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Original Article

Potential *anti*-biofilm producing marine actinomycetes isolated from sea sediments in Thailand

Kantinan Leetanasaksakul,[a, b, c] Arinthip Thamchaipenet[a, c, *]

[a] Department of Genetics, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand
[b] Interdisciplinary Graduate Program in Genetic Engineering, Graduate School, Kasetsart University, Bangkok 10900, Thailand
[c] Center for Advanced Studies in Tropical Natural Resources, Kasetsart University, Bangkok 10900, Thailand

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ABSTRACT

Marine actinomycetes are well known as novel resources of bioactive compounds including *anti*-biofilm agents with various therapeutic applications. In this study, one hundred and one marine actinomycetes were isolated from sea sediments collected from Andaman sea and the Gulf of Thailand. Morphological study and partial 16S rDNA sequence analysis revealed that most of isolates belong to genus *Streptomyces* (n = 90, 89.11%) and the rest are rare actinomycetes (n = 11, 10.89%) including *Actinopolymorpha, Actinomycetospora, Dietzia Nocardiopsis*, *Micromonospora* and *Mycobacterium*. Among them, ten and thirteen strains significantly reduced more than 60% of biofilm formation of *Escherichia coli* and *Staphylococcus aureus*, respectively. But no such activity was found against *Pseudomonas aeruginosa*. Further analysis of their supernatants indicated that most of marine actinomycetes secreted non-toxic *anti*-biofilm agents and some accompanied with protease activity. These are preferred characteristics of *anti*-biofilm substances that potentially restricted biofilm forming of *E. coli* and *S. aureus* and simultaneously prevented bacteria to develop resistance.

Introduction

In nature, bacteria grow in polymicrobial communities coexisted with other species. Thus, bacterial communication between the community members is crucial for bacterial survival in limited resources. For survival strategy, bacteria form complex architecture called biofilm that adhere microbial population to a solid surface by exopolysaccharide, proteins and nucleic acids (Flemming et al., 2007). Biofilm acts as a barrier of bacterial group to protect stresses around bacterial community by diminishing of antibiotics and host defenses which consequently caused bacterial perseverance in chronic infections (Savage et al., 2013).

At present, non-toxic biologically active compounds capable of inhibiting biofilms are of interested. One of attractive bioresources of novel bioactive compounds is marine actinomycetes, because they variously produce potential antibiotics, enzymes, enzyme inhibitors and pharmacological agents (Blunt et al., 2014). Marine ecosystem is one of the most productive habitats of a large number of organisms including bacteria, fungi, invertebrates and mammals (Costello and Chaudhary, 2017). Marine actinomycetes, in particular, are crucial sources of new natural products (Hong et al., 2009). Several reports revealed that marine actinomycetes produced *anti*-biofilm agents against different pathogenic bacteria. For example, extracts of *Streptomyces albus* inhibited biofilm formation of *Vibrio harveyi* (You et al., 2007); extracts of coral-associated *Streptomyces akiyoshiensis* inhibited biofilm formation of *Staphylococcus aureus* (Bakkiyaraj et al., 2010); *Rhodococcus* sp. BFI 332 inhibited *E. coli* O157:H7 biofilm formation (Lee et al., 2012); and extracellular proteases from marine actinomycetes inhibited and detached *S. aureus* biofilm formation (Park et al., 2012a, 2012b).

This study is aimed to isolate marine actinomycetes from sea sediments of Andaman and the Gulf of Thailand. All isolates were identified at genus level and showed diversity of this collection. Their supernatants were tested for *anti*-biofilm activity against *E. coli*, *Pseudomonas aeruginosa*, and *S. aureus*. Potential *anti*-biofilm activity from marine actinomycetes was discussed.

* Corresponding author. Department of Genetics, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
E-mail address: arinthip.t@ku.ac.th (A. Thamchaipenet).

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Material and methods

Sample collection and isolation of marine actinomycetes

A total of 7 samples from marine sediments (4–20 km off shore and 3–30 m depth) around the Gulf of Thailand at Chaophraya estuary, Chanthaburi, Phechaburi, Rayong, Sattahip, Tha Chin estuary, and from Andaman sea at Ranong were kindly obtained from Dr. Shettapong Meksumpun, Faculty of Fishery, Kasetsart University. One gram of wet sediment was mixed with 5 ml of artificial seawater then boiled at 55 °C for 60 min by a modified method of Mincer et al. (2002) and Magarvey et al. (2004). Fifty microliters of samples were mixed and spread onto 3% artificial seawater agar (SWA); and starch casein agar (SCA; Kuster and Williams, 1964) and starch yeast extract agar (SYA) (Mincer et al., 2002) supplemented with 3% artificial seawater. All media contained 100 μg/ml ampicillin and 50 μg/ml cycloheximide. Plates were incubated at 28 °C up to six weeks. Selected colonies were purified on ISP 2 agar (Shirling and Gottlieb, 1966) supplemented with 2% NaCl.

16S rRNA gene amplification and sequencing

Marine actinomycetes were grown on ISP 2 agar for 7 days. Total DNA was extracted following a protocol described by Hopwood et al. (1985). Amplification of 16S rRNA genes was performed using primers and PCR conditions described by Rachniyom et al. (2015). The amplified 16S rDNA fragment was purified using a GenepH low™ Gel/PCR Kit (Geneaid, Taiwan) and was directly sequenced by Macrogen (Korea). The sequences were compared with GenBank database and EzTaxon server (Yoon et al., 2017). The closely related 16S rDNA sequences were retrieved from the database and performed the multiple alignments. The phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) and the Mega 6 package (Tamura et al., 2013). The confidence values of branches of the tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1000 re-sampling.

Determination of anti-biofilm activity

E. coli ATCC 8739, *P. aeruginosa* ATCC 15442 and *S. aureus* ATCC 25923 were used as test strains for biofilm inhibition assay. Marine actinomycetes were grown in glucose starch soybean meal medium (GSS; Kim et al., 2012) at 28 °C, 150 rpm for 7 days. Cultures were centrifuged at 4 °C, 8000 g for 15 min. Their supernatants were transferred into new tubes under aseptic condition. Approximately 10^8 CFU of 40 μl culture of biofilm-forming test strains were inoculated into polystyrene 96-well microtiter plates containing 60 μl tryptic soy broth (TSB) supplemented with 0.25% glucose and 100 μl culture supernatant of marine actinomycete and incubated at 37 °C for 16 h. 100 μl of GSS medium was used instead of the supernatant as untreated control. Planktonic cells and supernatant were discarded, the attached cells (biofilm) were gently rinsed with distilled water 3 times then fixed by methanol for 15 min and allowed to air-dry. The biofilms were stained with 250 μl of 0.5% (w/v) crystal violet for 5 min, the dye was then discarded and the wells were rinsed twice with distilled water and allowed to air-dry before solubilization with 250 μl 30% glacial acetic acid. The optical density was measured at 595 nm by an enzyme-linked immunosorbent assay reader (Bio-Rad, USA). The percentage of biofilm formation was calculated using the following equation:

$$\%\ \text{Biofilm formation} = \frac{A_{595}\ \text{of biofilm with no supernatant} - A_{595}\ \text{of biofilm with supernatant}}{A_{595}\ \text{of biofilm with no supernatant}} \times 100$$

The percentage inhibition for each replicate was calculated for the mean value of each experiment. The mean and the standard error of the mean from three experiments were calculated.

Determination of antimicrobial activity

The antibacterial activity assay was modified from plate diffusion method (Collins et al., 1977). Marine actinomycetes were cultured on GSS agar at 28 °C for 7 days. Agar plugs were punched from the cultured agar and transferred onto nutrient agar (NA). *E. coli* and *S. aureus* were grown in nutrient broth (NB) at 37 °C, 250 rpm, overnight. The optical density at 600 nm was adjusted to 0.25, and then overlaid on NA placed with actinomycete agar plugs. Paper disc containing 1 mg/ml ampicillin was used as a positive control. The plates were incubated at 37 °C for 24 h and a presence of a zone of inhibition surrounding the plug was determined as positive antibacterial activity [measured by diameter of the inhibition zone (cm)].

Determination of proteolytic activity

Marine actinomycetes were grown in GSS medium at 28 °C, 150 rpm for 7 days and then centrifuged at 4 °C, 6500 g for 20 min. Twenty microliters of supernatant was transferred into a hole of skim milk agar plate (5% non-fat dry milk and 1% agar) and incubated at 37 °C for 24 h. Proteinase K (10 μg/ml) was used as a positive control. Protein digestion was observed by a clear zone surrounding the hole.

Statistical analysis

Each 96-well plate experiment was performed in at least three independent replicates. The reduction of biofilm formation was statistically analyzed using SPSS software version 16.0 (SPSS, Chicago, IL, USA). Differences between two mean values were calculated by *t*-test. Data was considered as a statistical significant at a $p < 0.05$ and $p < 0.01$.

Results

Isolation and identification of marine actinomycetes from sea sediments

A total of 101 isolates of marine actinomycetes were obtained from the sediments of Chanthaburi (n = 2, 1.96%), Chaophraya estuary (n = 7, 6.86%), Phetchaburi (n = 23, 22.55%), Ranong (n = 16, 15.69%), Rayong (n = 26, 25.74%), Sattahip (n = 22, 21.57%), and Tha Chin estuary (n = 5, 4.90%). Based on colony morphology, most of these isolates belong to genus *Streptomyces* (n = 90, 89.11%) and the

rest are rare actinomycetes (n = 11, 10.89%). Twenty representatives were chosen based on morphological differences to perform partial 16S rRNA sequencing and compared to those of type strains using EzTaxon web based tool (Yoon et al., 2017). The sequence data of all closely related type strains were used to construct phylogenetic tree. *Bacillus licheniformis* ATCC14580^T was used as an outgroup. The results indicated that 20 representatives formed distinct clade defined in 7 genera including *Actinomycetospo*ra, *Actinopolymorpha*, *Dietzia*, *Nocardiopsis*, *Micromonospora*, *Mycobacterium*, and *Streptomyces* (Fig. 1).

Determination of anti-biofilm, anti-bacterial and proteolytic activities

Supernatants of 101 marine actinomycetes were determined for *anti*-biofilm formation against *E. coli*, *P. aeruginosa* and *S. aureus*.

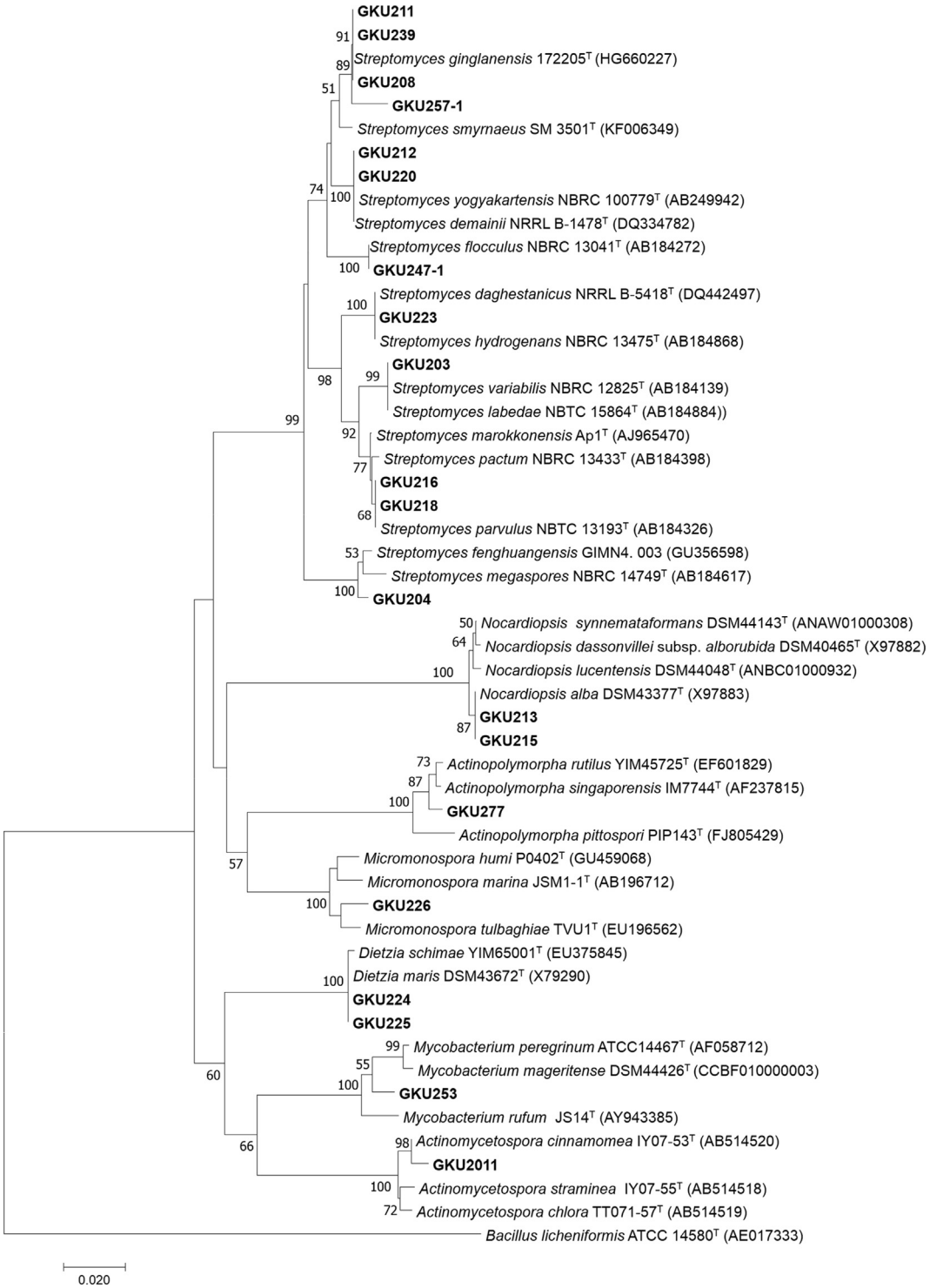

Fig. 1. Neighbor-joining phylogenetic tree based on partial 16S rRNA sequences of 20 isolates of marine actinomycetes and closely related type strains from EzTaxon database. Bootstrap values more than 50% indicated at branch point.

Monitoring biofilm adherence in well surface of 96-microtiter plates, the inhibiting biofilm formation was calculated to percentage of 0–20, 21–40, 41–60, and 81–100 (Fig. 2). There are 10 strains of actinomycetes showed potentially reducing biofilm formation of *E. coli* at 61–80% namely, *Streptomyces* sp. GKU 208, GKU 211, GKU 219, GKU 222, GKU 257-1, GKU 257-2, GKU 262, GKU 266, GKU 276, and GKU 279 (Fig. 2A and Table 1). Thirteen strains revealed potent of inhibiting biofilm formation of *S. aureus* more than 60% namely, *Nocardiopsis* sp. GKU 213, *Streptomyces* sp. GKU 218, GKU 220, GKU 221, GKU 222, GKU 223, GKU 237, GKU 258, GKU 260, GKU 264, GKU 267-1, GKU 297 and GKU 2008 (Fig. 2B and Table 2). Amongst these strains, *Streptomyces* sp. GKU 222 inhibited biofilm formation of both *E. coli* and *S. aureus*. Surprisingly, none of marine actinomycetes conferred *anti*-biofilm activity against *P. aeruginosa* more than 20% (Fig. 2C). Thus, the supernatants that inhibited biofilm formation against *E. coli* and *S. aureus* will be used for further screening of *anti*-bacterial and proteolytic activities.

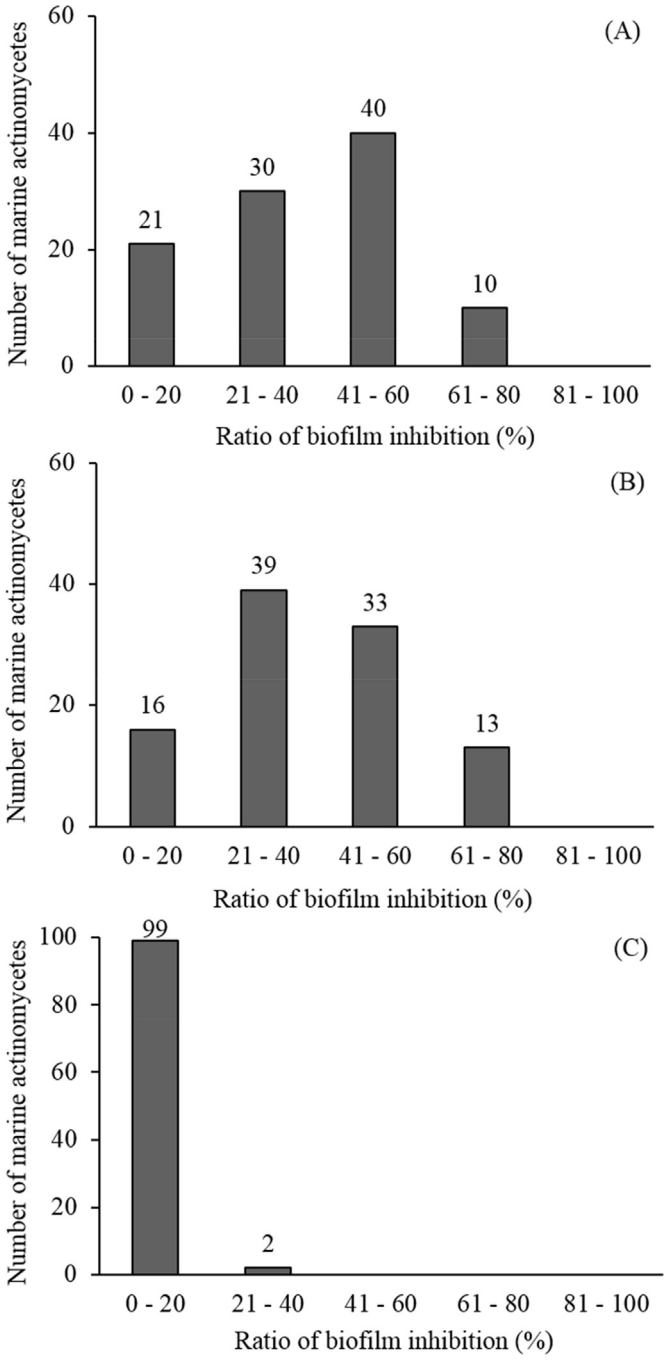

Fig. 2. Inhibition of biofilm formation at 0–20, 21–40, 41–60, 61–80, and 81–100 percentage by marine actinomycetes against (A) *E. coli*, (B) *S. aureus* and (C) *P. aeruginosa*. The numbers on the top of each bar indicate the number of marine actinomycete strains.

Those marine actinomycetes that inhibited biofilm formation of *E. coli* and *S. aureus* more than 60% were then investigated for their *anti*-bacterial activity against both species using agar plug assay. None of marine actinomycetes exhibited *anti*-bacterial activity against *E. coli* (Table 1), whereas *Streptomyces* sp. GKU 218, GKU 264, GKU 297, and GKU 2008 exhibited clear zone against *S. aureus* (Table 2). Furthermore, proteolytic activity of those marine actinomycetes was determined using skim milk agar assay. Only *Streptomyces* sp. GKU 262 that inhibited biofilm formation of *E. coli* carried protease activity, while *Streptomyces* sp. GKU 223, GKU 258, GKU 264, GKU 297 and GKU 2008 that inhibited biofilm formation of *S. aureus* showed protease activity (Tables 1 and 2). Amongst them, *Streptomyces* sp. GKU 264, GKU 297 and GKU 2008 exhibited both *anti*-bacterial activity against *S. aureus* and protease activity. The results revealed that most of secreted substances from marine actinomycetes inhibited biofilm formation of *E. coli* (10 strains) and *S. aureus* (9 strains) without *anti*-bacterial activity (Tables 1 and 2).

Discussion

Marine actinomycetes isolated from sea sediments of Andaman and the Gulf of Thailand showed predominant members of genus *Streptomyces* and minority in genera *Actinomycetospo*ra, *Actinopolymorpha*, *Dietzia*, *Nocardiopsis*, *Micromonospora* and *Mycobacterium*. Our results were in agreement with previous culture-dependent studies that common actinomycetes found in marine ecosystems were *Streptomyces* and *Micromonospora* (Goodfellow and Haynes, 1984; Jensen et al., 1991), *Dietzia*, *Nocardiopsis*, *Mycobacterium* and *Rhodococcus* (Colquhoun et al., 1998; Takami et al., 1999; Chun et al., 2000). The results were also consistent with previous reports that *Streptomyces* species were frequently found among actinomycetes isolated from sea sediments of mangrove areas (Mitra et al., 2008; Hong et al., 2009), deep sea (Paulus et al., 2017) and marine sponge (Jackson et al., 2018).

Biofilms are a consequence of growth adaptation of bacteria to environmental stresses including host immune system and antibiotics and, thus, are difficult to treat and eradicate (Hoiby et al., 2010; Vuotto et al., 2017). Hence, it is of interest to find non-antibacterial drugs that discourage biofilm forming but avoiding bacteria evolved resistance (Rabin et al., 2015). Our results demonstrated that *anti*-biofilm substances from marine actinomycetes carried various characteristics including lethal bioactive compounds, non-antibacterial active agents and proteolytic activity against *E. coli* and *S. aureus* biofilm formation. It is known that marine actinomycetes are important sources of natural products (Hong et al., 2009; Sangkanu et al., 2017) and are also potent sources of novel *anti*-biofilm substances (You et al., 2007). Our results showed that most of secreted substances from marine actinomycetes inhibited biofilm formation of *E. coli* and *S. aureus* but not causing lethal effect. Some strains that carried protease activity possibly inhibited biofilm formation by such proteolytic action which was in agreement with other reports that proteases produced by some marine *Streptomyces* efficiently removed biofilm of *S. aureus* (Park et al., 2012a, 2012b). It was indicated that extracellular proteins play multiple role in the biofilm, participating in structure and quorum-sensing functions (Flemming and Wingender, 2010) and as

Table 1
Anti-bacterial, proteolytic and *anti*-biofilm activities of marine actinomycetes against *E. coli*.

Strain	*Anti*-bacterial activity against *E. coli*[a]	Proteolytic activity[b]	% Inhibition of biofilm formation
Streptomyces sp. GKU 208	−	−	65.83 ± 10.70*
Streptomyces sp. GKU 211	−	−	64.70 ± 10.08*
Streptomyces sp. GKU 219	−	−	71.37 ± 9.17**
Streptomyces sp. GKU 222	−	−	66.94 ± 9.51*
Streptomyces sp. GKU 257-1	−	−	78.26 ± 2.19**
Streptomyces sp. GKU 257-2	−	−	66.47 ± 9.61*
Streptomyces sp. GKU 262	−	+	63.97 ± 4.54**
Streptomyces sp. GKU 266	−	−	62.10 ± 21.79
Streptomyces sp. GKU 276	−	−	68.34 ± 4.25**
Streptomyces sp. GKU 279	−	−	65.27 ± 13.56*

Asterisks indicate significant by comparison to *E. coli* (*$p < 0.05$, **$p < 0.01$).
[a] Antibacterial against *E. coli*; No clear zone (− means < 0.1 cm), Low (+ means ≥ 0.1–0.6 cm), High (++ means > 0.6–1.2 cm).
[b] Proteolytic activity; −, negative activity; +, positive activity.

Table 2
Anti-bacterial, proteolytic and *anti*-biofilm activities of marine actinomycetes against *S. aureus*.

Strain	*Anti*-bacterial activity against *S. aureus*[a]	Proteolytic activity[b]	% Inhibition of biofilm formation
Nocardiopsis sp. GKU 213	−	−	61.80 ± 6.87**
Streptomyces sp. GKU 218	+	−	71.32 ± 6.21**
Streptomyces sp. GKU 220	−	−	71.38 ± 9.10**
Streptomyces sp. GKU 221	−	−	62.46 ± 13.17*
Streptomyces sp. GKU 222	−	−	66.94 ± 16.98*
Streptomyces sp. GKU 223	−	+	63.76 ± 1.31**
Micromonospora sp. GKU 237	−	−	62.18 ± 14.79*
Streptomyces sp. GKU 258	−	+	66.04 ± 3.97**
Streptomyces sp. GKU 260	−	−	72.68 ± 16.07*
Streptomyces sp. GKU 264	+	+	78.79 ± 4.30**
Streptomyces sp. GKU 267-1	−	−	68.67 ± 18.64*
Streptomyces sp. GKU 297	++	+	70.26 ± 9.37**
Streptomyces sp. GKU 2008	++	+	67.42 ± 17.25*

Asterisks indicate significant by comparison to *S. aureus* (*$p < 0.05$, **$p < 0.01$).
[a] Antibacterial against *S. aureus*; No clear zone (− means < 0.1 cm), Low (+ means ≥ 0.1–0.6 cm), High (++ means > 0.6–1.2 cm).
[b] Proteolytic activity; −, negative activity; +, positive activity.

extracellular enzymes operating within the matrix which are presumably involved in the control of detachment process (Boles and Horswill, 2008; Park et al., 2012a, 2012b). However, marine actinomycetes in this study showed less *anti*-biofilm activity against *P. aeruginosa*. The results might due to permeability of the outer membrane of *P. aeruginosa* which is 12–100-fold lower than that of other Gram-negative bacteria such as *E. coli* (Hancock and Bell, 1988). This low permeability of the outer membrane of *P. aeruginosa* serves as barrier to restrict penetration of antibiotics, antimicrobial peptides and other small molecules (Drenkard, 2003; Taylor et al., 2014).

Several strains of marine actinomycetes in this study produced *anti*-biofilm substances against *E. coli* and *S. aureus* but not toxic to the bacteria. It was reported that marine actinomycetes secreted non-toxic compounds to potentially restrain biofilm formation. For instance, secreting substances of marine *Streptomyces* disturbed quorum-sensing system in *Vibrio harveyi* (You et al., 2007), *Streptococcus pyogenes* (Bakkiyaraj et al., 2010), and staphylococci (Oja et al., 2015; Balasubramanian et al., 2017). Moreover, *Rhodococcus* sp. BFI 332 and *Kribbella* sp. BFI 1562 produced indole-3-acetaldehyde to inhibit biofilm formation of *E. coli* O157: H7 (Lee et al., 2012) and *P. aeruginosa* (Kim et al., 2012), respectively. Marine *Streptomyces* sp. induced biofilm dispersal in *S. aureus* by production of D-amino acids and short peptides (Kolodkin-Gal et al., 2010; Takada et al., 2013). Remarkably, most of marine actinomycetes in this study secreted non-antibacterial *anti*-biofilm agents that potentially restrained biofilm forming of *E. coli* and *S. aureus* nevertheless, preventing bacteria to develop resistance. In addition, type stains of actinomycetes that showed closely related to our marine strains on phylogenetic tree have not yet been reported to produce any *anti*-biofilm substances. Therefore, the marine isolates are highly potential strains for discovery of new *anti*-biofilm agents.

This study signified that marine actinomycetes isolated from sea sediments from Andaman Sea and Gulf of Thailand are good sources of promising *anti*-biofilm producers. There are several potential candidate strains that produced *anti*-biofilm substances accompanying with proteolytic activity which caused no lethal effect to *E. coli* and *S. aureus*. Potential *anti*-biofilm agents including bioactive compounds, non-toxic substances and proteases have currently been under identification and characterization. Further study on molecular interaction of the secreted substances and biofilm formation is under way of investigation in a hope to sustainably utilize the agents in future pharmacologically and medicinally application.

Conflict of interest

There is no conflict of interest.

Acknowledgements

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