected at Poyu Town. Our results suggest that there might be a stable symbiotic relationship between ascidians and these high-abundance bacteria. In another study that investigated gut bacterial diversity in the ascidian Ciona intestinalis, the results showed that samples from three geographically isolated populations revealed a striking similarity in the abundant operational taxonomic unit (OTUs). These shared OTUs between disparate populations mask the effects of their highly variable environments [58]. Bacterial and chemical analysis of 32 different ascidians from a broad expanse of the tropical Pacific Ocean showed that ascidian-derived microorganisms were highly diverse, and this diversity does not correlate with geographical location or latitude. Ascidian-derived microorganisms were stable over time and space. The majority of these microorganisms are species-specific. Location-specific bacteria are found in low abundance in the ascidians and mostly represent widespread strains. Such species may be associated with changes in seawater or environment, and their specific association with ascidians is uncertain [59].

To date, 150 natural products have been isolated from ascidian-derived microorganisms, and many of them, such as polyketides, peptides, alkaloids, terpenoids, and other types, have been found to have antimicrobial activities [10]. Two new lipopeptides, peptidolipins B and E, which were isolated from an actinobacterium Nocardia sp. associated with the ascidian Trididemnum orbiculatum, showed antibacterial activities against both methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *Staphylococcus aureus* (MSSA) [60]. Arenimycin, which was isolated from an ascidian-derivated actinobacterium strain Salinispora arenicola, exhibited potent antimicrobial activities against drug-resistant *Staphylococci*, one *Mycobacterium* strain, and a variety of other Gram-positive microorganisms [61]. The development of multiple resistances among pathogenic microorganisms has become a public health problem, and the discovery of novel and efficient antimicrobial compounds has, therefore, become increasingly important. In this study, antimicrobial activities of bacteria associated with the ascidian *Styela clava* were screened for the first time. More than half of the strains showed antimicrobial activity and represent a potential source for novel active compounds. Most of the active strains showed inhibition against Gram-positive indicator bacteria, with comparatively fewer strains inhibiting Gram-negative bacteria. Similar results have previously been obtained from other ascidians, as well as other marine invertebrates including corals and sponges [62–64]. Gram-negative bacteria, as a consequence of their possessing an outer membrane barrier and multidrug efflux pumps, are typically more resistant to antibiotics than Gram-positive bacteria [65, 66]. The outer membrane barrier in Gram-negative bacteria, which comprises a lipid bilayer impermeable to large, charged molecules, limits the intracellular access of various antibiotic classes [67]. Multidrug efflux pumps are tripartite membrane-localized transport proteins capable of transporting a wide range of compounds, including drug molecules, out of cells, thus preventing the access of the drug to its target [68]. Nevertheless, we found several strains displaying potent antimicrobial activities against Gram-negative *V. anguillarum* and *V. parahaemolyticus*, including the two *Bacillus* strains HQB224 and HQB229. These results suggest that the ascidian *S. clava* is a potential source of antibiotic-producing bacteria. Studies are ongoing in our laboratory to identify the bioactive compounds responsible for the observed effects.

Ascidians are prominent sources of novel compounds with antimicrobial activities, including the well-known anticaner agents, didemnin B and ET-743, which have been proven to be of bacterial origin [7, 69]. To date, a quarter of all compounds isolated from ascidian-derived microorganisms have been shown to possess antiproliferative activity [70, 71].

### Table 5: Antiproliferative activities against HeLa cells of crude extracts from isolated strains associated with the ascidian *Styela clava* by MTT assay.

| Test strains | IC<sub>50</sub> (µg mL<sup>−1</sup>) | Test strains | IC<sub>50</sub> (µg mL<sup>−1</sup>) |
|--------------|-----------------|--------------|-----------------|
| HQA018       | 471.42±4.43     | HQB293       | 122.05±1.68     |
| HQA024       | 390.18±8.15     | HQB295       | 450.54±3.72     |
| HQA037       | 346.96±8.27     | HQB299       | 348.12±6.84     |
| HQA082       | 422.5±3.11      | HQB602       | 248.85±4.28     |
| HQA086       | 25.88±3.57      | HQB603       | 376.95±5.76     |
| HQA089       | 160.44±5.97     | HQB606       | 109.82±7.61     |
| HQB218       | 206.47±3.69     | HQB620       | 453.36±3.02     |
| HQB221       | 150.78±10.23    | HQB624       | 104.04±4.29     |
| HQB223       | 181.03±5.14     | HQB627       | 254.68±3.86     |
| HQB225       | 288.1±5.22      | HQB628       | 393.46±6.85     |
| HQB226       | 267.55±4.21     | HQB646       | 113.97±7.32     |
| HQB227       | 458.09±6.33     | HQB650       | 218.64±7.19     |
| HQB230       | 260.71±7.25     | HQB653       | 213.13±4.55     |
| HQB231       | 155.03±5.45     | HQB663       | 308.37±4.03     |
| HQB232       | 205.97±4.86     | HQB666       | 207.91±5.25     |
| HQB239       | 298.2±4.11      | HQB667       | 129.77±5.28     |
| HQB242       | 295.5±6.25      | HQB805       | 332.63±4.37     |
| HQB244       | 261.23±7.77     | HQB811       | 338.56±6.32     |
| HQB246       | 187.35±6.25     | HQB813       | 278.38±7.74     |
| HQB255       | 204.8±10.25     | HQB823       | 193.10±8.31     |
| HQB266       | 302.2±3.12      | HQB824       | 55.84±7.80      |
| HQB267       | 346.18±7.29     | HQB825       | 202.05±7.36     |
| HQB268       | 373.57±6.27     | HQB827       | 42.57±5.68      |
| HQB279       | 150.01±11.23    | HQB828       | 193.42±7.38     |
| HQB281       | 61.74±4.50      | HQB835       | 158.29±12.23    |
| HQB287       | 66.25±8.37      | HQB837       | 265.5±7.52      |
| HQB292       | 101.32±7.54     |              |                 |

Only the bacterial strains with antiproliferative activity IC<sub>50</sub> ≤500 µg mL<sup>−1</sup> were listed.

Data is expressed as mean ± SE of three or more experiments.
In this study, crude extracts from 129 out of the 269 isolated strains showed more than 50% growth inhibition in Bel 7402 or HeLa cells on treatment at 1 mg·mL$^{-1}$ (data listed in the Supplementary Material Tables S1 and S2). More than half of the 129 strains (51.94% of the strains) showed both antimicrobial and antiproliferative activities. Compared to extracts from four Bacillus strains and one Streptomyces strain that significantly suppressed the proliferation of Bel 7402 or HeLa cells with IC$_{50}$ values less than 100 μg·mL$^{-1}$, the extract of one Rhodococcus strain HQB281 showed stronger antiproliferative activity against HeLa cells, with an IC$_{50}$ of 61.74 μg·mL$^{-1}$. The genus Rhodococcus has been isolated from a broad range of environments including marine animals [72]. With a broad catabolic diversity and an array of unique enzymatic activities, the genus Rhodococcus exhibits significant potential for environmental and biotechnological applications including fossil fuel biodesulfurization, bioactive steroid production, and large-scale production of acrylamide and acrylic acid [73, 74]. However, there are limited studies on the antiproliferative activity of metabolites from the Rhodococcus strain. A particular Rhodococcus strain isolated from polluted soil was found to exhibit antiproliferative activity against two human cancer cell lines, hepatocellular carcinoma HepG2 and cervical carcinoma HeLa cells, with an IC$_{50}$ of 73.39 and 33.09 μg·mL$^{-1}$, respectively [73]. In this study, the antiproliferative activities of extracts from the Fictibacillus and Phaeobacter strains have been reported for the first time.

In this study, bacterial ascidian-derived strains from the genus Bacillus had the highest antimicrobial and antiproliferative activities, supporting the hypothesis that they might play a protective role in their hosts [75]. Bacillus strains are widely distributed in marine environments and are found to be associated with marine animals [76, 77]. They are culturable on many general-purpose media and have high proliferation rates and great adaptability [75]. Many secondary metabolites have been isolated from Bacillus, including peptides, terpenoids, polyketides, and isocoumarins [78]. These diverse compounds exhibit a wide range of biological properties, including antimicrobial, anticancer, and algicidal activities [79]. However, as fewer marine Bacillus strains have been examined for pharmaceutical activities, more studies are required.

Streptomyces is another dominant bacterial genus with bioactive activity found in this study. Some Streptomyces strains, such as strain HQA806 and HQA802, showed potent antiproliferative activity against both Bel 7402 and HeLa and showed antimicrobial activity against several indicator strains. Marine Streptomyces produce many well-known bioactive compounds, including the capoamycin-type antibiotic dioxamycin [80], the streptogramin etamycin [81], and the thiopptide antibiotic nosiheptide [82]. Moreover, a great number of new molecules were obtained from marine Streptomyces, for example, lobophorin [83], polyene acids [80], and polycyclic xanthenes [84]. Most compounds showed potent inhibition of common indicator strains, and some of them displayed anti-MRSA activity [85].

Rare actinobacteria, known as non-Streptomyces, are less frequently isolated than the common Streptomyces strains, even though this trend may not be reflected in the abundance of these strains in their ecological [86]. Rare actinobacteria are a promising source with potential novel metabolites of pharmaceutical relevance. Diverse new rare species, including novel genera and novel families of Actinobacteria, have been isolated from marine habitats (coastal, tidal, and deep-sea sediments), marine animals (sponges, corals, and ascidians), seawater, and mangrove forests [64, 85–88]. In our study, some rarely isolated actinobacteria showed potent antimicrobial activity. For example, three bacterial strains from the genus Micromonospora displayed inhibitory activity against E. coli, B. subtilis, S. aureus, and C. albicans. The genus Micromonospora can be easily found in marine invertebrates such as sponges [89]. Micromonospora is considered one of the most prolific producers of secondary metabolites among the Actinobacteria, which, as a group, display a rich chemical diversity and various pharmacologically and medically relevant bioactivities [90]. HQA030, belonging to the genus Actinoalloteichus, showed potent antimicrobial activity in our study. Two bioactive compounds, F1 (hydrophilic) and F2 (hydrophobic), were isolated from an Actinoalloteichus strain, which was isolated from a saline Saharan soil and showed antibacterial and antifungal activities against a broad spectrum of microorganisms known to be human and plant pathogens [91]. Further studies will be necessary to identify the bioactive substances produced by HQA030.

Bacterial strains with a 16S rRNA gene sequence similarity of less than 97% are considered to be separate species [92]. Recently, a similarity of 98.6% in the 16S rRNA gene sequence was suggested as a threshold for differentiating two species [93]. In our study, the strains HQB252, HQB272, and HQB233 shared similarities of less than 97% with other known species and may, therefore, be potentially new species of the genus Bacillus or Kocuria. To characterize these potentially new species, we will perform a polyphasic taxonomic study.

5. Conclusions

Our study reveals the diversity of bacteria associated with the ascidian S. clava and reports a broad spectrum of antimicrobial and antiproliferative activities displayed by these strains. Our results suggest that the culturable bacteria associated with the ascidian S. clava may constitute a promising source of novel bioactive compounds.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.
Acknowledgments

This work was supported by the National Natural Science Foundation of China [No. 31300009], Natural Scientific Research Innovation Foundation in Harbin Institute of Technology [No. HIT. NSRF. 2014127], and Discipline Construction Guide Foundation in Harbin Institute of Technology at Weihai [No. WH20150204 and No. WH20160205].

Supplementary Materials

Table S1: The comparison of 16S rRNA, antimicrobial activities, and antiproliferative activities of 125 strains associated with the ascidian Styela clava collected from Xiaoshi Island.

Table S2: The comparison of 16S rRNA, antimicrobial activities, and antiproliferative activities of 144 strains associated with the ascidian Styela clava collected from Poyu Town. (Supplementary Materials)

References

[1] R. C. Bruening, E. M. Oltz, J. Furukawa, K. Nakanishi, and K. Kustin, "Isolation of tunichrom B-1, a reducing blood pigment of the sea squirt, Ascidia nigra," *Journal of Natural Products*, vol. 49, no. 2, pp. 193–204, 1986.

[2] B. S. Davidson, "Ascidians: producers of amino acid derived metabolites," *Chemical Reviews*, vol. 93, no. 5, pp. 1771–1791, 1993.

[3] J. W. Blunt, B. R. Copp, R. A. Keyzers, M. H. Munro, and M. R. Prinsen, "Marine natural products," *Natural Product Reports*, vol. 34, no. 3, pp. 235–294, 2017.

[4] A. M. Mayer, K. B. Glaser, C. Cuevas et al., "The odyssey of marine pharmaceuticals: a current pipeline perspective," *Trends in Pharmacological Sciences*, vol. 31, no. 6, pp. 255–265, 2010.

[5] A. R. Carroll, B. R. Copp, R. A. Davis, R. A. Keyzers, and M. R. Prinsen, "Marine natural products," *Natural Product Reports*, vol. 36, no. 1, pp. 122–173, 2019.

[6] R. Beeso, V. Neergheen-Bhujun, R. Bhagooli, and T. Bahorun, "Apoptosis inducing lead compounds isolated from marine organisms of potential relevance in cancer treatment," *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 768, no. C, pp. 84–97, 2014.

[7] D. J. Newman and L. Giddings, "Natural products as leads to antitumor drugs," *Phytochemistry Reviews*, vol. 13, no. 1, pp. 123–137, 2014.

[8] G. D. Demetri, M. von Mehren, and R. L. Jones, "Efficacy and safety of trabectedin or dacarbazine for metastatic liposarcoma or leiomyosarcoma after failure of conventional chemotherapy: results of a phase III randomized multicenter clinical trial," *Journal of Clinical Oncology*, vol. 34, no. 8, pp. 786–793, 2016.

[9] A. Kawai, K. Yonemori, S. Takahashi, N. Araki, and T. Ueda, "Systemic therapy for soft tissue sarcoma: proposals for the optimal use of pazopanib, trabectedin, and eribulin," *Advances in Therapy*, vol. 34, no. 7, pp. 1536–1571, 2017.

[10] L. Chen, J. Hu, J. Xu, C. Shao, and G. Wang, "Biological and chemical diversity of ascidian-associated microorganisms," *Marine Drugs*, vol. 16, no. 10, p. 362, 2018.

[11] N. Franchi and L. Ballarin, "Immunity in protochordates: the tunicate perspective," *Frontiers in Immunology*, vol. 8, Article ID UNSP 674, 2016.

[12] T. P. Wyche, J. S. Piotrowski, Y. Hou et al., "Forazoline A: Marine-derived polyketide with antifungal in vivo efficacy," *Angewandte Chemie International Edition*, vol. 53, no. 43, pp. 11583–11586, 2014.

[13] G. Gribble, "Biological activity of recently discovered halogenated marine products," *Marine Drugs*, vol. 13, no. 7, pp. 4044–4136, 2015.

[14] S. J. Goldstien, L. Dupont, F. Viard et al., "Global phylogeny of the widely introduced north west pacific ascidian Styela clava," *PLoS ONE*, vol. 6, no. 2, article e16755, 2011.

[15] M. Davis, J. Lüttzen, and M. Davis, "The spread of Styela clava Herdman, 1882 (Tunicata, Ascidiae) in European waters," *Aquatic Invasions*, vol. 2, no. 4, pp. 378–390, 2007.

[16] G. Lambert, "Invasive sea squirts: A growing global problem," *Journal of Experimental Marine Biology and Ecology*, vol. 342, no. 1, pp. 3–4, 2007.

[17] B. Ju, B. Chen, X. R. Zhang, C. L. Han, and A. L. Jiang, "Purification and characterization of bioactive compounds from *Styela clava*," *Journal of Chemistry*, vol. 2014, Article ID 525441, 9 pages, 2014.

[18] S. C. Ko and Y. J. Jeon, "Anti-inflammatory effect of enzymatic hydrolysates from *Styela clava* flesh tissue in lipopolysaccharide-stimulated RAW 264.7 macrophages and in vivo zebrafish model," *Nutrition Research and Practice*, vol. 9, no. 3, pp. 219–226, 2015.

[19] S. Ko, J. Kim, S. Park, W. Jung, and Y. Jeon, "Antihypertensive peptide purified from *Styela clava* flesh tissue stimulates glucose uptake through AMP-activated protein kinase (AMPK) activation in skeletal muscle cells," *European Food Research and Technology*, vol. 242, no. 2, pp. 163–170, 2016.

[20] S. W. Taylor, A. G. Craig, W. H. Fischer, M. Park, and R. I. Lehrer, *"Styelin D, an extensively modified antimicrobial peptide from ascidian hemocytes,"* The Journal of Biological Chemistry, vol. 275, no. 49, pp. 38417–38426, 2000.

[21] O. N. Silva, I. C. Fensterseifer, E. A. Rodrigues et al., "Clavanin A improves outcome of complications from different bacterial infections," *Antimicrobial Agents and Chemotherapy*, vol. 59, no. 3, pp. 1620–1626, 2015.

[22] E. W. Schmidt, J. T. Nelson, D. A. Rasko et al., "Patellamide A and C biosynthesis by a microcin-like pathway in *Prochloron didemni*, the cyanobacterial symbiont of *Lissoclinum patella*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 20, pp. 7315–7320, 2005.

[23] P. R. Jensen, E. Gontang, C. Mafnas, T. J. Mincer, and W. Fenical, *"Culturable marine actinomycete diversity from tropical Pacific Ocean sediments,"* *Environmental Microbiology*, vol. 7, no. 7, pp. 1039–1048, 2005.

[24] S.-H. Yoon, S.-M. Ha, and S. Kwon, *"Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies,"* *International Journal of Systematic and Evolutionary Microbiology*, vol. 67, no. 5, pp. 1613–1617, 2017.

[25] J. D. Thompson, T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins, *"The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools,"* *Nucleic Acids Research*, vol. 25, no. 24, pp. 4876–4882, 1997.

[26] K. Tamura, G. Stecher, D. Peterson, A. Filipski, and S. Kumar, *"MEGA6: Molecular evolutionary genetics analysis version 6.0,"* *Molecular Biology and Evolution*, vol. 30, no. 12, pp. 2725–2729, 2013.
Saccharicrinis marinus Styela clava sea squirt (gen. nov., sp. nov., isolated from H. hattensis) and reclassification of Halocynthia roretzi, Antonie van Leeuwenhoek-Journal of Microbiology, vol. 52, no. 5, pp. 1767–1772, 2002.

[27] L. Chen, G. Wang, T. Bu et al., “Phylogenetic analysis and screening of antimicrobial and cytotoxic activities of moderately halophilic bacteria isolated from the Weihai Solar Saltern (China),” World Journal of Microbiology and Biotechnology, vol. 26, no. 5, pp. 879–888, 2010.

[28] L. Chen, C. Fu, and G. Wang, “Microbial diversity associated with ascidians: a review of research methods and application,” Symbiosis, vol. 71, no. 1, pp. 19–26, 2017.

[29] L. A. Romanenko, P. Schumann, M. Rohde, V. V. Mikhailov, L. Chen, C. Fu, and G. Wang, “Microbial diversity associated with ascidians,” Antonie van Leeuwenhoek-Journal of Microbiology, vol. 103, no. 6, pp. 1321–1327, 2013.

[30] Y. O. Kim, S. Park, B. H. Nam et al., “Ruegeria halocynthiae sp. nov., isolated from the sea squirt Halocynthia roretzi,” International Journal of Systematic and Evolutionary Microbiology, vol. 62, no. 4, pp. 925–930, 2012.

[31] Y. Kim, S. Park, B. Nam et al., “Tenacibaculum halocynthiae sp. nov., a member of the family Flavobacteriaceae isolated from sea squirt Halocynthia roretzi,” Antonie van Leeuwenhoek-Journal of Microbiology, vol. 103, no. 6, pp. 1321–1327, 2013.

[32] E. Harunari, M. Hamada, C. Shibata et al., “Streptomyces halotolerans sp. nov., isolated from a tunicate (Molgula manhattensis),” The Journal of Antibiotics, vol. 69, no. 3, pp. 159–163, 2016.

[33] D. C. Lu, J. X. Zhao, F. Q. Wang, Z. H. Xie, and Z. J. Du, “Labililbacter auranticus gen. nov., sp. nov., isolated from sea squirt (Styela clava) and reclassification of Saccharicrinis marinus as Labililbacter marinus comb,” International Journal of Systematic and Evolutionary Microbiology, vol. 67, no. 2, pp. 441–446, 2017.

[34] L. Chen, S. Wang, C. Ma, D. Zheng, Z. Du, and G. Wang, “Ascidiaceibacter salgoensis gen. nov., sp. nov., isolated from an ascidian,” Antonie van Leeuwenhoek-Journal of Microbiology, vol. 111, no. 9, pp. 1687–1695, 2018.

[35] H. Fukano, S. Wada, O. Kurata, K. Katayama, N. Fujisawa, and Y. Hoshino, “Mycobacterium stephanolepidis sp. nov., a rapidly growing species related to Mycobacterium chelonae, isolated from marine teleost fish, Stephanolepis cirrhifer,” International Journal of Systematic and Evolutionary Microbiology, vol. 67, no. 8, pp. 2811–2817, 2017.

[36] A. Veyisoglu, A. Sakaz, D. Cetin, K. Guven, and N. Sahin, “Saccharomonaspora amisovensis sp. nov., isolated from deep marine sediment,” International Journal of Systematic and Evolutionary Microbiology, vol. 63, pp. 3782–3786, 2013.

[37] D. White, L. Hird, and S. Ali, “Production and characterization of a trehalolipid biosurfactant produced by the novel marine bacterium Rhodococcus sp., strain PML026,” Journal of Applied Microbiology, vol. 115, no. 3, pp. 744–755, 2013.

[38] A. K. Singla, S. Mayliraj, T. Kudo et al., “Actinoalloteichus spitiensis sp. nov., a novel actinobacterium isolated from a cold desert of the Indian Himalayas,” International Journal of Systematic and Evolutionary Microbiology, vol. 55, pp. 2561–2564, 2005.

[39] W. Xiang, C. Liu, X. Wang, J. Du, L. Xi, and Y. Huang, “Actinoalloteichus nanhanensis sp. nov., isolated from the rhizosphere of a fig tree (Ficus religiosa),” International Journal of Systematic and Evolutionary Microbiology, vol. 61, no. 5, pp. 1165–1169, 2011.

[40] H. T. Zhang, W. Zheng, Huang J. Y. et al., “Actinoalloteichus hymeniacidonis sp. nov., an actinomycete isolated from the marine sponge Hymeniacidon perleve,” International Journal of Systematic and Evolutionary Microbiology, vol. 56, no. 10, pp. 2309–2312, 2006.

[41] S. Dupont, A. Carré-Mlouka, F. Descarrega et al., “Diversity and biological activities of the bacterial community associated with the marine sponge Phorbas tenacior (Porifera, Demospongiae),” Letters in Applied Microbiology, vol. 58, no. 1, pp. 42–52, 2014.

[42] S. Leiva, P. Alvarado, Y. Huang, J. Wang, I. Garrido, and A. Klier, “Diversity of pigmented Gram-positive bacteria associated with marine macroalgae from Antarctica,” FEMS Microbiology Letters, vol. 362, no. 24, Article ID fnv206, 2015.

[43] M. Yuan, Y. Yu, H. Li, N. Dong, and X. Zhang, “Phylogenetic diversity and biological activity of actinobacteria isolated from the chukchi shelf marine sediments in the arctic ocean,” Marine Drugs, vol. 12, no. 3, pp. 1281–1297, 2014.

[44] M. B. Nielsen, K. U. Kjeldsen, and K. Ingvorsen, “Description of Citricoccus nitrophilenicus sp. nov., a para-nitrophenol degrading actinobacterium isolated from a wastewater treatment plant and emended description of the genus Citricoccus Altenburger et al., 2002,” Antonie van Leeuwenhoek International Journal of General and Molecular Microbiology, vol. 99, no. 3, pp. 498–499, 2011.

[45] L. E. Petrovskaya, K. A. Novototskaya-Vlasova, E. V. Spirina et al., “Lipolytic enzymes of microorganisms from permafrost cryoeggs,” Doklady Biological Sciences, vol. 445, no. 1, pp. 279–282, 2012.

[46] F. Meng, G. Li, C. Fang et al., “Altererythrobacter aerophilus sp. nov., isolated from deep-sea water of the north-west Pacific,” International Journal of Systematic and Evolutionary Microbiology, vol. 69, no. 6, pp. 1689–1695, 2019.

[47] S. Park, J. Park, T. Oh, and J. Yoon, “Altererythrobacter insuiae sp. nov., a lipolytic bacterium isolated from a tidal flat,” International Journal of Systematic and Evolutionary Microbiology, vol. 69, no. 4, pp. 1009–1015, 2019.

[48] M. Matsumoto, D. Iwama, A. Arakaki et al., “Altererythrobac- terishigakensis sp. nov., an astaxanthin-producing bacterium isolated from a marine sediment,” International Journal of Systematic and Evolutionary Microbiology, vol. 61, no. 12, pp. 2956–2961, 2011.

[49] R. H. Dahal and J. Kim, “Altererythrobacter fulvus sp. nov., a novel alkalitolerant alphaproteobacterium isolated from forest soil,” International Journal of Systematic and Evolutionary Microbiology, vol. 68, no. 5, pp. 1502–1508, 2018.

[50] Z. Yan, P. Lin, K. Won et al., “Altererythrobacter deserti sp. nov., isolated from desert soil,” International Journal of Systematic and Evolutionary Microbiology, vol. 67, no. 10, pp. 3806–3811, 2017.

[51] C. Yuan, X. Chen, Z. Jiang et al., “Altererythrobacter lauratis sp. nov. and Altererythrobacter palmatus sp. nov., isolated from a Tibetan hot spring,” Antonie van Leeuwenhoek-Journal of Microbiology, vol. 110, no. 8, pp. 1077–1086, 2017.

[52] H. Xue, C. Piao, M. Guo, L. Wang, W. Fang, and Y. Li, “Description of Altererythrobacter aerius sp. nov., isolated from air, and emended description of the genus Altererythrobacter,” International Journal of Systematic and Evolutionary Microbiology, vol. 66, no. 11, pp. 4543–4548, 2016.

[53] Y. Singh, A. Sharma, P. Schumann, P. Kohli, and R. Lal, “Fictibacillus halophilus sp. nov., from a microbial mat of a hot spring atop the Himalayan Range,” International Journal of Systematic and Evolutionary Microbiology, vol. 66, no. 6, pp. 2409–2416, 2016.

[54] S. P. Glaeser, W. Dott, H. Busse, and P. Kampfer, “Fictibacillus phosphorivorans gen. nov., sp. nov. and proposal to reclassify...
Bacillus arsenicus, Bacillus barbaricus, Bacillus macaenusis, Bacillus ranhainensis, Bacillus rigui, Bacillus solisalts and Bacillus gelatini in the genus Fictibacillus,” International Journal of Systematic and Evolutionary Microbiology, vol. 63, no. Pt 8, pp. 2934–2944, 2013.

[55] Z. Zheng, J. Zheng, D. Peng, and M. Sun, “Complete genome sequence of Fictibacillus arsenicus G25-54, a strain with toxicity to nematodes,” Journal of Bacteriology, vol. 241, pp. 98–100, 2017.

[56] S. G. Dastager, R. Mawlankar, K. Srinivasan et al., “Fictibacillus enclensis sp. nov., isolated from marine sediment,” Antonie van Leeuwenhoek-Journal of Microbiology, vol. 105, no. 3, pp. 461–469, 2014.

[57] A. Muscholl-Silberhorn, V. Thiel, and J. F. Imhoff, “Abundance and bioactivity of cultured sponge-associated bacteria from the Mediterranean Sea,” Microbiol Ecology, vol. 55, no. 1, pp. 94–106, 2008.

[58] L. J. Dishaw, J. Flores-Torres, S. Lax et al., “A new marine-derived bacterium Bacillus arsenicus from a filamentous fungus,” Organic Letters, vol. 16, no. 18, pp. 4774–4777, 2014.

[59] Z. Zheng, J. Zheng, D. Peng, and M. Sun, “Complete genome sequence of Fictibacillus arsenicus AW25M09, isolated from the Hadsel Fjord, Northern Norway,” Genome Announcements, vol. 1, no. 2, Article ID e00055-13, 2013.

[60] X. Zhang, Z. Liu, J. Liu, Y. Zeng, G. Guo, and Q. Sun, “Antitumor activity of a Rhodococcus sp. Lut9010 isolated from polluted soil,” Tumor Biology, vol. 39, no. 6, Article ID T71661, 2017.

[61] R. van der Geize and L. Dijkhuizen, “Harnessing the catalytic diversity of rhodococci for environmental and biotechnological applications,” Current Opinion in Microbiology, vol. 7, no. 3, pp. 255–261, 2004.

[62] A. M. ElAhwany, H. A. Ghozlan, H. A. ElSharif, and S. A. Sabry, “Phylogenetic diversity and antimicrobial activity of marine bacteria associated with the soft coral Sarcophyton glaucum,” Journal of Basic Microbiology, vol. 55, no. 1, pp. 2–10, 2015.

[63] Z. Ma, N. Wang, J. Hu, and S. Wang, “Isolation and characterization of a new iturinic lipopeptide, mojavensin A produced by a marine-derived bacterium Bacillus mojavensis B0621A,” The Journal of Antibiotics, vol. 65, no. 6, pp. 317–322, 2012.

[64] A. Zuppa, S. Costantini, and M. Costantini, “Comparative sequence analysis of bacterial symbionts from the marine sponges Geodia cydonium and Irinia muscarum,” Bioinformation, vol. 10, no. 4, pp. 196–200, 2014.

[65] A. Hamdache, A. Lamarti, J. Aleu, and I. G. Collado, “Non-peptide metabolites from the genus Bacillus,” Journal of Natural Products, vol. 74, no. 4, pp. 893–899, 2011.

[66] M. Mondol, H. Shin, and M. Islam, “Diversity of secondary metabolites from marine Bacillus species: chemistry and biological activity,” Marine Drugs, vol. 11, no. 8, pp. 2846–2872, 2013.

[67] W. Xin, X. Ye, S. Yu, X. Lian, and Z. Zhang, “New capoamycin-type antibiotics and polyene acids from marine Streptomyces fradiae PTZ0025,” Marine Drugs, vol. 10, no. 12, pp. 2388–2402, 2012.

[68] N. M. Haste, V. R. Perera, K. N. Maloney et al., “Activity of the streptogramin antibiotic etamycin against mithicillin-resistant Staphylococcus aureus,” The Journal of Antibiotics, vol. 63, no. 5, pp. 219–224, 2010.

[69] N. M. Haste, W. Thienphrada, D. N. Tran et al., “Activity of the thiopede antibiotic nosiheptide against contemporary strains of mithicillin-resistant Staphylococcus aureus,” The Journal of Antibiotics, vol. 65, no. 12, pp. 593–598, 2012.

[70] H. Pan, S. Zhang, N. Wang et al., “New spiramycin antibiotic, Lobjoporin H and I, from a South China Sea-Derived Streptomyces sp. 12A35,” Marine Drugs, vol. 11, no. 10, pp. 3891–3901, 2013.

[71] L. Liu, Y. Xu, Z. Han et al., “Four new antibacterial xanthones from the marine-derived actinomycetes Streptomyces caelestis,” Marine Drugs, vol. 10, no. 12, pp. 2571–2583, 2012.
[85] G. Steinert, M. W. Taylor, and P. J. Schupp, “Diversity of actinobacteria associated with the marine ascidian *Eudistoma toezalensis*,” *Marine Biotechnology*, vol. 17, no. 4, pp. 377–385, 2015.

[86] R. Subramani and D. Sipkema, “Marine rare actinomycetes: a promising source of structurally diverse and unique novel natural products,” *Marine Drugs*, vol. 17, no. 5, Article ID 17050249, 2019.

[87] J. Vicente, A. Stewart, B. Song, R. T. Hill, and J. L. Wright, “Biodiversity of actinomycetes associated with caribbean sponges and their potential for natural product discovery,” *Marine Biotechnology*, vol. 15, no. 4, pp. 413–424, 2013.

[88] A. Azman, I. Othman, C. Fang, K. Chan, B. Goh, and L. Lee, “Antibacterial, anticancer and neuroprotective activities of rare actinobacteria from mangrove forest soils,” *Indian Journal of Microbiology*, vol. 57, no. 2, pp. 177–187, 2017.

[89] S. T. Khan, M. Takagi, and K. Shin-Ya, “Actinobacteria associated with the marine sponges *Cinachyra* sp., *Petrosia* sp., and *Ulosa* sp. and their culturability,” *Microbes and Environments*, vol. 27, no. 1, pp. 99–104, 2011.

[90] U. R. Abdelmohsen, K. Bayer, and U. Hentschel, “Diversity, abundance and natural products of marine sponge-associated actinomycetes,” *Natural Product Reports*, vol. 31, no. 3, pp. 381–399, 2014.

[91] F. Boudjelal, A. Zitouni, F. Mathieu, A. Lebrhi, and N. Sabao, “Taxonomic study and partial characterization of antimicrobial compounds from a moderately halophilic strain of the genus *Actinoalloteichus*,” *Brazilian Journal of Microbiology*, vol. 42, no. 3, pp. 835–845, 2011.

[92] E. Stackebrandt and B. M. Goebel, “Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology,” *International Journal of Systematic Bacteriology*, vol. 44, no. 4, pp. 846–849, 1994.

[93] M. Kim, H. Oh, S. Park, and J. Chun, “Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes,” *International Journal of Systematic and Evolutionary Microbiology*, vol. 64, pp. 346–351, 2014.