Bryophytes Harbor Cultivable Actinobacteria With Plant Growth Promoting Potential

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This study was designed to investigate the cultivable actinobacteria associated with bryophytes and their plant growth promoting ability. Thirteen actinobacteria were isolated and tested for their ability to promote growth of plant in vitro and in planta. All isolates were able to produce IAA and siderophores. Six isolates were identified as members of the genus Micromonospora. Five isolates belonged to the genus Streptomyces and one each of Microbispora and Mycobacterium. Micromonospora sp. CMU55-4 was inoculated to rare moss [Physcomitrium sphaericum (C. Ludw.) Fürnr.] and could increase the amount of carotenoid, fresh weight, and dry weight of this moss. In addition, this strain promoted capsule production, and rescued P. sphaericum’s gametophytes during acclimatization to land. Strain CMU55-4 was identified as Micromonospora chalcea based on whole genome sequence analysis. Its plant growth promoting potential was further characterized through genome mining. The draft genome size was 6.6 Mb (73% GC). The genome contained 5,933 coding sequences. Functional annotation predicted encoded genes essential for siderophore production, phosphate solubilization that enable bacteria to survive under nutrient limited environment. Glycine-betaine accumulation and trehalose biosynthesis also aid plants under drought stress. M. chalcea CMU55-4 also exhibited genes for various carbohydrate metabolic pathways indicating those for efficient utilization of carbohydrates inside plant cells. Additionally, predictive genes for heat shock proteins, cold shock proteins, and oxidative stress such as glutathione biosynthesis were identified. In conclusion, our results demonstrate that bryophytes harbor plant growth promoting actinobacteria. A representative isolate, M. chalcea CMU55-4 promotes the growth of P. sphaericum moss and contains protein coding sequences related to plant growth promoting activities in its genome.

Keywords: actinobacteria, bryophytes, Physcomitrium sphaericum, plant-microbes interaction, plant-growth promoting bacteria, genome mining, draft genome, whole genome sequencing
INTRODUCTION

Plant microbiomes are involved in the well-being of their host health by influencing the growth of plant pathogens and help plant to tolerate stress conditions (Gabrielle, 2016). Plant growth-promoting rhizobacteria (PGPR), which live around root area of plants, can be beneficial to plants by mediating plant growth through diverse biochemical mechanisms such as biosynthesis of indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, siderophore production, and phosphate solubilization. PGPR which are abundant in soil and rhizosphere can penetrate into root endosphere through cellular disjunction during lateral root emergence (Bulgarelli et al., 2013) to colonize inside plants. Actinobacteria, a phylum of Gram-positive bacteria that contained high amount of G+C in their DNA (>55 mol% of genomic DNA) are interesting due to their prolific metabolic capabilities (Yadav et al., 2018). Plant growth promoting actinobacteria (PGPA) are abundant in soil and can enhance nutrients availability, administer plant metabolism and reduce environmental stress in plants (Hamedi and Mohammadipanah, 2014). Bryophytes are group of plants that grow under specific climate conditions and their microbiomes are still not well studied. They are the oldest known land plants that lack true leaves, stem or true vascular system and considered as a remarkable reservoir of novel active compounds, natural products and antibiotics (Chandra, 2014). Actinobacteria associated with bryophytes could have some specific evolutionary adaptations to their host plants and may have potential of bioactive compounds production that need to be investigated. One of the easiest ways for bryophytes propagation is using tissue culture technique. However, the obtained in vitro plantlets are usually unable to compete with other native soil microbes when transfer to soil. In addition, micropropagated plants are struggled to cope with environmental conditions in the field which results in the morphological and anatomical changes of plantlets (Chandra and Bandopadhyay, 2010). The objectives of this study are

1. To investigate the cultivable actinobacteria associated with some bryophytes;
2. To study the plant growth promoting ability of actinobacteria from bryophytes.

One strain, Micromonospora chalcea CMU55-4 shows promising potential to promote the growth of tested bryophyte during acclimatization period in laboratory before transfer to the forest. Its taxonomic characterization and whole genome analysis are also reported.

MATERIALS AND METHODS

Sample Collection

Bryophyte samples were collected from Doi Inthanon National Park, Chiang Mai, Thailand. Various keys and checklists were used for identification such as Hattori et al. (1977), Eddy (1988, 1996), Zander (1993), and Li et al. (2007). Actinobacteria were isolated from five species of bryophytes, including mosses and liverwort. Four high altitude moss species were Bryum apiculatum Schwägr. (CMU15), Syntrichia gemmascens (P.C. Chen) R.H. Zander (CMU51), Campylopus involutus (Müll. Hal.) A. Jaeger (CMU55), and Plagiomnium maximovicii (Lindb.) T.J. Kop (CMU13). Liverwort was Frullania nepalensis (Spreng.) Lehmm. and Lindemb. (CMU12). The remaining bryophyte samples from isolation were kept at Chiang Mai University Herbarium (Herbarium code: CMUB).

Selective Isolation

Each bryophyte sample (1 g fresh weight) was surface sterilized (70% ethanol 1 min, 3% sodium hypochlorite 10 min, 3 times sterile distilled water). Sterile samples were crushed in sterile mortars, diluted with sterile distilled water up to 10^-3 and plated on starch casein agar (Küster and Williams, 1964), humic acid vitamin B agar (Hayakawa and Nonomura, 1987), R2A agar and water proline agar (1% proline). Plates were incubated for 30 days at 25°C. All colonies appeared on agar were picked and purified on ISP2 agar (Shirling and Gottlieb, 1966). The effectiveness of the surface sterilization method was evaluated by spread the final rinse water of each bryophyte sample on ISP2 agar.

Plant Growth Promoting Activity

IAA production was determined following the methods of Glickman and Dessaux (1995). In brief, actinobacteria were grown in ISP2 broth with and without 2 mg/ml L-tryptophan for 7 days in the dark. After that, the supernatant was mixed with Salkowski's reagent (1 ml of 0.5 M FeCl3 in 50 ml 35% HClO4), stored at room temperature for 30 min and measured the optical density at 530 nm. IAA production was also confirmed by HPLC (Fluorescent detector) with a Restek Ultra C18, 5 µm (150 × 4.6 mm) using 0.1 M acetic acid as mobile phase A and 0.1 M acetic acid in methanol as mobile phase B (1 ml/min flow rate). Standard methods were used to determine siderophore production quantitatively and qualitatively. Siderophore production was first screened on CAS agar (Schwyn and Neilands, 1987) using King’s B agar as a basal medium (King et al., 1954). The appearance of a yellow to orange zone around the actinobacterial colony after 7 days incubation in the dark was an indication of siderophore production. Quantitative determination of siderophore was carried out in liquid medium. Actinobacteria were grown in King’s B broth for 7 days. The culture supernatant was mixed with ferric perchlorate solution for hydroxamate siderophore (Atkin et al., 1970), and 0.5 M HCl, nitrite-molybdate reagent and 1 M NaOH for catecholate siderophore (Arnow, 1937). Phosphate solubilization was determined from clear zone formation on Pikovskaya (PVK) agar supplemented with 0.5% (w/v) tricalcium phosphate after 7 days incubation (Nautiyal, 1999). Drought tolerant ability of actinobacteria was also investigated using sorbitol as an osmotic adjustment in water agar medium (Hallsworth et al., 1998).

Taxonomic Characterization

DNA extraction was performed following the method of Kudo et al. (1998). Genomic DNA was used as the template for polymerase chain reaction (PCR) following the method of Lee et al. (2014). 27F (5’ AGAGTTTGTATCMTGCTCAG 3’)
TABLE 1 | IAA and siderophore production.

| Isolate      | IAA (µg/ml) | Siderophore production |
|--------------|-------------|------------------------|
|              | No L-tryptophan | 2 mg/ml L-Tryptophan | Halozone diameter (cm) | Halozone color | Hydroxamate (µMole/l) | Catecholate (µMole/l) |
| CMU15-1      | 0.70 ± 0.05abcd | 6.55 ± 1.43abc | 0.93 ± 0.39abcd | Clear | 60.00 ± 2.50abcd | 20.00 ± 3.80abcd |
| CMU15-2      | 2.12 ± 0.15abcd | 4.70 ± 0.61abcd | 1.11 ± 0.35abcd | Clear | 27.50 ± 0.00abcd | 4.21 ± 0.00abcd |
| CMU15-3      | 1.41 ± 0.22abcd | 6.62 ± 0.67abcd | 0.95 ± 0.33abcd | Yellow | 50.00 ± 2.50abcd | 25.79 ± 3.16abcd |
| CMU15-4      | 0.68 ± 0.31abcd | 1.50 ± 0.42abcd | 0.88 ± 0.24abcd | Yellow | 110.00 ± 10.6abcd | 5.00 ± 0.74abcd |
| CMU15-1-1    | 2.73 ± 1.25abcd | 3.33 ± 0.07abcd | 1.09 ± 0.24abcd | Yellow | 992.50 ± 50.76abcd | 484.47 ± 27.91abcd |
| CMU15-1-2    | 2.71 ± 0.71abcd | 3.42 ± 0.11abcd | 1.2 ± 0.16abcd | Yellow | 206.25 ± 12.37abcd | 77.11 ± 1.12abcd |
| CMU15-1-4    | 1.82 ± 0.36abcd | 3.45 ± 0.64abcd | 1.17 ± 0.43abcd | Yellow | 121.25 ± 1.7abcd | 98.68 ± 7.07abcd |
| CMU55-5      | 2.27 ± 0.37abcd | 3.53 ± 0.41abcd | 1.62 ± 0.18abcd | Yellow | 610.00 ± 17.6abcd | 40.53 ± 7.3abcd |
| CMU55-1-1    | 0.61 ± 0.05abcd | 3.5 ± 0.29abcd | 1.36 ± 0.16abcd | Yellow | 478.75 ± 65.4abcd | 11.05 ± 2.41abcd |
| CMU55-2      | 0.20 ± 0.07abcd | 3.7 ± 0.66abcd | 1.48 ± 0.31abcd | Clear | 42.50 ± 2.50abcd | 10.35 ± 1.85abcd |
| CMU55-3      | 1.45 ± 0.32abcd | 4.06 ± 0.66abcd | 1.27 ± 0.22abcd | Yellow | 808.75 ± 61.8abcd | 289.21 ± 42.80abcd |
| CMU55-4      | 1.20 ± 0.05abcd | 11.35 ± 3.34abcd | 0.97 ± 0.18abcd | Brownish yellow | 54.17 ± 8.7abcd | 10.18 ± 3.08abcd |
| CMU55-5      | 1.50 ± 0.04abcd | 5.00 ± 0.05abcd | 1.66 ± 0.22abcd | Orange yellow | 35.00 ± 4.33abcd | 1.05 ± 0.74abcd |

a–d indicated significant difference in statistical analysis tested by SPSS One-Way ANOVA and Tukey HSD test (p < 0.05), n = 3.

TABLE 2 | Taxonomic assignment of bryophytes associated actinobacteria based on EzBiocloud database.

| Bacterial strain | Isolation source | Media | DDBJ accession number | Different nt/Total nt | Similarity (%) | Top hit taxon |
|------------------|------------------|-------|------------------------|-----------------------|----------------|--------------|
| CMU15-1          | B. apiculatum    | Water proline agar | LC458843 | 5/1,373 | 99.63 | Micromonospora humi DSM 456477 |
| CMU15-2          | B. apiculatum    | Water proline agar | LC458844 | 7/1,343 | 99.48 | Micromonospora humi DSM 456477 |
| CMU15-3          | B. apiculatum    | Water proline agar | LC458845 | 11/1,362 | 99.19 | Micromonospora humi DSM 456477 |
| CMU15-4          | B. apiculatum    | Water proline agar | LC458846 | 11/1,366 | 99.19 | Micromonospora cirtae DSM 439037 |
| CMU51-1          | S. gemmascens    | Starch casein agar | LC458847 | 0/1,357 | 100 | Streptomyces fulvissimus DSM 405937 |
| CMU51-2          | S. gemmascens    | Starch casein agar | LC458848 | 2/1,395 | 99.85 | Streptomyces pratensis ch247 |
| CMU51-4          | S. gemmascens    | Water proline agar | LC458849 | 3/1,370 | 99.78 | Streptomyces pratensis ch247 |
| CMU55-5          | S. gemmascens    | R2A agar | LC458850 | 2/1,366 | 99.85 | Streptomyces fulvissimus DSM 405937 |
| CMU55-1          | C. involutus     | Humic acid vitamin B agar | LC458851 | 2/1,366 | 99.85 | Micromonospora tubagiae DSM 451427 |
| CMU55-2          | C. involutus     | Water proline agar | LC458852 | 0/1,339 | 100 | Mycolicibacterium iranicum DSM 455417 |
| CMU55-3          | C. involutus     | Humic acid vitamin B agar | LC458853 | 6/1,376 | 99.56 | Streptomyces fulvissimus DSM 405937 |
| CMU55-4          | C. involutus     | Water proline agar | LC458854 | 22/342 | 98.20 | Micromonospora chalcea DSM 430267 |
| CMU55-5          | C. involutus     | Humic acid vitamin B agar | LC438389 | 53/1,544 | 96.31 | Microbispora rosea subsp. rosea ATCC 129907 |

and 1492R (5′ TACGGYTACCTTGGTACGACT T 3′) primers were used to amplify 16S rRNA gene. The PCR amplicons were purified by GF-1 AmbiClean Kit (Vivantis®) following the manufacturer instruction. The 16S rRNA sequencing was performed by commercial service at First BASE Laboratories Sdn Bhd, Malaysia. Identification of all actinobacterial isolates was achieved by BLAST analysis of 16S rRNA gene sequences using EzBiocloud database1. Neighbor-joining phylogenetic tree was constructed using BioEdit Sequence Alignment Editor version 7.2 and MEGA7 (Kumar et al., 2016). Tree topology was evaluated using the bootstrap resampling method at 1000 bootstrap. Strain CMU55-4 was also identified based on whole genome sequence analysis. The ANI value was calculated and compared in JSpeciesWS (Ritcher et al., 2016), web server tool, using ANI-Blast (ANIIb) and ANI-MUMmer (ANIm) algorithms (Ritcher and Rosselló-Móra, 2009) within the web service. The Genome-To-Genome Distance Calculator (GGDC 2.1) with the BLAST + method (Meier-Kolthoff et al., 2013) was used to evaluate the digital DNA-DNA hybridisation (dDDH).

Growth Promotion on Physcomitrium sphaericum (C. Ludw.) Führnr.
Physcomitrium sphaericum (C. Ludw.) Führnr. was used as a moss model to determine beneficial effects of selected actinobacteria on bryophytes during transplantation. P. sphaericum is a rare moss species in Thailand with the risk from extinction. The re-introduction of this moss back to nature is badly needed. Spores of P. sphaericum were picked from healthy plants from the nature under stereo microscope using sterile forceps and placed into Hoagland agar (Hoagland and Arnon, 1950) and incubated for 3 months at 24°C, 35,000–40,000 lux intensity to let spore germinate and form protonema. Then, the moss protonema

1https://www.ezbiodiversity.org/
was transferred to Hoagland agar and incubated in the same condition for 3 months. The moss plantlets were transferred from agar media to soil. Strain CMU55-4 was chosen to be introduced to moss due to its high IAA production in vitro and fast growth within 3 days which is an important trait of PGPA to compete with other soil microbes. Strain CMU55-4 was grown in ISP2 broth for 7 days, 25°C, 150 rpm. Culture broth was centrifuged to collect cells. Cells were washed and resuspended in sterile distilled water and adjusted the concentration to OD_{600} = 1, which is equivalent to 10^6 cells/ml. A suspension of strain CMU55-4 (1 ml) was dropped into the autoclaved soil around the plants. Sterile distilled water was used as a control solution. The plants were maintained in the incubator at 24°C, 35,000–40,000 lux for 1 month. Chlorophyll and carotenoid contents were determined from fresh samples using standard method (Chappelle et al., 1992). Dry weight was obtained from plants that were dried in 60°C oven for 7 days.

### Genome Mining for Plant Growth Promoting Potential

Whole genome sequencing of *Micromonospora* sp. CMU55-4 was carried out by an Illumina Miseq platform (Illumina, Inc., San Diego, US-CA) using 2 × 250 bp paired-end reads. Raw reads quality was checked using FASTQC software (Andrews, 2010). Adaptors and poor-quality reads were removed using Trim Galore (Krueger, 2015), and the filtered reads were used as an input for Unicycler (Wick et al., 2017), genome assembly program. Annotation of assembled genome was done using Prokka Version 1.13 (Seemann, 2014), and NCBI Prokaryotic Genome Annotation Pipeline (PGAP). The draft genome sequence of strain CMU55-4 was mined using RAST annotation server (Aziz et al., 2008) and analyzed through SEED viewer (Overbeek et al., 2014) for genes responsible for plant growth promoting properties.
GenBank Accession Number
The accession number of the draft genome sequence is JAAOLH000000000.

Chemotaxonomic, Cultural, and Phenotypic Characterization of *Micromonospora* sp. CMU55-4
The type strain *Micromonospora chalcea* DSM 43026\textsuperscript{T} was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM) culture collection. *Micromonospora terminaliae* TMS7\textsuperscript{T} was kindly given by Dr. Onuma Kaewkla of Department of Biology, Faculty of Science, Mahasarakham University. Cultural characteristics were determined after 2 weeks at 30°C on ISP1-7 media (Shirling and Gottlieb, 1966), Nutrient agar (Difco), Tryptic Soy agar (Difco), Potato Dextrose agar (Difco), Modified Bennett agar (Jones, 1949), and ATCC172 agar. The ISCC-NBS color chart (Kelly, 1964) was used to determine the color of mycelia. pH range for growth (4.0–12.0 at intervals of 1.0 pH unit), tolerant to NaCl concentration (0–9% at 1% intervals) and temperature range for growth (4, 20, 25, 30, 37, 40, 45, 50, 55°C) were determined using ISP2 agar as a basal medium. H\textsubscript{2}S production, coagulation and peptonization, gelatin liquefaction, starch hydrolysis and nitrate reduction were determined on ISP6 agar, 10% skim milk (Difco), glucose-peptone-gelatin medium (2.0% glucose, 0.5% peptone, 20% gelatin pH 7.0), ISP4 agar and ISP8 broth (0.5% peptone, 0.3% beef extract, 0.1% KNO\textsubscript{3}, pH 7.0). The production of enzymes was determined using API ZYM kit (bioMérieux). The utilization of carbohydrates as sole carbon sources was obtained using ISP 9 (Nihon Pharmaceutical) as the basal medium supplemented with 1% (w/v) of each carbon source.
source. Spore morphology of 21-day-old culture on ISP2 agar was observed by scanning electron microscope (JSM-IT500HR; JEOL). For chemotaxonomic study, biomass of strain CMU55-4 was obtained from culture grown in ISP2 broth for 7 days at 30°C (160 rpm) and freeze dried. The analysis of whole cell reducing sugar and isomer of diaminopimelic acid on cell wall (A2mp) was determined on thin-layer-chromatography (TLC) following the method of Staneck and Robert (1974). Polar lipids profiles were extracted and identified using 2-dimensional TLC according to the method of Minnikin et al. (1984). Menaquinones were extracted according to the method of Collins et al. (1977) and were analyzed by UPLC (Agilent Technology 1290 Infinity II model: 67116B 1290MCT UV detector) with a µBondapak C18 column (Waters).

### Statistical Analysis

Statistical analysis was performed by SPSS statistics 17.0 program.

### RESULTS

#### Selective Isolation

A total of 13 actinobacteria were obtained from all four selective media. The highest number of isolates was recovered on water proline agar (6 isolates, 46.15%) followed by humic acid vitamin B agar (4 isolates, 30.76%), starch casein agar (2 isolates, 15.38%) and R2A gar (1 isolate, 7.69%) (Table 2). Most isolates were from *C. involutus* (38%). However, no bacteria were obtained from liverwort.

#### Plant Growth Promoting Activity

All isolates produced indole-3 acetic acid and siderophores at varying amount (Table 1). Isolates CMU51-1 and CMU55-4 produced the highest amount of IAA without L-tryptophan supplement (2.73 µg/ml) and in 2 mg/ml of L-tryptophan (11.35 µg/ml), respectively. For siderophores production, isolates CMU51-1 produced the highest amount of both hydroxamate type (992.50 ± 50.76 µMole/l) and catecholate type siderophores (484.47 ± 27.91 µMole/l) (Table 1). For drought tolerant ability, no growth was observed below aw 0.957 for all isolates. None of the obtained isolates could solubilize tricalcium phosphate on PVK agar.

#### Taxonomic Characterization

Comparison of 16S rRNA gene sequence similarity of the obtained actinobacteria in EzBiocloud database, assigned them as members of the following genera: *Micromonaspora*, *Streptomyces*, *Mycolicibacterium* and *Microbispora* (Table 2). The majority of isolates were *Micromonaspora* (46%) followed by *Streptomyces* (38%). The 16S rRNA gene similarity values were ranged from 96.31 to 100%. Phylogenetic analysis confirmed the assignment of these actinobacteria at genus level based on BLAST results (Figure 1). Strain CMU55-5 is closely related to *Microbispora rosea* subsp. *rosea* ATCC 12950T with low 16S rRNA gene sequence similarity value of 96.3%. Strain CMU55-4 is closely related to *Micromonaspora chalcea* DSM 43026T with 16S rRNA gene sequence similarity value of 98.2%. The AN1b values of 98.47% and digital DNA-DNA hybridization value of 91.8% were found between strain CMU55-4 and *M. chalcea* DSM 43026T.

### Growth Promotion on *Physcomitrium sphaerica*um (C. Ludw.) Fürnr.

*Physcomitrium sphaericum* (C. Ludw.) Fürnr. plantlets that were inoculated with *Micromonaspora* strain CMU55-4 showed better growth appearance compared to control plants. The control plants have more chlorosis leaves and showed growth retardation (Figure 2A). The plants that had been treated with strain CMU55-4 started to develop new leaves and showed the production of capsules (Figure 2B). Carotenoid, fresh weight and dry weight of *P. sphaericum* were significantly higher than the control. However, total chlorophyll content was not different in control and strain CMU55-4 inoculated plantlets (Figure 3).

### Genome Mining for Plant Growth Promoting Potential

From whole genome sequence data, the estimated genome size of *Micromonaspora* sp. CMU55-4 was 6.6 Mb. The GC content was 73.0%. The summary of sequence assembly and genome annotation is displayed in Table 3. Phylogenomic tree clearly classified isolate CMU55-4 in the same species and subspecies level with *M. chalcea* DSM 43026T (Figure 4). Prokka predicted 5,946 coding regions with 6,077 estimated genes (Table 4) contained 67 tRNAs, 3 rRNAs and 2 repeat regions. Gene annotation in RAST server showed that genes were grouped into 25 subsystems (Supplementary Material 1). The majority of genes play roles in amino acids, carbohydrate, and protein metabolisms. From various subsystems, essential roles of protein coding sequences according to their plant growth promoting traits was grouped in Table 5. Twenty-four genes were associated with siderophore production, namely siderophore assembly kit, siderophore desferrioxamine E, and siderophore aerobactin. Indole-3-glycerol phosphate synthase and tryptophan synthase plays role in IAA synthesis. Gene encoding exopolyphosphatase with possible role in phosphate solubilization was also found. In addition, protein coding sequences involved in nitrogen metabolism such as assimilatory nitrate reductase, nitrate/nitrite transporter, and ammonium transporter were also detected. Strain CMU55-4 was well equipped with protein coding sequences related to oxidative stress response such as SoxR, NsrR,
organic hydroperoxide reductase, glutathione peroxidases and trehalose synthesis genes. Glycine betaine transporter (OpuD) for osmotic adjustment under osmotic stress was also present. CspA protein family, DnaK and DnaJ chaperones responsible for cold or heat stress were identified. Strain CMU55-4 also exhibited genes involved in the utilization of saccharides found inside plant cells such as xylose, arabinose, mannose and D-galacturonate (Table 5).

**Chemotaxonomic, Cultural, and Phenotypic Characterizations of Micromonospora sp. CMU55-4**

Strain CMU55-4 grew well on TSA, modified Bennett’s, and ATCC172 media. The colors of substrate mycelia were strong orange on ISP2, deep yellowish-brown on ISP1 and ISP3, ISP5, and ISP7, strong orange yellow on ISP4, ISP6 and TSA. Dark olive brown on modified Bennett’s and vivid orange on ATCC172. No growth was observed on NA and PDA. The strain produced globular spores (0.8 μm in size) with warty surface (Figure 5). The strain grew at 20 to 45°C (optimally at 30°C), pH 5.0 to 12.0 (optimally pH at 8.0) and tolerated up to 4% (w/v) NaCl. Strain CMU55-4 utilized various type of carbohydrates such as L-arabinose, dulcitol, D-mannose, and D-mannitol and those found in plant cells including D-glucose, sucrose, cellulose, amygdalin, and starch (Table 6). Cell-wall peptidoglycan of strain CMU55-4 contained meso-diaminopimelic acid. Galactose, arabinose and xylose were detected as diagnostic sugars in the whole-cell hydrolysates. The predominant phospholipids were diphasphatidyglycerol (DPG), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG). Major menaquinones were
TABLE 4 | Summary of genome annotation of strain CMU55-4.

| Prokka | CMU55-4 |
|---|---|
| Number of predicted rRNA | 3 |
| Number of predicted CDS | 5,946 |
| Number of predicted tRNA | 67 |
| Number of predicted tmRNA | 1 |
| Number of predicted misc_rRNA | 1 |
| Number of repeat region | 2 |
| Number of predicted gene | 6,077 |
| NCBI PGAP |
| Genes (total) | 6,056 |
| CDSs (total) | 5,999 |
| Genes (coding) | 5,910 |
| CDSs (with protein) | 5,910 |
| Genes (RNA) | 57 |
| rRNAs | 1, 1, 1 (5S, 16S, 23S) |
| complete rRNAs | 1, 1, 1 (5S, 16S, 23S) |
| tRNAs | 51 |
| ncRNAs | 3 |
| Pseudo Genes (total) | 89 |
| CDSs (without protein) | 89 |
| Pseudo Genes (ambiguous residues) | 0 of 89 |
| Pseudo Genes (frameshifted) | 18 of 89 |
| Pseudo Genes (incomplete) | 74 of 89 |
| Pseudo Genes (internal stop) | 7 of 89 |
| Pseudo Genes (multiple problems) | 8 of 89 |
| CRISPR Arrays | 2 |

MK-9(H₄) (33.46%), MK-9(H₆) (13.63%), MK-9(H₈) (26.16%), and MK-10(H₈) (15.38%).

**DISCUSSION**

Actinobacteria from bryophytes are very rare. In the earliest report in 2007, small number of *Rothia*, *Arthrobacter*, *Micrococcus*, and *Plantibacter* isolates were reported as endophytic actinobacteria from *Sphagnum magellanicum* Brid. and *Sphagnum fallax* H. Klinggr (Opelt et al., 2007). Only three new species of actinobacteria were described from unidentified moss species namely *Streptomyces bryophytorum* (Li et al., 2015a) and *Microbispora bryophytorum* (Li et al., 2016). In this study, almost half of actinobacteria (46%) were identified as *Micromonospora*. Members of this genus have been isolated from various habitats including plants (Trujillo et al., 2010, 2015; Carro et al., 2012, 2013; Genilloud, 2015). Several new species of *Micromonospora* are endophytes of various plants as exemplified by the description of *M. costi* (Thawai, 2015), *M. globbae* (Kuncharoen et al., 2018), *M. phytophila* (Carro et al., 2018b), *M. luteiviridis* (Carro et al., 2018b), *M. oryzae* (Kittiwongwattana et al., 2015), and *M. terminaliae* (Kaewkla et al., 2017). *Streptomyces* isolates also dominant represent around 38.5%. Our results provide additional evidence that these two actinobacterial genera are also dominant in bryophytes. *Mycolicibacterium stellerae* was recently...

**TABLE 5 |** Protein coding sequences related with plant growth promoting traits of *M. chalcea* CMU55-4.

| PGP traits | Protein coding sequences conferring PGP traits |
|---|---|
| IAA | Indole-3-glycerol phosphate synthase (EC 4.1.1.48) |
| | Tryptophan synthase alpha chain (EC 4.2.1.20) |
| | Tryptophan synthase beta chain (EC 4.2.1.20) |
| Siderophore production | Siderophore assembly kit: |
| | 1. Isochorismatase (EC 3.3.2.1) of siderophore biosynthesis |
| | 2. Siderophore biosynthesis L-2,4-diaminobutyrate decarboxylase |
| | 3. Siderophore synthetase component, ligase |
| | 4. Ferric hydroxamate ABC transporter (TC 3.A.1.14.3), permease component FhuB |
| | 5. ABC-type Fe²⁺-siderophore transport system, ATPase component |
| | 6. ABC-type Fe³⁺-siderophore transport system, permease 2 component |
| | 7. Thioesterase in siderophore biosynthesis gene cluster |
| | 8. Siderophore synthetase small component, acetyltransferase |
| | 9. ABC-type Fe³⁺-siderophore transport system, permease component |
| | 10. Siderophore biosynthesis protein, monoxygenase |
| | 11. Putative ABC iron siderophore transporter, fused permease and ATPase domains |
| | 12. 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase (EC 1.3.1.28) of siderophore biosynthesis |
| | 13. Ferric hydroxamate ABC transporter (TC 3.A.1.14.3), ATP-binding protein FhuC |
| | 14. Ferric hydroxamate ABC transporter (TC 3.A.1.14.3), periplasmic substrate binding protein FnuD |
| | 15. Thioesterase in siderophore biosynthesis gene cluster |
| | 16. Siderophore synthetase small component, acetyltransferase |
| | 17. ABC-type Fe³⁺-siderophore transport system, permease component |
| | 18. Siderophore biosynthesis protein, monoxygenase |
| | 19. Putative ABC iron siderophore transporter, fused permease and ATPase domains |
| | 20. 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase (EC 1.3.1.28) of siderophore biosynthesis |
| | 21. Ferric hydroxamate ABC transporter (TC 3.A.1.14.3), ATP-binding protein FnuC |
| | 21. Ferric hydroxamate ABC transporter (TC 3.A.1.14.3), periplasmic substrate binding protein FnuD |
| Siderophore Desferrioxamine E: | |
| | 1. Desferrioxamine E biosynthesis protein DesA, DesB, DesC, DesD |
| Siderophore Aerobactin: | |
| | 1. Ferric hydroxamate ABC transporter (TC 3.A.1.14.3), permease component FhuB |
| | 2. Ferric hydroxamate ABC transporter (TC 3.A.1.14.3), ATP-binding protein FnuC |
| | 2. Ferric hydroxamate ABC transporter (TC 3.A.1.14.3), periplasmic substrate binding protein FnuD |
| Nitrate and nitrite ammonification: | |
| | 1. Assimilatory nitrate reductase large subunit (EC 1.7.99.4) |
| | 2. Nitrate/nitrite transporter |
| | 3. Nitrite reductase [NAD(P)H] small subunit (EC 1.7.1.4) |
| | 4. Nitrite reductase [NAD(P)H] large subunit (EC 1.7.1.4) |
| Ammonia assimilation: | |
| | 1. Ferredoxin-dependent glutamate synthase (EC 1.4.7.1) |
| | 2. Nitrogen regulatory protein P-II |
| | 3. Glutamate-ammonia-lyase adenylyltransferase (EC 2.7.7.42) |

(Continued)
described as endophytic actinobacteria from plant, *Stellera chamaejasme* L. in Yunnan (Nouioui et al., 2019b). However, this is the first time that member of this genus was found associated with a bryophyte. The remaining isolate, CMU55-5 was closely related to *Microbispora rosea* subsp. *rosea*. The low 16S rRNA gene similarity value (96.3%) between CMU55-5 and its closest neighbor, suggests that this strain may represent a new species. However, polyphasic taxonomic characteriz
is needed for its formal description which will be the subject of future investigation.

No actinobacteria was obtained from liverwort. This may be an effect of “oil bodies,” the highly distinctive organelle uniquely found in liverworts (He et al., 2013). This organelle is responsible for toxic compound accumulation that usually use for protection of liverworts from herbivore (Stahl, 1888), pathogens, low temperature and excessive light (Hieronymus, 1892). We opined that during the isolation of actinobacteria, the liverwort sample was crushed which may cause the release of toxic compounds from oil bodies and inhibit the growth of actinobacteria.

The use of water proline agar yielded the highest number of actinobacterial isolates in this study. Water proline agar is a low nutritional medium that simulate oligotrophic status in natural environments. Proline is also serving as a compatible solute that commonly found in plant cells. The composition of the minimal media is suggested to allow an easier adaptation for endophytes (Alain and Querllou, 2009) and allow slow growing endophytic strains a chance to develop (Eevers et al., 2015). Complex media composed of rich carbon and nitrogen sources are suggested to be unsuitable for the growth of slow-growing endophytic bacteria as they do not resemble the environment inside plant tissues (Eevers et al., 2015). Low nutritional media have been successfully used to isolate actinobacteria from various environmental samples (Janssen et al., 2002; Wang D.-S. et al., 2014; Ruttanasutja and Pathom-aree, 2015).

Strain CMU55-4 was classified into the genus Micromonospora of the family Micromonosporaceae based on the sequence of 16S rRNA gene and its unique phenotypic characteristics. It forms a characteristic orange colony at the early stage, which turns to dark olive brown color with age. The strain produced extensively branched substrate hyphae, lack of aerial mycelia which is a unique morphological characteristic of the genus Micromonospora (Örskov, 1923). The strain formed a single non-motile warty-globular spore on the vegetative mycelium similar to the closest type strains, M. chalcea DSM 43026T (Örskov, 1923). The formation of single spores is also the main morphological characteristic of the genus Micromonospora (Trujillo et al., 2015). A meso-diaminopimelic acid was found in the cell wall peptidoglycan of strain CMU55-4 with arabinose, xylose and galactose as diagnostic sugars in the whole-cell hydrolysates corresponding to the cell wall type II and sugar type D (Lechevalier et al., 1971). Strain CMU55-4 exhibited phospholipid type II, comprising of phosphatidylethanolamine (PE), diphosphatidylglycerol (DPG), and phosphatidylinositol (PI) as the major phospholipids (Lechevalier et al., 1977). The strain contained a large amount of MK-9(H4) and MK-9(H6) which generally found in the genus Micromonospora (Nouioui et al., 2018). Strain CMU55-4 shared 98.47% of ANib values and 91.8% digital DNA-DNA hybridization to M. chalcea DSM 43026T. The value of <95–96% ANI or 70% dDDH is proposed as a cutoff value for delineating of new species (Chun et al., 2018). Therefore, strain CMU55-4 was identified as M. chalcea CMU55-4.

Micromonospora have long been recognized as antibiotic producers (Hirsch and Valdes, 2010). Recently, endophytic Micromonospora have been regarded as plant growth promoting bacteria especially in legumes (Trujillo et al., 2014; Benito et al., 2017). Physcomitrium sphaericum moss is known to be distributed only in the temperate regions; North America, Europe, Russia, China, and Japan. Recently, it has been found in the tropical country and reported as new record to Thailand (Printarakul et al., 2014). The inoculation of M. chalcea CMU55-4 on P. sphaericum moss resulted in an increase of carotenoid content, fresh and dry weight significantly higher than the control (Figure 3). This promotion effect appears to be related to the production of phytohormone IAA as M. chalcea CMU55-4 produced the highest IAA among all tested actinobacteria. Interestingly, P. sphaericum inoculated with M. chalcea CMU55-4 developed new leaves and showed the production of capsules (Figure 2B). Capsule is an important part of bryophyte reproduction as it represent sporophyte stage and benefits in asexual reproduction through spores dispersion (Sundberg et al., 2014). It is likely that the moss-bacterium interaction between M. chalcea CMU55-4 and P. sphaericum may play a role in promote leaves and capsule development though the exact mechanism still required further investigation. Effect of moss associated bacteria on Pylaisiella selwynii moss development was reported that the obtained Gram-negative bacteria could promote protonemal growth and gametophore initiation of P. selwynii (Spiess et al., 1981). Recently, auxin has been shown to involve in the development of moss (Physcomitrella patens) including gametophore and sporophyte development (Thelander et al., 2018). Almost all works regarding plant growth promoting actinobacteria are carried out with vascular plants. For examples, the inoculation of Micromonospora strain SB3 promotes plant biomass, root length and increases the acclimatization rate of Lolium multiflorum plantlets after 4 weeks of in vitro culture (Della Mónica et al., 2018). Recently, an endophytic M. chalcea UAE1 from halophytic crop, Salicornia bigelovii has been reported to promote the growth of S. bigelovii mainly by ACC-deaminase production (El-Tarabily et al., 2019). To the best of our knowledge, this is the first report on potential of Micromonospora species on the growth and development of non-vascular plants such as mosses.

Approximate genome sizes of 40 Micromonospora type strains were reported to be ranged from 6.1 Mbp for Micromonospora marina DSM 45555T to 7.9 Mbp for M. carbonacea DSM 43168T with the average genome size for all of the Micromonospora strains was 7 ± 0.4 Mbp (Carro et al., 2018a). The estimated genome size of our M. chalcea CMU55-4 was within these previously reported ranges (6.6 Mbp) with 73.0% GC content. The genome size of M. chalcea DSM 43026T was 7.0 Mbp and 72.8% G+C content (Carro et al., 2018a). The slightly bigger genome size of 7.086 Mb was reported from plant growth promoting rhizosphere Micromonospora sp. strain MW-13 with a similar G+C content of 73.3% (Jahnshah et al., 2019).

Omics techniques, including genome sequencing, comparative genomics, microarray, next generation sequencing, metagenomics, and metatranscriptomics can be used to explain plant-endophyte relationship (Kaul et al., 2016). Endophytic lifestyle of M. chalcea CMU55-4 was supported by data from genome mining and phenotypic studies as this strain was
able to utilize various kind of saccharides and carbohydrates which found inside plant cells. Saccharides molecules were discharged from plant cell walls during elongation process and served as nutrients to promote the colonization ability of plant-associated bacteria (Etesami et al., 2015). *M. chalcea* CMU55-4 is also able to produce both catecholate and hydroxamate type siderophores *in vitro*. In addition, several predictive genes related to siderophore production were also detected in its genome for examples desferrioxamine E and aerobactin and genes encoding transporter proteins. Neilands and Leong (1986) suggested that siderophore systems may play a role in infections of plant tissue. Thus, the ability of *M. chalcea* CMU55-4 to produce siderophores may help this strain to enter and live inside plant cells. Metallophores producing *Micromonospora* strains were recently reported to promote the growth of *Arabidopsis thaliana* under heavy metal condition by playing roles in metal acquisition, iron metabolism and resistance to toxic compounds (Ortúzar et al., 2020). Genes mutually found in Ortúzar et al. (2020) and this study are ferric hydroxamate ABC transporter (FhuB, FhuC, FhuD), siderophore desferrioxamine E (DesA, DesB, DesC, DesD), and siderophore aerobactin (FhuB, FhuC, FhuD).

Previous studies on actinobacterial genome revealed genes related to plant growth promoting activities primarily production of IAA, siderophore, phosphate solubilization, and phytopathogen inhibition (Gupta et al., 2014; Liu et al., 2019; Nouioui et al., 2019a). Predictive genes obtained from genome mining in this study supported the experimental data on IAA and siderophore producing abilities of *M. chalcea* CMU55-4 *in vitro*. Tryp-dependent pathway could be a pathway for IAA synthesis in strain CMU55-4 as predictive genes for tryptophan synthase alpha chain (TSA1), tryptophan synthase beta chain (TSB), indole-3-glycerol phosphate synthase (IGS) were found. Indole-3-glycerol phosphate or indole is a branch point for Tryp-dependent pathway that directly leads to IAA synthesis (Spaepen and Vanderleyden, 2011; Di et al., 2016). IAA promotes plant growth by advocating cell division, cell elongation, stimulates primary growth, and plays role in stress resistant. IAA production trait is considered as a useful criterion for selection of endophytic and rhizospheric bacteria in rice growth promotion (Etesami et al., 2015). High IAA production without L-tryptophan was recorded in some isolates such as CMU51-1 and CMU51-5. This observation suggests the possibility that these isolates can produce IAA by Tryp-independent pathway. *M. chalcea* CMU55-4 failed to solubilize tricalcium phosphate on PVK agar though predictive gene for exopolypophosphatase was detected. Since only tricalcium phosphate was used for phosphate solubilizing property, we cannot rule out the possibility of *M. chalcea* CMU55-4 to solubilize other types of inorganic phosphates. *M. chalcea* CMU55-4 also genetically possessed abilities related to nitrogen metabolism in particular nitrate/nitrite ammonification and ammonia assimilation. These

**FIGURE 5** | Scanning electron micrograph showing *M. chalcea* CMU55-4 grown on ISP2 agar medium at 30°C for 21 days. Bar represents 1 µm.
abilities could enhance nitrogen uptake system of plant as nitrate (NO₃⁻) and ammonium (NH₄⁺) are the main resources of inorganic nitrogen (N₂) absorbed by the roots of higher plants (Wickert et al., 2007).

It is also interesting to note the gene sequences responsible for other functions, such as the production of antioxidants and heat shock/ cold shock proteins were detected though these properties had not been investigated under laboratory conditions in this study. M. chalcea CMU55-4 contained predictive genes involved with oxidative stress response such as SoxR, NsrR, organic hydroperoxide resistance protein, trehalose synthesis genes, glutathione S-transferase omega, and carotenoid production, which are the molecules help plant to tolerate to oxidative stress. Reactive oxygen species (ROS) created by the plant cells, are generally neutralized by the production of enzymes such as superoxide dismutases (SOD), catalases (CatA), peroxidases (POD), alkyl hydroperoxide reductases (AhpC), genes conferring nitrosative stress caused by reactive nitrogen species, and glutathione-S-transferases (GSTs) in endophytes (Khare et al., 2018). GST was found in the genome of Streptomyces scabrisporus NF3, an endophyte of Amphipterygium adstringens (Ceapă et al., 2018). The ability to alleviate oxidative stress is one attractive property of plant growth promoting bacteria. Forty-two genomes of Micromonospora strains including the type strain of M. chalcea, were analyzed to determine the presence of PGP genes and other characteristics related with plant growth promotion (Carro et al., 2018a). Genomes of Micromonospora strains contain genes related to stress responses such as cspA, cspC for cold shock response, dnaK and grpE for heat shock responses, and sod for oxidative stress. Similar observation was also found in this study.

Bryophytes require high water for growth. They are more sensitive to water deficit condition than other plants. Accumulation of compatible solute such as glycine betaine therefore can help bryophytes to maintain water balance. Glycine betaine transporter OpuD was found in M. chalcea CMU55-4 genome. The opuD gene product was reported to be essential for glycine betaine uptake and osmoprotection in E. coli (Kappes et al., 1996). Glycine betaine accumulation had been reported to increase the drought tolerant in Streptomyces chartreusis (Wang et al., 2019). Predictive genes involve in trehalose metabolism was also present in M. chalcea CMU55-4 genome including gene encoded for trehalose synthase. Trehalose is another compatible solute that can protect plant proteins and cellular membranes from inactivation or denaturation caused by a variety of stress conditions, including desiccation, dehydration, heat, cold, and oxidation (Elbein et al., 2003).

Bryophytes usually grow in low temperature and high humidity area. Cold shock protein may be essential for the adaptation of actinobacteria to live with bryophytes under such environment. M. chalcea CMU55-4 genome is equipped with CspA family of cold shock protein and several heat shock protein genes. This protein plays an essential role in inhibition of DNA replication during cold-adaptation in Streptomyces sp. AA8321 (Kim et al., 2007). Similarly, heat shock protein families (HSP) can be benefit to bryophytes for survival under high temperature. HSPs remove denatured proteins to prevent formation of large protein aggregates and cell death while DnaK/DnaJ/GrpE or GroEL/GroES chaperones are part of sigma-32 heat shock regulon that regulates cell during heat stress and maintains protein homeostasis in living cells (Schumann, 2016).

In conclusion, this study provides the first evidence of cultivable actinobacteria associated with three high altitude moss species, Bryum apiculatum, Syntrichia gemmascens and Campylypus involutus. These actinobacteria show plant growth promoting ability in vitro. The inoculation of the selected strain, M. chalcea CMU55-4, can promote the growth of rare moss species, P. sphaericum. The response of this moss to M. chalcea CMU55-4 suggested that actinobacteria of the genus Micromonospora might occur naturally in association with the moss and might commonly affect moss development in nature. Genome mining data also support plant growth promotion potential of M. chalcea CMU55-4 as a good candidate for breeding program of P. sphaericum especially those under acclimatization and other moss species.

### DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online the repositories. The names of the repository/repositories and
accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, JAAOLH000000000.

**AUTHOR CONTRIBUTIONS**

CI performed all experiments and wrote the manuscript. WP, NC, and ST supervised CI. CI, WP, NK, and NC revised the manuscript. WP conceived the idea and designed research outline. All authors contributed to the article and approved the submitted version.

**FUNDING**

The authors would like to acknowledge funding support from the Development and Promotion of Science and Technology Talents Project (DPST), Ministry of Education, Thailand, and Canada-ASEAN Scholarships and Educational Exchanges for Development (SEED) program, supported by the Government of Canada. This work was also partially supported by the Chiang Mai University.

**ACKNOWLEDGMENTS**

We thank to Dr. Narin Printarakul, Department of Biology, Faculty of Science, Chiang Mai University for bryophytes identification.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.563047/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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