TAXONOMY, BIOACTIVITIES, AND SECONDARY METABOLITES OF ENDOPHYTIC ACTINOMYCETES ISOLATED FROM MEDICINAL PLANTS

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อนุกรมวิธาน ฤทธิ์ทางชีวภาพ และสารเมแทบอไลต์ทุติยภูมิของแอคติโนมัยสีทเอนโดไฟท์ที่แยกจากพืชสมุนไพร

น.ส.ณัฐพร คล้ายคลึง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ณัฐพร คล้ายคลึง : อนุกรมวิธาน ฤทธิ์ทางชีวภาพ และสารเมแทบอไลต์ทุติยภูมิของแอคติโนมัยสีทเอนโดไฟท์ที่แยกจากพืชสมุนไพร. (TAXONOMY, BIOACTIVITIES, AND SECONDARY METABOLITES OF ENDOPHYTIC ACTINOMYCETES ISOLATED FROM MEDICINAL PLANTS) อ.ที่ปรึกษาหลัก : ศ. ดร.สมบูรณ์ ธนาศุภวัฒน์, อ.ที่ปรึกษาร่วม : ดร.ปัทมา พิทยขจรวุฒิ

แอคติโนมัยสีทเอนโดไฟต์ 50 สายพันธุ์ ที่แยกจากพืชสมุนไพร 6 ชนิด ในประเทศไทย พิสูจน์เอกลักษณ์ได้เป็น *Micromonospora* (34 สายพันธุ์), *Streptomyces* (8 สายพันธุ์), *Microbispora* (3 สายพันธุ์), *Nonomuraea* (2 สายพันธุ์), *Plantactinospora* (2 สายพันธุ์) และ *Amycolatopsis* (1 สายพันธุ์) โดยอาศัยลักษณะทางฟีโนไทป์ และลำดับนิวคลีโอไทด์บริเวณยีน 16S rRNA พบว่า 15 สายพันธุ์มีฤทธิ์ต้านแบคทีเรียแกรมบวก สายพันธุ์ CR1-09^{T} ที่เสนอเป็นสปีชีส์ใหม่ชื่อ *Microbispora catharanthi* มีฤทธิ์ต้าน *Staphylococcus aureus* ATCC 6538 และ *Kocuria rhizophila* ATCC 9341 *Streptomyces* sp. 3MP-10 และ 3MP-14 เป็นแอคติโนมัยสีทสปีชีส์ใหม่ที่มีฤทธิ์ต้าน *S. aureus* ATCC 6538 *K. rhizophila* ATCC 9341 และ *Bacillus subtilis* ATCC 6633 จากผลการศึกษาความเป็นพิษต่อเซลล์มะเร็งด้วยวิธี MTTพบว่า CR1-09 และ PA1-07 มีความเป็นพิษต่อเซลล์มะเร็ง HepG2 แต่ไม่มีความเป็นพิษต่อเซลล์ Vero ผลการศึกษาสารเมแทบอไลต์ทุติยภูมิเมื่อวิเคราะห์โดยใช้ LC/MS เทียบกับฐานข้อมูลสารใน Dictionary of natural products พบว่าสายพันธุ์ 3MP-10 และ 3MP-14 สร้างสาร ikarugamycin ($[M+H]^+$ = 479.2912 m/z; UV 224, 326 nm) fumaquinone ($[M+H]^+$= 303.1225 m/z; UV 220, 265, 304, 428 nm) และสารใหม่ $[M+Na]^+$ = 600.2576 m/z; UV 204, 246, 344 nm *Nonomuraea* sp. PA1-05 และ PA1-10 สร้าง dilactonmycin ($[M+H]^+$ = 539.3223 m/z; UV 222, 242, 316 nm) และ spithioneine A ($[M+H]^+$ = 492.1833 m/z; UV 250, 274, 336 nm) ตามลำดับ ในขณะที่ *Micromonospora* sp. BL1-08 สร้าง riboflavin ($[M+H]^+$ = 377.1466 m/z; UV 222, 268, 370, 446 nm) นอกจากนี้ยังพบว่าสายพันธุ์ที่แยกได้ทั้งหมดสามารถสร้างสารที่เป็น siderophore ได้แก่ nocardamine ($[M+H]^+$ = 565.1552 m/z; UV 214, 272, 336 nm) ด้วย การศึกษายีนที่เกี่ยวข้องกับการสร้างสารเมแทบอไลต์ทุติยภูมิที่พบบนร่างจีโนมของสายพันธุ์ 3MP-14 พบว่าสัมพันธ์กับการสังเคราะห์สารกลุ่มโพลีคีไทด์และสารกลุ่มนอนไรโบโซมอลเปปไทด์ ซึ่งเป็นยีนที่เกี่ยวกับเอนไซม์ PKS (polyketide synthase) ทั้งชนิดที่ 1, 2 และ 3 คิดเป็นร้อยละ 41.7 และ ยีนที่เกี่ยวกับเอนไซม์ NRPS (nonribosomal peptide synthetase)

สาขาวิชา	เภสัชเคมีและผลิตภัณฑ์ธรรมชาติ	ลายมือชื่อนิสิต .....
ปีการศึกษา	2562	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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Fifty endophytic actinomycetes isolated from six medicinal plants in Thailand were identified as *Micromonospora* (34 strains), *Streptomyces* (8 strains), *Microbispora* (3 strains), *Nonomuraea* (2 strains), *Plantactinospora* (2 strains), and *Amycolatopsis* (1 strain) based on phenotypic characteristics and 16S rRNA gene sequencing. Fifteen strains exhibited antimicrobial activity against Gram-positive bacteria. Strain CR1-09^{T}, proposed as *Microbispora cathalanthi* sp. nov. was active against *Staphylococcus aureus* ATCC 6538 and *Kocuria rhizophila* ATCC 9341. *Streptomyces* sp. 3MP-10 and 3MP-14, novel species were active against *S. aureus* ATCC 6538, *K. rhizophila* ATCC 9341, and *Bacillus subtilis* ATCC 6633. Strains CR1-09^{T} and PA1-07 showed cytotoxicity against HepG2 cells but not Vero cells based on MTT assay. *Streptomyces* sp. 3MP-10 and 3MP-14 produced ikarugamycin ($[M+H]^{+}$ = 479.2912 m/z; UV 224, 326 nm) and fumaquinone ($[M+H]^{+}$ = 303.1225 m/z; UV 220, 236, 304, 428 nm) while 3MP-14 produced a new structural compound, pudicin ($[M+Na]^{+}$ = 600.2576 m/z; UV 204, 246, 344 nm). *Nonomuraea* sp. PA1-05 and PA1-10 produced dilactonmycin ($[M+H]^{+}$ = 539.3223 m/z; UV 222, 242, 316 nm) and spithioneine A ($[M+H]^{+}$ = 492.1833 m/z; UV 250, 274, 336 nm), respectively. *Micromonospora* sp. BL1-08 produced riboflavin ($[M+H]^{+}$ = 377.1466 m/z; UV 222, 268, 370, 446 nm) based on liquid chromatography-mass spectrometry (LC/MS) with the dictionary of natural products. Moreover, all strains produced a siderophore as nocardamine ($[M+H]^{+}$ = 565.1552 m/z; UV 214, 272, 336 nm). The secondary metabolite biosynthetic gene clusters (BGCs) identified in the draft genome of strain 3MP-14 were related to the biosynthesis of polyketides by polyketide synthase (PKS-I, II, and III) as 41.7 % of all putative BGCs and non-ribosomal peptides by non-ribosomal peptide synthetase (NRPS).

Field of Study:	Pharmaceutical Chemistry and Natural Products	Student's Signature .....
Academic Year:	2019	Advisor's Signature .....
		Co-advisor's Signature .....

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CHAPTER I

INTRODUCTION

Actinomycetes are a group of Gram-positive filamentous bacteria with high guanine and cytosine contents in their chromosomal DNA. Most of them form mycelium and asexual spore on aerial or substrate mycelia (Lechevalier and Lechevalier, 1967). Actinomycetes inhabit in various environments such as soil, aquatic sediment, marine sediment, extreme environment, sponge, insect, lichen, and plant tissue. They decompose the organic matters or humus in the soil as saprophytes and draw the nutrients as nitrogen-fixing from rhizospheric soil. In addition, actinomycetes, especially *Streptomyces*, are major groups of microorganisms that produce many valuable bioactive secondary metabolites in clinical use, pharmaceutical industry, agriculture, and environment such as antibiotics, antifungals, antiprotozoals, antimalarials, anti-cancers, plant growth hormones, and enzymes (Caruso *et al.*, 2000; Strobel and Daisy, 2003; Bérdy, 2012; Takahashi and Nakashima, 2018; Ding *et al.*, 2019). The actinomycetes produced various secondary metabolites associated with one or many large secondary metabolism biosynthetic gene clusters (smBGCs) on their genomic DNA such as polyketide synthase genes (PKS; PKS I, PKS II and PKS III) and nonribosomal peptide synthetase genes (NRPS) (Janso and Carter, 2010; Doroghazi and Metcalf, 2013; Singh *et al.*, 2017; Jackson *et al.*, 2018; Genilloud, 2018).

Numerous studies exhibited that plants are potential resources for the discovery of new actinomycetes which leads to discover the new bioactive compounds (Matsumoto and Takahashi, 2017). Recently, the endophytic actinomycetes were discovered as the new actinomycetes and the new compound producers such as *Microbispora camponoti* isolated from the cuticle of *Camponotus japonicus* (Han *et al.,* 2016), *Plantactinospora soyae* isolated from soybean root (Guo *et al.*, 2016b), *Streptomyces roietensis* sp. nov., isolated from the stem of *Oryza sativa* KDML 105 (Kaewkla and Franco, 2017), *Micromonospora parathelypteridis* isolated from the root of *Parathelypteris beddomei* (Zhao *et al.*, 2017), *Nonomuraea stahlianthi* isolated from the stem of *Stahlianthus campanulatus* (Niemhom *et al.,* 2017), etc. Further, Thailand is one of the tropical countries that has been extremely rich in plant species and ethnic groups for various traditional medicinal knowledge so many medicinal plants have widely local ethnobotanical use in alternative medicine.

Thus, this study aims to study and report the biodiversity of endophytic actinomycetes isolated from medicinal plants in Thailand, their antimicrobial and anticancer activities, chemical structure elucidation and the related genes with their secondary metabolites.

The main objectives of this investigation are as follows:

1. To isolate and identify the endophytic actinomycetes from medicinal plants

2. To screen antimicrobial activity and cytotoxicity of the crude extracts

3. To isolate and characterize chemical structures of secondary metabolites of the selected strains related to their secondary metabolite genes

CHAPTER II

LITERATURE REVIEW

Actinomycetes are Gram-positive filamentous bacteria with high guanine and cytosine contents in their genomic DNA (more than 55 mol %) that belong to the class *Actinobacteria*. Most of them are aerobic or facultatively anaerobic bacteria that form branching filaments (diameter ranges from 0.4 to 1.2 μm) and asexual spores on aerial or substrate mycelia (Lechevalier and Lechevalier, 1967).

The life cycle of actinomycete begins with spore germination into the substrate. The colony responses to the low nutrition and other stress signals that produced the reproductive aerial hyphae and then differentiated to spores. The secondary metabolites were produced while hyphae differentiation, as shown in Figure 2.1.

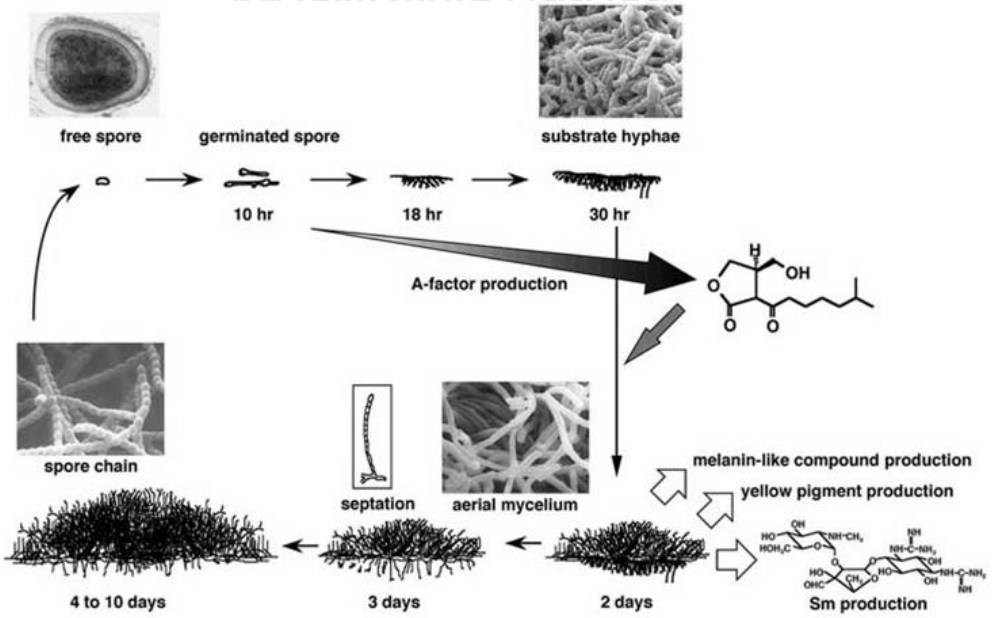

Figure 2.1 The life cycle of actinomycetes (Horinouchi and Beppu, 2007)

The actinomycetes divide into 2 major groups as *Streptomyces* and non-*Streptomyces* (rare-actinomycetes). They inhabit in various environments such as soil, aquatic sediment, marine sediment, extreme environment, sponge, insect, lichen, and plant tissue. In general, they are saprophytes that decompose organic matter for growth. They contribute to the degradation of lignin, organic matter and chitin, the formation and stabilization of humus, and the production of valuable bioactive secondary metabolites. Many studies revealed that they are usually harmless and particularly beneficial microorganisms in the pharmaceutical industry, agriculture and environment such as antibiotics, antifungals, antimalarials, anti-cancers, enzymes, immunosuppressants, pesticides, and plant growth hormones (Bérdy, 2005; Bérdy, 2012; Genilloud, 2017; Takahashi and Nakashima, 2018). The actinomycetes are the high potential resource of the discovery of new natural products and many valuable bioactive secondary metabolites. Almost 35,000 microbial bioactive secondary metabolites as almost 50 % of all microbial bioactive compounds are produced from actinomycetes, especially from the genus *Streptomyces* (Bérdy, 2005; Bérdy, 2012). However, some actinomycetes are human, animal or plant pathogens such as various *Nocardia species* causing an opportunistic infection (Palmieri *et al.*, 2014), *Streptomyces scabies* causing scab disease in potatoes (Emmert and Handelsman, 1999), etc.

2.1 Taxonomy of actinomycetes

Actinomycetes belong to the phylum *Actinobacteria* and class *Actinobacteria*, including 19 orders, 50 families, and 221 genera as shown in Figure 2.2 and described some genus in the below (Ludwig *et al.*, 2012; Bergey *et al.*, 2012; Barka *et al.*, 2016).

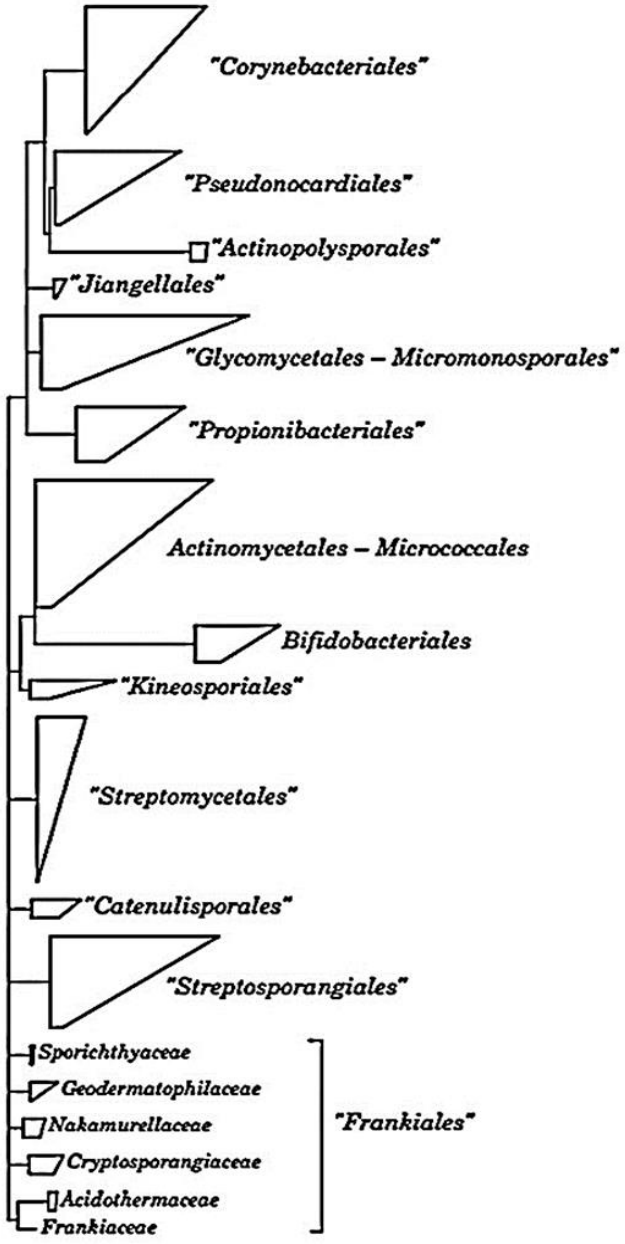

Figure 2.2 The road map of orders of class Actinobacteria (Ludwig *et al.,* 2012)

Streptomyces

The genus *Streptomyces*, first proposed in 1943 (Waksman and Henrici, 1943), belongs to the family *Streptomycetaceae*. The type species of this genus is *Streptomyces albus*. They form smooth, warty, rugose, spiny or hairy spore chains on an extensively branched substrate and aerial mycelia. The differentiated chemotaxonomic characteristics between the genus *Streptomyces* and the other genera belonging to the family *Streptomycetaeae* are shown in Table 2. 1. Their chemotaxonomic characteristics consist of *LL*- diaminopimelic acid in the cell wall peptidoglycan but lack diagnostic sugars in whole- cell hydrolysates (Cell wall chemotype I) (Lechevalier and Lechevalier, 1970). Predominant menaquinones are MK-9(H_6) and MK-9(H_8). The phospholipid profile contains phosphatidylethanolamine (PE) as a diagnostic phospholipid (Type PII phospholipid pattern) (Lechevalier *et al.*, 1977). The mycolic acid is absence. At the time of writing, this genus comprises 854 species and 38 subspecies with validly published names in the *List of Prokaryotic names* as distributed in soil, freshwater sediment, marine sediment, extreme environment, insect, lichen, and plant (www. bacterio. net/ streptomyces. html) . Recently, new *Streptomyces* are reported such as *S. amphotericinicus* isolated from ant (Cao *et al.*, 2017), *S. krungchingensis* isolated from soil (Sripreechasak *et al.*, 2017b), *S. roietensis* isolated from stem of jasmine rice (Kaewkla and Franco, 2017), *S. kalpinensis* isolated from marine (Ma *et al.*, 2017), etc.

Table 2.1 The differential characteristics of the genus *Streptomyces* with others belonging to the family *Streptomycetaeae*.

Genus	G+C (mol%	Fatty acid Type	Major menaquinone (s)	Polar lipid Type	Whole-cell sugar Type	DAP Type
Streptomyces	69-78	2	MK-9 (H_6, H_8)	PII	-	*LL*-DAP
Kitasatospora	69-73	2	MK-9 (H_6, H_8)	PII	C, E	*LL/meso*-DAP
Streptacidiphilus	70-72	2	MK-9 (H_6, H_8)	PII	E	*LL*-DAP

Micromonospora

The genus *Micromonospora*, first proposed in 1923 (Ørskov, 1923), belongs to the family *Micromonosporaceae*. *Micromonospora chalcea* is the type species of this genus. They form single conidial spore on substrate mycelium but lack aerial mycelium. The differentiated characteristics between the genus *Micromonospora* and the other genera in the family *Micromonosporaceae* are shown in Table 2.2. At the time of writing, this genus comprises 84 species and 7 subspecies with validly published names (www.bacterio.net/micromonospora.html) in the *List of Prokaryotic names* as distributed in soil (mangrove, sandy, rhizosphere soil), nickel-mining site, limestone quarry, marine (sea sediment, sea urchin), volcanic sediment, insect, and plant. Recently, new *Micromonospora* was reported such as *M. yasonensis* isolated from Black Sea sediment (Veyisoglu *et al.*, 2016), *M. fulva* isolated from soil (Lee and Whang, 2017), *M. parathelypteridis* isolated from the root of *Parathelypteris beddomei* (Zhao *et al.*, 2017), etc.

Plantactinospora

The genus *Plantactinospora,* first proposed in 2009 (Qin *et al.*, 2009b), belongs to the family *Micromonosporaceae*. *Plantactinospora mayteni* is the type species of this genus. They form smooth single or cluster spores on an extensively branching substrate mycelium. The differentiated characteristics between the genus *Plantactinospora* and the other genera belonging to the family *Micromonosporaceae* are shown in Table 2.2. At the time of writing, this genus comprises 6 species with validly published names in the *List of Prokaryotic names* distributed in soil and plant (www. bacterio. net/nonomuraea. html). Recently, new *Plantactinospora* is reported such as *P. soyae* isolated from soybean root (Guo *et al.*, 2016b).

Table 2.2 The differential characteristics of the genus *Micromonospora* with others belonging to the family *Micromonosporaceae*

Genus	G+C (mol%)	Fatty acid Type	Major menaquinone (s)	Polar lipid Type	Whole-cell sugar Type	DAP Type
Micromonospora	71-73	3b	MK-9(H_4,H_6), MK-10(H_4,H_6)	PII	D	*meso*-DAP
Actinoplanes	72-73	2d	MK-9(H_4), MK -10 (H_4,H_6)	PII	D	*meso*-DAP
Dactylosporangium	72-73	3b	MK-9(H_4,H_6, H_8)	PII	D	*meso*-DAP
Polymorphospora	70-71	2a	MK-9(H_4,H_6), MK-10(H_4,H_6)	PII	D	*meso*-DAP
Planactinospora	70-73	2d	MK-10(H_4,H_6,H_8)	PII	D	*meso*-DAP
Salinispora	70-73	3a	MK-10(H_4)	PII	D,A	*meso*-DAP
Spirilliplanes	69	2d	MK-10(H_4)	PII	D,A	*meso*-DAP
Verrucosispora	70	2b	MK-9(H_4)	PII	D	*meso*-DAP
Virgisporangium	71-72	2d	MK-10(H_4,H_6)	PII	D,A	*meso*-DAP

Microbispora

The genus *Microbispora*, first proposed in 1957 (Nonomura and Ohara, 1957), belongs to the family *Streptosporangiaceae*. *Microbispora rosea* is the type species of this genus. They form smooth ellipsoidal to circular paired spores on aerial mycelium. The differentiated characteristics between the genus *Microbispora* and the other genera belonging to the family *Streptosporangiaceae* are shown in Table 2.3. At the time of writing, the genus *Microbispora* comprises 10 species and 2 subspecies with validly published names as *M. amethystogenes*, *M. siamensis*, *M. bryophytorum*, *M. camponoti*, *M. corallina*, *M. hainanensis*, *M. soli*, *M. rosea* with two subspecies i.e. *M. rosea* subsp. *rosea* (containing five strains formerly reclassified as *M. chromogenes*, *M. diastatica*, *M. indica*, *M. karnatakensis*, and *M. parva*) and *M. rosea* subsp. *aerata* (containing two strains formerly reclassified as *M. thermodiastatica* and *M. thermorosea*), *M. triticiradicis*, and *M. tritici* (www.bacterio.net/microbispora.html) as distributed in soil and plant. Recently, new *Microbispora* is reported such as *M. camponoti* isolated from the cuticle of *Camponotus japonicus* (Han *et al.*, 2016).

Nonomuraea

The genus *Nonomuraea*, first established in 1998 (Zhang *et al.*, 1998) and subsequently corrected the spelling to *Nonomuraea* in 1999 (Chiba *et al.*, 1999), belongs to the family *Streptosporangiaceae*. *Nonomuraea pusilla* is the type species of this genus. They form straight, hooked or spiral spore chains that their surfaces are

smooth, spiny or warty on aerial mycelium and extensively branching substrate. The differentiated characteristics between the genus *Nonomuraea* and the other genera belonging to the family *Streptosporangiaceae* are shown in Table 2.3. At the time of writing, this genus comprises 51 species and 2 subspecies with validly published names in the *List of Prokaryotic names* distributed in soil, mangrove, limestone, and plant (www.bacterio.net/nonomuraea.html). Recently, new *Nonomuraea* was reported such as *N. rhodomycinica* isolated from peat swamp soil (Sripreechasak *et al.*, 2017a), *N. stahlianthi* isolated from the stem of *Stahlianthus campanulatus* (Niemhom *et al.*, 2017), etc.

Table 2.3 The differential characteristics of the genus belonging to the family *Streptosporangeaceae*.

Genus	G+C (mol%)	Fatty acid Type	Major menaquinone(s)	Polar lipid Type	Whole-cell sugar Type	DAP Type
Streptosporangium	69-71	3c	MK-9(H_2, H_4)	IV	B	*meso*-DAP
Microbispora	71-73	3c	MK-9(H_0, H_2, H_4)	IV	B, C	*meso*-DAP
Microtetraspora	69-71	3c	MK-9(H_2, H_4, H_6)	IV	B, C	*meso*-DAP
Nonomuraea	64-69	3c	MK-9(H_0, H_2, H_4)	IV	B, C	*meso*-DAP
Planotetraspora	71	3d	MK-9(H_2, H_4)	IV	B	*meso*-DAP

2.2 Identification methods of actinomycetes

A polyphasic approach, the characterization described in identification key of bacteria, including the phenotypic characteristics (based on the cultural, morphological, physiological, and biochemical characteristics), the chemotaxonomic analyses (based on the cell wall and cell membrane composition, including diaminopimelic acid, whole-cell sugars, cellular fatty acids, phospholipids, and menaquinone), and the genotypic characteristics (based on the 16S rRNA gene sequence, multilocus sequence, genome sequence, phylogenetic, and phylogenomic pattern) have been used for classification, identification, and taxonomic position of the genus and species levels of the actinomycetes as described in the below (Stackebrandt and Schumann, 2006; Prakash *et al.*, 2007; Das *et al.*, 2014; Nouioui *et al.*, 2018).

2.2.1. Phenotypic characteristics

Phenotypic characteristics comprise morphological, cultural, biochemical and physiological characteristics that use the characterization methods (Shirling and Gottlieb, 1966). The morphological characteristics concern about spore germination that forms substrate mycelium, aerial mycelium, and spore. For spore formation, the aerial mycelium develops to a spore that the shape, surface, arrangement of spore chain is the key characters for actinomycete identification into the genus level. The cultural, biochemical and physiological characteristics as the color of colony (substrate and aerial mycelium), soluble pigment production, carbon, and nitrogen utilization, pH, temperature, and NaCl tolerance growth, nitrate reduction, gelatin liquefaction, skim

milk peptonization and coagulation, starch hydrolysis, enzyme activities, and acid production are various characteristics in the species level (Arai *et al.*, 1975).

2.2.2. Chemotaxonomic characteristics

Chemotaxonomic characteristics classify the genus level of actinomycetes that determine the components of cell wall and cell membrane, including whole-cell hydrolysate analysis as diaminopimelic acid and whole-cell sugar (Staneck and Roberts, 1974), cellular fatty acid (Sasser, 1990), phospholipids (Minnikin *et al.*, 1984), and menaquinone (Collins *et al.*, 1977) as described below.

Cell wall composition

Peptidoglycan in bacterial cell wall is polymer of *N*-acetylglucosamine acid (NAG), *N*-acetylmuramic acid (NAM) and peptide moiety linked chain that three parts (based on the variation of peptide moiety, the types of diaminopimelic acid isomer, and the *N*-acyl types of muramic acid) have been used for the bacterial classification as shown in Figure 2.3 and Table 2.4 (Lechevalier and Lechevalier, 1970). The 2,6-diaminopimelic acid (DAP; A_2pm) is the majority peptide linked chain that has three stereoisomers (*LL*-, *DD*-, and *meso*-DAP), hydroxyl diaminopimelic acid (3-OH-DAP), and 3,4-dihydroxyl diaminopimelic acid (3,4-OH-DAP). The type of diaminopimelic acid in cell wall peptidoglycan as the key to chemotaxonomic analysis is one of the most important characters for classifying the genus *Streptomyces* and other rare actinomycete genera. For the actinomycetes, *LL*-DAP always found in the hydrolyzed cell wall of *Streptomyces* while *meso*-DAP, 3-OH-DAP, 3-4-OH-DAP, or the combination

of isomers found in the other rare actinomycetes (Matsumoto *et al.*, 2014). DAP isomers are analyzed by cellulose TLC (Staneck and Roberts, 1974).

Whole-cell sugar composition is used to classify the sporulated actinomycetes which have *meso*-DAP in the cell wall, as shown in Table 2.5 (Lechevalier and Lechevalier, 1970). Whole-cell sugars are analyzed by cellulose TLC (Staneck and Roberts, 1974) or HPLC (Mikami and Ishida, 1983).

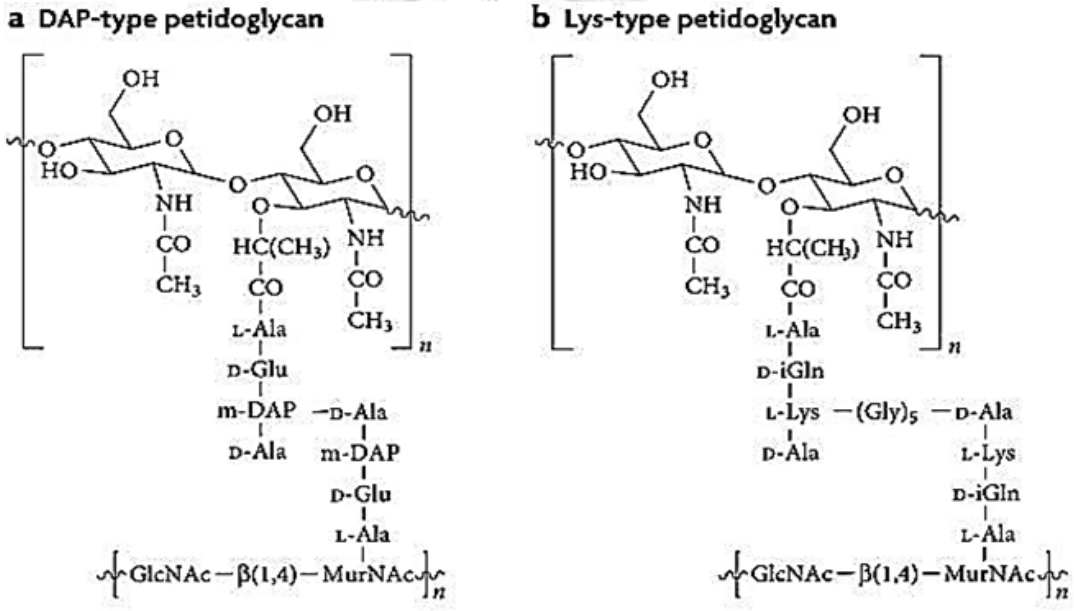

Figure 2.3 The structure of peptidoglycans contained in bacterial cell wall (Royet and Dziarski, 2007)

Table 2.4 The cell wall chemotypes of the actinomycetes.
(Lechevalier and Lechevalier, 1970)

Type	DAB*	lysine	ornithine	aspartic acid	glycine	*meso*- DAP	LL-DAP	arabinose	galactose
I	-	-	-	-	+	-	+	-	-
II	-	-	-	-	+	+**	-	-	-
II	-	-	-	-	-	+	-	-	-
I	-	-	-	-	-	+	-	+	+
V	-	+	+	-	*	-	-	-	-
V	-	+	-	+	*	-	-	-	-
VI	+	+	-	+	*	-	-	-	-
VII	-	-	-	+	*	-	-	-	-

*, Glycine is variably present in these groups; **, hydroxyl DAP may be present +, present; -, absent

Table 2.5 The whole-cell sugar patterns of the actinomycetes.
(Lechevalier and Lechevalier, 1970)

Patterns	Diagnostic sugars					Genera related
	Arabinose	Fucose	Galactose	Madurose	Xylose	
A	+	-	+	-	-	*Nocardia, Mycobacterium, Pseudonocardia, Micropolyspora*
B	-	-	-	+	-	*Actinomadura, Microbispora, Streptosporangium*
C	No diagnostic sugar					*Nocardiopsis, Saccharothrix*
D	+	-	-	-	+	*Micromonospora, Actinoplanes, Dactylosporangium*
E	-	+	-	-	-	*Frankia*

Cell membrane composition

Isoprenoid quinones in the bacterial cell membranes concerned the electron transport system of the cell respiration and oxidative phosphorylation that are menaquinone (2-methyl-3-polyprenyl-I,4-naphthoquinones). The number of isoprene units and the degree of hydrogenation of double bonds in the isoprenyl chain are the keys to bacterial identification as shown in Figure 2.4 (Komagata and Suzuki, 1988). Menaquinones are the predominant isoprenoid quinones of the actinomycete cell membranes as analyzed by HPLC (Collins *et al.*, 1977).

Phospholipids (Polar lipids) are in the lipid bilayer of bacterial cell membranes that relate to permeability and regulation at the membrane. The phospholipid patterns of actinomycetes relate to the genus level as shown in Table 2.6 and Table 2.7 (Lechevalier *et al.*, 1977; Busse *et al.*, 1996). Phosphatidylinositol-mannosides (PIMs), acyl phosphatidylglycerol (APG), phosphatidylethanolamine (PE), methylphosphatidyl-ethanolamine (PME), glycophospholipids, phosphatidylcholine (PC), and unidentified phospholipids containing glucosamine (GluNU) are the phospholipid markers to be useful for the actinomycete identification while diphosphatidylglycerols (DPG) and phosphatidylinositol (PI) are widely distributed in the member of actinomycetes that cannot be useful for classification and identification (Komagata and Suzuki, 1988). Polar lipid composition is analyzed by two-dimensional TLC (Minnikin *et al.*, 1984).

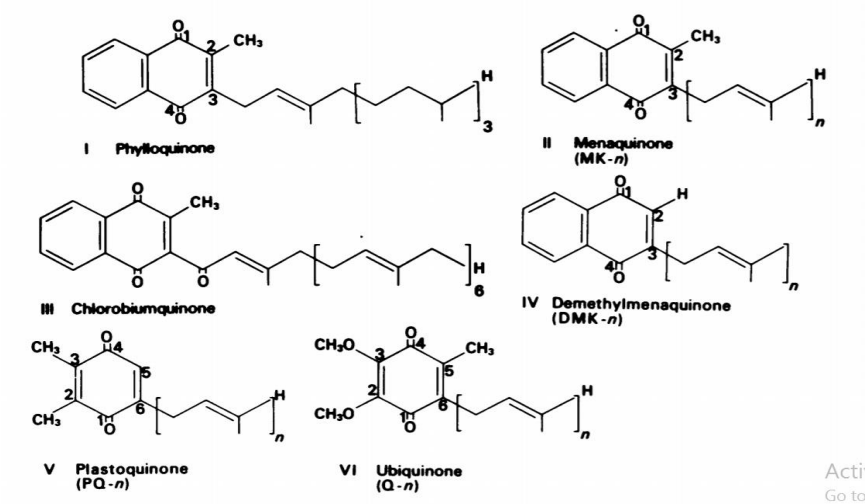

Figure 2.4 The structure of isoprenoid quinones (Busse *et al.*, 1996).

Table 2.6 The pattern types of phospholipids

Types	Phospholipids								
	PIMs	PI	PC	PG	PE	PME	GluNu	APG	DPG
I	+	+	-	v	-	-	-	v	v
II	+	+	-	v	+	-	-	v	+
III	v	+	+	v	v	+	-	v	v
IV	ND	+	-	-	v	v	+	-	+
V	ND	+	-	+	v	-	+	v	+

+, positive; -, absent; v, variable; ND, no data. (Lechevalier *et al.*, 1977)

Table 2.7 The phospholipid pattern types related to the genera of actinomycetes

Types	Phospholipids	Genera related
I	No nitrogenous phospholipids	*Actinomadura, Microtetraspora, Nocardioides*
II	Phosphatidylethanolamine	*Micromonospora, Actinoplanes, Nocardia, Streptomyces, Amycolatopsis*
III	Phosphatidylcholine	*Nocardia, Nocardiopsis, Pseudonocardia, Micropolyspora, Saccharopolyspora*
IV	GluNu*	*Actinomadura, Microbispora, Nonomuraea, Streptosporangium*
V	GluNU and phosphatidyl glycerol	*Oerskovia, Promicromonospora*

*, Unidentified phospholipids containing glucosamine (Lechevalier *et al.*, 1977)

Cellular fatty acids are long-chain fatty acids in the lipid bilayer of the bacterial cell membrane. The fatty acids divide into the 12-20 carbon atoms and the 20-80 carbon atoms (e.g. mycolic acid). The length of the carbon chain, the branching location of methyl groups (*iso*- or *anteiso*-), and the position of the double bond are used for the key to bacterial characterization (Minnikin, 1980). The cellular fatty acids are analyzed by GC (Sasser, 1990). Mycolic acid is analyzed by TLC (Tomiyasu, 1982).

2.2.3. Genotypic characteristics

Chromosomal DNA in most bacteria is a circular-double helix strand of polynucleotides that compose of four nucleotide bases with hydrogen bond as adenine (A) paired with thymine (T) and cytosine (C) paired with guanine (G). The genotypic characteristics assess based on the DNA based composition, 16S rRNA gene sequence, multilocus sequence, and genomic sequence to indicate the distinctive phylogenetic and phylogenomic relationships with the comparative microorganisms as classical (wet-lab) and bioinformatics (*in-silico* genome-to-genome comparison) methods. Recently, many genomic analysis tools of genetics, genomics, and computer sciences have changed the way of bacterial identification, their putative genes as secondary metabolite genes or virulent genes, and their natural product discovery by advances from the PCR-based capillary sequencing to whole genome-based next-generation sequencing (WGS) (Crusoe *et al.*, 2015; Genilloud, 2018; Caputo *et al.*, 2019).

DNA based composition

Chromosomal DNA of bacteria composes of four nucleotide bases as adenine (A), thymine (T), cytosine (C), and guanine (G). The DNA base composition is analyzed by HPLC and report to the calculated G+C content (% mol) as the molar ratio of [(G+C)/(G+C+A+T) x100]. The difference in DNA base composition can be assumed to the different of the genome and belong to the different species so the genomic G+C content is useful for the classification and identification of the genus and species level of the actinomycetes (Tamaoka, 1994).

16S rRNA gene sequence

16S ribosomal RNA (1,500 bp) is a part of the 30S small subunit of the bacterial ribosome. The 16S rRNA gene contained sufficient phylogenetic information as highly conserved regions of all bacteria that slowly evolved, therefore, the phylogenetic position of bacterial classification as the key to the identification in the genus level (Lane, 1991). The 16S rRNA gene sequence analysis based on Sanger sequencing or *in silico* method, compared the percent of pairwise sequence similarity with closely related species and constructed the phylogenetic tree, depicts all possible topological lines of evolutionary descent of different species by the Basic Local Alignment Search Tool (BLAST) searches and the pairwise global sequence alignments using EzBioCloud server (Kim *et al.*, 2012; Vetrovsky and Baldrian, 2013; Yoon *et al.*, 2017).

DNA-DNA hybridization

The classical DNA-DNA hybridization is the method for bacterial classification that DNA-DNA relatedness values are the key parameter in the species delineation. The values of DNA-DNA relatedness are determined using colorimetry. A species as a group of strains including the type strain has the values of at least 70% total genome DNA–DNA hybridization (Ezaki *et al.*, 1989).

The digital DNA–DNA hybridization (dDDH) was carried out using an Illumina Miseq platform (Illumina) by using 2x250 bp paired-end reads and then the assembly was accomplished using SPAdes 3.12 (Meier-Kolthoff *et al.*, 2013; Bankevich *et al.*, 2012). Moreover, the average nucleotide identity (ANI) values were calculated with pairwise genome alignment of the draft genome sequence of related type strain by using the ANI-BLAST (ANIb) and ANI-MUMmer (ANIm) algorithms implemented within the JSpeciesWS web service (Richter and Rosselló-Móra, 2009; Richter *et al.*, 2016).

2.3 Distribution of endophytic actinomycetes

Many previous studies showed that plants are potential resources of new actinomycetes and new bioactive compounds (Matsumoto and Takahashi, 2017). The host plants as special pressure and selective environment have led to endophytes that evolve to produce some new enzymes for adapting to their hosts which enable the biosynthesis of new secondary metabolites, especially the medicinal plants (Zhang *et al.*, 2006; Gunatilaka, 2006; Wilson and Brimble, 2009). Besides, there are nearly

300,000 species of plants on the earth that each individual plant has many endophytes (Strobel and Daisy, 2003). Thailand is one of the tropical countries that has been extremely rich in plant species and ethnic groups for various traditional medicinal knowledge so many medicinal plants have widely local ethnobotanical use in alternative medicine. Plants generally contain several endophytic actinomycetes that produce bioactive secondary metabolites similar with the compounds produced from host that indicated in co-evolution or genetic transfer or genetic recombination from the host plant into endophytes. This phenomenal supported in previous study that endophytic *Kitasatospora* sp. produced paclitaxel, an anticancer compound produced by the plant *Taxus brevivolia*, isolated from *Taxus baccata* plants (Caruso *et al.*, 2000).

The actinomycetes live within plant tissues as endosymbiosis that are called endophytic actinomycetes, but they are not the phytopathogens (Hasegawa *et al.*, 2006). Numerous novel endophytic actinomycetes and their bioactive secondary metabolites have been discovered since the genus *Frankia* was isolated from non-legume root nodules, indicating that actinomycetes were closely associated with plants in early 1886 (Dinesh *et al.*, 2017; Singh and Dubey, 2018). The common genera of endophytic actinomycetes are *Streptomyces*, *Streptosporangium, Pseudonocardia*, *Nocardiopsis Micromonospora,* and *Microbispora*. Many previous studies exhibited that plants, especially medicinal plants, are potential resources for the discovery of new actinomycetes which leads to discover the new bioactive compounds as shown in Figure 2.5 (Qin *et al.*, 2009a; Madhurama *et al.*, 2014; Matsumoto and Takahashi, 2017).

Recently, the endophytic actinomycetes were discovered to be the new species such as *Streptomyces roietensis* sp. nov., isolated from stem of *Oryza sativa* KDML 105 (Kaewkla and Franco, 2017), *Micromonospora globbae* sp. nov., isolated from roots of *Globba winitii* C. H. Wright (Kuncharoen *et al.*, 2018), *Microbispora triticiradicis* sp. nov. isolated from a root of wheat (Chuanyu Han *et al.*, 2018), etc. The diversity of the new endophytic actinomycete species in previous studies was reported as shown in Table 2.8.

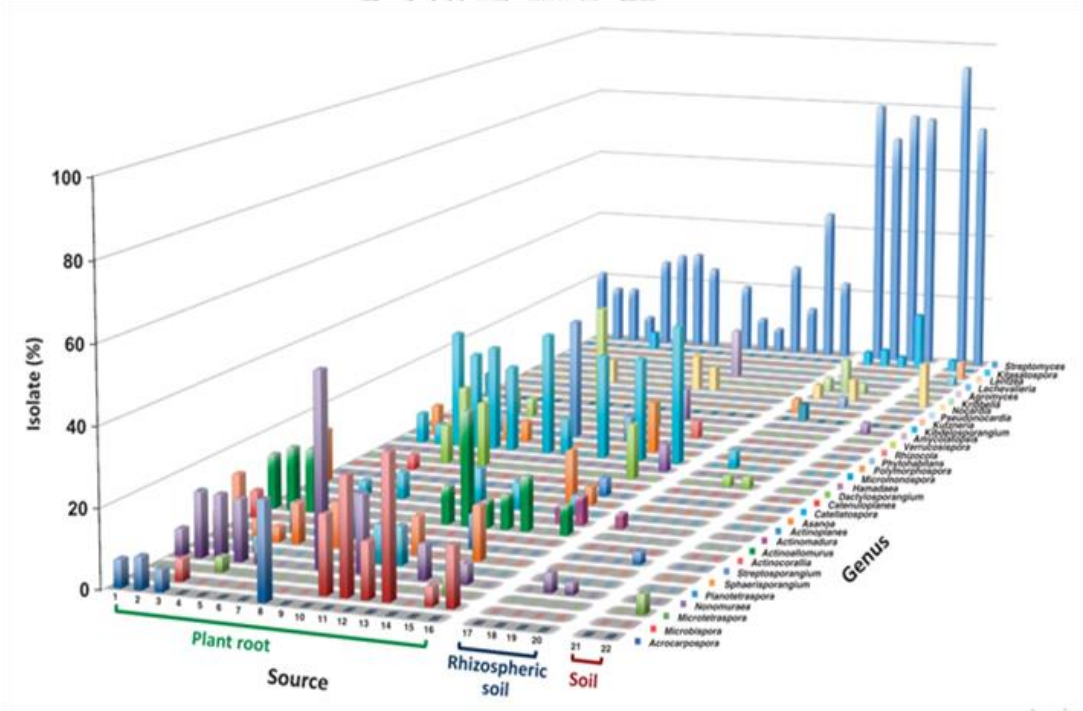

Figure 2.5 The diversity of actinomycetes isolated from plants and soils. (Matsumoto and Takahashi, 2017)

Table 2.8 Distribution of endophytic actinomycetes.

Strain	Host plants	References
Amycolatopsis stemonae	*Stemona* sp.	Klykleung *et al.*, 2015
Microbispora bryophytorum	*Bryophyta* (moss)	Li *et al.*, 2015
Micromonospora endophytica	*Oryza sativa*	Thanaboripat *et al.*, 2015
Micromonospora costi	*Costus speciosus*	Thawai, 2015
Streptomyces oryzae	*Oryza sativa*	Mingma *et al.*, 2015
Actinorhabdospora filicis	*Pteridophyta* (fern)	Mingma *et al.*, 2016
Micromonospora lycii	*Lycium chinense* Mill	Zhao *et al.*, 2016
Streptomyces polygonati	*Polygonatum odoratum*	Guo *et al.*, 2016a
Streptomyces pini	*Pinus sylvestris*	Madhaiyan *et al.*, 2016
Streptomyces phyllanthi	*Phyllanthus amarus*	Klykleung *et al.*, 2016
Kibdelosporangium kanagawaense		
K. rhizosphaerae	*Ophiopogon japonicus*	Mingma *et al.*, 2017
K. rhizovicinum		
Streptomyces roietensis	*Oryza sativa* KDML105	Kaewkla and Franco, 2017
Microbispora triticiradicis	*Triticum aestivum*	C. Han *et al.*, 2018
Micromonospora globbae	*Globba winitii* C.H. Wright	Kuncharoen *et al.*, 2018
Streptomyces populi	*Populus adenopoda*	Wang *et al.*, 2018
Streptomyces geranii	*Geranium carolinianum*	Li *et al.*, 2018
Microbispora tritici	*Triticum aestivum*	Han *et al.*, 2019
Microbispora catharanthi	*Catharanthus roseus*	Klykleung *et al.*, 2019a
Nonomuraea phyllanthi	*Phyllanthus amarus*	Klykleung *et al.*, 2019b

2.4 Secondary metabolite genes of actinomycetes

Nowadays in new drug discovery, the microbiologists are increasingly relying on sequencing genomes of a wide variety of microbes for rapidly and reliably pinpointing all the potential secondary metabolite gene clusters in the genome that relate to producing the secondary metabolites. All secondary metabolites of actinomycetes are genetically encoded by large secondary metabolism biosynthetic gene clusters (smBGCs), which are modular and can be highly repetitive. The relationship between secondary metabolite gene clusters and genome has been extremely challenging that has to use the specialized bioinformatic tools and necessary resources. Although many studies revealed that the actinomycetes have many natural product gene clusters on their genomes, it is difficult to understand relate these genes to their products.

The antiSMASH (Antibiotics and Secondary Metabolite Analysis Shell) algorithm is the first comprehensive pipeline capable of identifying biosynthetic loci covering the whole range of known secondary metabolite compound classes (polyketides, non-ribosomal peptides, terpenes, aminoglycosides, aminocoumarins, indolocarbazoles, lantibiotics, bacteriocins, nucleosides, butyrolactones, melanins, beta-lactams, siderophores, and others). It aligns the identified regions at the gene cluster level to their nearest relatives from a database containing all other known gene clusters and integrates or cross-links all previously available secondary metabolite specific gene analysis methods in one interactive view. Large scale comparisons of these clusters are difficult to perform due to the presence of highly similar repeated domains in the

most common biosynthetic machinery: polyketide synthases (PKSs; PKSI, PKSII, PKSIII) and nonribosomal peptide synthetases (NRPSs) (Dholakiya *et al.*, 2017). Recently, many studies revealed that the actinomycetes produced various secondary metabolites associated with one or many large secondary metabolism biosynthetic gene clusters (smBGCs) on their genomic DNA. The smBGCs in actinomycetes expressed the type I polyketide synthase (PKS-I) as 21-66 %, the type II PKS (PKS-II) as 45-82 %, and the NRPS as 33-100 %, respectively (Janso and Carter, 2010; Doroghazi and Metcalf, 2013; Singh *et al.*, 2017; Jackson *et al.*, 2018; Genilloud, 2018). Polyketide synthase (PKS) is a large protein and consists of a set of the module containing acyl carrier protein (ACP), ketosynthase (KS) and acyltransferase (AT) domains that extend the linear sequence of an intermediated two carbon atoms as produced the polyketides (Dutta *et al.*, 2014). Polyketides are synthesized by sequential reactions using a group of active enzymes called polyketide synthases (PKSs). The large multi-enzyme protein complexes of PKS contain a coordinated group of active sites. Their biosynthesis performs in a stepwise manner from the carbon building blocks such as acetyl-CoA and their activated derivatives, including malonyl-, methylmalonyl-, and ethylmalonyl-CoA. Type I PKSs consist of the large multi-fuctional or mutidomain proteins which carry all the active sites required for polyketide biosynthesis. Type II PKSs consist of the complexes of mono-functional proteins that the active sites are distributed among several smaller, typically mono-functional polypeptides for the formation of compounds that require aromatization and cyclization as involved in the biosynthesis of bacterial aromatic

natural products, but not extensive reduction or reduction/dehydration cycles. Type III PKSs synthesize of polyhydroxy phenols in bacteria that are comparatively small proteins with a single polypeptide chain and are involved in the biosynthesis of precursors for flavonoids. The NRPS modules contain an adenylation domain that activates the correct amino acid and loads the peptidyl carrier protein (PCP) domain. Internal modules contain a condensation domain that transfers the upstream amino acid or peptide to the newly loaded amino acid, extending the peptide length by one residue. (Miller *et al.*, 2016). The draft genome was used to identify open reading frames (ORFs) and provide functional annotation of predicted proteins, rRNA, and tRNA genes by the antiSMASH (http://antismash.secondarymetabolites.org) web platform for screening the smBGCs (Jackson *et al.*, 2018). and SMURF (Secondary Metabolite Unknown Regions Finder) algorithms. The smBGCs compare amongst strains in the search for novel sequences conferring the potential novel bioactive secondary metabolite production. These new annotations were leveraged to predict roles in secondary metabolism for genes lacking experimental characterization.

2.5 Secondary metabolites of endophytic actinomycetes

The actinomycetes decompose the organic matters or humus in the soil as saprophytes and draw the nutrients as nitrogen-fixing from rhizospheric soil. In addition, actinomycetes, especially *Streptomyces*, are major groups of microorganisms that produce many valuable bioactive secondary metabolites in clinical use, pharmaceutical industry, agriculture, and environment, including antibiotics (streptomycin, neomycin, chloramphenicol, erythromycin, tetracycline, vancomycin, gentamicin, lincomycin, etc.), antifungals (amphotericin B, nystatin, etc.), antiprotozoals (avermectin, ivermectin), antimalarials (salinosporamide A), anti-cancers (paclitaxel, anthracyclines, catechoserine, lupinacidin A and B, etc.), plant growth hormones (indole-3-acetic acid (IAA)), and enzymes (cellulase, hemicellulase, xylanase, chitinase, amylase, etc.) (Caruso *et al.*, 2000; Strobel and Daisy, 2003; Bérdy, 2012; Takahashi and Nakashima, 2018; Ding *et al.*, 2019; Bernardi *et al.*, 2019).

Nowadays, approximately 32,500 natural products are produced from microorganisms and over two-thirds of the antibiotics are produced from actinomycetes. Many studies of endophytic actinobacteria have shown the discovery of many new actinomycetes and new bioactive products with diverse biological activities such as paclitaxel, an anticancer from *Kitasatospora* sp. isolated from *Taxus baccata* (Caruso, 2000). Recently, the bioactive secondary metabolites produced from endophytic actinomycetes are listed in Table 2.9.

Table 2.9 Secondary metabolites and bioactivity of endophytic actinomycetes.

Actinomycete	Host plant	Secondary metabolite	Bioactivity	Reference
Polymorphospora rubra K07-0510	Orchid	trehangelins A, B and C	photo-oxidative hemolysis inhibitors	Nakashima *et al.*, 2013
Streptomyces sp. BT01	*Boesenbergia rotunda*	7-methoxy-3, 3',4',6-tetrahydroxyflavone; fisetin; naringenin; 3'-hydroxydaidzein; xenognosin B; 2',7-dihydroxy-4',5'-dimethoxyisoflavone	antibiotic	Taechowisan *et al.*, 2014
Actinoallomurus fulvus MK10-036	*Capsicum frutescens*	actinoallolides A–E	anti-trypanosomal	Inahashi *et al.*, 2015
Polymorphospora rubra K07–0510	*Goodyera procera*	Trehangelin	cytoprotective	Inahashi *et al.*, 2016
Allostreptomyces sp. K12-0794	Fern root	Hamuramicins A and B	antibiotic	Suga *et al.*, 2018
Streptomyces sp. KIB-H2054	*Campylotropis polyantha*	Naphthomycin	antimicrobial	Zhang *et al.*, 2019

CHAPTER III

RESEARCH METHODOLOGY

In this study, the endophytic actinomycetes were isolated from medicinal plants in Thailand and identified based on phenotypic, chemotaxonomic and genotypic characterization. All strains were screened for antimicrobial activity by paper disc diffusion and anticancer activity by MTT assay. The interesting strains, which showed the effective bioactivities of secondary metabolites, were further studied on their chemical structure using spectrometry and secondary metabolite gene analysis on their genome as shown in Figure 3.1.

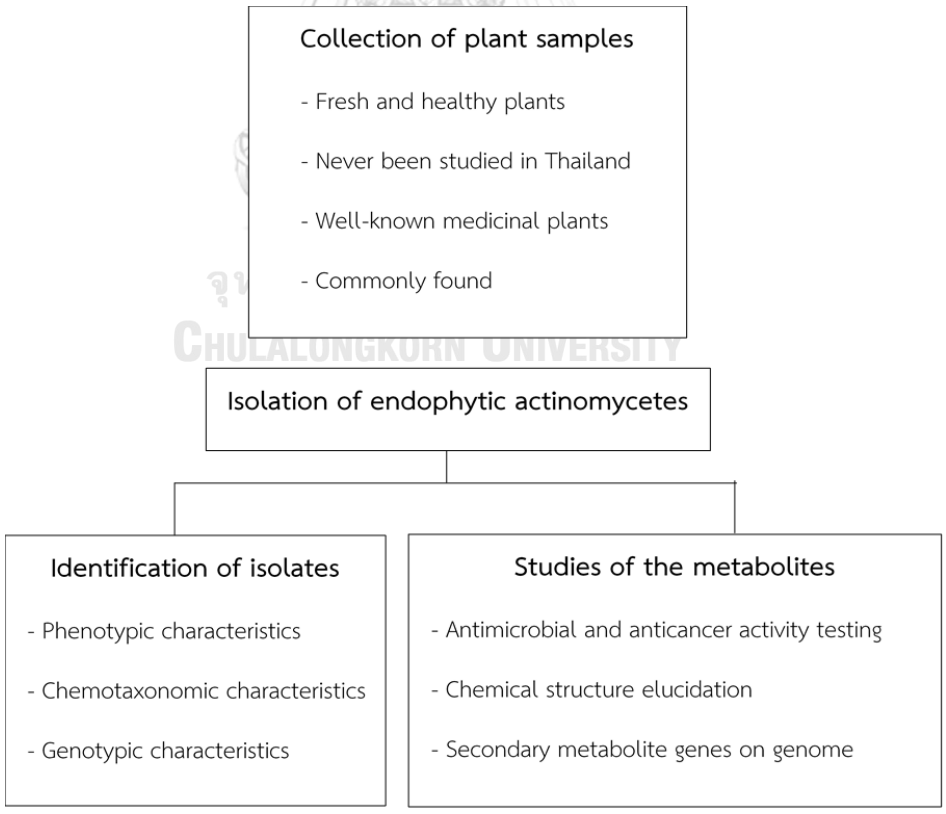

Figure 3.1 The diagram of experimentally designed in this study.

3.1 Plant sample collection

The seven healthy plant samples as *Catharanthus roseus* (แพงพวยฝรั่ง), *Phyllanthus amarus* (ลูกใต้ใบ), *Piper retrofractum* (ดีปลี), *Andrographis paniculata* (ฟ้าทะลายโจร), *Barleria lupulina* (เสลดพังพอนตัวผู้), and *Mimosa pudica* (ไมยราบ) were collected from Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok and other sources in Thailand. All samples were kept in plastic bags and stored at 4°C until the isolation.

3.2 Isolation of endophytic actinomycetes

The collected plant samples were washed to remove soil particles by running tap water for 1-2 min and were dried in the air on 3-5 days. And then, surface samples were sterilized by soaking in 1% (w/v) sodium hypochlorite for 2 min, followed by 70% ethanol for 2 min, and 10% (w/v) $NaHCO_3$ for 10 min before rinsing with sterilized water for three times and drying in laminar cabinet at the room temperature on 3-5 days (Klykleung *et al.*, 2015). After that, the surface-sterilized samples were aseptically ground and were done serial dilution with sterile normal saline (1:10, 1:100, 1:1,000). And then, the sample diluents were spread onto three isolation media included starch casein agar (SCA; Küster and Williams, 1964), humic acid-vitamin agar medium (HV; Hayakawa and Nonomura, 1987), water proline agar (1.0% proline in tap water, pH 7.0), and 2.5% water agar medium, containing 25 µg/mL nalidixic acid and 50 µg/mL cycloheximide to suppress the growth of gram-negative bacteria and fungi, respectively. The colony of strains was observed and purified on yeast extract-malt

extract agar (YMA, ISP no.2) after incubation at 28 ºC for 1-4 weeks. The pure cultures were preserved in ISP no.2 slant for a short-term period and as lyophilized cells in 10% skim milk for long-term preservation.

3.3 Identification methods

3.3.1 Phenotypic characterization

Morphological, cultural, physiological and biochemical characteristics of all strains were determined by the methods as described in the International *Streptomyces* Project (Shirling and Gottlieb, 1966; Arai *et al.*, 1975).

Morphological and cultural characteristics

All strains were observed the morphological characteristics in the 14-days old crosshatch-streak colony on ISP no.2 for the shape of mycelia, number and form of spore chain on hyphae using light and scanning electron microscopy. For cultural characteristics, all strains were inoculated in ISP no.2 broth at 28 ºC for 4-7 days before rinsed with sterile water and cultivated on different media as yeast extract-malt extract agar (ISP no.2), oatmeal agar (ISP no.3), inorganic salt-starch agar (ISP no.4), glycerol asparagine agar (ISP no.5), peptone-yeast extract ion agar (ISP no.6), tyrosine agar (ISP no.7), and nutrient agar at 28 ºC for 7-14 days (Shirling and Gottlieb, 1966). The color of the colony, diffusible pigment, and spore were detected using the NBS/IBCC color system (Kelly, 1964).

Physiological and biochemical characteristics

Physiological characteristics, all strains were observed including the properties of NaCl tolerance (0-10 % (w/v) NaCl), temperature tolerance (15, 20, 30, 37, 45 °C), and pH tolerance (pH at 4-10) on ISP no.2 agar for 7-14 days. For biochemical characteristics, all strains were determined by carbon utilization, starch hydrolysis, gelatin liquefaction, milk peptonization, and nitrate reduction that cultivated on various media (Shirling and Gottlieb, 1966; Arai *et al.*, 1975).

3.3.2 Chemotaxonomic characterization

The represented and selected strains of each genus were incubated in ISP no.2 broth on rotary shaker 200 rpm at 28 °C for 7-14 days. The cultured cells were collected and washed with sterile water three times before drying the cells by lyophilization.

Isomers of diaminopimelic acid analysis

The diaminopimelic acids (DAP) were analyzed by thin-layer chromatography (TLC) (Staneck and Roberts, 1974). Briefly, the dried cells (10 mg) were hydrolyzed with 1 mL of 6N HCl at 100 °C for 18 h. The hydrolyzed solution was filtrated and evaporated. The dry residue was re-dissolved with 300 µL of distilled water, after that, it was analyzed using a cellulose TLC plate (20 x 20 cm) with twice the developing solvent system as MeOH:H_2O:6N HCl:pyridine (80:26:4:10, v/v). The DAP isomer spot detected as dark-green spots by spraying with 0.2 % ninhydrin solution and heating at

100 °C for 5 min compared with the DAP standard solution as *meso*-DAP and LL-DAP isomers.

Whole-cell sugar analysis

The whole-cell sugars were analyzed by TLC (Staneck and Roberts, 1974). Briefly, the dried cells (50 mg) were hydrolyzed with 1 mL of 1N H_2SO_4 at 100 ºC for 2 h. After cooling, the hydrolysate was adjusted pH to 5.2-5.5 with $Ba(OH)_2$ solution and then centrifuged at 3,600 rpm for 10 min. The supernatant was collected and evaporated. The dry residue was dissolved with 200 µL of distilled water and developed on the cellulose TLC plate by using *n*-butanol:H_2O:pyridine:toluene (10:6:6:1, v/v) as the solvent system. The sugars were detected by spraying with acid aniline phthalate and heated at 100 °C for 4 min as yellowish-brown spots for the hexose sugars and maroon spots for the pentose sugars. The standard solution is the mixture sugar solution of galactose, arabinose, xylose, rhamnose, mannose, glucose, and ribose.

Polar lipid analysis

The polar lipids were analyzed by TLC (Minnikin *et al.*, 1984). Briefly, the dried cells (150-300 mg) were extracted with 3 ml of MeOH:0.3 % NaCl (100:10) and 3 mL of petroleum ether for 15 min. The lower layer was collected and added with 1 mL of petroleum ether for 5 min and heated at 100 °C for 5 min. After cooling to room temperature, 2.3 mL of $CHCl_3$:MeOH:H_2O (90:100:30, v/v) were added and mixed well

for 15 min. The lower layer was transferred to another tube and extracted again with 2.3 mL of $CHCl_3$:MeOH:H_2O (50:100:40, v/v). And then, the lower layer was transferred to the previous tube and added with 1.3 mL of each chloroform and water. The mixed solution was dried with N_2 gas (<37 ºC) and dissolved with 120 µL of $CHCl_3$:MeOH (2:1, v/v). The polar lipid extracts were determined by 2-dimension TLC technique. Briefly, 10 µl of the mixed solution was applied to the corner of the silica-gel TLC plate (10 x 10 cm) and developed on the first solvent system as $CHCl_3$:MeOH:H_2O (65:25:4, v/v) and the second solvent system as $CHCl_3$:CH_3COOH:MeOH:H_2O (40:7.5:6:2, v/v). The TLC plate was sprayed with specific reagents such as (A) molybdenum blue reagent for detection of lipids containing phosphate ester, (B) ninhydrin followed by heating at 110ºC for 10 min for detection of polar lipids contained free amino groups were phosphatidylethanolamine (PE) and its derivatives (lyso-PE, OH-PE and, methyl-PE), (C) Dragendorff's reagent for detection of phosphatidylcholine, (D) anisaldehyde reagent followed by heating at 110 ºC for 10 min for detection of glycolipids (green-yellow spot) and other lipid (blue spot), and (E) Dittmer and Lester reagent or phosphomolybdic acid reagent followed by heating 120 ºC for 10 min for detection of all lipids in the cells.

Cellular fatty acid analysis

The cellular fatty acids were analyzed by gas chromatography (GC) (Sasser, 1990). Briefly, the dried cells (40 mg) were saponified by well-suspending in 0.1 mL of the reagent 1 (NaOH 15 g, MeOH 50 mL, and milli-Q water 50 mL) and heating at 100

ºC for 30 min. After cooling to room temperature, the solution was methylated with 2 mL of the reagent 2 (6N HCl 65 mL, MeOH 55 mL) and heated at 80 ºC for 10 min. The solution was extracted with 1.25 mL of the reagent 3 (*n*-hexane 50 mL, methyl-tert-butyl ether 50 mL) and transferred the upper layer to another tube. The solution was mixed with 3 mL of the reagent 4 (NaOH 1.2 g, milli-Q water 100 mL) for 5 min and transferred the supernatant to a GC vial. The cellular fatty acids were analyzed using gas chromatography, according to the Microbial Identification System (Sherlock Microbial Identification System, MIDI).

Menaquinone analysis

The menaquinones (isoprenoid quinone) were analyzed by high-performance liquid chromatography-mass spectrometry (HPLC-MS) (Collins *et al.*, 1977). Briefly, the dried cells (100-500 mg) were extracted with 20 mL of $CHCl_3$:MeOH (2:1, v/v) overnight. The supernatant of suspension was filtrated and evaporated. The dried extract was dissolved with a small volume of acetone and developed on a silica gel TLC plate by using 100 % benzene as the solvent system. The band of menaquinones was detected with UV light at 254 nm, after that the band was scraped off and extracted with acetone. The suspension was filtrated through 0.45 µm membrane filter and dried up with N_2 gas. And then, the menaquinone sample was analyzed by HPLC-MS. The menaquinone analysis was compared with the reference strain. The standard solution is the mixture solution of menaquinones extracted from the reference strain and ubiquinone.

3.3.3 Genotypic characterization

The genotypic characteristics were analyzed by 16S rRNA gene sequencing, *gryB* gene sequencing, DNA base composition, and DNA-DNA hybridization. The genomic DNA was extracted from wet cells by aluminum oxide with micro-mixture for 16S rRNA gene sequencing and *gryB* gene sequencing. For DNA base composition and DNA-DNA hybridization, the genomic DNA was extracted from dried cells using mortar and pestle (Tamaoka, 1994).

16S rRNA gene sequencing and phylogenetic analysis

The chromosomal DNA for 16S rRNA gene sequencing was cultivated in ISP no.2 medium at 28 °C for 4-6 days. The cells were collected and washed twice with sterile water. The cells were lysed using aluminum oxide with a micromixer for 90 sec. And then, the lyzed cell was added with 300 mL of phenol:$CHCl_3$ (1:1, v/v) and mixed well for 5 min. The solution was centrifuged at 14,000 rpm for 15 min, the upper layer was transferred to the other tube and extracted with phenol:$CHCl_3$ (1:1, v/v). The upper layer was collected and precipitated the DNA.

The 16S rRNA gene sequence was amplified by polymerase chain reaction (PCR) (Lane, 1991; Suriyachadkun *et al.*, 2009). The PCR products were detected by gel electrophoresis and compared the particle size with 1 kb DNA marker. After that, the PCR products were analyzed the nucleotide sequence at the Macrogen, Korea. The universal primers were used for 16S rRNA gene sequencing as 27F (5′-AGAGTTTGATCMTGGCTCAG-3′)/1492R (5′-TACGGYTACCTTGTTACGACTT-3′) and 518F (5′-

CCAGCAGCCGCGGTAATACG-3′)/800R (5′-TACCAGGGTATCTAATCC-3′). The sequence homology was performed on the standard BLAST sequence similarity searching program as the EzBioCloud server (Yoon *et al.*, 2017). The multiple alignments of the sequences were carried out with the BioEdit program. The neighbor-joining (NJ) tree (Saitou and Nei, 1987), maximum-parsimony (Fitch, 1971), and maximum-likelihood (Felsenstein, 1981) were constructed using the MEGA 7.0 program (Kumar *et al.*, 2016). The topology was evaluated in a bootstrap analysis based on 1,000 replication (Felsenstein, 1985).

Primers of 16S rRNA gene

20F	5’-AGTTTGATCCTGGCTC-3’
1530R	5’-AAGGAGGTGATCCAGCC-3’
27F	5’-AGAGTTTGATCMTGGCTCAG-3’
1492R	5’-TACGGYTACCTTGTTACGACTT-3’
518F	5’-CCAGCAGCCGCGGTAATACG-3’
800R	5’-TACCAGGGTATCTAATCCC-3’
920F	5’-AAACTCAAATGAATTGACGG-3’
357R	5’-CTGCTGCCTCCCGTAG-3’

gyrB gene sequencing

The enzyme gyrase subunit B gene (*gryB*) amplification was carried out according to the method as previously described (Garcia *et al.*, 2010). The *gyrB* gene sequences which to of 1,100 nucleotides were overlapped and amplified to obtain from the two PCR products. The first fragment amplification (500-bp) was carried out using the primers GYF1 (5′-TCCGGYGGYCTGCACGGCGT-3′; position 19–38)/GYR1B (5′-CGGAAGCCCTCYTCGTGSGT-3′; position 548–567) and performed with an initial denaturation at 95 °C for 9 minutes, followed by 35 cycles with denaturation at 95 °C for 1 minute, annealing at 62 °C for 1 minute and extension at 72 °C for 2 minutes, followed by the last step at 72 °C for 7 minutes. The second fragment amplification (900-bp) was carried out using the primers GYF3 (5′-ACSGTCGACTTCGACTTCCA-3′, position 220–239)/GYR3B (5′-CAGCACSAYCTTGTGGTA-3′, position 1210–1226) and performed with an initial denaturation at 95 °C for 9 minutes, followed by 35 cycles with denaturation at 95 °C for 1 minute, annealing at 54 °C for 1 minute and extension at 72 °C for 2 minutes, followed by the last step at 72 °C for 7 minutes. Both PCR products were purified using the PCR purification kit (Gene aid) and were sequenced by Macrogen (Seoul, Korea). The first PCR product was sequenced using primers GYF1 and GYR1B while the second PCR product was sequenced using primers GYF3, GYR3B and GYF4 (5′-ACCCACGAGGAGGGCTTCCG-3′, position 548–567).

DNA base composition analysis

The 1 g of wet cells were suspended and inoculated in 2 ml of saline-EDTA (pH 8.0) with 5-10 mg of lysozyme at 37 ºC for 30 min. The solution was added with 8 ml of Tris-NaCl (0.1 M Tris and 0.1 M NaCl, pH 9.0) and 0.05-0.1 ml of 10 % SDS, and then heated at 55-60 ºC for 10 min. The extraction of proteins was carried out by adding 5 ml of phenol:$CHCl_3$ (1:1) and centrifuged at 10,000 rpm for 10 min. The supernatant was added with 5-10 ml of 95 % ethanol to precipitate the DNA. The DNA was spooled with a glass rod and dried at room temperature. After drying, the DNA was dissolved in 3 ml of 0.1 SSC (0.1 M NaCl and 0.015 M Na-Citrate, pH7) and eliminated the RNA by incubating in 0.3 mL of RNase A solution at 37 ºC for 20 min. The DNA solution was added with 0.5 mL of 10 x SSC and extracted with phenol:$CHCl_3$ (1:1) again. The DNA was precipitated, spooled with a glass rod, dried, and dissolved in 0.1 SSC. The DNA solution was measured for purity using a spectrophotometer at OD_{260}/OD_{280} (ratio as between 1.8 to 2.0). The DNA solution (10 µg/mL) was heated at 100 ºC for 10 min. The denatured DNA was hydrolyzed with 10 µL of nuclease P1 solution at 50 ºC for 1 h and incubated in 10 µL of alkaline phosphatase solution at 37 ºC for 1 h. The DNA G+C content was determined using HPLC (Tamaoka, 1994). The equimolar mixture was calculated as below.

$$\text{Mol \% G+C} = \frac{(G/Gs) + (C/Cs)}{(A/As) + (G/Gs) + (C/Cs) + (T/Ts)} \times 100$$

DNA-DNA hybridization

The purified genomic DNA (chromosomal DNA) solution was measured for purity using a spectrophotometer at OD_{260}/OD_{280} (ratio as between 1.8 to 2.0) (Tamaoka, 1994). The DNA-DNA relatedness was determined using the microplate hybridization method (Ezaki *et al.*, 1989).

The preparation of immobilized single-strand DNA on the 96-well plate (microplate), the DNA solutions (100 µg/mL in 0.1 x SSC) were boiled at 100 ºC for 10 min and was immediately be cooled on ice. After cooling, the DNA solutions were diluted to 10 µg/mL and were dispensed (100 µL) to each well of the 96-wells plate (a totally 1 µg of DNA per well). The plate was tightly sealed and incubated at 37 ºC for overnight. The calf thymus DNA was used as a control.

The preparation of DNA probe, 10 µL of DNA solution (100 µg/mL) was sonicated for 2 min and were added 10 µL of photobiotin solution. The DNA solution was exposed to the light (500 watts) for 30 min (in an icebox). Then, 127 µL of milli-Q water, 16 µL of 0.1 M Tris-HCl buffer and 160 µL were added into the DNA solution. This solution was partitioned twice with 160 µL of *n*-butanol and then the butanol-layer (upper layer) was removed. The lower layer (water phase) was boiled at 100 ºC for 10 min and will immediately be cooled on ice. After cooling, the DNA solution was diluted into the hybridization solution (Appendix B) to obtain the final concentration of DNA probe 1 µg/mL. The DNA probe was dispensed into each microplate well (100 µL). Finally, each well will contain 0.1 µg of the DNA probe.

For hybridization, the solution in the plate after incubation for overnight was discarded and were added 100 µL of the DNA probe solution to each well. The plate was tightly sealed with the plastic sticker and was incubated overnight at the optimal hybridization temperature. And then, the microplate will discard the solution and will wash with 0.2 mL of 0.2xSSC for three times. The microplate was added with 0.2 mL of the PBS-BSA-Triton solution (solution 1) and was incubated at room temperature for 10 min after that the solution 1 were discarded. The 0.1 mL of the streptavidin-β-galactosidase solution (solution 2) was added to each well of the microplate after that was incubated at 37 ^{o}C for 30 min and was discarded the solution 2. The microplate was analyzed with the colorimetric method by 100 µL of the peroxidase-streptavidin solution and discarded the solution after incubated at 37 ^{o}C for 30 min. And then, the microplate was washed twice with 300-400 µL of PBS and incubated in 100 µL of tetramethylbenzidine-H_2O_2 solution at 37 ^{o}C for 5 min. The enzyme reaction was stopped with 100 µL of H_2SO_4 after that measured at 450 nm using the microplate reader (Microplate Reader Wallac 1420, PerkinElmer^{TM}). The results were calculated for the percentage of DNA homology (Ezaki *et al.*, 1989).

$$\text{DNA-DNA relatedness (\%)} = \frac{\text{DNA sample} - \text{Calf thymus}}{\text{Labeled type strain} - \text{Calf thymus}} \times 100$$

Genome sequence analysis

Whole-genome sequencing of the selected strains was carried out using an Illumina Miseq platform (Illumina) by using 2×250 bp paired-end reads. Assembly of the reads to contigs was accomplished by using SPAdes 3.12 (Bankevich *et al.*, 2012). The draft assemblies of them had been submitted to the Gen-Bank and were publicly available. Phylogenomic tree based on whole-genome sequences was constructed on the Type (Strain) Genome Server (TYGS) (Meier-Kolthoff and Goker, 2019). Tree inferred with FastME 2.1.4 (Lefort *et al.*, 2015) from Genome BLAST Distance Phylogeny (GBDP) distances calculated from genome sequences. Branch lengths are scaled in terms of GBDP distance formula d_5. The genomes were annotated using the RAST server followed by the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) and a comparison performed in the SEED Viewer (Aziz *et al.*, 2008; Aziz *et al.*, 2012). Average nucleotide identity (ANI) values were calculated with pairwise genome alignment of the draft genome sequences of related type strains using the ANI-BLAST (ANIb) and ANI-MUMmer (ANIm) algorithms implemented within the JspeciesWS web service (Richter and Rosselló-Móra, 2009; Richter *et al.*, 2016). Calculation of the digital DNA–DNA hybridization (dDDH) values was achieved using the Genome-to-Genome Distance Calculator (GGDC 2.1) using the BLAST+ method (Meier-Kolthoff *et al.*, 2013). *In silico* G+C content of the selected strains was determined according to the genomic DNA sequences. Results were performed using the recommended formula 2 (identities/HSP length), which is useful when dealing with the incomplete draft genomes.

3.4 Screening for antimicrobial activity

Antimicrobial activity was determined using the paper disc diffusion assay (Boyle *et al.*, 1973).

3.4.1 Preparation of the sample

All strains were inoculated in 10 ml of the seed medium (301) at 28 ^{o}C for 3-5 days on a shaker at 200 rpm. The 0.1 mL of inoculums were incubated in 10 ml of the production media (ISP no.2 broth) at 28 ^{o}C for 7 days on a shaker at 200 rpm. The culture broth was extracted with EtOH in equal volume and centrifuged to discard the cells. The EtOH crude extract was screened for antimicrobial activity against the tested microorganisms using the paper disc diffusion method.

3.4.2 Paper disc diffusion assay for antimicrobial activity

The indicator strains, including *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Kocuria rhizophila* ATCC 9341, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were cultivated on trypticase soy agar (TSA) at 37 ^{o}C for 24 h, before antimicrobial testing. The tested yeast, *Candida albicans* ATCC 10231 were cultivated on Sabouraud dextrose agar (SDA) at 30 ^{o}C for 48 h. All tested microorganisms were suspended in 0.85 % normal saline solution and standardized the turbidity with McFarland solution No. 0.5, approximately 1.5 x10^{8} CFU of cell suspension. The tested plates were swabbed with tested microorganisms, TSA for the cultivation of the bacteria and SDA for the cultivation of the yeast. The dry discs were placed on the surface of tested plates and incubated at 37^{o}C 24 h for the bacteria and

30 °C, 48 h for the yeast. The 30 µL of chloramphenicol (1 mg/mL) was used as a positive control of antimicrobial activity testing for bacteria. The 20 µL of ketoconazole (1 mg/mL) was used to the positive control of antimicrobial activity testing for yeast. Methanol was used to the negative control of antimicrobial activity testing. The size of inhibition zones (in mm.) was measured using the Vernier calipers.

3.5 Screening for cytotoxicity

All crude extracts were evaporated and dissolved with cell culture media and screened for inhibitory effects on cancer cell proliferation using MTT assay.

3.5.1 Preparation of the sample

All strains were inoculated in 10 mL of the seed medium (301) at 28 ºC for 4-5 days on a shaker at 200 rpm. The 0.1 mL of inoculum was incubated in 10 mL of the production media (M60) at 28 ºC for 7 days on a shaker at 200 rpm. The 600 µL of culture broth was centrifuged to discard the cells at 4,000 x g for 10 min and filter-sterilized using a 0.22 µm membrane filter (Sigma, USA). The supernatant was concentrated by speed-vacuum drying (Speed vacuum: Rotational Vacuum Concentrator RVC 2-18, Germany) and re-suspended in an equal volume of serum-free culture media. The samples were kept in frozen at -20ºC until analysis for cell proliferation in colon carcinoma cells (Caco-2; ATCCHTB-37), human monocytic leukemia cells (U937; ATCC CRL-2367), human hepatocellular carcinoma cells (hepG2; ATCCHB-8065), and African green monkey kidney cells (Vero; ATCC CCL-81) by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).

3.5.2 MTT assay for anticancer activity

The hepG2, and Caco-2 were cultured in Dulbecco modified Eagle's minimal essential medium (DMEM) and the Vero cells were cultured in Medium 199 (M199), respectively. All culture media were supplemented with 10 % fetal bovine serum (FBS), penicillin (100 U/mL) mixed with streptomycin (0.1 mg/mL), and then incubated at 37°C with a humidified atmosphere containing 5 % CO_2. HepG2, Caco-2, and Vero cells were seeded in 96-well plates at a final density of 5 x 10^4 cells per well, respectively. HepG2, Caco-2, and Vero cells were incubated at 37 °C overnight before treatment with samples. 10 % v/v of the supernatants were treated into the cell line cultures which were incubated at 37°C for 24 h in 5 % CO_2 incubator. After incubation, MTT solutions were added to each well and were incubated at 37°C for 3 h in 5 % CO_2 incubator. The culture media of hepG2, Caco-2, and Vero cells were discarded before the dimethyl sulfoxide (DMSO) was added and were mixed gently. The optical density was measured at 595 nm using a microplate reader. Cell culture medium and M60 with cell culture medium (60 medium re-dissolved in cell culture media) were used as negative control. Cisplatin (50 μM) was used as positive control. This experiment was determined in three independent assays. The percentage of cell viability was calculated as follows:

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100$$

The experiments were performed three times in triplicate. Results were reported as means and standard error of the mean (SEM). Statistical analysis was evaluated by one-way ANOVA using the GraphPad Prism® version 5.01. Significant differences were considered significant at $P < 0.05$.

3.6 Chemical profile analysis of the secondary metabolites

3.6.1 Screening and fermentation of the selected strains

Screening for new secondary metabolites, the potency activity strains were selected to inoculate in the seed medium (301 broth; 2.4% starch, 0.1 % glucose, 0.3 % peptone, 0.3 % meat extract, 0.5 % yeast extract and 0.4 % $CaCO_3$ adjusted to pH 7.0 before sterilization) at 28 °C for 5 days on a shaker at 200 rpm. Approximately 1 µL of the inoculum were incubated in 10 mL of the production medium (M60 broth; 2 % soluble starch, 0.5 % glycerol, 1.0 % defatted wheat germ, 0.3 % meat extract, 0.3 % dry yeast and 0.3 % $CaCO_3$ adjusted to pH 7.0 before sterilization) at 28 °C for 7 days on the shaker at 200 rpm. The inoculum was extracted with 50 % EtOH and was separated by centrifugation at 3,000 x g for 10 min. The crude extract was dissolved with EtOH and was analyzed the chemical profile by HPLC-UV/VIS and HPLC-MS. The chemical profiles were compared with the Kitasato Institute of Life Science's in-house databases (Tokyo, Japan) and the Dictionary of Natural Product databases (http://dnp.chemnetbase.com). The strains which exhibited the unknown secondary metabolites were selected to optimize the productive condition based on the Kitasato

Institute of Life Science's in-house physicochemical (PC) screening and LC-MS/MS for the interesting peaks as the candidate new secondary metabolite.

Fermentation for new secondary metabolites, the strains which exhibited the unknown secondary metabolites were selected to inoculate in 100 mL of the seed medium 301 broth at 28 ^{o}C for 5 days on a shaker at 200 rpm. Approximately 1 mL of the inoculum was incubated in 100 mL of the optimal modified production medium 60 broth (total volume as 6 L) at 28 ^{o}C for 7 days on the shaker at 200 rpm before purification and chemical structure elucidation by spectrometry.

3.6.2 Extraction and purification of the secondary metabolites

After fermentation, the inoculum was separated by centrifugation at 3,000 x g for 10 min. The mycelium part was extracted with MeOH and was analyzed the interesting peak by LC-MS/MS before evaporated by rotary evaporator and weighed the mycelium crude extract part. The supernatant part was partitioned with ethyl acetate for three times. The ethyl acetate (EtOAc) layer was collected and analyzed the interesting peak by LC-MS/MS before evaporated by rotary evaporator and weighed the supernatant crude extract part.

The crude extract was separated into the fractions by the silica gel column chromatography with eluted systems (hexane:EtOAc or $CHCl_3$:MeOH, as stepwise separation) and the middle-pressure liquid chromatography (MPLC) with an ODS column and eluted system (Milli-Q:MeOH, as stepwise separation). After the stepwise separation, the fraction containing the interested peak was subjected to semi-

preparative HPLC (ODS column) using a solvent system (Milli-Q:MeOH) supplemented with 0.1% formic acid as isocratic solvent system.

3.6.3 Chemical profile analysis of the secondary metabolites

The purified extract was dissolved with EtOH and was analyzed the chemical profile by HPLC-UV/VIS and HPLC-MS. The chemical profiles were compared with the Kitasato Institute of Life Science's in-house databases (Tokyo, Japan) and the Dictionary of Natural Product databases (http://dnp.chemnetbase.com). The strains, showing the unknown secondary metabolites, were selected for purification and chemical structure elucidation by spectroscopic method. High-resolution electrospray ionization mass spectrometry (HRESIMS) or LC-ESI-MS spectra were measured using an AB Sciex QSTAR Hybrid LC/MS/MS Systems (AB Sciex, Framingham, MA, USA). Nuclear magnetic resonance (NMR) spectra were measured using a Bruker Avance III 400 MHz NMR spectrometer and Bruker AVANCE III HD 600 MHz NMR spectrometer (Bruker, Kanagawa, Japan), with ^{1}H NMR at 600 MHz and ^{13}C NMR at 150 MHz in CD_3OD. The chemical shifts are expressed in ppm and are referenced to residual CD_3OD in the ^{1}H-NMR spectra and in the ^{13}C-NMR spectra. Ultraviolet (UV) spectra were measured with a Hitachi U-2810 spectrophotometer (Hitachi, Tokyo, Japan). Optical rotation was measured on a JASCO model DIP-1000 polarimeter (Jasco, Tokyo, Japan). Infrared radiation (IR) spectra (KBr) were taken on a Horiba FT-710 Fourier transform IR spectrometer (Horiba, Kyoto, Japan).

3.7 Whole-genome sequencing and prediction of their smBGCs

The purified genomic DNA (chromosomal DNA) solution was measured for the purity by spectrophotometer at OD_{260}/OD_{280} (ratio as between 1.8 to 2.0) (Tamaoka, 1994). The genome sequence of the selected strain was carried out using a whole-genome shotgun strategy with an Illumina Miseq platform (2×250-bp paired-end reads). Assembly of the reads to contigs was accomplished by using SPAdes 3.12 (Bankevich *et al.*, 2012). The draft assemblies have been submitted to GenBank and show publicly available. The genomes were annotated by using the RAST server followed by the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) and a comparison performed in the SEED Viewer (Aziz *et al.*, 2008; Aziz *et al.*, 2012; Tatusova *et al.*, 2016). Average nucleotide identity (ANI) values were calculated with pairwise genome alignment of the draft genome sequences of related type strains by the ANI-BLAST (ANIb) and ANI-MUMmer (ANIm) algorithms implemented within the JSpeciesWS web service (Richter and Rosselló-Móra, 2009; Richter *et al.*, 2016). Calculation of the digital DNA–DNA hybridization (dDDH) values was performed using the Genome-to-Genome Distance Calculator (GGDC 2.1) using the BLAST+ method (Meier-Kolthoff *et al.*, 2013). The G+C content was determined according to the genomic DNA sequences. Results were evaluated by the recommended formula 2 (identities/HSP length), which is useful when dealing with the incomplete draft genomes.

The draft genome was used to identify secondary metabolite gene clusters (smBGCs), the annotating will use antiSMASH (antibiotics and Secondary Metabolite

Analysis Shell) algorithm to identify potential clusters in genomic strain (Medema *et al.*, 2011).

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Plant sample collection

In this study, the six healthy medicinal plant samples were collected from the botanical garden, Faculty of Pharmaceutical Sciences at Chulalongkorn University, Bangkok (6 samples) such as *Catharanthus roseus*, *Phyllanthus amarus*, *Piper retrofractum, Barleria lupulina, Andrographis paniculate*, and from Kanchanaburi Province at 14°31'12" N 98°49'15" E (1 sample) such as *Mimosa pudica* as shown in Table 4.1. They were collected and kept in a clean plastic bag at 4 °C before the isolation of the endophytic actinomycetes.

Table 4.1 Plant sample, collected date, and location

Plant sample		Collected date	Location
Genus or scientific name	**Family name**		
Catharanthus roseus	*Apocynaceae*	14/01/2013	Bangkok
Phyllanthus amarus	*Euphorbiaceae*	11/02/2014	
Piper retrofractum	*Piperaceae*	24/06/2017	
Barleria lupulina	*Acanthaceae*	24/06/2017	
Andrographis paniculate	*Acanthaceae*	11/09/2017	
Mimosa pudica	*Fabaceae*	11/09/2017	Kanchanaburi

4.2 Isolation of endophytic actinomycetes

Fifty actinomycetes, divided into forty-nine strains were isolated from roots, stems and leaves of seven healthy medicinal plant samples after incubation at 30 °C in 14-30 days using five different media, including humic acid-vitamin agar, starch-casein agar, Pridham's agar, water proline agar, and 2.5% water agar supplemented with cycloheximide (50 µg/mL) and nalidixic acid (25 µg/mL), as shown in Table 4.2. All strains were preserved as a colony on ISP no. 2 slants and lyophilized in 10 % skim milk. In this study, strain ST1-08 from *Stemona tuberosa*, identified in the previous study, was studied in secondary metabolites (Klykleung *et al.*, 2015).

Three strains, CR1-01, CR1-08, and CR1-09 isolated from leaf and roots of *Catharanthus roseus*. Three strains, PA1-05, PA1-07, and PA1-10 isolated from root, stem, and leaf of *Phyllanthus amarus*. Three strains, PR1-03, PR1-10, and PR1-12 isolated from stems of *Piper retrofractum*. Eleven strains, BL1-01, BL1-03, BL1-04, BL1-05, BL1-06, BL1-07, BL1-08, BL1-10, BL1-11, BL1-13, and BL1-16 isolated from roots, stems, and leaves of *Barleria lupulina*. Eight strains, AP1-03, AP1-06, AP1-08, AP1-10, AP1-18, AP1-19, AP1-20, and AP1-21 isolated from roots, stems, and leaves of *Andrographis paniculate*. Twenty-one strains, 3MP-10, 3MP-11, 3MP-12, 3MP-13, 3MP-14, 3MP-34, 3MP-35, 4WP-20, 4WP-21, 4WP-22-1, 4WP-22-2, 4WP-23, 4WP-24, 4WP-25, 4WP-27, 7MP-06, 8MP-16, 8MP-31, 8MP-36, 10MP-01, and 10MP-05 isolated from roots, stem, and leaves of *Mimosa pudica*.

Table 4.2 Plant, media, part of plant, isolation date, and isolate number of actinomycetes

Plant (Isolation date)	Agar medium	Isolate no.		
		Root	Stem	Leaf
Catharanthus roseus	HV	-	-	CR1-01
(14/01/2013)	SC	CR1-08, CR1-09	-	-
Phyllanthus amarus	HV	-	PA1-07	-
(18/02/2014)	SC	PA1-05	-	PA1-10
Piper retrofractum	PD	-	PR1-10	-
(24/06/2017)	WP	-	PR1-03, PR1-12	-
Barleria lupulina	SC	-	BL1-01, BL1-07	BL1-03, BL1-04, BL1-05
(24/06/2017)	PD	BL1-06, BL1-08, BL1-16	-	-
	WP	BL1-10, BL1-11	BL1-13	-
Andrographis paniculate	SC	AP1-18, AP1-19, AP1-20	-	AP1-21
(18/09/2017)	WA	-	AP1-06	-
	PD	AP1-03	-	-
	WP	AP1-08, AP1-10	-	-
Mimosa pudica	PD	3MP-10, 3MP-11, 3MP-12, 3MP-14, 3MP-34, 10MP-01	7MP-06, 8MP-36, 10MP-05	3MP-13, 3MP-35, 8MP-16, 8MP-31
(21/09/2017)	WP	4WP-21, 4WP-24	4WP-20, 4WP-22-1, 4WP-22-2,	4WP-23, 4WP-25, 4WP-27

HV; humic acid-vitamin agar, SC; starch casein agar, WA; 2.5% water agar, PD; Pridham's agar medium, and WP; water proline agar.

CR; *Catharanthus roseus*, ST; *Stemona tuberosa,* PA; *Phyllanthus amarus*, PR; *Piper retrofractum*, BL; *Barleria lupulina*, AP; *Andrographis paniculate*, and MP; *Mimosa pudica*

4.3 Identification of endophytic actinomycetes

All strains were selected based on morphological characteristics. On the basis of the 16S rRNA gene sequence and phylogenetic tree, they were belonged to six genera including *Micromonospora* (34 strains, 68 %), *Streptomyces* (8 strains, 16 %), *Microbispora* (3 strains, 6 %), *Plantactinospora* (2 strains, 4 %), *Nonomuraea* (2 strains, 4 %), and *Amycolatopsis* (1 strain, 2 %) as described below and showed in Tables 4.3, 4.4, and Figure 4.1.

Ten strains, candidate novel species, strain CR1-09 isolated from the root of *Catharanthus roseus* was closely related to *Microbispora tritici* (99.5 % similarity), strain PA1-10 isolated from the leaf of *Phyllanthus amarus* was related to *Nonomuraea candida* (98.4 % similarity), strain PA1-05 isolated from the root of *Phyllanthus amarus* was related to *N. monospora* (99.3 % similarity), strains 3MP-10 and 3MP-14 isolated from the root of *Mimosa pudica* was related to *S. zhaozhouensis* (98.9 and 98.8 % similarity, respectively), strain 8MP-36 isolated from the stem of *Mimosa pudica* was related to *Micromonospora eburnean* (98.8 % similarity), strain 10MP-04 isolated from the root of *Mimosa pudica* was related to *Mn. terminaliae* (98.9 % similarity), strain BL1-01 isolated from the stem of *Barleria lupulina* was related to *Mn. aurantica* (99.1 % similarity), strain AP1-03 isolated from the root of *Andrographis paniculate* was related to *Mn. equina* (99.0 % similarity) while strain AP1-08 isolated from the root of *Andrographis paniculate* was related to *Mn. rhizophaerae* (98.7 % similarity).

Table 4.3 16S rRNA gene sequence similarity (%) of the representative strains.

Isolate no.	Closely related strain	Similarity (%)	Length (bp)
PR1-03	*Micromonospora costi*	100.0	1333
PR1-12	*Micromonospora schwarzwaldensis*	99.9	1317
AP1-03	***Micromonospora equina***	**99.0**	**1380**
AP1-06	***Micromonospora citrea***	**99.3**	**1349**
AP1-08	***Micromonospora citrea***	**98.7**	**1386**
AP1-10	*Micromonospora yasonensis*	99.6	1325
AP1-20	*Micromonospora soli*	99.7	1334
3MP-11	*Micromonospora aurantiaca*	100.0	1343
3MP-34	*Micromonospora tulbaghiae*	99.9	1358
3MP-35	*Micromonospora aurantiaca*	100.0	1322
4WP-21	*Micromonospora schwarzwaldensis*	99.9	1317
4WP-22-1	*Micromonospora costi*	100.0	1322
4WP-22-2	*Micromonospora costi*	100.0	1333
4WP-24	*Micromonospora musae*	99.9	1379
4WP-25	*Micromonospora chersina*	99.6	1292
8MP-36	***Micromonospora yasonensis***	**99.0**	**1373**
7MP-06	*Micromonospora tulbaghiae*	99.9	1323
10MP-04	***Micromonospora terminaliae***	**98.9**	**1362**
10MP-05	*Micromonospora soli*	99.7	1334
BL1-01	***Micromonospora aurantiaca***	**99.1**	**1388**
BL1-05	*Micromonospora aurantiaca*	99.9	1363
BL1-11	*Micromonospora aurantiaca*	99.7	1355
BL1-13	*Micromonospora tulbaghiae*	99.9	1323
PA1-07	*Streptomyces phyllanthi* sp. nov.	100.0	1462
AP1-18	*Streptomyces seoulensis*	99.9	1320
AP1-19	*Streptomyces seoulensis*	99.9	1325
AP1-21	*Streptomyces seoulensis*	99.9	1319
3MP-10	***Streptomyces zhaozhouensis***	**98.9**	**1425**
3MP-12	*Streptomyces albidoflavus*	99.7	1340
3MP-14	***Streptomyces zhaozhouensis***	**98.8**	**1457**
BL1-06	*Streptomyces albidoflavus*	99.8	1354
CR1-01	***Microbispora rosea* subsp. *rosea***	**99.3**	**1369**
CR1-08	*Microbispora rosea* subsp. *rosea*	99.5	1485
CR1-09	*Microbispora catharanthi* sp. nov.	100.0	1469
PA1-05	***Nonomuraea monospora***	**99.3**	**1372**
PA1-10	*Nonomuraea phyllanthi* sp. nov.	100.0	1473
BL1-16	*Plantactinospora endophytica*	99.7	1467
8MP-16	*Plantactinospora endophytica*	99.4	1370

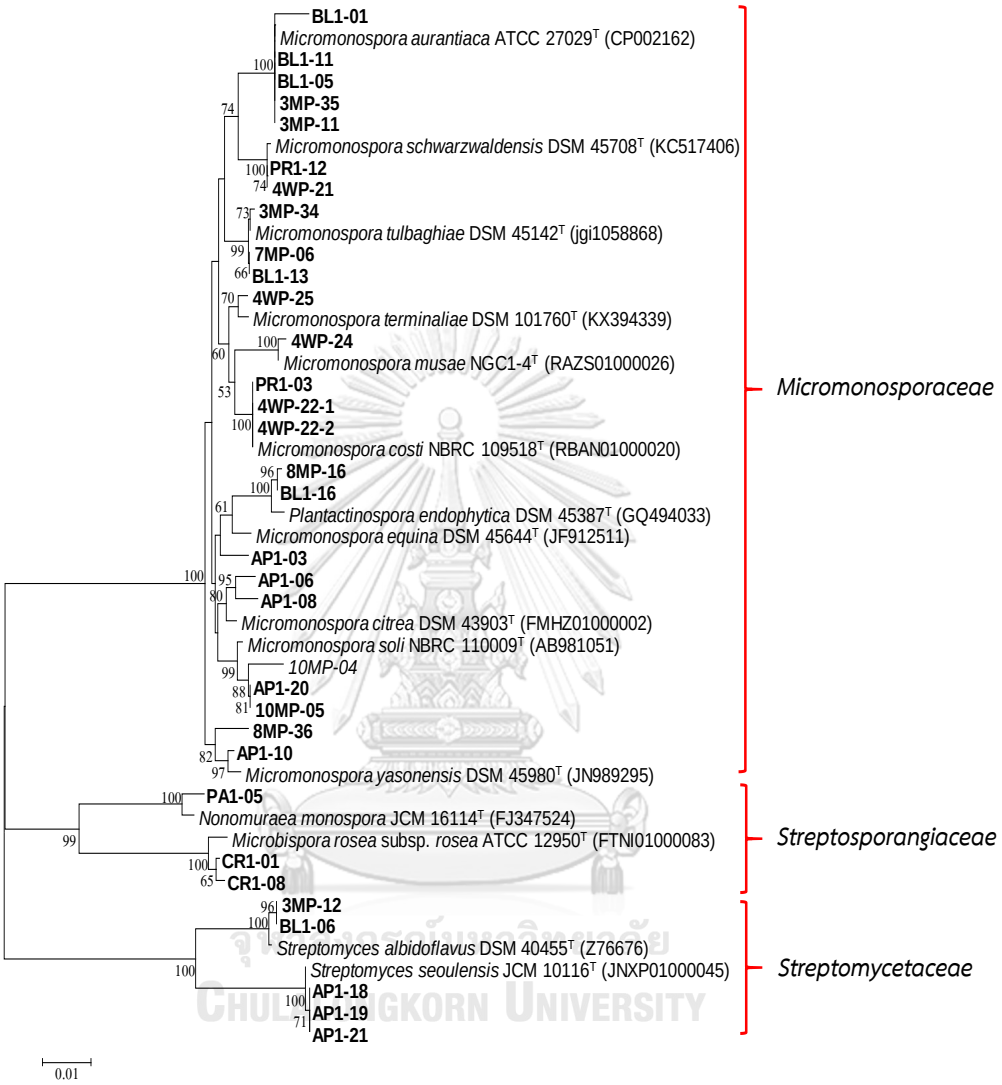

Figure 4.1 Neighbor-joining phylogenetic tree based on 16S rRNA gene sequence of the representative strains and related type strains

Table 4.4 Identification of strains based on 16S rRNA gene sequences

Host plant	Sample No.	Part	Identification
Catharanthus roseus	CR1-01	Leaf	*Microbispora* sp.
	CR1-08	Root	*Microbispora* sp.
	CR1-09	Root	*Microbispora catharanthi*
Phyllanthus amarus	PA1-05	Root	*Nonomuraea* sp.
	PA1-07	Stem	*Streptomyces phyllanthi*
	PA1-10	Leaf	*Nonomuraea phyllanthi*
Mimosa pudica	3MP-10	Root	*Streptomyces* sp.
	3MP-12	Root	*S. albidoflavus*
	3MP-14	Root	*Streptomyces* sp.
	3MP-11	Root	*Micromonospora aurantiaca*
	3MP-34	Root	*Mn. tulbaghiae*
	3MP-35	Leaf	*Mn. aurantiaca*
	4WP-21	Root	*Mn. schwarzwaldensis*
	4WP-22-1	Stem	*Mn. costi*
	4WP-22-2	Stem	*Mn. costi*
	4WP-24	Root	*Mn. musae*
	4WP-25	Leaf	*Mn. chersina*
	8MP-16	Leaf	*Plantactinospora endophytica*
	8MP-36	Stem	*Micromonospora* sp.
	7MP-06	Stem	*Mn. tulbaghiae*
	10MP-04	Root	*Micromonospora* sp.
	10MP-05	Stem	*Mn. soli*

HV; humic acid-vitamin agar, SC; starch casein agar, WA; 2.5% water agar, PD; Pridham's agar medium, and WP; water proline agar.

Table 4.4 Identification of strains based on 16S rRNA gene sequences (continued)

Host plant	Sample No.	Part	Identification
Barleria lupulina	BL1-01	Stem	*Micromonospora* sp.
	BL1-05	Leaf	*Mn. aurantiaca*
	BL1-11	Root	*Mn. aurantiaca*
	BL1-13	Stem	*Mn. tulbaghiae*
	BL1-16	Root	*Plantactinospora endophytica*
Andrographis paniculata	AP1-03	Root	*Micromonospora* sp.
	AP1-06	Stem	*Micromonospora* sp.
	AP1-08	Root	*Micromonospora* sp.
	AP1-10	Root	*Mn. yasonensis*
	AP1-20	Root	*Mn. soli*

HV; humic acid-vitamin agar, SC; starch casein agar, WA; 2.5% water agar, PD; Pridham's agar medium, and WP; water proline agar.

4.3.1 Characterization of *Micromonospora* strains (Group I)

Thirty-four strains PR1-03, PR1-10, PR1-12, BL1-01, BL1-03, BL1-04, BL1-05, BL1-07, BL1-08, BL1-10, BL1-11, BL1-13, AP1-03, AP1-06, AP1-08, AP1-10, AP1-20, 3MP-11, 3MP-13, 3MP-34, 3MP-35, 4WP-20, 4WP-21, 4WP-22-1, 4WP-22-2, 4WP-23, 4WP-24, 4WP-25, 4WP-27, 7MP-06, 8MP-31, 8MP-36, 10MP-01, and 10MP-05 were identified as *Micromonospora* that belonged to the family *Micromonosporaceae* based on morphology and 16S rRNA gene sequence (Figure 4.3). Strains PR1-03, 4WP-22-1, and 4WP-22-2 were identified as *Micromonospora costi* (Thawai, 2015), PR1-12 and 4WP-21 were *Mn. schwarzwaldensis* (Gurovic *et al.*, 2013); 3MP-11, 3MP-35, BL1-05, and BL1-11 were *Mn. aurantiaca* (Sveshnikova *et al.*, 1970); AP1-10 was *Mn. yasonensis*

(Veyisoglu *et al.*, 2016); AP1-20 and 10MP-05 were *Mn. soli* (Thawai *et al.*, 2016); 4WP-24 was *Mn. musae* (Kuncharoen *et al.*, 2019); 4WP-25 was *Mn. chersina* (Tomita *et al.*, 1992); 3MP-34, 7MP-06 and BL1-13 were *Mn. tulbaghiae* (Kirby and Meyers, 2010) while strains AP1-03, AP1-06, AP1-08, 8MP-36, 10MP-04, and BL1-01 were identified as *Micromonospora* sp.

They produced monomeric spores on the non-fragmented branched substrate mycelia but not produced aerial mycelia on various agar media. In this study, strain 4WP-24 was select to represent the genus *Micromonospora* that formed rugose spores on substrate mycelium cultivated on ISP4 agar 30 °C for 14 days (Figure 4.2).

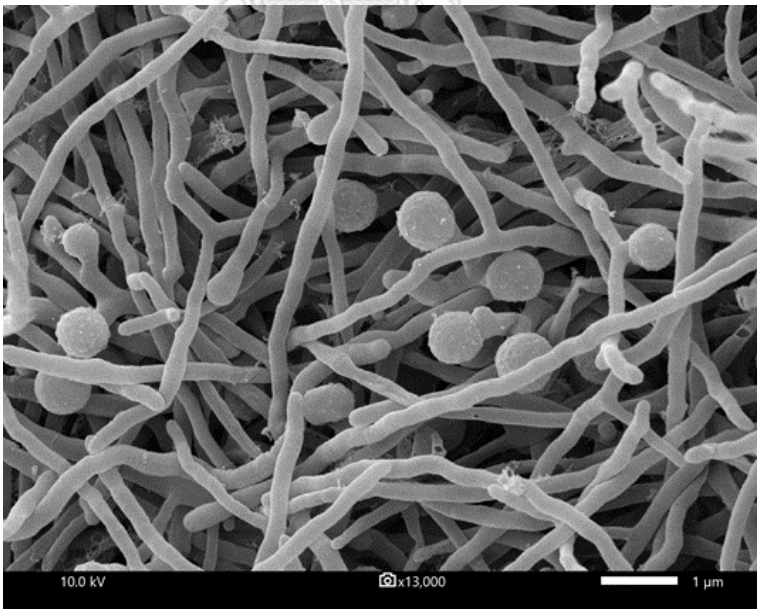

Figure 4.2 Scanning electron micrograph of *Micromonospora* sp. 4WP-24 after incubation on ISP4 agar at 30 °C for 14 days.

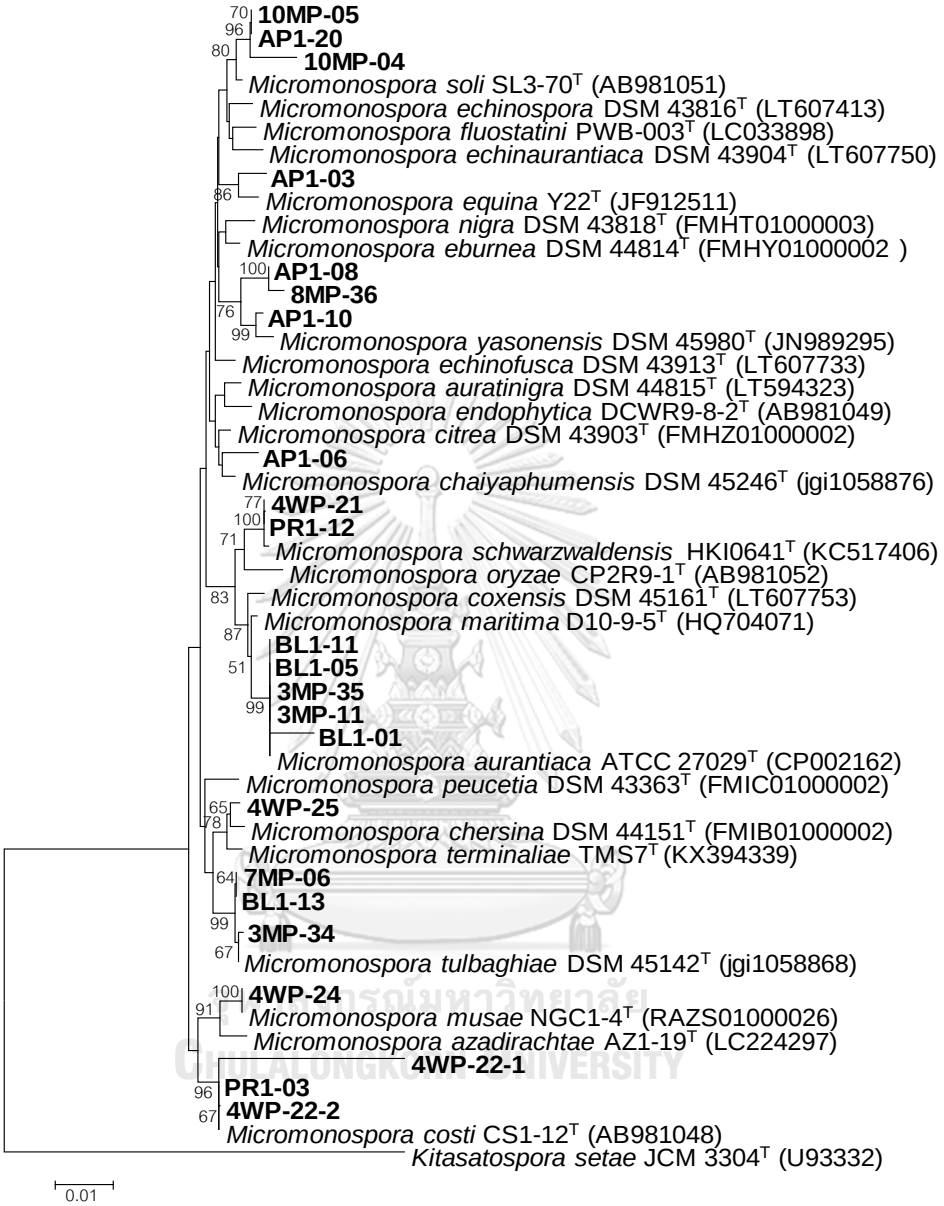

Figure 4.3 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequence between the isolated strains and related type strains of the genus *Micromonospora*.

The colonies of strain 4WP-24 were moderate orange and grew well on ISP2 agar medium. It grew at 20-40 °C, pH 4-10, and 3 % (w/v) maximum of NaCl. Nitrate reduction, starch hydrolysis, and skim milk peptonization were positive while skim milk coagulation and gelatin liquefaction were negative. It used D-glucose, *myo*-inositol, D-mannose, D-mannitol, and L-rhamnose as sole carbon sources. In API ZYM, positive activities were alkaline phosphatase, esterase (C4), esterase lipase (C8), valine aminopeptidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol- AS- BI-phosphohydrolase, and β-galactosidase. Weak positive activities were leucine aminopeptidase, cystine aminopeptidase, and α-glucosidase whereas negative activities were lipase (C14), α-fucosidase, α-galactosidase, β-glucuronidase, β-glucosidase, and α-mannosidase.

Its whole-cell hydrolysate composed of *meso*-diaminopimelic acid, glucose, xylose, mannose, and ribose. Major menaquinones were MK-9 (H_6) (27.3 %), MK-10 (H_8) (26.7 %), MK-10 (H_6) (25.9 %), and MK-9 (H_8) (19.4 %) while MK-9 (H_4) (7.3 %), and MK-10 (H_4) (3.6 %) were minor menaquinones. The phospholipid profile contained phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), diphosphatidylglycerol (DPG), unidentified phospholipids (PLs), and unidentified glycolipids (GLs) as corresponding to phospholipid type II (Lechevalier *et al.*, 1977). Predominant cellular fatty acids were iso-$C_{15:0}$ (31.2 %), *anteiso*-$C_{15:0}$ (14.9 %), *anteiso*-$C_{17:0}$ (12.2 %), $C_{17:0}$ (10.7 %), and *iso*-$C_{17:0}$ (9.0 %).

Almost complete 16S rRNA gene sequence of strain 4WP-24 (1379 nt) was closest related to *Micromonospora musae* NGC1-4 (99.85 %). The draft genome sequence of strain 4WP-24 (VSFB00000000) was 6,841,654 bp in size with the average *in silico* DNA G+C content of 72.8 mol %. The dDDH value between the genomes of strain 4WP-24, *M. musae* NGC1-4 (RAZS00000000), *M. musae* MS1-9^T (RAZT00000000) were 77.1 % (C.I. model 74.1-79.9 %) and 76.9 % (C.I. model 73.9-79.7 %), respectively. The ANIb values of the draft genomes between the strain 4WP-24, *M. musae* NGC1-4, and *M. musae* MS1-9^T were 97.20 and 97.19 %, respectively. The ANIm values of the draft genomes between the strain 4WP-24, *M. musae* NGC1-4, and *M. musae* MS1-9^T were 97.43 and 97.40 %, respectively. Phylogenetic tree and phylogenomic tree based on 16S rRNA gene, *gryB* gene, and whole-genome sequences indicated that strain 4WP-24 shared a clade with *M. musae* NGC1-4 and *M. musae* MS1-9^T (Figure 4.5, 4.6, and 4.4, respectively).

On the basis of a polyphasic approach, including phenotypic and genotypic characteristics as mentioned above, the strain 4WP-24 could be classified in the genus *Micromonospora*. The dDDH value between strain 4WP-24 (VSFB00000000) and closely related strains as *M. musae* NGC1-4 (RAZS00000000) and *M. musae* MS1-9^T (RAZT00000000) were significantly more than 70% (the threshold value for assigning in the same species) but lower than 79 % (the cutoff to distinguish subspecies) (Meier-Kolthoff *et al.*, 2014; Chun *et al.*, 2018). Although, both ANI values of strain 4WP-24 were more than the 95–96 % (the range of % cut-off for species delineation) that

indicate the strain 4WP-24, isolated from the root of *Mimosa pudica*, could be proposed to the new subspecies of *M. musae,* which named *Micromonospora musae* subsp. *mimosae* (Kuncharoen *et al.*, 2019; Chun *et al.*, 2018; Richter and Rosselló-Móra, 2009).

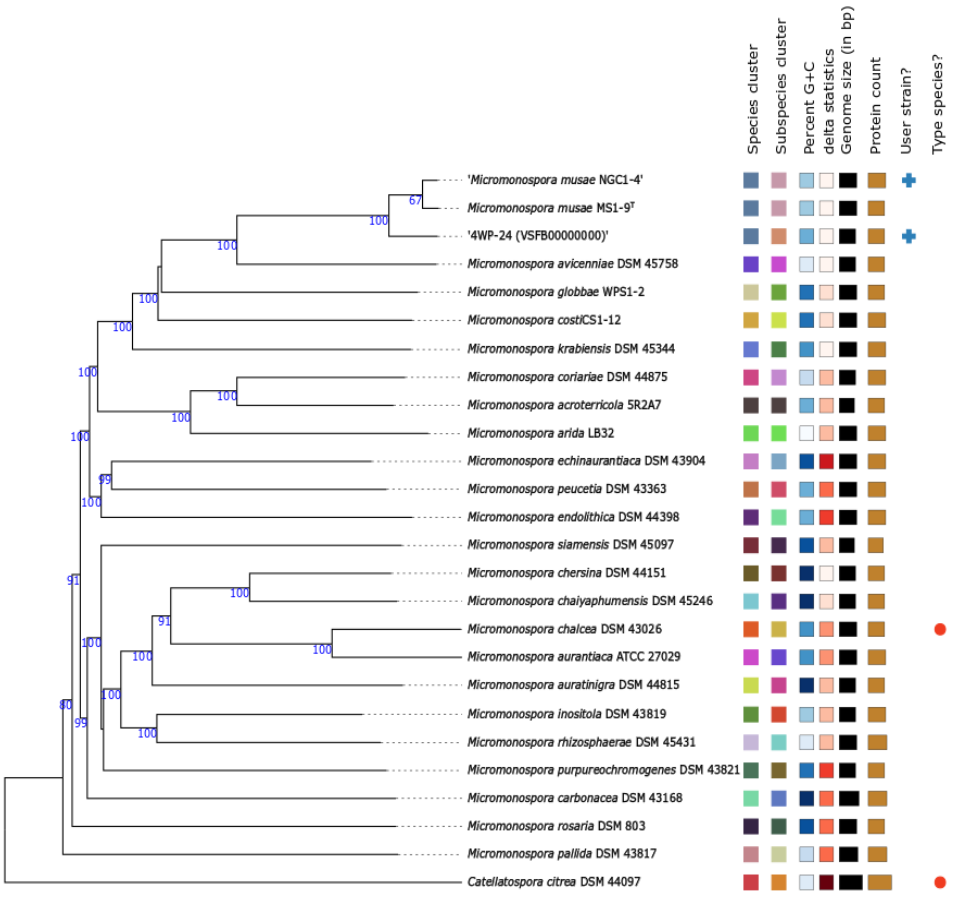

Figure 4.4 Phylogenomic tree based on TYGS result showing the relationship between the strain 4WP-24 and related type strains. The numbers above branches are GBDP pseudo-bootstrap support values from 100 replications.

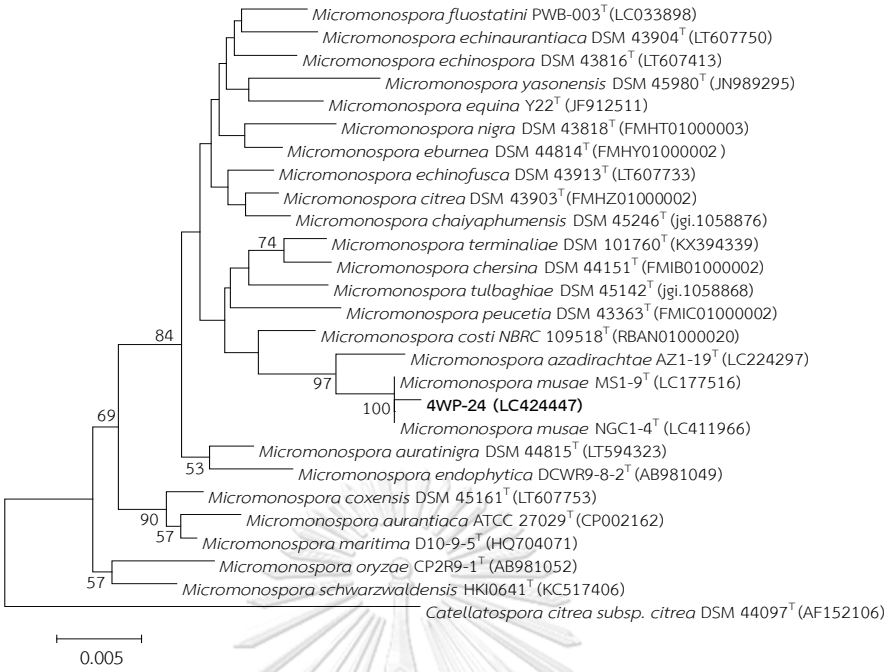

Figure 4.5 Neighbour-joining phylogenetic tree based on 16S rRNA gene between strain 4WP-24 and related strains of the genus *Micromonospora.*

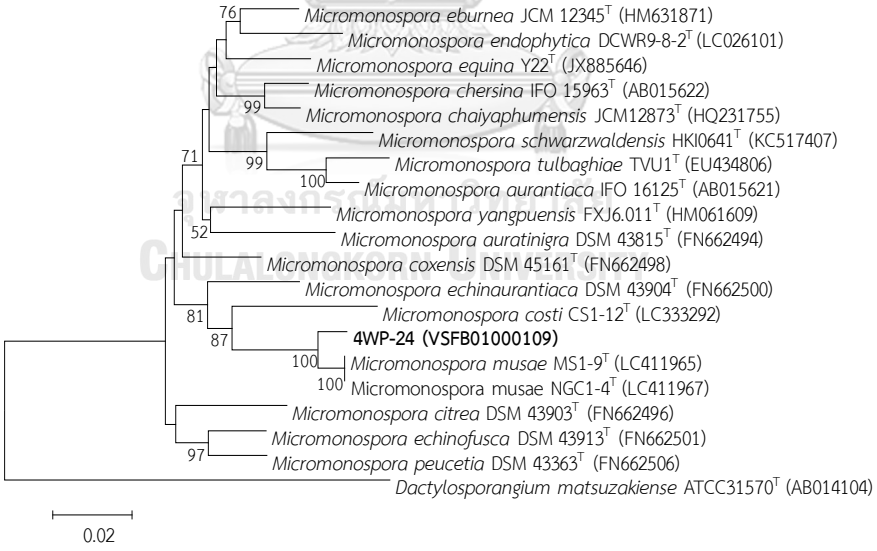

Figure 4.6 Neighbour-joining phylogenetic tree based on *gryB* gene between strain 4WP-24 and related strains of the genus *Micromonospora.*

4.3.2 Characterization of *Streptomyces* strains (Group II)

Eight strains PA1-07, 3MP-10, 3MP-12, 3MP-14, AP1-18, AP1-19, AP1-21, and BL1-06 were identified as *Streptomyces* that belonged to the family *Streptomycetaceae* based on morphology and 16S rRNA gene sequence (Figure 4.7). Strain PA1-07 was identified as *Streptomyces phyllanthi* (Klykleung *et al.*, 2016); strains 3MP-12 and BL1-06 were *S. albidoflavus* (Rong *et al.*, 2009); strains AP1-18, AP1-19, and AP1-21 were *S. seoulensis* (Chun *et al.*, 1997) while strains 3MP-10 and 3MP-14 were identified as *Streptomyces* sp.

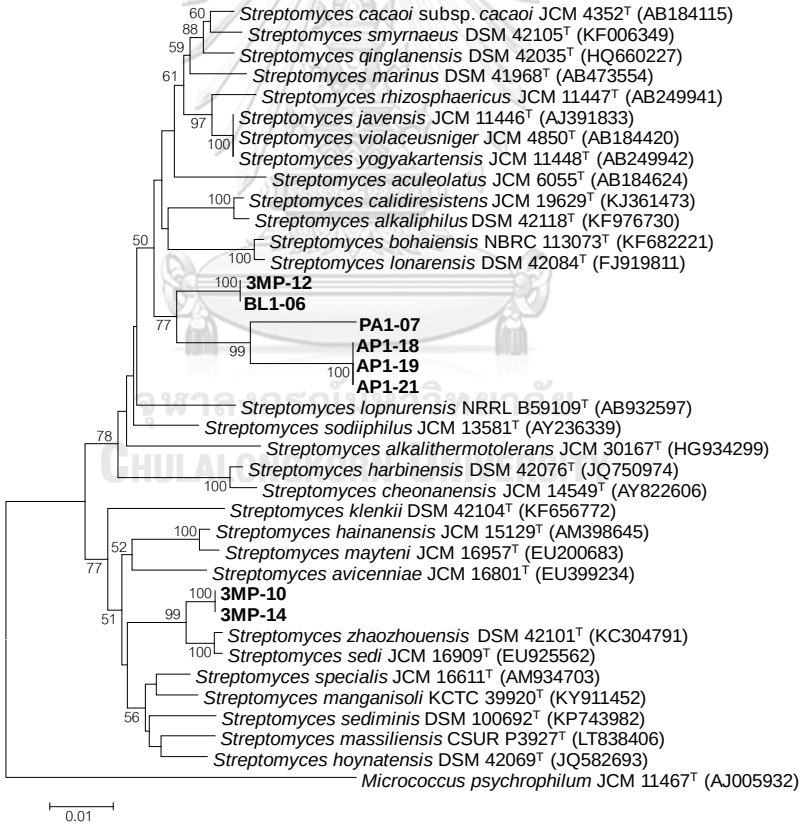

Figure 4.7 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequence between the isolated strains and related type strains of the genus *Streptomyces*.

They produced spore chains on the branched aerial and substrate mycelia on various media. In this study, strain 3MP-10 and 3MP-14 were selected to represent the genus *Streptomyces* that formed spiral smooth spore chains on aerial and substrate mycelium cultivated on ISP4 agar 27 °C for 30 days (Figure 4.8).

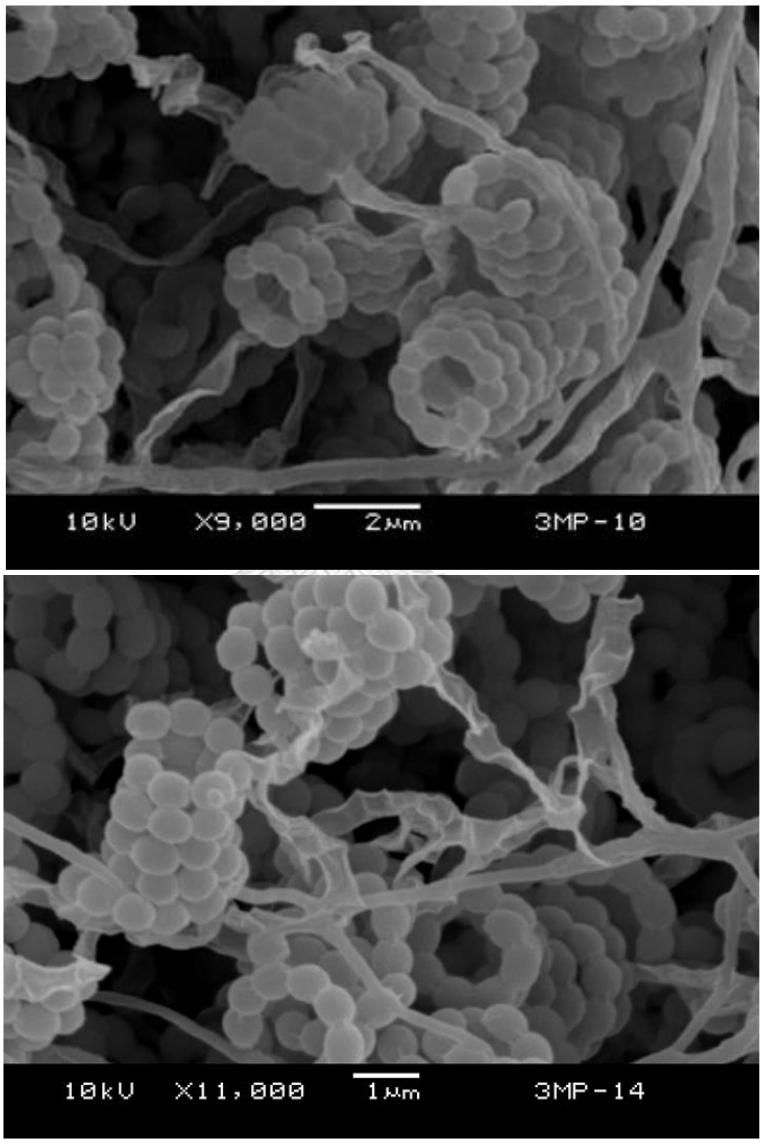

Figure 4.8 Scanning electron micrograph showed the spiral spore chains on aerial mycelia of strain 3MP-10 (A) and 3MP-14 (B) on ISP4 agar at 27 °C for 30 days.

Strain 3MP-10 and 3MP-14 grew well on ISP4 and nutrient agar, moderately on ISP2, ISP3, and nutrient agar whereas poorly on ISP5, ISP6, and ISP7 media**.** White aerial masses were formed on ISP3 and ISP4 media. The soluble pigment was not formed on tested media. Spiral spore chains with 4 to 6 turns, circular to elliptical shape (0.5-0.7 x 0.6-0.8 µm in size) and smooth on surface spores, were formed on ISP4 medium (Figure 4.8). The whole-cell hydrolysates of these strains contained *LL*-diaminopimelic acid, glucose, and ribose. The menaquinones were MK-9 (H_8) (15.8 %), MK-10 (H_6) (23.6 %), and MK-10 (H_8) (60.5 %). The major cellular fatty acid profile (>10 %) of strain 3MP-10^T comprised *iso*-$C_{16:0}$ (36.2 %), *anteiso*-$C_{15:0}$ (21.8 %), and *anteiso*-$C_{17:0}$ (13.0 %) while strain 3MP-14 was *iso*-$C_{16:0}$ (50.8 %). The phospholipid profiles consisted of phosphatidylethanolamine (PE), diphosphatidylglycerol (DPG), phosphatidylinositol (PI), phosphatidylglycerol (PG), and phosphatidylinositol mannoside (PIMs). Growth of these strains occurred at 20-37 °C (optimum 30-37 °C), pH 6.0-11.0 (optimum pH 7.0-9.0), and 0-3 % (w/v), respectively. Their typical characteristics showed consistent with other members of the genus *Streptomyces*. They could be distinguished from related type strains based on phenotypic characteristics, including colony color, maximum NaCl tolerance, pH range and temperature for growth, starch hydrolysis, gelatin liquefaction, nitrate reduction, as well as utilization of L-arabinose, *myo*-inositol, D-fructose, D-galactose, glucose, lactose, maltose, D-mannose, mannitol, D-rhamnose, and D-ribose. In API ZYM, positive activities were observed for esterase (C4), esterase lipase (C8), lipase (C14), chymotrypsin, leucine aminopeptidase, valine

aminopeptidase, cystine aminopeptidase, acid phosphatase, phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, trypsin, and α-glucosaminidase. Weak positive activities were alkaline phosphatase and α-fucosidase while negative activity was β-glucuronidase (Table 4.5 to 4.7).

The 16S rRNA gene sequences of strain 3MP-10 and 3MP-14 revealed that they closely related to *S. zhaozhouensis* DSM 42101^T (98.87 and 98.76 %, respectively) and *S. sedi* JCM 16909^T (98.56 and 98.45 %, respectively). Phylogenetic tree and phylogenomic tree showed that they were on the same node and shared the cluster with *S. zhaozhouensis* DSM 42101^T and *S. sedi* JCM 16909^T (Figures 4.9 and 4.10). The draft genome sequences of strain 3MP-10 (VDLY00000000) and 3MP-14 (VDLZ00000000) were 7,198,341 and 7,197,316 bp in size, respectively, with their average *in silico* DNA G+C content of 73.4 mol %. The dDDH value between the genomes of the strain 3MP-10 and 3MP-14 was 100.00 % (C.I. model 100.0-100.0 %) while the dDDH values between the genomes of these strains and related species, including *S. zhaozhouensis* DSM 42101^T (OCNE00000000) and *S. sedi* JCM 16909^T (VDGT00000000) were 29.9 % (C.I. model 27.5-32.4 %) and 29.6 % (C.I. model 27.2-32.1 %), respectively**.** These values exhibited significantly lower than the threshold value of 70 % commonly used to delineate separated species status (Chun *et al.*, 2018). In addition, ANIb and ANIm values of the draft genomes between the strain 3MP-10 and 3MP-14 were 100.00 %. ANIb values between the strains 3MP-10, 3MP-14, and *S. zhaozhouensis* DSM 42101^T were 84.94 and 84.88 % while ANIb values between the

strain 3MP-10, 3MP-14, and *S. sedi* JCM 16909^T were 84.77 and 84.74 %. ANIm values between these strains and related species, *S. zhaozhouensis* DSM 42101^T and *S. sedi* JCM 16909^T were 88.01 and 87.92 %, respectively (Table 4.8). Both ANI values were (B) clearly lower than the 95–96 % cut-off for species delineation (Richter and Rosselló-Móra, 2009).

On the basis of phenotypic and chemotaxonomic characteristics, 16S rRNA gene analysis as well as genome analysis, strains 3MP-10 and 3MP-14 have represented the same novel species for which the name *Streptomyces mimosae* is proposed, with type strain 3MP-10^T (= JCM 33328^T = TISTR 2646^T).

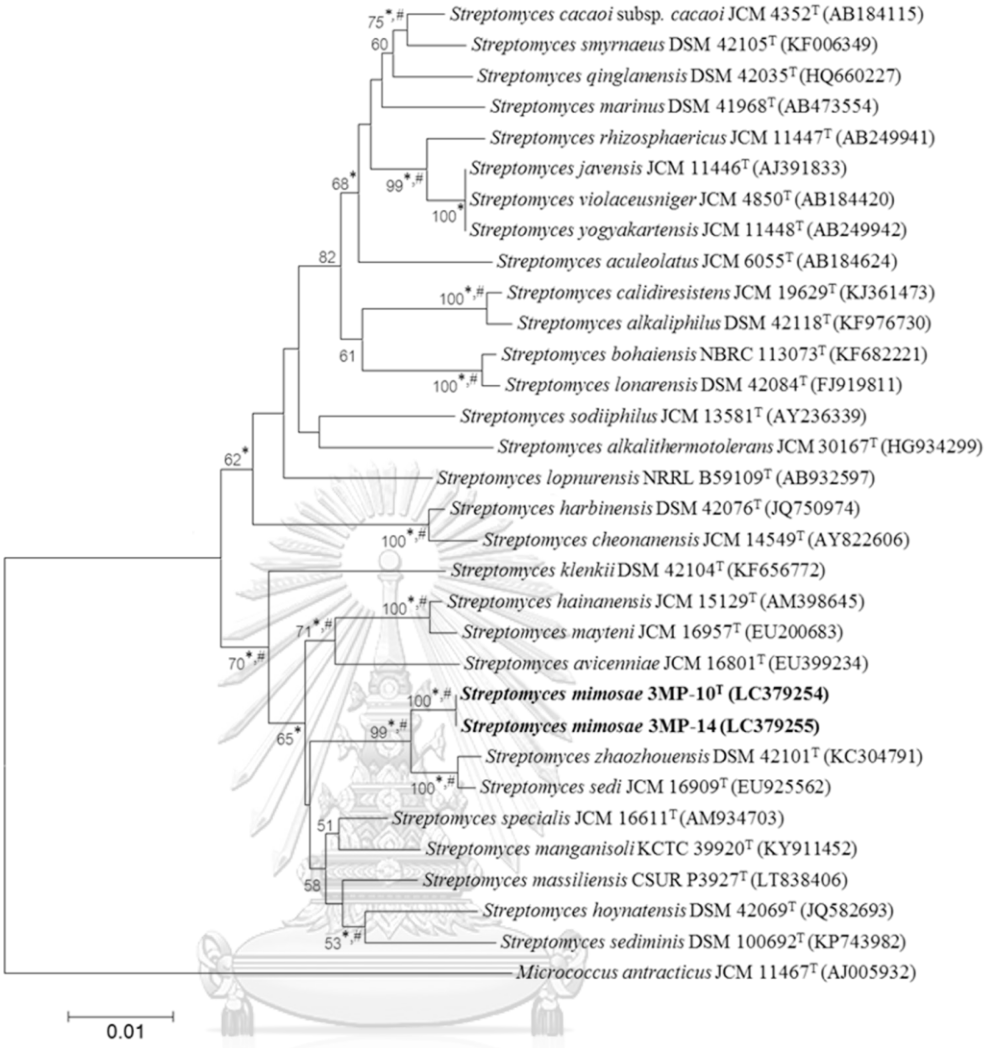

Figure 4.9 Neighbour-joining phylogenetic tree based on 16S rRNA gene between strain 3MP-10, 3MP-14, and related strains of the genus *Streptomyces. Micrococcus antarcticus* JCM 11467^{T} was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

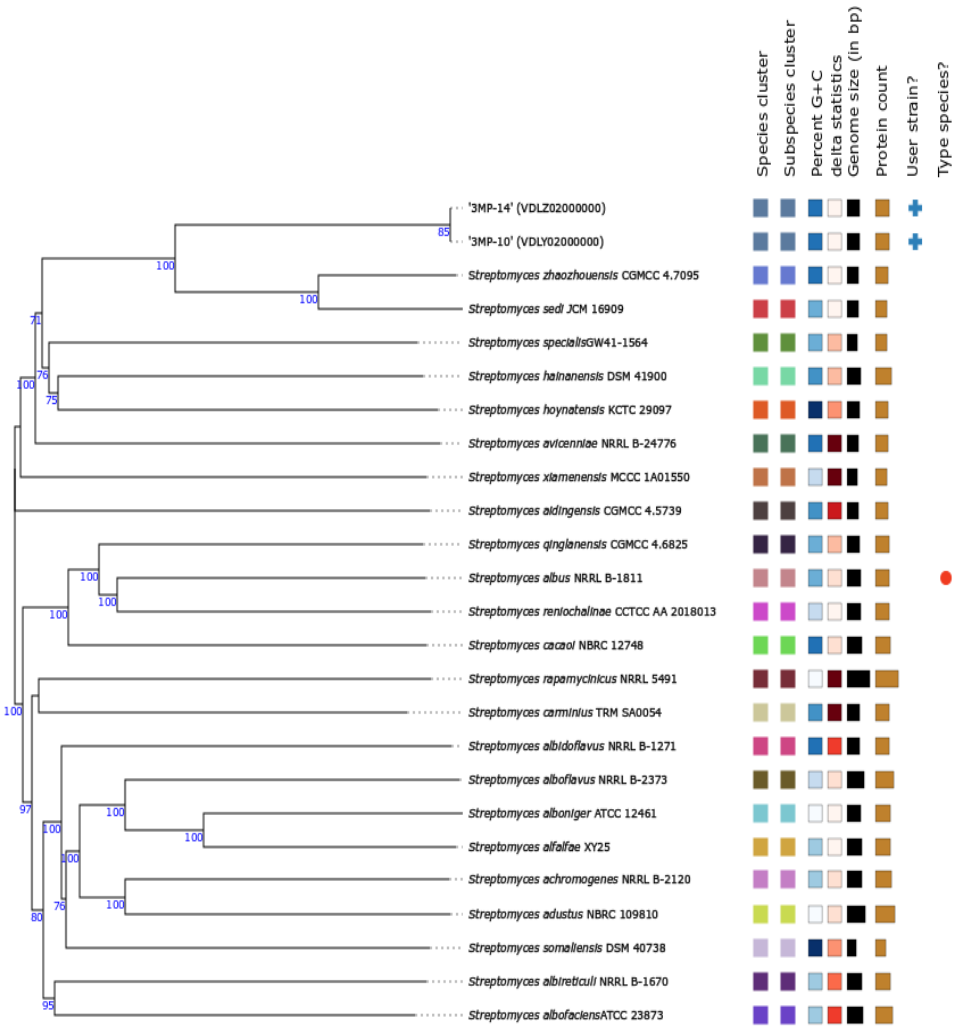

Figure 4.10 Phylogenomic tree based on TYGS result showing the relationship between the strain 3MP-10, 3MP-14, and related type strains. The numbers above branches are GBDP pseudo-bootstrap support values from 100 replications.

Table 4.5 Cultural characteristics of 3MP-10 and 3MP-14.

Medium	Characteristic	3MP-10	3MP-14
ISP 2	Growth	Moderate	Moderate
	Aerial mycelium	Pale yellow	Pale yellow
	Substrate mycelium	Vivid yellowish green	Vivid yellowish
	Soluble pigment	None	None
ISP 3	Growth	Moderate	Moderate
	Aerial mycelium	White	White
	Substrate mycelium	Pale yellowish	Pale yellowish
	Soluble pigment	None	None
ISP 4	Growth	Good	Good
	Aerial mycelium	White	White
	Substrate mycelium	Pale yellowish	Pale yellowish
	Soluble pigment	None	None
ISP 5	Growth	Poor	Poor
	Aerial mycelium	Pale yellow	Pale yellow
	Substrate mycelium	Light yellow green	Light yellow green
	Soluble pigment	None	None
ISP 6	Growth	Poor	Poor
	Aerial mycelium	Pale yellowish	Pale yellowish
	Substrate mycelium	Pale yellow	yellow
	Soluble pigment	None	None
ISP 7	Growth	Poor	Poor
	Aerial mycelium	Pale yellow	Pale yellow
	Substrate mycelium	yellow	yellow
	Soluble pigment	None	None
NA	Growth	Good	Good
	Aerial mycelium	Pale yellow	Pale yellow
	Substrate mycelium	Pale yellowish	Pale yellowish
	Soluble pigment	None	None

Table 4.6 Differential characteristics of 3MP-10, 3MP-14 and related type strains. Strains: 1, 3MP-14; 2, 3MP-10; 3, *S. zhaozhouensis* DSM 42101^{T}; and 4, *S. sedi* JCM 16909^{T}. +, positive; w, weakly positive; -, negative.

Characteristic	1	2	3	4
Soluble pigment on ISP3	None	None	None	Dark green
NaCl tolerance (%, w/v)	0-3	0-3	0-7	0-5
pH range for growth	6-11	6-11	6-10	7-8
Growth temperature (°C)	20-37	20-37	15-37	15-37
Nitrate reduction	-	-	+	-
Starch hydrolysis	+	+	+	-
Gelatin liquefaction	-	-	+	-
Utilization of:				
myo-Inositol	+	w	-	-
D-Fructose	+	+	-	+
Lactose	+	+	+	-
D-Rhamnose	+	+	-	-
D-Ribose	+	+	-	-
Maltose	W	w	+	+
Arabinose	+	+	-	-
Galactose	+	+	+	-
Mannitol	+	+	+	-
Mannose	+	+	-	-
Enzyme activity of:				
Esterase (C4)	+	+	+	-
Leucine	+	+	w	+
Valine aminopeptidase	+	+	+	+
Cystine	w	+	+	w
Trypsin	+	+	-	-
Chymotrypsin	w	+	+	-
Alkaline phosphatase	w	w	-	-
Acid phosphatase	+	+	+	-
α-Galactosidase	+	+	-	w
β-Galactosidase	+	+	-	+
α-Glucosidase	+	+	+	-
β-Glucosidase	+	+	+	-
α-Glucosaminidase	+	+	-	w
α-Mannosidase	+	+	-	w
α-Fucosidase	+	w	-	-

Table 4.7 Cellular fatty acid compositions (%) of strain 3MP-10, 3MP-14, and related type strains.

Strains: 1, 3MP-10; 2, 3MP-14; 3, *S. zhaozhouensis* DSM 42101^T; and 4, *S. sedi* JCM 16909^T.

Fatty acid	1	2	3	4
Saturated fatty acids				
$C_{16:0}$	5.8	2.0	6.7	4.3
$C_{17:0}$	-	2.0	1.2	2.2
$C_{17:0}$ cyclo	-	1.4	-	1.7
$C_{18:0}$	2.0	-	1.7	-
Unsaturated fatty acids				
$C_{17:1}$ **ω**8c	-	-	3.4	3.7
$C_{18:1}$ **ω**9c	-	-	1.8	-
Branched fatty acids				
i-$C_{10:0}$	-	4.5	-	-
i-$C_{14:0}$	1.9	-	-	-
i-$C_{15:0}$	4.1	2.6	-	1.3
a-$C_{15:0}$	21.8	2.8	3.1	2.4
i-$C_{16:0}$	36.2	50.8	31.6	45.9
i-$C_{16:0}$ H	-	-	-	-
i-$C_{17:0}$	3.5	1.7	-	1.8
i-$C_{17:0}$ 3OH	-	1.8	-	-
a-$C_{17:0}$	13.0	7.2	14.4	14.6
i-$C_{18:0}$	1.0	1.2	-	1.9
3OH-$C_{8:0}$	-	1.7	-	-
Unsaturated Branched				
a-$C_{15:1}$ A	-	-	1.2	-
i-$C_{16:1}$ G	-	7.7	10.0	6.1
a-$C_{17:1}$ **ω**9c	3.4	5.3	8.9	5.0
i-$C_{18:1}$ H	-	-	1.2	-
Summed feature 3[a]	-	-	6.6	1.8
Summed feature 5[b]	1.3	-	-	-
Summed feature 8[c]	-	-	1.5	-
Summed feature 9[d]	1.9	3.2	1.2	2.5

Values are percentages of total cellular fatty acids. -, not detected.

[a]Summed feature 3, $C_{16:1}$ **ω**7c / $C_{16:1}$ **ω**6c; [b]Summed feature 5, anteiso-$C_{18:0}$ / $C_{18:2}$ **ω**6,9c; [c]Summed feature 8, $C_{18:1}$ **ω**7c; [d]Summed feature 9, 10Me-$C_{16:0}$

Table 4.8 ANIb and ANIm values (%) and the digital (*in silico*) DNA-DNA hybridization (dDDH) values between the draft genomes of strain 3MP-10, 3MP-14, and related type strains.

Draft genomes: 1, Strain 3MP-10; 2, Strain 3MP-14; 3, *S. zhaozhouensis* DSM 42101^{T}; and 4, *S. sedi* JCM 16909^{T}.

Query genome	Reference genome	ANIb	ANIm	% dDDH (Formula 2*)	Model C.I.	Distance	Prob. DDH >= 70 %	G+C difference
1	2	100.00	100.00	100.0	100 – 100 %	0.0000	98.30	0.00
1	3	84.94	88.01	29.9	27.5 – 32.4 %	0.1429	0.10	0.02
1	4	84.77	87.92	29.6	27.2 – 32.1 %	0.1443	0.09	0.48
2	1	100.00	100.00	100.0	100 – 100 %	0.0000	98.30	0.00
2	3	84.88	88.01	29.9	27.5 – 32.4 %	0.1429	0.10	0.02
2	4	84.74	87.92	29.6	27.2 – 32.1 %	0.1443	0.09	0.48

*Recommended formula (identities/HSP length), which is liberated of genome length and is thus prosperous against the use of incomplete draft genome.

4.3.3 Characterization of *Microbispora* strains (Group III)

Three strains CR1-01, CR1-08, and CR1-09 were identified as *Microbispora* that belonged to the family *Streptosporangiaceae* based on morphology and 16S rRNA gene sequence (Figure 4.11). Strain CR1-09 was identified as *Microbispora catharanthi* (Klykleung *et al.*, 2019a) while strains CR1-01 and CR1-08 were *Microbispora* sp. They produced paired-spores on the branched aerial and substrate mycelia on various media. In this study, strain CR1-09^{T} were selected to represent the genus *Microbispora* that formed smooth paired-spores on aerial and substrate mycelium cultivated on ISP4 agar 30 °C for 14 days (Figure 4.11).

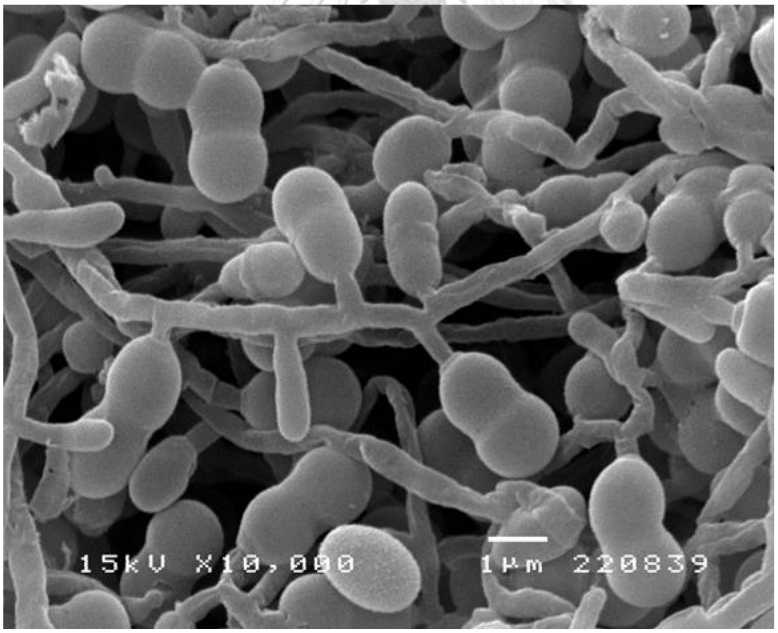

Figure 4.11 Scanning electron micrograph of *Microbispora* sp. CR1-09^{T} after incubation on ISP2 agar at 30 °C for 14 days.

Strain CR1-09^T grew well on yeast extract-dextrose, ISP2 and ISP3 media, moderately on ISP7, Czapek's agar, and nutrient agar whereas poorly on ISP 4, ISP5 and ISP6 media. Pinkish white aerial masses were formed on various agar media. Spores chains, a pair of ovular to circular shape and smooth on surface spores, were observed only on aerial mycelia (Figure 4.11). Moderate to very dark purple pigments could be observed on ISP2, ISP3, and ISP4 media. It reduced nitrate and peptonized milk. It grew at 20-45 °C, at pH 6-10 and in the presence of 3 % (w/v) NaCl. It did not coagulate the skimmed milk, gelatin liquefaction, and hydrolyze starch. Utilizes D-galactose, D-raffinose, myo-Inositol, and D-sorbitol but does not utilize L-arabinose, glycerol, lactose, and L-rhamnose as sole carbon sources (Table 4.9).

Strain CR1-09^T showed typical characteristics consistent with those members of the genus *Microbispora*. The cell wall peptidoglycans of strain CR1-09^T contained *meso*-diaminopimelic acid. Madurose, glucose, galactose and a small amount of xylose, rhamnose, and ribose were detected in whole-cell hydrolysates. The phospholipids are diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), hydroxyl phosphatidylethanolamine (OH-PE), phosphatidylinositol mannosides (PIMs), phosphatidylglycerol (PG), phosphatidylinositol (PI), and unidentified ninhydrin positive phosphoglycolipids (NPPGs). Based on the presence of undescribed ninhydrin phosphoglycolipids, this polar lipid pattern could be classified as type PIV (Lechevalier *et al.*, 1977). The menaquinones were MK-9 (H_4) (50 %), MK-9 (H_2) (34 %), MK-9 (11 %),

and MK-9 (H_6) (5 %). The major cellular fatty acid profile (>10 %) comprised *iso*-$C_{16:0}$ (44.3 %), $C_{17:0}$ (12.4 %), and $C_{16:0}$ (11.9 %). The G+C content of genomic DNA was 71.2 mol % (Table 4.10).

The BLAST analysis based on almost complete 16S rRNA gene (1469 nt) revealed the strain closely related to *M. tritici* DSM 104650^T (99.5 %), *M. triticiradicis* DSM 104649^T (99.3 %), *M. hainanensis* DSM 45428^T (99.0 %), *M. bryophytorum* DSM 46710^T (98.8 %), *M. camponoti* DSM 100527^T (98.6 %), *M. siamensis* JCM 17237^T (98.5 %), and *M. corallina* DSM 44682^T (98.4 %), respectively. The phylogenetic tree showed that the strain was shared the cluster with *M. tritici* DSM 104650^T and *M. triticiradicis* DSM 104649^T (Figure 4.12). Based on the highest 16S rRNA gene sequence similarity and phylogenetic relationship, *M. tritici* DSM 104650^T and *M. triticiradicis* DSM 104649^T were selected for comparative purposes to confirm the novel species status of this strain. The draft genome sequence of strain CR1-09^T (VDMA00000000) was 9.24 Mb in size with an average *in silico* DNA G+C content of 71.2 mol %. Phylogenomic tree based on TYGS result showed the relationship between the strain CR1-09^T with related type strains (Figure 4.13) indicated the strain CR1-09^T shared the same node with *M. bryophytorum* DSM 46710^T as provided on 2019-09-12. The ANIb and ANIm values of the draft genomes between strain CR1-09^T and its related species, *M. tritici* DSM 104650^T (85.5 and 89.2 %, respectively), *M. triticiradicis* DSM 104649^T (85.6 and 89.2 %, respectively), and *M. bryophytorum* DSM 46710^T (91.89 and 93.87 %, respectively) using

JSpeciesWS (Richter *et al.*, 2016). Both ANI values were clearly lower than the 95–96 % cut-off for species delineation (Richter and Rosselló-Móra, 2009). In addition, the dDDH values between the genomes of the strain CR1-09^{T} and its related species as *M. tritici* DSM 104650^{T} (VSEX00000000), *M. triticiradicis* DSM 104649^{T} (QFZU00000000), and *M. bryophytorum* DSM 46710^{T} (VIRL00000000) were 34.9 % (C.I. model 32.4 -37.5 %), 33.9 % (C.I. model 31.5-36.4 %), and 52.6 % (C.I. model 49.9-55.3 %) as shown in Table 4.11. These values exhibited significantly lower than the threshold value of 70 % commonly used to delineate separated species status (Chun *et al.*, 2018).

On the basis of phenotypic and chemotaxonomic characteristics, 16S rRNA gene analysis as well as DNA-DNA hybridization, strain CR1-09^{T} (= JCM 30045^{T} = TISTR 2273^{T}) could be classified as a member of the genus *Microbispora* and represented the novel species of the genus *Microbispora* for which the name *Microbispora catharanthi* is proposed. The etymology of *M. catharanthi* is can.thar.an'thi. N.L. gen. n. *catharanthus* of the host plant genus *Catharanthus*.

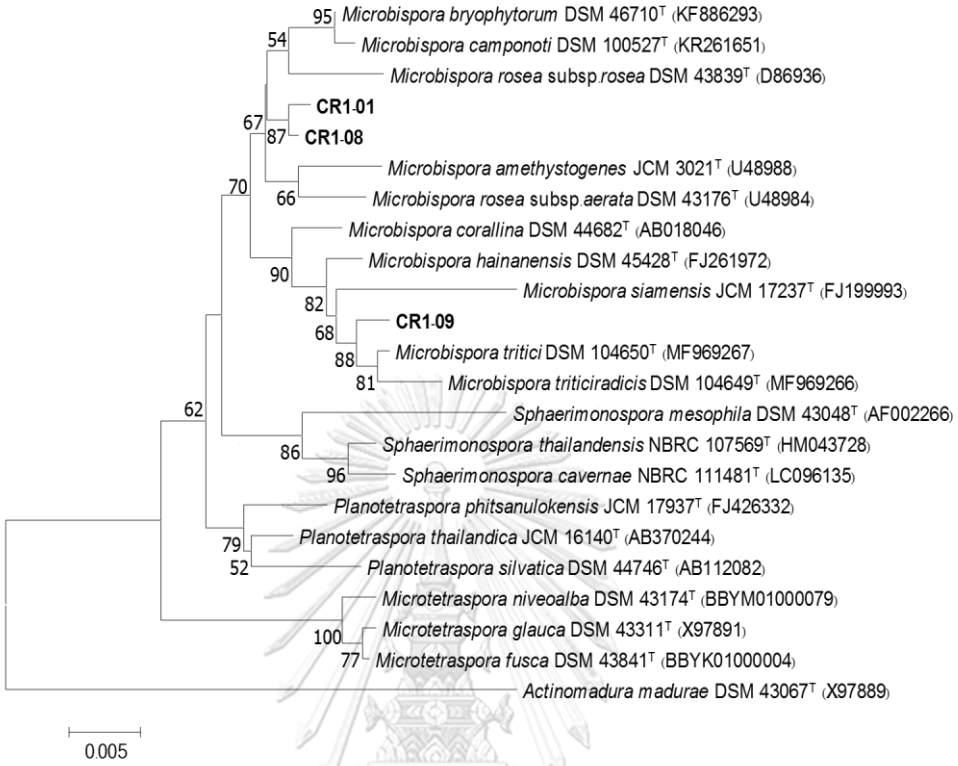

Figure 4.12 Neighbour-joining phylogenetic tree based on almost complete 16S rRNA gene showing the relationship between strain CR1-01, CR1-08, CR1-09^{T}, and other members of the genus *Microbispora.*

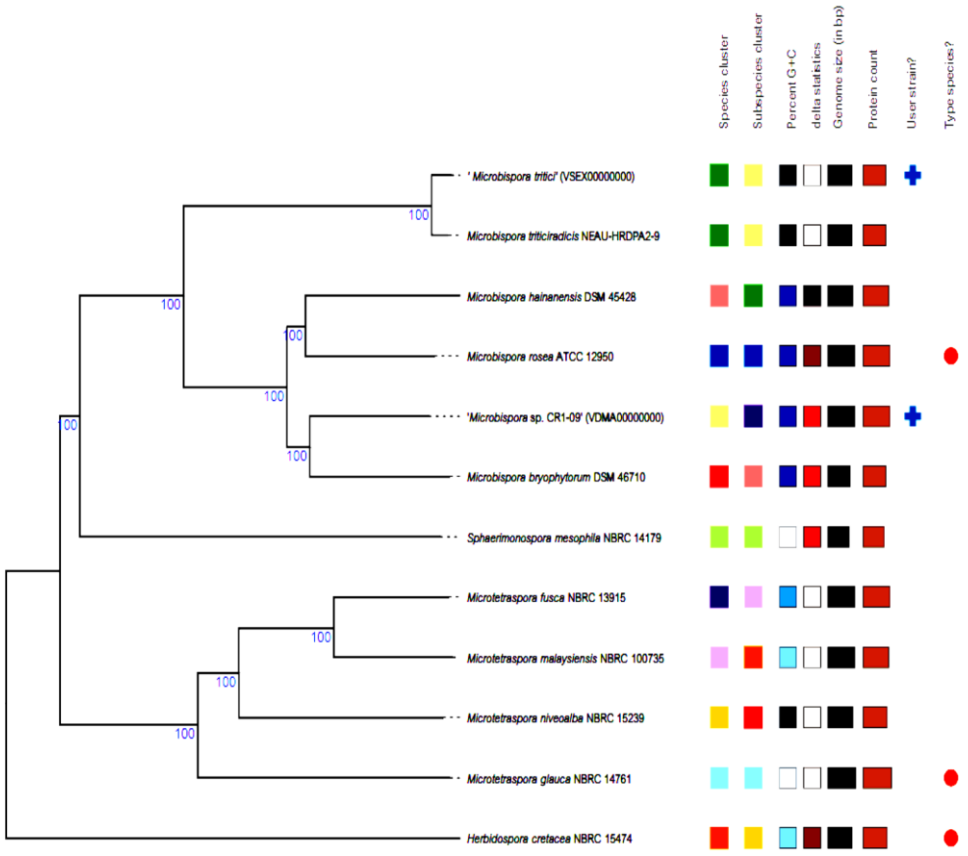

Figure 4.13 Phylogenomic tree based on TYGS result showing the relationship between strain CR1-09^{T} and related type strains. The numbers above branches are GBDP pseudo-bootstrap support values from 100 replications.

Table 4.9 Differential characteristics of CR1-09^T and related *Microbispora* species. Strains: 1, CR1-09^T; 2, *M. tritici* DSM 104650^T; and 3, *M. triticiradicis* DSM 104649^T. +, positive; w, weakly positive; -, negative.

Characteristic	1	2	3[a]
Colony color on ISP2	Blackish red	Yellowish brown	Dark reddish brown
Soluble pigment on ISP2	Very dark purple	-	-
NaCl tolerance (%w/v)	0-3	0-2	0-3.5
pH range for growth	6-10	5-8	5-10
Growth temperature (℃)	20-45	20-50	20-45
Nitrate reduction	+	-	-
Starch hydrolysis	-	+	-
Utilization of:			
myo-Inositol	+	-	-
Lactose	-	+	-
D-Raffinose	+	+	-
D-Sorbitol	+	w	+
D-Ribose	w	-	-
D-Xylose	w	-	+

. [a]Data from the previous study (C. Han *et al.*, 2018).

Table 4.10 Cellular fatty acid compositions (%) of CR1-09^T and related *Microbispora* species.

Strains: 1, CR1-09^T; 2, *M. tritici* DSM 104650^T; and 3, *M. triticiradicis* DSM 104649^T. Data are from this study.

Fatty acid	1	2	3[c]
Saturated fatty acids			
$C_{14:0}$	2.5	2.5	-
$C_{15:0}$	-	-	1.3
$C_{16:0}$	11.9	-	27.9
$C_{17:0}$	12.4	7.9	-
$C_{18:0}$	1.9	3.4	-
Unsaturated fatty acids			
$C_{16:1}$ **ω**7c	-	-	6.9
$C_{17:1}$ **ω**6c	1.3	-	-
$C_{17:1}$ **ω**7c	-	-	3.8
$C_{17:1}$ **ω**8c	2.9	3.5	-
$C_{18:1}$ **ω**9c	-	-	3.9
Branched fatty acids			
i-$C_{14:0}$	2.4	1.3	-
i-$C_{15:0}$	4.2	13.1	-
a-$C_{15:0}$	1.3	5.0	-
i-$C_{16:0}$	44.3	26.9	30.7
i-$C_{16:0}$ H	1.2	-	-
i-$C_{17:0}$	1.2	4.8	1.5
a-$C_{17:0}$	2.0	7.8	4.8
10Me-$C_{17:0}$	6.4	10.6	19.2
10Me-$C_{18:0}$TBSA	-	2.1	-
Summed feature 3[a]	-	3.9	-
Summed feature 9[b]	4.2	7.1	-

Values are percentages of total cellular fatty acids. -, not detected

[a]Summed feature 3, $C_{16:1}$**ω**7c/ iso-C_{15} 2OH/ $C_{16:1}$ **ω**6c;

[b]Summed feature 9, $C_{16:0}$ 10-methyl; [c]Data from the previous study (C. Han *et al.*, 2018).

Table 4.11 ANIb and ANIm values (%) and the digital (in silico) DNA-DNA hybridization (dDDH) values between the draft genome of strain CR1-09^T and its closest related type strains.

Draft genomes: 1, Strain CR1-09^T; 2, *M. tritici* DSM 104650^T; 3, *M. triticiradicis* DSM 104649^T; and 4, *M. bryophytorum* DSM 46710^T.

Query genome	Reference genome	ANIb	ANIm	% dDDH (Formula 2*)	Model C.I.	Distance	Prob. DDH >= 70 %	G+C difference
1	2	83.45	89.28	34.9	32.4 – 37.5 %	0.1026	1.07	0.49
1	3	83.45	89.28	33.9	31.5 – 36.4 %	0.1228	0.46	0.46
1	4	91.89	93.87	52.6	49.9 – 55.3 %	0.0659	17.91	0.09

*Recommended formula (identities/HSP length), which is liberated of genome length and is thus prosperous against the use of incomplete draft genome.

4.3.4 Characterization of *Nonomuraea* strains (Group IV)

Two strains PA1-05 and PA1-10^T were identified as *Nonomuraea* that belonged to the family *Streptosporangiaceae* based on morphology and 16S rRNA gene sequence (Figure 4.15). Strain PA1-10 was identified as *Nonomuraea phyllanthi* (Klykleung *et al.*, 2019b) while PA1-05 was identified as *Nonomuraea* sp. They produced spore chain or pseudosporangia on the branched aerial and substrate mycelia on various media. In this study, strain PA1-10^T was selected to represent the genus *Nonomuraea* that formed the rugose spiral spore chain and pseudosporangia on aerial and substrate mycelium cultivated on ISP3 agar plate at 30°C for 14 days (Figure 4.14).

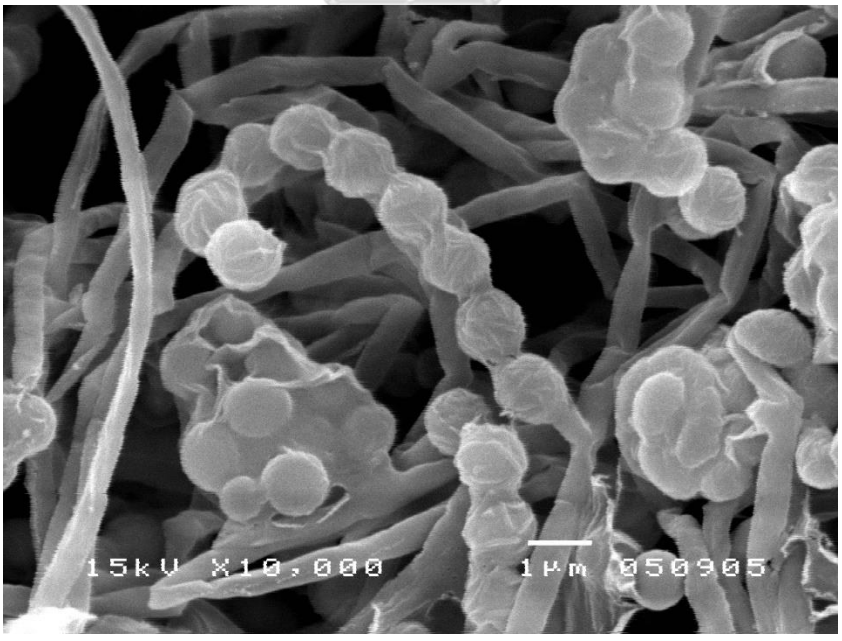

Figure 4.14 Scanning electron micrograph of *Nonomuraea* sp. PA1-10^T after incubation on ISP3 agar at 30 °C for 14 days.

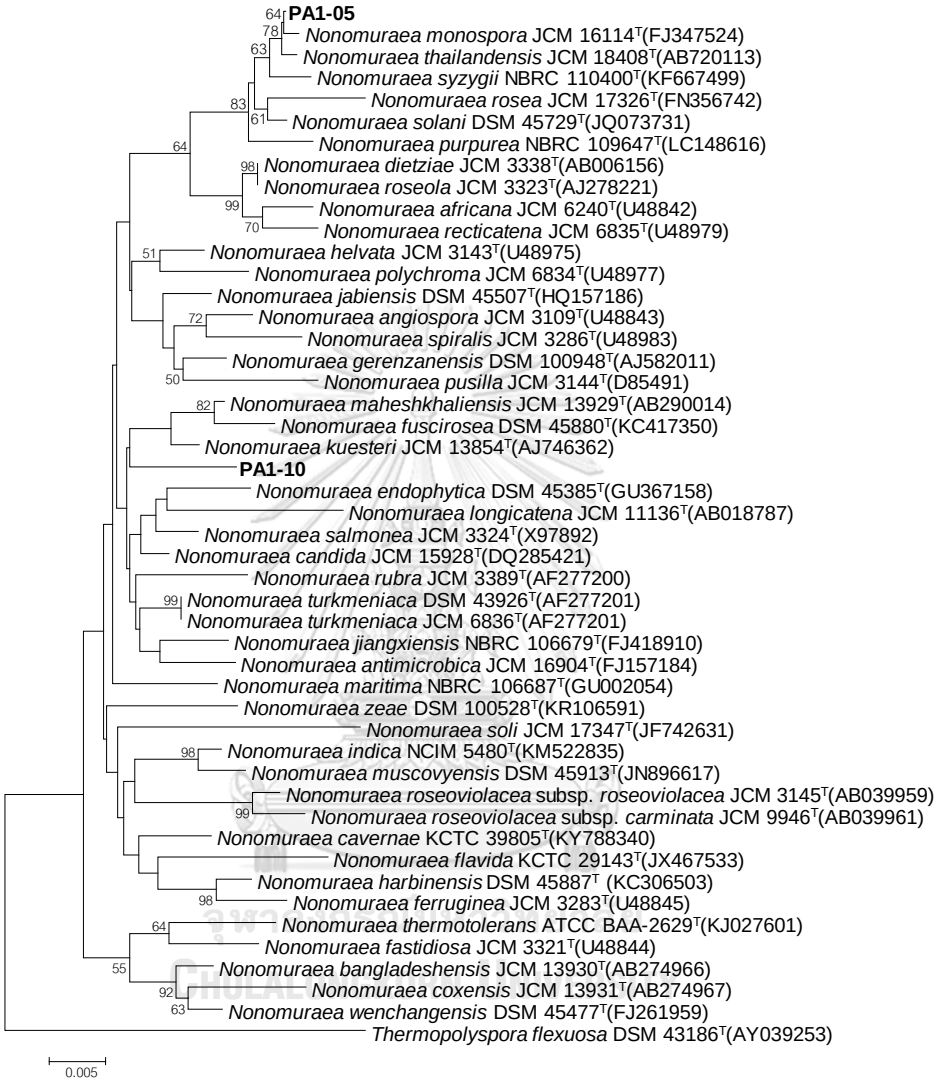

Figure 4.15 Neighbour-joining phylogenetic tree based on almost complete 16S rRNA gene showing the relationship between strain PA1-05, PA1-10^{T}, and other members of the genus *Nonomuraea.*

The strain PA1-10^T formed abundant white aerial mycelia and yellow to brown vegetative mycelia on yeast extract-malt extract (ISP 2), oatmeal (ISP 3), inorganic salts-starch (ISP 4), glycerol-asparagine (ISP 5), peptone-yeast extract iron (ISP 6), tyrosine (ISP 7) and nutrient agar plates. It grew at 15-40 °C, pH 5-9 and on 4 % (w/v) NaCl. Nitrate reduction was positive. Gelatin liquefaction, starch hydrolysis, skim milk coagulation and skim milk peptonization were negative. It utilized D-glucose, *myo*-inositol, D-mannose, D-melibiose, L-raffinose, sucrose but did not use L-arabinose, cellobiose, cellulose, fructose, glycerol, D-mannitol, L-rhamnose and xylose (Table 4.12). This strain contained *meso*-diaminopimelic acid, galactose, glucose, madurose, mannose, and ribose in whole-cell hydrolysates. Predominant menaquinones were MK-9 (H_4) (78.3 %), MK-9 (H_2) (14.6 %), and MK-9 (H_6) (7.1 %). Major cellular fatty acid was *iso*-$C_{16:0}$ (32.2 %) and $C_{17:0}$ 10-methyl (11.2 %) while other fatty acids were $C_{16:0}$ (8.3 %), *iso*-$C_{15:0}$ (6.9 %), $C_{17:0}$ (5.6 %), $C_{17:1}\omega 8C$ (5.4 %), and *anteiso*-$C_{17:0}$ (5.0 %) (Table 4.13). The strain showed the same fatty acids profiles with the related type strains but only the amounts were different. The phospholipid profiles composed of phosphatidylethanolamine (PE), methylphosphatidylethanolamine (PME), *lyso*-phosphatidylethanolamine (*lyso*-PE), phosphatidylinositolmannoside (PIM), phosphatidylglycerol (PG), and diphosphatidylglycerol (DPG).

The BLAST search of 16S rRNA gene sequence revealed that the strain PA1-10^T showed 98.35 % similarity with *Nonomuraea candida* JCM 15928^T and less than 98.0%

with other *Nonomuraea* species. The NJ tree of 16S rRNA gene sequences revealed that the strain PA1-10^T formed a node with *Nonomuraea maritima* JCM 18321^T (Figure 4.16). Based on both of the highest 16S rRNA gene sequence similarity and phylogenetic relationship, *N. candida* JCM 15928^T, and *N. maritima* JCM 18321^T were selected for further comparative purposes to confirm the novel species status of the strain PA1-10^T. The draft genome sequence of the strain PA1-10^T (VDLX02000000) is 11.0 Mb in size with an average *in silico* DNA G+C content of 71.2 mol %. The ANIb and ANIm values of the draft genomes between the strain PA1-10^T and its closest related species, *N. candida* JCM 15928^T (82.7 and 81.7 %, respectively) and *N. maritima* JCM 18321^T (87.3 and 86.38 %, respectively). Both ANI values were clearly lower than the 95–96 % cut-off for species delineation (Richter and Rosselló-Móra, 2009). The dDDH values between the genomes of the strain PA1-10^T and its closest species, *N. candida* JCM 15928^T (JOAG00000000) and *N. maritima* JCM 18321^T (FNFB00000000) were 29.8 % (C.I. model 27.4–32.3 %) and 27.5 % (C.I. model 25.1–30.0 %) as shown in Table 4.14. These values exhibited significantly lower than the threshold value of 70 % commonly used to delineate separated species status (Chun *et al.*, 2018).

In addition, based on DNA-DNA hybridization using colorimetric method, the strain PA1-10^T showed low levels of DNA-DNA relatedness to *N. candida* JCM 15928^T (49.6 $\pm$ 3.5 %) and *N. maritima* JCM 18321^T (40.9 $\pm$ 1.8 %) which were significantly lower than the threshold value of 70 % commonly used to delineate separated species status (Richter *et al.*, 2016; Wayne *et al.*, 1987). Phylogenomic tree based on TYGS

result showing the relationship between the strain PA1-10^T with related type strains (Figure 4.17) These results confirmed that the strain PA1-10^T was distinct from its related *Nonomuraea* species. The strain PA1-10^T was differentiated from other members of the genus *Nonomuraea* based on its physiological and biochemical characteristics.

On the basis of phenotypic, chemotaxonomic, and genotypic characteristics, including DNA–DNA relatedness evidence showed a novel species of the genus *Nonomuraea*, for which the name *Nonomuraea phyllanthi* is proposed. The type strain is PA1-10^T (=JCM 33073^T =NBRC 112774^T =TISTR 2497^T). The etymology of *N. phyllanthi* is phyll.an'thi. N.L.gen. n. *phyllanthi* of *Phyllanthus*, referring to the host plant genus *Phyllanthus*.

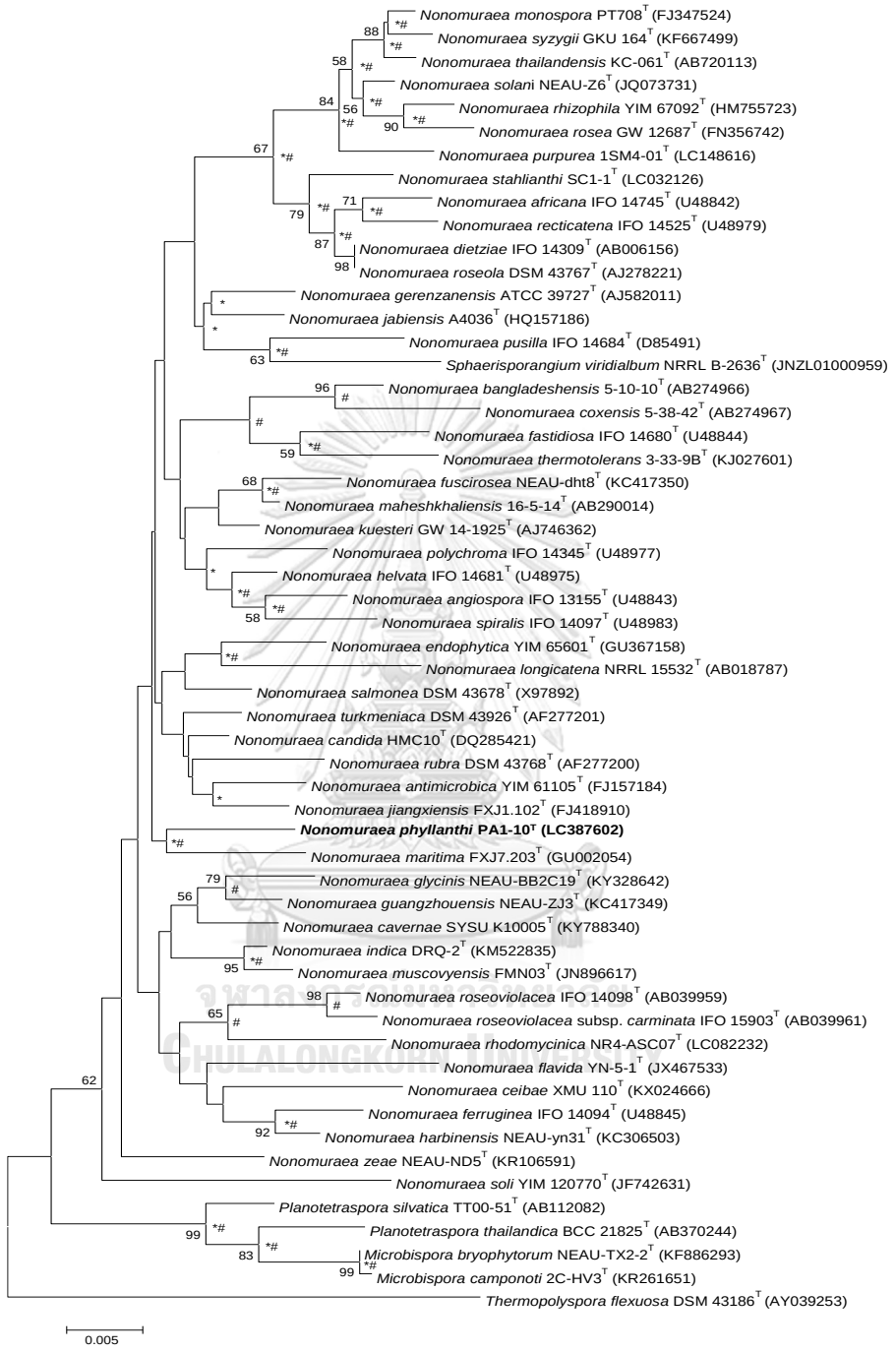

Figure 4.16 Neighbour-joining phylogenetic tree based on 16S rRNA gene showing the relationship between strain PA1-10^{T} and other members of the genus *Nonomuraea.*

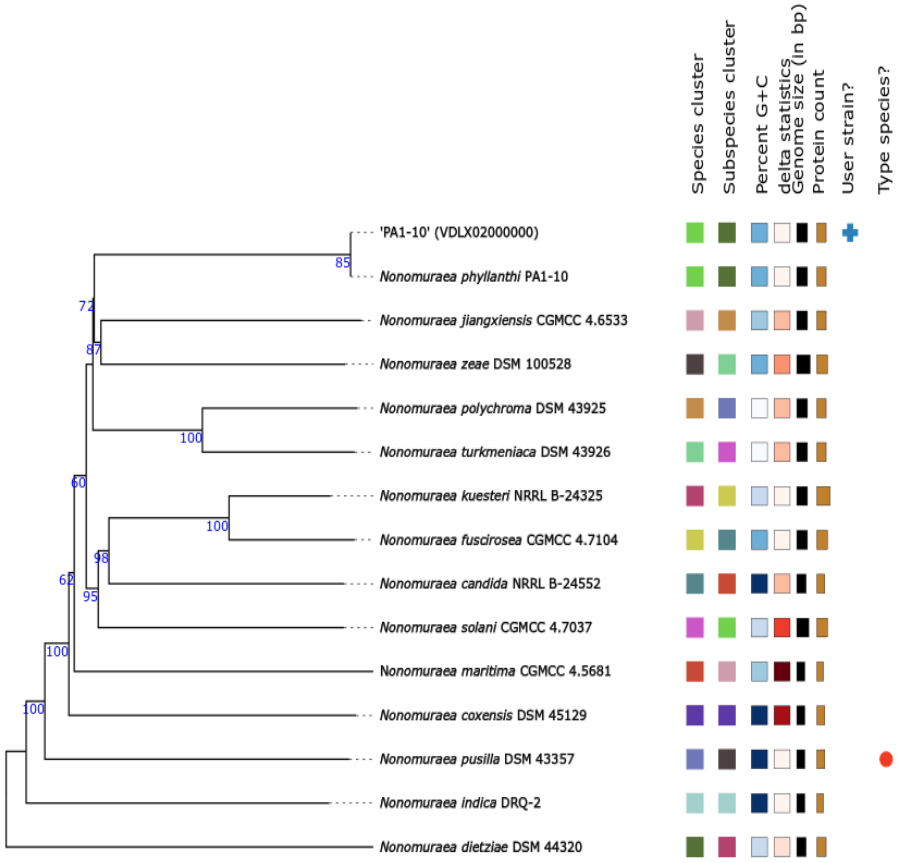

Figure 4.17 Phylogenomic tree based on TYGS result showing the relationship between strain PA1-10^{T} and related type strains. The numbers above branches are GBDP pseudo-bootstrap support values from 100 replications.

Table 4.12 Differential characteristics of PA1-10^T and closely related strains.

Strains: 1, PA1-10^T; 2, *N. candida* JCM 15928^T; 3, *N. maritima* JCM 18321^T. All data were examined in this study. +, positive; w, weakly positive; -, negative. H, Hooks, curled; Psp, Pseudosporangia; S, Spirals of one or two turns; Sp, Spirals of three to five turns.

Characteristic	**1**	**2**	**3**
Spore chain morphology	S, Psp	H	Sp
Spore ornamentation	Rough	Smooth	Smooth to
Growth at 45 °C	-	+	-
Nitrate reduction	+	-	w
Milk peptonization	-	w	w
Starch hydrolysis	-	w	-
Gelatin liquefaction	-	+	-
Utilization of:			
L-Arabinose	-	w	+
Cellobiose	-	w	+
D-Fructose	-	+	+
Glycerol	-	w	-
myo-Inositol	+	+	-
D-Mannose	+	+	w
D-Raffinose	+	w	w
L-Rhamnose	-	+	+
Enzyme activity:			
N-Acetyl-β-glucoseamidase	+	w	+
Cystine arylamidase	w	-	w
α-Galactosidase	w	+	+
β-Glucuronidase	-	-	w
β-Glucosidase	w	+	w
Lipase (C14)	-	-	w
α-Mannosidase	w	+	w
Phosphatase acid	w	+	+
Trypsin	w	+	+

Table 4.13 Cellular fatty acid composition of PA1-10^T and closely related strains. Strains: 1, PA1-10^T; 2, *N. candida* JCM 15928^T; 3, *N. maritima* JCM 18321^T. Values are percentages of total cellular fatty acids. -, not present. Fatty acids amounting to less than 0.5% in all strains were omitted.

Fatty acid	1	2	3
Straight-chain fatty acids			
$C_{14:0}$	1.2	2.2	-
$C_{15:0}$ 2-OH	0.7	-	-
$C_{16:0}$	8.3	7.9	3.1
$C_{16:0}$ 2-OH	0.6	1.2	-
$C_{17:0}$	5.6	5.6	7.3
$C_{17:0}$ 10-methyl	11.2	13.4	14.6
$C_{18:0}$	1.9	4.1	1.3
$C_{18:0}$ 10-methyl, TBSA	1.4	3.5	-
Unsaturated fatty acids			
$C_{17:1}$ ω8c	5.4	2.8	4.7
$C_{18:1}$ ω9c	1.8	1.4	-
Branched fatty acids			
iso-$C_{14:0}$	0.7	-	1.0
iso-$C_{15:0}$	6.9	8.1	3.1
anteiso-$C_{15:0}$	2.1	1.0	0.7
iso-$C_{16:0}$	32.2	30.1	51.2
iso-$C_{17:0}$	2.5	2.8	1.8
anteiso-$C_{17:0}$	5.0	3.2	2.3
iso-$C_{18:0}$	1.4	2.5	2.2
Unsaturated branched fatty acids			
iso-$C_{16:1}$ G	-	2.6	-
iso-$C_{16:1}$ H	2.2	-	1.9
anteiso-$C_{17:1}$ω9c	0.5	-	-
Summed in feature 3*	3.1	1.7	0.7
Summed in feature 8*	-	0.8	-
Summed in feature 9*	4.4	3.2	1.7

*Summed feature 3 comprised $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$.

*Summed feature 8 comprised $C_{18:1}\omega7c$.

*Summed feature 9 comprised $C_{16:0}$ 10-methyl.

Table 4.14 ANIb and ANIm values (%) and the digital (*in silico*) DNA-DNA hybridization (dDDH) values between the draft genomes of PA1-10^{T} and its related strains.

Draft genomes: 1, Strain PA1-10^{T}; 2, *N. candida* JCM 15928^{T}; 3, *N. maritima* JCM 18321^{T}. All data were determined in this study.

Query genome	Reference genome	ANIb	ANIm	% dDDH (Formula 2*)	Model C.I.	Distance	Prob. DDH >= 70%	G+C difference
1	2	82.71	87.30	29.8	[27.4 – 32.3%]	0.1431	0.1	0.92
1	3	81.73	86.38	27.5	[25.1 – 30.0%]	0.1569	0.03	0.32

*Recommended formula (identities/HSP length), which is liberated of genome length and is thus prosperous against the use of incomplete draft genome

4.3.5 Characterization of *Plantactinospora* strains (Group V)

Two strains BL1-16 and 8MP-16 were identified as *Plantactinospora* that belonged to the family *Micromonosporaceae* based on morphology and 16S rRNA gene sequence (Figure 4.19). Strains BL1-16 and 8MP-16 were identified as *Plantactinospora endophytica* (Zhu *et al.*, 2012). They produced single or cluster spores on substrate mycelia. In this study, the strain BL1-16 was selected to represent the genus *Plantactinospora* that formed the single spores on substrate mycelia cultivated on V8 agar (V8 Juice 20 mL, $CaCO_3$ 0.3 g, agar 1.5 g, tap water q.s. to 100 mL) at 27 °C for 14 days (Figure 4.18).

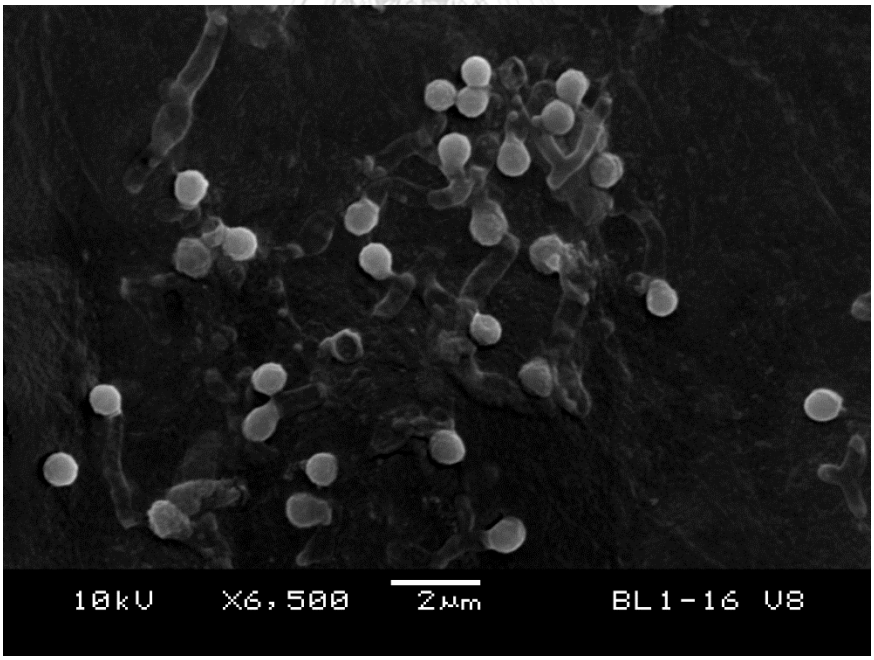

Figure 4.18 Scanning electron micrograph of *Plantactinospora* sp. BL1-16 after incubation on V8 agar at 27 °C for 14 days.

The strain BL1-16, isolated from the root of *Barleria lupulina*, formed smooth single spores on substrate mycelia that grew well on ISP 2, ISP 3, ISP 6, and NA agar, moderately well on ISP 4, ISP 5, and ISP7. The color of colony on ISP 2 was orange–yellow. Grows at 15-40 °C, pH 5-9 and on 3 % (w/v) NaCl. It contained *meso*-diaminopimelic acid, galactose, xylose, and arabinose in whole-cell hydrolysates. Predominant menaquinones were MK-10 (H_4) (57.97 %) and MK-10 (H_6) (42.03 %). Phospholipids were phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylinositolmannoside (PIM), diphosphatidylglycerol (DPG). The 16S rRNA gene sequence revealed that strain BL1-16 showed closely related to *Plantactinospora endophytica* DSM 45387^T (99.7 % similarity).

On the basis of the phenotypic and genotypic characteristics as mentioned above, the strain BL1-16 is classified as a member of the genus *Plantactinospora*.

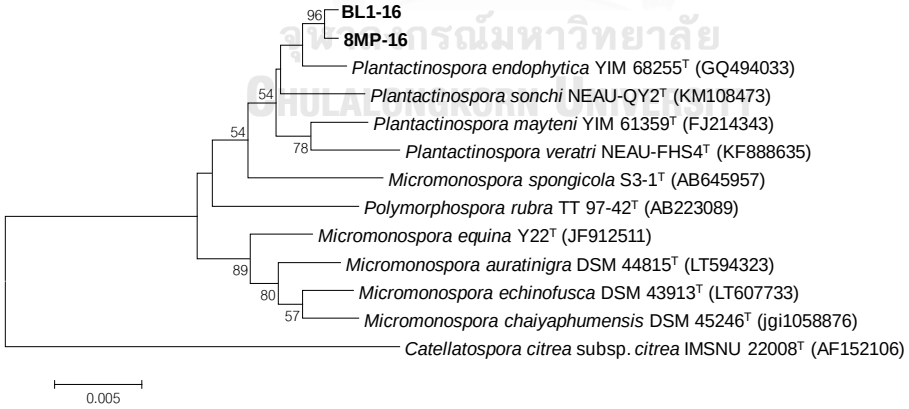

Figure 4.19 Neighbour-joining phylogenetic tree based on 16S rRNA gene between BL1-16, 8MP-16, and members of the genus *Plantactinospora*.

4.4 Screening for antimicrobial activity

All strains, incubated in ISP no.2 broth, were screened for antimicrobial activity using disc diffusion method. The ethanol extract of fifteen strains exhibited the antimicrobial activities against Gram-positive tested microorganisms as shown in Table 4.15. The *Microbispora* sp. strain CR1-01 and CR1-08 showed inhibitory effect against *S. aureus* ATCC 25923 and *K. rhizophila* ATCC 9341 while CR1-09 showed inhibitory effect against *S. aureus* ATCC 25923 and *K. rhizophila* ATCC 9341. The *Streptomyces* sp. strains 3MP-10 and 3MP-14 showed inhibitory effect against *S. aureus* ATCC 25923, *K. rhizophila* ATCC 9341, and *B. subtilis* ATCC 6633. The *Micromonospora* sp. strains 3MP-11 and AP1-08 showed inhibitory effect against *S. aureus* ATCC 25923, *K. rhizophila* ATCC 9341, and *B. subtilis* ATCC 6633 while the strain BL1-13 showed inhibitory effect against *S. aureus* ATCC 25923, *B. subtilis* ATCC 6633, and *C. albicans* ATCC 10231. The *Nonomuraea* sp. strain PA1-05 and *Micromonospora* sp. strain 8MP-36 showed inhibitory effect against *K. rhizophila* ATCC 9341. The *Micromonospora* sp. strain 4WP-22-1 showed inhibitory effect against *S. aureus* ATCC 25923 and *B. subtilis* ATCC 6633 while the strain PR1-12 showed inhibitory effect against *B. subtilis* ATCC 6633. In this study, no EtOH crude extract showed biological activity against Gram-negative tested microorganisms.

Table 4.15 Screening for antimicrobial activity of EtOH extracts based on disc diffusion method

Genus	Strain no.	Inhibition zone (mm)					
		S. aureus ATCC 25923	*B. subtilis* ATCC 6633	*K. rhizophila* ATCC 9341	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
Microbispora	CR1-01	17.4	-	12.6	-	-	-
Microbispora	CR1-08	25.3	-	11.4	-	-	-
Microbispora	CR1-09	15.8	-	17.3	-	-	-
Streptomyces	3MP-10	16.4	12.0	11.4	-	-	-
Micromonospora	3MP-11	13.4	14.1	15.3	-	-	-
Streptomyces	3MP-14	16.5	15.7	12.7	-	-	-
Micromonospora	BL1-13	19.5	15.5	-	-	-	11.4
Micromonospora	AP1-08	13.0	9.3	17.0	-	-	-
Streptomyces	AP1-18	22.7	16.8	15.3	-	-	-
Streptomyces	AP1-19	21.3	17.4	16.7	-	-	-
Streptomyces	AP1-20	22.1	17.0	15.1	-	-	-
Nonomuraea	PA1-05	-	-	11.2	-	-	-
Micromonospora	8MP-36	-	-	9.3	-	-	-
Micromonospora	4WP-22-1	14.3	12.0	-	-	-	-
Micromonospora	PR1-12	-	21.8	-	-	-	-

The other strains as PA1-07, PA1-10, PR1-10, PR1-03, BL1-01, BL1-03, BL1-04, BL1-05, BL1-06, BL1-07, BL1-08, BL1-10, BL1-11, BL1-16, AP1-20, AP1-06, AP1-03, AP1-10, 3MP-12, 3MP-13, 3MP-34, 3MP-35, 4WP-21, 4WP-24, 4WP-20, 4WP-22-2, 4WP-23, 4WP-25, 4WP-27, 7MP-06, 8MP-16, 8MP-31, 10MP-01, and 10MP-05, did not show antimicrobial activity by disc diffusion method. +, positive that showed clear zone on tested medium; -, negative.

4.5 Screening for cytotoxicity

The crude extracts of all strains were screened for cytotoxicity for Caco-2, HepG2, and Vero cells using MTT assay. In this study, the crude extract of strains CR1-08, CR1-09, 3MP-10, 3MP-14, 8MP-16, 8MP-36, and PA1-07 showed cytotoxicity against HepG2 cells compared with positive control (50 μM cisplatin) and negative control (60 medium re-dissolved in cell culture media) while CR1-09 and PA1-07 did not show cytotoxicity against Vero cells as the cytotoxic agent as bold letters shown in Table 4.16. One-way analysis of variance confirmed their crude extracts were statistically significant at 95 % confidence interval. The other strains did not show cytotoxicity against Caco-2, HepG2, and Vero cells.

Table 4.16 The cytotoxicity against HepG2 and Caco-2 cells of some strains

Genus	Strain no.	Cell viability (% ± SD)		
		Vero cell	HepG2	Caco-2
Microbispora	CR1-08	32.4 ± 0.1	27.0 ± 0.8	84.0 ± 0.0
Microbispora	**CR1-09**	**91.0 ± 0.2**	**41.0 ± 0.7**	**21.0 ± 0.0**
Streptomyces	3MP-10	68.4 ± 1.6	52.0 ± 0.3	114.0 ± 0.0
Streptomyces	3MP-14	66.5 ± 0.5	18.0 ± 0.0	96.1 ± 0.0
Micromonospora	8MP-16	32.1 ± 0.2	58.0 ± 0.2	102.0 ± 0.0
Micromonospora	8MP-36	39.9 ± 0.1	56.0 ± 0.4	33.1 ± 0.0
Streptomyces	**PA1-07**	**91.0 ± 0.2**	**36.0 ± 0.5**	**26.0 ± 0.1**
-	Positive	28.0 ± 0.1	22.8 ± 0.6	88.0 ± 0.0
-	Negative	100.0 ± 1.0	100.0 ± 0.1	100.0 ± 0.0

4.6 Secondary metabolites of endophytic actinomycetes

All strains were incubated on M60 and were screened for the new secondary metabolites based on LC-MS/MS spectrum compared with the natural product databases in the Dictionary of Natural Products (http://dnp.chemnetbase.com). Three candidate strains of endophytic actinomycetes, including 3MP-14, CR1-09, and PA1-05, exhibited the interesting peaks while other strains exhibited the peaks of the known compound as described below and shown in Table 4.17.

Streptomyces sp. strain 3MP-10 and 3MP-14 showed three major peaks, (A) peak of the candidate new compound, designed pudicin with antioxidant activity; $[M+Na]^+$ 600.2576 *m/z*; UV_{254} at 10.6 min; 245, 343 nm, (B) peak of fumaquinone with antimicrobial activity and cytotoxicity; $[M+H]^+$ 303.1225 *m/z*; UV_{254} at 11.1 min; 220, 304, 428 nm, and (C) peak of ikarugamycin with antimicrobial and antiprotozoal activities; $[M+H]^+$ 479.2912 *m/z*; UV_{254} at 11.8 min; 224, 326 nm (Ito and Hirata, 1977). In plants (family *Fabaceae*), napthoquinones are widespread associated with antibacterial, fungicidal, antiparasitic and insecticidal (Babula *et al.*, 2009). Fumaquinone, one of napthoquinones, was also produced by *Streptomyces fumanu* which previously isolated from alluvial soil (Charan *et al.*, 2005).

Nonomuraea sp. strain PA1-05 showed the candidate new compound; $[M+H]^+$ 261.1448 *m/z*; UV_{254} at 5.5 min; 206, 260 nm.

Microbispora sp. strain CR1-09 showed two major peaks, (A) peak of deferoxamine as iron chelating compound; $[M+H]^+$ 561.3609 *m/z*; UV_{254} at 6.0 min;

270 nm, (B) peak of the candidate new compound with antimicrobial activity against Gram-positive bacteria; $[M+H]^+$ 925.2790 *m/z*; UV_{254} 10.3, 10.4 min; 236, 282, 446 nm.

Table 4.17 Secondary metabolites of some selected strains incubated in M60 at 200 rpm, 27°C for 7 days based on LC-UV/MSMS and the databases of the Dictionary of natural products (2014)

Genus	Strain no.	$t_{R\ uv254}$ (min)	$[M+H]^+$ (m/z)	UV spectrum (nm)	Compound	Activity
Streptomyces	3MP-10, 3MP-14	10.6	600.2576 $[M + Na]^+$	245, 343	New	Antioxidant
		11.1	303.1225	220,304,428	Fumaquinone	Antimicrobial, Cytotoxicity
		11.8	479.2912	224,326	Ikarugamycin	Antimicrobial, Antiprotozoal
Micromonospora	3MP-11	4.7	315.0210	222,270	Anthramycin	Antimicrobial, Cytotoxicity
Micromonospora	3MP-13	7.5	305.1030	210,257,307	Coryoctalactone E	Aytotoxicity
Micromonospora	AP1-08	4.6	205.0968	218,276	Caulilexin	Antimicrobial, Antioxidant
Micromonospora	AP1-10	8.9	295.0154	206, 275, 356	Cupalaurenol	Antimicrobial
Nonomuraea	PA1-05	5.5	261.1448	206,260	Candidate	N/A
Nonomuraea	PA1-10	4.7	492.1833	266	Spithioneine A	Antimicrobial
Planactinospora	8MP-16	11.4	702.3034	206,272,332	Paulomycin F	Cytotoxicity
Micromonospora	8MP-36	6.4	377.1466	220,268,352,446	Riboflavin	Vitamin B2
Microbispora	CR1-09	6.0	561.3609	270	Deferoxamine	Iron chelating
		10.3,10.4	925.2790	236,282,446	Candidate	Antimicrobial
Micromonospora	BL1-05	6.4	377.1466	220,268,352,446	Riboflavin	Vitamin B2
Micromonospora	BL1-08	6.4	377.1466	220,268,352,446	Riboflavin	Vitamin B2

N/A; no data. Many strains produced nocardamine ($[M+H]^+$ = 565 m/z; UV 214, 272, 336. nm).

The strain 3MP-14 was selected to scale-up in jar fermenter using the production medium (modified M60; 2.5 % soluble starch, 1.0 % dry yeast, 1.0 % wheat germ B, 0.5 % glycerol, 0.3 % meat extract, and 0.3 % $CaCO_3$) that was optimized the condition based on physicochemical (PC) screening. On the basis of spectroscopy and MS, the major secondary metabolites of strain 3MP-14 were identified as two known antimicrobial ikarugamycin (*m/z* 479.2912 calculated value for $C_{29}H_{39}O_4N_2$ 479.2910; UV λ_{max} at 224 and 326 nm) with antiprotozoal activity and fumaquinone (*m/z* 303.1225 $[M+H]^+$ calculated value for $C_{17}H_{19}O_5$ 303.1232; UV λ_{max} at 220, 265, 304, and 428 nm) with cytotoxicity activity while the major secondary metabolite of strain 3MP-14 was identified as a new compound designated pudicin (1) as a unique structure with *N*-acetylcysteine moiety as mild antioxidant activity by DPPH assay.

Pudicin (1): pale yellow amorphous solid; $[\alpha]_D^{30}$ -114.1° (*c* = 0.1, MeOH), IR (KBr) ν_{max} cm^{-1}: 3350, 2908, 2873, 2850, 1715, 1647, 1614, 1566, 1454, 1376, 1342, 1308, 1239, 1214, 1123; UV λ_{max} nm (ε): 204 (680,000), 246 (1,190,000), 344 (640,000); ^1H and ^{13}C NMR data are shown in Table 3; HRESI-MS *m/z* 600.2576 $[M + Na]^+$ (calculated value for $C_{30}H_{43}NO_8SNa$: *m/z* 600.2607).

The HRESI-TOF-MS of pudicin (**1**) showed the $[M+H]^+$ at *m/z* 600.2576 $[M + Na]^+$ (calculated value for $C_{30}H_{43}NO_8SNa$: *m/z* 600.2607), indicating the molecular formula was $C_{30}H_{43}NO_8S$. Analysis of the ^1H NMR (Table 4.18 and Figure D1 in Appendix D) and HSQC data (Figure D3 in Appendix D) revealed four methyls at δ_H 0.90 (H-20), 1.13 (H-

19), 1.18 (H-17), and 1.50 (H-18), an acetyl group at δ_H 2.00 (H-5′), six methylenes at δ_H 0.89, 1.81 (H-7), 0.90, 1.76 (H-5), 1.29, 1.87 (H-4), 1.32, 1.79 (H-15), 2.90, 3.08 (H-3′), and 2.96 (H-14), five methine protons at δ_H 1.46 (H-6), 1.83 (H-7a), 1.95 (H-9a), 2.39 (H-3), and 2.81 (H-12), a oxymethine proton at 3.81 (H-16), a nitrogen bonded proton at δ_H 4.59 (H-2′) and three olefinic protons at δ_H 5.72 (H-8), 5.74 (H-9), 5.86 (H-11). The ^{13}C NMR (Table 4.18 and Appendix D) and HSQC data showed the presence of 30 carbons classified into an ketone group at δ_C 205.2 (C-1), two carbonyl carbons at δ_C 173.3 (C-4′), and 173.8 (C-1′), three sp^2 quaternary carbons including an oxygenated carbon at δ_C 104.8 (C-1a), 137.8 (C-10a), and 183.1 (C-2) and three sp^2 methine carbons at δ_C 117.8 (C-11), δ_C 123.5 (C-9), δ_C 136.4 (C-8), six sp^3 methylene carbons at δ_C 29.1 (C-4), 34.2 (C-6), 36.0 (C-3′), 37.1 (C-7), 40.1 (C-15), and 41.8 (C-14) three sp^3 quaternary carbons at 43.2 (C-2a), 71.2 (C-10), and 79.0 (C-13), seven sp^3 methine carbons including two heteroatom bonded methine carbons at δ_C 34.2 (C-6), 36.0 (C-3′), 37.1 (C-7), 40.1 (C-15), 40.6 (C-12), 54.3 (C-2′′), and 67.7 (C-16), and five sp^3 methyl carbons including an acetyl carbon at δ_C 19.3 (C-19), 23.3 (C-17), 22.5 (C-5′), 22.9 (C-20), and 25.0 (C-18). The gross structure of pudicin (1) was confirmed by analysis of two-dimensional (2D) NMR data, including ^{1}H–^{1}H COSY, HMBC spectra in CD_3OD (Figure D4 and D5 in Appendix D). The ^{1}H–^{1}H COSY data indicated partial structures **a** (C-5–C-7a), **b** (C-8–C-9a), **c** (C-11–C-17), and **d** (C-2′–C-3′) (Figure 4.20). The partial structure **a** and the HMBC correlations from H-3 to C-7 and from H-20 to C-5, C-6, and C-7 revealed

methylcyclohexane. The partial structure **b** and the HMBC correlations from H-3 to C-8, from H-8 to C-3, C-7a, and C-9a, from H-9 to C-2a and C-7a and from H-9a to C-8 revealed cyclohexene, confirming the connectivity of cyclohexene and methylcyclohexane at C-3 and C-7a. This bicyclic structure was also supported by the TOCSY correlations (Figure D6 in Appendix C). The partial structure **c** and the HMBC correlations from H-11 to C-1a and C-13, from H-12 to C-1 and C-10a, and from H-15 to C-13 revealed 5-propylcyclohex-3-en-1-one. The partial structure **d** and the HMBC correlations from H-2′ to C-4′, from H-3′ to C-1′, and from H-5′ to C-4′ revealed *N*-acetylcysteine moiety. The connectivity of this moiety at C-13 through C-14 was revealed the HMBC correlations from H-3′ to C-14, from H-14 to C-3′, C-1, C-12, and C-13, and from H-12 to C-14. The HMBC correlations from C-11 to C-10, from H-11 and H-9 to C-2 (^4J HMBC), from H-18 to C-9a, C-10, and C-10a, and from H-19 to C-2, C-2 revealed the connectivity of two partial structures, 5-(2-hydroxypropyl)cyclohex-3-en-1-one and cyclohexene, through 4,6-dimethylcyclohex-1-en-1-ol. Finally, four hydroxy groups were attached at C-2, C-10, C-13, and C-16 by accounting for the molecular formula and chemical shifts. The central part of this structure was also supported by the data of 1D and 2D NMR measured in $CDCl_3$ (Figure D7 to D10 in Appendix D). Figure D7 in Appendix D, ^{1}H NMR showed a downfield proton signal of 2-OH (14.9 ppm). The HMBC correlations (Figure 5.11) from 2-OH to C-1a, C-2 and C2a also supported this structure. Thus, the planar structure was determined as shown in Figure 4.20.

Table 4.18 ^{1}H and ^{13}C NMR chemical shifts of pudicin (1).

3MP-14 (CD_3OD)		
Position	d_H	d_C
1		205.2
1a		104.8
2		183.1
2a		43.2
3	2.39 (m,1H)	42.6
4	1.29 (m, 1H)	29.1
	1.87 (m, 1H)	
5	0.90 (overlap, 1H)	37.1
	1.76 (m, 1H)	
6	1.46 (m, 1H)	34.2
7	0.89 (overlap, 1H)	42.7
	1.81 (overlap, 1H)	
7a	1.83 (overlap, 1H)	39.9
8	5.72 (dd, J = 10.4, 10.4 Hz, 1H)	136.4
9	5.74 (dddd, J = 10.4, 10.2, 4.2, 1.8 Hz, 1H)	123.5
9a	1.95 (m, 1H)	52.3
10		71.2
10a		137.8
11	5.86 (d, J = 7.2 Hz, 1H)	117.8
12	2.81 (m, 1H)	40.6
13		79.0
14	2.96 (d, J = 7.2 Hz, 2H)	41.8
15	1.32 (overlap, 1H)	40.1
	1.79 (overlap, 1H)	
16	3.81 (q, J = 6.6 Hz, 1H)	67.7
17	1.18 (d, J = 6.6 Hz, 3H)	23.3
18	1.50 (s, 3H)	25.0
19	1.13 (s, 3H)	19.3
20	0.90 (d, J = 6.6 Hz, 3H)	22.9
1′		173.8
2′	4.59 (m, 1H)	54.3
3′	2.90 (m, 1H)	36.0
	3.08 (m, 1H)	
4′		173.3
5′	2.00 (s, 3H)	22.5

Recorded for ^{1}H-NMR at 600 MHz, for ^{13}C-NMR at 150 MHz in methanol-d_4 (CD_3OD).

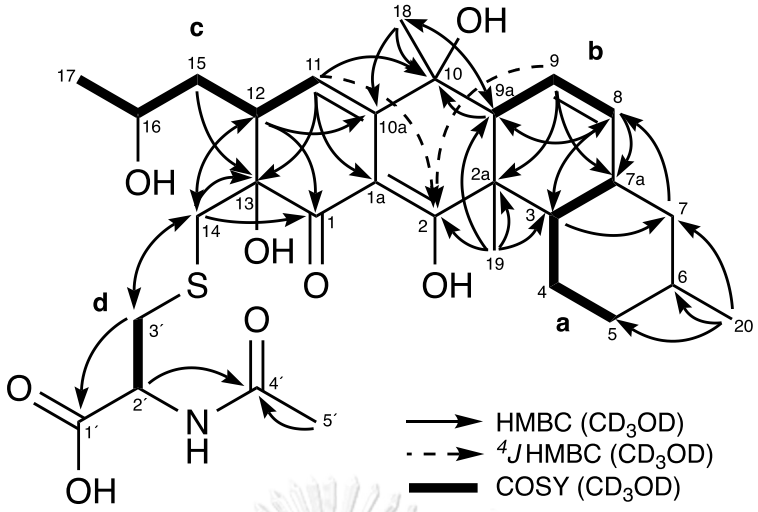

Figure 4.20 Selected 2D-NMR correlations of pudicin (1).

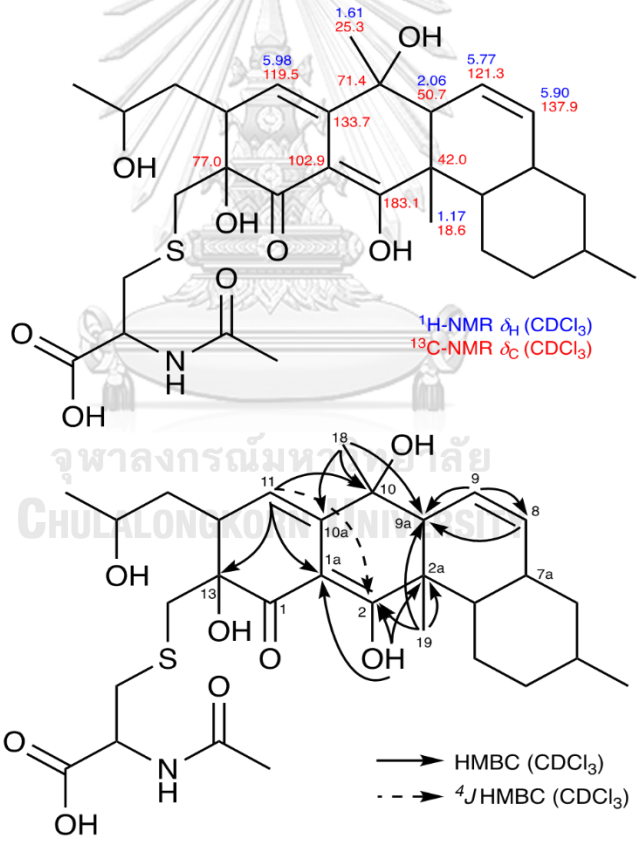

Figure 4.21 Partial ^{1}H and ^{13}C NMR chemical shifts and HMBC correlations of pudicin (600 MHz, $CDCl_3$)

4.7 Secondary metabolite genes of endophytic actinomycetes

The BGCs of eight strains, CR1-09, 3MP-14, 3MP-10, 4WP-24, 8MP-36, AP1-08, PA1-05, and PA1-10 are potentially involved in the biosynthesis of secondary metabolites that are predicted by antiSMASH algorithm.

The genome of *Streptomyces* sp. strain 3MP-14 was assembled to generate the 7,614,683 bp with the genome GC content was 73.4 %. A total of 6,177 coding genes from the draft genome of strain 3MP-14 was predicted by RAST, which 34 % were hypothetical. Genomic and bioinformatics analysis by antiSMASH of strain 3MP-14 revealed the presence of at least 24 synthetic gene clusters (BGCs). Twenty-four putative secondary metabolite biosynthetic gene clusters (BGCs), predicted in the draft genome of strain 3MP-14, comprised 10 different types of BGCs, including non-ribosomal peptide synthetase (NRPS), polyketide synthetase (PKS), thiopeptide, lanthipeptide, terpenes, butyrolactone, bacteriocin, siderophores, ectoine, and tRNA-dependent cyclodipeptide synthases. The related biosynthetic secondary metabolite genes have emphasized as polyketide synthase (PKS) type I, II, III, and non-ribosomal peptide synthetase (NRPS), especially PKS cluster and hybrid PKS cluster with the others as showed up to 41.7 % clusters of all putative BGCs that assigned to be high potency strain to produce antibiotics and complex secondary metabolites (Mizuno *et al.*, 2013). Cluster 16 on the draft genome of strain 3MP-14 was identified as nrps-t1pks (NRPS and T1PKS) gene clusters which is close to that of ikarugamycin gene cluster of 12 % identities by antiSMASH database as shown in Table 4.19 (Blin *et al.*, 2019). However, the putative nrps-t1pks gene cluster on the draft genome of *Streptomyces* sp. strain 3MP-14 related to produce ikarugamycin. Among them, ectoine and naringenin biosynthetic gene clusters have been characterized completely 100 %

identities in cluster 22 and cluster 9, respectively. The related biosynthetic secondary metabolite genes of strain 3MP-14 have emphasized as many enzymes in gene clusters, including NRPS, PKS type I, II, III, or terpene clusters as presented in Table 4.19.

Table 4.19 Potential BGCs in the genome of strain 3MP-14

No.	Type	Length (bp)	Similar BGC (gene name)	Similarity[a] (%)
1	T1PKS	36,422	Maklamicin	13
2	hglE-KS	46,138	-	-
3	T2PKS, T1PKS, PKS-	72,486	Polyketomycin	83
4	Terpene	21,218	Chlortetracycline	8
5	Bacteriocin	10,849	-	-
6	Siderophore, T1PKS	90,234	Tirandamycin	33
7	LAP, thiopeptide	24,706	-	-
8	Terpene	21,841	Hopene	30
9	T3PKS	29,740	Naringenin	100
10	CDPS	20,822	Marinophenazine	7
11	Terpene	22,406	Geosmin	100
12	Lanthipeptide	37,024	Cahuitamycins	8
13	Butyrolactone	9,942	Murayaquinone	3
14	Butyrolactone, PKS-	31,646	Lactonamycin	3
15	NRPS, Butyrolactone	60,372	Azicemicin	8
16	T1PKS, NRPS	49,372	Ikarugamycin	12
17	Terpene	19,277	Isorenieratene	42
18	Terpene	21,221	Isorenieratene	25
19	T1PKS	33,735	Meoabyssomicin/Abyssomicin	25
20	T3PKS, other	43,440	Furaquinocin	34
21	Siderophore	8,791	Desferrioxamine	50
22	Ectoine	10,396	Ectoine	100
23	T1PKS	12,066	Laidlomycin	28
24	T1PKS	10,531	Versipelostatin	14

[a] The percentage of genes in reference BGC showing similarity to the BGC, which is calculated by antiSMASH.

T1PKS: Type I PKS (Polyketide synthase); T2PKS: Type II PKS; T3PKS: Type III PKS; PKS-like: Other types of PKS cluster; Bacteriocin: Bacteriocin or other unspecified ribosomally synthesized and post-translationally modified peptide product (RiPP) cluster; Siderophore: Siderophore cluster; LAP: Linear azol(in)e-containing peptides; CDPS: tRNA-dependent cyclodipeptide synthases; NRPS: Non-ribosomal peptide synthetase cluster; other: Cluster containing a secondary metabolite-related protein that does not fit into any other category.

CHAPTER V

CONCLUSION

Endophytic actinomycetes are the potential resources of novel species and new secondary metabolites for the new drug discovery of natural products. In this study, a total of fifty endophytic actinomycetes were isolated from six medicinal plants in Thailand such as *Catharanthus roseus*, *Phyllanthus amarus*, *Piper retrofractum*, *Barleria lupulina, Andrographis paniculate* and *Mimosa pudica*. On the basis of morphology and 16S rRNA gene sequence, they belonged to six genera of 4 families, including Family *Streptomycetaceae* as *Streptomyces* (8 strains, 16 %), Family *Micromonosporaceae* as *Micromonospora* (34 strains, 68 %) and *Plantactinospora* (2 strains, 4 %), Family *Streptosporangiaceae* as *Microbispora* (3 strains, 6 %) and *Nonomuraea* (2 strains, 4 %), and Family *Pseudonocardiaceae* as *Amycolatopsis* (1 strain, 2%). Strains PR1-03, 4WP-22-1, and 4WP-22-2 were identified as *Micromonospora costi*, PR1-12 and 4WP-21 were *Mn. schwarzwaldensis*; strains 3MP-11, 3MP-35, BL1-05, and BL1-11 were *Mn. aurantiaca*; 3MP-34 was *Mn. tulbaghiae*; AP1-10 was *Mn. yasonensis*; AP1-20 and 10MP-05 were *Mn. soli*; 4WP-24 was *Mn. musae*; 4WP-25 was *Mn. chersina*; 8MP-36 was *Mn. yasonensis*; 7MP-06 and BL1-13 were *Mn. tulbaghiae* while strains AP1-03, AP1-06, AP1-08, 10MP-04, and BL1-01 were identified as *Micromonospora* sp. Strain PA1-07 was identified as *Streptomyces phyllanthi*; strains 3MP-12 and BL1-06 were *S. albidoflavus*; strains AP1-18, AP1-19, and AP1-21 were *S.*

seoulensis while strains 3MP-10 and 3MP-14 were identified as *Streptomyces* sp. Strain CR1-09 was identified as *Microbispora catharanthi* while strains CR1-01 and CR1-08 were *Microbispora* sp. Strain PA1-10 was identified as *Nonomuraea phyllanthi* while PA1-05 was identified as *Nonomuraea* sp. Strains BL1-16 and 8MP-16 were identified as *Plantactinospora endophytica*. Strain ST1-08 was identified as *Amycolatopsis stemonae*.

Fifteen strains exhibited the antimicrobial activity against Gram-positive bacteria, including strains CR1-01 and CR1-08 showed against *S. aureus* ATCC 25923 and *K. rhizophila* ATCC 9341 while CR1-09 showed against *S. aureus* ATCC 25923 and *K. rhizophila* ATCC 9341. The strains 3MP-10 and 3MP-14 exhibited against *S. aureus* ATCC 25923, *K. rhizophila* ATCC 9341, and *B. subtilis* ATCC 6633. The crude extract of CR1-09 and PA1-07 showed potential cytotoxicity against HepG2 and Caco-2 cells while they did not show cytotoxicity against Vero cells.

The partial chemical composition related to the metabolite was detected by LC/MS/MS. In the course of chemical investigation for the strain 3MP-14 conducted by physicochemical screening (PC screening), a new compound with antioxidant activity, designated pudicin (1), was discovered and two known antimicrobials as ikarugamycin and fumaquinone, were identified based on LC-MS/MS. The planar structure of pudicin (1) revealed a unique structurally new compound with *N*-acetylcysteine moiety using 1D and 2D NMR, and HRESI-MS. For secondary metabolite gene analysis, twenty-four putative secondary metabolite biosynthetic gene clusters (BGCs) were also predicted

in the draft genome of strain 3MP-14. The related biosynthetic secondary metabolite genes have emphasized as polyketide synthase gene (PKS) type I, II, III, and non-ribosomal peptide synthetase gene (NRPS), especially PKS cluster and hybrid PKS cluster with the others as showed up to 41.7 % clusters of all putative BGCs that assigned to be high potency strain to produce antibiotics and complex secondary metabolites (Mizuno *et al.*, 2013).

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APPENDIX A

CULTURE MEDIA

All media were sterilized in autoclave at 121 °C, 15 pounds pressure for 15 min, except skim milk and media for carbon utilization test that were sterilized in autoclave at 110 °C, 15 pounds pressure for 10 min. All media were prepared in 100 mL of distilled water.

1. Humic acid vitamin agar (HV)

Humic acid (dissolved in 1 mL of 0.2 N NaOH)	0.1	g
Na_2HPO_4	0.05	g
KCl	0.171	g
$MgSO_4 \cdot 7H_2O$	0.005	g
$FeSO_4 \cdot 7H_2O$	0.001	g
$CaCO_3$	0.002	g
Vitamin B solution	1	mL
agar	1.5	g

Vitamin B solution

Thiamine-HCl	0.005	g
Riboflavin	0.005	g
Nicothinate (Niacin)	0.005	g
Pyridoxine-HCl	0.005	g
Inositol	0.005	g
Ca-Pantothenate	0.005	g
p-Aminobenzoate	0.005	g

d-Biotin	0.0025	g

To add vitamin B solution, cycloheximide (50 mg/L) and nalidixic acid (25 mg/L) that was filter-sterilized.

2. Starch-casein nitrate agar (SCN)

Soluble starch	1	g
Sodium caseinate	0.3	g
KNO_3	0.2	g
Agar	1.5	g

To add cycloheximide (50 mg/L) and nalidixic acid (25 mg/L) that was filter-sterilized.

3. Pridham's agar medium

Starch	1	g
Glycerol	1	g
$(NH_4)_2SO_4$	0.2	g
$CaCO_3$	0.2	g
K_2HPO_4	0.1	g
$MgSO_4 \cdot 7H_2O$	0.1	g
NaCl	0.1	g
Agar	1.5	g

To add cycloheximide (50 mg/L) and nalidixic acid (25 mg/L) that was filter-sterilized.

4. Water proline agar

Agar	1.5	g
Proline	1	g
Tap water q.s. to	100	mL

To add cycloheximide (50 mg/L) and nalidixic acid (25 mg/L) that was filter-sterilized.

5. 2.5% Water agar

Agar	2.5	g
Tap water q.s. to	100	mL

To add cycloheximide (50 mg/L) and nalidixic acid (25 mg/L) that was filter-sterilized.

6. Starch-yeast extract agar

Soluble starch	1	g
Yeast extract	0.2	g
Agar	1.5	g

7. 301 Seed medium

Soluble starch	2.4	g
Glucose	0.1	g
Peptone	0.3	g
Meat extract	0.3	g
Yeast extract	0.5	g
$CaCO_3$	0.4	g

8. Yeast extract-glucose broth

Yeast extract	1	g
Glucose	1	g

9. Production medium no. 54 (M60)

Soluble starch	2	g
Glycerol	0.5	g
Defatted wheat germ (B)	1	g

Meat extract	0.3	g
Yeast extract	0.3	g
$CaCO_3$	0.3	g

10. Production medium no. 30

Soluble starch	2.4	g
Glucose	0.1	g
Peptone	0.3	g
Meat extract	0.3	g
Yeast extract	0.5	g
$CaCO_3$	0.4	g

Trace salts solution A

$FeSO_4 \cdot 7H_2O$	0.1	g
$MnCl_2 \cdot 4H_2O$	0.1	g
$ZnSO_4 \cdot 7H_2O$	0.1	g
distilled water	100	mL

11. Yeast extract-malt extract agar (ISP no.2), pH 7.2 ± 0.2

Yeast extract	0.4	g
Malt extract	0.1	g
Glucose (Dextrose)	0.4	g
Agar	1.5	g

12. Oatmeal agar (ISP no.3), pH 7.2 ± 0.2

Oatmeal	2	g
Agar	1.5	g

13. Inorganic salts-starch agar (ISP no.4), pH 7.2 ± 0.2

Soluble starch	0.1	g
K_2HPO_4	0.1	g
$MgSO_4 \cdot 7H_2O$	0.1	g
NaCl	0.1	g
$(NH_4)_2SO_4$	0.2	g
$CaCO_3$	0.2	g
Trace salts solution A	0.1	mL
Agar	1.5	g

14. Glycerol-asparagine agar (ISP no.5), pH 7.2 ± 0.2

Glycerol	1	g
L-Asparagine	0.1	g
K_2HPO_4	0.1	g
Trace salts solution A	0.1	mL
Agar	1.5	g

15. Peptone-yeast extract iron agar (ISP no.6), pH 7.2 ± 0.2

Peptone Iron agar, dehydrated	0.36	g
Yeast extract	0.01	g
Agar	1.5	g

16. Tyrosine agar (ISP no.7), pH 7.2 ± 0.2

Glycerol	1.5	g
L-Tyrosine	0.05	g
L-Asparagine	1	g
K_2HPO_4	0.05	g
$MgSO_4 \cdot 7H_2O$	0.05	g

NaCl	0.05	g
$FeSO_4 \cdot 7H_2O$	0.01	g
Trace salts solution A	0.1	mL
Agar	1.5	g

Trace salts solution A

$FeSO_4 \cdot 7H_2O$	0.1	g
$MnCl_2 \cdot 4H_2O$	0.1	g
$ZnSO_4 \cdot 7H_2O$	0.1	g
Distilled water	100	mL

17. Glucose asparagine agar (GluA), pH 7.2 ± 0.2

Glucose (Dextrose)	1	g
Asparagine	0.05	g
K_2HPO_4	0.05	g
Agar	1.5	g

18. Czapek's sucrose agar (Czk), pH 7.2 ± 0.2

Sucrose	3	g
K_2HPO_4	0.1	g
$MgSO_4$	0.05	g
KCl	0.05	g
$FeSO_4$	0.001	g
Agar	1.5	g

19. Nutrient agar (NA), pH 7.2 ± 0.2

Meat extract	1	g
Peptone	1	g
NaCl	0.1	g
Agar	1.5	g

20. Carbon utilization medium (ISP no.9), pH 7.2 ± 0.2

Carbohydrate	1	g
$(NH_4)_2SO_4$	0.264	g
$K_2HPO_4 \cdot 3H_2O$	0.565	g
KH_2PO_4 anhydrous	0.238	g
$MgSO_4 \cdot 7H_2O$	0.1	g
Trace salts solution B	0.1	mL
Agar	1.5	g

Trace salts solution B (Pridham and Gottlieb trace salts)

$CuSO_4 \cdot 5H_2O$	0.64	g
$FeSO_4 \cdot 7H_2O$	0.11	g
$MnCl_2 \cdot 4H_2O$	0.79	g
$ZnSO_4 \cdot 7H_2O$	0.15	g
Distilled water	100	mL

21. Bouillon gelatin broth, pH 7.2 -7.8

Peptone	1	g
Meat extract	0.5	g
NaCl	0.5	g
Gelatin	15	g

22. Peptone KNO_3 broth, pH 7.2 -7.8

Peptone	1	g
KNO_3	0.1	g
NaCl	0.5	g

23. 10 % Skim milk

Skim milk	10	g

To sterilize at 110 °C for 10 min

24. Mueller-Hinton agar (Difco)

Beef infusion form	3	g
Casamino acid, technical	1.75	g
Starch	0.15	g
Agar	1.5	g

25. Sabouraud's dextrose agar (Difco)

Neopeptone	1	g
Glucose (Dextrose)	4	g
Agar	1.5	g

APPENDIX B

REAGENTS AND BUFFER

1. Nitrate reduction test reagent

Sulphanilic acid solution

Sulphanilic acid	0.8	g
5 N Acetic acid	100	mL

To dissolve by gentle heating in fume hood.

N,N-dimethyl-1-naphthylamine solution

N,N-dimethyl-1-naphthylamine	0.5	g
5 N Acetic acid	100	mL

To dissolve by gentle heating in fume hood.

2. 2 N H_2SO_4

conc. H_2SO_4	2	mL
Distilled water	34	mL

To add conc. H_2SO_4 into the distilled water for cell hydrolysis process in the whole-cell sugar analysis.

3. 6 N HCl

conc. HCl	50	mL
Distilled water	50	mL

To add conc. HCl (12 N) into the distilled water for cell hydrolysis process in the diaminopimelic acid analysis.

4. Dittmer & Lester reagent

Solution A

MoO_3	4.011	g
25 N H_2SO_4	100	mL

To dissolve MoO_3 in hot acid.

Solution B

Molybdenum powder	0.178	g
Solution A	50	mL

To add molybdenum powder in solution A and boil for 15 minutes. After cooling, remove the precipitate by decantation. And then, mix solution A (50 mL), solution B (50 mL) and water (100 mL) before use.

5. Anisaldehyde reagent

EtOH	90	mL
H_2SO_4	5	mL
p-Anisaldehyde	5	mL
Acetic acid	1	mL

6. Ninhydrin solution

Ninhydrin	0.3	g
n-Butanol	100	mL
Glacial acetic acid	3	mL

7. Dragendroff's reagent

Solution A

Basic bismuth nitrate	1.7	g
Acetic acid	20	mL
Distilled water	80	mL

Solution B

KI	40	g
Distilled water	100	mL

To mix solution A (10 mL), solution B (10 mL) and acetic acid (10 mL), before use.

8. Reagents for fatty acid analysis

Reagent 1 (Saponification reagent)

NaOH	15	g
MeOH	50	mL
Ultrapure water	50	mL

Reagent 2 (Methylation reagent), pH below 1.5

6N HCl	65	mL
MeOH	55	mL

Reagent 3 (Extraction solvent)

n-Hexane	50	mL
Methyl-tert-butyl ether	50	mL

Reagent 4 (Base wash reagent)

NaOH	1.2	g
Ultrapure water	100	mL

Reagent 5 (Saturated sodium chloride)

Sodium hydroxide saturated in ultrapure water.

9. Phenol : Chloroform (1 : 1, v/v)

Crystalline phenol (melted)	50	mL
Chloroform	50	mL

To melted crystalline phenol in water bath at 65 °C and mix with chloroform. The solution was stored in a light tight bottle.

10. 0.2 M Tris-HCl buffer, pH 8.5

Tris	24.22	g
Distilled water	700	mL

To adjust pH 8.5 with conc. HCl and adjust volume to 1 L with distilled water.

11. DNA extraction buffer (Grind method)

0.2 M Tris-HCl buffer, pH 8.5	900	mL
NaCl	14.61	g
EDTA·2Na	9.31	g
SDS	5	g

To adjust volume to 1 L with 0.2 M Tris-HCl buffer. The solution was sterilized by autoclaving at 121°C, 15 lb/in^2 for 15 minutes.

12. 3 M NaCl

NaCl	17.55	g
Distilled water	100	mL

The solution was autoclaved at 121°C, 15 lb/in^2 for 15 minutes.

13. RNase A solution

RNase A	20	mg
0.15 M NaCl	10	mL

To dissolve RNase A in 0.15 M NaCl and heat at 95°C for 5-10 minutes. To keep this solution in –20°C.

14. 0.1 M Tris-HCl buffer, pH 7.5

Tris	1.2	g
Distilled water	70	mL

To adjust pH 7.5 with conc. HCl and adjust volume to 100 mL with distilled water. The solution was sterilized by autoclaving at 121°C, 15 lb/in^2 for 15 minutes.

15. RNase T_1 solution

RNase T_1	80	μL
0.1 M Tris-HCl, pH 7.5	10	mL

To mix RNase T_1 in 0.1 M Tris-HCl (pH 7.5) and heat at 95°C for 5 minutes. To keep this solution in –20°C.

16. 100 x Denhardt solution

Bovine serum albumin	2	mL
Polyvinylpyrrolidone	2	mL
Ficoll 400	2	mL
Distilled water	700	mL

17. 20 x SSC solution

NaCl	175.3	g
Citrate·3Na·2H_2O	88.2	g
Distilled water	700	mL

To adjust pH to 7.0 with 10 M NaOH and adjust volume to 1 L with distilled water. The solution was autoclaved at 121°C, 15 lb/in^2 for 15 minutes.

18. 10 X PBS

Na_2HPO_4	5.68	g
KH_2PO_4	1.02	g
NaCl	40.07	g
KCl	1	g
Distilled water	1	L

To adjust pH 7.0 with 1N NaOH or 1N HCl. The solution was autoclaved at 121°C, 15 lb/in^2 for 15 minutes.

19. 10 mg/mL Salmon sperm DNA

Salmon sperm DNA	10	mg
10 mM TE buffer, pH7.6	1	mL

To boil for 10 minutes. After immediately cooling in ice, sonicate for 3 mins.

20. Proteinase K

Proteinase K	4	mg
50 mM Tris-HCl, pH 7.5	1	mL

Use freshly prepared solution.

21. 40 mM CH_3COONa

CH_3COONa	3.2812	g
Distilled water	1	L

22. 12 mM $ZnSO_4$

$ZnSO_4$ (anhydrous)	1.9376	g
Distilled water	1	L

23. Nuclease P1 solution

Nuclease P1	0.1	mg
40 mM CH_3COONa	500	µL
12 mM $ZnSO_4$, pH 5.3	500	µL

To store at 4°C.

24. Alkaline phosphatase solution

Alkaline phosphatase	2.4	units
0.1 M Tris-HCl (pH 8.1)	1	mL

25. TE buffer

10 mM Tris HCl, pH 8.0	10	mL
1 m M Na_2-EDTA, pH 8.0	10	mL
Distilled water	980	mL

To autoclave at 121 °C, 15 lb/in^2 for 15 minutes.

26. 10 mg/mL TMB in 10 % DMSO

3,3',5,5'-Tetramethylbenzidine (TMB)	0.001	g
DMSO	10	µL
Distilled water	90	µL

27. 0.3 % H_2O_2

3% H_2O_2	10	µL
Distilled water	90	µL

28. 0.1 M Citric acid in 10 % DMSO

Citric acid	0.05	g
DMSO	250	µL
Distilled water	2.25	mL

29. 0.2 M Na_2HPO_4 in 10 % DMSO

Na_2HPO_4	0.07	g
DMSO	250	µL
Distilled water	2.25	mL

30. TE buffer + RNase A

TE buffer	960	mL
Rnase A (2 mg/mL)	100	µL

31. Reagent and buffer for DNA-DNA hybridization

Prehybridization solution

100 x Denhardt solution	5	mL
10 mg/ml Salmon sperm DNA	1	mL
20 x SSC	10	mL
Formamide	50	mL
Distilled water	34	mL

Hybridization solution

Prehybridization solution	100	mL
Dextran-sulfate	5	g

Solution I

Bovine serum albumin (Fraction V)	0.25	g
Titron X-100	50	µL
1X PBS	50	mL

Solution II

Streptavidin-POD	1	µL
Solution I	4	mL

Solution III, pH 6.2

10 mg/mL TMB in 10 % DMSO	100	µL
0.3 % H_2O_2	100	µL
0.1 M Citric acid in 10 % DMSO	2.5	mL
0.2 M Na_2HPO_4 in 10 % DMSO	2.5	mL

2M H_2SO_4

conc. H_2SO_4	22	mL
Distilled water	178	mL

To autoclave at 121 °C, 15 lb/in^2 for 15 minutes.

32. Ethidium bromide solution (10 mg/mL)

Ethidium bromide	1	g
Distilled water	100	mL

To store in light-tight container at room temperature.

33. 5 X Tris-acetate EDTA buffer (5 X TAE)

Tris	5.4	g
Boric acid	2.75	g
Na_2-EDTA	0.47	g
Distilled water	100	mL

34. Agarose gel

Agarose	0.8	g
1 x TAE buffer	100	mL

35. 0.1 M $MgCl_2$ in 1 x PBS (PBSM)

$MgCl_2$ (anhydrous)	0.95211	mg
10 x PBS	10	mL
Distilled water	90	mL

APPENDIX C

16S rRNA gene sequence of strains

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>AP1-20

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APPENDIX D

NMR SPECTRA

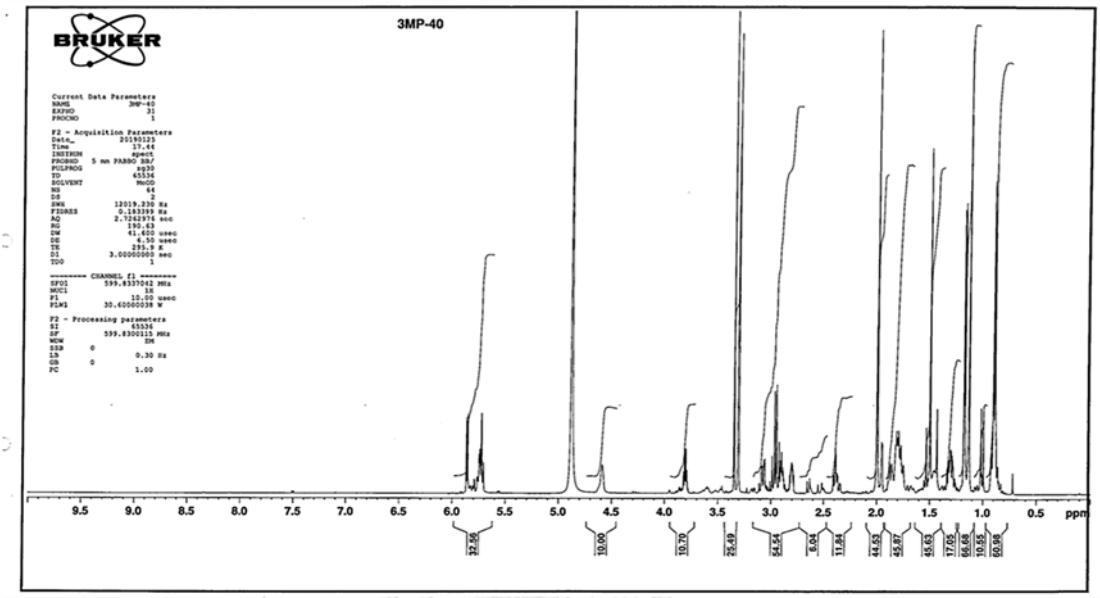

Figure D1 ^{1}H NMR spectrum of 3-NA14608 (600 MHz, CD_3OD)

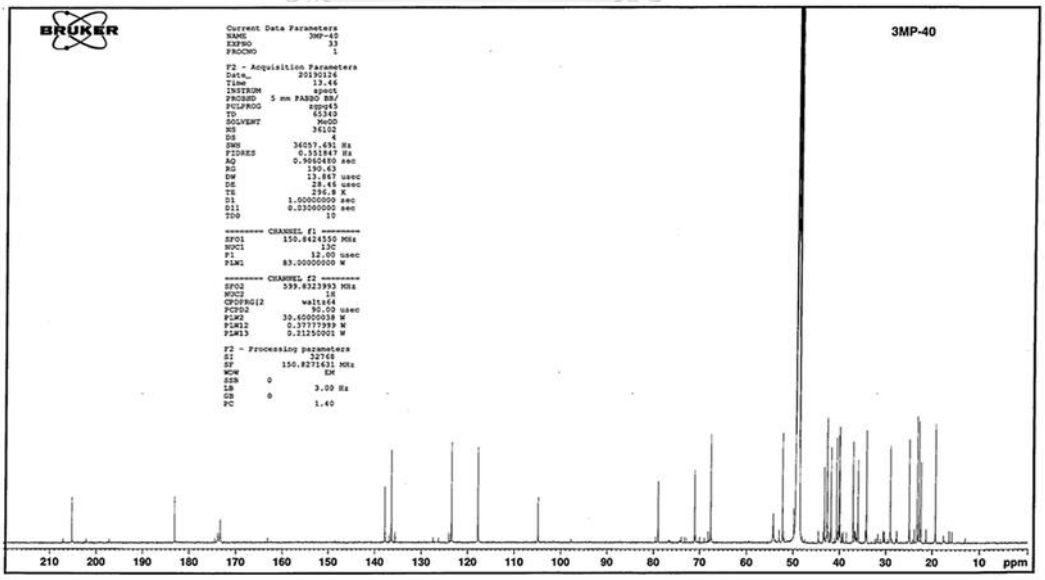

Figure D2 ^{13}C NMR spectrum of 3-NA14608 (150 MHz, CD_3OD)

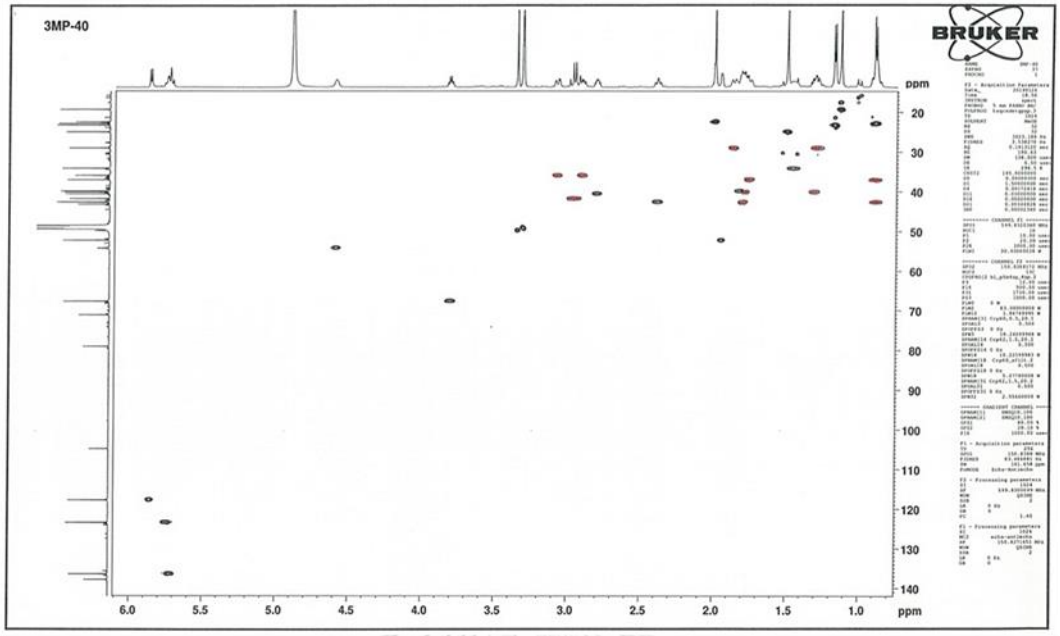

Figure D3 HSQC spectrum of 3-NA14608 (600 MHz, CD_3OD)

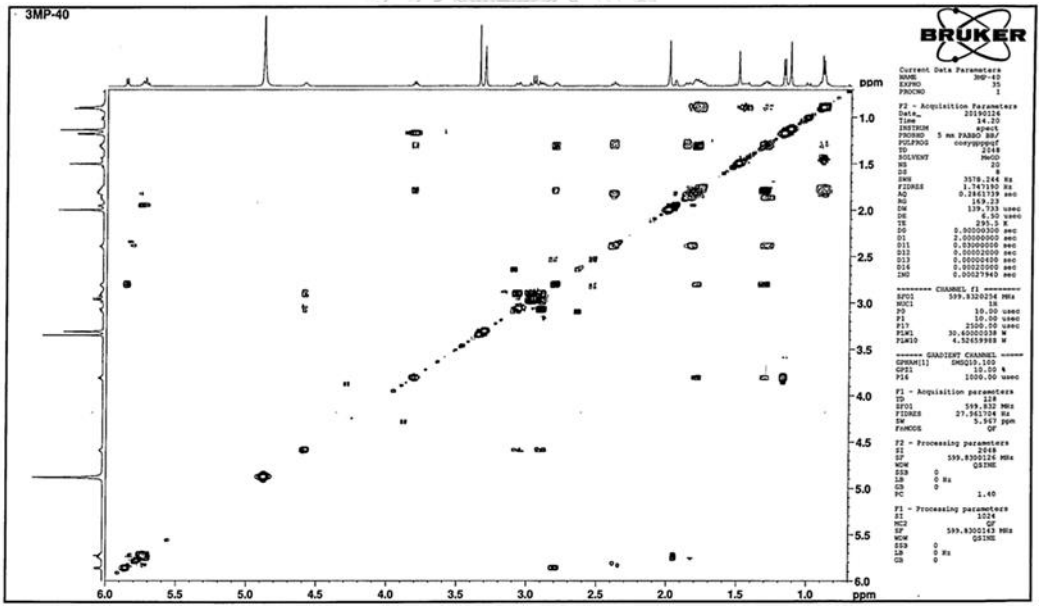

Figure D4 ^{1}H ^{1}H COSY spectrum of 3-NA14608 (600 MHz, CD_3OD)

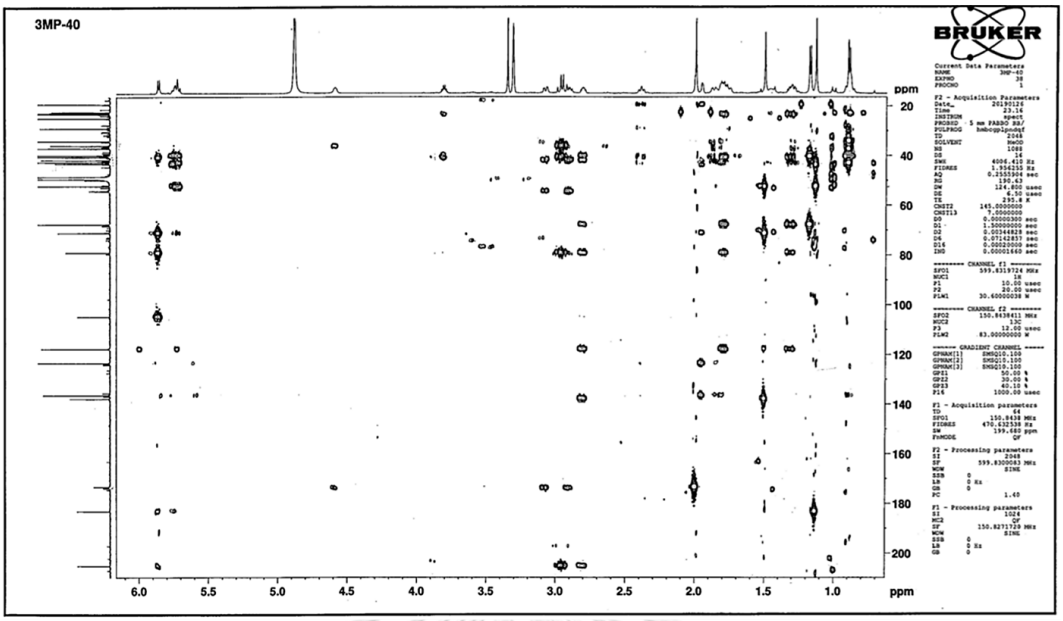

Figure D5 HMBC spectrum of 3-NA14608 (600 MHz, CD_3OD)

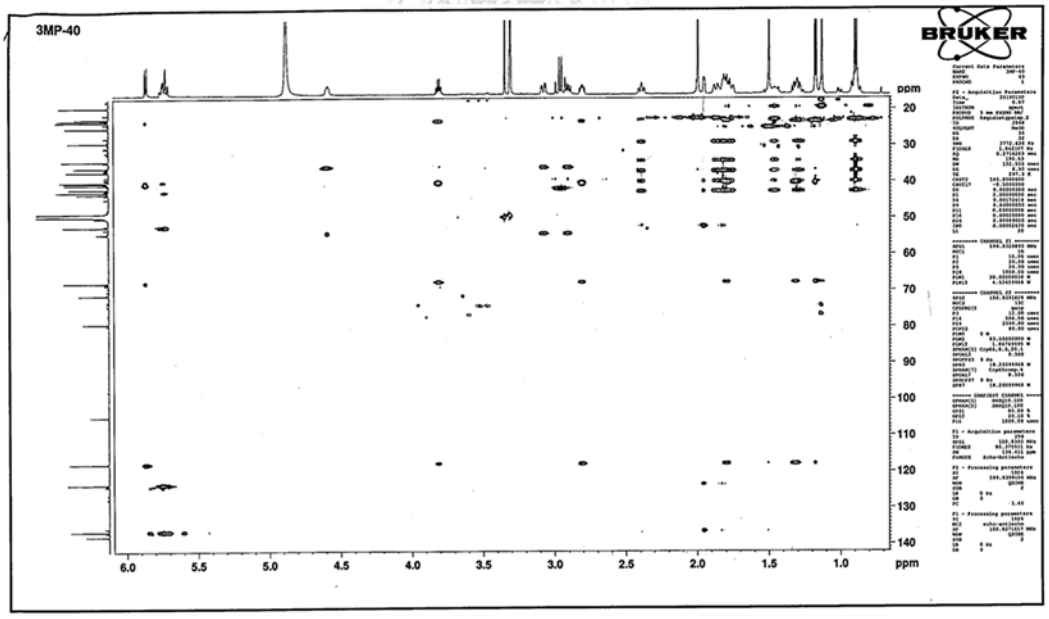

Figure D6 TOCSY spectrum of 3-NA14608 (600 MHz, CD_3OD)

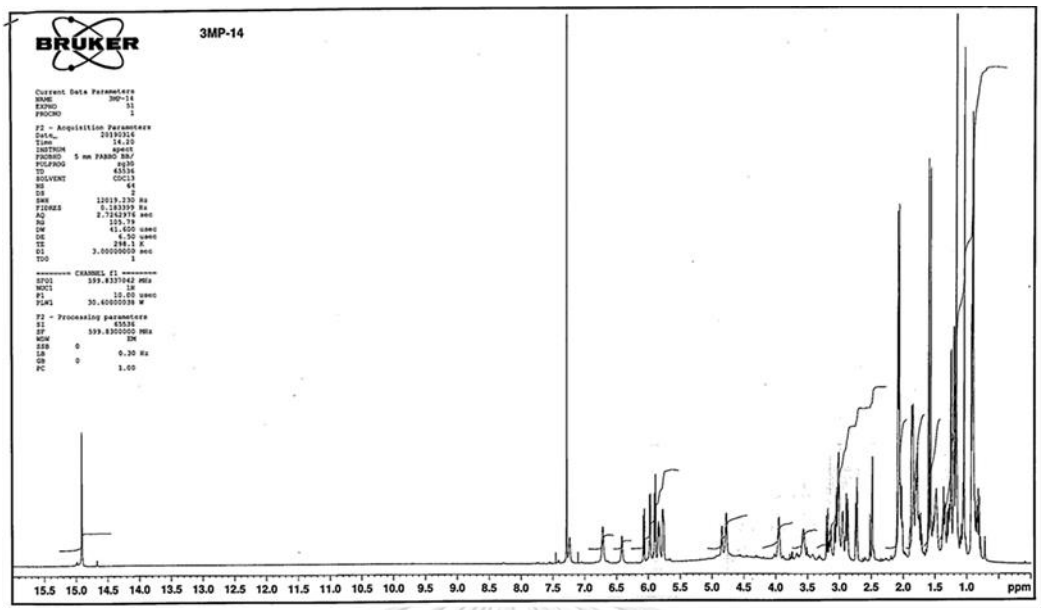

Figure D7 ^{1}H NMR spectrum of 3-NA14608 (600 MHz, $CDCl_3$)

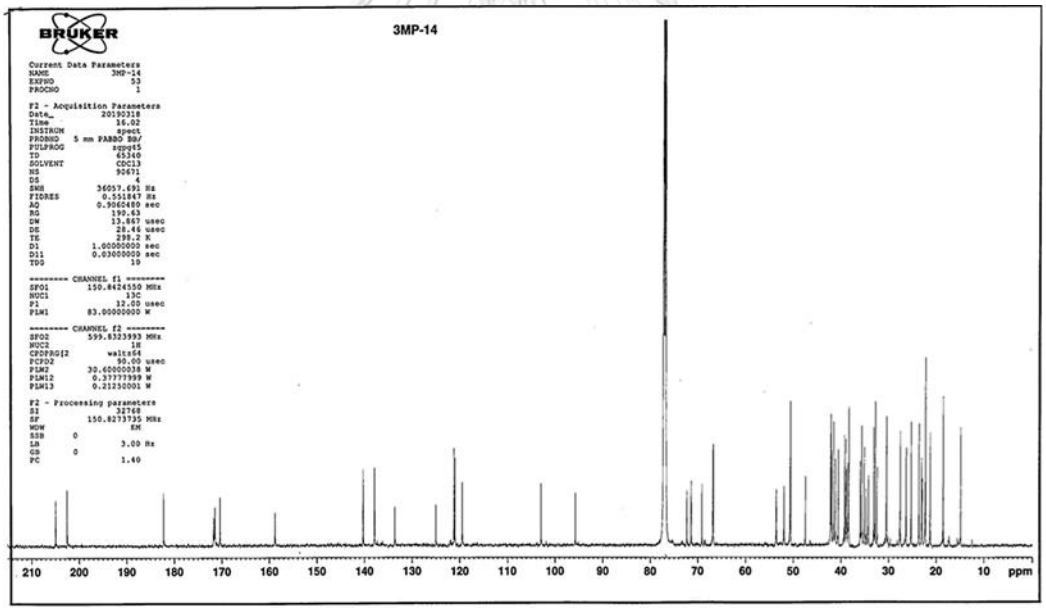

Figure D8 ^{13}C NMR spectrum of 3-NA14608 (150 MHz, $CDCl_3$)

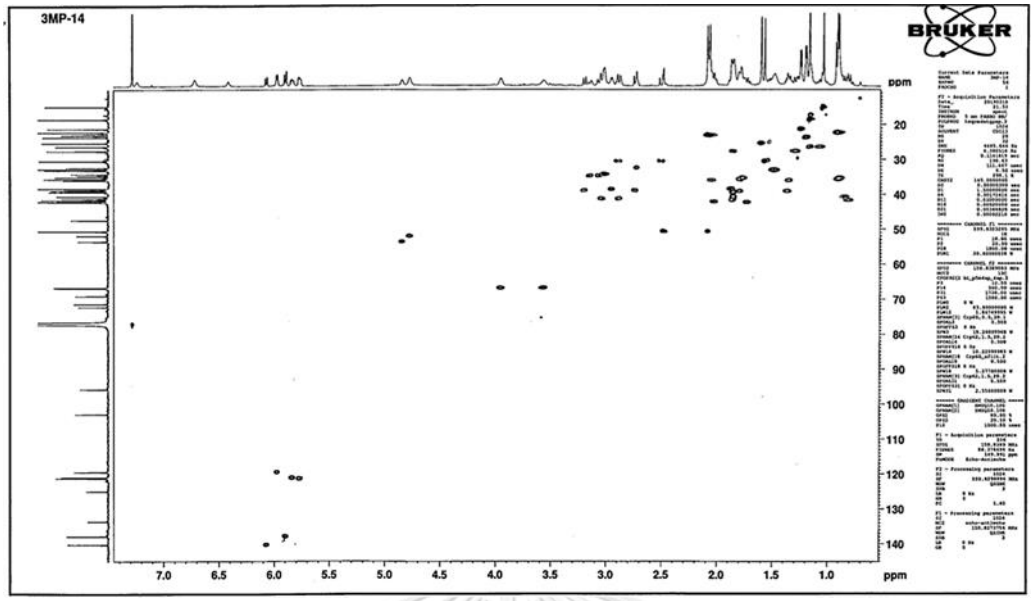

Figure D9 HSQC spectrum of 3-NA14608 (600 MHz, $CDCl_3$)

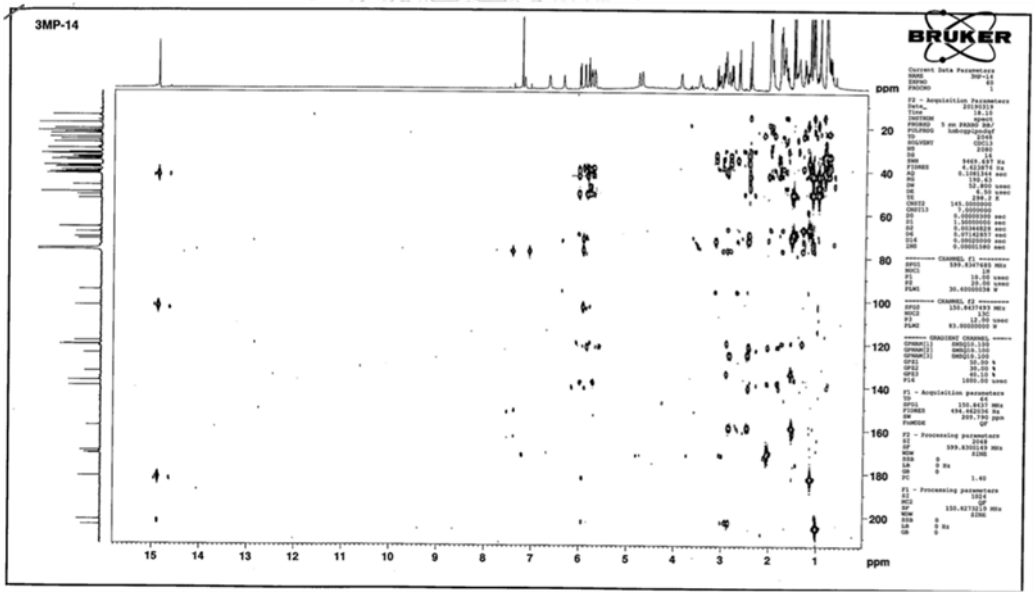

Figure D10 HMBC spectrum of 3-NA14608 (600 MHz, $CDCl_3$)

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Klykleung, N., Yuki, M., Kudo, T., Ohkuma, M., Phongsopitanun, W., Pittayakhajonwut, P., and Tanasupawat, S. (2019). *Microbispora catharanthi* sp. nov., a novel endophytic actinomycete isolated from the root of *Catharanthus roseus*. *Int J Syst Evol Microbiol.* (in press) doi:10.1099/ijsem.0.003858.

Presentation:

Klykleung, N., Nakashima, T., Takahashi, Yoko., Kudo, T., Ohkuma, M., Pittayakhajonwut, P., Taweechotipatr, M., and Tanasupawat, S. Taxonomy, Bioactivities, and Secondary Metabolites of Endophytic Actinomycetes Isolated from Medicinal Plants. The TSRI CONGRESS 2019. August 8, 2019 at Royal Paragon Hall 3, Siam Paragon, Bangkok, Thailand. (poster presentation)

Klykleung, N., Nakashima, T., Takahashi, Yoko., Kudo, T., Ohkuma, M., Pittayakhajonwut, P., Taweechotipatr, M., and Tanasupawat, S. Taxonomy, Bioactivities, and Secondary Metabolites of Endophytic Actinomycetes Isolated from Medicinal Plants.

The TSRI CONGRESS 2019. August 9, 2019 at the M floor, Pathumwan Princess, Bangkok, Thailand. (oral presentation)

Internship:

2018: Fermentation and structural elucidation of the secondary metabolites of actinomycetes. Takuji Nakashima, Ph.D. Kitasato Institute for Life Sciences (Research Division), Kitasato University. September 1, 2018-February 28, 2019. The Thailand Research Fund through the 2017 Royal Golden Jubilee Ph. D. program (PHD/0089/2559) and the Institute for Fermentation, Osaka (IFO), Japan.

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PUBLICATION	1) Klykleung, N., Yuki, M., Kudo, T., Ohkuma, M., Phongsopitanun, W., Pittayakhajonwut, P., and Tanasupawat, S. (2019). Nonomuraea phyllanthi sp. nov., an endophytic actinomycete isolated from the leaf of Phyllanthus amarus. Arch. Microbiol. (in press) doi:10.1007/s00203-019-01717-w. 2) Klykleung, N., Yuki, M., Kudo, T., Ohkuma, M., Phongsopitanun, W., Pittayakhajonwut, P., and Tanasupawat, S. (2019). Microbispora catharanthi sp. nov., a novel endophytic actinomycete isolated from the root of Catharanthus roseus. Int J Syst Evol Microbiol. (in press) doi:10.1099/ijsem.0.003858. 3) Klykleung, N., Nakashima, T., Takahashi, Yoko., Kudo, T., Ohkuma, M., Pittayakhajonwut, P., Taweechotipatr, M., and Tanasupawat, S. Taxonomy, Bioactivities, and Secondary Metabolites of Endophytic Actinomycetes Isolated from Medicinal Plants. The TSRI CONGRESS 2019. August 8 and 9, 2019 at Royal Paragon Hall 3, Siam Paragon and the M floor, Pathumwan Princess, Bangkok, Thailand. (poster and oral)