



Article

Marine Sediment-Derived *Streptomyces* Strain Produces Angucycline Antibiotics against Multidrug-Resistant *Staphylococcus aureus* Harboring SCCmec Type 1 Gene

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Abstract: The Philippine archipelago is geographically positioned in the tropics with rich areas of marine biodiversity. Its marine sediments harbor actinomycetes that exhibit antibacterial activity. Screening of actinomycetes isolated from marine sediments collected near the coast of Islas de Gigantes, Iloilo showed one isolate that exhibited high activity against the multidrug-resistant *Staphylococcus aureus* (MRSA) strain carrying the Staphylococcal Cassette Chromosome *mec* (SCCmec) type 1 gene, a biomarker for drug resistance. The isolate was identified as *Streptomyces* sp. strain DSD011 based on its 16s rRNA and protein-coding genes (*atpD*, *recA*, *rpoB*, and *trpB*) sequences, and was found to be a new species of salt-tolerant marine *Streptomyces*. Further, the strain harbors both non-ribosomal peptide synthetase (NRPS) and type II polyketide synthase (PKS) in its genome. The targeted chromatographic isolation and chemical investigations by Liquid Chromatography Mass Spectrometry-Time of Flight (LCMS-TOF), tandem mass spectrometry (MS/MS), and Global Natural Product Social molecular networking (GNPS) of the antibiotics produced by the strain afforded the two polycyclic aromatic polyketide angucycline glycosides, fridamycin A (**1**) and fridamycin D (**2**), which are products of type II PKS biosynthesis. Compounds **1** and **2** displayed antibacterial activity against MRSA with minimum inhibitory concentration (MIC) of 500 µg/mL and 62.5 µg/mL, respectively. These results suggest that the underexplored marine sediments near the coast of Islas de Gigantes, Iloilo offer access to undiscovered *Streptomyces* species that are invaluable sources of antibiotic leads.

Keywords: marine sediments; *Streptomyces*; antibiotic angucycline glycosides; multidrug-resistant *Staphylococcus aureus*; SCCmec gene; type II polyketide synthase

1. Introduction

The seafloor covers about 70% of the Earth's surface and is carpeted by marine sediments, which are mixtures of complex organic and inorganic particles that accumulated due to accretion and erosion of the continents, oceanic biological activities, volcanic eruptions, and chemical processes within the ocean [1,2]. Given their vast coverage, marine sediments harbor remarkable diverse microbial communities accounting for 12–45% of the total microbial biomass or ~0.6–2% of the whole biosphere [3–6]. Remarkably, members of the soil-dwelling Actinobacteria (the order of actinomycetes in particular) are widely distributed in marine sediments found in intertidal zones, near shores [7–10], and deep-sea [10–13]. Actinomycetes, known for its antibiotic-producing ability [14], spend the majority of their life cycle as dormant spores [15] that are continually washed in large numbers from surrounding terrestrial niches through river runoffs into the marine environment where they can remain dormant [8,16]. These marine actinomycetes can survive in a very demanding, nutrient deficient, competitive, and hostile environment that is inundated with high salinity and high pressure [17–19]. As a result, some metabolic changes occur in these bacteria that trigger the production of secondary metabolites that are distinct from their terrestrial counterparts [19,20]. The diversity of marine sediment-derived actinomycetes is indisputable [7,21,22], with decreasing genetic diversity patterns in proportion with depth [13]. This enormous diversity and metabolic changes yield new or novel core structures with potential pharmaceutical applications [23–28] as antibacterial [24,27,29–31] and antitumor [32,33] agents. In particular, Salinosporamide A (Marizomib), a novel rare bicyclic beta-lactone gamma-lactam isolated from an obligate marine actinobacterium, *Salinispora tropica* [34], has been a significant representation of compound derived from marine actinobacteria leading to clinical trials in the treatment of cancer in humans [35,36].

Efforts in searching for bioactive compounds from marine sediment-derived actinomycetes gained the attention of many researchers in the last five years [7,12,37–44]. Some examples of marine actinomycetes that produce bioactive compounds include the marine sediment-derived *Streptomyces* sp. RKND004 isolated from Prince Edward Island, Canada that produces the anticancer polycyclic polyether natural products terrosamycins A and B [31]; a marine sediment-derived *Streptomyces* sp. EG1 collected from the North Coast of the Mediterranean Sea of Egypt which produces tetracene antibiotic mersaquinone [27], and deep-sea sediment-derived *Streptomyces* sp. SCSIO 11791 that produces two new chlorinated bis-indole alkaloid antibiotics namely, dionemycin and 6-OMe-7',7''-dichlorochromopyrrolic acid [45]. Most recently, our group discovered the antibiotic anthracycline shunt metabolites bisanhydroaklavinone and 1-hydroxybisanhydroaklavinone from a Philippine marine sediment-derived *Streptomyces griseorubens* strain DSD069, which destroys the cell membrane integrity of multidrug-resistant *Staphylococcus aureus* [24]. These anthracyclines shunt metabolites are precursors of anticancer anthracyclines such as doxorubicin, daunorubicin, and cinerubins. They were accumulated in the growth medium during the fermentation of *Streptomyces griseorubens* strain DSD069, which is a rare event occurring in a non-genetically modified *Streptomyces* strain [24]. This recent finding shows that Philippine marine sediment-derived actinomycetes are a potentially rich source of new or novel secondary metabolites.

As part of our continuing program to explore the biological and chemical profiles of Philippine marine sediment-derived actinomycetes for antibiotics against multidrug-resistant *S. aureus* (MRSA), we screened the actinomycete strains isolated from tropical marine sediments collected from Islas de Gigantes, Iloilo (Figure 1a). The Islas de Gigantes is an island chain within the larger Western Visayas archipelago in the Visayan Sea and Jintotolo Channel. We report here the isolation of marine sediment-derived actinomycete strain from Islas de Gigantes and its bioactivity against

multidrug-resistant *S. aureus* (MRSA), a causative pathogen of various community and hospital-acquired infections that carries the *SCCmec* type 1 gene as a drug-resistant biomarker. The actinomycete isolate was identified as a *Streptomyces* sp. strain DSD011 based on phylogenetic marker genes using small subunit rRNA (i.e., 16s rRNA) and protein-coding genes (*atpD*, *recA*, *rpoB*, and *trpB*) sequence analyses and was found to belong to a new species of salt-tolerant marine *Streptomyces*. Further, the *Streptomyces* sp. strain DSD011 was screened for the presence of type I and II polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) biosynthetic genes. Our results showed that the *Streptomyces* sp. strain DSD011 harbors both type II polyketide synthase (PKS) and NRPS in its genome. The targeted chromatographic isolation and chemical investigations by LCMS, MS/MS, and Global Natural Product Social molecular networking (GNPS) of the antibiotics present in the crude fermentation extract of *Streptomyces* sp. strain DSD011 led to the discovery of type II PKS that are polycyclic aromatic polyketide angucycline glycosides fridamycin A (**1**) and fridamycin D (**2**) with activities against MRSA strain harboring the *SCCmec* gene. These results suggest that the under-explored marine sediments near the coast of Islas de Gigantes, Iloilo offer access to undiscovered *Streptomyces* species, which are valuable sources of antibiotic leads.

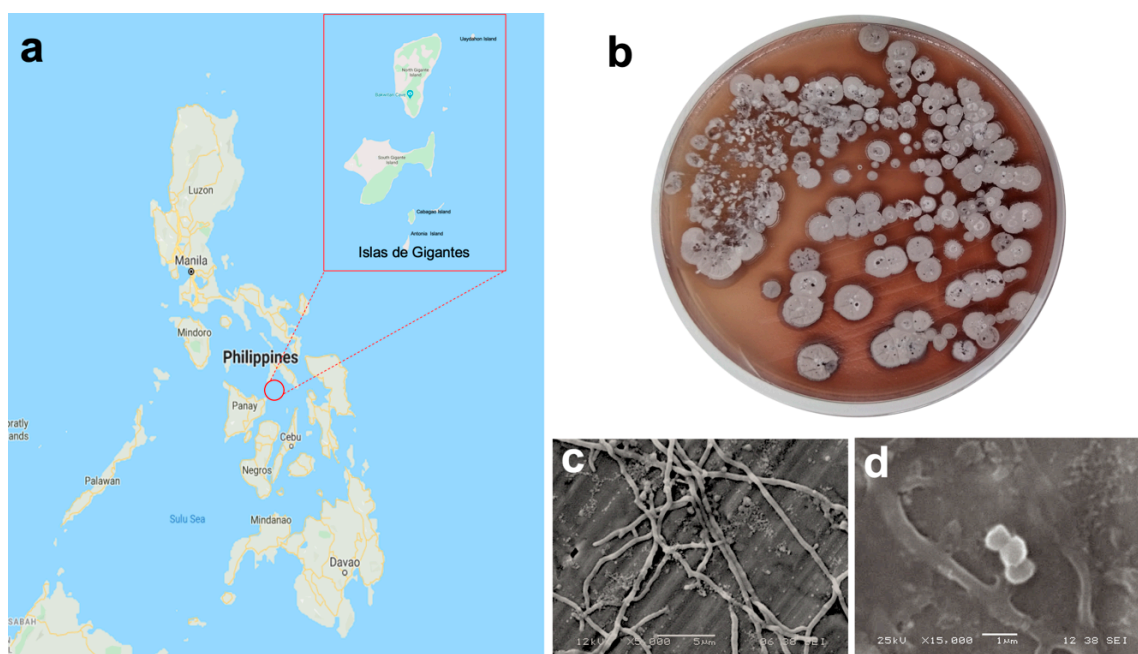


Figure 1. (a) Map of the collection site (11°35′39″ N, 123°20′11″ E); (b) *Streptomyces* sp. strain DSD011 in MM1 marine media; scanning electron micrograph of strain DSD011 showing (c) branched hyphae; and (d) chain of smooth-surfaced spores, when grown in marine medium 1 (MM1) broth at 28 °C for 14 days.

2. Materials and Methods

2.1. Collection of Marine Sediments

Marine sediments were collected from Islas de Gigantes (11°35′39″ N, 123°20′11″ E) group of islands in March 2016. The locations sampled included the islands of Antonia, Bantigue, Cabugao, and Uaydahon. The marine sediment samples were collected by self-contained underwater breathing apparatus (SCUBA) diving from a depth of approximately 20 to 30 m and a distance of 300 to 500 m away from the shore of the nearest island. Samples of marine sediments were collected by digging a shallow trench of 0.3 m using a hand trowel technique. The habitats encountered at each sampling location were categorized as sand, silt, or reef. The sediments were collected in sterile 50 mL tubes and kept on ice until processed in the laboratory.

2.2. Culture-Dependent Isolation

The sediment samples were air-dried for 8 h in a biosafety hood prior to inoculation in the solid medium of ISP4 (International Streptomyces Project Medium 4) (Difco) with filtered seawater using the dry stamp method (DSM) and heat shock method (HSM) [19,24,46]. The inoculated plates were incubated at room temperature (25 to 28 °C) for 15 to 30 days.

2.3. Identification of Strain DSD011 by Phylogenetic Marker Genes Using Small Subunit rRNA (i.e., 16S rRNA) and Protein Coding Genes (*atpD*, *recA*, *rpoB*, and *trpB*) Sequence Analyses

The DNA of actinomycete strain was extracted using a DNeasy blood and tissue kit (Qiagen, Germany) according to the manufacturer's instruction. For 16S rRNA gene amplification, universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3'), 518F (5'-CCAGCAGCCGCGGTAATACG-3'), 800R (5'-TACCAGGGTATCTAATCC-3'), and 1492R (5'-TACGGCTACCTTGTTACGACTT-3') were used in the PCR reaction. The reaction mix of 50 µL contained 5 µL Taq Polymerase (5 units/µL), 5 µL MgCl₂ (50 mM), 5 µL Taq Buffer, 5 µL dNTPs (10 mM), 3 µL 27F (10 µM), 3 µL 1492R (10 µM), 19 µL nuclease-free H₂O and 100 ng DNA template. The PCR conditions were as follows: initial denaturation at 98 °C for 3 min; 35 cycles at 98 °C for 10 s, 60 °C for 10 s and 72 °C for 60 s; a 10-min final extension at 72 °C using a thermocycler (Bio-Rad T100™ Thermal Cycler, Bio-Rad Laboratories, Inc., Singapore). The amplification products were cleaned up using a QIAquick PCR Purification Kit (Qiagen, Germany) according to the manufacturer's protocol. The universal primers 27F, 518F, 800R, and 1492R were used to obtain the almost complete 16S rRNA sequences of the isolate (1401 bp). The species-level affiliation of the sequences was validated using sequences from the BLAST server from National Center for Biotechnology Information (NCBI) (U.S. National Library of Medicine, Rockville Pike, Bethesda, MD, USA) [47]. The phylogenetic tree was constructed using a multiple alignment with maximum likelihood method in Mega 7.0 software [48] and the resultant tree topologies were evaluated by bootstrap analysis based on 1000 replicates. The evolutionary distance was calculated using the Kimura two-parameter model for nucleotide sequences [49].

In addition to 16S rRNA, the multilocus sequence analysis (MLSA) was employed using four housekeeping genes, namely *atpD* (ATP synthase F1, beta subunit), *recA* (recombinase A), *rpoB* (RNA polymerase beta subunit), and *trpB* (tryptophane B, beta subunit) were amplified and sequenced. The primers shown in Supplemental Table S1 were used to amplify *atpD*, *recA*, *rpoB*, and *trpB* genes [50]. Each 50 µL PCR reaction contains 25 µL SYBR green supermix, 3 µL forward primer (20 µM), 3 µL reverse primer (20 µM), 14 µL nuclease-free H₂O, and 5 µL DNA template. The PCR conditions were as follows: initial denaturation at 98 °C for 3 min; 35 cycles at 98 °C for 10 s, 60 °C for 10 s and 72 °C for 60 s, and a 5-min final extension at 72 °C using a thermocycler (Bio-Rad CFX96™ Real-Time System, Bio-Rad Laboratories, Inc., Singapore). The amplification products were cleaned up using a QIAquick PCR Purification Kit (Qiagen, Germany) according to the manufacturer's protocol and sequenced. Gene sequences were combined to create a concatenated sequence for MLSA. Concatenated sequence alignment was done using MUSCLE and corrected using MEGA 7.0 Software (Pennsylvania State University, PA, USA) [48]. The resultant tree topologies were also evaluated by bootstrap analysis based on 1000 replicates. The evolutionary distance was calculated using the Kimura two-parameter model for nucleotide sequences [49].

2.4. PCR-Based Screening for Secondary Metabolite Biosynthetic Genes

Specific degenerate primers used in this study were designed to detect conserved regions of ketosynthase (KS) and adenylation (AD) domains necessary for polyketides and non-ribosomal peptides, respectively using the primers shown in the Supplemental Table S2 [51–56]. PCR amplification using the genomic DNA was carried out in a final volume of 20 µL containing 10 µL SYBR green, 1 µL 10 µM forward and 1 µL 10 µM reverse primers, 1 µL DMSO, and 140 ng DNA template. Three reaction mixes were prepared for every DNA sample. To amplify biosynthetic genes targeting type I polyketide synthase β-ketoacyl synthase (KS) domain fragments, two sets of primers were used: KSMAF and

KSMBR, and KSF and KSR [51,52]. For the PCR protocol using PKS-I KSMAF and KSMBR primers, the reaction started with an initial denaturation at 95 °C for 5 min, followed by 40 cycles at 95 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min, a 5 min final extension at 72 °C using a thermocycler (Bio-Rad CFX96TM Real-Time System, Bio-Rad Laboratories, Inc., Singapore). Gene amplification using PKS-I KSF and KSR primers was executed as follows: initial denaturation at 95 °C for 15 min, one cycle of 95 °C for 1 min, 65 °C for 1 min and 72 °C for 1 min, followed by 35 cycles of 95 °C for 1 min, 62 °C for 1 min and 72 °C for 1 min, with a 10-min final extension at 72 °C.

PKS-II ketoacyl synthase domain fragments were amplified using three sets of primers: KS1F and KS1R; KS α and KS β ; 540F and 1100R [53–55]. PCR protocol in detecting PKS-II type of polyketides were as follows: initial denaturation at 95 °C for 5 min; 40 cycles at 95 °C for 1 min, 58 °C for 1 min and 72 °C for 2 min; and a 10-min final extension at 72 °C. Meanwhile, for the amplification of AD gene fragments, NRPS A3F and A7R primers were used [56]. The PCR condition included an initial denaturation of 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s and 59 °C for 1.5 min, and 72 °C for 1 min, with a 10 min final extension at 72 °C. The amplified PCR products were viewed in 2% agarose gel using a Quantum CX5 Edge Gel Doc automated gel imaging and documentation systems (Vilber Lourmat, Collégien, France).

2.5. Phenotypic Characterization

2.5.1. Salt Tolerance

Pure culture of strain DSD011 was inoculated in Marine Medium 1 (MM1) [19,24] prepared with artificial seawater varying in NaCl concentrations (0%, 3%, 5%, 7%, 10%, 12%, 15%, and 20%) [57]. The plates were incubated at room temperature (25 to 28 °C) for 7 days and checked periodically for the growth of actinomycetes. Detection of diffusible pigments, aerial, and substrate mycelia were observed and determined the optimal salt concentration that the strain could tolerate.

2.5.2. Carbon Utilization Test

Cells of strain DSD011 were inoculated unto five marine media containing different carbon sources: glucose (0.05%), mannitol (0.05%), raffinose (0.05%), soluble starch (1.0%), and trehalose (0.05%) in *w/v*. Thereafter, the plates were incubated at room temperature (25 to 28 °C) for 7 days. Morphological characteristics were identified according to aerial mycelium, mycelium substratum, and diffusible pigments. The medium having prolific growth of the strain was determined as the best medium to utilize a particular carbon source for growth development [58].

2.5.3. Scanning Electron Microscopy

The strain DSD011 was grown on marine medium 1 (MM1) broth at 25–28 °C for 14 days and spore morphology was observed using a scanning electron microscope (JEOL JSM 5510LV, JEOL Ltd., Tokyo, Japan). The sample was fixed using a 2.5% glutaraldehyde solution at 4 °C for 2 h. Cells were precipitated using poly-L-lysine in aluminum foil, oven-dried for 10 min and washed with PBS. The sample was dehydrated by a series of gradient exchange using ethanol solutions (30%, 50%, 85%, 95%, and 100%), and stored again at 4 °C for 10 min before adding t-butyl alcohol. The sample was freeze-dried at –20 °C for 10 min prior to mounting of samples in metal swab to remove ice crystals and avoid damage of cells. Once the sample was mounted by sputter coating, the metal stub was viewed at an accelerating voltage from 10 kV to 25 kV and intact spore structures were then selected for examination up to 15,000 \times magnification.

2.6. Cultivation and Extraction of Biomass

The strain DSD011 was inoculated and allowed to grow in the marine medium 1 (MM1) agar and broth according to the published method and protocols [19,24]. The MM1 agar inoculated with the strain was incubated for 14 days at 25–28 °C. After 14 days of incubation, the biomass was harvested.

The agar with the cells was extracted three times with ethyl acetate. The extract was concentrated *in vacuo* using a rotary evaporator, dried in vacuum, and stored in -20°C until tested for antibacterial assay and purified by chromatographic separations.

2.7. Antibacterial Assay

2.7.1. Multidrug-Resistant *Staphylococcus aureus* (MRSA)

The target pathogen used in this study was *S. aureus* ATCC BAA-44. This is an Iberian clone of MRSA containing SCC_{mec} type 1 gene and resistant to various types of antibiotics such as ampicillin, amoxicillin/clavulanic acid, ciprofloxacin, cephalothin, doxycycline, gentamicin, erythromycin, imipenem, methicillin, penicillin, tetracycline, oxacillin, azithromycin, clindamycin, ceftriaxone, rifampin, amikacin, and tobramycin (*Staphylococcus aureus* subsp. *aureus* (ATCC[®] BAA-44[™]) [59]. These characteristics make *S. aureus* ATCC BAA-44 an ideal target in searching for an antibiotic that can combat multidrug-resistant pathogens.

It has to be emphasized that although tetracycline was listed as one of the antibiotics with resistance against *S. aureus* ATCC BAA-44, this antibiotic was used as the positive control in all the antibacterial assays covered in this work. According to the protocol described in this work, the *S. aureus* ATCC BAA-44 has an MIC value of 31.25–50 $\mu\text{g/mL}$ for tetracycline [24].

2.7.2. Disk Diffusion Assay of the Crude Extract

The fermented crude extract was tested for preliminary antibacterial screening against multidrug-resistant *S. aureus* ATCC BAA-44 (MRSA) [59]. Briefly, the bacterial cells of MRSA were adjusted to 1×10^6 CFU/mL and were flooded accordingly into Mueller Hinton Agar (MHA) plates. Negative control (10 μL MeOH), positive control (0.5 mg tetracycline), and crude extracts (5 mg) were then dispensed in 6 mm circular disks and impregnated into agar plates with the pathogen. The plates were incubated for 18–24 h at 37°C before measuring the zone of inhibition in mm. The assay was done in triplicates.

2.7.3. Antibacterial Activity by Microbroth Susceptibility Assay

Antibacterial activity was confirmed using microbroth susceptibility assay against MRSA using the protocol and method of Dalisay et al. [19] and Paderog et al. [24]. Accordingly, a volume of 5 μL of 5 mg/mL extract concentration was dispensed into a 96 well plate and 195 μL of Mueller Hinton Broth (MHB) seeded with test pathogen with an optical density of 1×10^6 CFU/mL. Tetracycline (5 mg/mL) was used as a positive control, dimethyl sulfoxide (DMSO) as a negative control, and MHB as blank control. Plates were incubated for 18–24 h at 37°C . The optical density was measured at 600 nm using an absorbance microplate reader (BioTek[™] ELx808[™], Biotek, Winooski, Vermont, USA). The threshold used for an acceptable antibacterial activity was 50% growth inhibition. The assay was done in triplicates.

2.7.4. Minimum Inhibitory Concentration

Serial dilution of the extracts was carried out by two-fold dilution starting from an initial test concentration of 1000 $\mu\text{g/mL}$ to 1.9 $\mu\text{g/mL}$ (compound 1) and 125 $\mu\text{g/mL}$ to 0.122 $\mu\text{g/mL}$ (compound 2). DMSO and tetracycline (125 $\mu\text{g/mL}$) were used as negative and positive controls, respectively. The bacterial suspension (195 μL) with an optical density of 1×10^6 CFU/mL was added into the wells. The negative control used in this assay was DMSO and the positive control was tetracycline (125 to 0.122 $\mu\text{g/mL}$). The plates were incubated for 18–24 h at 37°C . The optical density was measured at 600 nm using an absorbance microplate reader (BioTek[™] ELx808[™], Biotek, Winooski, VT, USA). The last concentration showing more than 90% growth inhibition was considered as the MIC₉₀ ($\mu\text{g/mL}$) of the extract [24,60]. The assay was done in triplicates.

2.8. Targeted Chromatographic Isolation

2.8.1. Gel Filtration Chromatography

Approximately 450 mg of ethyl acetate crude extract was dissolved in 4.0 mL MeOH and centrifuged prior to loading to a 3.4 cm × 38 cm column Sephadex® LH-20. A total of 21 batches of gel filtration chromatography were performed giving a total of 7055.9 mg crude extract purified in Sephadex® LH-20. The column was eluted with MeOH and the fractions were collected at 2 min intervals. Each fraction was subjected to TLC analysis using 9.5:0.5 DCM/MeOH as the mobile phase. The eluted fractions were pooled according to their TLC profiles as observed under UV (254 and 365 nm). A total of 12 final gel filtration chromatography fractions (G1–G12) were obtained and dried *in vacuo* for succeeding antibacterial testing.

2.8.2. Flash Column Chromatography

To purify the active fractions (i.e., G6, 506.1 mg and G7, 143.5 mg) from the gel filtration chromatography, Isolera® One (Biotage, Uppsala, Sweden) normal-phase flash column chromatography system was used. The antibacterial fraction, G6 (106.2 mg representing only 20% total yield of Fraction G6), was dissolved in 500 µL MeOH and was directly applied on a 1 g samplelet, dried under vacuum for 20 min and packed in a 10 g SNAP KP-SIL cartridge. The column volume of the cartridge was 15 mL and was eluted with dichloromethane/methanol (DCM/MeOH) gradient (0% to 100% MeOH) at a flow rate of 36 mL/min. Each fraction was collected, pooled according to their TLC profiles at 254 and 365 nm, and dried *in vacuo* for succeeding antibacterial testing. The same methods and conditions were applied for the separation of G7 (114.5 mg loaded in the column). Each individual fraction was collected, pooled under the absorbance range of 254 and 365 nm, and dried *in vacuo* for succeeding antibacterial screening.

2.8.3. High-Performance Liquid Chromatography (HPLC)

The antibacterial fractions from Section 2.8.2 (i.e., G6I5, 17 mg; G7I3-6, 38.1 mg) were purified by reversed-phase HPLC (Shimadzu Prominence) equipped with a photodiode array detector. For Fraction G6I5, the purification was performed using a reversed-phase C₁₈ column (Phenomenex Synergi™ Hydro-RP, 250 mm × 10 mm, 10 µm, 80 Å). The mobile phase used was water/methanol (H₂O/MeOH) in gradient at a flow rate of 5 mL/min from 0% to 100% MeOH. Compound 1 (4.0 mg, *t_R* = 20 min) was obtained under these conditions. Similarly, Fraction G7I3-6 was purified using the same column as above but was eluted with water/acetonitrile (H₂O/ACN) in gradient at a flow rate of 5 mL/min from 0% to 100% ACN to yield a semi-pure fraction, which was further purified using the same conditions in H₂O/ACN in gradient at a flow rate of 3 mL/min from 20% water to 100% ACN to afford compound 2 (1.2 mg, *t_R* = 14 min).

2.9. LCMS, MS/MS and Global Natural Product Social Molecular Networking (GNPS)

LCMS analyses were performed using a high-resolution mass spectrometer (Waters Xevo® G2-XS QTOF) equipped with StepWave™ ion source technology, XS collision cell technology, QuanTOF™ technology, UPLC/TOF™ for high transmission mode, and UPLC/MSe for data acquisition. The stationary phase used was a Waters 100 Å, 1.8 µm, 1 × 150 mm column. Samples were reconstituted to 0.15 mg/mL and an optimized injection volume of 2 µL was adopted to avoid column overloading. The solvent system was water with 0.1% (*v/v*) formic acid (solvent A) and ACN with 0.1% (*v/v*) formic acid (solvent B). A flow rate of 0.22 mL/min was used with a column temperature of 50 °C. The interface used for MS^c was electron spray ionization (ESI) with a collision energy of 6.0 eV for low energy and 10 to 50 eV for high energy ramp. The scan time was set to 0.250 s using polarity positive or negative mode with a mass range scan of *m/z* 50–1500. MS data were analyzed using Waters UNIFI Scientific Information System R and Chemspider™ Database.

Mass spectra for the GNPS MASST Search tool were acquired on a Shimadzu™ LCMS-TQ 8045 instrument equipped with a Prominence HPLC system (Shimadzu Corporation, Japan) (SIL-20A HT autosampler, LC-20AD pump system, SDP-M20A UV Vis Detector) using Phenomenex Synergy™ column (particle size: 4 µm, diameter: 100.0 × 2.0 mm) in a gradient elution system from 100% water (solvent A) to 100 % ACN (solvent B) in 0.1% formic acid. The sample load was 5 µg of dried HPLC fractions. The instrument parameters for the elution of extract was performed in a solvent flow rate of 0.4 mL/min and with oven temperature at 40 °C, starting with 100% solvent A at 0.01 to 0.5 min, gradually increasing to 100% solvent B at 21 min to 26 min, then back to 100% solvent A at 28 min to 30 min. Data acquisition was stopped at 30 min. The interface used for MS was electron spray ionization (ESI). The block temperature was 400 °C, and DL temperature was 250 °C. The nebulizing gas flow rate was 3.0 L/min nitrogen while the drying gas flow rate was 10.0 L/min nitrogen. The event time was 0.1 s at Q1 scan positive mode and Q3 scan negative mode. The masses were analyzed in both positive and negative ion modes with range m/z 200–1500. MS/MS experiments were performed on the peaks of interest at 35 V collision energy.

A library search was performed using the online workflow at GNPS through MASST Search Tool (University of California, San Diego, San Diego, CA, USA) [61]. A single MS/MS spectrum was matched against all public spectral libraries that includes peptides, lipids, small molecules, and pharmacologically active natural products. The spectral match is required to have a parent mass tolerance of 2.0 Da, ion tolerance of 1.0 Da, score threshold of 0.59, and at least 4 matched peaks.

3. Results and Discussion

3.1. Isolation and Phenotypic Characterization of Actinomycete Strain DSD011

Actinomycete strain DSD011 was isolated from the marine sediments collected in the coast of Uaydahon Island (11°38'14.5" N, 123°22'18.5" E) in the Islas de Gigantes group of islands (Figure 1a). The strain was recovered in ISP4 marine media using the dry stamp method (DSM). The colony on MM1 agar at 14 days of incubation exhibited a tough, leathery, and filamentous colony with diffusible reddish-brown pigmentation (Figure 1b). The colony produced a red-brown substrate mycelium with branched hyphae (between 0.5–1.0 µm in diameter) (Figure 1c) grey aerial mycelium which differentiated into chains of smooth-surfaced spores (between 0.8 to 1 µm in diameter) (Figure 1d). These observed morphological features of strain DSD011 are diagnostic characteristics and typical morphological properties of the genus *Streptomyces*.

The strain DSD011 exhibited good growth in glucose and soluble starch and exhibited moderate growth in mannitol, trehalose, and raffinose as carbon sources (Table 1). Good growth of strain DSD011 occurred with NaCl concentrations of 0–5% (w/v) and moderate growth at 7% NaCl (Table 2). The morphological characteristics of strain DSD011 were affected by the culture conditions that led to the development of different substrate mycelium, aerial mycelium formation, and pigments. After 7 days of growth in a NaCl enriched medium, strain DSD011 produced white aerial mycelium and brownish substrate mycelium (Supplemental Table S3). A series of yellow to brown, light brown, and white substrate mycelium was observed in the carbon source utilization test. Sporulating aerial mycelium was white and gray in mannitol and glucose, respectively, while a yellowish-brown substrate mycelium was observed in starch. Interestingly, raffinose, soluble starch, and glucose did not produce aerial mycelia and diffusible pigment. Instead, white, light brown, and yellow substrate mycelia were observed, respectively (Supplemental Table S4).

Table 1. Growth of *Streptomyces* sp. strain DSD011 in various carbon sources (% w/v).

Strain	Starch (1.0%)	Mannitol (0.05%)	Glucose (0.05%)	Trehalose (0.05%)	Raffinose (0.05%)
DSD011	+++	++	+++	++	++

Good = +++, Moderate = ++, Poor = +, Nil = −.

Table 2. Growth of *Streptomyces* sp. strain DSD011 in various NaCl concentrations (% w/v).

Strain	0	3	5	7	10	12	15	20
DSD011	+++	+++	+++	++	-	-	-	-

Good = +++, Moderate = ++, Poor = +, Nil = −.

The fermentation crude extract of strain DSD011 showed activity against SCCmec type 1-harboring MRSA strain [59] with a 17 mm zone of inhibition at 5 mg/disk concentration (Supplemental Table S5). MRSA gives rise to a variety of infections acquired in community and hospital settings and affects vulnerable populations. Treatment of MRSA infections is difficult due to the limited range of effective antibiotics; hence it poses a significant health threat [62]. This indicates that new antibiotic leads are needed to overcome MRSA treatment challenges.

3.2. Genomic Characterization and Phylogenetic Analysis of Antibiotic Producing Actinomycete Strain DSD011

The molecular identification of strain DSD011 was performed with the sequence of the small subunit rRNA, i.e., 16S rRNA as a genetic marker. The phylogenetic analysis of the nearly complete 16S rRNA gene sequence (1401 bp) led to the identification of strain DSD011 (GenBank Accession No. MT820508), which is related to type strains assigned in the genus *Streptomyces* hence denoted as *Streptomyces* sp. strain DSD011. BLAST analysis revealed the highest 16S rRNA gene sequence similarity to *Streptomyces cellulosae* strain NBRC 13027 (99.93%, GenBank NR_112346.1) and *Streptomyces pseudogriseolus* strain NRRL B-3288 (99.79%, GenBank NR_043835.1). Both of these strains are terrestrial in origin. The related species of *Streptomyces* sp. strain DSD011 in the GenBank was used to create a phylogenetic tree (Figure 2) to visualize the relatedness of the species with the level of confidence calculated from 1000 replicates each for bootstrap analysis using the maximum-likelihood method [63]. The analysis revealed that the 16S rRNA gene sequence has a mean pairwise evolutionary distance of 0.037. It also revealed that the *Streptomyces* sp. strain DSD011 formed a distinct lineage among the most closely related species: *Streptomyces cellulosae* strain NBRC 13027 (99.93%, GenBank NR_112346.1) and *Streptomyces cellulosae* strain NRRL B-2889 (99.93%, GenBank NR_043815.1). However, it showed low bootstrap values indicating less significant separation of *Streptomyces* sp. strain DSD011 and related species.

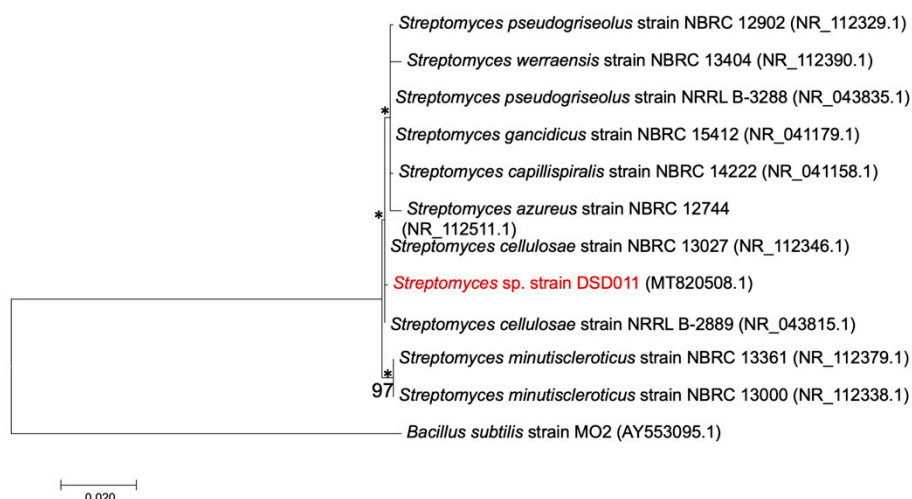


Figure 2. Maximum-likelihood phylogenetic tree based in the nearly complete 16S rRNA gene sequences (1401 bp) of strain DSD011 showing the relationship with members of the genus *Streptomyces* with homology values of more than 99%. *Bacillus subtilis* strain MO2 (AY553095.1) was used as an outgroup. Asterisks denote branches that were also recovered using the neighbor-joining method. Bootstrap values above 50% (based on 1000 replications) are shown in the branch points. Bar, 0.020 substitutions per nucleotide position.

The close relationships between *Streptomyces* sp. strain DSD011 with *Streptomyces cellulosae* strain NBRC 13027 and *Streptomyces cellulosae* strain NRRL B-2889 were further confirmed by the multi-locus sequence analysis (MLSA). While most of the recent studies on MLSA analysis of *Streptomyces* species are based on five housekeeping genes—*atpD* (ATP synthase F1, beta subunit), *gyrB* (DNA *gyrB* subunit), *recA* (recombinase A), *rpoB* (RNA polymerase beta subunit), and *trpB* (tryptophane B, beta subunit) [64–66], our MLSA work covers four housekeeping genes: *atpD*, *recA*, *rpoB*, and *trpB*, which are also useful in studying the systematics of *Streptomyces* species [67]. We tested *gyrB* in strain DSD011, but a well-amplified product was not obtained; hence this gene was not included in the study. All the partial gene sequences of four housekeeping genes, *atpD*, *recA*, *rpoB*, and *trpB* that were obtained in this study were deposited in Genbank with accession numbers: MT844068, MT844069, MT844070, MT844071.

The concatenation of the four gene sequences (*atpD*, *recA*, *rpoB*, and *trpB*) yielded an alignment of 3567 nt. A concatenated MLSA tree (Figure 3) was constructed based on 1000 replicates each for bootstrap analysis using Kimura’s maximum-likelihood method [63]. The concatenated alignment of the four housekeeping genes has a mean pairwise evolutionary distance of 0.240 within the phylogenetic tree. MLSA provides an improved framework and a more stable topological structure supported by high bootstrap values ranging from 62–100%. The *Streptomyces* sp. strain DSD011 notably separated itself from its nearest lineage as indicated by the evolutionary distance value of 0.015 and 0.014 to its close neighboring species, *Streptomyces* sp. WAC 01438 (98.82% percent identity) and *Streptomyces variabilis* strain ARRS001 (99.57% percent identity), respectively. This result is consistent with the MLSA evolutionary genetic distances, which showed that nucleotide sequence distance with ≥ 0.007 value should retain the species status [64]. Our MLSA analysis showed that *Streptomyces* sp. strain DSD011 diverged from the related species on the database and showed good discriminatory ability to classify *Streptomyces* sp. strain DSD011 and related species into a more resolved separation on the phylogenetic tree, thus indicating that the strain DSD011 formed a new *Streptomyces* species.

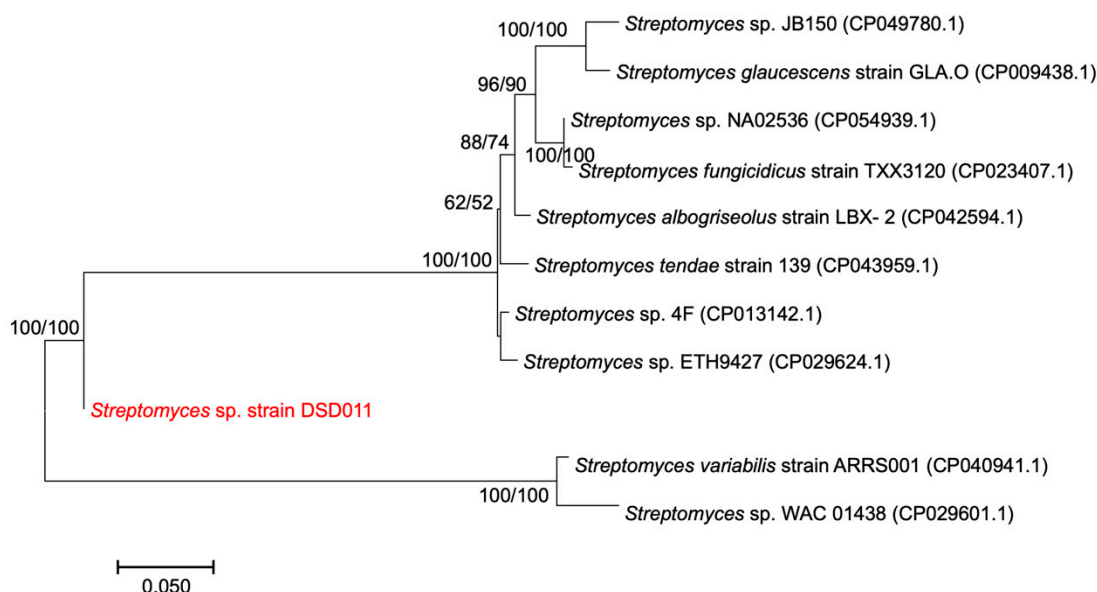


Figure 3. Maximum-likelihood phylogenetic tree based on concatenated sequences of four housekeeping genes, *atpD*, *recA*, *rpoB*, and *trpB*, showing the phylogenetic relationship of strain DSD011 and its relative within the genus *Streptomyces*. Bootstrap values above 50% (based on 1000 replications) are shown in the branch points for maximum-likelihood (left) and maximum-parsimony (right) analyses. Bar, 0.050 substitutions per nucleotide position.

In this study, we have demonstrated that the 16S rRNA phylogeny along with multi-locus analysis (MLSA) of four housekeeping genes (*atpD*, *recA*, *rpoB*, and *trpB*) provided evidence to examine fine-scale interrelationships of *Streptomyces* sp. strain DSD011 and its reference strains match in the GenBank. Considered as a highly conserved gene with a minimal rate of nucleotide changes, 16S rRNA as a sole basis for bacterial phylogeny is weak and has a low differentiation power when dealing with closely-related species [68]. The MLSA used in this study are universal genes that serve as alternatives to 16S rRNA by using individually or concatenated analyzed sequences [64]. The phylogenetic analysis constructed based upon the 16S rRNA gene sequences and concatenated MLSA revealed differences between the phylogenetically coherent branches of *Streptomyces* sp. strain DSD011 and its reference matches. The *Streptomyces* sp. strain DSD011 was closely related to *Streptomyces cellulosae* strain NBRC 13027 (99.93% percent identity) based on the 16S rRNA phylogeny, as compared to that of the MLSA tree where *Streptomyces variabilis* strain ARRS001 (99.57% percent identity) was observed to be the closest relative. Moreover, individual housekeeping gene phylogenies demonstrated slight dissimilarities where the *Streptomyces* sp. strain DSD011 share close affinity with *Streptomyces* sp. 4F (98–99% percent identity) for genes associated with *atpD*, *recA* and *rpoB*, but was more affiliated only with *Streptomyces variabilis* strain ARRS001 (96.8% percent identity) for *trpB* gene. Thus, the incongruent results of two phylogenetic analyses indicate that *Streptomyces* sp. strain DSD011 may represent a new taxon.

To note, the closely related species of *Streptomyces* sp. strain DSD011 are terrestrial in origin. More interestingly, one related strain, *Streptomyces* sp. strain 4F, was isolated from the highly saline environment in the Great Salt Plains, Oklahoma [69]. It is possible that *Streptomyces* sp. strain DSD011 originated from terrestrial soil and its spores are washed in the marine environment and reside in marine sediments as dormant spores. It is noteworthy that *Streptomyces* sp. strain DSD011 grew well at 0 to 5% NaCl and showed moderate growth at 7% NaCl as shown in Table 2, suggesting that the strain developed growth adaptation in the marine environment and has evolved as a salt-tolerant *Streptomyces*.

3.3. Screening for Polyketide Synthase (PKS) and Non-Ribosomal Peptide Synthetase (NRPS) Secondary Metabolite Biosynthetic Genes

As major producers of natural products, *Streptomyces* are often surveyed for the presence, abundance, and novelty of PKS and NRPS genes to evaluate its potential as secondary metabolite producers. To determine the presence of type I and type II PKS and NRPS biosynthetic genes in *Streptomyces* sp. strain DSD011, we screened its genome by targeting the adenylation (AD), type I ketoacyl synthase (KS), and KS α and KS β domains, respectively using PCR amplification approach [51–56]. The biosynthetic genes were considered present when a strong unambiguous amplicon could be observed in the agarose gel.

Our results revealed that *Streptomyces* sp. strain DSD011 harbors both NRPS and type II PKS in its genome. The presence of a distinct band at 700 base pair (bp) size indicated positive amplification of AD of NRPS. For type II PKS gene clusters, three sets of degenerate primers targeting different conserved regions of KS α and KS β domain were used to screen for its presence: (1) KS1-F and KS1-R (with 613 bp PCR product) that target the KS α domain; (2) KS α and KS β (with 800–900 bp PCR product) that target the KS α and KS β domain complex; and (3) 540F and 1100R (with 554 bp PCR product) that target the 5' of KS α domain. PCR screening showed that *Streptomyces* sp. strain DSD011 harbors all target regions for the type II PKS gene as indicated by the distinct bands with an amplicon size of 554–700 bp (Figure 4). Interestingly, *Streptomyces* sp. strain DSD011 harbors type II PKS genes targeting the KS α and KS β domain complex at 600–700 bp, which is in contrast to the reported 800–900 expected length of target region [53]. Conversely, *Streptomyces* sp. strain DSD011 does not harbor type I PKS in this PCR screening as supported by the absence of distinct amplicon bands in 613 bp for the two target conserved sequences in the KS domain (Figure 4).

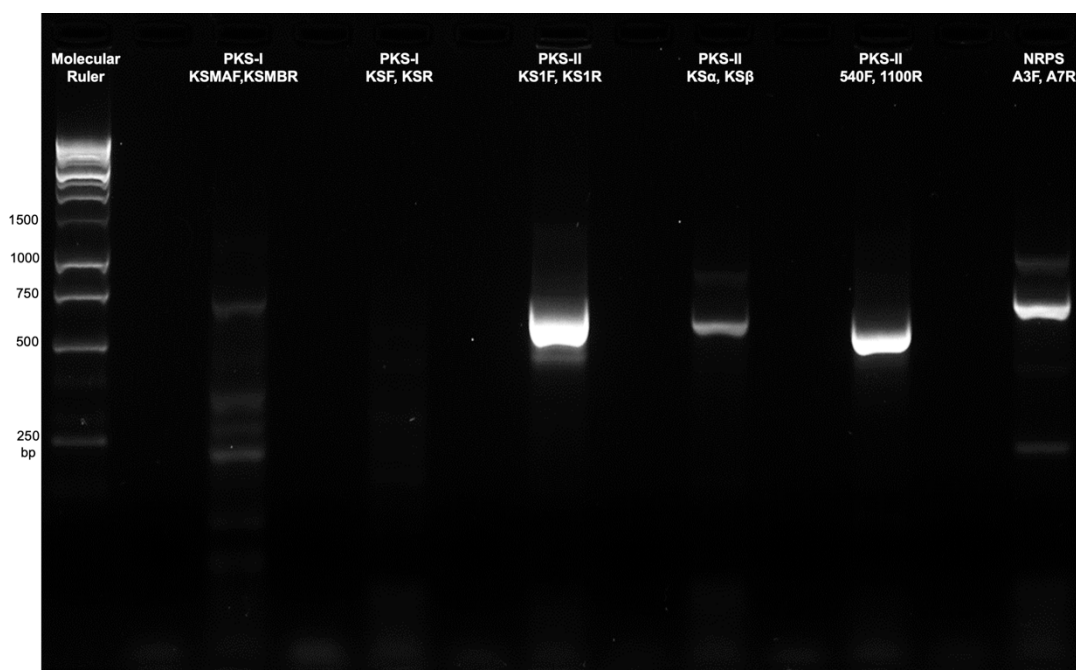


Figure 4. Amplified PCR products of *Streptomyces* sp. strain DSD011 using degenerate primers to detect conserved regions of types I and II polyketide synthetase (PKS) and non-ribosomal peptide synthase (NRPS) domains.

Here, the PCR detected type II PKS gene of ketosynthase domains in *Streptomyces* sp. strain DSD011 indicates a high possibility of aromatic polyketides potentially produced by the strain. Notably, contrary to the expected length, KS α and KS β gene fragment were amplified at 600–700 bp suggesting that type II PKS KS α domains were abundant in *Streptomyces* strain sp. DSD011 which can be associated with complex and diverse type II PKS compounds. The type II PKSs are mainly responsible for

producing polycyclic aromatic polyketides compounds harboring at least one aromatic ring [70]. These compounds are an important type of natural product because of their potential pharmaceutical applications such as antibacterial, anticancer, and antiviral activities [71]. Although type II PKS and NRPS are the major enzymes for secondary metabolite biosynthesis found in *Streptomyces* sp. strain DSD011, their presence does not indicate expression or functionality but rather increases the likelihood of producing bioactive polyketides and non-ribosomal peptide compounds.

3.4. Isolation and Identification of Antibiotic Compounds 1 and 2

To identify the structures of the antibacterial compounds from *Streptomyces* sp. strain DSD011, the crude extract was fractionated by series of chromatography including gel filtration chromatography, flash chromatography, and semi-preparative reversed-phase HPLC, which afforded two pure compounds with UV activity at 254 nm. QTOF-MS and MS/MS data in combination with GNPS analysis revealed molecular ion peaks at m/z 487.1606 $[M + H]^+$ (predicted molecular formula: $C_{25}H_{27}O_{10}$, calc. mass = 487.1604, DBE = 13) and 597.1977 $[M + H]^+$ (predicted molecular formula: $C_{31}H_{33}O_{12}$, calc. mass: 597.1972, DBE = 16) that correspond to compounds 1, fridamycin A (Figure 5, Supplemental Table S6 and S7) and 2, fridamycin D (Figure 6, Supplemental Tables S8 and S9), respectively. Compounds 1 and 2 displayed antibacterial activity against multidrug-resistant *S. aureus* ATCC BAA-44 with MIC of 500 and 62.5 $\mu\text{g/mL}$, respectively (MIC of tetracycline = 31.25 $\mu\text{g/mL}$).

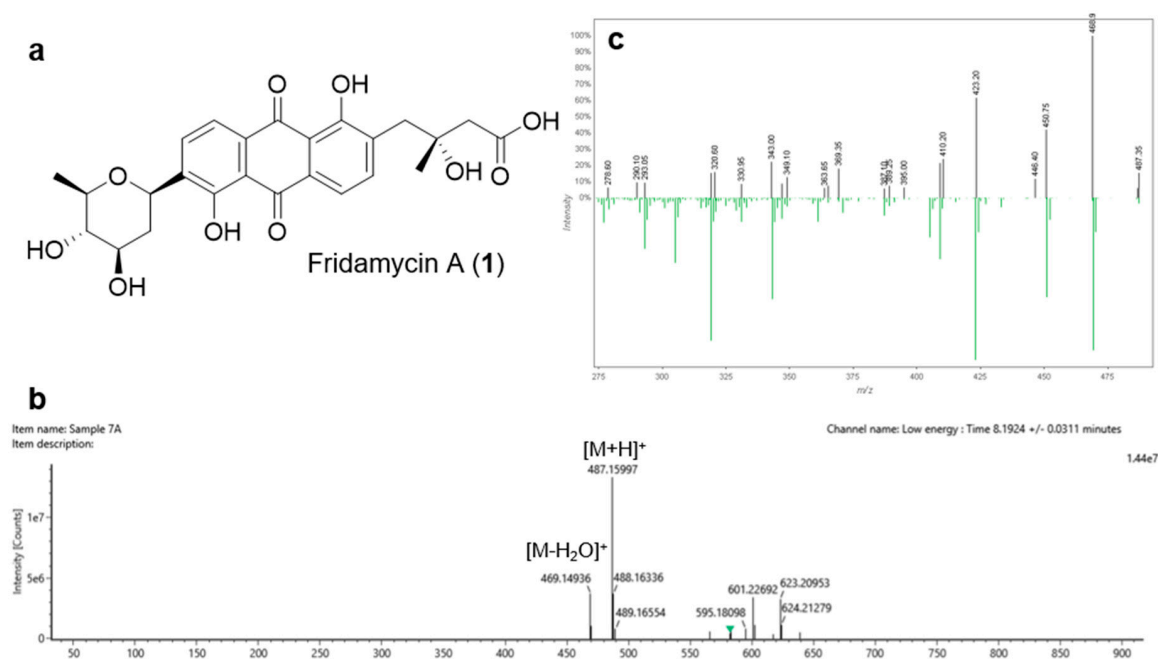


Figure 5. (a) Chemical structure of the angucycline glycoside fridamycin A (1); (b) HRMS spectrum of 1; (c) Global Natural Product Social molecular networking (GNPS) mass mirror view of MS/MS from experimental data and GNPS Library Spectrum (bottom spectrum) that are associated to the structure of 1.

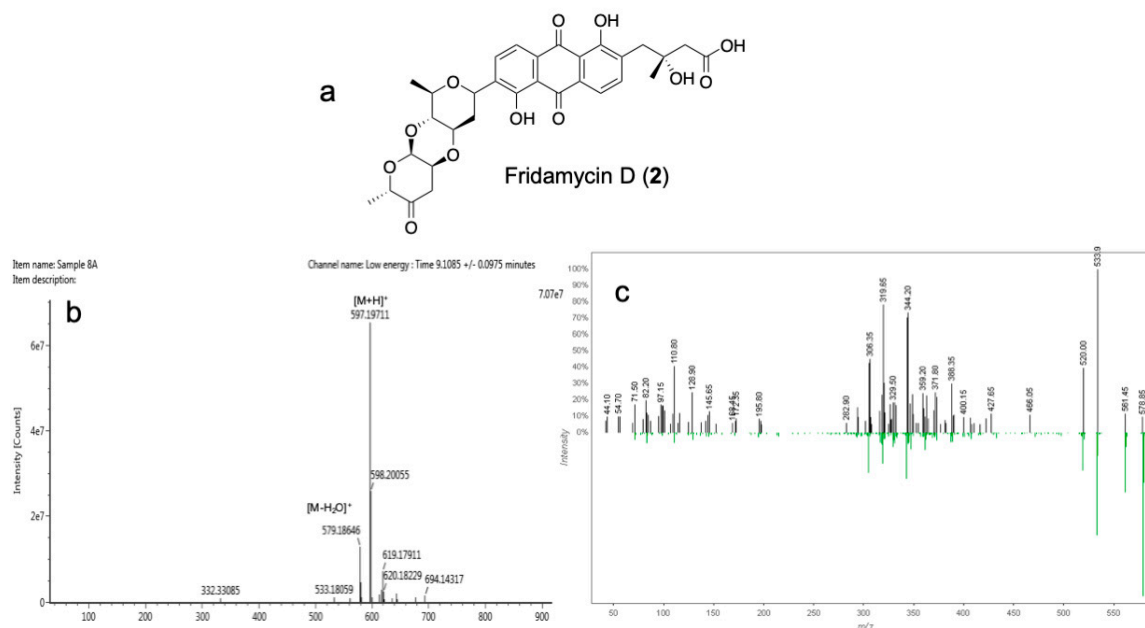


Figure 6. (a) Chemical structure of the angucycline glycoside fridamycin D (2); (b) HRMS spectrum of 2; and (c) GNPS mass mirror view of MS/MS from experimental data and GNPS Library Spectrum (bottom spectrum) that are associated to the structure of 2.

In this work, we isolated two angucycline glycoside antibiotics, fridamycin A (1) and fridamycin D (2) that belongs to the angucycline class of natural products—the largest group of polycyclic aromatic polyketides produced by actinomycetes, mostly from *Streptomyces* species. These compounds are known to possess diverse pharmacological activities including cytotoxicity and antitumor antibacterial and antiviral properties [72,73]. Structurally, the angucycline compounds have a rich core structure that contains an angular benz[a]anthracene moiety that is glycosylated [72,73]. Fridamycin A (1) and fridamycin D (2) are non-classic congeners of glycosylated angucycline compounds with rearranged linear tetracyclic or tricyclic core units [72]. The biosynthetic origin of all angucyclines are polyketide derived, with type II polyketide synthases (PKS II) responsible for the initial decaketide backbone which is followed by ring closure reactions to form the benz[a]anthracene scaffold [73]. The isolation of 1 and 2 in this work is supported by the results on PKS screening, wherein *Streptomyces* sp. strain DSD011 showed unambiguous amplicon of type II PKS genes.

While older studies report that terrestrial *Streptomyces* produces angucycline glycosides, recent reports have shown that marine sediment-derived *Streptomyces* are also prolific producers of angucycline natural products [9,74–77]. Numerous angucycline compounds along with 1 and 2 that were isolated from marine sediment-derived *Streptomyces* were found to be cytotoxic against breast cancer cells (MCF-7, MDA-MB-231, and BT-474) [76,77], Jurkat cells [75], and hepatoma carcinoma cells (HepG-2, SMMC-7721, and plc-prf-5) [9,77]. Our work demonstrates for the first time that compounds 1 and 2 from marine sediment-derived *Streptomyces* sp. are antibacterial against multidrug-resistant *S. aureus* harboring the SCCmec gene, which is a biomarker of drug resistance. While the mode of action of angucycline glycosides as an anticancer is known for its DNA cleaving activity [73], there are no reports that describe the mechanism of their antibacterial activity. Hence, the promising antibacterial activities of compounds 1 and 2 against multidrug-resistant *S. aureus* warrant more research to investigate their antibacterial mechanism of action.

4. Conclusions

In conclusion, we have isolated and identified the *Streptomyces* sp. strain DSD011 from marine sediments of Islas de Gigantes, Iloilo, Philippines that possesses antibacterial property against multidrug-resistant *S. aureus* carrying the antibiotic drug resistance marker SCCmec type I gene.

The *Streptomyces* sp. strain DSD011 is a new species of salt-tolerant marine *Streptomyces* and harbors type II PKS gene clusters responsible for the production of two polycyclic aromatic polyketide angucycline antibiotics, fridamycin A (1) and fridamycin D (2). This study demonstrates that marine sediments in the Philippine archipelago contain new or novel *Streptomyces* species that produce antibiotics. Further work that includes genomics for mining of novel biosynthetic genes and metabolomics for the detection of new chemical scaffolds will be needed for future studies.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2077-1312/8/10/734/s1>. Supplemental Table S1. Primers for Multilocus Sequence Analysis [50]. Supplemental Table S2. Degenerate Primers for PCR Screening of Polyketide Synthase and Non-Ribosomal Synthetase. Supplemental Table S3. Growth Characteristics of *Streptomyces* sp. strain DSD011 in various NaCl concentrations. Supplemental Table S4. Growth Characteristics of *Streptomyces* sp. strain DSD011 in various carbon sources (% w/v) after 7 days of incubation. Supplemental Table S5. Antibacterial Activity of strain DSD011 crude fermented extract. Supplemental Table S6. Latest Library Spectrum Information on GNPS match for fridamycin A, 1 (m/z 487 [M + H]⁺). Supplemental Table S7. Waters UNIFI Scientific Information System Report for fridamycin A, 1 (m/z 487 [M + H]⁺). Supplemental Table S8. Latest Library Spectrum Information on GNPS match for fridamycin D, 2 (m/z 597 [M + H]⁺). Supplemental Table S9. Waters UNIFI Scientific Information System Report for fridamycin D, 2 (m/z 597 [M + H]⁺).

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