ORIGINAL ARTICLE

Isolation, identification and antimicrobial activities of actinobacteria associated with fire ant, *Solenopsis geminata*

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Abstract. Actinobacteria, Gram-stain positive with high G+C content bacteria, have been well known as the antibiotic producer. To find out the new habitat for searching new actinobacteria, we isolated the actinobacteria from the fire ant, *Solenopsis geminata*, collected in Thailand. Totally, 6 actinobacteria were isolated and identified using 16S rRNA gene, as *Streptomyces* (2 isolates) and *Nocardia* (4 isolates) species. Both *Streptomyces* isolates showed antimicrobial activities against tested microorganisms used in this study. Moreover, *Nocardia* isolate SE2 showed low 16S rRNA gene similarity to those known actinobacterial species and represented the undescribed species.

Keywords: Actinobacteria, Fire ant, *Solenopsis geminata*, Antimicrobial activities, Diversity.

1. Introduction

Microorganisms, especially Actinobacteria, Gram-stain positive with high% of G+C content bacteria, are a primary resource of critical bioactive compounds. However, it has recently become challenging to find the novel compounds because of the re-isolation of the antibiotic-producing actinobacteria (Matsumoto and Takahashi 2017).

Insect-bacterial symbioses have been well known since the discovery of the symbiosis association between fungus-growing ants and Actinobacteria (Currie et al. 1999). The insects, especially social insects, are often used the antibiotic-producing bacterial symbionts to protect their nests (Beemelmanns et al. 2016). Consequently, these social insects become promising sources for the natural product discovery. In the past decade, there are several novel compounds isolated from actinobacteria associated with social insects including amycomycins A-B (Guo et al. 2012), pseudonocardones A-C (Carr et al. 2012a), fasamycins C-E (Qin et al. 2017), formicamycins A-M (Qin et al. 2017), macrotermycins A-D (Beemelmanns et al. 2017)[7], microtermolides A-B(Carr et al. 2012b), sceliphorolactam (Oh et al. 2011), and termisoflavones A-C (Kang et al. 2016).

In this study, the actinobacteria from the fire ant, *Solenopsis geminata*, collected in Thailand were isolated and the antimicrobial activities from the isolates were screened. We have attempted to find a novel source for isolating novel actinobacteria to solve the re-isolation strain problem The new habitat to discoveri the new actinobacterial strains are expected.

2. Materials and Methods

2.1 Sample collection and Isolation of actinobacteria

The ant samples, *Solenopsis geminata*, were collected in Chonburi Province, Thailand. Individual ants were collected in killing jar saturated with acetone. Then, ant samples were washed with sterile water and further used for actinobacterial isolation. Actinobacteria were isolated from ants using standard serial dilution methods. Briefly, five ants were ground using a pestle. Then, 0.5 ml of sterile distilled water was added to the sample and vortex for 1 minute. The sample

solution was spread on humic acid vitamin agar (HV agar) supplemented with nalidixic (50 μg ml^{-1}) acid and cycloheximide (25 μg ml^{-1}) (Hayakawa and Nonomura 1987) and incubated at 30°C for 14 days. The actinobacterial colonies were collected and purified on ISP2 agar (Shirling and Gottlieb 1966). The pure cultures were preserved in glycerol solution (15% w/v) at -20 °C.

2.2 Characterization of actinobacteria

2.2.1 Cultural and Morphological characteristics

Cultural characteristics of the isolates were determined on the culture gown ISP2 agar media at 30 °C for 14 days. The morphology of the isolates was observed using light microscopy (Shirling and Gottlieb 1966).

2.2.2 16S rRNA gene and phylogenetic analysis

DNA was extracted from the actinobacterial mycelia, obtained from the culture grown in yeast-dextrose broth at 30 °C for 4-7 days, using the method described by Tamaoka (1944). The 16S rRNA gene amplification was carried out using primers 20F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1500R (5′-GTTACCTTGTTACGACTT-3′) (Suriyachadkun et al. 2009). PCR products were purified using Gel/PCR kit (Geneaid). Nucleotide sequencing of PCR products was carried out using universal primers (Lane 1994) (Macrogen, Seoul, Korea). BLASTN analysis of the 16S rRNA sequences was performed using EzBioCloud server (https://www.ezbiocloud.net) (Yoon et al. 2017). The sequences of all actinobacterial isolates were aligned with selected sequences obtained from the GenBank/EMBLDDBJ database using CLUSTAL W (Thompson et al. 1997). The maximum-likelihood (ML) (Felsenstein 1981) phylogenetic tree was constructed using MEGA 7.0 (Kumar et al. 2016). All gaps were eliminated before using for tree calculation. The confidence values of tree nodes were evaluated using the bootstrap resampling method based on 1,000 replications (Felsenstein 1985).

2.3 Screening of antimicrobial activity

Antimicrobial activity screening was determined using cross streak method. The actinobacteria were streaked on one side of the ISP2 agar plates and incubated at 30°C for 14 days. Then, the tested microorganisms, including *Bacillus subtilis* ATCC 6633, *Kocuria rhizophila* ATCC 4341, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 10231, were inoculated on the plates by a single streak with 90° angles to the actinobacteria and incubated at 37 °C for 24 hour. Finally, the inhibition area was recorded.

3. Results

Six actinobacteria were isolated from fire ant samples. Based on morphology and 16S rRNA gene analysis, the isolates could be clearly classified into two groups.

Group 1 including SE1, SE2, SE7, and SE13 showed yellowish white to pale orange yellow color on aerial mycelia. The morphological observation revealed that the substrate mycelia of these isolates were fragmented (Table 1). The BLAST search of 16S rRNA gene showed that isolates SE1, SE7 and SE13 were closely related to *Nocardia thailandica* NBRC 100428^T with similarity of 99.86, 99.93% and 100%, respectively. Meanwhile, isolates SE2 was closely related to *Nocardia pseudobrasiliensis* NBRC 108224^T with similarity of 98.00%. Therefore, the member in this group was identified as *Nocardia* (Table 2).

Group II including SE4 and SE11. Both isolates produced long aerial mycelia. The spiral spore chain was observed on SE11 but not SE4. Based on cultural characteristic on ISP2 agar, SE4 produced yellowish white aerial mass and dark grayish yellow substrate mycelia while SE11 produced pale green aerial mass and dark grayish yellow substrate mycelia. The 16S rRNA gene analysis revealed that isolate SE4 and SE11 are closely related to *Streptomyces roseofulvus* $NBRC13194^T$ and *Streptomyces nogalater* JCM 4799^T with similarity of 99.65% and 99.79%, respectively. Therefore, these isolates were members of the genus *Streptomyces*.

Based on Figure 1, isolates SE1, SE2, SE7 and SE13 shared the clade with members of the genus *Nocardia*. Meanwhile, isolates SE4 and SE11 shared the clade with members of

the genus *Streptomyces*. The phylogenetic tree (ML) is clearly confirmed the BLAST results that these actinobacteria were the members of the genus *Streptomyces* and *Nocardia*.

The results of antimicrobial activities screening showed that none of *Nocardia* isolates in this study showed antimicrobial activities against the tested microorganism. In the other hand, both *Streptomyces* SE4 and SE11 showed some antimicrobial activities. SE4 exhibited anti-Gram positive bacteria against *K. rhizophila* ATCC 4341 and *S. aureus* ATCC 25923, but not against *B. subtilis* ATCC 6633,others Gram-negative bacteria and yeast. For SE11, it displayed activity against *Candida albicans* ATCC 10231, but not against any other tested bacteria (Table 2).

4. Discussion

This study is the first report of the culture-dependent isolation of actinobacteria from the fire ant, *Solenopsis geminata*, in Thailand. However, the culture-dependent methods are known to underestimate the true diversity of microbes in the sample. With respect to the culture-independent method, the previous study of the bacterial diversity in *Solenopsis geminata* in the USA by using 16S amplicon 454 pyrosequencing found that the major bacterial group of *So. geminata* worker was Spiroplasma (Ishak et al. 2011). Moreover, the small amount of the actinobacteria members were found without any detection of the *Nocardia* species in those fire ant samples studied in the USA. Thus, the culture-dependent methods should be useful when we have the goal to isolate specific culturable microbes.

In the past decade, several new actinobacterial species, for example, *Microbispora camponoti* (Han et al. 2016), *Micromonospora polyrhachis* (Xiang et al. 2014), *Nocardia lasii* (Liu et al. 2016), *Streptomyces formicae* (Bai et al. 2016), and *Streptomyces polyrhachii* (Yu et a. 2013), were isolated from ant species. In this study, the isolate SE2 showed 98.00% similarity of 16S rRNA gene to those known *Nocardia* species. This value is lower than the value of 98.7% of 16S rRNA gene similarity recommended by Stackebrandt & Ebers for testing the genomic uniqueness of a novel isolates (Stackbrandt and Eber 2006). Hence, SE2 represents the candidate of novel *Nocardia* species status. However, additional studies by using polyphasic taxonomy and the comparative genome analysis are required to prove this hypothesis.

In this study, only two actinobacterial genera were isolated from the target fire ant samples. Typically, the most abundant actinobacteria in the soil are *Streptomyces* species. However, four from six actinobacterial isolates obtained from this study were *Nocardia* spp. Some members of the genus *Nocardia* can cause *Nocardiosis*, the *Nocardia* infection, but some species are non-pathogenic. In the previous report, *N. thailandica* and *N. pseudobrasiliensis* were firstly isolated from clinical specimen (Ruimy et al. 1996; Kageyama et al. 2004). The re-isolation of these two *Nocardia* species including one candidate new *Nocardia* species from fire ant should be mentioned in the role of *Nocardia* associated with ant. Unfortunately, in this investigation, we collected ant samples from only one ant colony. Therefore, the comparison between colonies will be further performed

Both *Streptomyces* isolates obtained in this study exhibited antimicrobial activities against the tested microorganisms. Normally, *S. roseofulvus* and *S. nogalater* have been reported as the producers for frenolicin B (Iwai et al. 1987) and nogalamycin (Ylihonko et al. 1996), respectively,It is possible that the antimicrobial activities of SE4 and SE11are derived from these compounds.

In conclusion, the results from this study confirm that the phrase “new habitat new microbe” still works.

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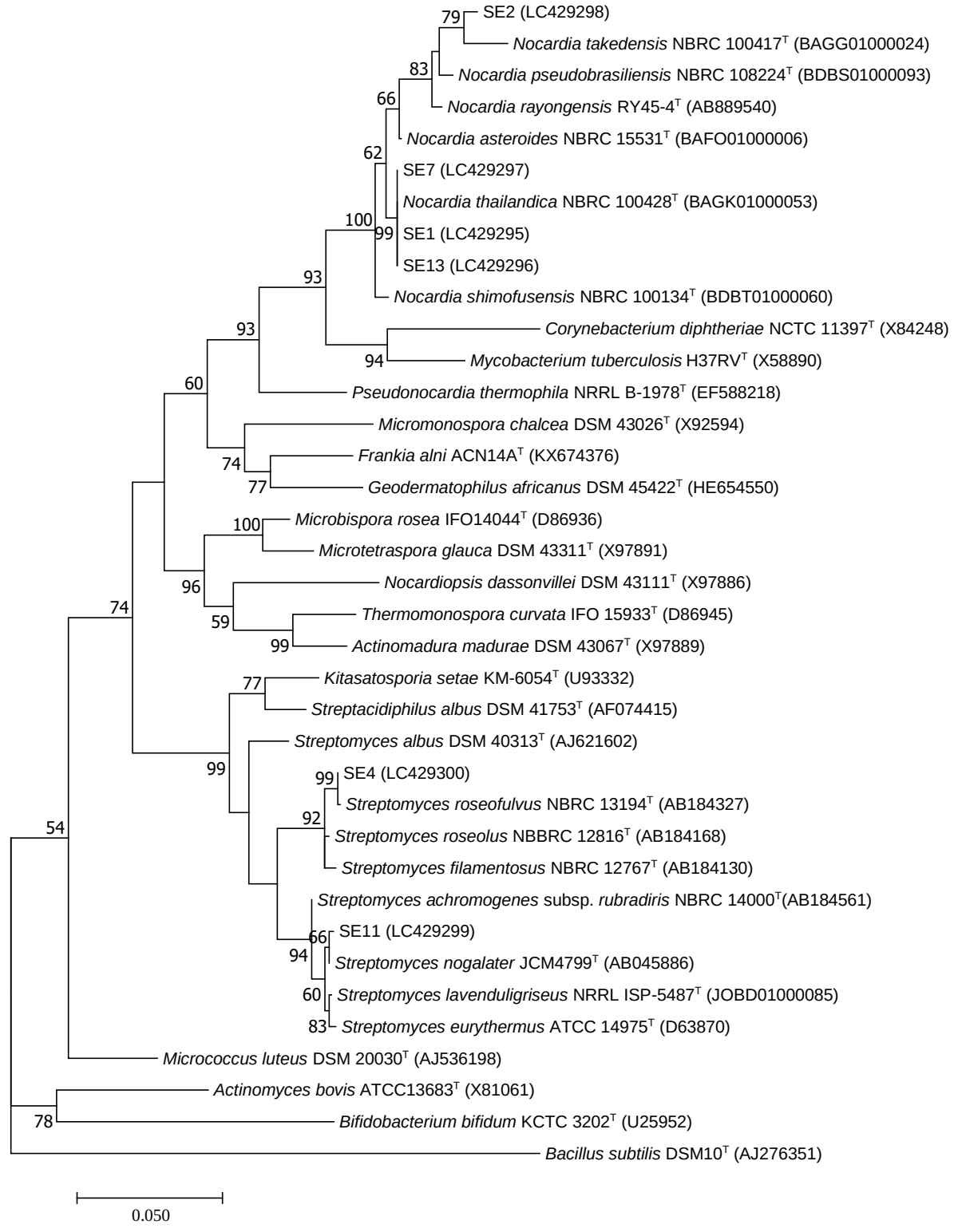

Figure 1. Phylogenetic tree of actinobacterial isolates and type strains/type species of the Phylum Actinobacteria. *Bacillus subtilis* DSM10^T was used as the out group. The number of branch nodes indicate bootstrap percentage derived from 1,000 replication (only values > 50% are shown). Bar, 0.050 substitutions per nucleotide position.

Table 1. Cultural and morphological characteristics of actinobacterial isolates

Isolate no.	Cultural characteristic on ISP2 agar					Morphology
	Growth	Aerial mycelia color	Substrate mycelia color	Colony color	Soluble pigment	
SE1	Good	Pale orange yellow	Light orange yellow	Moderate orange yellow	-	Fragmented substrate mycelia
SE2	Good	Pale orange yellow	Strong orange yellow	Moderate orange yellow	-	Fragmented substrate mycelia
SE4	Good	Yellowish white	Dark grayish yellow	Dark grayish yellow	-	Long aerial mycelia
SE7	Good	Yellowish white	Light orange yellow	Light orange yellow	-	Fragmented substrate mycelia
SE11	Good	Pale green	Dark grayish yellow	Colorless	Moderate olive brown	Spiral spore chain on aerial mycelia
SE13	Good	Yellowish white	Light orange yellow	Strong yellow	-	Fragmented substrate mycelia

Table 2. 16S rRNA gene analysis of the actinobacterial isolates and their antimicrobial activities

Isolate no.	Top-Hit taxon	Similarity (%)	Accession no.	Inhibition zone (mm)*					
				S	B	K	E	P	C
SE1	*Nocardia thailandica* NBRC 100428^T	99.86	LC429295	-	-	-	-	-	-
SE2	*Nocardia pseudobrasiliensis* NBRC 108224^T	98.00	LC429298	-	-	-	-	-	-
SE4	*Streptomyces roseofulvus* NBRC 13194^T	99.65	LC429300	42	-	26	-	-	-
SE7	*Nocardia thailandica* NBRC 100428^T	99.93	LC429297	-	-	-	-	-	-
SE11	*Streptomyces nogalater* JCM 4799^T	99.79	LC429299	-	-	-	-	-	6
SE13	*Nocardia thailandica* NBRC 100428^T	100	LC429296	-	-	-	-	-	-

* -, no activity; S = *Staphylococcus aureus* ATCC 25923; B = *Bacillus subtilis* ATCC 6633; K = *Kocuria rhizophila* ATCC 4341; E = *Escherichia coli* ATCC 25922; P = *Pseudomonas aeruginosa* ATCC 27853; C = *Candida albicans* ATCC 10231

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